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CMFRI Training Manual Series No.15/2018

Training Manual

In the frame work of the project:
DBT sponsored Three Months National Training in

Molecular Biology and Biotechnology for Fisheries Professionals

2015-18



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This is a limited edition of the CMFRI Training Manual provided to participants of the “DBT sponsored Three Months National Training in Molecular Biology and Biotechnology for Fisheries Professionals” organized by the Marine Biotechnology Division of Central Marine Fisheries Research Institute (CMFRI), from 2nd February 2015 - 31st March 2018.

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Foreword

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Central Marine Fisheries Research Institute (CMFRI), Kochi along with CIFE, Mumbai and CIFA, Bhubaneswar within the Indian Council of Agricultural Research (ICAR) and Department of Biotechnology of Government of India organized a series of training programs entitled “DBT sponsored Three Months National Training in Molecular Biology and Biotechnology for Fisheries Professionals”. The scope of this training is to promote development of trained human resource for application of molecular tools to research problems in fisheries and aquaculture, to help them adapt to such facilities and work programs and to include analyses that comply with worldwide regulatory acts in the field of biotechnology.

At present, mostly traditional methods are being used in the fisheries sector and only a few researchers are applying molecular methodologies. The emphasis of this training program is to enhance the capabilities of research personnel already employed in fisheries Institutes, Universities and Colleges, but working in the area of marine biology/fisheries/aquaculture. The contents of this training course are intended to teach molecular techniques to laboratory personnel with a good level of analytical knowledge, but with no or little expertise in this specific domain.

The 3 months training program comprises of theory classes with hands-on practical sessions and research work. All basic molecular biology, genetic engineering and molecular genetics techniques are included in the course along with their applications in various aspects of aquaculture and fisheries. Each institute offers specialized modules based on areas of expertise. The course content at CMFRI is designed to cover all kinds of applications of molecular methods in fisheries. The emphasis is on hands-on experience and skill development of the participants. Technical details were provided to trainees as oral presentations and brief written outlines. Aware of the need for a permanent source of information, the Marine Biotechnology Division of CMFRI developed this manual as background information for course participants and is intended to provide the theoretical and practical information on methodologies and protocols currently used, which describes some of the techniques used in our laboratory.

It is our hope that the structure and content of this manual will help course participants (as well as other users) in the diffusion and dissemination of the acquired skills in the context of the different working environments according to needs. This manual aims to complement existing information in the specialised literature.

Dr. P. Vijayagopal supervised the preparation of this manual and the contributors are mentioned in the Table of contents. A special recognition and acknowledgment to all personnel who, even not individually mentioned, contributed to the successful preparation of the manual. Thanks are also extended to Dr. Reynold Peter, Ms. Adithya C. and Ms. Swetha Damodharan P.V. for their support for the preparation of this manual.

Dr. A. Gopalakrishnan
Director, CMFRI

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Marine Biology

Introduction to Marine Biodiversity

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Introduction

The Convention on Biological Diversity (CBD) defined biodiversity as being the variability among living organisms from all sources including, among others, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems.

An ecosystem is a dynamic complex community of plant, animal, microorganism and the non-living environment interacting as a functional unit. Biological components are crucial in proper ecosystem functioning, which provides essential ecosystem services to human beings. Ecosystem diversity is the variation of different biological communities and their interaction with the biotic and abiotic environment.

Biological diversity comprises species, genetic and ecosystem diversity. Species diversity is the diversity of all the species on earth from single celled bacteria and protists to the species of the multicellular kingdom. Diversity in species shows the variation of species due to evolutionary and ecological adaptations of the species to the entire geographical range. Genetic diversity is the variation within species due to geographical separation and intraspecific variation within the population.

Ecosystem services from Marine and Coastal Ecosystems

Marine ecosystems provide a wide variety of services to nature which is essential for the well-being of the human population. The ecosystem services are classified into four *i.e.* provisioning services, regulating services, supporting services and cultural

and amenity services. Provisioning services means the products obtained from ecosystem in the form of food, natural products, fuel wood, medicines, genetic and ornamental resources, energy resources, and product from bioprospecting. Regulating services include the shoreline stabilization, flood prevention, storm protection, climate regulation, hydrological services, nutrient regulation, carbon sequestration, detoxification of polluted waters and waste disposal. Supporting services are mainly the habitat provision, nutrient cycling, migration, seed dispersal, primary productivity and soil formation. Cultural and amenity services include the culture, tourism and recreation.

Provisioning services

Food provisioning in the form of fish landings and aquaculture products is one of the most important services obtained from the marine ecosystems. Mangroves are important in supporting fisheries due to their function as fish nurseries. Mangroves help to increase fish production in the inshore waters near to it. Coral reefs also provide services like protection of breeders and larvae for the better survival and recruitment success of the resources. They form an important source of fisheries products for coastal residents and export markets. The coral reefs of the Gulf of Mannar, Andaman Nicobar Islands, Lakshadweep Islands and Gulf of Kutch contribute substantially to the total marine finfish catch of India. Other ecosystems like rocky intertidal, near shore mudflats, seagrass beds, mud bank areas, seamounts, brackish water, lagoons, estuaries, marshy areas and beaches also helps in the production of fish as food in one way or another.

The total marine fish landings from India were

estimated at 3.63 million tonnes during 2016. Fisheries sector plays an important role in the Indian Economy, contributing about 1% to the national GDP. The sector provides livelihood to about 4 million fisher folk population along the coastal line of 8129 Km. The value of total marine fish landings at landing center level was estimated at Rs. 48381 crore during 2016. Since 1950 the marine fish production in India has gradually increased from mere 5.8 lakh tonnes (1950) to 3.63 million tonnes (2016) showing six fold increase. History of the development of the Indian fisheries sector has gone through three phases. First phase (>1965) is characterized by non-mechanized indigenous crafts and gears and the landings remained below one million tonnes during this phase. Second phase (1965-1985) showed an increase in the mechanization of crafts, use of advanced gear materials, introduction of motorization of traditional crafts, and expansion of export trade. Third phase (1986-2015) is featured intensification of mechanization, motorization of traditional crafts, multi-day and multi-gear stay over fishing and introduction of deep sea fishing. A total of about 2000 marine species is caught from the Indian seas. They are categorized into 29 resource groups. Out of the 26 groups studied by CMFRI, 20 were found to be under the abundant category, four under less abundant category and one each under declining, depleted and collapsed category. Elasmobranchs, threadfins, ribbonfishes, mullets and flatfishes are the four resource groups falling under less abundant category. Big-jawed jumper falls under declining category, flying fishes under depleted category and unicorn cod is the one that falls under collapsed category (Sathianandan *et al.*, 2011). Many of the marine and coastal ecosystems provide coastal communities with construction materials and building materials from the mining of coral reefs. Mangroves provide coastal and Island community with building materials for boat construction.

According to Marine Products Export Development Authority export of marine products from India reached an all-time high of Rs. 30213 crores for quantity of 9.8 lakh tonnes (2014). Among the products exported, shrimp product formed the major

share about 3.0 lakh tonnes which forms about 64% of the total value realized. Increased export demand often leads to expansion of mariculture practices. Coastal areas provide the foundation for the marketers which produce fisheries products from prawn, crab and fish. The factors affecting the marine fish production are the overexploitation, species extinctions and use of destructive methods of fishing. The proportion of marine fish stocks that are over exploited and depleted are increasing over the last 30 years. It is reported that 133 extinctions of regional and global marine species occurred over the last 30 years. The major cause of the extinction was overexploitation (55%) and rest of habitat loss and other reasons.

Bio prospecting is the exploration of biodiversity for new biological resources of social and economic value. It yielded several products from species in marine and coastal ecosystems. Coral reefs are important reservoirs of natural bioactive products many of which exhibit structural features not found in the terrestrial natural products. The pharmaceutical industry has discovered several potentially useful substances among sponges, jellyfish and Mollusca.

Regulating services

Regulating services are the benefits people obtained from the regulation of ecosystem processes, including air quality maintenance, erosion control, regulation of human diseases and water purification. The mangroves, sea grass, coral reefs, rocky intertidal, mudflats, and deltas play key role in shoreline stabilization, protection from storms, floods and soil erosion, processing pollutants and stabilizing land in the event sea level rise. Mangroves have great capacity to absorb heavy metals and other toxic substances, coral reefs buffer land from storms and prevent beach erosion. Estuaries, lagoons, marshes, brackish water areas play a key role in maintaining hydrological balance and filtering water of pollutants. Marine ecosystems play significant roles in climate regulation. CO₂ is continuously exchanged between the atmosphere and ocean; it dissolves in surface waters and is then transported to the deep ocean. Marine plants fix CO₂ during photosynthesis in the ocean and return it via respiration.

Supporting Services

Many species use coastal areas like estuaries, mangroves, sea grasses as nurseries. Estuaries are particularly important as nursery areas for fisheries and other species and they form one of the strongest linkages between coastal, marine and freshwater ecosystems and the ecosystem services they provide. The success of the prawn fishery mainly depends on the migration of prawns through the estuary. Mangroves provide nursery for many species as well as give links to sea grass beds with associated coral reefs. Decline in the area of mangroves can interrupt these linkages and cause biodiversity loss which results in lower productivity from the reef and sea grass beds.

Sea grasses are important in providing nursery areas and it provides habitat for coral reef fishes and invertebrates. It is an important source of food for many species of coastal and marine species. Sea horses, sea cow, and turtles are the species associated with sea grass beds. Kelp beds and other Macro algae provide nursery habitat for some species. They support many fish species and invertebrates like sea urchins. Estuaries provide a range of habitats to sustain diverse flora and fauna. There are many more estuarine dependent species than estuarine resident species. Mudflats are also critical habitat for migrating shorebirds and many marine organisms including the commercially important clam species. Sea mounts forms another important habitat provides nursery to several species of fishes. All of the these ecosystems the beaches, sandy shores, dune systems, salt marshes, estuaries, and mudflats provide feeding and nesting habitats to numerous species of birds, fishes, molluscs, crustaceans, and other organisms.

Cultural and Amenity Services

Cultural services include tourism and recreation, aesthetic and spiritual services, traditional knowledge and education and research services. Most important cultural services provided by coastal and marine ecosystems are tourism and recreation. Natural amenities are highly valued by people and contribute to human welfare, thus providing significant economic

value. Stretches of beach, rocky cliffs, estuarine and coastal marine waterway are the places where people make frequent visits for sightseeing and recreation. Some species are of considerable cultural importance, for example cultural significance of *Turbinella pyrum* (Sacred Chank) as it is used temples and bangle industry. The sea shores are also of great spiritual importance as so many temples are situated along the coast. Marine and coastal ecosystems are areas that received attention through research. Rocky intertidal habitats main focus of research and provided some of the foundation principles of ecology.

Status of Marine Resources

The ecosystem goods and services provided by the fauna and flora and the interrelationship between the biodiversity and ecological processes are the fundamental issues in the sustainability and the equilibrium of the ecosystem. Ecosystem goods from the marine realm included the fin fishes, crustaceans, molluscs and seaweeds. The important flora and fauna falling to the two major kingdoms such as Animal and Plant kingdom recorded from the Indian region and their present status are discussed below.

Kingdom: Plantae

Mangroves: Mangroves trees up to medium size and shrubs that grow in saline coastal sediment habitats in the tropics and subtropics. Asia has the largest amount (42%) of the world's mangroves (Kathiresan and Rajendran, 2005).

Seagrasses: Sea grasses are flowering plants from one of four plant families (Posidoniaceae, Zosteraceae, Hydrocharitaceae, or Cymodoceaceae), all in the order Alismatales grow in marine fully saline environments. A total of 14 species of sea grasses in six genera is reported from Indian seas (Venkataraman and Wafer, 2005).

Macro algae (Sea weeds): Sea weeds are large multicellular plants that resemble vascular plants but lack the complex array of tissues used for reproduction and water transport. They are found in red (Rhodophyta), green (Chlorophyta) and brown

(Phaeophyta) divisions. A total of 1010 species of macro algae has been reported from India. They are most abundant along the Gujarat, Kerala and TamilNadu coasts and around the Andaman and Lakshadweep Islands. A large number of seaweed species known from the Indian seas are edible and serve various industrial purposes. The edible seaweeds are known to be rich in protein (20 to 25%) Carbohydrates (16 to 24%), lipids (6 to 11%) vitamins and amino acids.

Kingdom: Animalia

Phylum: Porifera (Sponges)

Sponges are multicellular organisms which have bodies have pores and channels, allowing water to circulate through them, consisting of jellylike mesohyl sandwiched between two thin layers of cells. About 519 species of sponges are known to occur in the Indian seas. About 34 species of coral boring sponges have been reported from the Gulf of Mannar and Island system of India (Thomas, 1996 a). Sponges are the major components of the benthic fauna and are distributed from the intertidal to the hadal depths and are a potential source of many new bioactive compounds. In India out knowledge of the identity, biology, availability, population structure and possibilities of commercial exploitation of sponges is meagre and requires prioritization.

Ctenophora (Comb jellies): Ctenophora are live in marine waters and distinctive feature is the groups of cilia (comb) they use for swimming. They are the largest animals that swim by means of cilia. A total of 20 species of comb jellies has been reported from India.

Phylum: Cnidaria

Class: Scyphozoa (True jellyfish)

Scyphozoa is referred as the true jellyfish. Their stings may cause skin rashes, muscle cramps, or even death. A total of 30 species of Scyphozoans has been reported from India.

Class: Hydrozoa (Jelly fish)

Hydrozoans are small, predatory animals, some solitary and some colonial, and marine. The colonies are large, and in some cases the specialized individual animals

cannot survive outside the colony. The Portuguese Man of War (*Physalia physalis*) and *Crambionella stulhamani* are important jelly fish species. About 116 species of hydrozoans belonging to 13 families have been reported from India.

Class: Anthozoa

Octocorallia (Soft corals): Octocorallia is belonging to the subclass of Anthozoa. It includes the blue coral, soft corals, sea pens and gorgonians (sea fans and sea whips) within three orders: Alcyonacea, Helioporacea, and Pennatulacea. Their life cycle includes a motile phase as plankton and later a sessile phase. About 300 species of soft corals have been reported from India. Gorgonids are abundant in the Gulf of Mannar and distributed almost all along the Indian coasts including Andaman Sea. About 22 species belonging to 7 families and 15 genera were reported from India (Thomas, 1996 b). The biomedical versatility of the gorgonids, popularly known as the sea fans, attracted great attention to this resource. The four species which have already shown symptoms of depletion include *Echinomuricea indica*, *Heterogorgia flabellum*, *Echinogorgia complexa* and *Gorgonella umbraculum* (Thomas and Ranimary George, 1987).

Ceriantharia (Tube – dwelling anemones):

Tube-dwelling anemones, which are similar to sea anemones, but belong to the subclass of anthozoans. They are solitary, living buried in soft sediments. Tube anemones live and can withdraw into tubes, which are made of a fibrous material, which is made from secreted mucus and threads of nematocyst like organelles known as ptychocysts. The diversity included about 20 species in India.

Actiniaria (Sea anemones): The Actiniaria belongs to the class Anthozoa which includes the true sea anemones. They are water-dwelling, predatory animals. They have large polyps that allow for digestion of larger prey and also lack a medusa stage. They are related to corals, jellyfish, tube-dwelling anemones, and Hydra. Sea anemone *Heteractis magnifica* Quoy and Gairmad, 1833 is associated with clown fish. Actinarian diversity included about 30 species in India.

Corallimorpharia (Coral anemones):

Corallimorpharia is closely related to the true sea anemones (Actiniaria). The tentacles are usually short and arranged in rows radiating from the mouth. They resemble the stony corals, except for the absence of a stony skeleton. They occur in a wide range of marine habitats, and are associated with phase shifts in coral reefs that change from hard-coral dominated to soft-coral dominated. Diversity of Corallimorpharia includes about 10 species along the Indian coast.

Zoanthidea (Mat anemones): Zoanthids are commonly found in coral reefs, the deep sea and many other marine environments around the world. They may be in the form individual polyps, attached by a fleshy stolon or a mat that can be created from small pieces of sediment, sand and rock. A total of 8 species of Zoanthids has been reported from India.

Scleractinia (Reef building corals): Scleractinia are marine corals that form a hard skeleton. Most of the modern coral reefs are formed by scleractinians. About 200 species Scleractinia from the diversity of India (Pillai, 1996).

Antipatharia (Black corals): Black corals are tree-like corals related to sea anemones and found in deeper depths. There are about 230 known species of black corals in 42 genera of this 10 species occur in India (Pillai, 1996). Though black coral's living tissue is brilliantly coloured, it takes its name from the distinctive black or dark brown colour of its skeleton.

Phylum: Platyhelminthes (Flat worms)

Platyhelminthes are bilaterally symmetrical, unsegmented, soft-bodied invertebrate worms. They don't have body cavity, and circulatory and respiratory systems, which made them to in a flattened shape. The digestive cavity has only one opening for both the ingestion and ejection as a result; the food cannot be processed continuously. About 100 species of flatworms have been reported from India. Research on the Platy helminthes of India is less as compared to the Annelids of India (Venkataraman and Wafer, 2005).

Phylum: Echiura (Spoon worms)

The Echiura are a small group of marine animals. They lack the segmented structure found in other Annelids of this group. Recent studies show they may be included in the phylum Annelida. About 43 species under 14 genera have been reported from India.

Phylum: Sipuncula (Peanut worms)

The Sipuncula are bilaterally symmetrical worms and contains about 144-320 species. They live in shallow waters, either in burrows or in discarded as molluscan shells. Some bore into solid rocks to make a shelter for themselves. About 35 species under 10 genera have been reported from India. They are concentrated mainly along the Andaman and Nicobar Islands, Lakshadweep Islands, Gulf of Mannar and Gulf of Kutch.

Phylum: Annelida**Class: Polychaeta (Clam worms)**

The Polychaeta are generally marine, and belong to phylum Annelida. The body has a pair of fleshy protrusions called parapodia that bear many bristles, called chaetae, which are made of chitin. The annelid worm diversity includes about 300 species in India (Venkataraman and Wafer, 2005).

Class: Clitella

Oligochaeta (Earthworms): The Oligochaeta is different types of aquatic and terrestrial worms. Earthworms are semi aquatic or fully aquatic. There are several interstitial marine worms. About 10 species reported from India.

Phylum: Nemertea (Ribbon worms)

Nemertea (Nemertini, Nemertinea, Rhynchocoela) is a group of invertebrate animals and known as ribbon worms or proboscis worms. They have an unsegmented body, thin and elongated with no differentiated head. Ribbon worm diversity includes about 60 species in India.

Phylum: Arthropoda

Subphylum: Chelicerata

Class: Merostomata

Horseshoe crab: Horseshoe crab belonging to the family Limulidae and known as Living Fossil because their origin is 450 million years ago. The marine king crab of the species *Tachypleus gigas* and *Carcnoscorpius rotundicauda* occurs in the deltaic regions of Ganges and Mahanadi along the northeast coast. They are considered as living fossil and hence care should be taken to preserve them in the nature. Recently because of biotic interference there has been a decline in the numbers of these animals in Orissa. The chemical reagent lysat is produced from the blood of this crab. This medicine has got a wide usage in the treatment of several diseases.

Subphylum: Crustacea

India is endowed with rich diversity of crustaceans and several of them supporting commercial fisheries since ancient times.

Class: Maxillopoda

Cirripedia (Barnacles): They have a calcareous shell composed of several pieces. They are known as curl footed because of their curved legs. A total of 36 species of cirripedia have been reported from India.

Class: Malacostraca

Order: Amphipoda (Land hoppers)

Amphipoda is have no carapace and generally with laterally compressed bodies. Amphipods range in size from 1 to 340 millimeters and are mostly detritivorous or scavengers. They live in marine aquatic environments. A total of 132 species of Amphipods belonging to 54 genera has been reported from India.

Order: Isopoda (Pill bugs, sow bugs)

The Isopoda are small crustaceans with seven pairs of legs in the size of above 300 micrometers. They have dorso-ventrally, flattened body, without carapace. There are about 33 species belonging to 13 genera have been reported from India.

Order: Stomatopoda (Mantis shrimp)

Stomatopods are marine crustaceans and they occur in a variety of different colours, from shades of browns to bright neon colours. These aggressive and typically solitary sea creatures spend most of their time hiding in rock formations or burrowing indicate passageways in the sea bed. Unlike most crustaceans, stomatopods hunt, chase, and kill their prey. Most species live in tropical and subtropical seas, although some live in temperate seas. The stomatopod diversity includes about 30 species along the Indian coast.

Order: Decapoda

Dendrobranchiata (Shrimp, prawns): Dendrobranchiata are decapod crustaceans, known as shrimp or prawns. There are 540 extant species in seven families. They differ from related animals, such as Caridea and Stenopodidea, from the branching form of the gills and by the fact that they do not brood their eggs, but release them directly into the water. They are widely fished and farmed for human consumption. About 10 species have contributed to the diversity in India.

Caridea (Caridean shrimp): The Caridean shrimp is an infraorder of shrimp within the order Decapoda. They are found widely around the world in both fresh and salt water. Carideans are found in every kind of aquatic habitat, with the majority of species being marine. About 150 specimens included in the caridean shrimp diversity of India.

Palinura (Lobsters): Lobsters have a cylindrical, sub ovoid or dorso-ventrally compressed carapace and flattened abdomen. The group includes the spiny lobsters and slipper lobsters. The abdomen is flattened.

Thalassinidea (Ghost shrimps, mud shrimps): Thalassinidea include crustaceans, which live in burrows in muddy bottoms of the sea. Thalassinids typically live in deep and sometimes complex burrows. Shallow water local species typically remain deep in the burrow and suspension feed (filtering plankton and organic particles from the water) by beating their pleopods to create a current. About 20 species of Thalassinides has

been reported from the Indian Ocean.

Anomura (Hermit crabs, sand crabs): Anomura is a group of decapod crustaceans, including hermit crabs and others. All true crabs are in the sister group to the Anomura. A total of 20 species Anomuran crabs has been reported from India.

Brachyura (Crabs): Crabs are decapod crustaceans with a typically very short tail, usually entirely hidden under the thorax. A total of 250 species of crabs has been reported from Indian coast.

Phylum: Mollusca

Molluscs in general had a tremendous impact on Indian tradition and economy and were popular among the common man as ornaments, currency, as a part of spiritual activities even at the inception of human culture and civilization. A total of 3271 species of molluscs distributed among 220 families and 591 genera, of which 1900 are gastropods, 1100 bivalves, 210 cephalopods, 41 polyplacophora and 20 scaphopods. Among these 8 species of oysters, 2 species of mussels, 17 species of clams, 3 species of pearl oysters, 3 species of giant clams, 1 species of windowpane oyster and gastropods such as Sacred Chank, Trochus, Turbo and 15 species of Cephalopods are exploited from the Marine sector of India. The species like *Cassis cornuta*, *Charonia tritonis*, *Conus milneedwardsi*, *Cypraea rufa*, *Nautilus pompilius*, *Hippopus hippopus*, *Tridacna maxima*, *Tridacna squamosa* etc. are the some of the molluscs protected under the Wildlife (Protection) Act, 1972 Schedule I.

Phylum: Echinodermata

Class: Echinoidea (Sea urchin)

Sea urchins move slowly, feeding mostly on algae. Sea otters, wolf eels, triggerfish, and other predators feed on them. A total of 60 species of sea urchins was showed occurrence in the Indian seas.

Class: Holothuroidea (Sea cucumbers)

Total number of holothurians species is 1,250 in the world and maximum number being in the Asia Pacific region. Many of these are utilized for

human consumption and some species are used in aquaculture systems. Sea cucumbers serve a useful purpose in the marine ecosystem as they help recycle nutrients, breaking down detritus and other organic matter after which bacteria can continue the degradation process. About 150 species of sea cucumber have been reported from India. About 12 species of sea cucumber are economically important and have commercial value (James, 1996).

Class: Asteroidea (Starfish)

Starfish is among the most familiar and diverse group of marine invertebrates. They have a central disc and five arms, and some species have more than five arms. The ochre sea star (*Pisaster ochraceus*) and the reef sea star (*Stichaster australis*) are widely known as examples of the keystone species concept in ecology. A total of 180 species of starfishes belonging to 81 genera have been reported from India.

Class: Ophiuroidea (Brittle stars)

They have a disk and generally have five long, slender, whip-like arms which may reach up to 60 centimeters in length on the largest specimens. A total of 150 species of brittle stars belonging to 79 genera have been reported from India.

Class: Crinoidea (Sea lilies)

They live both in shallow water and in depths up to 6,000 meters. Sea lilies in their adult form are attached to the sea bottom by a stalk. They have a mouth on the top surface that is surrounded by feeding arms. Crinoids usually have a stem used to attach them to a substrate, but many live attached only as juveniles and become free-swimming as adults. A total of 95 species belonging to 43 genera have been reported from India.

Phylum: Phoronida (Horseshoe worms)

They live in most of the oceans and seas, including the Arctic Ocean but excluding the Antarctic Ocean, and between the intertidal zone and about 400 meters down. About 5 species of phoronids were reported from India.

Phylum: Brachiopoda (Lamp shells)

They have hard shells on the upper and lower surfaces, unlike the left and right arrangement in bivalve molluscs. There are two types are recognized, articulate and inarticulate. Articulate brachiopods have toothed hinges and simple opening and closing muscles, while inarticulate brachiopods have untoothed hinges and a more complex system of muscles used to keep the two halves aligned. About 5 species of Brachiopods were found in India.

Phylum: Bryozoa (Moss animals)

They are known as Polyzoa, Ectoprocta or moss animals are aquatic invertebrate animals. Size range from 0.5 millimeters long, and are filter feeders. Over 4,000 living species are known. One genus is solitary and the rest colonial. There is a rich biodiversity in India with about 500 species are reported so far. Several collections and descriptions in the past, enriched the knowledge about the Bryozoans occurring along the Indian coast (Venkataraman and Wafer, 2005).

Phylum: Hemichordata (Acorn worms)

Acorn worms are solitary live in burrows and are deposit feeders, and species are filter feeders. About 12 species of hemichordates have been reported from India as compared to global species of 102. The balanoglossus, *Phychodera fluva* is a unique living fossil that links vertebrates and invertebrates occurs in the Gulf of Mannar area of India.

Phylum: Chaetognatha (Arrow worms)

Arrow worms are predatory marine worms that form a major component of plankton worldwide. About 20% of species are benthic, and can attach to algae and rocks. They range in size from 2 to 120 millimeters. A total of 30 species have been reported from India. They are abundant all along the Indian coast. Extensive studies along the Malabar Coast, Vishakhapatnam Coast, Andhra coast revealed the presence of 30 species occurring in India (Venkataraman and Wafer, 2005).

Phylum: Chordata

Class: Thaliacea (Pelagic tunicates)

Thaliaceans are free-floating for their entire lifespan. They include both solitary and colonial species. Thaliaceans have 30% carbon by mass. Therefore, their dense bodies sink to the bottom of the oceans when they die and this may be a major part of the worldwide carbon cycle. A total of 40 species was reported from India.

Class: Ascidiacea (Sea squirts)

Ascidians are found all over the world, usually in shallow water with salinities over 2.5% the members of the Thaliacea and Larvacea swim freely like plankton, sea squirts are sessile animals. A total of 50 species belonging 21 genera have been reported from India against 2000 species of Asidian in the world.

Class: Pisces

Elasmobranchs: The elasmobranchs consists of sharks, sawfishes, rays, skates and guitar fishes. The protected elasmobranchs as per the Wildlife (Protection) Act, 1972, Schedule I are *Rhincodon typus* (Whale shark), *Anoxyprisits cuspidate* (Pointed saw fish), *Prisitis microdon* (Large tooth sawfish), *Prisitis zijron* (Long comb sawfish), *Carcharhinus hemiodon* (Pondicherry shark), *Glyphis gangeticus* (Ganges shark), *Glyphis glyphis* (Speer tooth shark), *Himantura fluviatilis* (Gangetic sting ray), *Rhynobatus djiddensis* (Giant guitarfish) and *Urogymnus asperimus* (Thorny ray).

Ornamental fish: The Gulf of Mannar, Palk bay, Gulf of Kutch, South West coast and the Lakshadweep and Andaman group of Islands are known to be rich in Ornamental fishery. The Wrasses, damsel fish, Surgeon, Butterfly fish, Moorish idol, Squirrel fish, Trigger fish, Rabbit fish, Parrot fish, Angels, Goat fish and Puffer fish are the major aquarium fishes represented by about 180 species (Murty *et al.*, 1989; Murty, 2002). CITES have listed all the sea horse in the Appendix I to stop the trade of these organisms. Indian wild Life Act 2002 also protects the sea horse by putting them in Schedule list I. Dried sea horse has got a high demand in Singapore and China for making soup and for medicinal purposes.

Class: Reptilia

Marine reptiles: Marine reptiles are air-breathing, ectothermic, poikilothermic vertebrates. Their skin is covered with dry scales and lays their egg on land. Out of the 700 living species only few species of snakes, turtles, and crocodiles are seen in the ocean.

Order: Chelonia

Sea Turtles: Five species of sea turtles were reported in India which include, Olive Ridley (*Lepidochelys olivacea*) Green Turtle (*Chelonia mydas*), Leather back (*Dermocheylus olivacea*), Hawksbill (*Eretmocheylus imbricate*) and Logger head (*Caretta caretta*). Green turtles (*Chelone mydas*) are found in coastal water and feed mainly as sea grasses and sea weeds. The hawksbill turtle (*Erectmocheylus imbricata*) feed on encrusting animals like sponges, sea quirts, barnacles and sea weeds. The largest sea turtle the leather back (*Dermocheylus coriacea*) have a series of shells and an oceanic species. They have scissor-like jaws for capturing and they feed on jelly fish. Other species feed on soft, bottom invertebrates like sponges, soft corals, jelly fishes and crabs. Prey-predator relationship in the ecosystem is one of the important factors in limiting as well as proliferation of organisms due to the decline of the one of the components in the trophic relations (Joshi, 2012). All species of marine turtles are in the endangered category, and are therefore, protected under the Indian Wildlife Act, 1972.

Order: Squamata

Sea snakes: Sea snakes occur in the tropical and sub-tropical waters of the Indian Ocean from the east coast of Africa to Australia. They occur in shallow coastal waters, estuary, lakes and fresh water in the rivers away from the sea. They feed on fish, fish eggs, crustacean and tuna. The genus *Laticauda* is oviparous and all other sea snakes are viviparous. The Sea snake bite is dangerous and it is neurotoxic like terrestrial snakes like krait and cobra. There are about 80 species, sea snakes belonging to three families inhabiting the world oceans and estuaries. In Indian waters, about 22 species of marine snakes belonging to three families have been documented.

Order: Crocodilia

Salt water crocodiles: Salt water crocodile, *Crocodylus porosus* (Schneider, 1801) is the largest reptile in the present world with about six meter lengths and up to one metric ton weight. They can live in saltwater, but usually occurs in mangrove swamps, estuaries, deltas, lagoons and lower stretches of rivers. The species is endangered due to hunting, loss of habitat and breeding sites. Marsh crocodile or Indian swamp crocodile is *Crocodylus palustris* (Lesson, 1831) found in rivers, swamps, lakes and saltwater lagoon. Indian Gharial is *Garialis gangeticus* (Gmelin, 1789) are found mainly in river Ganga, Brahma putra and Mahanadi.

Class: Aves

Sea birds: Sea birds are long lived, with very low natural mortality. These biological traits and human induced adult mortality potentially damaging population decreases and collapse of the population. Ecosystem services that birds provide are mainly indirect and supporting services. It includes trophic effects, seed dispersal, nutrient cycling, aesthetics, and recreation. Also most of the tropical sea bird species feed in association with tuna stocks which derive their prey to the surface, thereby bringing within the reach of the sea birds. The depletion of the tuna stocks could therefore have impacts on their dependent species like sea birds. The cascade effects of reduce tuna or shark on the ecosystem are not known.

Common birds are Grey heron (*Ardea cinerea*), Pond heron (*Ardeola grayii*), Large erget (*Egretta alba*), Little erget (*E. garzetta*), Painted stork (*Ibis leucocephalus*), Spoon bill (*Platalea leucocordia*), Flamingo (*Phoenicopterus roseus*), Parian kite (*Milvus nigrans*), Golden plover (*Pluvuailis dominica*), Black headed Gull (*Larus ridibundus*), Gull billed Tern (*Geolchelidon nilotica*), Caspian tern (*Hydroprogne caspia*), Little tern (*Sterna abilfrons*) and Sandwich tern and (*Sterna sandvicensis*), Three species of Albatross are endangered (IUCN) two species near threatened and one is critically endangered (IUCN). Sea birds occur along the Gulf of Kutch, Gulf of Mannar, Chilka Lake, Coringa Wild life Sanctuary and the Sundarbans, Islands of Laccadive such as Pitti and Batapari are the

colonies of sea birds. Sundarbans is important staging and wintering area of gulls and terns.

Class: Mammalia

Dolphins: The species diversity of dolphins in India is one among the richest in the world. A total of five species, dolphins was recorded from our seas. Important species are *Stenella longirostris* (Spinner dolphin), *Sousa chinensis* (Humpback dolphin), *Delphinus delphis* (Common dolphin), *Tursiops truncatus* (Bottlenose dolphin) and Risso's dolphin (*Grampus griseus*).

Whales: Whales constitute the most dominant groups of marine mammals. They usually occupy in the temperate and polar oceanic waters, they migrate to tropical waters for breeding and avoid extreme climatic conditions during certain seasons. Whales are classified into Odontoceti (toothed whales) and mysticeti (baleen whales). All the Cetaceans are included in the list of protected animals. A total of about 10 species has been reported from the Indian seas. They are *Indopacetus pacificus* (Longman's Beaked whale), *Balaenoptera borealis*, *Balaenoptera musculus*, *Balaenoptera acutorostrata*, *Pseudorca crassidens*, *Physeter macrocephalus*, *Ziphius cavirostris* and *Balaenoptera* sp.

Sea Cow: The sea cow, Dugong dugon inhabits in the Gulf of Mannar and Palk bay area and is included in the List of protected animals as per the Wildlife (Protection) Act, 1972 Schedule I.

Conclusion

The marine organisms provide ecosystem services, but are not valued properly or unappreciated. It has necessary to ecosystem services been better studied and valued properly for the conservation and

sustainable use of biodiversity not only for the present generation but for the future generations. Despite the huge role of marine ecosystems and organisms very little research has been done to the ecosystem-species interactions. A thorough knowledge about the comprehensive taxonomy of the marine organism from Phytoplankton to Marine mammals will form the basis of such ecosystem-species interaction research. Further, human activities are the major causes for the loss of biodiversity and degradation of marine and coastal habitats, which needs immediate attention and comprehensive action plan to conserve the biodiversity for living harmoniously with nature.

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Introduction to Mariculture

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It is well accepted the hunger is the most vital issue of mankind. The global population has grown from 1.5 billion in 1900 to 7 billion now and is expected to reach about 10 billion by the year 2050. It is estimated that about 925 million are undernourished with the major chunk at the Asia-Pacific Region. Micronutrient deficiencies affect > 2 billion; 250 million children at risk of vitamin A deficiency; equal number suffer from deficiency of minerals (iron, zinc, calcium, etc.). Hence food and nutritional security assumes highest priority for future prosperity of mankind. Food security is not just producing food, but also providing access to food and is linked with poverty and rural development. It is evident that economic access to food is only when households generate sufficient income. In this context, Fisheries and Aquaculture is a major sector producing not only food but also generating employment opportunities leading to accessibility of food.

Fish is considered as the "Rich food for Poor" and the cheapest source of animal protein. It provides over 20% of animal protein to 2.6 billion people globally. In developed countries it contributes 13%, while in developing countries > 30% of the animal protein. Fish forms the major source of animal protein in regions where animal protein in diets is below world average. It provides at least half of animal protein intake for 400 million poor in S. Asia & Africa. Fish is a rich source of protein, essential fatty acids, vitamins and minerals. Some fishes are high in calcium, zinc, vitamin A and iron. Globally over 540 million (8% of population) are involved in fisheries & aquaculture; the growth in the sector is more than population & employment in traditional agriculture. The present global production of food fish is estimated as 158 million tonnes (capture and culture). The demand for fish has increased at twice population growth over

last 50 years. Estimated additional 20-30 million tons are required to meet demand by 2020. The per capita consumption has increased from 11.5kg in 1970 to 12.5kg in 1980 to 14.4kg in 1990 to 19.2kg in 2012.

A global review of the marine capture fisheries scenario reveals that 80% of the world's fish stocks for which assessment information is available are reported as fully exploited and thus requiring effective and precautionary management. The maximum wild capture fisheries potential from world's oceans have almost been exploited and a more closely controlled approach to fisheries management is required. The current marine capture fisheries scenario in India is also characterized by increased and excessive fishing effort, overexploitation of certain resources from the inshore grounds and increased conflicts among the different stakeholders in the sector. Due to the larger dependency on inshore fisheries over the years, the production from nearshore waters has reached asymptotic level and hence ensuring sustainability is inevitable in our marine fisheries policy.

Aquaculture the farming and husbandry of aquatic animals is the promising area for increasing aquatic food production in future years. It is the fastest growing food production sector with annual growth of >6% in last two decades. It increased from <1 million tonne in 1950 to 55 million tonnes in 2009. About 80% of the production comes from 20 million small-holder farms (<2ha) in developing countries. The environmental demands for unit biomass of protein produced are lower as compared to poultry, piggery and beef. Aquaculture provides primary source of income. Aquaculture can be a starting point for alleviation of poverty in rural areas. Due to all these advantages aquaculture has been growing rapidly - faster than any other food production sector - over the past three decades, and is continuing. It is

clear that the bulk of fish required to feed the world in the coming decades will come from aquaculture. It is also time to put “nutrition security” at par with “food security”. Fish, wherever it comes from, is a global commodity of key significance due to its potential to improve human health and nutrition, its accessibility by the poor, and the low environmental impact of its production compared to that of other animal source foods.

Relevance of Mariculture

It is widely accepted that the exploited marine fisheries in India has reached a maximum sustainable level as reflected in the recent total fish production. The exploitation pattern over a long term is indicative of the fact that the additional demand for marine fish in future years cannot be met from the capture fisheries. In this context, mariculture – the farming and husbandry of marine plants and animals in the marine environment- is a promising sector by which the additional marine fish requirement can be met in the future years. It is also the fastest growing sub-sector of aquaculture. At global level, mariculture produces many high value finfish, crustaceans, and molluscs like oysters, mussels, clams, cockles and scallops. In 2012 mariculture has contributed around 24.7 million tonnes of foodfish globally which formed about 35.7% of the aquaculture production. (World aquaculture production was 90.4 million tonnes in 2012 – contributed 42.2% to the total fish production, supplied 9.4kg of foodfish per person). Molluscs dominated the global mariculture production (60.3%) followed by finfish (22.5%), crustaceans (15.9%) and others (1.3%). In addition about 23.8 tonnes of macro algae and seaweeds were also produced by mariculture.

Indian scenario of Mariculture Research and Development

In India the potential of mariculture production largely remains untapped. The mariculture activities are confined only to coastal brackish water aquaculture, chiefly shrimp farming. The other coastal aquaculture activities are green mussel farming which is confined to Malabar coast in Kerala producing around 15,000 tonnes and seaweed farming along Ramanathapuram, Puthukottai, Tanjore, Tuticorin and Kanyakumari

districts of Tamilnadu producing about 17000 tonnes wet weight annually.

The Central Marine Fisheries Research Institute (CMFRI) has been pioneering in the development and standardization of several commercially viable coastal aquaculture technologies viz. mussel farming, oyster farming, sea cage farming of marine finfish, seaweed farming, clam culture, pen culture, integrated farming, pearl oyster culture and pearl production. Many of these technologies are very simple, eco-friendly and use only locally available infrastructure facilities for construction of farm, feed and seed and hence the entire farming can be practiced by traditional fishermen.

Brackish water shrimp farming and the lessons learnt

Brackish water shrimp farming started in a big way in India in the early 90s especially in the coastal districts of Andhra Pradesh and Tamil Nadu. So far, shrimp remains as the single largest and maximum value earner among the seafood exported from the country. Shrimp farming in India, till 2008, was synonymous with the mono culture of tiger shrimp, *Penaeus monodon*. Since 1995, culture of *P. monodon* is affected by White Spot Syndrome Virus (WSSV) and the development of shrimp farming has become stagnant. Most of the Southeast Asian countries like Thailand, Vietnam and Indonesia shifted to culture of the exotic white leg shrimp, *Litopenaeus vannamei*. The successful development of Specific Pathogen Free (SPF) and Specific Pathogen Resistant (SPR) broodstock of *L. vannamei* also favoured the large scale expansion of its farming. However, in India, pilot-scale introduction of *L. vannamei* was initiated in 2003 and after risk analyses large-scale introduction was permitted in the year 2009. Of late *L. vannamei* farming is being threatened by outbreak of new diseases namely Early Mortality Syndrome (EMS), Acute Pancreatic and Haematopoietic Necrosis Syndrome (APHNS) and many viral diseases. The very fact is that these diseases are common to many of the shrimp species, the aqua farmers are now desperately looking for an additional species for farming. Hence, species diversification with viable finfish can be one of the best options for a

long term solution for sustaining the aquaculture sector. The major constraints for initiating and developing marine finfish farming in the country is the lack of seed production technologies for suitable high value species and the non-availability of commercially viable farming techniques. Now, with the development of indigenous technology for seed production and farming of cobia and silver pompano by CMFRI, and seabass by CIBA and RGCA, there is great scope for the aqua farmers to diversify their aquaculture practices.

Marine Finfish

Seed Production and farming of Cobia

Fast growth rate, adaptability for captive breeding, low cost of production, good meat quality and high market demand are some of the attributes that make cobia, *Rachycentron canadum* an excellent species for aquaculture. In recent years the seed production and farming of cobia is rapidly gaining momentum in many Asian countries. Envisaging the prospects of cobia farming in India, CMFRI has developed for the first time in the country the broodstock development, breeding and seed production of cobia and several successful seed production trials were conducted and the technology is now standardised at its Mandapam Regional Centre.

The farming protocols for the hatchery produced cobia fingerlings in sea cages with different feeding strategies were developed, tested and validated. Based on the trials, an economically viable farming model has been evolved. This farming method has been adopted by private entrepreneurs, fishermen groups and farmers. In a recent demonstration conducted at Mandapam, nursery reared juveniles were transferred to the grow-out sea cages. The stocking density was maintained at 3.0-5.0kg/m³ or 750 nos of juvenile cobia per cage of 6m diameter and 3 metre depth. The entire grow-out culture was carried out for a period of 6- 7 months. The juveniles reached an average weight of 1.0kg in 4 months and 2.5 – 3.0kg in 6- 7 months. The grow-out fishes could attain an average weight of 7.0kg with a maximum weight of 8.0kg in one year.

Seed Production and Farming of Silver pompano

Among the many high value marine tropical finfish that could be farmed in India, the silver pompano, *Trachinotus blochii* is one of the topmost, mainly due to its fast growth rate, good meat quality and high market demand. The silver pompano is caught only sporadically in the commercial fishery and hence its availability is rather scarce. It is a much sought after species and hence the demand can only be met through aquaculture. The farming can be successfully carried out in ponds, tanks and floating sea cages. The species is pelagic, very active and is able to acclimatize and grow well even at a lower salinity of about 10 ppt and hence is suitable for farming in the vast low saline waters of our country besides its potential for sea cage farming.

CMFRI has successfully developed and standardised the broodstock development, induction of spawning, larviculture and fingerling production of silver pompano for the first time in India. The first farming demonstration from the hatchery produced seed was carried out in a coastal aquaculture pond at Anthervedi Village, East Godavari District, Andhra Pradesh. The growth performance, survival and productive capacity of silver pompano, *Trachinotus blochii*, were evaluated in a brackishwater pond. A total of 3,400 fingerlings of silver pompano (30.59 ± 0.24 mm mean length and 2.00 ± 0.04 g mean weight) were stocked into a one acre pond (0.4047 hectare) with salinity of 8 ± 1.2 ppt. The salinity gradually raised to $24 + 1.8$ ppt during the farming period due to high saline intake water. Fishes were fed with extruded floating pellet feed containing 30% to 50% crude protein and 6 % to 10 % crude fat. After 240 days culture, 1305kg of silver pompano were harvested and the survival rate was 91.32%. The mean length of the harvested fishes was 296.88 ± 6.27 mm and mean body weight 464.65 ± 10.25 g. The absolute growth was 1.93 g/day and specific growth rate was 2.27%/day. Based on the experience gained from the above demonstration, farming protocols were evolved.

Ornamental Fish Culture

The marine ornamental fish trade has been expanding in recent years and has grown into a multimillion

dollar enterprise. The ornamental animals are the highest valued products that are mostly harvested from coral reef environments. The global marine ornamental trade is estimated at US\$ 200-330 million. The trade is operated throughout the tropics. Philippines, Indonesia, Solomon Islands, Sri Lanka, Australia, Fiji, Maldives and Palau supplied more than 98% of the total number of marine ornamental fish exported in recent years. It is a multi-stakeholder industry ranging from specimen collectors, culturists, wholesalers, transhippers, retailers, and hobbyists to researchers, government resource managers and conservators and hence involves a series of issues to be addressed and policies to be formulated for developing and expanding a sustainable trade. It is well understood that a long term sustainable trade of marine ornamental fishes can be developed only through the development and commercialization of hatchery production technologies for the species which are in high demand in the trade.

Global scenario

In recent years it has been reported that nearly 1500 species of marine ornamental fishes are traded globally and most of these are associated with coral reefs. Nearly 98% of the marine ornamental fishes marketed are wild collected from coral reefs of tropical countries. Among the most commercially traded families of reef fishes, family Pomacentridae dominate, accounting for nearly 43% of all fish traded. The family contains about 235 species worldwide. They are followed by species belonging to Pomacanthidae (8%), Acanthuridae (8%), Labridae (6%), Gobiidae (5%), Chaetodontidae (4%), Callionymidae (3%), Microdesmidae (2%), Serranidae (2%) and Blennidae (2%). In recent years the blue green damselfish (*Chromis viridis*), the clown anemone fish (*Amphiprion ocellaris*), the whitetail Dascyllus (*Dascyllus aruanus*), the sapphire devil (*Chrysiptera cyanea*) and the three spot damsel (*Dascyllus trimaculatus*) are among the most commonly traded species.

Hatchery production Technologies

Indiscriminate exploitation of ornamental fishes from the coral reef areas has been threatening the long term sustainability of the trade. Hence hatchery production of selected marine ornamental fishes

is the only option for the development of a long term sustainable trade. The Central Marine Fisheries Research Institute (CMFRI) has been focusing on this vital aspect for the past few years. The Institute was able to develop hatchery production methods of the following species of ornamental fishes which are in high demand in the international trade.

Table 1. Marine Ornamental Fishes

<i>Amphiprion percula</i>	Orange clown
<i>A. ocellaris</i>	False clown
<i>A. sebae</i>	Sebae clown
<i>A. nigripes</i>	Maldive's clownfish
<i>A. ephippium</i>	Red saddleback clownfish
<i>A. perideraion</i>	Pink skunk
<i>A. clarkii</i>	Clark's anemonefish
<i>Premnas biaculeatus</i>	Maroon clown(spine cheek anemonefish)
<i>Pomacentrus cearuleus</i>	Blue damsel
<i>P.pavo</i>	Peacock damsel
<i>Dascyllus trimaculatus</i>	Three spot damsel
<i>Dascyllus aruanus</i>	Humbug damsel
<i>Chromis viridis</i>	Bluegreen damsel
<i>Neopomacentrus nemurus</i>	Yellowtail damsel
<i>N.cyanomos</i>	Filamentous tail damsel
<i>Chrysiptera cyanea</i>	Sapphire devil damsel

The damaging fishing methods which destroy the fragile corals and over harvesting of the species in demand are the vital problems associated with the trade. It is widely accepted that the ultimate answer to a long term sustainable trade of marine ornamental trade can be achieved only through the development of hatchery production technologies. In this context it is imperative to develop commercially viable seed production techniques of species which are in demand. It is well accepted as an environmentally sound way to increase the supply of marine ornamentals by reducing the pressure on wild population and producing juvenile and market sized fish of wide variety of fish year round. In addition hatchery produced fish are hardier and fair better in captivity and survive longer. The methodologies developed by CMFRI can be scaled up for commercial level production and a hatchery produced marine ornamental fish trade could be developed.

Marine Finfish Brood bank

The availability of required quantities of bio-secure seed is the major prerequisite for the initiation and

expansion of marine finfish farming in the country. The major bottleneck in achieving commercial level seed production is the non-availability of a facility where the bio-secure broodstocks can be maintained and controlled spawning can be obtained year round. Broodstock management usually includes collection, selection and domestication of brooders as well as control of maturation, spawning and egg production. The broodstocks of large growing fishes like cobia is mostly developed in sea cages. However, the broodstock developed in sea cages are susceptible to mortality due to the changes in the water quality of the cage site, disease problems and impact of harmful algal blooms. In addition, the broodstock developed in sea cages is not bio-secure and hence can lead to spreading of diseases while farming is taken up on a commercial basis. If the broodstock can be maintained onshore in controlled facilities the loss of broodstock can be minimised and controlled breeding by manipulating the photo thermal regimes and spawning all through the year can be achieved.

Recirculating Aquaculture System (RAS)

Closed-system aquaculture presents a new and expanding commercial opportunity. Recirculating aquaculture Systems (RAS) are tank-based systems in which fish can be grown at high density under controlled environmental conditions. They are closed-loop facilities that retain and treat the water within the system. In an RAS, water flows from a fish tank through a treatment process and is then returned to the tank, hence the term Recirculating Aquaculture System. These systems use land based units to pump water in a closed loop through fish rearing tanks and consist of a series of sub-systems for water treatment which include equipment for solids removal, biological filtration, heating or cooling, dissolved gas control, water sterilization and photo-thermal control. Sustainable production of bio-secure fish seed all through the year employing photo-thermal conditioning is possible only by recirculating systems.

Sea cage farming

The sea cage farming has been expanding in recent

years on a global basis and it is viewed by many stakeholders in the industry as the aquaculture system of the millennium. Cage culture has made possible the large-scale production of commercial finfish in many parts of the world and can be considered as the most efficient and economical way of rising fish. The rapid growth of the industry in most countries can be attributed to (i) availability of suitable sites for cage culture (ii) well established breeding techniques that yield a sufficient quantity of various marine and freshwater fish juveniles (iii) availability of supporting industries such and feed, net manufactures, fish processors etc. (iv) strong research and development initiatives from institutions, governments and universities and (v) the private sector ensuring refinement and improvement of techniques/ culture systems, thereby further developing the industry.

Commercial cage culture was pioneered in Norway in the 1970s with the rise and development of salmon farming. As in terrestrial agriculture, the move within aquaculture towards the development and use of intensive cage farming systems was driven by a combination of factors, including the increasing competition faced by the sector for available resources (including water, land, labour, energy), the drive for increased productivity per unit area and the need for the sector to access and expand into new untapped open water culture sites such as lakes, reservoirs, rivers and coastal brackish and marine offshore waters. The cage aquaculture sector has grown very rapidly during the past 20 years and is presently undergoing rapid changes in response to pressures from globalization and growing demand for aquatic products. Total reported cage aquaculture production from 62 countries and provinces/regions from where data is available amounted to 2412167 tonnes (excluding China) On the basis of the reported information, the major cage culture producers in 2005 included– Norway (652306 tonnes), Chile (588 060 tonnes), Japan (272 821 tonnes), United Kingdom (135 253 tonnes), Vietnam (126 000 tonnes), Greece (76 577 tonnes), Turkey (78 924 tonnes), and the Philippines (66 249 tonnes). Currently on a global basis commercial cage culture has been restricted to the culture of high value, compound feed fed finfish species, including salmon, Japanese amberjack, red sea bream, yellow croaker, European sea bass,

gilthead sea bream, cobia and groupers. Cage culture systems employed by farmers vary from traditional family owned cage farms (Asian countries) to modern commercial large scale salmon and trout cage farms in Northern Europe and Americas. The rapid rise and success of the salmon cage farming industry has been due to a combination of interlinked factors such as the development and use of an easily replicated and cost effective technology (including hatchery seed production), access to large areas of suitable waters, good species selection, market acceptability, increased corporate investment and a good and supportive government regulatory environment. This can be taken as a model for cage culture development.

Marine cage farming is relatively new in Asia and was developed initially in Japan for species such as yellowtail (*Seriola quinqueradiata*) and red sea bream *Pagrus major*. Over the last twenty years the cage farming practice has spread almost throughout Asia. The major cage farming countries are China, Indonesia, Taiwan Province of China and Vietnam. A large number of finfish species are farmed in cages in Asia viz. groupers, snappers, carangids, seabass and cobia. In most countries individual operations are not large, and often a clustering of farming activities, which is due to limited site availability in coastal waters is seen.

When compared to many countries in the Asia-Pacific Region, India is still in its infancy in sea cage farming. For the first time in India as part of R & D a marine cage of 15 m diameter with HDPE frame was successfully launched in 2007 and operated at Visakhapatnam, in the east coast of India by the Central Marine Fisheries Research Institute. Since then, a lot of innovations on designing and fabrication of cages and mooring systems were made which led to the development of better designs of cages of 6m diameter with improved mooring systems that can withstand rough sea conditions. Subsequently demonstrations of cage farming were undertaken along different parts of the Indian coast under a participatory mode with the local coastal fishermen. Successful sea cage farming demonstrations were conducted at Kanyakumari, Vizhinjam, Kochi, Mangalore, Karwar, Veraval, Mandapam, Chennai and Balasore. Cobia,

Sea bass and spiny lobsters were the major groups employed for farming. These demonstrations have created an awareness regarding the prospects of sea cage farming in India. Many entrepreneurs, fishermen and farmers are coming forward to take up this venture.

Mussel Farming

The Institute has developed technologies for culture of bivalves viz. raft method (in bays, inshore waters), rack method (in brackishwater, estuaries) or long line method (open sea). These methods are commonly adopted for mussel farming (*Perna indica* and *P. viridis*). Mussel seeds of 15-25 mm size collected from intertidal and sub tidal beds are attached to coir/nylon ropes of 1-6 m length and enveloped by mosquito or cotton netting. Seeds get attached to rope within a few days while the netting disintegrates. The seeded ropes are hung from rafts, racks or longlines. A harvestable size of 70-80 mm is reached in 5-7 months and production of 12-14kg mussel (shell on) per metre of rope can be obtained. Innovations such as automatic seeding machines and depuration protocols were also evolved. The farming of mussels is currently being practised commercially at Malabar Coast, Kerala.

Edible Oyster Farming

CMFRI has developed methods for edible oyster (*Crassostrea madrasensis*) culture and has produced a complete package of technology, which is presently being widely adopted by small scale farmers in shallow estuaries, bays and backwaters. In the adopted rack and ren method, a series of vertical poles are driven into the bottom in rows, on top of which horizontal bars are placed. Spat collection is done mainly from the wild on suitable cultch materials. Spat collectors consist of clean oyster shells (5-6 Nos.) suspended on a 3 mm nylon rope at spaced intervals of 15-20 cm and suspended from racks, close to natural oyster beds. Spat collection and further rearing is carried out at the same farm site and harvestable size of 80 mm is reached in 8-10 months. Harvesting is done manually with a production rate of 8-10 tonnes/ha. Oyster shells are also in demand by local cement and lime industry.

Molluscan shellfish (mussels, oysters and clams) are

much sought after and widely consumed throughout the world as gourmet food. But in India these nutritious seafood have not found much acceptance. Currently farmed mussel and oyster production in India is between 15,000 and 20,000 tonnes. In order to create an awareness on the general public on molluscan shellfish as a highly nutritious food, ShellCon 2014 was organised by CMFRI in which shellfish food festival and programmes for the popularisation of the consumption of molluscan shellfish were conducted.

Mabe Pearl Production

CMFRI successfully developed and standardised a simple technique for value added marine pearls, called mabe pearls. A mabe pearl is a dome shaped or image pearl produced by placing a miniature image against the side of the oyster shell interior. The result is an exquisite pearly nacre coated image. The main advantage is the very short gestation period (2 months) and the superior quality of the nacre of Indian pearl oyster *Pinctada fucata*.

Seaweed Culture

Around 60 species of commercially important seaweeds with a standing crop of one lakh tonne occur along the Indian coast. Seaweed products like agar, algin, carrageenan and liquid fertilizer are in demand in global markets and some economically viable seaweed cultivation technologies have been developed in India. CMFRI has developed technology to culture seaweeds by either vegetative propagation using fragments of seaweeds collected from natural beds or spores (tetraspores/ carpospores). It has the potential to develop in large productive coastal belts. The rate of production of *Gelidiella acerosa* from culture amounts to 5 tonnes dry weight per hectare, while *Gracilaria edulis* and *Hypnea* production is about 15 tonnes dry weight per hectare. Recently the culture of the carageenan yielding sea weed *Kappaphycus alvarezii* has become very popular due to its fast growth and less susceptibility to grazing by fishes and is being cultivated extensively along the Ramanathapuram, Pudukkottai, Tanjore, Tuticorin and Kanyakumari districts of Tamil Nadu producing about 17000 t wet weight annually.

Way Forward

Seed availability is the major constraint for the initiation of commercial level farming of marine finfishes and shellfishes. The huge demand for cobia and pompano seeds received at CMFRI from fish farmers and entrepreneurs is indicative of the need of the sector. Hence there is an urgent need to establish marine finfish hatcheries by fisheries development agencies /private sector to ensure the seed availability. In addition, it is required to intensify research programmes for the development of seed production techniques for at least one dozen species of high value marine fishes. In this context, CMFRI has already taken up broodstock development and seed production of orange spotted Grouper *Epinephelus coioides*, Indian Pompano *Trachinotus mookalee* and Malabar Red Snapper *Lutjanus argentimaculatus*. Initial success has already been obtained in the broodstock development and seed production of *E. coioides* and *T. mookalee* at the Vishakapatnam Research Centre of CMFRI. Broodstock development of *L. argentimaculatus* is being pursued. If seed production technologies of more species are available, the farmers will be able to select the species as per the need of the locality.

The commercial level farming of lucrative shellfish species like the sand lobster, *Thenus unimaculatus* and the blue swimmer crab *Portunus pelagicus* can also be practiced if hatchery produced seeds are available. CMFRI is able to succeed in the seed production of both the species and now research is being focused on the standardization of these techniques. Similarly seed production techniques are already developed by the Institute for edible oyster, pearl oyster and green mussel. The methods can be scaled to commercial level production as per the requirement of the sector.

The development of farming systems especially the sea cage farming deserves prime attention. To promote sea cage farming in the country, identification of suitable sites with proper depth, water quality and water current are required. Site selection survey and identification of at least a dozen sites suitable for cage farming by the entrepreneurs and farmers deserves urgent attention. Availability of logistic support for cage farming should be given

careful consideration if a profitable business is to be established. Cage farming has to be promoted away from the human settlements, discharge points of industrial and municipal waste, so as to maintain ideal water quality for sea farming. Further, policy for leasing the suitable sites, bank finance, and governmental support through subsidy assistance are the needs of the hour.

The bivalve farming which is already being practiced at a few locations can be further expanded. The carrying capacity assessment, low value availability of hatchery produced seed and the feasibility of open sea farming of bivalves require attention by the R&D sector. Similarly the expansion of sea weed farming offers immense scope. A concerted effort by the developmental agencies for popularization of sea weed farming is warranted.

On a global basis, the mariculture practices are dominated by intensive monocultures which have led to sustainability problems, environmental degradation and consequent disease problems. In this context, the idea of bio-mitigation of the environment along with increased biomass production integrating commercially important species of different trophic levels is emerging as an innovation in aquaculture. Integrated Multi trophic aquaculture (IMTA) is the practice which combines in appropriate proportions the cultivation of fed aquaculture species (E.g. fin fish / shrimp) with organic extractive aquaculture species (e.g. shell / herbivorous fish) and inorganic extractive aquaculture species (e.g. seaweed) to create

balanced systems for environmental stability (bio-mitigation) economic stability (product diversification and risk reduction) and social acceptability (better management practices). IMTA is well recognized as a mitigation approach against the excess nutrients / organic matter generated by intensive aquaculture activities especially in marine waters, since it incorporates species from different trophic levels in the same system. In addition, it is also relevant in the implementation of ecosystem approach to aquaculture (EAA) propagated by FAO. IMTA can also increase the production capacity of a particular site. It is well understood that increasing use of coastal waters worldwide coupled with rapid growth and expansion of mariculture demand for more sustainable practices and hence the concept of IMTA has much relevance and scope. The development of IMTA in marine and coastal environments, has not been demonstrated as a viable enterprise in India and hence there is an urgent need to impart front line demonstration on this potential sector of mariculture to different stake holders.

The development of commercial level seed production technologies for a few species of high market value finfish and shellfish, establishment of hatcheries by fisheries development agencies, identification of appropriate cage/coastal farming sites, development of economically viable farming protocols, formulation of suitable grow-out feeds, health management protocols, development of mariculture policies and appropriate marketing strategies can go a long way to promote mariculture as a substantial contributor of sea food production of India.

Introduction to Marine Aquaculture

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Introduction

Aquarium keeping is amongst the most popular of hobbies with millions of enthusiasts worldwide, and the aquarium industry is also a multimillion dollar business in the world. Profit of marine aquarium industry is threefold increase than freshwater. However, 90% of income from marine industry is from wild caught only. The main drawback of this industry is difficulties endowed with various stages in marine fish rearing under captive conditions. The acquisition of marine ornamental fish has also greatly increased in recent years. Presently, 30 million coral reef fish belonging to 1,000 species and 100 species of invertebrate are collected annually to supply private and public aquaria around the world. The majority of these specimens come from coral reefs and associated habitats, with about 45 countries supplying the ornamental market. Many fish collectors in tropical and subtropical countries are employing cyanide to stun tropical fish, making it easier to collect them, but widespread cyanide application harms coral reefs and their ecosystems and threatens the food source of the local population. Therefore, in the last few years, a number of scientists have studied the reproduction of some of the species which are most commonly used in the aquarium trade for the purpose of rearing and breeding them in captivity (Thresher, 1884; Holt, 2003; Olivotto *et al.*, 2003, 2004). Marine ornamental fish production is now considered as one of the most important trade in international markets. Due to the popularity of aquariums in households in many parts of the world, aquaculture play a growing role in the international fish trade. The total value of wholesale ornamental trade is estimated at close to US\$ 1 billion, and retail trade about US\$ 3 billion (Olivotto, 2005).

Considering many pressures currently faced by reefs, it is vital that ornamental fisheries are to be investigated and monitored, and management strategies are need to be formulated to ensure that they are sustainable. This requires research, monitoring, training, use of non-damaging collecting methods and adoption of conservation strategies for controlling catch, such as reserves, quotas and closed seasons. Such measures include limiting collecting effort, establishment of species-based or overall quotas, restrictions on rare and/or endemic species, temporary closures and establishment of fishery break reserves. There are also a number of possibilities for enhancing the fishery, such as mariculture and construction of artificial reefs. It is well understood that India has a wealth of marine ornamental animals in our island ecosystems of Lakshadweep and Andaman- Nicobar, besides many areas of mainland. In the context of the expanding global marine ornamental fish trade in recent years it appears that India has the potential to develop a lucrative marine ornamental fish trade. A critical assessment of the current global scenario of marine ornamental trade can provide much insight into the complexities and conservational issues associated with trade, which will be of much relevance while formulating policies for the development of a marine ornamental industry. The Philippines, Indonesia, Soloman Islands, SriLanka, Australia, Fiji, the Maldives and Palau supplied the major share of marine ornaments during the recent years. The United States, the United Kingdom, the Netherlands, France and Germany were the most important countries of destination. It is well accepted that the trade developed from tank reared fish and other ornamentals is the final solution for a long term sustainable trade. The economic viability of ornamental fish production is more lucrative when

compared to other mariculture species, due to their high unit value.

In India, till date no organized trade of marine ornamentals has been initiated. But it is a fact that a great deal of illegal collection of marine ornamentals is in vogue in many parts of our reef ecosystem and this is a matter of great concern due to the indiscriminate nature of exploitation and eco hostile methods of collection which damage the reef ecosystem. In addition to this, lack of knowledge on appropriate post-harvest husbandry practices leads to large scale mortality of the collected animals. It is time to evolve a marine ornamental fisheries policy for developing an organized trade of marine ornamentals in many countries. It is felt that even though the ideal situation is to develop a sustainable trade of marine ornamentals through tank reared species, it has to be admitted that development of commercial level breeding technologies of all the species of demand will take a very long time and if you have to wait till then, we may fail to enter into this lucrative global trade in the near future. A critical analysis of current global trade of the marine ornamentals from wild collections reveals many ecological concerns which require policy interventions.

Captive breeding of marine ornamentals in India

More than 200 varieties of export oriented marine ornamental fishes are available in Indian waters, and it is widely accepted that their wild collection from the flimsy reef ecosystem will lead to habitat damage and overexploitation of the species which are in high demand. In these scenario, development of a long term sustainable trade of marine ornamental fishes through hatchery production is the only alternative. The decline of exploited marine fishery resources due to increasing fishing pressure, the setback in shrimp farming due to disease outbreak and the impact of tsunami have adversely affected the livelihood of Indian coastal villagers, and an alternative livelihood option is felt very essential. Considering these situations, during the past few years, the Central Marine Fisheries Research Institute (C.M.F.R.I.) has intensified research activities on breeding and culture of marine ornamental fishes. The success in the

hatchery production of clown fish and few damsel fishes (Gopakumar *et al.*, 2002, Ignatius *et al.*, 2001, Madhu and Rema Madhu, 2002) were reported first time in India with an objectives to generate scientific knowledge on ornamental fish maintenance, behaviour, influence of social status on sex change, pair formation, breeding, influence of lunar periodicity in spawning, parental care, egg incubation and hatching, developments of egg, larvae, and juveniles. These investigations have resulted in the development of hatchery technology for 20 species of marine ornamental fishes such as clown fishes *Amphiprion percula* (True pecula/ clown anemone fish); *A. ocellaris* (Common Clown/ False clown anemonefish) Rema *et al.*, 2012; *A. sandaracinos* (Yellow Skunk Clown) Rema and Madhu, 2012 ; *A. frenatus* (Tomato clown) Madhu *et al.*, 2011, *A. clarkii* (Clark's Anemonefish) *A. nigripes* (Maldives Anemonefish) (Madhu and Rema Madhu, 2006; Madhu *et al.*, 2006a, b, c; Rema Madhu, *et al.*, 2007; Madhu *et al.*, 2008; Madhu and Rema, 2011) *A. perideraion* (Pink anemone fish) (Anil *et al.*, 2012), *Amphiprion ephippium* (redsaddle back anemone fish), *A. sebae* (Sebae clown) (Gopakumar, *et al.*, 2007, 2009); and *Premnas biaculeatus* (Maroon clown/ Spine cheek anemone fish) Madhu *et al.*, 2012 and dottyback *Pseudochromis dilectus* (Redhead Dottyback) and *Nemateleotris decora* (Madhu and Rema, 2014). The species such as damsels *Dascyllus trimaculatus* (Three spot damsel); *D. aruanus* (Striped damsel); *Pomacentrus caeruleus* (Blue damsel); *P. pavo* (Sapphire or Peacock Damsel); *Neopomacentrus nemurus* (Yellow tail damsel); *N. filamentosus* (Filamentous tail damsel); *Chrysiptera cyanae* (Sapphire devil); *C. unimaculata* (One spot damsel) and *Chormis viridis* (Green chromis) (Gopakumar, *et al.*, 2007, 2009, Syda Rao *et al.*, 2010). At present tank reared species contribute only 1-2% of the trade. Culture of marine ornamental fish is well accepted as an environmentally sound way to increase the supply of such organisms by reducing the pressure on wild populations and producing juveniles of a wide variety of species year round. In addition, hatchery produced fish are hardier which are survive better in captivity and survive longer. The high unit value of ornamentals makes them more commercially viable than marine food fish culture. Hence in future, hatchery reared fish will become a significant part of marine ornamental fish trade in India and also globally.

Major requirements in Aquaculture

Filtration: Efficient filtration is mandatory in a marine aquarium. There are two basic types of contaminants in aquarium water – suspended physical particles and dissolved chemical compounds. The dissolved contaminants are created from the metabolic waste materials of fish, invertebrates and plants, and also develop from the activity of bacteria on waste organic matter produced in the tank. These dissolved chemical compounds include ammonia, nitrite, nitrate, urea, proteins, fatty acids, phenols, dyes and many other less abundant compounds. Filtration can be classified into three types. i) Mechanical filtration ii) Chemical filtration & iii) Biological Filtration (Under gravel filtration)

Adequate aeration: The purpose of aeration is not only for oxygen but also keep the water moving and exchanging gases with the air. This occurs in the surface, not between the bubbles and the water unless these are very dense. Aeration is often combined with filtration but it is better to provide air stones to add the effects of filters. Air stones come in all shapes and sizes, but what is most important is that they should give medium sized bubbles between ½ and 1 mm in diameter and these should move the water most efficiently. Very fine bubbles are good but form a mist in open water. A good brand of diaphragm pump with a volume control can be used for both air stones and filters.

Formulation of synthetic sea water salts: There are a number of sea salts readily available in the market. Synthetic seawater differs from natural seawater in which the concentrations of the major inorganic salts are not exactly the same, inorganic trace elements are not the same in number or concentration and there are no dissolved organics.

Availability of suitable feeds: (Live and artificial).

Treatment facilities: for common diseases of marine fishes.

Size of the tanks: A large tank is more stable in the constitution and temperature of water than a

smaller one. A tank of the size 90cm x 40cm x 50cm can be fabricated with 6mm glass and larger tanks of 500 litres and 1000 litres can be fabricated with glass plates of thickness 8mm and 12mm respectively. A shallow tank is more advisable because the water surface is the place where oxygen enters and the carbon-di-oxide leaves.

Lighting: The aquarium should not be installed in a place where there is no much day light, in particular direct sunlight, because it may overheat the water of the tank. Lighting is usually provided by a fluorescent tube or tubes with a reflecting hood. If you want to grow sea-anemones, sea-weeds and live corals, more light is needed and it must be provided in sufficient intensity by special lamps which can emit lights of red and blue wave lengths of visible spectrum. The light should be on for at least 12 hours per day.

Temperature: As temperature fluctuations may be detrimental to many species, it is important to have a heater / thermostat combination submersible and guaranteed suitable for salt water in the tanks. Power of heaters should be 100 watt for a 100 litre tank, 150 watt for a 200 litre tank and 200 watt for 300 litre tank. A tropical marine tank generally needs a temperature range of 25 – 28°C.

Quality water: Quality seawater should have dissolved salts, trace elements and dissolved gases. Only seven salts *viz.*, sodium chloride, magnesium chloride, magnesium sulphate, calcium sulphate, potassium sulphate, calcium carbonate and potassium or sodium bromide make up over 99.5% of all the conservative salts in sea water. The remaining 0.5% of the inorganic solids is made up of at least 60 elements found in such tiny amounts that they are called trace elements. Eventhough the trace elements are present in extremely small amounts, some of them especially zinc, copper, iodine, strontium, vanadium, cobalt, molybdenum and arsenic are essential to many living organisms. The dissolved organic substances are compounds such as amino acids, proteins, enzymes, vitamins and pigments. Inshore water carries a greater load of dissolved organics than clear offshore waters.

Seawater treatments: The best way to treat the water after collection is to store it in the dark to

2-3 weeks prior to using it in the aquarium. Another treatment of collected seawater is chlorination and de-chlorination. Chlorine kills all lives in the collected water including bacteria and oxidizes the organic matter dissolved in natural seawater, including toxins. For chlorination and de-chlorination we require bleaching powder, sodium thiosulphate and a test kit for chlorine.

New tank syndrome

A new tank which has been carefully set up and fishes are introduced without maturation of the tank will become unhealthy after a few days and fish mortality will result. Matured tanks will have a balanced bacterial population with the nitrogen cycle proceeding satisfactorily, and hence generation of ammonia or nitrites will be less. As ammonia is produced, it is converted rapidly into nitrites which in turn are converted into nitrates which are comparatively nontoxic. In a new tank there will not be an inadequate population of any type of bacteria and the first thing is the growth of bacteria which decompose organic matters such as fish faeces, uneaten food or any kind of decaying matter. The end product of such decomposition is ammonium hydroxide.

Aquarium Accessories

- Powered Canister Filters
- Protein Skimmer
- Denitrator
- Ozonizer
- UV Sterilizers:
- Other auxiliary equipments (Test kits for measuring salinity, pH, ammonia, nitrite and nitrate concentration; hand nets, siphons, etc.)

Rearing/ breeding of fishes in aquarium

Pair formation and broodstock developments:

The basic requirement is to have a sufficient number of broodstocks or breeding pairs which

can either be collected from the coral reef habitat or can be purchased from the pet shop depending upon the availability. In case mated pairs are not available, the fishes having different size groups can be collected and made to pair under captive condition through pair formation. In order to make breeding pairs from the juveniles groups, fishes can be collected from the wild and transported to the laboratory in healthy condition. In the case of clownfishes, fishes and sea anemones should be kept in separate plastic transportation bags during transportation. For the pair formation, five fishes of each sex of different size groups need to be stocked together along with single host sea anemone in a 500 L FRP tanks fitted with biological filter to reduce the aggression. The pair formation tanks need to be maintained in the hatchery where an incident light intensity of 2500 to 3000 lux was available as the sea anemones require sunlight for its better survival under laboratory condition. The fishes and anemones should be fed two times per day with wet feeds such as meat of shrimp, mussel and clam at the rate of 15% of their body weight and live feeds like *Brachionus plicatilis*, artemia nauplii and adult artemia. Environmental parameters such as temperature 26 to 29° C, salinity 33 to 36 ppt, dissolved oxygen 4.6 to 6.2 ml/L and pH 8.1 to 8.9 are need to be maintained in all the rearing tanks. After a period of 3 to 4 months rearing in the pair formation, in each tank one pair grew ahead of others and became the spawning pair. As the newly formed pairs will be very aggressive and spending time for fleeing the other subordinates rather than reproductive activity, it is very essential to stock each breeding pairs in separate broodstock tanks (250 to 500 L capacity) with single healthy pair and host sea anemone. An ideal tank would be a 3ft x 2 ft x 2 ft with a layer of coral sand on the bottom, a few live rocks, a healthy anemone, bright lighting and good filtration, preferably an efficient protein skimmer to reduce the ammonia and organic materials from the fish. A trickle filter could be used with regular water changes to keep the nitrates low enough for the anemone to do well. Since the gonad development and spawning of fishes are influenced by moon phases, the broodstocks/ spawning tanks need to be kept in an apt place where the fish receive a regular day/

night lighting cycle (moon phase). An anemone is generally not required to breed clownfish under captive condition. The pairs formed through pair formation should then be transferred to separate glass aquaria for broodstock development. The broodstocks need to be fed with wet feeds such as meat of green mussel, shrimp, clam and fish egg mass, and can also be provided formulated feeds enriched with vitamins, minerals and algal powder at the rate of 10% of their body weight and supplied at an interval of every 3 hrs during day time. Apart from these, the broodstocks were also fed with enriched rotifer 800 to 1000 nos/ml and artemia nauplii (200-400 nos/ml) and adult artemia (3 to 5 nos/ ml) every day. Provision of enriched live feeds which apparently improved egg quality and hatchability than the brooders fed with non-enriched live feeds.

Water quality maintenance

As a measure for this, the seawater need to be filtered through a series of sand filters before being taken to the rearing tanks. The temperature in all the breeding tanks need to be maintained between 26 to 30°C, and level of dissolved oxygen (4.8 to 6.3 ml/L), pH (8.0 to 8.9), salinity (32 to 36 ppt) and the water needs to be recirculated to ensure water movement and provided good water quality with the aid of a specially devised filter system during the period of rearing. Once in a week 25% of the water should be exchanged to avoid stress like a rapid increase in plasma cortisol concentration, depression of gonadal steroidogenesis, and subsequent development of gonadal atresia.

Substrate for egg deposition

As many coral fishes are laying attached eggs, it is very essential to provided suitable substratum preferably tiles or earthen pots or shells of edible oyster or PVC pipes which will also be helpful for the transfer of deposited egg without any mechanical injury to hatching tank.

Breeding of clownfishes

After broodstock rearing, each pair will start breeding

within a period of 4 to 6 months rearing under captive condition if the broodstocks are provided nutritious food and provided suitable rearing conditions. Few days prior to spawning, the male selected a suitable site near to sea anemone for laying the egg and cleared algae and debris with its mouth and on the day of spawning both the parents spent considerable time for the cleaning of site which indicated that spawning may occur within few hours. Under laboratory condition, the spawning can be obtained between 0500 hrs to 1530 hrs during day time and the spawning lasted for one hr to one and a half hour. Each female lays 300 to 1000 capsule shaped eggs at every 12 to 15 days interval depending on the species of clown fish, size of fish and previous experience. Generally the egg size of clown fishes ranges between 1.5 mm to 3.0 mm in length with a width of 0.8 to 1.84 mm and adhered to the provided substratum with stalk. An average of two spawning per lunar month per pair resulting in an estimated annual fecundity of 7200 to 24000 eggs/ breeding pair/ year can be obtained under laboratory condition. Parental care and egg morphology As parental care is inevitable for hatching out of the larvae, the parents should be allowed to remain in the parental tank itself till hatching. During incubation period, both the parents carefully look after the eggs during day time and it involved two basic activities viz. fanning by fluttering the pectoral fins and mouthing to remove the dead or weakened eggs and dust particles.

Egg hatching and larval rearing of clown fishes

On the expected day of hatching, two hours before sunset, the eggs along with substratum were transferred from the parental tank to hatching tanks (100 L) and provided with complete darkness for accelerating the hatching. The larvae broke the egg capsule and the hatchling emerged tail first and the hatching occurred soon after sunset and the peak hatching took place between 1900 to 2030 hrs under darkness. The newly hatched larvae measured 3 to 4mm in length and each had a transparent body, large eyes, visible mouth, and a small yolk sac and remained at the bottom of the tank for a few seconds and soon after became free swimming. The larval rearing can

be carried out under green water system and feeding with super small rotifer *B. rotundiformis* and newly hatched artemia nauplii. The larval period of clown fishes generally last for maximum of 20 days and then after most of the fry resembled juvenile adult fish and began to shift from partially pelagic to epibenthic and started eating minced shrimp, fish flesh, mussel meat, clam meat and formulated diets.

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An Overview of the Fish Diversity of Indian Waters

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There are 20,000-30,000 species of fish in a multitude of diverse marine aquatic ecosystems worldwide, and in freshwater environments many new fish species continue to be 'discovered' by science. About 22000 species of fishes have been recorded in the world; of which, about 11% are found in Indian waters. Out of the 2200 species so far listed, 73 (3.32%) belong to the cold freshwater regime, 544 (24.73%) to the warm fresh waters domain, 143 (6.50%) to the brackish waters and 1440 (65.45%) to the marine ecosystem.

Fishing is one of the oldest human activities and it developed gradually, when our ancestors moved from the collection of plants and animals to hunting by using tools and weapons. The oldest fishing implements so far identified are harpoons, found in the territory of Congo, and dating about 90,000 years. Interestingly, these harpoons were found associated with the bones of a species of now extinct giant catfish. In India too, it is believed that the development of fishing must have been parallel. There are reports that fishes were grown in reservoirs as early as 320 BC. There are several evidences of fish capture and culture since then. There were evidences to indicate over-fishing in the River Ganges as early as 1785. Russell made the first systematic study of the Indian fish fauna from 1785 to 1789 AD. Sir Francis Day studied the systematics of Indian fishes for over 20 years and listed 351 genera and 1418 species of marine, brackish water and freshwater fishes in 1868. Later, Alcock added 86 new genera and 200 species to the list. Jones and Kumaran (1980) recorded 603 species of fish from the Laccadive archipelago. Of the 603 species of marine fishes belonging to 126 families that are reported from the islands, at least 300 species belong to the

ornamental fish category. At present, of the recorded 24,000 species of finfishes in the world, about 2364 species are known to occur in India (www. fishbase. org). Rao (2009) recorded 1371 species in 77 families from the Andaman and Nicobar islands.

Fish biodiversity

The Indian fish fauna is divided into two classes, viz., Chondrichthyes and Osteichthyes. The Chondrichthyes are represented by 131 species under 67 genera, 28 families and 10 Orders in the Indian region. The Indian Osteichthyes are represented by 2,415 species belonging to 902 genera, 226 families and 30 orders.

Classification and Diversity

Class Actinopterygii-the ray finned fishes

Numerically, actinopterygians are the dominant class of vertebrates, comprising nearly 99% of the over 30,000 species of fish. Traditionally actinopterygians have been divided into the subclasses Chondrostei and Neopterygii. Neopterygii, in turn, have been divided into the infraclasses Holostei and Teleostei.

Important characters of the class are

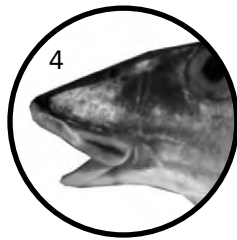
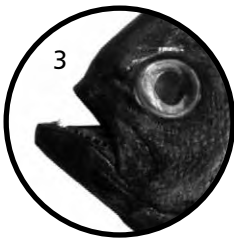
- Scales ganoid, cycloid, or ctenoid
- Spiracles usually absent
- Pectoral radials attached to the scapula-coracoid complex except in Polypteriformes
- Interopercle and branchiostegal rays usually present

Infraclass teleostei

Teleost are the most species rich and diversified group of all the vertebrates

Though teleosts are a group wide variation is noticed in the parts of the fish.

Mouth



1. Terminal
2. Sub Terminal
3. Superior
4. Inferior
5. Protrusible

Different mouth patterns are noticed in fish. They are

Terminal: Fish with a terminal mouth position have a mouth in the middle, or center of the head. These fish are mostly predators who either chase their food or feed on what is seen in front of them. The terminal mouth position is the “normal” position of mouth for most of the fishes inhabiting the middle levels of the water column of oceans or lakes.

Superior: This kind of fish has scoop-like mouth which is designed to feed on prey that swims above the fish (on the surface of the water), such as insects or plankton.

Inferior: Bottom feeding fish generally have inferior or sub-terminal mouths. Mouths located under the fishes head that are adapted for scavenging or grazing on algae, molluscs or bottom dwelling invertebrates.

Protrusible: Protrusible or protractile mouth in fish is a structural arrangement of the jaws that enables the animal to extend the mouth at will. When fully protruded, the cavity of the mouth is enlarged to form a funnel-like space facilitating the uptake of food. Fishes which feeds on small invertebrates in hidings has protrusible mouth.

Teeth

These serve as a very important taxonomic character. Generally, five types of teeth are recognised in fish based on their structure-cardiform, villiform, caniniform, incisiform and molariform.



Teeth types: The following teeth patterns are encountered in the fishes seen in tropical waters.

Canine teeth: They are sharp, highly pointed teeth seen in predatory fishes which are seen to attack and hold prey in their sharp teeth. The teeth are also used to tear off flesh from the prey. Sharks are best examples of fishes with canine teeth.

Incisor teeth: Incisors are used for cutting and they come in variety of shapes. These are flattened tooth with chisel like or saw edges.

Molar teeth: These are blunt, rounded, broad tooth adapted for crushing and grinding shellfish. They are generally found in bottom dwelling fish.

Villiform teeth: Villiform teeth are elongated teeth they are very long, slender and crowded having the appearance of velvet or fine bristles of a brush.

They are more common on deep sea fishes used for stabbing and direction.

Dental plates: Teeth fused to form beak like plates.

There are four different types of fish scale, each with their own characteristics and variations.

Placoid Scales: Placoid scales are formed of a rectangular base plate that is embedded within the skin of the fish and some of spine externally. The interior of the scale is a pulp that receives blood from the fish's vascular system, while the outside is made of an enamel-like substance called vitrodentine. The shape of the spines can vary greatly depending on species. However, almost all give the fish a rough texture. Sharks and rays are examples of fish with placoid scales.

Cosmoid Scales: Cosmoid scales have evolved from placoid scales fusing together. This is because cosmoid scales have two base plates and similar external spines composed of vitrodentine. The base plates are made from bone and new bone is added as the fish grows. Lungfishes and coelacanth have cosmoid scales.

Ganoid Scales: Ganoid scales have a bony base layer similar to that of cosmoid scales. and are modified cosmoid scales. However, they differ in that their outer layer is made of an inorganic bone salt called ganoine and that they are diamond-shaped and interconnected. Between ganoid scales are peg-and-socket joints that articulate. Ganoid scales are found on sturgeons, bowfish, paddlefishes and gars.

Cycloid and Ctenoid Scales: Cycloid and ctenoid scales have different shapes but the same composition and positioning. Both are composed of collagen and calcium carbonate, rather than bone, and both are overlapping. This means that they are more flexible than the other types of scales. While the edges of cycloid scales are smooth, those of ctenoid scales have tiny teeth-like protrusions called ctenii, giving them a rougher texture. The majority of bony fish have cycloid or ctenoid scales.

Caudal Fin types

The caudal fin is the tail fin, located at the end of the caudal peduncle and is used for propulsion. Types of

caudal fin encountered in the present study are

Heterocercal: the vertebrae extend into the upper lobe of the tail, making it longer. Eg., sharks.

Homocercal: the vertebrae extend for a very short distance into the upper lobe of the fin, but the fin appears superficially symmetric. Most modern fishes are homocercal tailed fishes. In homocercal tail patterns, the following subpatterns are noticed.

Emarginate: ending in a slight inward curve

Lunate: ending in crescent shape

Forked: ending in two prolonged edges

Deeply forked: with the caudal fin deeply cut into two

Truncate: ending in vertical edge

Rounded: ending in round shape

Rhomboid: ending in rhomboid shape.

Notched or double emarginate: with two inward curves and a central point.

Pointed: trailing to a final point.

The list presented is tentative and adopted from Froese and Pauly (2006)

Order: Anguilliformes (Apodes)

The Order Anguilliformes, or true eels, contains 20 families and about 820 species. Species are usually elongate and slender, with single dorsal and anal fins that are continuous with the caudal fin (if present) in most species. All species lack fins and skeleton while some groups lack pectoral fins. Scales are usually absent, or if present, are cycloid and embedded in skin. All have *leptocephalus* larvae.

Most true eels are predators and belong to one of three families Congridae (Conger eels), Muraenidae (Moray Eels) and Ophichthidae (snake eels and worm eels). Some species are excellent food fish and form the basis of very important commercial fisheries.

In August 2011, a new family, the Protoanguillidae, comprising a single genus and species, with a separate caudal fin and many other primitive characters was described. Johnson *et al.* (2011) reports that

based on morphological and molecular data this species is the most primitive living member of the order Anguilliformes

Family Anguillidae (Freshwater eels)

Members in this family undertake long migrations to breed offshore in the deep water (catadromous); body elongate, one genus with 15 species. In India, 2 species has been reported.

- *Anguilla bengalensis bengalensis*-Indian longfin eel, Indian mottled eel
- *Anguilla bicolor bicolor*-Shortfin eel, Indonesian shortfin eel

Family Colocongridae (Short tail Eels)

Body stubby, snout blunt, pectoral fins present. One genus, *Coloconger* with about 5 species; one species reported from India

- *Coloconger raniceps*-Froghead eel

Family Congridae (Conger and garden eels)

Body elongate, lack scales, possess pectoral fins. These feed on crustaceans and small fish and do not migrate to breed.

Three subfamilies with 32 genera and roughly 160 species; 11 species recorded from Indian waters.

Subfamily Heterocongrinae (Garden Eels) with 2 genera-*Gorgasia* and *Heteroconger*

Subfamily Bathymyrinae with about 5 genera-*Ariosoma*, *Bathymyrus*, *Chiloconger*, *Parabathymyrus* and *Paraconger*.

Subfamily Congrinae with about 25 genera.

Species recorded from India

- *Ariosoma anago*
- *Bathymyrus echinorhynchus*
- *Conger cinereus*-Longfin African conger
- *Gorgasia maculata*-Whitespotted garden eel
- *Promyllantor purpureus*
- *Uroconger lepturus*-Slender conger
- *Xenomystax trucidans*

Family Moringuidae (Worm or spaghetti eels)

Small eels with a maximum size of 50 cm. Body moderately or very elongate, cylindrical except near tip of tail. Scales absent, eyes small covered with skin.

Species recorded from India

- *Moringua abbreviata*
- *Moringua bicolor*-Bicolor spaghetti eel
- *Moringua javanica*-Java spaghetti eel
- *Moringua microchir*-Lesser thrush eel

Family Muraenesocidae (Pike congers)

Elongated body with large eyes, covered with skin; dorsal fin origin over or slightly before the pectoral base. Teeth well developed; conspicuous lateral line

Four genera, *Congresox*, *Cynoponticus*, *Muraenesox* and *Sauromuraenesox* with about eight species recorded worldwide. Four species recorded from India.

Species recorded from India

- *Congresox talabon*-Yellow pike conger
- *Congresox talabonoides*-Indian pike conger
- *Muraenesox bagio*-Common pike conger, pike eel
- *Muraenesox cinereus*-Dagger-tooth pike conger

Family Muraenidae (Moray eels)

Efficient predators on reefs and rocky shores. Smaller sized eels like *Gymnothorax* are reported to be involved in ciguatera fish poisoning. This is said to be due to the eels feeding on a ciguatoxic fish mainly plant eaters feeding on a particular algae. About 15 genera with about 185 species reported worldwide; 35 species said to occur in India.

Family Muraenidae is divided into 2 subfamilies- sub family Uropterygiinae with four genera *Anarchias*, *Channomuraena*, *Scuticaria* and *Uropterygius* and subfamily Muraeninae with about 11 genera, *Echidna*, *Enchelycore*, *Enchelynassa*, *Gymnomuraena*, *Gymnothorax*, *Siderea*, *Strophidon* and *Thyrsoidea*

Species recorded from India

- *Anarchias allardicei*-Allardice's moray

- *Anarchias cantonensis*-Canton Island moray
- *Echidna delicatula*-Mottled moray
- *Echidna leucotaenia*-Whiteface moray
- *Echidna nebulosa*-Snowflake moray
- *Echidna polyzona*-Barred moray
- *Enchelynassa canina*-Viper moray
- *Gymnomuraena zebra*-Zebra moray
- *Gymnothorax buroensis*-Vagrant moray
- *Gymnothorax enigmaticus*-Enigmatic moray
- *Gymnothorax favagineus*-Laced moray
- *Gymnothorax fimbriatus*-Fimbriated moray
- *Gymnothorax flavimarginatus*-Yellow-edged moray
- *Gymnothorax hepaticus*-Liver-colored moray eel
- *Gymnothorax javanicus*-Giant moray
- *Gymnothorax meleagris*-Turkey moray
- *Gymnothorax monochrous*-Drab moray
- *Gymnothorax monostigma*-One-spot moray
- *Gymnothorax pictus*-Peppered moray
- *Gymnothorax pseudothyroideus*-Highfin moray
- *Gymnothorax punctatofasciatus*-Bars'n spots moray
- *Gymnothorax punctatus*-Red Sea whitespotted moray
- *Gymnothorax reticularis*
- *Gymnothorax richardsonii*-Richardson's moray
- *Gymnothorax rueppellii*-Banded moray
- *Gymnothorax thyroideus*-Greyface moray
- *Gymnothorax tile*-Freshwater moray
- *Gymnothorax undulatus*-Undulated moray
- *Scuticaria tigrina* (native) Tiger reef-eel
- *Strophidon sathete*-Gangetic moray, slender giant moray
- *Uropterygius concolor*-Unicolor snake moray
- *Uropterygius macrocephalus*-Needle-tooth moray
- *Uropterygius marmoratus*-Marbled reef-eel

Family Nemichthyidae (Snipe eels)

Snipe eels are found in every ocean and generally occupy depths of 300-600 m. Body elongate with extremely long, upper and lower jaws and large eyes. Three genera reported in the family *Avocettina*, *Labichthys*, *Nemichthys* with about nine species

Species recorded from India

- *Nemichthys scolopaceus*-Slender snipe-eel

Family Nettastomatidae (Duckbill eels)

Body elongate with slender tail which is regenerated

when broken. Mouth large. Family represented by six genera, *Facciolella*, *Hoplunnis*, *Nettastoma*, *Nettenchelys*, *Saurenchelys* and *Venefica*, with about 38 species.

Species recorded from India

- *Nettenchelys taylori*

Family Ophichthidae (Snake eels)

Ophichthids are found worldwide in tropical to warm temperate waters. They inhabit a wide range of habitats, from coastal shallows, and even rivers, to depths of above 750 m (2,460 ft). Most species are bottom dwellers, hiding in mud or sand to capture their prey of crustaceans and small fish, but some are pelagic. The family is supported by two subfamilies-subfamily Myrophinae (worm eels) with 11 genera, *Benthenchelys*, *Ahlia*, *Asarcenchelys*, *Glenoglossa*, *Mixomyrophis*, *Muraenichthys*, *Myrophis*, *Neenchelys*, *Pseudomyrophis*, *Schismorhynchus* and *Schultzidia* and Subfamily Ophichthinae (Snake eels) with 41 genera

Species recorded from India

- *Bascanichthys deraniyagalai*-Indian longtailed sand-eel
- *Bascanichthys longipinnis*
- *Caecula pterygera*-Finny snake-eel
- *Callechelys catostoma*-Black-striped snake eel
- *Lamnostoma orientalis*-Oriental worm-eel, Oriental sand-eel
- *Lamnostoma polyopthalma*-Ocellated sand-eel
- *Leiuranus semicinctus*-Saddled snake eel
- *Muraenichthys schultzei*-Maimed snake eel
- *Myrichthys colubrinus*-Harlequin snake eel
- *Neenchelys buitendijki*-Fintail serpent eel
- *Ophichthus altipennis*-Highfin snake eel
- *Ophichthus apicalis*-Bluntnose snake-eel
- *Ophichthus cephalozona*-Dark-shouldered snake eel
- *Pisodonophis boro*-Rice-paddy eel
- *Pisodonophis cancrivorus*-Longfin snake-eel
- *Scolecenchelys macroptera*-Slender snake eel
- *Skythrenchelys zabra*-Angry worm eel
- *Xestochilus nebulosus*-Nebulous snake eel

Order Atheriniformes

The order has six families in 2 suborders -

Suborder Atherinopsoidei

Family Atherinopsidae-new world silversides

11 genera and about 108 species in two subfamilies

Suborder Atherinoidei

Family Notocheiridae-Surf sardines

Species recorded from India

- *Iso natalensis*-Surf sprite

Family Atherinidae-Silversides

The Atherinidae is a large family of principally marine and euryhaline (living in varied salinities) fishes that is circumglobal in distribution. A few species are strictly confined to fresh water.

Species recorded from India

- *Atherinomorus duodecimalis*-Tropical silverside
- *Atherinomorus lacunosus*-Hardyhead silverside
- *Atherinomorus pinguis*-Narrow-banded hardyhead silverside
- *Hypoatherina barnesi*-Barnes' silverside
- *Hypoatherina valenciennesi*-Sumatran silverside

Family Melanotaeniidae-rainbow fishes and blue eyes

Seventeen genera with 113 species recorded worldwide.

Order Aulopiformes

Suborder Chlorophthalmoidei includes 5 families

Family Bathysauroididae-Bathysauroidids

One species *Bathysauroides gigas* (not reported from India)

Family Chlorophthalmidae-Greeneyes

Large eye with teardrop-shaped pupil and distinctive lensless space anteriorly. A hermaphroditic species.

Species recorded from India

- *Chlorophthalmus agassizi*-Shortnose greeneye
- *Chlorophthalmus bicornis*-Spiny jaw greeneye

Family Ipnopidae-Deepsea tripod fishes

The family Ipnopidae includes five genera *Bathymicrops*, *Bathypterois*, *Bathytyphlops*, *Discoverichthys* and *Ipnops* and 29 species of slender deep-sea fishes (Nelson, 2006) distributed worldwide demersally in tropical and temperate seas, at depths between 476 and 6000 m (McEachran & Fechtel 1998). Eyes minute or plate like, directed dorsally.

Species recorded from India

- *Bathypterois atricolor*-Attenuated spider fish
- *Bathypterois guentheri*-Tribute spiderfish
- *Bathypterois insularum*

Family Paraulopidae-Cucumber Fishes

One genus *Paraulopus*, with 10 species; no species recorded from Indian waters.

Family Synodontidae-Lizardfishes

These are generally small with a slender cylindrical body and head that superficially resemble those of lizards. They have a dioecious mode of reproduction. Worldwide, four genera with about 57 species have been recorded.

In India 22 species have been reported in three genera-*Harpadon*, *Saurida*, *Trachinocephalus* and *Synodus*

Subfamily Synodontinae (Lizard Fishes)

Two genera, *Synodus* and *Trachinocephalus* with about 37 species

Species recorded from India

- *Synodus englemani*-Engleman's lizardfish
- *Synodus indicus*-Indian lizardfish
- *Synodus jaculum*-Lighthouse lizardfish
- *Synodus macrocephalus*
- *Synodus macrops*-Triplecross lizardfish
- *Synodus variegatus*-Variegated lizardfish
- *Trachinocephalus myops*

Subfamily Harpadontinae- Bombay Ducks

Two genera *Harpadon* and *Saurida*, with about 20 species

Species recorded from India

- *Harpadon nehereus*-Bombay duck
- *Saurida gracilis*-Gracile lizardfish
- *Saurida isarankurai*-Shortjaw saury
- *Saurida longimanus*-Longfin lizardfish
- *Saurida micropectoralis*-Shortfin lizardfish
- *Saurida nebulosa*-Clouded lizardfish
- *Saurida tumbil*-Greater lizardfish
- *Saurida undosquamis*-Brushtooth lizardfish
- *Saurida wanieso*-Wanieso lizardfish

Family Aulopidae (Flagfins)

Two genera *Aulopus* and *Hime*

Family Pseudotrichonotidae (Sandiving Lizardfishes)

Family Cimolichthyidae

one genus *Cimolichthys*

Family Enchodontidae

With five genera *Enchodus*, *Eurypholis*, *Palaeolycus*, *Parenchodus*, *Rharbichthys*, *Saurorhamphus*

Family Scopelarchidae

Pearleyes with four genera, *Benthalbella*, *Rosenblattichthys*, *Scopelarchoides* and *Scopelarchus*

Family Evermannellidae (Sabertooth Fishes)

Three genera, *Coccorella*, *Evermannella* and *Odontostomus* with seven species

Family Alepisauridae (Lancetfishes)

Slender elongated body with a large mouth and strong teeth. Two genera, *Alepisaurus* and *Omosudis* reported worldwide (Nelson 2006). However as per Eschmeyer (2015), only one genus *Alepisaurus* has been recorded.

Species recorded from India

- *Alepisaurus ferox*-Longnose lancetfish

Order Batrachoidiformes

Family Batrachoididae (Toadfishes)

Species recorded from India

- *Allenbatrachus grunniens* (native) Frog fish, Grunting toadfish
- *Austrobatrachus dussumieri* (native) Flat toadfish

Order Beloniformes

Family Adrianichthyidae (Ricefishes)

Species recorded from India

- *Horaichthys setnai*-Thready top-minnow, Malabar ricefish
- *Oryzias carnaticus*
- *Oryzias dancena*

Family Belonidae (Needlefishes)

Needlefishes have slender, stream-lined bodies, and very long jaws filled with very sharp teeth. They are voracious predators and feed mostly on small fishes. In most species, the upper jaw only reaches its full length in adulthood, so the juveniles have a half-beak appearance, with an elongated lower jaw, but a much smaller upper one. During this stage of their lifecycle, they eat plankton, switching to fish once the beak fully develops. Needlefish reproduce through mating and lay eggs. The male usually rides the female on the waves as they mate.

5 genera are recorded from India with 9 species

Species recorded from India

- *Ablennes hians*-Flat needlefish
- *Platybelone argalus platyura*-Keeled needlefish
- *Strongylura incisa*-Reef needlefish
- *Strongylura leiura*-Banded needlefish
- *Strongylura strongylura*-Spottail needlefish
- *Tylosurus acus melanotus*-Keel-jawed needle fish
- *Tylosurus crocodilus*-Houndfish, Crocodile needlefish
- *Xenentodon cancila*-Freshwater garfish

Family Exocoetidae (Flyingfishes)

Elongate bodied fishes with cylindrical shape, flattened ventrally in some species. Head short. Snout blunt. Worldwide, five subfamilies with 52 species in 8 genera has been reported. Represented in Indian waters by 18 species in 6 genera.

Species recorded from India

- *Cheilopogon abei*-Abe's flyingfish
- *Cheilopogon cyanopterus*-Margined flyingfish
- *Cheilopogon furcatus*-Spotfin flyingfish
- *Cheilopogon intermedius*
- *Cheilopogon nigricans*-African flyingfish
- *Cheilopogon spilopterus*-Manyspotted flyingfish
- *Cheilopogon suttoni*-Sutton's flyingfish
- *Cypselurus naresii*-Pharao flyingfish
- *Cypselurus oligolepis*-Largescale flyingfish
- *Exocoetus monocirrhus*-Barbel flyingfish
- *Exocoetus volitans*-Tropical two-wing flyingfish
- *Hirundichthys coromandelensis*-Coromandel flyingfish
- *Hirundichthys oxycephalus*-Bony flyingfish
- *Hirundichthys speculiger*-Mirrorwing flyingfish
- *Parexocoetus brachypterus*-Sailfin flyingfish
- *Parexocoetus mento*-African sailfin flyingfish
- *Prognichthys brevipinnis*-Shortfin flyingfish

Family Hemiramphidae (Halfbeaks)

Elongate fishes with a prolonged lower jaw and a short triangular upper jaw. Spines absent in fins. Represented in Indian waters by 17 species in 7 genera.

Species recorded from India

- *Dermogenys brachynotopterus*-Gangetic halfbeak
- *Dermogenys pusilla*-Freshwater halfbeak, Wrestling halfbeak
- *Euleptorhamphus viridis*-Ribbon halfbeak
- *Hemiramphus archipelagicus*-Jumping halfbeak
- *Hemiramphus far*-Blackbarred halfbeak
- *Hemiramphus lutkei* -Lutke's halfbeak
- *Hyporhamphus affinis* -Tropical halfbeak
- *Hyporhamphus balinensis* -Balinese garfish
- *Hyporhamphus dussumieri* -Dussumier's halfbeak
- *Hyporhamphus limbatus*-Congaturi halfbeak, *Hyporhamphus quoyi*-Quoy's garfish
- *Hyporhamphus sindensis* -Sind halfbeak

- *Hyporhamphus unicuspis* -Simpletooth halfbeak
- *Hyporhamphus xanthopterus* -Red-tipped halfbeak
- *Oxyporhamphus micropterus micropterus* -Bigwing halfbeak
- *Rhynchorhamphus georgii* -Long billed half beak
- *Rhynchorhamphus malabaricus* -Malabar halfbeak
- *Zenarchopterus buffonis*-Buffon's river-garfish
- *Zenarchopterus dispar* -Viviparous half beak, Feathered river-garfish
- *Zenarchopterus ectuntio* -Ectuntio halfbeak
- *Zenarchopterus gilli* -Viviparous halfbeak
- *Zenarchopterus pappenheimi* -Bangkok halfbeak
- *Zenarchopterus striga* -Hooghly halfbeak

Order Beryciformes

The Order has 7 families with 29 genera and 144 species. All species are marine. Four families represented in Indian waters.

Family Berycidae (Alfonsinos)

Dorsal fin without notch, with 4-7 spines increasing in length from first to last, and 12-20 soft rays. 2 genera with about 9 species.

Species recorded from India

- *Beryx decadactylus*-Alfonsino
- *Beryx splendens*-Splendid alfonsino
- *Centroberyx rubricaudus*-Red alfonsino

Family Holocentridae (Squirrelfishes, soldierfishes)

Species with a long dorsal fin with spiny portion and soft rayed portion divided by a notch. Holocentrids (squirrelfish and soldierfish) are vocal reef fishes whose calls and sound-producing mechanisms have been studied in some species only. Worldwide, eight genera with 78 species has been reported. In India, 18 species in 4 genera have been recorded.

Species recorded from India

- *Myripristis adusta*-Shadowfin soldierfish
- *Myripristis botche*-Blacktip soldierfish
- *Myripristis hexagona*-Doubletooth soldierfish
- *Myripristis murdjan*-Pinecone soldierfish
- *Neoniphon argenteus*-Clearfin squirrelfish

- *Neoniphon opercularis*-Blackfin squirrelfish
- *Neoniphon sammara*-Sammara squirrelfish
- *Ostichthys acanthorhinus*
- *Ostichthys japonicus*-Brocade perch
- *Sargocentron caudimaculatum*-Silverspot squirrelfish
- *Sargocentron diadema*-Crown squirrelfish
- *Sargocentron ittodai*-Samurai squirrelfish
- *Sargocentron microstoma*-Smallmouth squirrelfish
- *Sargocentron praslin*-Dark-striped squirrelfish
- *Sargocentron punctatissimum*-Speckled squirrelfish
- *Sargocentron rubrum*-Redcoat
- *Sargocentron spiniferum*-Sabre squirrelfish
- *Sargocentron violaceum*-Violet squirrelfish

Family Monocentridae (Pinecone fishes)

The family contains just four species in two genera, one of which is monotypic. Worldwide two genera with four species recorded. In India, one species in one genus has been recorded.

Species recorded from India

- *Monocentris japonica* -Japanese pinecone fish

Order Carcharhiniformes

Family Carcharhinidae (Requiem sharks)

- *Carcharhinus altimus*-Bignose shark
- *Carcharhinus amblyrhynchoides*-Graceful shark
- *Carcharhinus amboinensis*-Pigeye shark
- *Carcharhinus brevipinna*-Spinner shark
- *Carcharhinus dussumieri*-Whitecheek shark
- *Carcharhinus falciformis*-Blackspot shark, Silky shark
- *Carcharhinus hemiodon*-Pondicherry shark
- *Carcharhinus leucas*-Bull shark
- *Carcharhinus limbatus*-Blacktip shark
- *Carcharhinus longimanus*-Oceanic whitetip shark
- *Carcharhinus macloti*-Maclot's shark
- *Carcharhinus melanopterus*-Blacktip reef shark
- *Carcharhinus sealei*-Blackspot shark
- *Carcharhinus sorrah*-Sorrah, Spottail shark
- *Galeocerdo cuvier*-Tiger shark
- *Lamiopsis temminckii*-Broadfin shark
- *Loxodon macrorhinus*-Sliteye shark
- *Negaprion acutidens*-Sicklefin lemon shark
- *Prionace glauca*-Blue shark
- *Rhizoprionodon acutus*-Milk shark
- *Rhizoprionodon oligolinx*-Grey dog shark

- *Scoliodon laticaudus*-Spadenose shark
- *Triaenodon obesus*-Whitetip reef shark

Family Hemigaleidae (Weasel sharks)

- *Chaenogaleus macrostoma*-Hooktooth shark
- *Hemipristis elongata*-Elliot's grey shark, Snaggletooth shark

Family Proscylliidae (Finback catsharks)

- *Eridacnis radcliffei*-Pygmy ribbontail catshark

Family Scyliorhinidae (Cat sharks)

- *Apristurus investigatoris*-Broadnose catshark
- *Atelomycterus marmoratus*-Marbled cat shark, Coral catshark
- *Cephaloscyllium silasi*-Indian swellshark
- *Halaelurus hispidus*-Bristly catshark
- *Halaelurus quagga*-Quagga catshark

Family Sphyrnidae (Hammerhead sharks)

- *Eusphyrna blochii*-Winghead shark
- *Sphyrna lewini*-Scalloped hammerhead
- *Sphyrna mokarran*-Great hammerhead
- *Sphyrna zygaena*-Round-headed hammerhead

Family Triakidae (Houndsharks)

- *Iago omanensis*-Bigeye houndshark
- *Mustelus mosis*-Arabian smooth-hound

Order Clupeiformes

Herrings are certainly among the most valuable commercial fishes in the world, being important food fishes in many countries and serving as a chief source of fish meal for animal feeds. The order Clupeiformes include anchovies, herrings, sardines, shads, gizzard shads, wolf herrings and their relatives. The current classification by Nelson (2006), divided the Clupeoidei into four families: the Engraulidae with two subfamilies with 139 species in 16 genera, the Pristigasteridae with two subfamilies and 37 species in 9 genera, the Chirocentridae with 2 species in one genus and the Clupeidae with five subfamilies with 216 species in 66 genera.

Family Chirocentridae (Wolf herring)

Species recorded from India

- *Chirocentrus dorab*-Dorab wolf-herring
- *Chirocentrus nudus*-Whitefin wolf-herring

Family Clupeidae (Herring, shads and sardines)

The family consists over 31 species in 16 genera.

Species recorded from India

- *Amblygaster clupeoides*-Bleeker smoothbelly sardinella
- *Amblygaster leiogaster* -Smooth-belly sardinella
- *Amblygaster sirm*-Spotted sardinella
- *Anodontostoma chacunda* -Chacunda gizzard shad
- *Dayella malabarica* -Day's round herring
- *Ehirava fluviatilis*-Malabar sprat
- *Escualosa thoracata* -White sardine
- *Gonialosa manmina* -Ganges river gizzard shad
- *Gudusia chapra*-Indian river shad
- *Herklotsichthys quadrimaculatus* -Bluestripe herring
- *Hilsa kelee* -Kelee shad
- *Nematalosa galathea*-Galathea gizzard shad
- *Nematalosa nasus* -Hairback, Bloch's gizzard shad
- *Opisthopterus tardoore* -Tardoore
- *Sardinella albella* -White sardinella
- *Sardinella brachysoma*-Deepbody sardinella
- *Sardinella fimbriata* -Fringescale sardinella
- *Sardinella gibbosa* -Goldstripe sardinella
- *Sardinella jussieu* -Mauritian sardinella
- *Sardinella longiceps* -Indian oil sardine
- *Sardinella melanura*-Blacktip sardinella
- *Sardinella sindensis* -Sind sardinella
- *Spratelloides delicatulus*-Delicate round herring
- *Spratelloides gracilis* -Silver-stripe round herring
- *Tenualosa ilisha*-Ilish
- *Tenualosa toli*-Toli shad

Family Dussumieriidae (Round herring)

Though two genera are recorded worldwide, one genus with two species are reported.

Species recorded from India

- *Dussumieria acuta*-Rainbow sardine
- *Dussumieria elopsoides*-Slender rainbow sardine

Family Engraulidae (Anchovies)

The anchovies are small herring-like fishes; but they are easily distinguishable from the herrings by the fact that their mouths are much larger and gape much farther back, but are on the lower side of the head, and are overhung by the upper jaw, which projects like a short piglike snout in some species.

Though 16 genera with 139 species are recorded worldwide, five genera with 35 species are recorded from India.

Species recorded from India

- *Coilia dussumieri*-Goldspotted grenadier anchovy
- *Coilia grayii*-Gray's grenadier anchovy
- *Coilia neglecta*-Neglected grenadier anchovy
- *Coilia ramcarati*-Ramcarat grenadier anchovy
- *Encrasicholina devisi*-Devis' anchovy
- *Encrasicholina heteroloba*-Shorthead anchovy
- *Encrasicholina punctifer*-Buccaneer anchovy
- *Setipinna breviceps* Shorthead hairfin anchovy
- *Setipinna brevifilis* (endemic) Short-hairfin anchovy
- *Setipinna phasa* (endemic) Gangetic hairfin anchovy
- *Setipinna taty*-Scaly hairfin anchovy
- *Setipinna tenuifilis*-Common hairfin anchovy
- *Stolephorus andhraensis*-Andhra anchovy
- *Stolephorus baganensis*-Bagan anchovy
- *Stolephorus commersonii*-Commerson's anchovy
- *Stolephorus dubiosus*-Thai anchovy
- *Stolephorus indicus*-Indian anchovy
- *Stolephorus insularis*-Hardenberg's anchovy
- *Stolephorus waitei*-Spotty-face anchovy
- *Thryssa baelama*-Baelama anchovy
- *Thryssa dayi*-Day's thryssa
- *Thryssa dussumieri*-Dussumier's thryssa
- *Thryssa encrasicholoides*-False baelama anchovy
- *Thryssa gautamiensis*-Gautama thryssa
- *Thryssa hamiltonii*-Hamilton's thryssa
- *Thryssa kammalensoides*-Godavari thryssa
- *Thryssa malabarica*-Malabar thryssa
- *Thryssa mystax*-Moustached thryssa
- *Thryssa polybranchialis*-Humphead thryssa
- *Thryssa purava*-Oblique-jaw thryssa
- *Thryssa setirostris*-Longjaw thryssa
- *Thryssa spinidens*-Bengal thryssa
- *Thryssa stenosoma*-Slender thryssa
- *Thryssa vitrirostris*-Orangemouth anchovy

Family Pristigasteridae (Pristigasterids)

- *Ilisha elongata*-Elongate ilisha
- *Ilisha filigera*-Coromandel ilisha
- *Ilisha kampeni*-Kampen's ilisha
- *Ilisha megaloptera*-Bigeye ilisha
- *Ilisha melastoma*-Indian ilisha
- *Ilisha striatula*-Banded ilisha
- *Pellona dayi*-Day's pella
- *Pellona ditchela*-Indian pella

Order Elopiformes

Family Elopidae (Tenpounders)

- *Elops machnata* (native) Ladyfish, tenpounder

Family Megalopidae (Tarpons)

- *Megalops cyprinoides* (native) Oxeye tarpon, Indo-Pacific tarpon

Order Gadiformes

Family Bregmacerotidae (Codlets)

- *Bregmaceros maclellandi* (native) Spotted codlet

Family Macrouridae (Grenadiers or rattails)

- *Bathygadus furvescens* (native)
- *Caelorinchus flabellispinnis* (native)
- *Caelorinchus parallelus* (native) Spiny grenadier
- *Coryphaenoides hextii* (native)
- *Coryphaenoides macrolophus* (native)
- *Coryphaenoides woodmasoni* (native)
- *Gadomus multifilis* (native)
- *Sphagemacrurus pumiliceps* (native)

Order Gasterosteiformes

Family Pegasidae (Seamoths)

- *Eurypegasmus draconis* (native) Short dragonfish
- *Pegasus laternarius* (native)
- *Pegasus volitans* (native) Longtail seamo

Family Chanidae (Milkfish)

Chanos chanos (native) Milkfish

Order Hexanchiformes

Family Hexanchidae (Cow sharks)

- *Heptranchias perlo* (native) Sharpnose sevengill shark

Order Lamniformes

Family Alopiidae (Thresher sharks)

- *Alopias pelagicus* (native) Pelagic thresher
- *Alopias superciliosus* (native) Bigeye thresher
- *Alopias vulpinus* (native) Thintail thresher

Family Lamnidae (Mackerel sharks or white shark)

- *Isurus oxyrinchus* (native) Shortfin shark, Shortfin mako

Family Odontaspidae (Sand tigers)

- *Carcharias taurus* (native), Sand tiger shark
- *Carcharias tricuspidatus* (native) Blue nurse sand-tiger, Indian sand tiger

Order Lampriformes

Family Lophotidae (Crestfishes)

- *Eumecichthys fiski* (native), Unicorn crestfish

Family Veliferidae (Velifers)

- *Velifer hypselopterus* (native), Sailfin velifer

Order Lophiiformes

Family Antennariidae (Frogfishes)

- *Antennarius coccineus* (native), Scarlet frogfish
- *Antennarius hispidus* (native), Shaggy angler
- *Antennarius indicus* (native), Indian frogfish
- *Antennarius nummifer* (native), Spotfin frogfish
- *Antennarius pictus* (native), Painted frogfish
- *Antennarius striatus* (native), Striated frogfish
- *Histrio histrio* (native), Sargassum fish

Family Chaunacidae (Sea toads)

- *Chaunax pictus* (native), Pink frogmouth

Family Diceratiidae (Double anglers)

- *Diceratias bispinosus* (native), Two-rod anglerfish

Family Lophiidae (Goosefishes)

- *Lophiodes gracilimanus* (native)
- *Lophiodes mutilus* (native), Smooth angler

- *Lophiomus setigerus* (native), Blackmouth angler

Family Ogcocephalidae (Batfishes)

- *Dibranchius nasutus* (native)
- *Halicmetus ruber* (native)
- *Haliutaea coccinea* (native)
- *Haliutaea indica* (native), Indian handfish
- *Haliutaea stellata* (native), Starry handfish

Family Oneirodidae (Dreamers)

- *Lophodolos indicus* (native)

Order Myctophiformes

Family Myctophidae (Lanternfishes)

- *Bolinichthys pyrsobolus* (native)
- *Benthoosema petrotum* Skinnycheek lantern fish
- *Diaphus luetkeni* (native)
- *Diaphus splendidus* (native)
- *Hygophum reinhardtii* (native), Reinhardt's lantern fish
- *Myctophum affine* (questionable), Metallic lantern fish
- *Myctophum aurolaternatum* (native), Golden lanternfish
- *Myctophum indicum* (native)
- *Myctophum spinosum* (native), Spiny lantern fish
- *Symbolophorus evermanni* (native), Evermann's lantern fish

Order Notacanthiformes

Family Halosauridae (Halosaurs)

- *Aldrovandia affinis* (native), Gilbert's halosaurid fish
- *Aldrovandia mediostris* (native)
- *Aldrovandia phalacra* (native), Hawaiian halosaurid fish
- *Halosaurus parvipennis* (native)

Order Ophidiiformes

Family Bythitidae (Viviparous brotulas)

- *Dinematichthys iluocoeteoides* (native), Yellow pigmy brotula

Family Carapidae (Pearlfishes)

- *Carapus boraborensis* (native), Pinhead pearlfish

- *Carapus mourlani* (native), Star pearlfish
- *Encheliophis gracilis* (native), Graceful pearlfish
- *Encheliophis homei* (native), Silver pearlfish

Family Ophidiidae (Cusk-eels)

- *Bassozetus glutinosus* (native)
- *Brotula multibarbata* (native), Goatsbeard brotula
- *Dicrolene introniger* (questionable), Digitate cusk eel
- *Enchelybrotula paucidens* (native)
- *Glyptophidium argenteum* (native)
- *Holcomycteronus pterotus* (native)
- *Monomitopus conjugator* (native)
- *Monomitopus nigripinnis* (native)
- *Neobythites steatiticus* (native)
- *Porogadus trichiurus* (native)
- *Tauredophidium hextii* (native)

Order Orectolobiformes

Family Ginglymostomatidae (Nurse sharks)

- *Nebrius ferrugineus* (native) Giant sleepy shark, Tawny nurse shark

Family Hemiscylliidae (Bamboo sharks)

- *Chiloscyllium arabicum* (native), Arabian carpetshark
- *Chiloscyllium griseum* (native) Grey bambooshark
- *Chiloscyllium indicum* (native) Slender bambooshark
- *Chiloscyllium plagiosum* (native), Whitespotted bambooshark
- *Chiloscyllium punctatum* (native), Brownbanded bambooshark

Family Rhincodontidae (Whale shark)

- *Rhincodon typus* (native) Whale shark, Whale shark

Family Stegostomatidae (Zebra sharks)

- *Stegostoma fasciatum* (native) Zebra shark, Zebra shark

Order Osmeriformes

Family Alepocephalidae (Slickheads)

- *Aulastomatomorpha phospherops* (native)
- *Bathytroctes squamosus* (questionable)

- *Narcetes erimelas* (native)

Family Platyroctidae (Tubeshoulders)

- *Platyroctes apus* (native), Legless searsid
- *Platyroctes mirus* (native), Leaf searsid

Order Perciformes

Family Acanthuridae (Surgeonfishes, tangs, unicornfishes)

- *Acanthurus gahhm*, Black surgeonfish
- *Acanthurus leucosternon* (native), Powderblue surgeonfish
- *Acanthurus lineatus* (native) Blue lined surgeonfish
- *Acanthurus mata* (native), Elongate surgeonfish
- *Acanthurus nigricans* (native), Whitecheek surgeonfish
- *Acanthurus nigrofuscus* (native), Brown surgeonfish
- *Acanthurus nigroris* (native), Bluelined surgeonfish
- *Acanthurus tennentii* (native), Doubleband surgeonfish
- *Acanthurus thompsoni* (native), Thompson's surgeonfish
- *Acanthurus triostegus* (native), Convict surgeonfish
- *Acanthurus xanthopterus* (native), Yellowfin surgeonfish
- *Ctenochaetus striatus* (native), Striated surgeonfish
- *Ctenochaetus strigosus* (questionable), Spotted surgeonfish
- *Naso brachycentron* (native), Humpback unicornfish
- *Naso brevirostris* (native), Spotted unicornfish
- *Naso lituratus* (misidentification), Orangespine unicornfish
- *Naso tonganus* (native), Bulbnose unicornfish
- *Naso tuberosus* (native), Humpnose unicornfish
- *Naso unicornis* (native), Bluespine unicornfish
- *Naso vlamingii* (native), Bignose unicornfish
- *Paracanthurus hepatus* (native), Palette surgeonfish
- *Zebrasoma flavescens* (questionable), Yellow tang

- *Zebrasoma veliferum*
- *Zebrasoma xanthurum* (native), Yellowtail tang

Family Acropomatidae (Lanternbellies,

- *Acropoma japonicum* (native), Glowbelly
- *Synagrops philippinensis* (native)

Family Ambassidae (Asiatic glassfishes)

- *Ambassis ambassis* (native) Commerson's glassy perchlet
- *Ambassis buton* (native) Buton glassy perchlet
- *Ambassis dussumieri* (native) Malabar glassy perchlet
- *Ambassis gymnocephalus* (native) Bald glassy perchlet
- *Ambassis interrupta* (native) Interrupta glassy perchlet, Long-spined glass perchlet
- *Ambassis kopsii* (native) Singapore glassy perchlet, Freckled hawkfish
- *Ambassis macracanthus* (native), Estuarine glass perchlet
- *Ambassis miops* (native) Myops glassy perchlet, Flag-tailed glass perchlet
- *Ambassis nalua* (native) Nalua-chanda, Scalloped perchlet
- *Ambassis urotaenia* (native) Banded-tail glassy perchlet, Banded-tail glassy perchlet
- *Chanda nama* (native) Elongate glass-perchlet
- *Parambassis dayi* (endemic) Day's glass fish
- *Parambassis lala* (native) Highfin glassy perchlet
- *Parambassis ranga* (native) Indian glassy fish
- *Parambassis thomassi* (endemic) Western Ghat glassy perchlet
- *Pseudambassis baculis* (native) Himalayan glassy perchlet

Family Ammodytidae (Sand lances)

- *Bleekeria kallepis* (native)

Family Anabantidae (Climbing gouramies)

- *Anabas cobojus* (native) Gangetic koi
- *Anabas testudineus* (native) Climbing perch

Family Apogonidae (Cardinalfishes)

- *Apogon coccineus* (native), Ruby cardinalfish
- *Apogon fasciatus* (native), Broad-banded cardinalfish

- *Apogon fleurieu* (native), Cardinalfish
- *Apogon fraenatus* (native), Bridled cardinalfish
- *Apogon guamensis* (native), Guam cardinalfish
- *Apogon holotaenia* (native), Copperstriped cardinalfish
- *Apogon kallopterus* (native), Iridescent cardinalfish
- *Apogon leptacanthus* (native), Threadfin cardinalfish
- *Apogon moluccensis* (native), Moluccan cardinalfish
- *Apogon novemfasciatus*, Sevenstriped cardinalfish
- *Apogon oxina* (native)
- *Apogon poecilopterus* (native), Pearly-finned cardinalfish
- *Apogon quadrifasciatus* (native), Twostripe cardinal
- *Apogon sangiensis* (native), Sangi cardinalfish
- *Apogon savayensis* (native), Samoan cardinalfish
- *Apogon taeniophorus* (native), Reef-flat cardinalfish
- *Apogonichthys ocellatus* (native), Ocellated cardinalfish
- *Archamia bleekeri* (native)
- *Archamia fucata* (native), Orangelined cardinalfish
- *Cheilodipterus arabicus* (native), Tiger cardinal
- *Cheilodipterus quinquelineatus* (native), Five-lined cardinalfish
- *Foa brachygramma* (native), Weed cardinalfish
- *Fowleria aurita* (native), Crosseyed cardinalfish
- *Gymnapogon africanus* (questionable), Crystal cardinal
- *Pseudamia gelatinosa* (native), Gelatinous cardinalfish
- *Rhabdamia cypselura* (native), Swallowtail cardinalfish
- *Rhabdamia gracilis* (native), Luminous cardinalfish

Family Ariommatidae (Ariommatids)

Ariomma indica (native), Indian ariomma

Family Badidae

- *Badis assamensis* (native)
- *Badis badis* (native) Blue perch
- *Badis blosyrus* (native)
- *Badis kanabos* (native)

- *Badis tuivaiei* (native)
- *Dario dario* (native) Scarlet badis

Family Bathyclupeidae

- *Bathyclupea hoskynii* (native)

Family Blenniidae (Cometooth blennies)

- *Alticus kirkii* (native) Kirk's blenny
- *Andamia reyi* (native) Suckerlip blenny
- *Antennablennius bifilum* (native) Horned rockskipper
- *Aspidontus tractus* (native)
- *Blenniella leopardus* (native)
- *Blenniella periophthalmus* (native) Blue-dashed rockskipper
- *Cirripectes castaneus* (native) Chestnut eyelash-blenny
- *Cirripectes filamentosus* (native) Filamentous blenny
- *Cirripectes perustus* (native) Flaming blenny
- *Cirripectes polyzona* (native)
- *Cirripectes quagga* (native) Squiggly blenny
- *Cirripectes stigmaticus* (native) Red-streaked blenny
- *Cirripectes variolosus* (questionable) Red-speckled blenny
- *Ecsenius midas* (native) Persian blenny
- *Ecsenius pulcher* (native)
- *Enchelyurus kraussii* (native) Krauss' blenny
- *Entomacrodus striatus* (native) Reef margin blenny
- *Entomacrodus vermiculatus* (native) Vermiculated blenny
- *Exallias brevis* (native) Leopard blenny
- *Haptogenys bipunctata* (native)
- *Hirculops cornifer* (native) Highbrow rockskipper
- *Istiblennius dussumieri* (native) Streaky rockskipper
- *Istiblennius edentulus* (native) Rippled rockskipper
- *Istiblennius lineatus* (native) Lined rockskipper
- *Istiblennius spilotos* (native) Spotted rockskipper
- *Meiacanthus smithi* (native) Disco blenny
- *Mimoblennius atrocinctus* (native)
- *Omobranchus elongatus* (native) Cloister blenny
- *Omobranchus fasciolatus* (native) Arab blenny
- *Omobranchus ferox* (native) Gossamer blenny
- *Omobranchus obliquus* (native)
- *Omobranchus punctatus* (native) Muzzled blenny
- *Omobranchus zebra* (native) Zebra blenny
- *Parablennius thysanius* (native) Tasseled blenny
- *Petroscirtes breviceps* (native) Striped poison-fang blenny mimic
- *Petroscirtes mitratus* (native) Floral blenny

- *Petroscirtes xestus* (native) Xestus sabretooth blenny
- *Plagiotremus rhinorhynchus* (native) Bluestriped fangblenny
- *Plagiotremus tapeinosoma* (native) Piano fangblenny
- *Salarias fasciatus* (native) Jewelled blenny
- *Salarias reticulatus* (*sp. nov.*)[6]
- *Scartella emarginata* (native) Maned blenny
- *Xiphasia setifer* (native) Hairtail blenny

Family Caesionidae (Fusiliers)

- *Caesio caerulea* (native), Blue and gold fusilier
- *Caesio cuning* (native), Redbelly yellowtail fusilier
- *Caesio lunaris* (native), Lunar fusilier
- *Caesio teres* (native), Yellow and blueback fusilier
- *Caesio varilineata* (native), Variable-lined fusilier
- *Caesio xanthonota* (native), Yellowback fusilier
- *Dipterygonotus balteatus* (native), Mottled fusilier
- *Gymnoaesio gymnoptera* (native), Slender fusilier
- *Pterocaesio chrysozona* (native), Goldband fusilier
- *Pterocaesio digramma* (questionable), Double-lined fusilier
- *Pterocaesio pisang* (native), Banana fusilier
- *Pterocaesio tessellata* (native), One-stripe fusilier
- *Pterocaesio tile* (native), Dark-banded fusilier

Family Callionymidae (Dragonets)

- *Bathycallionymus kaianus* (native)
- *Callionymus carebares* (native), Indian deepwater dragonet
- *Callionymus erythraeus* (native), Smallhead dragonet
- *Callionymus fluviatilis* (native) River dragonet, River dragonet
- *Callionymus japonicus* (questionable)
- *Callionymus kotthausi* (native)
- *Callionymus margaretae* (native), Margaret's dragonet
- *Callionymus megastomus* (native)
- *Callionymus sagitta* (native) Arrow headed dragonet, Arrow dragonet
- *Eleutherochir opercularis* (native) Indian dragonet, Flap-gilled dragonet

Family Carangidae (Jacks and pompanos)

- *Alectis ciliaris* (native), African pompano
- *Alectis indicus* (native), Indian threadfish
- *Alepes djedaba* (native), Shrimp scad
- *Alepes kleinii* (native), Razorbelly scad

- *Alepes melanoptera* (native), Blackfin scad
- *Alepes vari* (native), Herring scad
- *Atropus atropos* (native), Cleftbelly trevally
- *Atule mate* (native), Yellowtail scad
- *Carangoides armatus* (native), Longfin trevally
- *Carangoides chrysophrys* (native), Longnose trevally
- *Carangoides ciliaris* (questionable)
- *Carangoides coeruleopinnatus* (native), Coastal trevally
- *Carangoides dinema* (native), Shadow trevally
- *Carangoides ferdau* (native), Blue trevally
- *Carangoides fulvoguttatus* (native), Yellowspotted trevally
- *Carangoides gymnostethus* (native), Bludger
- *Carangoides hedlandensis* (native), Bumpnose trevally
- *Carangoides humerosus* (native), Duskyshoulder trevally
- *Carangoides malabaricus* (native), Malabar trevally
- *Carangoides oblongus* (native), Coachwhip trevally
- *Carangoides orthogrammus* (native), Island trevally
- *Carangoides plagiotænia* (native), Barcheek trevally
- *Carangoides praeustus* (native) Brown-backed trevally, Brownback trevally
- *Carangoides talamparoides* (native), Impostor trevally
- *Caranx heberi* (native), Blacktip trevally
- *Caranx hippos* (questionable) Blacktailed trevally, Crevalle jack
- *Caranx ignobilis* (native) Giant kingfish, Giant trevally
- *Caranx lugubris* (native), Black jack
- *Caranx melampygus* (native), Bluefin trevally
- *Caranx papuensis* (native), Brassy trevally
- *Caranx sexfasciatus* (native), Bigeye trevally
- *Caranx tille* (native), Tille trevally
- *Decapterus macarellus* (native), Mackerel scad
- *Decapterus macrosoma* (native), Shortfin scad
- *Decapterus russelli* (native), Indian scad
- *Elagatis bipinnulata* (native), Rainbow runner
- *Gnathanodon speciosus* (native), Golden trevally
- *Megalaspis cordyla* (native) Torpedo scad, Torpedo scad
- *Naucrates ductor* (native), Pilotfish
- *Parastromateus niger* (native) Brown pomfret, Black pomfret
- *Scomberoides commersonianus* (native), Talang queenfish
- *Scomberoides lysan* (native) Double-spotted

- queenfish, Doublespotted queenfish
- *Scomberoides tala* (native), Barred queenfish
- *Scomberoides tol* (native), Needlescaled queenfish
- *Selar boops* (native), Oxeye scad
- *Selar crumenophthalmus* (native), Bigeye scad
- *Selaroides leptolepis* (native), Yellowstripe scad
- *Seriola lalandi* (native), Yellowtail amberjack
- *Seriola rivoliana* (native), Almaco jack
- *Seriolina nigrofasciata* (native), Blackbanded trevally
- *Trachinotus bailloni* (native), Smallspotted dart
- *Trachinotus blochii* (native), Snubnose pompano
- *Trachinotus botla* (native), Largespotted dart
- *Trachinotus mookalee* (native), Indian pompano
- *Ulua mentalis* (native), Longrakered trevally
- *Uraspis helvola* (native), Whitemouth jack
- *Uraspis secunda* (native), Cottonmouth jack
- *Uraspis uraspis* (native), Whitetongue jack

Family Centrogenyidae

- *Centrogenys vaigiensis* (native), False scorpionfish

Family Centrolophidae (Medusafishes)

- *Psenopsis cyanea* (native), Indian ruff

Family Cepolidae (Bandfishes)

- *Acanthocephala indica* (native)

Family Chaetodontidae (Butterflyfishes)

- *Chaetodon andamanensis* (native)
- *Chaetodon auriga* (native), Threadfin butterflyfish
- *Chaetodon bennetti* (native), Bluelashed butterflyfish
- *Chaetodon citrinellus* (native), Speckled butterflyfish
- *Chaetodon collare* (native), Redtail butterflyfish
- *Chaetodon decussatus* (native), Indian vagabond butterflyfish
- *Chaetodon falcula* (native), Blackwedged butterflyfish
- *Chaetodon kleinii* (native), Sunburst butterflyfish
- *Chaetodon lunula* (native), Raccoon butterflyfish
- *Chaetodon melannotus* (native), Blackback butterflyfish
- *Chaetodon meyeri* (native), Scrawled butterflyfish
- *Chaetodon octofasciatus* (native), Eightband butterflyfish
- *Chaetodon punctatofasciatus* (questionable), Spotband butterflyfish
- *Chaetodon speculum* (native), Mirror butterflyfish
- *Chaetodon trifascialis* (native), Chevron butterflyfish

- *Chaetodon trifasciatus* (native), Melon butterflyfish
- *Chaetodon vagabundus* (native), Vagabond butterflyfish
- *Chaetodon xanthocephalus* (native), Yellowhead butterflyfish
- *Chelmon rostratus* (native), Copperband butterflyfish
- *Forcipiger longirostris* (native), Longnose butterflyfish
- *Hemitaurichthys zoster* (native), Brown-and-white butterflyfish
- *Heniochus acuminatus* (native) Pennant coral fish, Pennant coralfish
- *Heniochus chrysostomus* (native), Threeband pennantfish
- *Heniochus monoceros* (native), Masked bannerfish
- *Heniochus pleurotaenia* (native), Phantom bannerfish
- *Heniochus singularis* (native), Singular bannerfish
- *Parachaetodon ocellatus* (native), Sixspine butterflyfish

Family Champsodontidae

- *Champsodon capensis* (questionable), Gaper
- *Champsodon vorax* (questionable)

Family Cichlidae (Cichlids)

- *Eetroplus maculatus* Orange Chromide
- *Eetroplus canarensis* (endemic) Canara pearlspot
- *Eetroplus maculatus* (native) Orange chromide
- *Eetroplus suratensis* (native) Green chromide
- *Oreochromis mossambicus* (introduced) Mozambique cichlid
- *Oreochromis niloticus* (introduced), Nile tilapia

Family Cirrhitidae (Hawkfishes)

- *Cirrhitichthys aureus* (native), Yellow hawkfish
- *Cirrhitichthys bleekeri* (native)
- *Cirrhitus pinnulatus* (native), Stocky hawkfish
- *Paracirrhites forsteri* (native), Blackside hawkfish

Family Coryphaenidae (Dolphinfishes)

- *Coryphaena hippurus* (native), Common dolphinfish

Family Datnioididae

- *Datnioides polota* (native) Four-barred tigerfish

Family Drepaneidae (Sicklefishes)

- *Drepane longimana* (native), Concertina fish

- *Drepane punctata* (native) Spotted sicklefish

Family Echeneidae (Remoras)

- *Echeneis naucrates* (native), Live sharksucker
- *Phtheichthys lineatus* (native), Slender suckerfish
- *Remora osteochir* (native), Marlin sucker
- *Remora remora* (native) Common remora
- *Remorina albescens* (native), White suckerfish

Family Eleotridae (Sleepers)

- *Bostrychus sinensis* (native), Four-eyed sleeper
- *Butis amboinensis* (native), Olive flathead-gudgeon
- *Butis butis* (native) Duckbill sleeper
- *Butis gymnopomus* (native)
- *Butis koilomatodon* (native), Mud sleeper
- *Butis humeralis* (native) Blackspot sleeper
- *Eleotris fusca* (native) Dusky sleeper
- *Eleotris lutea* (native) Lutea sleeper
- *Eleotris melanosoma* (native) Broadhead sleeper
- *Incara multisquamatus* (native) Incara
- *Odonteleotris macrodon* (native) Gangetic sleeper
- *Ophiocara porocephala* (native) Flathead sleeper

Family Emmelichthyidae (Rovers)

- *Erythrocles schlegelli*

Family Ehippidae (Spadefishes, batfishes and scats)

- *Ehippus orbis* (native), Orbfish
- *Platax teira* (native), Tiera batfish

Gempylidae (Snake mackerels)

- *Epinnula magistralis* (native), Domine
- *Gempylus serpens* (native), Snake mackerel
- *Neopinnula orientalis* (native), Sackfish
- *Promethichthys prometheus* (native), Roudi escolar
- *Rexea bengalensis* (native), Bengal escolar
- *Rexea prometheoides* (native), Royal escolar
- *Ruvettus pretiosus* (native), Oilfish

Gerreidae (Mojarras)

- *Gerres erythrourus* (native), Deep-bodied mojarra
- *Gerres filamentosus* (native) Whiptail silver-biddy
- *Gerres limbatus* (native), Saddleback silver-biddy
- *Gerres longirostris* (native) Strongspine silver-biddy
- *Gerres macracanthus* (native)
- *Gerres oblongus* (native), Slender silverbiddy
- *Gerres oyena* (native) Common silvery-biddy

- *Gerres setifer* (native) Small Bengal silver-biddy
- *Pentaprion longimanus* (native), Longfin mojarra

Gobiidae (Gobies)

- *Acentrogobius bontii* (native)
- *Acentrogobius caninus* (native), Tropical sand goby
- *Acentrogobius cyanomos* (native)
- *Acentrogobius griseus* (endemic) Grey goby
- *Acentrogobius masoni* (native)
- *Acentrogobius viridipunctatus* (native), Spotted green goby
- *Amblyeleotris gymnocephala* (native), Masked shrimp goby
- *Amblygobius albimaculatus* (native), Butterfly goby
- *Amblyotrypauchen arctocephalus* (native)
- *Acentrogobius madraspatensis* (native)
- *Apocryptes bato* (native)
- *Apocryptodon madurensis* (native)
- *Asterropteryx semipunctata* (native), Starry goby
- *Awaous grammepomus* (native), Scribbled goby
- *Awaous guamensis* (native)
- *Awaous melanocephalus* (native), Largesnout goby
- *Awaous ocellaris* (native)
- *Bathygobius cyclopterus* (native), Spotted frillgoby
- *Bathygobius fuscus* (native), Dusky frillgoby
- *Bathygobius niger* (native), Black minigoby
- *Bathygobius petrophilus* (questionable)
- *Bathygobius smithi* (native)
- *Boleophthalmus dussumieri* (native)
- *Brachyamblyopus brachysoma* (native)
- *Brachygobius nunus* (native) Bumblebee goby
- *Callogobius hasseltii* (native), Hasselt's goby
- *Callogobius seshaiyai* (endemic)
- *Ctenogobiops crocineus* (native), Silverspot shrimp goby
- *Paratrypauchen microcephalus* (native) Comb goby
- *Drombus globiceps* (native) Bighead goby
- *Egglestonichthys melanoptera* (native)
- *Pseudogobiopsis oligactis* (native)
- *Eviota distigma* (native), Twospot pygmy goby
- *Exyrias puntang* (native), Puntang goby
- *Favonigobius reichei* (native), Indo-Pacific tropical sand goby
- *Fusigobius neophytus* (native), Common fuse goby
- *Glossogobius giuris* (native) Tank goby
- *Glossogobius kokius* (native)
- *Glossogobius mas* (native)
- *Pseudogobiopsis oligactis* (native)

- *Gobiodon citrinus* (native), Poison goby
- *Gobiodon rivulatus* (native), Rippled coralgoby
- *Gobiopsis canalis* (native), Checkered goby
- *Gobiopsis macrostomu* (native), Longjaw goby
- *Gobiopsis woodsi* (native)
- *Gobiopterus chuno* (native), Glass goby
- *Hemigobius hoevenii* (questionable)
- *Heteroleotris zonata* (native), Goggles
- *Istigobius diadema* (native)
- *Istigobius ornatus* (native), Ornate goby
- *Istigobius spence* (native), Pearl goby
- *Mahidolia mystacina* (native) Smiling goby, Flagfin prawn goby
- *Obliuogobius cometes* (native)
- *Odontamblyopus roseus* (native)
- *Odontamblyopus rubicundus* (native) Rubicundus eelgoby
- *Oligolepis acutipennis* (native) Sharptail goby
- *Oligolepis cylindriceps* (native)
- *Oxuderces dentatus* (native)
- *Oxyurichthys dasi* (native)
- *Oxyurichthys formosanus* (native)
- *Oxyurichthys microlepis* (native) Maned goby
- *Oxyurichthys ophthalmonema* (native), Eyebrow goby
- *Oxyurichthys paulae* (native) Jester goby
- *Oxyurichthys tentacularis* (native)
- *Aulopareia ocellatus* (native)
- *Parachaeturichthys polynema* (native) Taileyed goby
- *Paragobiodon echinocephalus* (native), Redhead goby
- *Parapocryptes rictuosus* (native)
- *Parapocryptes serperaster* (native)
- *Periophthalmodon schlosseri* (native), Giant mudskipper
- *Periophthalmodon septemradiatus* (native)
- *Periophthalmus argentilineatus* (native), Barred mudskipper
- *Periophthalmus barbarus* (questionable), Atlantic mudskipper
- *Periophthalmus chrysospilos* (native)
- *Periophthalmus minutus* (native)
- *Periophthalmus novemradiatus* (native) Pearse's mudskipper
- *Periophthalmus waltoni* (questionable), Walton's mudskipper
- *Periophthalmus weberi* (questionable) Weber's mudskipper
- *Pleurosicya bilobata* (native), Bilobed ghost goby
- *Priolepis eugenius* (native), Noble goby
- *Priolepis inhaca* (native), Brick goby
- *Psammogobius biocellatus* (native) Sleepy goby
- *Pseudapocryptes elongatus* (native)
- *Pseudogobius javanicus* (native)
- *Pseudogobius gastrospinos*
- *Pseudogobius poicilosoma* (native)
- *Pseudotrypauchen multiradiatus* (native)
- *Scartelaos cantoris* (native)
- *Scartelaos histophorus* (native), Walking goby
- *Scartelaos tenuis* (questionable), Indian Ocean slender mudskipper
- *Schismatogobius deraniyagalai* (native), Redneck goby
- *Sicyopterus griseus* (native)
- *Sicyopterus microcephalus* (native)
- *Silhouettea indica* (native)
- *Stenogobius gymnopomus* (native)
- *Stenogobius laterisquamatus* (questionable)
- *Stigmatogobius minima* (native)
- *Stigmatogobius sadanundio* (native)
- *Taenioides anguillaris* (native) Anguilla eelgoby
- *Taenioides buchanani* (native) Burmese gobyeel
- *Taenioides cirratus* (native) Hooghly gobyeel
- *Taenioides gracilis* (native), Slender eel goby
- *Trimma annosum* (native), Greybearded pygmy goby
- *Trypauchen vagina* (native) Burrowing goby
- *Trypauchenichthys sumatrensis* (native)
- *Valenciennea muralis* (native), Mural goby
- *Valenciennea sexguttata* (native), Sixspot goby
- *Valenciennea strigata* (native), Blueband goby
- *Yongeichthys nebulosus*
- *Yongeichthys tuticorinensis* (native)

Haemulidae (Grunts)

- *Plectorhinchus pictum* (native), Painted sweetlips
- *Plectorhinchus albivittatus* (native), Two-striped sweetlips
- *Plectorhinchus chubbi* (native), Dusky rubberlip
- *Plectorhinchus diagrammus* (questionable), Striped sweetlips
- *Plectorhinchus gibbosus* (native), Harry hotlips
- *Plectorhinchus lineatus* (native), Yellowbanded sweetlips
- *Plectorhinchus picus* (native), Painted sweetlip
- *Plectorhinchus schotaf* (native), Minstrel sweetlip
- *Pomadasy argenteus* (native) Silver grunt
- *Pomadasy argyreus* (native) Bluecheek silver grunt

- *Pomadasys commersonnii* (native) Spotted grunter
- *Pomadasys furcatus* (native), Banded grunter
- *Pomadasys guoraca* (native)
- *Pomadasys kaakan* (native), Javelin grunter
- *Pomadasys maculatus* (native), Saddle grunt
- *Pomadasys multimaculatum* (native), Cock grunter
- *Pomadasys olivaceus* (native), Olive grunt
- *Pomadasys stridens* (native), Striped piggy

Istiophoridae (Billfishes)

- *Istiophorus platypterus* (native) Sailfish, Indo-Pacific sailfish
- *Istiompax indica* (native) Short nosed sword fish, Black marlin
- *Makaira mazara* (native), Indo-Pacific blue marlin
- *Tetrapturus angustirostris* (native), Shortbill spearfish

Kraemeriidae (Sand darters)

- *Kraemeria samoensis* (native), Samoan sand dart

Kuhliidae (Aholeholes)

- *Kuhlia mugil* (native) Barred flagtail
- *Kuhlia rupestris* (native) Rock flagtail

Kurtidae (Nurseryfishes)

- *Kurtus indicus* (native) Indian humphead, Indian hump head

Kyphosidae (Sea chubs)

- *Kyphosus bigibbus* (native), Grey sea chub
- *Kyphosus cinerascens* (native), Blue seachub
- *Kyphosus vaigiensis* (native), Brassy chub

Labridae (Wrasses)

- *Anampses caeruleopunctatus* (native), Bluespotted wrasse
- *Anampses meleagrides* (native), Spotted wrasse
- *Bodianus neilli* (native), Bay of Bengal hogfish
- *Cheilinus chlorourus* (native), Floral wrasse
- *Cheilinus fasciatus* (native), Redbreast wrasse
- *Cheilinus oxycephalus* (native), Snooty wrasse
- *Cheilinus trilobatus* (native), Tripletail wrasse
- *Cheilinus undulatus* (native), Humphead wrasse
- *Cheilio inermis* (native), Cigar wrasse
- *Choerodon anchorago* (native), Orange-dotted tuskfish
- *Choerodon robustus* (native), Robust tuskfish
- *Cirrhilabrus exquisitus* (native), Exquisite wrasse

- *Coris aygula* (native), Clown coris
- *Coris formosa* (native), Queen coris
- *Coris gaimard* (questionable), Yellowtail coris
- wrasse
- *Gomphosus caeruleus* (native), Green birdmouth wrasse
- *Gomphosus varius* (native), Bird wrasse
- *Halichoeres hortulanus* (native), Checkerboard wrasse
- *Halichoeres marginatus* (native), Dusky wrasse
- *Halichoeres nebulosus* (native), Nebulous wrasse
- *Halichoeres nigrescens* (native), Bubblefin wrasse
- *Halichoeres scapularis* (native), Zigzag wrasse
- *Halichoeres timorensis* (native), Timor wrasse
- *Halichoeres zeylonicus* (native), Goldstripe wrasse
- *Hemigymnus fasciatus* (native), Barred thicklip
- *Hemigymnus melapterus* (native), Blackeye thicklip
- *Hologymnosus annulatus* (native), Ring wrasse
- *Hologymnosus doliatus* (native), Pastel ringwrasse
- *Iniistius pavo* (native), Peacock wrasse
- *Labroides dimidiatus* (native), Bluestreak cleaner wrasse
- *Leptojulius cyanopleura* (native), Shoulder-spot wrasse
- *Macropharyngodon meleagris* (native), Blackspotted wrasse
- *Novaculichthys taeniourus* (native), Rockmover wrasse
- *Oxycheilinus bimaculatus* (native), Two-spot wrasse
- *Oxycheilinus digramma* (native), Cheeklined wrasse
- *Pseudocheilinus hexataenia* (native), Sixline wrasse
- *Pseudodax moluccanus* (native), Chiseltooth wrasse
- *Pteragogus flagellifer* (native), Cocktail wrasse
- *Stethojulis albobittata* (native), Bluelined wrasse
- *Stethojulis balteata* (questionable), Belted wrasse
- *Stethojulis strigiventer* (native), Three-ribbon wrasse
- *Stethojulis trilineata* (native), Three-lined rainbowfish
- *Thalassoma amblycephalum* (native), Bluntheaded wrasse
- *Thalassoma hardwicke* (native), Sixbar wrasse
- *Thalassoma janseni* (native), Jansen's wrasse
- *Thalassoma lunare* (native), Moon wrasse
- *Thalassoma purpurium* (native), Surge wrasse
- *Thalassoma quinquevittatum* (native), Fivestripe wrasse
- *Choerodon typus* (native), Blue-banded wrasse
- *Iniistius bimaculatus* (native), Two-spot razorfish

- *Xyrichtys cyanifrons* (native)
- *Iniistius pentadactylus* (native), Fivefinger wrasse
- *Novaculops rajagopalani* (native)

Lactariidae (False trevallies)

- *Lactarius lactarius* (native) Big-jawed jumper

Latidae (Lates perches)

- *Lates calcarifer* (native) Barramundi, Barramundi
- *Psammoperca waigiensis* (native) Waigiu seaperch, Waigiu seaperch
- Leionathidae (Slimys, slipmouths, or ponyfishes)
- *Gazza achlamys* (native), Smalltoothed ponyfish
- *Gazza minuta* (native) Toothpony, Toothpony
- *Gazza rhombea* (native), Rhomboid toothpony
- *Leionathus berbis* (native), Berber ponyfish
- *Photopectoralis bindus* (native) Orangefin ponyfish
- *Nuchequula blochii* (native) Twoblotch ponyfish
- *Leionathus brevirostris* (native), Shortnose ponyfish
- *Leionathus daura* (native), Goldstripe ponyfish
- *Nuchequula decorus* (native) Shortnose ponyfish
- *Karalla dussumieri* (native), Dussumier's ponyfish
- *Equuilites elongatus* (native), Slender ponyfish
- *Leionathus equula* (native) Common ponyfish
- *Aurigequula fasciatus* (native), Striped ponyfish
- *Equuilites leuciscus* (native), Whipfin ponyfish
- *E. lineolatus* (native), Ornate ponyfish
- *Aurigequula longispinis* (questionable)
- *Eubleekeria splendens* (native), Splendid ponyfish
- *Leionathus striatus* (native)
- *Deveximentum insidiator* (native) Pugnose ponyfish
- *Secutor ruconius* (native) Deep pugnose ponyfish

Lethrinidae (Emperors or scavengers)

- *Gnathodentex aureolineatus* (native), Striped large-eye bream
- *Gymnocranius elongatus* (native), Forktail large-eye bream
- *Gymnocranius grandoculis* (native), Blue-lined large-eye bream
- *Gymnocranius griseus* (native), Grey large-eye bream
- *Lethrinus conchyliatus* (native), Redaxil emperor
- *Lethrinus erythracanthus* (native), Orange-spotted emperor
- *Lethrinus harak* (native), Thumbprint emperor
- *Lethrinus lentjan* (native) Pig-face bream, Pink ear emperor
- *Lethrinus mahsena* (native), Sky emperor

- *Lethrinus microdon* (native), Smalltooth emperor
- *Lethrinus miniatus* (questionable), Trumpet emperor
- *Lethrinus nebulosus* (native), Spangled emperor
- *Lethrinus obsoletus* (native), Orange-striped emperor
- *Lethrinus olivaceus* (native), Longface emperor
- *Lethrinus ornatus* (native), Ornate emperor
- *Lethrinus rubrioperculatus* (native), Spotcheek emperor
- *Lethrinus semicinctus* (native), Black blotch emperor
- *Lethrinus variegatus* (native), Slender emperor
- *Lethrinus xanthochilus* (native), Yellowlip emperor
- *Monotaxis grandoculis* (native), Humpnose big-eye bream
- *Wattsia mossambica* (native), Mozambique large-eye bream

Lobotidae (Tripletails)

- *Lobotes surinamensis* (native) Tripletail

Lutjanidae (Snappers)

- *Aphareus furca* (native), Small toothed jobfish
- *Aphareus rutilans* (native), Rusty jobfish
- *Aprion virescens* (native), Green jobfish
- *Apsilus fuscus* (questionable), African forktail snapper
- *Etelis carbunculus* (native), Ruby snapper
- *Etelis coruscans* (native), Flame snapper
- *Etelis radiosus* (native), Scarlet snapper
- *Lipocheilus carnolabrum* (native), Tang's snapper
- *Lutjanus argentimaculatus* (native), Mangrove red snapper
- *Lutjanus bengalensis* (native), Bengal snapper
- *Lutjanus biguttatus* (native), Two-spot banded snapper
- *Lutjanus bohar* (native), Two-spot red snapper
- *Lutjanus carponotatus* (native), Spanish flag snapper
- *Lutjanus decussatus* (native), Checkered snapper
- *Lutjanus ehrenbergii* (native) Blackspot snapper
- *Lutjanus erythropterus* (native), Crimson snapper
- *Lutjanus fulviflamma* (native), Dory snapper
- *Lutjanus fulvus* (native), Blacktail snapper
- *Lutjanus gibbus* (native), Humpback red snapper
- *Lutjanus gilcheri* (native), Yellowfin red snapper
- *Lutjanus johnii* (native) John's snapper
- *Lutjanus kasmira* (native), Common bluestripe snapper

- *Lutjanus lemniscatus* (native), Yellowstreaked snapper
- *Lutjanus lunulatus* (native), Lunartail snapper
- *Lutjanus lutjanus* (native), Bigeye snapper
- *Lutjanus madras* (native), Indian snapper
- *Lutjanus malabaricus* (native), Malabar blood snapper
- *Lutjanus monostigma* (native), Onespotsnapper
- *Lutjanus quinquelineatus* (native), Five-lined snapper
- *Lutjanus rivulatus* (native), Blubberlip snapper
- *Lutjanus rufolineatus* (questionable), Yellow-lined snapper
- *Lutjanus russellii* (native), Russell's snapper
- *Lutjanus sanguineus* (native), Humphead snapper
- *Lutjanus sebae* (native), Emperor red snapper
- *Lutjanus vitta* (native), Brownstripe red snapper
- *Macolor niger* (native), Black and white snapper
- *Paracaesio sordida* (native), Dirty ordure snapper
- *Paracaesio xanthura* (native), Yellowtail blue snapper
- *Pinjalo lewisi* (native), Slender pinjalo
- *Pinjalo pinjalo* (native), Pinjalo
- *Pristipomoides filamentosus* (native), Crimson jobfish
- *Pristipomoides multidentatus* (native), Goldbanded jobfish
- *Pristipomoides sieboldii* (native), Lavender jobfish
- *Pristipomoides typus*, Sharptooth jobfish
- *Pristipomoides zonatus*, Oblique-banded snapper

Malacanthidae (Tilefishes)

- *Hoplostethus atlanticus* (native), Pastel tilefish

Menidae (Moonfish)

- *Mene maculata* (native), Moonfish

Monodactylidae (Moonyfishes or fingerfishes)

- *Monodactylus argenteus* (native) Silvery moony
- *Monodactylus falciformis* (native) Full moony

Mugilidae (Mullet)

- *Crenimugil crenilabis* (native), Fringelip mullet
- *Planiliza carinata* (native), Keeled mullet
- *Planiliza klunzingeri* (native), Klunzinger's mullet
- *Planiliza macrolepis* (native) Largescale mullet
- *Planiliza mandapamensis* (native), Indian mullet

- *Planiliza melinoptera* (native) Giantscale mullet, Otomebora mullet
- *Planiliza parsia* (native) Goldspot mullet
- *Planiliza subviridis* (native) Greenback mullet
- *Planiliza tade* (native) Tade mullet, Tade mullet
- *Ellochelon vaigiensis* (native) Squaretail mullet
- *Mugil cephalus* (native) Flathead mullet
- *Plicomugil labiosus* (native), Hornlip mullet
- *Rhinomugil corsula* (native) Corsula mullet
- *Minimugil cascasia* (native) Yellowtail mullet
- *Sicamugil hamiltonii* (questionable) Burmese mullet
- *Crenimugil buehleri* (native) Bluetail mullet
- *Osteomugil cunnesius* (native) Longarm mullet
- *Crenimugil seheli* (native) Bluespot mullet
- *Osteomugil speigleri* (native) Speigler's mullet

Mullidae (Goatfishes)

- *Mulloidichthys flavolineatus* (native), Yellowstripe goatfish
- *Parupeneus barberinus* (native), Dash-and-dot goatfish
- *Parupeneus ciliatus* (native), Whitesaddle goatfish
- *Parupeneus cyclostomus* (native), Goldsaddle goatfish
- *Parupeneus heptacanthus* (native), Cinnabar goatfish
- *Parupeneus indicus* (native), Indian goatfish
- *Parupeneus macronemus* (native), Longbarbel goatfish
- *Parupeneus margaritatus* (questionable), Pearly goatfish
- *Parupeneus multifasciatus* (native), Manybar goatfish
- *Parupeneus pleurostigma* (native), Sidespot goatfish
- *Parupeneus trifasciatus* (native), Doublebar goatfish
- *Upeneus japonicus*, Bensasi goatfish
- *Upeneus luzonius*, Dark-barred goatfish
- *Upeneus moluccensis* (native), Goldband goatfish
- *Upeneus sulphureus* (native), Sulphur goatfish
- *Upeneus sundaicus* (native), Ochre-banded goatfish
- *Upeneus taeniopterus* (native), Finstripe goatfish
- *Upeneus tragula* (native), Freckled goatfish
- *Upeneus vittatus* (native), Yellowstriped goatfish

Nemipteridae (Threadfin breams, Whiptail breams)

- *Nemipterus bipunctatus* (native), Delagoa threadfin bream

- *Nemipterus furcosus* (native), Fork-tailed threadfin bream
- *Nemipterus japonicus* (native), Japanese threadfin bream
- *Nemipterus marginatus* (questionable), Red filament threadfin bream
- *Nemipterus mesoprion* (questionable), Mauvelip threadfin bream
- *Nemipterus nematophorus* (native), Doublewhip threadfin bream
- *Nemipterus nemurus* (questionable), Redspine threadfin bream
- *Nemipterus peronii* (native), Notchedfin threadfin bream
- *Nemipterus randalli* (native), Randall's threadfin bream
- *Nemipterus zysron* (native), Slender threadfin bream
- *Parascolopsis aspinosa* (native), Smooth dwarf monocle bream
- *Parascolopsis boesemani* (endemic), Redfin dwarf monocle bream
- *Parascolopsis eriomma* (native), Rosy dwarf monocle bream
- *Parascolopsis inermis* (native), Unarmed dwarf monocle bream
- *Scolopsis bilineata* (native), Two-lined monocle bream
- *Scolopsis bimaculata* (native), Thumbprint monocle bream
- *Scolopsis ciliata* (native), Saw-jawed monocle bream
- *Scolopsis frenata* (native), Bridled monocle bream
- *Scolopsis ghanam* (native), Arabian monocle bream
- *Scolopsis lineata* (native), Striped monocle bream
- *Scolopsis margaritifera* (questionable), Pearly monocle bream
- *Scolopsis taeniata* (native), Black-streaked monocle bream
- *Scolopsis taenioptera* (questionable), Lattice monocle bream
- *Scolopsis vosmeri* (native), Whitecheek monocle bream
- *Scolopsis xenochrous* (native), Oblique-barred monocle bream

Nomeidae (Driftfishes)

- *Cubiceps whiteleggu* (native), Indian driftfish

- *Psenes cyanophrys* (native), Freckled driftfish

Pempheridae (Sweepers)

- *Parapriacanthus ransonneti* (native), Pigmy sweeper
- *Pempheris mangula* (native), Black-edged sweeper
- *Pempheris molucca* (questionable)
- *Pempheris oualensis* (native), Silver sweeper
- *Pempheris vanicolensis* (native), Vanikoro sweeper

Percophidae (Duckbills)

- *Bembrops caudimacula* (native)
- *Bembrops platyrhynchus* (native), Natal duckbill

Pinguipedidae (Sandperches)

- *Parapercis alboguttata* (native), Whitespot sandsmelt
- *Parapercis hexophthalma* (native), Speckled sandperch
- *Parapercis maculata* (native), Harlequin sandperch
- *Parapercis pulchella* (native), Harlequin sandsmelt
- *Parapercis punctata* (native)
- *Parapercis quadrispinosa* (native)
- *Parapercis tetracantha* (native), Reticulated sandperch

Plesiopidae (Roundheads)

- *Acanthoplesiops indicus* (native), Scottie
- *Plesiops coeruleolineatus* (native), Crimsontip longfin
- *Plesiops corallicola* (native), Bluegill longfin

Polynemidae (Threadfins)

- *Eleutheronema tetradactylum* (native) White salmon, Fourfinger threadfin
- *Filimanus heptadactyla* (native) Sevenfinger threadfin
- *Filimanus similis* (native)
- *Filimanus xanthonema* (native), Yellowthread threadfin
- *Leptomelanosoma indicum* (native) Indian threadfin, Indian threadfin
- *Polydactylus macrochir* (native), King threadfin
- *Polydactylus microstoma* (native), Small-mouthed threadfin
- *Polydactylus mullani* (native)
- *Polydactylus plebeius* (native), Striped threadfin
- *Polydactylus sexfilis* (native), Sixfinger threadfin
- *Polydactylus sextarius* (native) Blackspot threadfin
- *Polynemus dubius* (questionable) Borneo threadfin, Eastern paradise fish

- *Polynemus paradiseus* (native) Paradise threadfin, Paradise threadfin

Pomacanthidae (Angelfishes)

- *Apolemichthys xanthurus* (native), Yellowtail angelfish
- *Centropyge bicolor* (native), Bicolor angelfish
- *Centropyge multispinis* (native), Dusky angelfish
- *Chaetodontoplus melanosoma* (native), Black-velvet angelfish
- *Pomacanthus annularis* (native) Ringed angle fish
- *Pomacanthus imperator* (native), Emperor angelfish
- *Pomacanthus semicirculatus* (native), Semicircle angelfish
- *Neopomacentrus taeniurus* (native), Freshwater demoiselle
- *Plectroglyphidodon dickii* (native), Blackbar devil
- *Plectroglyphidodon lacrymatus* (native), Whitespotted devil
- *Plectroglyphidodon leucozonus* (native), Singlebar devil
- *Pomacentrus pavo* (native), Sapphire damsel
- *Premnas biaculeatus* (native), Spinecheek anemonefish
- *Pristotis obtusirostris* (native), Gulf damselfish
- *Stegastes albifasciatus* (native), Whitebar gregory
- *Stegastes lividus* (native), Blunt snout gregory
- *Stegastes nigricans* (native), Dusky farmerfish
- *Stegastes obreptus* (native), Western gregory

Pomacentridae (Damsel-fishes)

- *Abudefduf bengalensis* (native), Bengal sergeant
- *Abudefduf septemfasciatus* (native), Banded sergeant
- *Abudefduf sexfasciatus* (native), Scissortail sergeant
- *Abudefduf sordidus* (native), Blackspot sergeant
- *Abudefduf vaigiensis* (native), Indo-Pacific sergeant
- *Amphiprion bicinctus*, Twoband anemonefish
- *Amphiprion chrysogaster*, Mauritian anemonefish
- *Amphiprion ephippium* (native), Saddle anemonefish
- *Amphiprion frenatus*, Tomato clownfish
- *Amphiprion nigripes*, Maldive anemonefish
- *Amphiprion ocellaris* (native), Clown anemonefish
- *Amphiprion percula*, Orange clownfish
- *Amphiprion sebae* (native), Sebae anemonefish
- *Cheiloprion labiatus* (native), Big-lip damsel
- *Chromis dimidiata* (native), Chocolatedip chromis
- *Chromis opercularis* (native), Doublebar chromis
- *Chromis ternatensis* (native), Ternate chromis
- *Chromis viridis* (native), Blue green damselfish
- *Chromis weberi* (native), Weber's chromis
- *Chrysiptera biocellata* (native), Twin-spot damselfish
- *Chrysiptera brownriggii* (native), Surge damselfish
- *Chrysiptera cyanea* (native), Sapphire devil
- *Chrysiptera glauca* (native), Grey demoiselle
- *Chrysiptera unimaculata* (native), Onespot demoiselle
- *Dascyllus aruanus* (native), Whitetail dascyllus
- *Dascyllus trimaculatus* (native), Threespot dascyllus
- *Dischistodus perspicillatus* (native), White damsel
- *Dischistodus prosopotaenia* (native), Honey-head damsel
- *Lepidozygus tapeinosoma* (native), Fusilier damselfish

Pomatomidae (Bluefishes)

- *Pomatomus saltatrix* (native), Bluefish

Priacanthidae (Bigeyes or catalufas)

- *Heteropriacanthus cruentatus* (native), Glasseye
- *Priacanthus hamrur* (native), Moontail bullseye
- *Priacanthus macracanthus* (native), Red bigeye
- *Priacanthus prolixus* (native), Elongate bulleye
- *Priacanthus tayenus* (native), Purple-spotted bigeye
- *Pristigenys nipponia* (native), Japanese bigeye

Pseudochromidae (Dottybacks)

- *Congrogadus subducens* (native), Carpet eel-blenny
- *Halidesmus thomaseni* (native), Thomasen's snakelet
- *Pseudochromis caudalis* (native)
- *Pseudochromis tapeinosoma* (native), Blackmargin dottyback

Ptereleotridae

- *Ptereleotris evides* (native), Blackfin dartfish
- *Ptereleotris microlepis* (native), Blue gudgeon

Rachycentridae (Cobia)

- *Rachycentron canadum* (native), Cobia

Scaridae (Parrotfishes)

- *Calotomus spinidens* (native), Spinytooth parrotfish
- *Calotomus viridescens*, Viridescent parrotfish
- *Chlorurus enneacanthus* (native), Captain parrotfish
- *Chlorurus gibbus* (native), Heavybeak parrotfish
- *Chlorurus oedema* (native), Knothead parrotfish
- *Chlorurus sordidus* (native), Daisy parrotfish
- *Hipposcarus harid* (native), Candellamo parrotfish

- *Leptoscarus vaigiensis* (native), Marbled parrotfish
- *Scarus ghobban* (native), Blue-barred parrotfish
- *Scarus globiceps* (native), Globehead parrotfish
- *Scarus niger* (native), Dusky parrotfish
- *Scarus prasiognathos* (native), Singapore parrotfish
- *Scarus psittacus* (native), Common parrotfish
- *Scarus quoyi* (native), Quoy's parrotfish
- *Scarus rubroviolaceus* (native), Ember parrotfish
- *Scarus russelii* (native), Eclipse parrotfish
- *Scarus scaber* (native), Fivesaddle parrotfish
- *Scarus tricolor* (native), Tricolour parrotfish

Scatophagidae (Scats)

- *Scatophagus argus* (native) Spotted scat

Schindleriidae

- *Schindleria pietschmanni* (questionable)
- *Schindleria praematura* (questionable), Schindler's fish

Sciaenidae (Drums or croakers)

- *Argyrosomus amoyensis* (native), Amoy croaker
- *Argyrosomus hololepidotus* (misidentification), Madagascar meagre
- *Argyrosomus japonicus* (native), Japanese meagre
- *Atrobucca alcocki* (native)
- *Atrobucca antonbruun* (native)
- *Atrobucca nibe* (native), Longfin kob
- *Atrobucca trewavasae* (native)
- *Bahaba chaptis* (native) Chaptis bahaba
- *Chrysochir aureus* (native), Reeve's croaker
- *Daysciaena albida* (native) Two-bearded croaker, Bengal corvina
- *Dendrophysa russelii* (native) Goatee croaker
- *Johnius amblycephalus* (native), Bearded croaker
- *Johnius belangerii* (native) Belanger's croaker
- *Johnius borneensis* (native), Sharpnose hammer croaker
- *Johnius carouna* (native) Caroun croaker
- *Johnius carutta* (native) Karut croaker
- *Johnius coitor* (native) Coitor croaker
- *Johnius dussumieri* (native) Sharptooth hammer croaker
- *Johnius elongatus* (native), Spindle croaker
- *Johnius gangeticus* (endemic) Gangetic bola
- *Johnius glaucus* (native), Pale spotfin croaker
- *Johnius macropterus* (native), Largefin croaker
- *Johnius macrorhynchus* (native), Big-snout croaker
- *Johnius mannarensis* (native), Mannar croaker

- *Johnius plagiostoma* (native), Large-eye croaker
- *Kathala axillaris* (native), Kathala croaker
- *Nibea maculata* (native), Blotched croaker
- *Nibea soldado* (native), Soldier croaker
- *Otolithes cuvieri* (native), Lesser tigertooth croaker
- *Otolithes ruber* (native), Tiger-toothed croaker
- *Otolithoides biauritus* (native) Bronze croaker
- *Otolithoides pama* (native), Pama croaker
- *Panna heterolepis* (native) Hooghly croaker
- *Panna microdon* (misidentification) Panna croaker
- *Paranibea semiluctuosa* (native), Half-mourning croaker
- *Pennahia anea* (native), Greyfin croaker
- *Pennahia macrocephalus* (questionable), Big-head Pennah croaker
- *Pennahia ovata* (native)
- *Protonibea diacanthus* (native) Spotted croaker
- *Pterotolithus maculatus* (native) Blotch
- *Umbrina canariensis* (native), Canary drum

Scombridae (Mackerels, tunas, bonitos)

- *Acanthocybium solandri* (native), Wahoo
- *Auxis rochei* (native), Bullet tuna
- *Auxis thazard* (native), Frigate tuna
- *Euthynnus affinis* (native) Mackerel tuna, Kawakawa
- *Grammatorcynus bicarinatus* (questionable), Shark mackerel
- *Gymnosarda unicolor* (native) Dogtooth tuna, Dogtooth tuna
- *Katsuwonus pelamis* (native), Skipjack tuna
- *Rastrelliger brachysoma* (native), Short mackerel
- *Rastrelliger faughni* (native), Island mackerel
- *Rastrelliger kanagurta* (native), Indian mackerel
- *Sarda orientalis* (native) Oriental bonito, Striped bonito
- *Scomber japonicus* (native), Chub mackerel
- *Scomberomorus commerson* (native) King seer
- *Scomberomorus guttatus* (native) Spotted Spanish mackerel, Indo-Pacific king mackerel
- *Scomberomorus lineolatus* (native) Streaked seer
- *Thunnus albacares* (native) Yellow fin tuna
- *Thunnus obesus* (native), Bigeye tuna
- *Thunnus orientalis* (native), Pacific bluefin tuna
- *Thunnus tonggol* (native) Blue fin tuna, Longtail tuna

Serranidae (Sea basses: groupers and fairy basslets)

- *Aethaloperca rogaea* (native), Redmouth grouper

- *Anyperodon leucogrammicus* (native), Slender grouper
- *Cephalopholis argus* (native) Balufana, Peacock hind
- *Cephalopholis aurantia* (native), Golden hind
- *Cephalopholis boenak* (native), Chocolate hind
- *Cephalopholis formosa* (native), Bluelined hind
- *Cephalopholis leopardus* (native), Leopard hind
- *Cephalopholis miniata* (native), Coral hind
- *Cephalopholis sexmaculata* (native), Sixblotch hind
- *Cephalopholis sonnerati* (native), Tomato hind
- *Cephalopholis urodeta* (native), Darkfin hind
- *Chelidoperca investigatoris* (native)
- *Cromileptes altivelis* (native), Humpback grouper
- *Diploprion bifasciatum* (native), Barred soapfish
- *Epinephelus areolatus* (native), Areolate grouper
- *Epinephelus bleekeri* (native), Duskytail grouper
- *Epinephelus chabaudi* (native), Moustache grouper
- *Epinephelus chlorostigma* (native), Brownspotted grouper
- *Epinephelus coeruleopunctatus* (native), Whitespotted grouper
- *Epinephelus coioides* (native), Orange-spotted grouper
- *Epinephelus corallicola* (questionable), Coral grouper
- *Epinephelus diacanthus* (native), Spinycheek grouper
- *Epinephelus epistictus* (native), Dotted grouper
- *Epinephelus erythrurus* (native), Cloudy grouper
- *Epinephelus fasciatus* (native), Blacktip grouper
- *Epinephelus faveatus* (native), Barred-chest grouper
- *Epinephelus flavocaeruleus* (native), Blue and yellow grouper
- *Epinephelus fuscoguttatus* (native), Brown-marbled grouper
- *Epinephelus hexagonatus* (native), Starspotted grouper
- *Epinephelus lanceolatus* (native) Bridlebass, Giant grouper
- *Epinephelus latifasciatus* (native), Striped grouper
- *Epinephelus longispinis* (native), Longspine grouper
- *Epinephelus macropsilos* (native), Snubnose grouper
- *Epinephelus maculatus* (native), Highfin grouper
- *Epinephelus malabaricus* (native) Malabar rockcod, Malabar grouper
- *Epinephelus marginatus* (questionable), Dusky grouper
- *Epinephelus melanostigma* (native), One-blotch grouper

- *Epinephelus merra* (native), Honeycomb grouper
- *Epinephelus morrhua* (native), Comet grouper
- *Epinephelus octofasciatus* (native), Eightbar grouper
- *Epinephelus poecilnotus* (native), Dot-dash grouper
- *Epinephelus polylepis* (native), Smallscaled grouper
- *Epinephelus polyphekadion* (native), Camouflage grouper
- *Epinephelus quoyanus* (native), Longfin grouper
- *Epinephelus radiatus* (native), Oblique-banded grouper
- *Epinephelus rivulatus* (native), Halfmoon grouper
- *Epinephelus spilotoceps* (native), Foursaddle grouper
- *Epinephelus tauvina* (native) Greasy rockcod, Greasy grouper
- *Epinephelus undulosus* (native), Wavy-lined grouper
- *Plectropomus areolatus* (native), Squartail coral grouper
- *Plectropomus maculatus* (questionable), Spotted coral grouper
- *Pogonoperca sp.* (native), Soapfish
- *Pseudanthias conspicuus* (native)
- *Pseudanthias squamipinnis* (native), Sea goldie
- *Variola louti* (native), Yellow-edged lyretail
- *Variola albimarginata*

Siganidae (Rabbitfishes)

- *Siganus argenteus* (native), Streamlined spinefoot
- *Siganus canaliculatus* (native), White-spotted spinefoot
- *Siganus corallinus* (native), Blue-spotted spinefoot
- *Siganus fuscescens* (native), Mottled spinefoot
- *Siganus guttatus* (native), Orange-spotted spinefoot
- *Siganus javus* (native), Streaked spinefoot
- *Siganus lineatus* (native), Golden-lined spinefoot
- *Siganus punctatus* (questionable), Goldspotted spinefoot
- *Siganus spinus* (native), Little spinefoot
- *Siganus stellatus* (native), Brownspotted spinefoot
- *Siganus vermiculatus* (native), Vermiculated spinefoot
- *Siganus virgatus* (native), Barhead spinefoot

Sillaginidae (Smelt-whitings)

- *Sillago aeolus* (native), Oriental sillago
- *Sillago chondropus* (native), Clubfoot sillago
- *Sillago indica* (endemic), Indian sillago
- *Sillago ingenuua* (native), Bay sillago

- *Sillago intermedius* (native), Intermediate sillago
- *Sillago maculata* (questionable), Trumpeter sillago
- *Sillago parvisquamis* (questionable), Small-scale sillago
- *Sillago sihama* (native) Silver sillago
- *Sillago soringa* (endemic), Soringa sillago
- *Sillago vincenti* (endemic) Estuarine whiting

Sparidae (Porgies)

- *Acanthopagrus berda* (native) Riverbream, Picnic seabream
- *Acanthopagrus bifasciatus* (native), Twobar seabream
- *Acanthopagrus latus* (native) Yellow seabream
- *Argyrops spinifer* (native), King soldierbream
- *Cheimerius nufar* (native), Santer seabream
- *Crenidens crenidens* (native), Karenteen seabream
- *Rhabdosargus sarba* (native) Natal stumpnose
- *Sparidentex hasta* (native), Sobaity seabream

Sphyraenidae (Barracudas)

- *Sphyraena acutipinnis* (native), Sharpfin barracuda
- *Sphyraena barracuda* (native), Great barracuda
- *Sphyraena flavicauda* (native), Yellowtail barracuda
- *Sphyraena forsteri* (native), Bigeye barracuda
- *Sphyraena jello* (native), Pickhandle barracuda
- *Sphyraena obtusata* (native), Obtuse barracuda
- *Sphyraena putnamae* (native) Sawtooth barracuda
- *Sphyraena qenie* (native), Blackfin barracuda
- *Pampus argenteus* (native) Silver pomfret
- *Pampus chinensis* (native) Chinese pomfret

Symphysanodontidae

- *Symphysanodon andersoni* (native)

Terapontidae (Grunters or tigerperches)

- *Pelates quadrilineatus* (native) Fourlined terapon
- *Helotes sexlineatus* (questionable), Six-lined trumpeter
- *Terapon jarbua* (native) Jarbua terapon
- *Terapon puta* (native) Smallscale terapon
- *Terapon theraps* (native) Banded grunter

Toxotidae (Archerfishes)

- *Toxotes chatareus* (native) Spotted archerfish
- *Toxotes jaculatrix* (native) Banded archerfish

Trichiuridae (Cutlassfishes)

- *Benthodesmus oligoradiatus* (native), Sparse-rayed frostfish
- *Eupleurogrammus glossodon* (native), Longtooth hairtail
- *Eupleurogrammus muticus* (native), Smallhead hairtail
- *Lepidopus caudatus* (questionable), Silver scabbardfish
- *Lepturacanthus pantului* (native) Coromandal ribbonfish
- *Lepturacanthus savala* (native) Small headed ribbon fish
- *Trichiurus auriga* (native), Pearly hairtail
- *Trichiurus gangeticus* (native) Gangetic ribbonfish
- *Trichiurus lepturus* (native), Largehead hairtail
- *Trichiurus russelli* (native), Short-tailed hairtail

Trichonotidae (Sand divers)

- *Trichonotus cyclograptus* (native)
- *Trichonotus setiger* (native), Spotted sand-diver

Tripterygiidae (Threefin blennies)

- *Enneapterygius elegans* (native), Hourglass triplefin
- *Enneapterygius fasciatus* (native)
- *Enneapterygius pusillus* (native), Highcrest triplefin
- *Helcogramma ellioti* (native)

Uranoscopidae (Stargazers)

- *Ichthyoscopus lebeck* (native), Longnosed stargazer
- *Uranoscopus crassiceps* (native)
- *Uranoscopus guttatus* (native)

Xenisthmidae

- *Gobiopterus smithi* (endemic)

Xiphiidae (Swordfish)

- *Xiphias gladius* (native), Swordfish

Zanclidae (Moorish idol)

- *Zanclus cornutus* (native), Moorish idol

Pleuronectiformes

Bothidae (Lefteye flounders)

- *Arnoglossus aspilos* (native), Spotless lefteye flounder
- *Arnoglossus tapeinosoma* (native)
- *Bothus leopardinus* (questionable), Pacific leopard flounder

- *Bothus myriaster* (native), Indo-Pacific oval flounder
- *Bothus pantherinus* (native), Leopard flounder
- *Chascanopsetta lugubris* (native), Pelican flounder
- *Engyprosopon grandisquama* (native), Largescale flounder
- *Grammatobothus polyophthalmus* (native), Threespot flounder

Cynoglossidae (Tonguefishes)

- *Cynoglossus arel* (native), Largescale tonguesole
- *Cynoglossus bilineatus* (native), Fourlined tonguesole
- *Cynoglossus carpenteri* (native), Hooked tonguesole
- *Cynoglossus cynoglossus* (native), Bengal tongue sole
- *Cynoglossus dubius* (native), Carrot tonguesole
- *Cynoglossus lida* (native) Shoulder spot tongue, Roughscale tonguesole
- *Cynoglossus lingua* (native) Long tonguesole
- *Cynoglossus macrostomus* (native) Malabar-sole
- *Cynoglossus puncticeps* (native) Speckled tonguesole
- *Cynoglossus semifasciatus* (native) Malabar sole
- *Paraplagusia bilineata* (native) Fingerlip tonguesole, Doublelined tonguesole
- *Paraplagusia blochii* (native), Bloch's tonguesole

Paralichthyidae (Large-tooth flounders)

- *Cephalopsetta ventrocellatus* (native)
- *Pseudorhombus arsius* (native) Largetooth flounder
- *Pseudorhombus duplici-cellatus* (native), Ocellated flounder
- *Pseudorhombus elevatus* (native), Deep flounder
- *Pseudorhombus javanicus* (native), Javan flounder
- *Pseudorhombus malayanus* (native), Malayan flounder
- *Pseudorhombus natalensis* (native), Natal flounders
- *Pseudorhombus triocellatus* (native) Three sopt flounder

Pleuronectidae (Righteye flounders)

- *Poecilopsetta colorata* (native), Coloured righteye flounder
- *Poecilopsetta praelonga* (native), Alcock's narrow-body righteye flounder

Psettodidae (Psettodids)

- *Psettodes erumei* (native) Indian halibut

Samaridae (Flounders)

- *Samaris cristatus* (native), Cockatoo righteye flounder
- *Samariscus longimanus* (native), Longfinned flounder

Soleidae (Soles)

- *Aesopia cornuta* (native), Unicorn sole
- *Brachirus orientalis* (native) Oriental-sole
- *Brachirus pan* (native) Pan sole
- *Heteromycteris oculus* (native), Eyed sole
- *Liachirus melanospilos* (native)
- *Pardachirus marmoratus* (native), Finless sole
- *Pardachirus pavoninus* (native), Peacock sole
- *Solea elongata* (native) Elongate sole, Elongate sole
- *Solea ovata* (native), Ovate sole
- *Soleichthys heterorhinos* (native)
- *Dagetichthys albomaculatus* (native) Kaup's sole
- *Dagetichthys commersonii* (native), Commerson's sole
- *Zebrias altipinnis* (native)
- *Zebrias annandalei* (native)
- *Zebrias keralensis* (native)
- *Zebrias quagga* (native), Fringefin zebra sole
- *Zebrias synapturoides* (native), Indian zebra sole
- *Zebrias zebra* (questionable), Zebra sole

Polymixiiformes

Polymixiidae (Beardfishes)

- *Polymixia fusca* (native)
- *Polymixia japonica* (questionable), Silver eye

Pristiformes

Pristidae (Sawfishes)

- *Anoxypristis cuspidata* (native), Knifetooth sawfish
- *Pristis microdon* (native) Smalltooth sawfish, Largetooth sawfish
- *Pristis zijsron* (native), Longcomb sawfish

Rajiformes

Dasyatidae (Stingrays)

- *Hemirhynchus bennetti* (native), Bennett's stingray
- *Negatrygon kuhlii* (native), Bluespotted stingray
- *Megatrygon microps* (native), Smalleye stingray
- *Dasyatis pastinaca* (questionable), Common stingray

- *Telatrygon zugei* (native) Pale-edged stingray
- *Pateobatis bleekeri* (native) Whiptail stingray
- *Himantura chaophraya* (questionable), Freshwater whipray
- *Himantura fai* (native), Pink whipray
- *Maculabatis gerrardi* (native), Sharpnose stingray
- *Brevitrygon imbricata* (native) Scaly stingray
- *Pateobatis jenkinsii* (native), Pointed-nose stingray
- *Himantura marginatus* (native) Blackedged stingray
- *Himantura uarnak* (native) Leopard stingray, Honeycomb stingray
- *Himantura undulata* (native), Leopard whipray
- *Brevitrygon walga*, Dwarf whipray
- *Pastinachus sephen* (native) Feathertail stingray, Cowtail stingray
- *Taeniura lymma* (native), Bluespotted ribbontail ray
- *Taeniura meyeni* (native), Blotched fantail ray
- *Urogymnus asperrimus* (native), Porcupine ray

Gymnuridae (Butterfly rays)

- *Gymnura tentaculata* (native), Tentacled butterfly ray
- *Aetoplatea zonura* (native), Zonetail butterfly ray
- *Gymnura japonica*, Japanese butterflyray
- *Gymnura micrura*, Smooth butterfly ray
- *Gymnura poecilura* (native), Longtail butterfly ray

Myliobatidae (Eagle and manta rays)

- *Aetobatus flagellum* (native) Plain eagleray
- *Aetobatus narinari* (native) Spotted eagleray
- *Aetobatus ocellatus* (native)
- *Aetomylaeus maculatus* (native), Mottled eagle ray
- *Aetomylaeus milvus* (native)
- *Aetomylaeus nichofii* (native) Nieuhof's eagle ray, Banded eagle ray
- *Mobula birostris* (native), Giant manta
- *Mobula eregoodootenkee* (native), Pygmy devilray
- *Mobula japonica* (native), Spinetail mobula
- *Mobula kuhlii* (native), Shortfin devil ray
- *Mobula mobular* (questionable), Devil fish
- *Rhinoptera javanica* (native), Javanese cownose ray

Plesiobatidae (Deepwater stingray)

- *Plesiobatis daviesi* (native), Deepwater stingray

Rajidae (Skates)

- *Dipturus johannisdavisi* (native), Travancore skate
- *Fenestrata mamillidens* (native), Prickly skate
- *Orbiraja powelli* (native), Indian ringed skate

Rhinobatidae (Guitarfishes)

- *Rhina ancylostoma* (native), Bowmouth guitarfish
- *Rhinobatos annandalei* (native) Annandale's guitarfish
- *Glaucostegus granulatus* (native), Sharpnose guitarfish
- *Glaucostegus halavi* (native), Halavi's guitarfish
- *G. obtusus* (native), Widenose guitarfish
- *G. thouin* (native), Clubnose guitarfish
- *G. typus* (questionable), Giant shovelnose ray
- *Acroteriobatus variegatus* (native), Stripenose guitarfish
- *Rhynchobatus djiddensis* (native), Giant guitarfish

Salmoniformes

Salmonidae (Salmonids)

- *Oncorhynchus mykiss* (introduced) Rainbow trout
- *Salmo trutta fario* (introduced) Brown trout
- *Salmo trutta* (introduced), Sea trout
- *Salvelinus fontinalis* (introduced) Brook trout

Scorpaeniformes

Apistidae

- *Apistus carinatus* (native), Ocellated waspfish

Aploactinidae (Velvetfishes)

- *Acanthosphex leurynnis* (native)
- *Cocotropus roseus* (native)

Caracanthidae (Orbicular velvetfishes)

- *Caracanthus maculatus* (native), Spotted coral croucher
- *Caracanthus unipinna* (native), Pygmy coral croucher

Dactylopteridae (Flying gurnards)

- *Dactyloptena gilberti* (native)
- *Dactyloptena macracantha* (native), Spotwing flying gurnard
- *Dactyloptena orientalis* (native), Oriental flying gurnard
- *Dactyloptena peterseni* (native), Starry flying gurnard

Peristediidae (Armored searobins or armored gurnards)

- *Scalicus investigatoris* (native)
- *Satyrichthys laticeps* (native)

Platycephalidae (Flatheads)

- *Cociella crocodilus* (native) Crocodile flathead
- *Sunagocia carbunculus* (native), Papillose flathead
- *Grammoplites scaber* (native) Rough flathead
- *Grammoplites suppositus* (native), Spotfin flathead
- *Inegocia japonica* (native), Japanese flathead
- *Kumococius rodericensis* (native), Spiny flathead
- *Platycephalus indicus* (native) Bartail flathead
- *Rogadius asper* (native), Olive-tailed flathead
- *Rogadius serratus* (native), Serrated flathead
- *Rogadius tuberculata* (native), Tuberculated flathead
- *Suggrundus macracanthus* (native), Large-spined flathead
- *Sunagocia otaitensis* (native), Fringelip flathead
- *Thysanophrys chiltonae* (native), Longsnout flathead

Scorpaenidae (Scorpionfishes or rockfishes)

- *Brachypterois serrulata* (native)
- *Dendrochirus brachypterus* (native), Shortfin turkeyfish
- *Ebosia falcata* (native)
- *Parascorpaena picta*, Northern scorpionfish
- *Pteroidichthys amboinensis* (native)
- *Pterois antennata* (native), Broadbarred firefish
- *Pterois mombasae* (native), Frillfin turkeyfish
- *Pterois radiata* (native), Radial firefish
- *Pterois russellii* (native) Russell's fire fish, Plaintail turkeyfish
- *Pterois volitans* (native), Red lionfish
- *Scorpaenodes parvipinnis* (native), Lowfin scorpionfish
- *Scorpaenopsis gibbosa* (native), Humpback scorpionfish
- *Taenianotus triacanthus* (native), Leaf scorpionfish

Setarchidae

- *Setarches guentheri* (native), Deepwater scorpionfish

Synanceiidae (Stonefishes)

- *Choridactylus multibarbus* (native), Orangebanded stingfish
- *Inimicus caledonicus* (native), Chinese ghoull
- *Inimicus sinensis* (native), Spotted ghoull
- *Minous dempsterae* (native), Obliquebanded stingfish
- *Minous inermis* (native), Alcock's scorpionfish

- *Minous monodactylus* (native) Grey goblin fish
- *Synanceia verrucosa* (native), Stonefish
- *Trachicephalus uranoscopus* (native), Stargazing stonefish

Tetrarogidae (Wasp fishes)

- *Ocosia ramaraoi* (native)
- *Paracentropogon longispinis* (native), Wispy waspfish
- *Pseudovespicula dracaena* (native), Draco waspfish
- *Richardsonichthys leucogaster* (native), Whiteface waspfish
- *Snyderina guentheri* (native), Günther's waspfish
- *Tetraroge niger* (native)

Triglidae (Searobins)

- *Lepidotrigla bispinosa* (native), Bullhorn gurnard
- *Lepidotrigla faurei* (native), Scalybreast gurnard
- *Lepidotrigla longipinnis* (native)
- *Lepidotrigla omanensis* (native), Oman gurnard
- *Pterygotrigla hemisticta* (native), Blackspotted gurnard

Siluriformes

Ariidae (Sea catfishes)

- *Arius arius* (native) Threadfin sea catfish
- *Netuma bilineatus* (native), Bronze catfish
- *Plicofollis tonggol* (native), Roughback sea catfish
- *Plicofollis dussumieri* (native), Blacktip sea catfish
- *Arius gagora* (native) Gagora catfish
- *Arius maculatus* (native) Spotted catfish
- *Nemapteryx nenga* (native)
- *Arius parvipinnis* (native)
- *Plicofollis platystomus* (native), Flatmouth sea catfish
- *Hexanematichthys sagor* (native) Sagor catfish
- *Hemiarius sona* (native) Sona sea-catfish
- *Arius subrostratus* (native), Shovelnose sea catfish
- *Hemiarius sumatranus* (native), Goat catfish
- *Plicofollis layardi* (native) Thinspine sea catfish
- *Netuma thalassina* (native), Giant seacatfish
- *Arius venosus* (native), Veined catfish
- *Batrachcephalus mino* (native) Frog-headed catfish

Dalatiidae (Sleeper sharks)

- *Centroscyllium ornatum* (native), Ornate dogfish

Echinorhinidae (Bramble sharks)

- *Echinorhinus brucus* (native), Bramble shark

Stomiiformes**Gonostomatidae (Bristlemouths)**

- *Cyclothone signata* (questionable), Showy bristlemouth
- *Gonostoma elongatum* (native), Elongated bristlemouth fish

Sternoptychidae

- *Polyipnus spinosus* (questionable)
- Stomiidae (Barbeled dragonfishes)
- *Chauliodus pammelas* (native)
- *Chauliodus sloani* (native), Sloane's viperfish
- *Stomias affinis* (native), Günther's boafish

Synbranchiformes**Chaudhuriidae****Synbranchidae (Swamp-eels)**

- *Monopterus albus* (native) Rice swampeeel
- *Monopterus cuchia* (native) Cuchia
- *Monopterus digressus* (native)
- *Monopterus eapeni* (endemic)
- *Monopterus fossorius* (endemic) Malabar swampeeel
- *Monopterus hodgarti* (endemic) Indian spaghetti-eel
- *Monopterus indicus* (endemic) Bombay swampeeel
- *Monopterus roseni* (native)
- *Ophisternon bengalense* (native) Bengal mudeel

Syngnathiformes**Aulostomidae (Trumpetfishes)**

- *Aulostomus chinensis* (native), Chinese trumpetfish

Centriscidae (Snipefishes and shrimpfishes)

- *Centriscus scutatus* (native), Grooved razor-fish

Fistulariidae (Cornetfishes)

- *Fistularia petimba* (native), Red cornetfish

Solenostomidae (Ghost pipefishes)

- *Solenostomus cyanopterus* (native), Ghost pipefish

Syngnathidae (Pipefishes and seahorses)

- *Choeroichthys brachysoma* (native), Short-bodied pipefish
- *Choeroichthys sculptus* (native), Sculptured pipefish
- *Doryrhamphus excisus* (native), Bluestripe pipefish

- *Hippichthys cyanospilos* (native), Blue-spotted pipefish
- *Hippichthys penicillus* (native), Beady pipefish
- *Hippichthys spicifer* (native), Bellybarred pipefish
- *Hippocampus fuscus* (questionable) Chilka seahorse, Sea pony
- *Hippocampus histrix* (native), Thorny seahorse
- *Hippocampus kelloggi* (native), Great seahorse
- *Hippocampus kuda* (native), Spotted seahorse
- *Hippocampus trimaculatus* (native), Longnose seahorse
- *Microphis brachyurus* (native) Short-tailed pipefish
- *Microphis cuncalus* (native) Crocodile-tooth pipefish
- *Microphis insularis* (native) Andaman pipefish
- *Phoxocampus belcheri* (native), Rock pipefish
- *Syngnathoides biaculeatus* (native), Alligator pipefish
- *Trachyrhamphus serratus* (native)

Tetraodontiformes**Balistidae (Triggerfishes)**

- *Abalistes stellaris* (native), Starry triggerfish
- *Balistapus undulatus* (native), Orange-lined triggerfish
- *Balistes rotundatus* (native)
- *Balistes vetula*, Queen triggerfish
- *Balistoides conspicillum* (native), Clown triggerfish
- *Balistoides viridescens* (native), Titan triggerfish
- *Melichthys niger* (native), Black triggerfish
- *Odonus niger* (native), Redtoothed triggerfish
- *Pseudobalistes flavimarginatus* (native), Yellowmargin triggerfish
- *Pseudobalistes fuscus* (native), Yellow-spotted triggerfish
- *Rhinecanthus aculeatus* (native), Blackbar triggerfish
- *Rhinecanthus rectangulus* (native), Wedge-tail triggerfish
- *Sufflamen chrysopterum* (native), Halfmoon triggerfish
- *Sufflamen fraenatum* (native), Masked triggerfish

Diodontidae (Porcupinefishes)

- *Cyclichthys orbicularis* (native), Birdbeak burrfish
- *Cyclichthys spilostylus* (native), Spotbase burrfish
- *Diodon holocanthus* (native) Bloched porcupine fish, Long-spine porcupinefish
- *Diodon hystrix* (native) Spotted porcupine fish
- *Lophodiodon calori* (native), Four-bar porcupinefish

Molidae (Molas or Ocean Sunfishes)

- *Mola mola* (native), Ocean sunfish

Monacanthidae (Filefishes)

- *Aluterus monoceros* (native), Unicorn leatherjacket
- *Aluterus scriptus* (native), Scrawled filefish
- *Anacanthus barbatus* (native), Bearded leatherjacket
- *Lalmohania velutina* (native)
- *Oxymonacanthus longirostris* (native), Harlequin filefish
- *Paraluteres prionurus* (native), Blacksaddle filefish
- *Paramonacanthus choirocephalus* (questionable) Pig faced leather jacket
- *Paramonacanthus japonicus* (native), Hairfinned leatherjacket
- *Paramonacanthus oblongus* (native), Hair-finned filefish

Paramonacanthus tricuspidus (native)

Thamnaconus modestoides (native), Modest filefish

Ostraciidae (Boxfishes)

- *Lactoria cornuta* (native), Longhorn cowfish
- *Ostracion cubicus* (native), Yellow boxfish
- *Ostracion meleagris* (native), Whitespotted boxfish
- *Ostracion nasus* (native), Shortnose boxfish
- *Tetrosomus gibbosus* (native), Humpback turretfish

Tetraodontidae (Puffers)

- *Arothron hispidus* (native) White spotted blow fish, White-spotted puffer
- *Arothron immaculatus* (native) Immaculate blow fish
- *Arothron meleagris* (native), Guineafowl puffer
- *Arothron nigropunctatus* (native) Black spotted blow fish
- *Arothron reticularis* (native) Reticulated blowfish
- *Arothron stellatus* (native) Star blaasop
- *Canthigaster amboinensis* (native), Spider-eye puffer
- *Canthigaster bennetti* (native), Bennett's sharpnose puffer
- *Canthigaster coronata* (native), Crowned puffer
- *Canthigaster margaritata* (native)
- *Carinotetraodon imitator* (endemic)
- *Carinotetraodon travancoricus* (endemic) Malabar pufferfish
- *Lagocephalus guentheri* (native), Diamondback puffer
- *Lagocephalus inermis* (native) Smooth-

backed blowfish

- *Lagocephalus lagocephalus* (native), Oceanic puffer
- *Lagocephalus lunaris* (native) Moontail blaasop, Green rough-backed puffer
- *Lagocephalus spadiceus* (native) Chinese blaasop
- *Takifugu oblongus* (native) Lattice blaasop, Lattice blaasop
- *Leiodon cutcutia* (native) Ocellated pufferfish, Ocellated pufferfish
- *Dichotomyctere fluviatilis* (native) Green pufferfish
- *Dichotomyctere nigroviridis* (native) Burmese pufferfish
- *Torquigener hypselogeneion* (native), Orange-spotted toadfish
- *Tylerius spinosissimus* (native), Spiny blaasop

Triacanthidae (Triplespines)

- *Pseudotriacanthus strigillifer* (native) Long spined tripod fish
- *Triacanthus biaculeatus* (native) Short-nosed tripodfish, Short-nosed tripodfish
- *Triacanthus nieuhofii* (native), Silver tripodfish
- *Tripodichthys oxycephalus* (native), Short-tail tripodfish

Triacanthodidae (Spikefishes)

- *Macrorhamphosodes platycheilus* (native), Trumpetsnout spikefish
- *Triacanthodes ethiops* (native), Shortsnout spikefish

Order Torpediniformes

Narcinidae (Numbfishes)

- *Benthobatis moresbyi* (native), Dark blind ray
- *Narcine brunnea* (native), Brown numbfish
- *Narcine lingula* (native), Chinese numbfish
- *Narcine prodorsalis* (questionable), Tonkin numbfish
- *Narcine timplei* (native), Blackspotted numbfish
- *Narke dipterygia* (native), Spottail sleeper ray

Torpedinidae (Electric rays)

- *Torpedo fuscomaculata* Black-spotted torpedo
- *Torpedo panthera* (native), Panther electric ray
- *Torpedo sinuspersici* (native), Marbled electric ray

Zeiformes

Caproidae (Boarfishes)

- *Antigonia indica* (native)

- *Antigonia rubescens* (questionable), Indo-Pacific boarfish

Parazenidae (Parazen)

- *Cyttopsis rosea* (native), Rosy dory

Zeidae (Dories)

- *Zenopsis conchifer* (native), Silvery John dory
- *Zenopsis nebulosa* (native), Mirror dory

Suggested readings

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Crustacean Diversity

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The Ocean has always fascinated mankind, the quest for unravelling its mystery leading to several exploratory surveys. The most notable as far as marine crustaceans are concerned was the exploratory surveys by the Indian Royal Marine Survey Ship 'Investigator' (1888–1892) which brought to the fore several marine crustacean species till then unknown. Majority of the fauna collected came from depths between 100-1900 fathoms. Later, mechanization of crafts and gears and extension of fishing into deeper waters enhanced our knowledge of the biodiversity of crustaceans. Crustacean biodiversity is as vast and deep as the oceans itself, difficult to comprehend. Crustaceans first appeared in the fossil records half a billion years ago. They have been important components of marine biodiversity and compose virtually the entire fossil arthropod fauna through the Mesozoic and Cenozoic Eras. The most abundant crustaceans in the ocean are copepods and they have no fossil record.

Amphipods

They have a laterally compressed body without carapace. They are distributed in marine, brackish



water, freshwater and terrestrial environments. In India, there is lot of information on their diversity

both from the east and west coasts of the country by several workers. They are fed upon by fishes and other crustaceans.

Isopods

They are a morphologically diverse group of crustaceans that live in sea, in freshwater and on



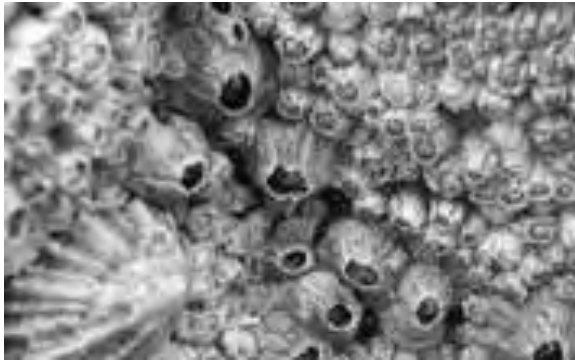
land. They have segmented and rigid exoskeleton and vary in shape. Smaller ones are parasitic on fishes and some larger species inhabit the deep sea. Some live in coastal and shelf waters. The largest isopod is in the genus *Bathynomus* found in deep sea.

Barnacles

They are highly specialized crustaceans belonging to the group known as Cirripedia and are exclusively marine forms. Most of the species live attached to inanimate objects, some to living objects like sponge and bury into the flesh of whales and turtles. They attach to substratum with their cement glands. They are mostly found at depths less than 100 m and also in the intertidal zone. Common barnacles are the goose barnacle (*Lepas anserifera*), *Balanus* spp., *Sacculina* spp. *Sacculina* is parasitic on crabs. It lives



Lepas sp



Balanus sp



Sacculina in crab

inside crabs and produce hormones that lead to feminization of crabs (changes the host from male to female) which causes the crabs to nurture the barnacle egg mass as its own.

Mysids / Opossum shrimp

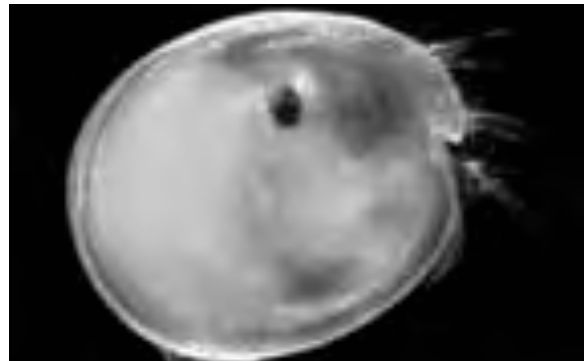
They are commonly called the opossum shrimps as they have a pouch or marsupium in females. Their larvae are reared in this pouch and are not free swimming. They may be pelagic or benthic and are filter feeders feeding on algae, detritus and zooplankton. They are found in both marine and freshwater environments. They are used as feed in aquaculture.



A female opossum shrimp carrying eggs

Ostracods

They range from 0.1 to 32 mm in size. Body is flattened from side to side and protected by a bivalve



like calcareous valve or shell. They are also called seed shrimps. Besides marine, there are freshwater and terrestrial species also.

Copepods

They are found in sea and in freshwater. Their body is cylindrical or round, the head is fused with the first or two thoracic segments. They may



be free living, symbiotic or internal or external parasite. They mainly feed on algae. Among the zooplankton community they are usually the dominant members and are the major food of fishes, seabirds and Krills.

Brine shrimp



They inhabit saltwater lakes and belong to the genus *Artemia*. They can produce dormant cysts which can be stored and hatched when required. The young ones from hatched cysts are used as live feed in aquaculture industry.

Euphasiids



They are marine crustaceans occupying surface to at least 4000 m depth and are distributed from Arctic to Antarctic waters. *Euphasia* species are fished commercially in Japan, Canadian and Russian waters for aquarium feed, pharmaceutical purpose and as food for humans. They are the main prey of baleen whales.

Shrimps/prawns

The species of shrimps and prawns are placed under four infra orders- Penaeidea, Stenopodidea, Caridea

and Axiidea. The majority of the shrimps and prawns belong to the superfamily Penaeoidea. Around 120



Fenneropenaeus indicus



Melicertus latisulcatus



Metapenaeopsis stridulans

species of penaeoid shrimps are recorded from the Indian coast.

Penaeid shrimps

The commercially important genera in Indian waters belonging to family penaeidae are represented by *Fenneropenaeus*, *Penaeus*, *Melicertus*, *Marsupenaeus*, *Parapenaeus*, *Metapenaeopsis*, *Penaeopsis*, *Metapenaeus*, *Trachypenaeopsis*, *Atypopenaeus*, *Parapenaeopsis*, *Megokris* and *Trachysalambria*. Genera *Solenocera* represent the family Solenoceridae and Aristeidae by *Aristaeomorpha*, and *Aristeus*. Under the family Penaeidae there are 71 species recorded from Indian waters.

Carideans

a. Alpheid shrimps



They are commonly known as the pistol shrimps or snapping shrimps having asymmetrical claws, the larger of the claw produces snapping sound. They dig burrows and inhabit coral reefs. Maximum species are under the genera *Alpheus* and *Synalpheus*. They are caught as bycatch in trawls.

b. Crangonid shrimps

The genera *Aegaeon*, *Pontocaris* and *Parapontocaris* are known from Indian waters like *Aegaeon lacazei* and *Pontocaris pennata*. The genera *Aegaeon*



Pontocaris pennata

are found from shallow waters to up to 800 m, *Parapontocaris* at and beyond 200 m depth and *Pontocaris* from shallow to deeper waters.



Glyphocrangon investigatoris

c. Glyphocrangonid shrimps

They are a single genus—*Glyphocrangon* under the family Glyphocrangonidae. Around 10 species have been recorded from Indian waters. Body is hard, thick and with spines or tubercles. They are deep sea inhabitants found at depths of 300 m and beyond.



Plesionika quasigrandis

d. Pandalid shrimps

A number of pandalid shrimp species are fished on a commercial scale for human consumption from the southern coasts of India. Notable are *Plesionika quasigrandis*, *Plesionika martia*, *Heterocarpus*



Acantheephyra armata



Acantheephyra sanguinea



Oplophorus typus

gibbosus and *Heterocarpus woodmasoni* landed by deep sea trawlers.

e. Oplophorids

The species that have commercial value are *Acantheephyra sanguinea*, *Acantheephyra armata*, *Oplophorus typus* and *Oplophorus gracillirostris*. They form a minor fishery. They are found at depths of 300 m and beyond.



Nematopalaemon tenuipes

f. *Palaemonid* shrimps

They are found in freshwater and also in marine habitats. The species that are usually encountered in the fishery are *Nematopalaemon tenuipes* and



Expalaemon styliferus.

g. *Stenopid* shrimps

They are usually associated with reefs. They have enlarged third pair of walking legs. Members of the genus *Stenopus* are commonly known as cleaner shrimps. *Stenopus hispidus* is a common aquarium pet under the family stenopodidae. This banded cleaner shrimp or banded coral shrimp uses its three pairs of claws to remove parasites, fungi and damaged tissue from fish.



Mud shrimp species

h. *Mud shrimps* (*Thalassinideans*)

They construct burrows in intertidal and subtidal soft sediments. Their burrows are considered to be the deepest and complex among the crustaceans. They use the burrows for shelter, reproduction and feeding. They have a brief pelagic larval stage but

spend most part of their life in burrows. The largest individuals may reach 10 cm (excluding appendages). They have an exceedingly large claw which may be on the right or left side.



Podophthalmus vigil



Calappa calappa



Calappa japonica



Galene bispinosa



Liagore rubromaculata



Jonas indica



Cryptopodia fornicata



Parthenope longimanus



Dorippe quadridens

Brachyuran crabs

The brachyuran crabs or true crabs belonging to the

infraorder Brachyura are the most diverse groups among the decapod crustaceans. They lack tail and their abdomen are reduced and curved or tucked under their body. Around 991 species of brachyuran crabs have been recorded from Indian waters. Commercially important crabs belong to the family Portunidae. The important commercial brachyuran crabs are *Portunus pelagicus*, *Portunus sanguinolentus*, *Charbydis feriata*, *Charybdis lucifera* and *Scylla spp.* Lesser important crabs belonging to family Portunidae are *Portunus gladiator*, *Portunus argentatus*, *Podophthalmus vigil*, *Charybdis natator*, and *Charybdis smithii*. There are several species of crabs caught as bycatch in trawlers in India belonging to different families – Calappidae, Matutidae, Dorippidae, Dromiidae, Leucosiidae, Xanthidae, Corystidae, Galenidae, Majidae, Grapsidae, Geryonidae, Ocypodidae etc.

Anomurans

The anomurans include the hermit crabs, porcelain crabs, mole crabs or sand crabs, hairy stone crab, king crab and squat lobsters. They have telson and uropods which the brachyuran crabs lack. Majority of anomurans live in tropical and temperate marine habitats



Hermit crabs

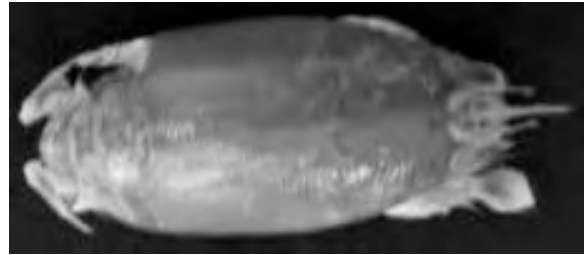


Hermit crabs

They have a soft body and carry a shell to protect their-self. Their entire body can be retracted into the shell. The shells used by them are mostly that of snails and also bivalve shells. As they grow they change their shell to accommodate themselves. They range in size from a few millimeters to the size of a coconut. The hermit crabs belong to the superfamily Paguroidea of the infraorder Anomura. They inhabit both aquatic and terrestrial habitats. Generally they have only three pair of walking legs with the last pair of legs being



Albunea



Emerita

very small and positioned under the abdomen hence not visible externally.

Mole crab/sand crab

They are placed under the family Hippidae. They have an oval shaped body and are filter feeders burrowing in sand in the intertidal zone. *Albunea spp.*, *Hippa spp.*, *Emerita spp.* are common and are fed upon by seabirds.



Eumunida funambulul



Munidopsis scobina

These organisms are also consumed by humans.

Squat lobsters

In countries like Argentina, Mexico and New Zealand the squat lobsters are considered as potential fishery resource. They are found in great abundance in several countries as bycatch and in India too several species have been recorded- *Munidopsis scobina*, *Munida squamosa*, *Munida japonica*, *Munida heteracantha*) etc.

Genus *Eumunida* is mostly represented by deep water squat lobsters. In India, *Eumunida funambulul* was



reported from off Arabian Sea, coast of Tamil Nadu and Lakshadweep. Their first pair of appendage is much longer than their body length.

Porcelain crabs

The porcelain crabs are small in size usually less than 15 mm in carapace width. They are fragile and have the habit of shedding their limbs when attacked by predators, hence the name Porcelain crabs. These shed appendages often grow back after moulting. They use their fifth pair of pereiopods for cleaning and chelate appendages for territorial struggle.

Lobsters

Palinurid lobsters

The Palinurid lobsters are moderate to large sized, lacking rostrum and having spines or granules on the carapace and are placed under the infra order Achelata. Most of the species are brightly coloured have bands



Palinustus waguensis



Palinustus waguensis



Peurulus sewelli



Panulirus versicolor



Thenus unimaculatus



Petractus rugosus

or spots. They are mostly coastal and species of the genera *Linuparus*, *Peurulus* are found beyond 300 m depth. They are usually caught in traps and also form bycatch in trawls. The genera *Panulirus* in Indian waters (including Andaman & Lakshadweep) is represented by *Panulirus homarus*, *P. versicolor*, *P. ornatus*, *P. longipes*, *P. pencillatus* and *P. polyphagus*, all being commercially exploited. The deep water lobster *Peurulus sewelli* is fished along the southern coasts of India. *Linuparus somniosus* is distributed in Andaman waters.

Scyllarid lobsters

They have flattened firm body, lacking rostrum or with rudimentary rostrum. They also are members of the infra order Achelata. They range in total length from a few to 40 cm. Antennae is short and broad, plate like or flattened lacking flagella, hence they are also called shovel nosed lobsters. Body colour is usually in various shades of brown. *Thenus unimaculatus* is fished commercially in Indian waters (Kerala, Tamil Nadu & Maharashtra). Certain other species recorded are *Petractus rugosus*, *Scyllarides elisabethae* and *Scyllarides tridacnophaga*. *Petractus rugosus* have ornamental potential and *Scyllarides tridacnophaga* grow to large size and are edible.

Polychelids

They possess extremely long and chelate first



Enoplometopus macrodontus

pereiopod and are inhabitants of very deep water, hence are blind eg *Stereomastis nana* from Indian waters. All five pairs of pereiopods may be clawed from which they get the name polycheles which means many clawed.

Astacids

The reef lobsters comprise single family and one genus *Enoplometopus*. Only two species are recorded from Indian waters – *Enoplometopus macrodontus* and



Nephropsis stewartii

Enoplometopus occidentalis. They usually inhabit reefs and can be distinguished from the Nephropsid lobsters by the presence of fully developed claws on the first pair of pereiopods, the second and third pair of pereiopods being only subchelate. They are caught from a depth of 90 to 200 m.

Members of the family Nephropidae have tubular body with well-developed rostrum, first pair of legs larger than the other pairs. The first three pairs of legs are with pincers. *Nephropsis stewartii* and *Nephropsis carpenteri* are recorded from Indian waters and have commercial value in some countries.

Stomatopods

Stomatopods are commonly known as praying mantis shrimp. They are predatory, the second maxilliped modified as large raptorial appendages. The last three segments of the second maxilliped fold against each other forming the raptorial claw similar to the praying mantis insect. There are 65 species of stomatopods recorded from Indian waters. Maximum numbers (38) have been recorded from Tamil Nadu, 22 from west Bengal and 17 from Maharashtra. They are



Squilloides leptosquilla



Oratosquilla woodmasoni



Lysiosquilla tredecimdentata

found to inhabit sandy or muddy bottom in the littoral region up to a depth of 100 m. One species *Squilloides leptosquilla* was recorded from a depth of 150 to 300 m. Some species belonging to the family Gonodactylidae inhabit coral reefs. Several species form commercial fishery in the Mediterranean Sea. In East Asia, *Oratosquilla oratoria* and in Indo-Pacific various species of squillids and lysiosquillids are fished.

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Molluscan Diversity

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Introduction

Phylum Mollusca is the second most diverse group under the animal Kingdom. They are soft bodied invertebrates often enclosed in a hard exoskeleton or shell. Therefore the word *mollusc* is derived from the Latin word *mollis* with the meaning *soft*. The branch of zoology that deals with the study of mollusc, known as *malacology*, originates from the Greek word for soft '*malacos*'.

Molluscs are among the most successful of the animal phyla in terms of numbers of species and also in terms of the wide range of habitats to which they have become adapted. They occupy a vast range of habitats, both aquatic and terrestrial, from the arctic seas to small tropical streams, from valleys to mountainsides and few are adapted to live in deserts while some are parasitic. In the sea they occur from the deepest ocean trenches to the intertidal zone.

Molluscs also exhibit an enormous range in size, from species which are almost microscopic to the largest of all invertebrates, the giant squid which can weigh 270kg and measures up to 12 m in the body length, with tentacles as much as another 50 m in length. The diversity of specific shapes within the phylum makes it difficult to define *mollusc* in terms of any distinctive trait. Structures that are prominent in some groups may be completely absent in others. One of the most characteristic features of molluscs is the possession of a hard calcareous shell by the majority of species offering protection, shape and rigidity to the soft visceral mass. These shells are long lasting and have been collected by human beings for thousands of years.

Classification

In terms of taxonomic rank, phylum Mollusca consists of two subphyla, namely Aculifera and Conchifera. Aculifera includes all molluscs that either primitively lack a shell (aplacophora) or have a series of plates instead of a single shell (polyplacophora). The subphylum Aculifera includes the class Polyplacophora (chitons) as well as the classes Caudofoveata and Solenogastres (or Solenogasters), along with their fossil relatives. The subphylum Conchifera (shell-bearers) comprises of five classes, which includes the

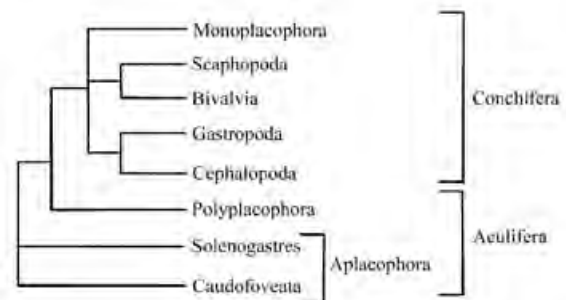


Fig. 1. Molluscan phylogeny based on morphological and larval features (Adapted from von Salvini-Plawen (1990a, b))

dominant molluscan classes the Gastropoda, Bivalvia and Cephalopoda.

There are various estimates of the number of species of molluscs from different parts of the world. Appeltan *et al.*, (2011) places the number of marine molluscs between 135,887 and 164,107 (Table. 1) in a recent compilation. Their estimates of the number of extant described marine molluscs vary from 43,689 to 51,689. The number of marine species described per year continues to rise for Bivalvia and Gastropoda among several other marine taxonomic groups.

In India, major studies have been carried out mainly on the commercially important molluscan groups.

Nearly, 5,070 species of molluscan species are recorded under the phylum Mollusca. Among the molluscan biodiversity, India contributes 7.21% of the world's biodiversity (UNEP-GBA, 1995). Within the number of molluscs recorded from India, 3,370 species of molluscs are recorded from marine habitat (Venkataraman and Wafar, 2005).

Solenogastres, Caudofoveata and Polyplacophora

Two groups of aplacophorans, Caudofoveata (Chaetodermomorpha) and Solenogastres, are universally recognized but their current classification is still contentious with two different systems in use. One system treats Caudofoveata and Solenogastres as subclasses of a class Aplacophora, which is accepted to be monophyletic while the other system consider Solenogastres and Caudofoveata to be paraphyletic. The latter view is largely corroborated by studies comparing morphological characteristics and molecular analyses, but the interrelationship between the two remains ambiguous with recent studies supporting either of the classification systems. The classification recognizing Caudofoveata and Solenogastres as separate classes is considered here. The two classes are however still known collectively as aplacophorans. Though they are not reported from Indian Sea, about 263 species of Solenogastres

and 133 species of Caudofoveata are described so far (Table 1).

Solenogastres are worm-like marine animals without shells, having a narrow, ciliated, gliding sole located in a ventral groove (possibly related to the foot of other mollusc) on which they crawl on hard or soft substrates, or on the cnidarian colonies on which they feed. The solenogastres range in size from less than a millimeter (0.8 mm) in body length (*Meiomenia swedmarki*) to more than 30 cm long (*Epimonia babaï*) and often colorful. Caudofoveata are worm-like lacking a ventral groove and foot. They are covered by cuticle and aragonitic scales. They are infaunal, feeding on detritus or selectively on foraminiferans. Hence they are adapted to burrowing in the mud with their oral shield. Caudofoveates range in length from 2 mm (*Prochaetoderma raduliferum*) to 14 cm (*Chaetoderma Productum*).

The representatives of Polyplacophora are commonly referred to as the Chitons. They are dorsoventrally flattened, exclusively marine molluscs characterized by the presence of eight dorsal aragonitic shell plates (valves) and a broad ventral ciliated foot. There is thick marginal girdle surrounding the dorsal shell plates covered by a chitinous cuticle. About 930 living species are reported from the marine intertidal or sublittoral, including deep-sea species ranging from 3mm to 43 cm in size. Nearly 21 species are reported from the Indian Seas.

Table 1. Estimates of Known and Unknown global marine molluscan species diversity (Adapted from Appeltans *et al.*, 2012)

	Total known (Described)	% Synonyms	Undescribed collected	Undiscovered (Morphospecies)	Total unknown (Experts)	Total unknown (Model)	Total estimated	% Known	New spp. (1999-2008)
Bivalvia	9000	55	2000	3000	5000	**	135,887-164,107	64	
Caudofoveata	133	8	Not estimated	500	500	No data		21	
Cephalopoda	761	No data	150	500	650	No data		54	
Gastropoda	32000-40000	69-75	35000-45000	50000-60000	85000-105000	**		23-32	
Monoplacophora	30	No data	3	50	53	No data		36	
Polyplacophora	930	52	50	50-100	100-150	No data		86-90	
Scaphopoda	572	33	55	500	555	No data		51	
Solenogastres	263	21	20-30	320-480	340-510	No data		34-44	
Total Mollusca	43,689-51,689		37,278-47,288	54,920-65,130	92,198-112,418			28-36	4022

** rate of discovery still rising

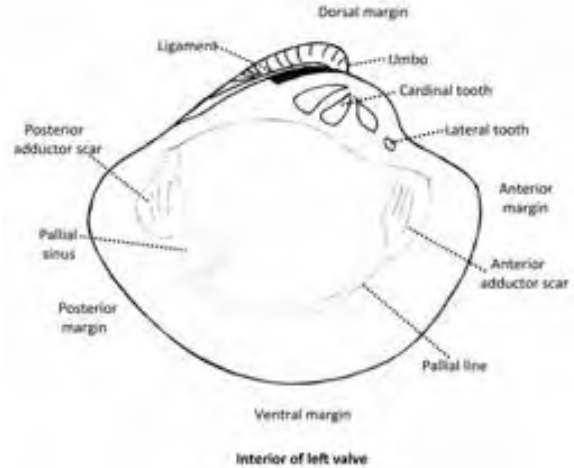
Monoplacophora

Extant Monoplacophora live in deep cold waters and resembles chitons in several characters. They are molluscs with a cap-shaped shell. They are not reported from Indian Seas. Thirty species are described under the class Monoplacophora. They are smaller in size ranging from 1.5 to 37 mm.

Scaphopoda

Scaphopods are inhabitants of the sea floor. The shell is tubiform to barrel-shaped and is open at both ends. The foot is pointed and cylindrical. The foot extends from the anterior (front) opening, with which scaphopods dig into the sandy sea floor. Tusk shells are exclusively marine and 18 species are reported from India. They range in size from 2-150 mm, eg. *Dentalium (Dentalium) aprinum*, *Dentalium elp*.

Class Bivalvia (Lamellibranchia or Pelecypoda)



(Adapted from Poutiers, 1998a)

Marine bivalve families reported from India

Solemyidae	Pectinidae	Semelidae	Lyonsiidae
Nuculidae	Spondylidae	Dreissenidae	Myochamidae
Nuculanidae	Anomiidae	Trapezidae	Pandoridae
Yoldiidae	Placunidae	Glossidae	Penicillidae
Malletiidae	Lucinidae	Vesicomomyidae	Poromyoidea
Arcidae	Galeommatidae	Corbiculidae	Ungulinidae
Noetiidae	Kelliidae	Veneridae	Unionidae
Cucullaeidae	Carditidae	Petricolidae	Verticordiidae
Limopsidae	Chamidae	Turtoniidae	
Glycymerididae	Crassatellidae	Corbulidae	
Mytilidae	Cardiidae	Myidae	
Pteriidae	Tridacnidae	Gastrochaenidae	
Malleidae	Mactridae	Pholadidae	
Isognomonidae	Mesodesmatidae	Teredinidae	
Pinnidae	Solenidae	Astartidae	
Limidae	Pharidae	Cuspidariidae	
Gryphaeidae	Tellinidae	Cyrenidae	
Ostreidae	Donacidae	Euciroidae	
Plicatulidae	Psammobiidae	Glauconomidae	
Propeamussiidae	Solecurtidae	Laternulidae	

Characteristics of important bivalve families (marine) (Source: Poutiers, 1998a)

The Bivalvia is the second largest class of the molluscs. They show much variation in body form yet

share a basic morphology. The bivalves are bilaterally symmetrical, laterally compressed molluscs, with extensive mantle lobes which secrete a single shell composed of two valves. The bivalves are mainly marine, but a few species are found in freshwater habitats, although none have invaded the land.

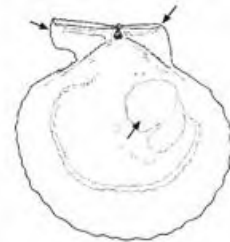
Pteriidae (Pearl oysters, wing oysters)

Pteriidae is a bivalve family of great economic importance. The dorsal shell margin is often produced at each end into a wing-like ear, sometimes very long behind. Shell slightly inequivalve. Right valve is with a byssal notch anteriorly. Hinge toothless or with denticles. Interior brilliantly nacreous. Only one adductor muscle scar. Pallial line without a sinus. In Indian Seas 12 species are reported under this family including the six species of pearl oysters, eg. *Pinctada fucata*, *P. margaritifera*, *P. chemnitzii*, *P. sugillata*, *Pteria* spp.



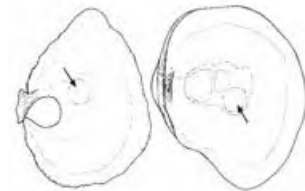
Pectinidae (Scallops)

Pectinidae have circular shells with radiating ribs, shell more or less inequivalve, ovate to subcircular with a straight dorsal margin forming wing-like ears. A byssal notch and a ctenolium at right valve. Ligament internal, in a small trigonal pit pointing under the umbones. Hinge without teeth. A single adductor muscle scar. Pallial line without a sinus. Thirty one species are reported from Indian waters, eg. *Chlamys tranquebaricus*



Anomiidae (Jingle shell)

The shells are inequivalve, often irregular, adhering to substrate by means of a calcified byssus passing through a hole-like embayment of right valve. Ligament internal. Hinge without teeth. Central area of the interior thickened, with 1 or 2 retractor muscle scars in left valve, in addition to the single adductor scar. No pallial sinus. Four species are reported from India, eg. *Anomia ehippium*



Placunidae (Windowpane oysters)

Placunidae includes only a single genus and single species. Shell thin, rounded to saddle-shaped, very compressed laterally, slightly inequivalve. Ligament internal, forming an inverted V-shaped structure. Hinge without teeth. A single adductor muscle scar. Pallial line without a sinus, eg. *Placuna placenta*



Ostreidae (Oysters)

Ostreidae and Gryphaeidae are known as the true oysters. Shell inequivalve, cemented to substrate by the left valve, right valve quite flat. Ligamental area with a shallow median groove and 2 lateral thickenings. Hinge without teeth. A single adductor muscle scar, median in position or nearer to the ventral margin. Internal margins smooth or with simple short marginal crenulations. About 11 species are recorded from India, eg. *Crassostrea madarsensis*, *C. gryphoides*, *C. rivularis*, *Saccostrea cucullata*.



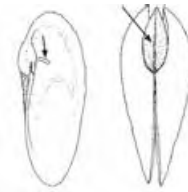
Gryphaeidae (Honeycomb oyster)

Gryphaeidae is having only a single species reported from India, the giant honeycomb oyster (*Hyotissa hyotis*). Shell more or less inequivalve, cemented to substrate by the left valve, with a microscopic vesicular structure. Ligamental area with a shallow median groove. Hinge without teeth. A single adductor muscle scar, closer to the hinge.



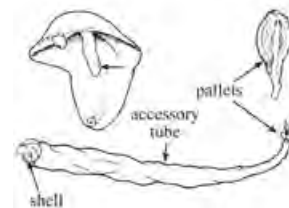
Pholadidae (Angelwings, paddocks)

Pholadidae have uniquely evolved shells. They burrow a cavity into wood, rock and other materials for protection. Shell subequivalve, gaping. Dorsal margin forming an umbonal reflection. A number of accessory calcareous plates about the main shell. Ligament reduced. Hinge without teeth. A finger-like internal apophysis. Three adductor muscle scars. Pallial line deeply sinuated, eg. *Martesia striata*



Teredinidae (Shipworms)

Teredinidae are known for boring into wood structures that are immersed into seawater. Shell reduced, equivalve, widely gaping. Anteroventral margin with a deep, right-angled notch. Dorsal margin forming an umbonal reflection. Ligament reduced. Hinge without teeth. A finger-like internal apophysis. An internal umbonoventral ridge, with a knob at both ends. Three adductor muscle scars. Accessory calcareous tube lining burrow long, closed by a pair of pallets. eg. *Uperotus panamensis*. *Teredora malleolus*.



Mytilidae (Mussels)

Mytilidae include the green mussel *Perna viridis*, and *P. indica*, important mariculture species which also contribute to the fishery. Thirty two species are recorded from India, of which 5 species are endemic to India. Shell equivalve and very inequilateral, with a byssal gape. Umbones at or near anterior end. Periostracum prominent. Ligament external, deep-set, supported by a whitish ridge. Hinge teeth absent or reduced. Adductor muscle scars unequal, the anterior one small to absent. Pallial line without a sinus. Inner side with an extensive nacreous layer, eg., *Perna* spp., *Modiolus* spp.



Pinnidae (Pen shells)

The pen shells are large, brittle, equivalve, subtrigonal, ventrally and posteriorly gaping; very inequilateral, pointed in front. Anterior end eroded and internally closed by small transverse partitions. Ligament linear. Hinge without teeth. Interior with a thin nacreous layer, restricted to the anterior half. Two unequal adductor muscle scars, eg. *Pinna* spp. Seven species are recorded from India.



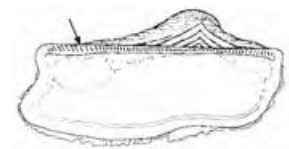
Tridacnidae (Giant clams)

The family includes the largest living bivalve species, the giant clams, *Tridacna* spp. Four species are reported from India. Shell equivalve, thick, heavy and often very large, with strongly scalloped free margins. Umbones ventral, free margins of the valves dorsal-most in position. Byssal gape, when developed, internally plicate. Outer surface with strong radial folds. Ligament external. Hinge with ridge-like cardinal and lateral teeth. A single adductor muscle scar, associated with a pedal retractor scar, submedian in position. Pallial line without a sinus.



Arcidae (Ark shells)

About 37 species are recorded from Indian Seas under the family Arcidae. This includes the commercially important blood clam, *Anadara granosa*. Shell equivalve or slightly inequivalve, mostly longer than high, more or less inequilateral. Umbones prosogyrate, on top of a wide cardinal area. Ligament external, often with V-shaped grooves. Hinge elongate, almost straight, with numerous small transverse teeth. Two subequal adductor muscle scars. Pallial line without a sinus.



Mactridae (Trough shells)

The shell is shaped like a rounded-cornered equilateral triangle and there is a slight gape at the posterior, shell equivalve. Umbones prosogyrate. Internal ligament well developed, in a trigonal pit of hinge plate. Hinge characteristic, with 2 cardinal teeth and lateral teeth; cardinal teeth of the left valve forming an inverted V-shaped process. Two adductor muscle scars. Pallial line with a well-developed sinus. The superfamily Mactroidea, which includes Mactridae and Mesodesmatidae contains 32 species, of which 3 species are endemic to India. eg. *Mactra violacea*.



Mesodesmatidae (Wedge clams)

Shell equivalve, inequilateral, subtrigonal to wedge-shaped. Umbones opisthogyrate. Internal ligament in a deep pit of hinge plate. One or 2 cardinal teeth and lateral teeth. Two adductor muscle scars. Pallial line with a short sinus. eg. *Mesodesma glabaratum*.



Solenidae (Razor shell)

Members of the genus *Solen* are with a narrowly elongate shape, gaping at both ends. Umbones more or less near the anterior end. Ligament external. Hinge feeble. Two adductor muscle scars, the anterior one larger. Pallial sinus relatively shallow, eg., *Solen* spp.



Corbiculidae (Basket clams)

Shell equivalve, solid, umbones prosogyrate. No lunule or escutcheon. Periostracum conspicuous. Ligament external. Three diverging cardinal teeth in each valve, and strong anterior and posterior lateral teeth. Two adductor muscle scars. Pallial sinus reduced to absent. The family includes the commercially important black clam, *Villorita cyprinoidea*, the mangrove clam, *Polymesoda erosa*.



Veneridae (Venus clams)

The superfamily Veneroidea having three families (Petricolidae, Turtonidae, Veneridae) is the second largest after the Tellinids. Ninety species are reported under this superfamily from India. Shell mostly solid, equivalve, inequilateral, with prosogyrate umbones. Lunule and/or escutcheon usually present. Ligament external. Three cardinal teeth in each valve, anterior lateral teeth sometimes present. Two adductor muscle scars. Pallial sinus usually present. eg. *Meretrix* spp., *Paphia* spp.



Cardiidae (Cockles)

Twenty five species are recorded from Indian Seas. One species is endemic to Andaman and Nicobar. Shell equivalve, inflated, oval to subquadrate, sometimes heart-shaped. Umbones prominent. External sculpture mostly radial. Ligament external. Hinge characteristic, with teeth curving outwards; 2 cardinal teeth and lateral teeth in each valve; cardinal teeth cruciform in arrangement. Two adductor muscle scars. Pallial line without a sinus. eg. *Cardium flavum*, *Cardium asiaticum*.



Carditidae (Carditas)

Carditidae family is represented by 7 species in Indian Seas. Shell equivalve, stout and inflated, inequilateral. Exterior mostly with radial ribs. Ligament external. Two cardinal teeth, unequal and with fine transverse striations; lateral teeth frequently reduced to absent. Two adductor muscle scars. Pallial line without a sinus. eg. *Cardita antiquata*



One of the most widely accepted classification systems for the class Bivalvia was that which employed a grouping system based on the shell shape, microstructures and hinge configuration by Newell (1965, 1969). Because features such as hinge morphology, dentition, mineralogy, shell morphology and shell composition change slowly over time, therefore these characteristics are used to define major taxonomic groups.

Among the 652 species of marine bivalves reported from India, 88 species are endemic to Indian waters (Tripathy and Mukhopadhyay, 2014).

Class Gastropoda

Gastropods are the most successful group of molluscs not only in terms of the number of species, but also in the wide range of habitat in which they may be found. Marine gastropod species have become adapted to living on all types of substratum and some have even adopted a pelagic existence. Generally a univalve spirally coiled shell is present in the majority of gastropods, although the shell may be poorly developed or lacking in the opisthobranchs. Their soft body is divided into 4 main regions: **the head**, which normally protrudes anteriorly from the shell; **the foot**, a muscular ventral organ with a flattened base used for locomotion (creeping or burrowing); **the visceral mass**, which fills dorsally the spire of the shell, and contains most organ systems; **the mantle**, a collar-like tegument which lines and secretes the shell, and forms a mantle cavity normally provided with respiratory gills in aquatic species. The noteworthy asymmetry of the internal anatomy of gastropods results from a twisting through 180° called the "torsion", which occurs in the first few hours of larval development. Part of the paired organs of the visceral mass cease developing, and the animal begins to be asymmetrical. This internal asymmetry persists in the adult, even when a subsequent detorsion occurs.

The taxonomy of the Gastropoda was revised by Philippe Bouchet and Jean-Pierre Rocroi in 2005. They have grouped both living and extinct gastropods, as well as some fossils as clades, derived from research on molecular phylogenetics. This is in contrast to the taxonomic schemes relying on morphological

features of the animals. In the Bouchet and Rocroi taxonomy, clades are unranked and used between the rank of class and the rank of superfamily. They use six main clades: Patellogastropoda, Vetigastropoda, Cocculiniformia, Neritimorpha, Caenogastropoda, and Heterobranchia. The first three of these major clades have no nesting clades within them: the taxonomy goes immediately to the superfamily level. Within the Caenogastropoda there is one extra clade.

Cladogram showing gastropod clads, groups and informal groups (Bouchet and Rocroi, 2005)

1. **Patellogastropoda**
2. **Vetigastropoda**
3. **Cocculiniformia**
4. **Neritimorpha**
 - ★ Paleozoic Neritimorpha of uncertain systematic position (fossil)
 - ★ Cyrtoneritimorpha (fossil)
 - ★ Cycloneritimorpha
5. **Caenogastropoda**
 - ★ Caenogastropoda of uncertain systematic position
 - ★ Architaenioglossa
 - ★ Sorbeoconcha
 - ★ Hypsogastropoda
 - ✦ Littorinimorpha
 - ✦ Ptenoglossa
 - ✦ Neogastropoda
6. **Heterobranchia**
 - ★ Lower Heterobranchia
 - ★ Opisthobranchia
 - ✦ Cephalaspidea
 - ✦ Thecosomata
 - ✦ Gymnosomata
 - ✦ Aplysiomorpha
 - ✦ Acochliidae
 - ✦ Sacoglossa
 - ✦ Cyndrobullida
 - ✦ Umbraculida
 - ✦ Nudipleura
 - ▲ Pleurobranchomorpha
 - ▲ Nudibranchia
 - » Eucteniidae
 - » Dexiarchia
 - Pseudoeucteniidae
 - Cladobranchia
 - ◇ Euarminida
 - ◇ Dendronotida
 - ◇ Aeolidida
- ★ **Pulmonata**
 - ✦ Basommatophora
 - ✦ Eupulmonata
 - ▲ Systellommatophora
 - ▲ Stylommatophora
 - » Elasmognatha
 - » Orthurethra
 - » Sigmurethra

In contrast, within the Heterobranchia, for some of the nudibranch groups there are six separate clades above the level of superfamily, and in the case of most of the land snails, there are four clades above the level of superfamily. Since the publication of this taxonomic system in 2005, various proposals for changes have been published by other authors.

Patellogastropoda, are the true limpets, historically called Docoglossa. They are found attached to hard surfaces in the intertidal zone, and are capable of locomotion (Patellidae, Nacellidae, Pectinodontidae).

Vetigastropoda are considered among the most primitive living gastropods, and are widely distributed from the intertidal areas to the deep-sea in all oceans of the world. They include the keyhole limpets, abalones, top shells, turban shells and other families (Trochidae, Turbinidae).

Cocculiniformia includes the deep-sea limpets (Cocculinidae).

Neritimorpha includes the terrestrial, marine

and freshwater snails and some deepwater limpets (Neritidae).

Caenogastropoda is a large diverse group of sea snails, land snails and freshwater snails, which includes 60% of the extant gastropods. They include the shelled marine gastropods, the periwinkles, cowries, moon snails, murexes, cone snails, turrids and other families.

Heterobranchia includes families which were historically placed in many different class of gastropods. This includes three informal groups; the lower Heterobranchia (shelled marine and freshwater species), the Ophisthobranchia (mostly marine species) and the Pulmonata (land snails and slugs; many freshwater snails and few marine species).

Marine gastropod diversity

Nearly 32,000-40,000 marine gastropods are described globally and an additional 35,000-45,000 more are collected but undescribed. In India, there are 1487 listed marine gastropod species along the east coast of India,

Characteristics of important gastropod families (marine) (Source: Poutiers, 1998b)

Haliotidae (Ear shells)

Shell ear-shaped, not permanently cemented to a substrate depressed and loosely coiled. Spire eccentric. A spiral row of holes on body whorl. Aperture occupying most of the underside. Interior nacreous. No operculum. *Haliotis varia* (variable abalone)



Trochidae (Top shells)

Shell conical to globose, often with a flattened base. Aperture without a siphonal canal, nacreous within. Operculum corneous, nearly circular. *Trochus radiates*, *T. niloticus* (Commercial top), *Umbonium vestiarium*, (Common button top)



Turritellidae (Screw shells)

Shell elongate, sharply conical, with numerous whorls and a small aperture. Whorls sculptured with spiral ribs or keels. Siphonal canal absent. Operculum corneous, rounded. *Turritella attenuata*, *T. acutangula*



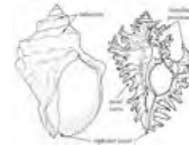
Buccinidae (Babylon shells)

Shell with a fairly high spire and large body whorl. Outer surface smooth or with sculpture, without axial varices. Siphonal canal rather short. Operculum corneous. *Babylonia spirata*, *B. zeylanica*, *Nassaria* sp.



Muricidae (Murex snails)

Shell variably shaped, generally with a raised spire and strong sculpture with axial varices, spines, tubercles or blade-like processes. Periostracum absent. Aperture with a well-marked siphonal canal. Operculum corneous. *Murex* sp. *Haustellum*, *Haustellum*, *Thais* sp., *Drupa* sp., *Rapana rapiformis*



Cerithiidae (Horn shells)

Shell sharply conical, with a high, many-whorled spire and rather small aperture. Sculpture variable. Aperture with a siphonal canal. Outer lip somewhat expanded. Operculum ovate, corneous, with a few spiral coils. *Cerithium* spp.



Cassidae (Helmet shells)

Shell thick and solid, with a large body whorl and rather small, conical spire. Sculpture variable, axial varices sometimes present. Aperture elongate, with a short siphonal canal, recurved dorsally. Outer lip thickened. Inner lip with a shield-like callus. Operculum quite small, corneous. *Cypraecassis* (*C.*) *rufa*, *Phalium glaucum*, *Semicassis* (*S.*) *bisulcata*



Tonnidae (Tun shells)

Shell thin, globose, with a short spire and very inflated body whorl. Sculpture only spiral. Siphonal canal short. Operculum absent. *Tonna dolium*, *T. cumingii*, *T. tessellata*



Conidae (Cones)

Shell cone-shaped, with a low spire and a well-developed body whorl tapering towards the narrow anterior end. Aperture very long, with a short siphonal canal. Operculum corneous, quite small. *Conus* sp.



Olividae (Olive shells)

Shell elongate-ovate, with a short spire, a large body whorl and channeled sutures. Surface smooth, highly polished. Aperture elongate, with a short siphonal canal. Inner lip calloused, with oblique grooves anteriorly. Operculum absent. *Oliva* sp.



Ranellidae

Shell ovate-fusiform, with a strong sculpture and axial varices. Periostracum frequently well developed and hairy. Aperture with a siphonal canal. Operculum corneous. *Cymatium (L.) lotorium*, *C. (M.) pileare*.



Ficidae (Fig shells)

Shell thin, pear-shaped, drawn out anteriorly into a long, tapered and gracefully curved siphonal canal. Operculum absent. *F. gracilis*, *F. investigatoris*, *F. Ficus*.



Volutidae (Volutes)

Shell variable in shape, often glossy and brightly coloured. Aperture long, with a short siphonal canal. Inner lip with strong folds, weaker posteriorly. Operculum horny, often absent. *Voluta lapponica*, *Melo melo*, *Voluta sp.*



Turbinellidae (Chank shells)

Shell thick and heavy, biconical to fusiform, often nodulose to spinose on shoulder. Periostracum conspicuous. Siphonal canal present. Inner lip with strong folds. Operculum corneous. *Turbinella pyrum*



Bursidae (Frog shells)

Shell ovate, often slightly dorsoventrally compressed, with 2 strong axial varices per whorl. Periostracum obsolete. Aperture with a short siphonal canal and a distinct posterior canal. Operculum corneous. *Bufonaria crumena*, *B. echinata*, *B. margaritula*



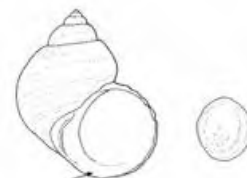
Naticidae (Moon snails)

Shell globular to ovate-conical. Outer surface smooth or with reduced sculpture. Aperture large, semicircular. Siphonal canal absent. Umbilicus open or closed, sometimes with an internal rib. Operculum corneous or calcified. *Natica sp.*



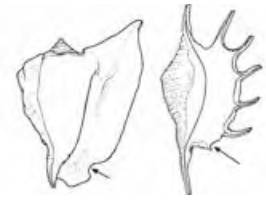
Turbinidae (Turban shells)

Shell thick, turbanate to conical. Outer sculpture often spiral to nodular. Aperture rounded, without a siphonal canal, nacreous within. Operculum strongly calcified. *Turbo marmoratus* (Great green turban) *T. (M.) radiates*, *T. intercostalis*, *T. (M.) brunneus* (Brown Pacific turban)



Strombidae (True conches)

Shell thick and solid, with a relatively large body whorl. Aperture with a well-marked siphonal canal. A distinct notch along the anterior margin of the outer lip. Operculum corneous, claw-like. *Tibia curta*, *T. deliculata*, *Lambis (Lambis) lambis* (common spider conch), *Strombus (Laevistrombus) canarium* (dog conch), *S. (Dolomena) marginatus* (marginate conch), *Terebellum terebellum*



Cypraeidae (Cowries)

Shell ovate or oblong, spire concealed under body whorl. Surface highly polished, smooth. Aperture long and narrow, channeled at both ends. Both lips with teeth. No operculum. *Cypraea sp.*



Ovulidae

Shell globular to spindle-shaped, with more or less expanded extremities. Spire concealed under body whorl. Surface often smooth, porcellaneous. Aperture very long, channeled at both ends. Inner lip smooth. No operculum. *Volva volva*



Mitridae (Mitre shells)

Shell fusiform-ovate, with a predominantly spiral sculpture. Aperture notched by a short siphonal canal. Outer lip not lirated inside. Columella with strong folds, larger posteriorly. No operculum. *Mitra sp.*



Potamididae (Horn shells)

Shell high-conical, with many spire whorls. Sculpture generally coarse. Aperture relatively small, with a short siphonal canal. Outer lip often flaring. Operculum rounded, corneous, with many spiral coils. *Telescopium telescopium*, *Cerithidea (Cerithideopsis) cingulate*, *Terebralia sp.*



of which 222 are repeated and 7 species are freshwater gastropods (Tripathy and Mukhopadhyay, 2014).

Class Cephalopoda

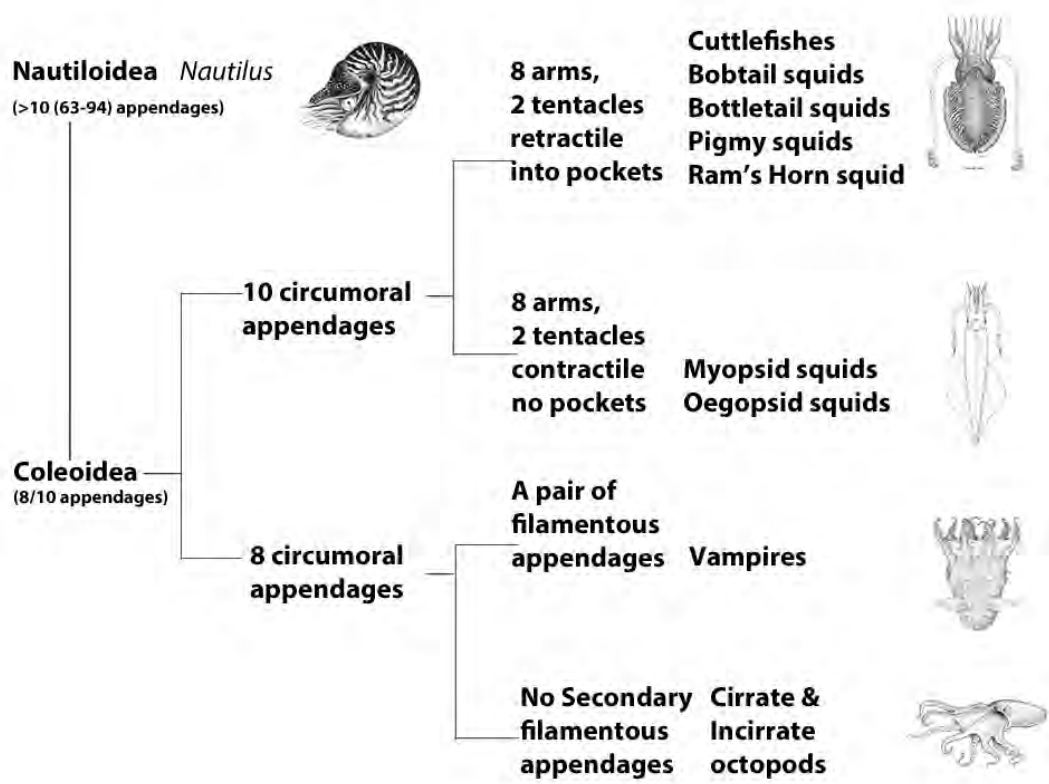
The class Cephalopoda is the most complex in the phylum Mollusca, and indeed, in all of the invertebrate phyla. It includes exclusively marine animals represented by the squids, octopuses and cuttlefishes. At the present time the status and understanding of the Systematics

and Classification of the recent Cephalopoda is under considerable discussion. The families of living cephalopods are, for the most part, well resolved and relatively well accepted. Species-level taxa usually can be placed in well-defined families. The higher classification, however, still is not resolved. Jereb and Roper (2005) have used an 'operational breakdown' for classification. For practical purposes they have separated the cephalopods into several groups, without assigning or implying taxonomic relationships.

The living cephalopods are at present not the most successful of the molluscan groups, although there is fossil evidence to suggest that they were once a much more important group. There are only about 761 living species (Appeltans *et al.*, 2012) described compared to 7500 fossil species so far discovered.

Cephalopods are soft-bodied animals with a well-developed head, bearing an anterior circumoral (surrounding the mouth) crown of appendages (arms, tentacles) (Jereb and Roper, 2005). This characteristic feature reflects the origin of the name Cephalopoda, which derives from the union of the two Greek words: 'kefale', head, and 'pous', feet. Arms and tentacles bear suckers and/or hooks (except in Nautilus), which are powerful tools to seize prey. The mouth has a pair of chitinous jaws (the beaks) and, as in other molluscs, a chitinous tongue-like radula (band of teeth) occurs in most cephalopod species. The ancestral mollusc shell is variously modified, reduced, or absent in living coleoids. It is a calcium carbonate structure in cuttlefishes, reduced to a rigid chitinous structure

in squids (the gladius or pen) and to a cartilaginous structure in finned octopods. In some sepiolids no vestige of shell is found. A true external shell occurs only in the nautilus, although a shell-like egg case is produced and carried by female argonauts (pelagic octopods often misnamed 'paper nautilus'). The loss of the external shell allowed the development of a powerful muscular mantle that became the main locomotory organ for fast swimming, via water jetting from the funnel. The funnel (siphon) is a unique, multifunctional, muscular structure that aids in respiration and expulsion of materials in addition to locomotion. Oxygenated water is drawn through the mantle opening around the head (neck) into the mantle cavity, where it bathes the gills for respiration. Mantle muscular contraction expels the deoxygenated water from the mantle cavity through the ventrally located funnel. The discharge jet serves to eliminate nephridial and digestive wastes, as well as to complete the respiratory cycle and for locomotion. Female reproductive products (eggs, egg masses) also are discharged through the funnel. Most coleoids produce



Cephalopod classification (Modified from Jereb and Roper, 2005)

ink, a dark, viscous fluid to decoy potential predators, or a cloud to obscure the escaping cephalopod.

communicates with the exterior via 3 openings. Chitinous shell present.

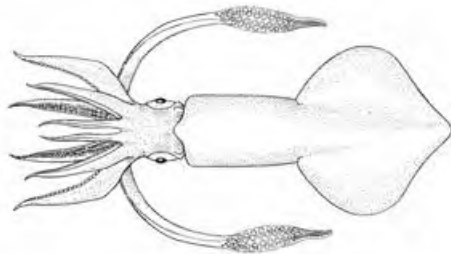
Cephalopod Groups

Squid (Source: Jereb et al., 2010)

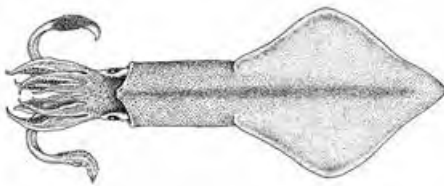
Suckers (and/or hooks) present; no external shell. Suckers stalked with chitinous rings; 10 circumoral appendages, 8 arms and 2 ventrolateral tentacles; contractile not retractile, no pockets. Mantle cavity

Family: Loliginidae

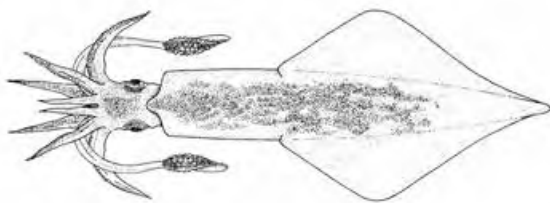
Internal shell straight, feather or rod-shaped, chitinous; tentacles contractile, not retractile, no pockets; fins usually joined posteriorly; mantle edge near mantle cartilages with small projections or 'angles'. Eye covered by transparent membrane (cornea); Four longitudinal rows (series) of suckers on



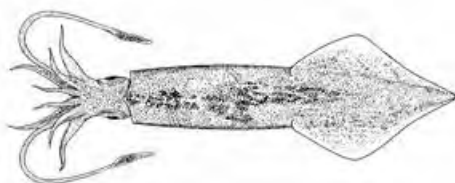
Uroteuthis (Photololigo) duvauceli



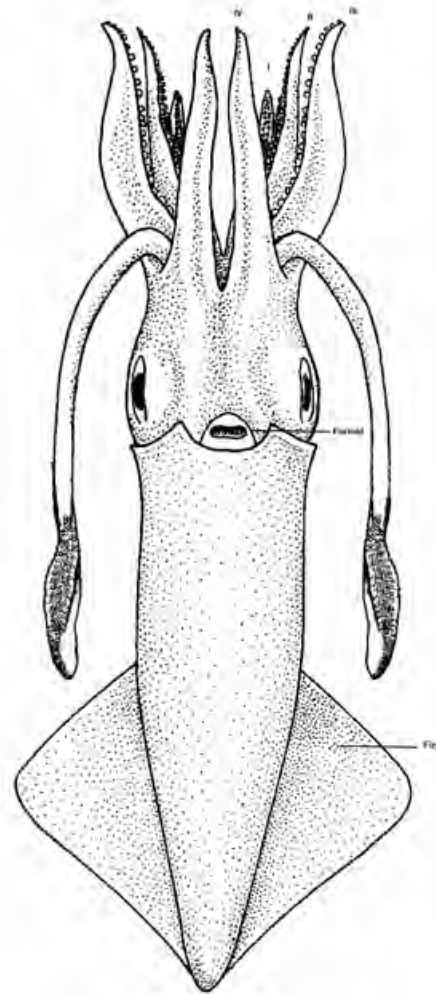
Uroteuthis (Photololigo) chinensis



Uroteuthis (Photololigo) edulis



Uroteuthis (Photololigo) sinhalensis

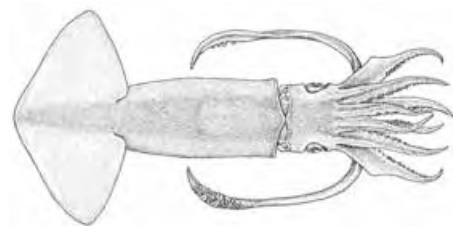
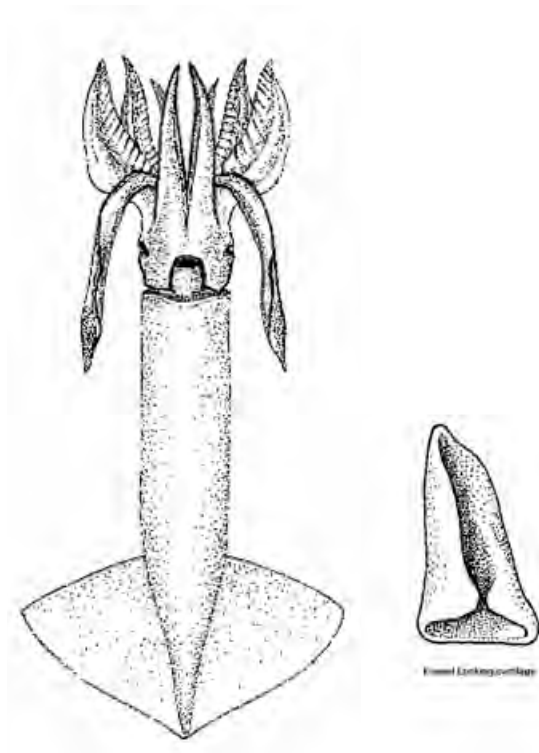


Ventral view

manus of tentacular clubs; fins united at posterior end of mantle; medial posterior border of fins concave.

Family: Ommastrephidae

Internal shell straight, feather or rod-shaped, chitinous; tentacles contractile, not retractile, no pockets; fins usually joined posteriorly; mantle edge near mantle cartilages with small projections or 'angles'. Eye without cornea; lens in open contact

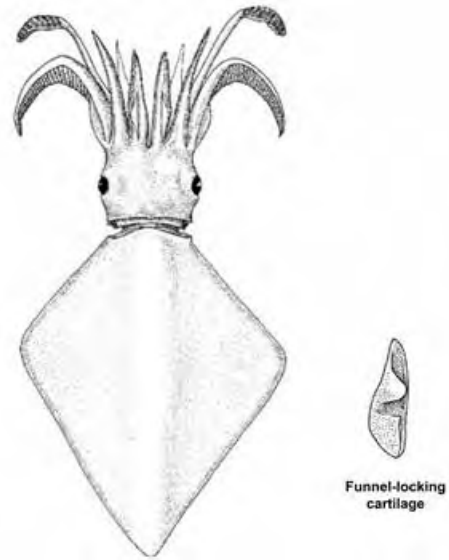


Stenoteuthis oualaniensis

with seawater. Funnel free from mantle; funnel-mantle locking apparatus present; Funnel-locking apparatus not a simple, straight groove and ridge; Funnel-locking cartilage with a longitudinal and a transverse groove shaped.

Family: Thysanoteuthidae

Internal shell straight, feather-or rod-shaped, chitinous; tentacles contractile, not retractile, no



Thysanoteuthis rhombus

pockets; fins usually joined posteriorly; mantle edge near mantle cartilages with small projections or 'angles'. Eye without cornea; lens in open contact with seawater. Funnel free from mantle; funnel-mantle locking apparatus present; Funnel-locking cartilage with a longitudinal groove from which a shorter groove branches medially, shaped; fins more than 80% of mantle length

Cuttlefish (Source: Reid et al., 2005)

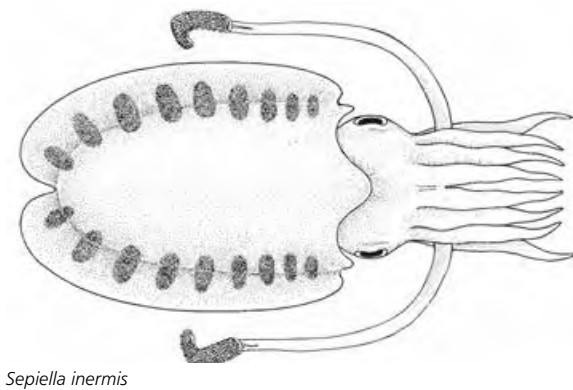
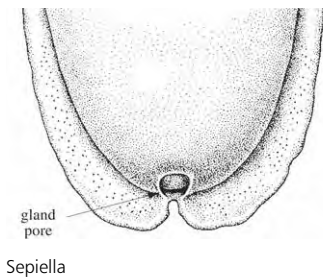
Suckers (and/or hooks) present; no external shell, suckers stalked with chitinous rings; 10 circumoral appendages, 8 arms and 2 ventrolateral tentacles. Mantle cavity communicates with the exterior through 3 openings. Internal shell straight, laminate, calcified; tentacles contractile and retractile into pockets between arms III and IV; fins not joined posteriorly; mantle edge near mantle cartilages straight.

Family Sepiidae:

Sepia: Cuttlebone outline elliptical to lanceolate;

cuttlebone length approximately equal to mantle length; dorsal anterior edge of mantle usually with tongue-like projection. Gland and gland pore absent; mantle-locking apparatus semicircular, without triangular projection, cuttlebone inner cone with relatively long limbs; outer cone usually calcareous, not obviously spatulate posteriorly. **Species:** *Sepiella inermis*, *Sepia elliptica*, *S. prashadi*, *S. trygonina*, *S. aculeata*, *S. brevimana*, *S. arabica*.

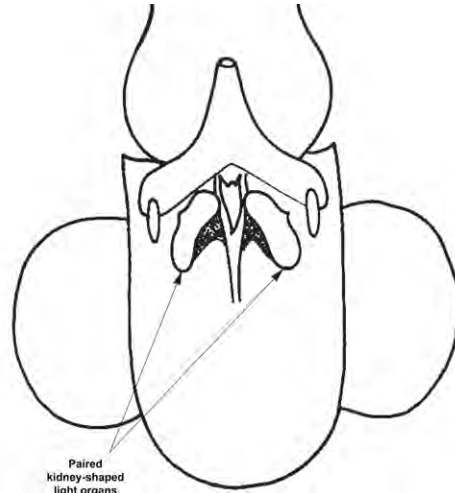
Sepiella: Cuttlebone outline elliptical to lanceolate; cuttlebone length approximately equal to mantle length; dorsal anterior edge of mantle usually with tongue-like projection. A gland and gland pore located on the ventral side of the posterior end of the mantle; mantle-locking apparatus with triangular projection;



cuttlebone inner cone with very short limbs; outer cone a wide, spatulate, chitinized border around posterior end of cuttlebone. **Species:** *Sepiella inermis*

Family: Sepiolidae

Internal shell straight and chitinous; tentacles contractile and retractile into pockets between arms III and IV; fins not joined posteriorly; mantle edge near mantle cartilages straight.



Sepioloidea: Paired, kidney-shaped light organs on anterior surface of ink sac; tentacular club suckers usually in 4 to 8 transverse rows.

Octopus

Suckers without stalks, bases sometimes constricted in finned (cirrate) octopods, without chitinous rings; 8 arms, no ventrolateral tentacles. Mantle cavity communicates with the exterior via one opening, rarely 2. Octopods have short, sac like body, eight circum oral arms connected at the base by a numerous web, no tentacles. Octopods are divided into two suborders. Cirrata, mostly deep sea forms possess cirri along the arms and paddle shaped fins dorsal lateral to the mantle. Incirrata, mostly shallow living forms instead of cirri have one or two series of non-stalked suckers along the arms; and no fins.

Family: Octopodidae

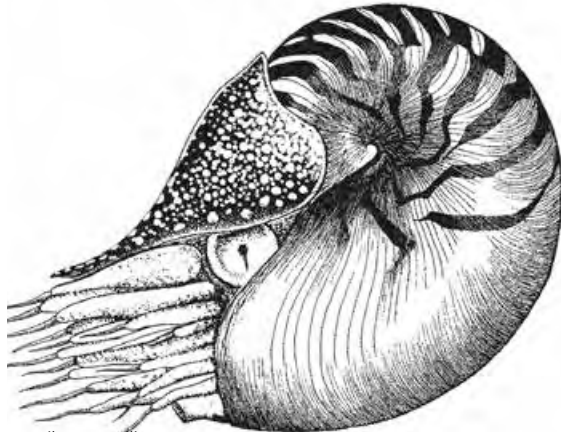
Funnel-locking apparatus absent; water pores on head absent; males not very much smaller than females, with left or right ventrolateral arm hectocotylized (never in pocket), with spoon-shaped, non-filamentous tip; females without dorsal arm flaps or permanent reticulate sculpturing of ventral mantle

Species: *Octopus vulgaris*, *Amphioctopus marginatus*, *Amphioctopus aegina*, *Amphioctopus neglectus*, *Amphioctopus rex*, *Cistopus indicus*

Nautilus (Source: Jereb, (2005))

Family Nautilidae

Nautilus possess more than ten (63-94) circumoral appendages, without suckers. The shell is external and coiled with chambers. The living nautilus are limited belonging to 1 family and 2 genera.



Nautilus pompilius

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Bryozoa – Taxonomy and Diversity

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Introduction

Bryozoans are an ancient, aberrant phylum of microscopic coelomate and often beautiful animals that build intricate colonies. Bryozoan phylum contains around 3,500 living species and 15,000 extinct species. They are found both in marine and freshwater environments, living at all latitudes and at depths ranging downward to 8,500 meters. They have a well recorded fossil history due to their zooecial nature and have been around since the Ordovician era. The bryozoans are almost always sedentary and sessile animals that live exclusively in colonies, normally adhered to a substratum. The only exception to this colonial existence is *Monobryozoon*, which contains species that may be solitary, depending upon conditions.

History

Early naturalists classified Bryozoans as members of the plant kingdom and Linnaeus invented the name 'zoophytes'. In early 16th century, Imperato asserted the animal nature of the zoophytes and noted the pores on the colonies. The 'pores' that he noted as the unique features of these organisms, are still retained in many generic names of the phylum like *Retepora*, *Membranipora* etc. Despite this, until the latter part of 18th century, naturalists like Linnaeus, Cuvier etc. continued to use the term Zoophyta to describe Bryozoans. By 19th century, they began to be considered as the polyps of cnidarians. In the second quarter of 19th century, Thompson and Ehrenberg independently found out that they are not ordinary polyps and that they have a separate digestive tract with two openings. Thompson

gave the name POLYZOA (Many animals) as the colonies are composed of numerous connected units called zooids. Ehrenberg named them as BRYOZOA—"moss animals" (Greek *Bryo* – moss and *Zoion* – animal). Hyman renamed it as ECTOPROCTA as the animals had their anus outside the crown of tentacles (Greek *ecto* – outside, *proktos* – anus). The controversy over which name to be selected still continues, and today, the phylum has three names—PHYLUM BRYOZOA or ECTOPROCTA or POLYZOA.

Habitat

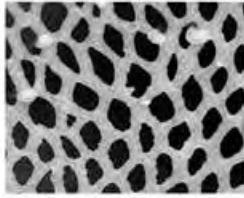
Bryozoans are benthic sessile forms found from continental shelf to bottom depths. In 1995, the first free-swimming bryozoan colonies were recorded from Weddell Sea, Antarctica - 400m deep and some distance from the normal bryozoan habitats. They resembled floating brown golf balls in an area free of sea ice for most of the year. It is suspected that the colonies may have been released from under the sea ice because of ice break-up. Another possibility is that the colonies may feed on the microscopic algae that grow on the under-layer of sea-ice in this area. A further advantage is that mobile species are able to exploit patchy food resources which are some distance apart. This is important because the plankton levels in the Weddell Sea are very seasonal. Scientists currently speculate that the new bryozoan may be a juvenile bryozoan of the genus *Alcyonidium*.

Bryozoans are well distributed in the Indian waters. They show exceptional levels of species diversity than any other organisms known today (Menon, 1967; Menon and Menon, 2006).

FORMS OF THE COLONY



Calcified coral-like *Schizoporella violacea*
(Tubular Horn Bryozoan)



Calcified, reticulate (fenestrated or net-like)
and lace-like - *Reteporella*



Stoloniferous zooids borne on creeping
stolons singly, regular row - *Scruparia*



Stoloniferous zooids borne on erect stolons,
in pairs, spirals - *Bogula*



Flat, encrusting, sheet like - *Canopenum*
reticulum



Eurystomella (orange encrusting Bryozoan)



Gelatinous with minimum calcification -
Adeona sp.

(Adapted from Poutiers, 1998a)

Basic Morphology of Bryozoa

The phylum name, Bryozoa, literally means 'moss animals' and refers to the bushy, moss-like colonies of some species. Flat encrusting forms are sometimes called sea mats. Erect, lacy forms are often called lace corals, a name that could also be applied to the thin, lace-like sheets that encrust kelp fronds.

Bryozoans are defined as microscopic, sessile, colonial coelomates that are permanently fastened in exoskeletal cases or gelatinous material of their own secretion, that are provided with a circular or crescentic lophophore and a recurved digestive tract bringing the anus near the mouth, and that lack nephridia and a circulatory system. The colony can be arborescent or frondose or can very often form flat spreading incrustations on objects, or sometimes become adherent or erect by stolons bearing the

zooids. The colony is composed of individuals (zooids), each of which is typically enclosed in a secreted exoskeletal case. The case is termed zoecium; the zoecia of a colony has an opening to the exterior called orifice, provided with a closing apparatus called the operculum. The ectoproct individual or zooid consists of two main parts, the tentacular crown or lophophore, protrusible through the orifice, and the trunk, permanently fastened in the zoecium. In the marine forms or gymnolaemates, the lophophore is circular but has the shape of a horseshoe in the fresh-water or phylactolaemate forms. Lophophore always embraces the mouth but never the anus. Lophophore is raised above the zooid on a slender extension of the body wall (the tentacle sheath, or introvert). When not spread for feeding, the tentacles are withdrawn into the coelom by the action of paired retractor muscles. Eversion of the tentacle sheath and tentacles is effected by raising the hydrostatic pressure of the body fluid.

The colony is composed of polymorphic zooids.

- **Autozooid:** zooids responsible for feeding and excretion.
- **Heterozooid:** specialized non-feeding zooids which get nutrients from autozooids through channels.
- **Avicularia:** small heterozooids in which the zoecium and operculum form a beak-like, snapping structure—defensive in function.
- **Vibracula:** zooids that bear long setae, or bristles—clean the bryozoan colony and supposed to aid in chemoreception.
- **Kenozooids:** small heterozooids that strengthen and support the colony as well as fill space.
- **Ooecia / ovicells:** zooids specialised for reproductive activities, they act as brood chambers, producing and holding safe the eggs until they are ready to hatch.

Also there are individuals that serve as stolons, holdfasts and attachment discs.

Bryozoans are filter feeders. They have a U-shaped gut and a lophophore of ciliated tentacles. Retraction and protrusion of the lophophore aids in feeding. Tentacles generate a water current and trap small particles of food on a constantly moving stream

of mucous. This mucous stream moves down the tentacles and into the digestive tract, taking anything it has caught with it. The digestive tract consists of a pharynx, oesophagus, a stomach and an intestine which terminates in an anus. The re-curved digestive tract hangs freely in the coelom with few attachments to the body wall. In some forms, proximal part of stomach is altered into a gizzard that helps in grinding the siliceous and calcareous exoskeleton of diatoms and dinoflagellates.

The nervous system consists of a main ganglionic mass situated between the mouth and the anus encircles the pharynx. The nerves ascend into the tentacles and descend along the digestive tract and other parts of the trunk. Most ectoprocts are hermaphroditic. Circulatory, respiratory and excretory systems as organic assemblages are wanting.

Bryozoans can reproduce both sexually and asexually. Most of the zooids are hermaphrodite, but the testes and ovary usually do not mature at the same time. Some species shed both eggs and sperm directly into the water where they fuse. But the majority of species brood their eggs within the zooecium or in special chambers known as ovicells, and capture free-swimming sperm with their tentacles to fertilize the eggs.

The fertilized eggs divide and develop into free-swimming ciliated larvae called *Cyphonautes*. These animals exist as colonies that are typically derived by asexual reproduction from a single progenitor (ancestrula), originating by the metamorphosis of a sexually produced larva. In those forms where embryo develops in the ovicell, larvae escape from the brood chamber and swim away. The escape of larva appears to be a response to illumination (positive phototaxis). These larvae eventually settle on a suitable substrate and metamorphose into a new zooid. The first formed zooid or the parent zooid is called ancestrula. Ancestrula initiates colony formation by asexual reproduction.

Asexual reproduction occurs by budding and is the main way by which a colony expands in size. Proliferation proceeds according to a pattern characteristic of the species. If a piece of a bryozoan

colony breaks off, the piece can continue to grow and will form a new colony. In old and senile zooids, the whole body of the zooid retracts to form a small ball, which then degenerates to form a mass of non-living debris. This mass is often brownish and is called a "brown body." A short time after this process, either the organism regenerates around the brown body, which is often incorporated into it or the brown body is incorporated into the gut and expelled from the new individual.

In fresh water forms, during winter, the colonies become dormant or inactive. At this point colonies form statoblasts which are modified unopened zooid buds. They are cold resistant and survive the freezing waters when the rest of the zooid colony dies off. When the water warms up, each statoblast germinates into a functional zooid with the ability to form between one and five newer buds.

Peculiarities in Reproduction

Selfing vs Outcrossing

Studies have shown that selfing (sexual reproduction involving egg and sperm from the same zooid) is not routinely occurring in Bryozoans as the fitness of selfed offspring is significantly reduced compared to outcrossed offsprings. However, in certain situations like the following, selfing in Bryozoans could lead to the production of viable, self-compatible offspring (Johnson, 2010).

- If the population within a given locale were founded by few individuals, the selection for selfing would be greater, where solitary colonies were indeed able to reach reproductive maturity, self, and release offspring and result in the establishment and subsequent propagation of self-compatible individuals.
- Results from investigations with *Celleporella hyalina* showed a differential ability to self among geographically distinct populations (Hughes *et al.*, 2009).

Cloning

Prolific polyembryony (the splitting of a single sexually produced embryo into many clonal copies), is reported

in marine bryozoans of the order Cyclostomata. Microsatellite genotyping of brooded embryos and maternal colonies conclusively demonstrated polyembryony. The characteristically voluminous brood chamber of cyclostomes is judged to be an adaptation linked to larval cloning and hence an indicator of polyembryony. Embryos are always genetically identical within broods but genetically distinct among broods and from their mother. Each brood, therefore, result from vegetative budding of a primary embryo, itself derived from a zygote resulting from outcrossed mating via water-borne sperm. The reasons of such cloning is not sure, but it is speculated that either

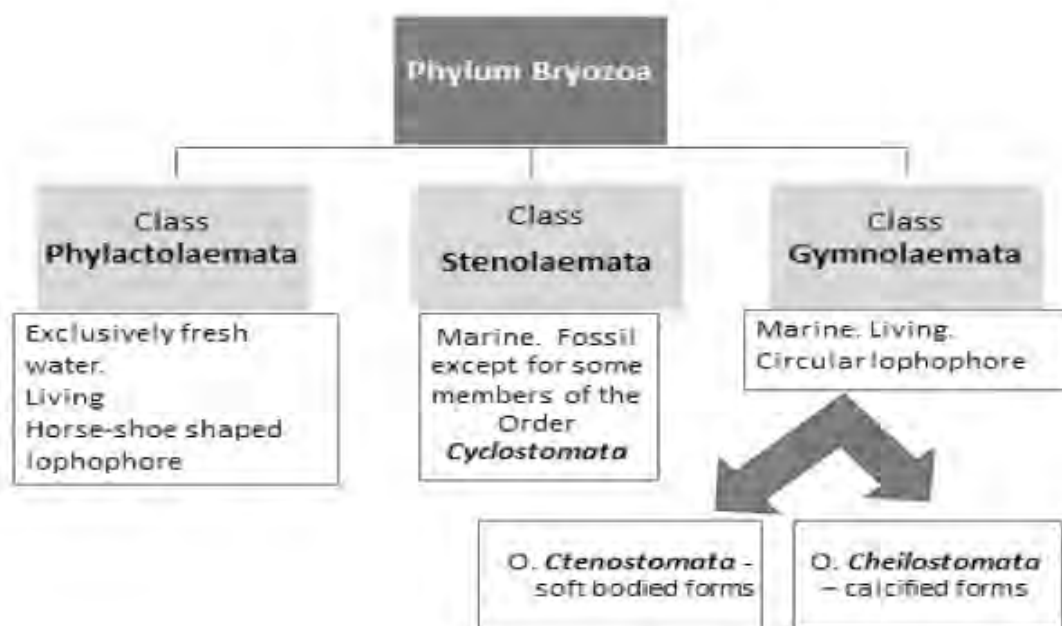
- Low sperm output from colonies (Pemberton *et al.*, 2011) or
- Unpredictable environmental conditions or
- Phylogenetic constraint (Hughes *et al.*, 2005) may be responsible.

Classification

The marine bryozoans, the Gymnolaemata, may or may not be calcified, at least to some extent, this helps in classifying this group, which is subdivided into two living orders, based on the complexity involved by the calcification of the exoskeleton. The two living orders are the ctenostomes and cheilostomes in the

sequential order of the morphological complexity.

- The ctenostomes are the gymnolaemates with a simple, flexible, uncalcified zoecium composed of chitinous cuticle. Usually represented by stoloniferous vase like zooids, which are greatly misunderstood as the coelenterates. The orifice lacks a closing apparatus called the operculum but the diaphragm usually bears a pleated collar which when folded blocks the vestibule.
- The Cheilostomata are the calcified group and the most dominant group in a bryozoan assemblage. The colony usually consists of box-like contiguous zooids arranged as branches, continuous incrustations or lamellate expansions. They are unique in having an operculum, the closing apparatus of the orifice. Polymorphism, a phenomenon of variation in zooidal structure and function within a species reaches its paramount in this group. Based on the extent of calcification of the exoskeleton and the presence or absence of ascopore, they are subdivided into Anasca and Ascophora.
 - » Anasca having a less calcified membranous front which itself regulates the in and out movements of their feeding organ—the lophophore.



- » Ascophora has a distinctly calcified front due to which the in and out movements of the lophophore is facilitated by the possession of a compensation sac—the ascus.

The family Cribrillinidae needs to be mentioned, which is the evolutionary link between the Anascans and Ascophorans, is featured by its calcified costulate armour with partially exposed front and possesses characters of both the classes.

Class Stenolaemata has one extant order named Cyclostomata and two extinct orders.

- The cyclostomes are more complex group possessing fully calcified tubular zooids. Lophophore protrusion is by the action of annular muscles and is devoid of avicularia and vibracula but heterozooids occur in the form of gonozooids, nanozooids and kenozooids

Palaentology

The Bryozoa have a long and eventful fossil history. However, records do not appear in Cambrian (when most metazoans appeared) or late Pre-Cambrian rocks. Bryozoa might have existed in the Cambrian but were soft-bodied or not preserved for some reason. A poorly preserved fossil called *Archaeotrypa* from the upper Cambrian is perhaps the oldest Bryozoan. The oldest known fossil bryozoans appeared in the Early Ordovician (diversity of marine invertebrates), 470 million years ago. Freshwater bryozoans are virtually unknown as fossils, presumably because they did not have mineralized skeletons. From the time of their appearance in Ordovician, they evolved rapidly into many diverse forms. During Ordovician, the rate of appearance of genera was practically equalled by the rate of extinction. For the remainder of the Palaeozoic Era (until 251 mya) they were abundant in shallow marine environments and were an important component of coral reefs formed during this time. Dominant Palaeozoic orders all belonged to the Class Stenolaemata and included trepostomes, cystoporates, cryptostomes and fenestrates. The last of these orders became extinct at the end of the Permian and the other three did not survive beyond the Triassic. The massive extinctions

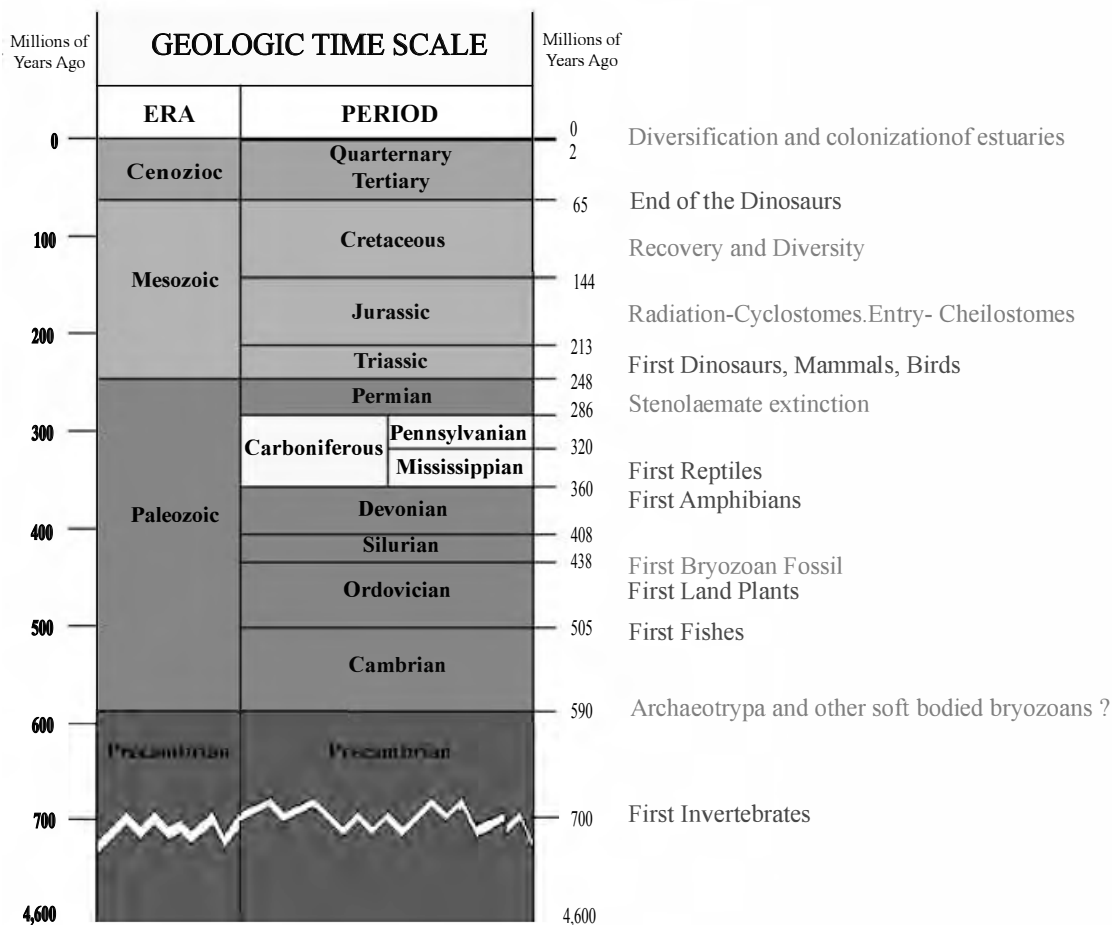
in marine creatures at the end of the Palaeozoic greatly affected bryozoans. During the following Mesozoic Era (251-65 mya) they recovered and new major groups appeared, including those groups that are most common today. During the Caenozoic Era (less than 65 mya) bryozoans continued to increase in variety. Cyclostomes, a stenolaemate order of minor importance in the Palaeozoic, radiated in the Jurassic and are extant today. Another extant order—cheilostomes—first appeared in the Late Jurassic and by the end of the Cretaceous had surpassed cyclostomes in both diversity and abundance in fossil assemblages. Cheilostomes belong to the Class Gymnolaemata and represent an independent origin of a calcareous skeleton from a non-mineralized ancestor belonging to the paraphyletic Order Ctenostomata. Bryozoan diversity today may be greater than at any time in the geological past, except perhaps during the Pliocene before the cooling and other changes that accompanied Pleistocene glaciation.

Economic Importance

Fresh water Bryozoans (*Plumatella species*) are one of the major foulers in pipelines. They disrupt the public water service in many countries. Many techniques like Sodium hypochlorite (commercial bleach) washing, painting the pipelines with Cu containing paints and spraying with water of increased velocity (> 1m/sec) have been tried, but the following three factors hinder control efforts:

1. statoblasts that tolerate harsh physical and chemical treatments;
2. regeneration of bryozoan colonies from pockets of living tissue;
3. easy dispersal of bryozoans through air and water.

Marine Bryozoa are serious foulers on the hulls of ships. Many fouling bryozoans are even resistant to Cu containing anti-fouling paints. Through ships and through ballast water, they reach new environments and colonise there as invasive alien species. Tufted forms like *Bugula* and *Zoobotryon* foul the intake pipes of power station and ships' cooling systems. *Bugula neretina*, an inhabitant of tropical waters have



reached the British seas and has invaded the area and established itself as a dominant species.

Species like *Alcyonidium* is common in N. Sea and gets trapped in piles in trawling nets. Repeated handling of this can cause allergic dermatitis with painful rash and weeping blisters.

Bryozoans are biochemically important and have been proved to be a rich source of novel compounds or bioactive agents. Bryostatin-1, a macrocyclic lactone compound is isolated from the bryozoan *Bugula neritina*. It has antineoplastic activity. Bryostatin-1 binds to and inhibits the cell-signaling enzyme protein kinase C, resulting in the inhibition of tumor cell proliferation, the promotion of tumor cell differentiation, and the induction of tumor cell apoptosis. This agent may act synergistically with other chemotherapeutic agents. A draft developed from this has already

reached the pharmaceutical markets (September, 2005). Over 20 different bryostatins have been isolated from this particular species (Faulkner, 1990, 1991, 1992, 1993, 1994).

According to scientists at the Blanchette Rockefeller Neurosciences Institute (BRNI) and the Marine Biological Laboratory, a cancer drug may stimulate the production of proteins needed for long-term memory, suggesting the compound may be a possible treatment for Alzheimer's disease. Both these drugs are in the stage of Phase II clinical trials.

Scientists at BRNI have discovered that Bryostatin and a related class of drugs discovered at BRNI administered 24 hours after stroke can rescue and repair brain tissue. These findings are markedly advanced compared to current stroke treatments that must be administered within three hours and are unable to repair damaged brain tissue.

B. dentata was shown to contain an anti-microbial blue pigment (Matsunaga *et al.* 1986).

A series of brominated alkaloids have been isolated from *Flustra foliacea* (Wright, 1984) of which Flustramines A and B having muscle relaxant activity and Dihydroflustramine exhibiting strong antimicrobial activity.

Chartella papyracea and *Cribricellina cribraria* are biochemically important for its biological activities including anti-tumor and antifungal activities (Prinsep *et al.*, 1991). The calcium carbonate of these animals is in a highly pure form for the utilisation in dentistry. Chitin extraction from bryozoan is another field that is developing. These chemicals open up an important field in biotechnology research of pharmaceutical importance. The experimental studies to understand cloning and mapping of genes is a recent field of research in which bryozoans are being used extensively by genetic engineers.

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Seaweeds and Marine Biotechnology

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Introduction

Seaweeds or marine macroalgae consist of taxonomically distinguished groups of Chlorophyta (green seaweeds), Phaeophyta (brown seaweeds) and Rhodophyta (red seaweeds). They are generally found attached to rocks, pebbles or other aquatic plants in the intertidal or subtidal regions of the sea. Seaweeds are valued for the natural source of phycocolloids such as agar-agar, algin and carrageenan. A number of tropical seaweeds including green algae (*Ulva*, *Enteromorpha*, *Monostroma*, *Caulerpa*) brown seaweeds (*Dictyota*, *Laminaria*, *Cladosiphon*, *Padina*) and red seaweed (*Gracilaria*, *Porphyra*, *Eucheuma*) are eaten directly (sea vegetables) for their minerals, vitamins, proteins, essential aminoacids and low fat content. The major economic significance of seaweeds is the polysachharides (agar, algin, carrageenan, agarose etc) certain red and brown seaweed species contain. Mariculture of seaweeds is essential for the steady supply of raw materials to seaweed industries and to reduce the exploitation pressure being faced by the seaweed beds along the coast. Already countries like China, Japan, Philippines, Korea are widely cultivating seaweeds and wild harvests are regulated.

Seaweed Resources

Economically important seaweed resources of the world, as per the harvests made during 1971-1973 is estimated to 2.105 million tones wet weight (about 1460 million tones of brown algae; 261 million tones of red algae) dominated by brown seaweeds (Michanek, 1975). The south east and north west coasts of India and the Andaman- Nicobar and Laccadive archipelagoes harbour a variety of seaweeds with rich biomass and species diversity. Luxuriant growth of seaweeds is found in southern coast of

Tamilnadu, Gujarat, Lakshadweep and Anadaman-Nicobar Archipelagos. Rich seaweed beds occur at Dwaraka, Okha, Mumbai, Ratnagiri, Goa, Karwar, Thikodi, Varkala, Vizhinjam, Rameswaram and Chilka Lakes. There are about 40 seaweed industries functioning in India producing algin and agar, depending only on natural resources.

In a checklist of marine algae (Oza and Zaidi, 2001) 844 species of marine algae have been reported from India, comprising 216 species of Chlorophyta, 191 species of Phaeophyta, 434 species of Rhodophyta and 3 species of Xanthophyta, which is again revised to 896 species from 250 genera (Umamaheswara Rao, 2011) indicating a considerable increase in the species of seaweeds of India.

Seaweed utilization

Agar is the major constituent of the cell wall of certain red algae(Rhodophyceae), especially the members of families Gelidiaceae, Gelidiellaceae and Gracilariaceae. Agar-agar is the Malay word a gelling substance extracted from *Eucheuma*, but now known to be carrageenan. The term agar is now generally applied to those algal galactans which have agarose, the disachharide agarobiose as their repeating unit. Raw materials for the production of agar are red algae such as *Gelidiella acerosa*, *Gracilaria edulis*, *G. verrucosa* and species of *Gelidium*, *Pterocladia* and *Ahenfeltia*.

Agar is an important colloid used extensively if biomedical laboratories and in R& D labs as a basal medium for the culture of microbes, cells and tissue. In food sector, agar is used for gelling and thickening in the confectionary and bakery purposes and as

a stabilizer for the preparation of cheese. In fish and meat processing industries, agar is applied for canned products as a protective coating against the effect of metal containers. In brewery agar is used as a clarifying agent for wines, beer and liquors. In pharmaceutical industry agar is used as a laxative for chronic constipation, as a drug vehicle. Agar is an ion exchanger and is used in the manufacture of ion exchange resins. In cosmetic industry agar serves as a constituent of skin creams and ointments. Agar is also employed in paper and textile industries as finishing and sizing agents.

Algin or alginic acid is a membrane mucilage and a major constituent of all alginates. The various salts of alginic acid are termed "alginates" (eg., sodium alginate, calcium alginate etc). In pharmaceutical industry alginic acid is used as emulsifiers in watery emulsions with fats, oils and waxes as filters in the manufacture of tablets, pills and as base of any ointments. An alginate gauze is used as a blood stopping plaster. As a slimming agent, the alginate forms a jelly in the stomach which produces the feeling of satiation. Ammonium alginate wool is used as a filter for microorganisms for laminar flowhood.

Carrageenan is a sulphated galactan polymer obtained from various red seaweeds belonging to families such as Gigartinaceae, Soliriaceae and Hypneaceae. In food industry carrageenan finds its use in bakery, confectionery and for the culinary purposes especially in the preparation of condiments, syrups, whipped creams, ice disserts, cheese etc. Carrageenan is used for clarifying fruit juices and other beverages. Quality of wheat flour is improved for making spaghetti and parotta by adding carrageenan. The food sector accounts for nearly 70% of the world market for carrageenan.

Mannitol is an important sugar alcohol of the hexite series found in the cell sap of brown algae. Mannitol also occurs as mannitan. The chief raw material for the extraction of mannitol are *Fucus vesiculosus*, *Bifurcaria brassiformis*, *Sargassum* spp, *Turbinaria* spp etc. In pharmacy mannitol is used for the preparation of tablets, for making diabetic diet, chewing gum etc. Mannitol is also used in explosives and other

pyrotechniques. Mannitol finds its use as plasticizers for the production of resins

Liquid seaweed fertilizer Seaweed extract is made into mineral rich liquid seaweed fertilizer (LSF) and marketed under various trade names. Studies have proved that extracts of *Sargassum wightii*, *Ulva lactuca*, and *Spathoglossum asperum* at 1% strength show favourable response on the germination, seedling vigour, fruit setting and on the weight of the fruit in crops such as groundnut, maize, gingelly, tomato and ber. Liquid seaweed extract was first patented in the year 1912. Another patent was offered in 1962 by Maxicrop Ltd and marketed as "Maxicrop" and "Bioextract". When foliar feeding became an orthodox method of plant nutrition in the 1950s 'Marinure', 'SM-3' and 'Trident' brands were made in the UK in 1966 and 'Algifert' in Norway in 1970. In India SPIC manufactures and markets LSF in the name of 'Cytozyme'.

Cattle feed from seaweeds Shrinkage of cultivable land due to urbanization and shortage of water limit the possibility of producing more feed and fodder to livestock from land. Sea remains untapped and the seaweed resources has got immense potential to fill the gap in India. Seaweeds are the marine macrophytic thallophytes and as animal feed had been in use as early as first century BC by the Greeks. Seaweed has been used by farmers living near the sea in Europe. In Norway *Ascophyllum* is used as pigmeal. *Rhodymenia palmata* a red seaweed is called cow weed in Brittany and horse weed in Norway. Dried and processed seaweeds have been used as animal feed in Europe and North America.

Seaweeds are rich in protein (20- 25%), carbohydrate (50-70%), vitamins, minerals and certain drugs. When used in animal feed, cows produced more milk, chicken eggs became better pigmented and horses and pets became healthier (White and Keleshian, 1994). Feed supplemented with *Gracilaria* and/or *Spirulina* to layer chicks (white leghorn) increased the number of eggs, size and colour of yolk (Chaturvedi *et al.* 1985). Dave *et al.* (1977) assessed the possibility of seaweeds being used as supplementary animal feed and they reviewed the feeding trials of farm animals with seaweeds conducted in Japan, Germany, the UK and Norway

Seaweed farming

Seaweed farming is encouraged in developing countries as it provides employment for poor fishermen in growing the seaweed as well as in processing industries. One of the biggest exporters of cultured seaweed is China where the industry employs between 100,000-120,000 people. Seaweeds are cultivated for their commercial importance of phycocolloids such as agar, algin and carrageenan besides, their use as food, source of enzymes, dyes, drugs, antibiotics etc.

Cultivation of seaweeds in India

Seaweed mariculture in India mostly dealt with cultivation of *Gracilaria edulis* due to its high regenerative capacity. Very recently the cultivation of *Kappaphycus*, an exotic carrageenophyte introduced in Indian waters found to be very encouraging and shall help overcome the shortage for raw materials for extraction of carrageenan and will offer livelihood security to the coastal fishers. In India mariculture of seaweed was attempted by Central marine Fisheries Research Institute, Central Salt and Marine Chemicals Research Institute and National Institute of Oceanography.

In 1964 seaweed culture experiments were conducted for the first time in ponds at Porbander by attaching small plants of brown alga *Sargassum* to coir nets (Thivy, 1964). It was during the Second World War, due to the shortage of agar, that the Board of Scientific and Industrial Research started manufacture of agar in India at the Research Department of Kerala University (the erstwhile Travancore University). Since then, much stride has been made in these lines on the economic utilization of algae and the Central Marine Fisheries Research Institute developed a cottage industry method for the manufacture of agar from *Gracilaria* spp. and *Gelidium micropterum* (Thivy, 1960).

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in ponds at Porbander by attaching small plants of brown alga *Sargassum* to coir nets (Thivy, 1964). The plants of *Sargassum* grew to a height of 15-52 cm in 40 days from the initial height of 5-10 cm. This experiment revealed good possibilities for cultivation of *Sargassum* and other seaweeds in India. Agar yielding seaweed *Gracilaria edulis* was first cultured by long line rope method in a sandy lagoon on the eastern side of the Kurusadi Island (Rameswaram).

Since 1972 the Central Marine Fisheries Research Institute is engaged in the cultivation of several economically important seaweeds such as *Gracilaria edulis*, *Gelidiella acerosa*, *Sargassum wightii*, *Acanthophora spicifera* and *Ulva lactuca*. There are two methods for cultivation of seaweeds; one by means of vegetative propagation using fragments from mother plants and the other by different kinds of spores such as zoospores, monospores, tetraspores and carpospores. In the vegetative propagation method, the fragments are inserted in the twists of ropes, tied to nylon twine or polypropylene straw and cultured in the inshore areas of the sea. The fragments are also cultured by broadcasting them in outdoor ponds and tanks.

Vegetative propagation

Through the vegetative propagation method, where seaweed thallus (fronds) is the seed material and these can be collected from the natural bed from the intertidal area during the low tide. Seaweeds are cultivated on substrata such as 2 x 2 m nets (20 cm mesh) made of either nylon or coir (obtained from coconut husks) and on 10 m long ropes. Approximately 5 g of fronds is inserted or sandwiched between the twists of the rope at a distance of 10 cm in the long line or at each mesh in the 2 sq m nets. These "seeded" ropes or nets attached to floating rafts or bottom set fixed structures in the sea, especially protected bays, lagoons or shallow coast. The seeded ropes/nets are kept afloat in water either at surface or at the subsurface, suitable to the species cultivated by series of floats and sinkers.

There are many techniques of seaweed farming through vegetative propagation method to suit the location, season of farming and the species cultivated.

- Inserting the fragments to the twist of the nylon or coir long line rope or net and allow them to grow in natural environment. They can be placed in sea either by stationary structure or by floating raft (*Gracilaria*).
- Placing the fragments in the nylon net bag and tied to 10mm HDP rope at a fixed interval of distance (*Eucheuma*)
- Broadcasting the fragments in ponds, raceways and tanks.
- Tying the fragments in sand filled polyethylene tube (*Gracilaria*).
- Fronds can be fixed to small coral stones or pebbles and these 'seeded' stones are broadcast on the shallow sea bottom.

Reproductive method

The propagation of seaweed through reproductive method can be carried out by using the reproductive units like zoospores, carpospore, tetraspore, conchospore etc. In India, reproductive propagation of seaweed was successfully done in *Gracilaria edulis* liberating the carpospores on different substrata like nylon twine, cement blocks, HDP rope and old fishing net (Reeta and Ramoorthy, 1997). The spores liberated on the substrata were allowed to grow to germling stage in a nursery and then transplanted to natural environment during favourable period of growth. The spores reached to germling stage within 13-17 days of liberation and attachment to the substrata. Three consecutive harvest can be made from the same seed after 105 days till 135 days of culture period. It was observed that the growth is very encouraging after first and successive harvest by hand pruning. The favourable period of growth for cultivation of *Gracilaria* in southeast and south west coast was found to be from November to March.

Mariculture of *Kappaphycus*

The Central Salt and Marine Chemical Research Institute (CSMCRI) introduced this fast growing species of seaweed in the Diu coast (Gujarat) in 1995 for experiments in confined waters from the Philippines. After successful introduction and acclimatization it transferred the material and the technology to PepsiCo only after convincing itself

that its cultivation would be ecologically safe. The species is a source of carrageenan, a gel-forming agent widely used in the pharmaceutical and food industries. This algae is commercially cultivated in the open sea in a large area by the Pepsi Foods Limited (PFL) with the help of fishermen with whom the company has a "buy back" agreement. *Kappaphycus* also gives a liquid fertilizer that the company intends to market.

Seaweed farming and carbon sequestration

There has been a 35% increase in CO₂ emission worldwide since 1990 (IPCC, 2007). Carbon fixation by photoautotrophic algae has the potential to diminish the release of CO₂ into the atmosphere. Phytoplankton, seaweeds and seagrasses are excellent carbon sequestering agents than their terrestrial counterparts (Zou, 2005). It was estimated that the seaweed biomass occurring along the Indian coasts is capable of utilizing 9052 t of CO₂ day⁻¹ against emission of 365 t CO₂ day⁻¹ indicating strong sequestration of 8687 t of CO₂ day⁻¹ by seaweeds (Kaladharan *et al.*, 2009). Large scale mariculture of seaweeds along the Indian continental shelf is recommended as one of the positive anthropogenic activities to sequester CO₂ that can check global warming to a larger extent and in turn can check the ocean acidification.

Total estimated CO₂ absorbed (t/day) and emitted (t/day) by seaweed biomass along the Indian coasts

Seaweed industry in India

The seaweed industry in India is mainly a cottage industry and is based only on the natural stock of agar-yielding red seaweeds, such as *Gelidium acerosa* and *Gracilaria edulis*, and algin yielding brown seaweeds species such as *Sargassum* and *Tubinaria*. The production of total seaweeds in India in 2000 was approximately 600,000 tons (wet weight). India produces 110-132 tons of dry agar annually utilizing about 880-1100 tons of dry agarophytes. Annual algin production is 360 to 540 tons from 3,600 to 5,400 tons dry alginophytes. Perhaps, the first large scale commercial cultivation of seaweeds in India

Type of seaweeds	Standing crop (t)	Efficiency to absorb (mg/g/h)	CO ₂ absorbed (t/day)	Efficiency to emit (mg/g/h)	CO ₂ emitted (t/day)
Red	36523	1.60	584	1.0	365
Brown	41740	2.35	981	0	0
Green	182613	4.10	7487	0	0
Total	260876	8.05	9052	1.0	365

has been embarked upon by Pepsi Foods Ltd. (PFL) along a 10 km stretch of the Palk Bay side towards Mandapam (Ramanathapuram Dist.) in Tamil Nadu, with technical support from Marine Algal Research Center, CSMCRI, Mandapam. Furthermore, many agar, algin and carrageenan extracting industries have been established in different places along the maritime states of Tamil Nadu, Andhra Pradesh, Kerala, Karnataka and Gujarat. The seaweed industry is certainly on its way marching towards socio economic development of our nation.

Biotechnological interventions in seaweed

- Barcoding seaweeds for eliminating ambiguity in species identification
- Improvement of species for better yield of phycocolloids and for fast growth
- Isolation of growth promoters, pigments, enzymes, drugs and nutraceuticals
- Isolation and culture of protoplasts for propagation and strain improvement
- Production of biofertilizers and animal feed
- Bioremediation and water quality management and
- Production of biofuel and bioenergy from seaweeds

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Seagrass Diversity

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Seagrasses are the marine monocots, which constitute about 0.01% of flowering plants and have adapted to the submerged marine habitat. They occur in most shallow, soft-bottomed marine coastlines, estuaries and lagoons. They are submerged marine angiosperms and mainly distributed in Southeast Asian countries, Australian and Caribbean coasts. Seagrass ecosystem is associated with several faunal and floral assemblages such as algae, sponges, corals, crustaceans, molluscs and fishes. The growth and distribution of seagrasses are controlled by a number of physical parameters such as temperature, salinity, light regime, sediment type and availability of nutrients. Seagrasses form the nursery and feeding ground for a number of marine organisms. They are highly involved in the detritus food web and play an important role in the recycling of nutrients.

Seagrass ecosystem forms one of the important coastal ecosystems of tropical and temperate regions. This ecosystem is conspicuous and often dominant habitats in shallow water coastal areas (den Hartog, 1970). This ecosystem is well known for its high primary and secondary productivity, ability to stabilize sediments, production of vast quantities of detritus and support of diverse faunal and floral communities (Phillips and Mc Roy, 1980). Seagrasses stabilize and hold sediments, thus preventing erosion. Due to the shallow water existence, seagrasses are generally subjected to anthropogenic activities, such as water sports, dredging, sewage disposal, etc. When considering all these, seagrass bed monitoring demands prime importance in the integrated coastal zone management.

Seagrass meadows may include mono-specific or multi-species communities. They exhibit a variety of leaf shapes, shoot densities and rhizome

characteristics (Fig. 1). Many meadows are not uniform in appearance, due to biological and physical disturbances. Seagrasses grow in soft sediments, from the low water mark to the depths of about 3-5 m and are inhabited by a rich associated biota. At the deeper end of the seagrass meadows, light becomes a limiting factor, strongly affecting photosynthesis and the lower limit is usually related to light irradiance. As the rhizome system grows and extends laterally, shoots may be sent up. A well-developed seagrass bed

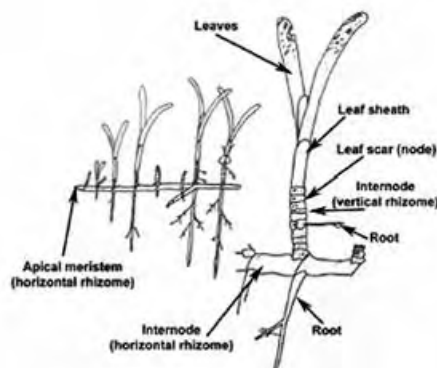


Fig. 1. Key morphological features of seagrass (adapted from Hemminga and Duarte, 2000)

may extend laterally into bare sediments by means of the rhizome system. Dissolved nutrients are taken up by the rhizomes and roots mainly from the pore water present in sediments.

Seagrasses are flowering plants and the pollens are transported through the water currents. They produce seeds and are borne by water currents. Seagrasses appear to reproduce more by asexual method, through the rhizome system. Colonization of new areas by seedlings is difficult unless the sediment is already physically stable and rich in dissolved nutrients. This can be accomplished by the presence of other plants such as seaweeds, which stabilize the sediments and add nutrients.

Thus, through succession a patch of bare sand may change to a bed of seagrass.

The complex ecology and multiple roles (Fig. 2) that seagrass communities carry out thrust the need for maintaining and improving these communities. Like mangrove and salt marsh communities, seagrasses are important primary producers. The abundance and diversity of ichthyo-fauna in seagrass meadows is well known. The roles of benthic algae are less understood,

proposal of a gradual transition of hydrophytic species into saline habitats was the prevailing view until den Hartog (1970) suggested that fossils from cretaceous deposits in Japan (*Archeozostera*) and the Netherlands (*Thalassocharis*) represent primitive seagrasses. According to him, seagrasses evolved from xerophytic plants that tolerated salt, they then would have to become tolerant of a hydrophytic habitat. Seagrasses probably arose in the mid- to late Cretaceous (65 to 40 million years) after angiosperms

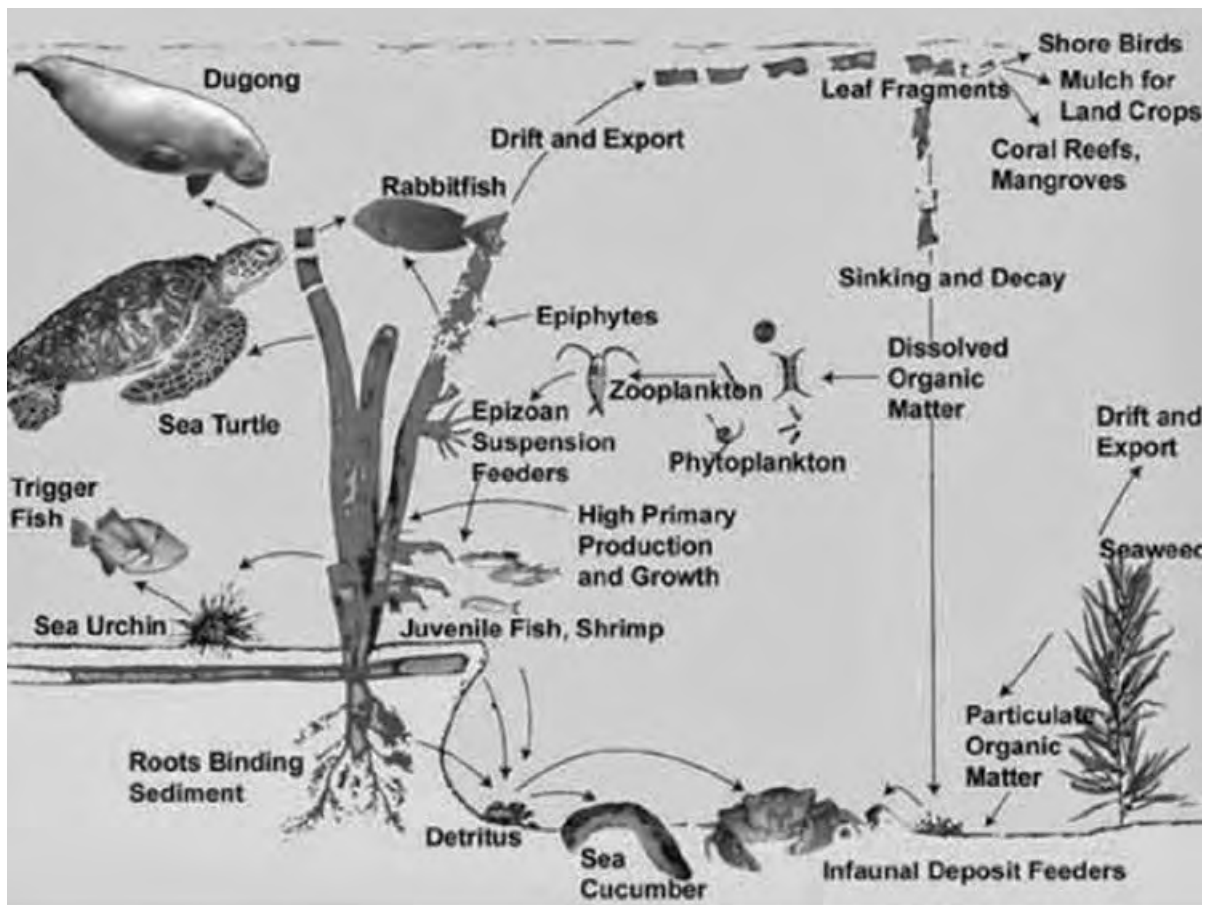


Fig. 2. Summary of seagrass ecosystem (adapted from Fortes, 1990)

although drift species are known to serve as habitats and food source for gammaridean amphipods. Further, these submerged flowering plants can be used to monitor the health of coastal ecosystems. So the need for conservation and management of seagrass meadows is evident when their extensive ecological roles are considered.

They have originated from freshwater and estuarine hydrophytic relatives (Arber, 1920) or from xerophytic salt marsh like plants (den Hartog, 1970). The

began to evolve and spread on land, in the earlier portion of this period (120 million years).

Seagrasses belong to the families Hydrocharitaceae and Cymodoceaceae. den Hartog (1970) recorded 49 species and 12 genera in 2 families of the class **Helobiae** (Monocotylidoneae). Kuo and Mc Comb (1989) recorded 58 species and 12 genera, which are placed in 4 families, 2 orders (Hydrocharitales and Potamogetonales) and 1 class Liliopsida. They include 3 genera in the family Hydrocharitaceae, 5

genera in the family Cymodoceaceae, 1 genus in the family Posidoniaceae and 3 genera in Zosteraceae, Ruppiaceae also included in seagrass community. The genera *Enhaulius*, *Halophila*, and *Thalassia* belong to the family Hydrocharitaceae; *Syringodium*, *Halodule Cymodocea*, *Amphibolis* and *Thalassodendron* belong to the family Cymodoceaceae. Family Zosteraceae include *Zostera*, *Heterozostera* and *Phyllospadix*, and *Posidonia* included in the family Posidoneaceae. Lee Long *et al.*, (2000) described 60 species worldwide within 12 genera, 4 families and 2 orders. According to him, *Halophila* and *Thalassia* belong to the family Hydrocharitaceae and order Hydrocharitales; *Cymodocea*, *Halodule* and *Syringodium* belong to the family Cymodoceaceae and the order Potamogetonales.

Five characteristics have contributed to making seagrasses the most successful tropical shallow marine community. These characteristics (den Hartog, 1970) are:

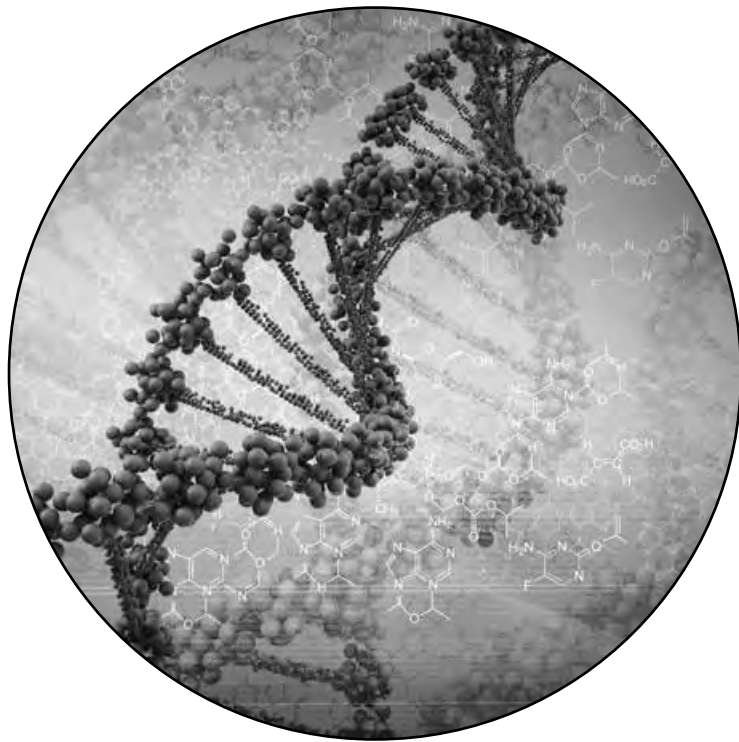
1. The ability to live in a saline medium. Seagrasses are actually killed in low salinities.
2. The ability to function physiologically while fully submerged, unlike mangroves, which rely on air exposure and pneumatophores for gas exchange.
3. A well developed anchoring system and the ability to slow near bottom currents that aids in the accumulation of sediments.
4. The ability to reproduce while submerged.
5. The ability to compete with other marine organisms for space and resources.

The importance of seagrasses in coastal and near shore environments and their contribution to the productivity of the world's oceans has become increasingly recognized over the last four decades. They play a significant role in the processes and resources of near shore coastal ecosystems. They having adapted to the submerged aquatic environment, show morphological and anatomical

features, which obviously form the constraints in their geographic distribution and speciation. They possess a well-developed creeping rhizome and an erect shoot bearing several foliage leaves. A well-developed seagrass system may develop laterally into bare sediment by means of rhizome system. Thus bare sand may change by succession to a seagrass bed and appear to reproduce more by vegetative method *i.e.*, through the rhizome system. Distribution of seagrass in the deeper areas of the water body is limited by intensity of light, since they depend on light for photosynthesis.

For the protection and management of seagrass meadows, information about their ecosystem services is essential. It is important to document floral and faunal species diversity, distribution and abundance in seagrass meadow to identify the areas requiring conservation measures. In such a point of view, this study forms the baseline information about the seagrass meadow of Minicoy lagoon. Responsive management based on adequate information will help to prevent any further significant areas or species being lost.

Seagrass ecology has evolved as a major part of aquatic ecology, from a descriptive stage, focused on the distribution and biology of the plants, to a quantitative, process-oriented stage. Research efforts over the past four decades have generated widespread awareness of the importance of seagrass meadows as marine ecosystems, thereby placing seagrass ecosystems as primary targets for marine conservation and restoration programmes. These achievements have resulted from the efforts of a growing community of seagrass ecologists. The scientific studies on seagrass ecology are still limited compared to many marine ecosystems. Moreover, understanding the ecology of seagrass meadows would enable a better basis to sustainably manage these ecosystems, because seagrass meadows are still being lost from the world's coastal ocean at alarming rates.



Molecular Biology

Standard Operating Procedure (SOP)

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Introduction

The primary aim and idea behind formulating a code of conduct classified as standard operating procedure in a biotechnology laboratory is to ensure the safety and security of persons involved in research in different capacities. Such a protocol would certainly ensure the judicious use of sensitive and expensive chemicals and enzymes in a completely secured manner. One can easily assess the level of understanding and to the extent of practising SOP through a self-valuation procedure. The questions to be answered in this process are the following.

- Are you aware about the security and safety rules to be followed in a biological laboratory using sensitive and hazardous chemicals?
- Do you know the procedure for storing and handling hazardous materials?
- Are you practising scientific protocols during handling and disposal of biological and chemical materials?
- What is your level of understanding about the health hazards which could cause by the chemicals in regular use?
- What sort of personal protective equipment is required to protect you from hazardous chemicals?
- How do you respond in the event of causality?
- Are you well aware about the medical attention to be followed during emergency?

If you are successful in responding properly to the above queries, you could assess yourself as proficient in SOP.

Laboratory Security

As biotechnology Institutes and Industries use variety of hazardous materials, the proper care and security is to

be given to avoid the access of the same in the hands of unwanted social elements. Further proper inventory register and a record of the usage of such materials is to be maintained. Unauthorised handling or removal of hazardous materials is to be restricted in the laboratory. Students who are new to the system are to be trained in the safety protocols to be followed to avoid the theft of such hazardous materials as the same is important from the angle of National security. The protocols related to security are to be designed and practiced based on the situations in each laboratory with the due approval of certifying agencies. Laboratory group is to be trained and responsibilities are to be entrusted to ensure the error free practice of the protocols related to security. Each personal working in laboratory should be aware about an emergency plan and security protocols for reporting immediately in the event of any kind of incidents leads to causality. Proper training should be given to operate fire extinguishers and other emergency handling devices.

Inventory of equipment, Operational knowledge and Periodical servicing

The laboratory manager or the personal concerned should be aware of the instruments and other minor equipment available. The new recruits should be trained properly on the operational protocols which should be displayed for speedy reference. A log book is to be maintained to record the use of the instruments with adequate instructions to note remarks. Periodic service of the instruments should be monitored to ensure the break down free service. All documents related such correspondences should be made available in the laboratory as the same may be reviewed in social auditing. Adequate power supply depending on the power

requirements of the instruments should be made available in the laboratory.

Storage & handling of chemicals, enzymes and reagents

There is no universal formula or procedures for handling sensitive materials like chemical and enzymes used in a biotechnology laboratory. Some common methodologies to be followed by one and all include proper labelling of all chemicals so that even a person who is new to the system would find it easy. There should be a Material Safety Data Sheets (MSDS) in file for each chemicals and enzymes available in the laboratory. In many countries MSDS is a legally required technical document which is normally being provided by the suppliers.

Specific parameters to be included in the MSDS sheet are as follows

- Identity of the chemical along with contact information's of manufacturer.
- The identity of hazard ingredients
- Physical and Chemical characteristic of the product.
- Nature of reactivity –This is vital information to determine the place of storage
- Examples- The acids and bases reacts each other and should not be stored in adjacent places). Certain other chemicals with chances to react with water should be stored with desiccants.
- Health related details- include the toxicological properties like carcinogenicity, lethal dose (LD50).
- Precautions and how to handle the chemical safely.
- Details regarding the personal protective equipment (PPE) suggested wearing.

Safety precautions to handle hazardous chemicals

One should be well aware about the general route through which a toxic chemical can enter into the body. There exist primarily four routes.

- Skin and eye –avoid with the use of lab coats, gloves and goggles.
- Ingestion–keep away the habit of eating and

drinking inside the laboratory and also avoid leaving the lab with gloves and without washing hands.

- Inhalation–Use masks and fume hoods.
- Injection- Ensure the Proper disposal of all broken glasses and needles.
- Always try to remove gloves while using phones and touching light switches *etc.*..

List of common hazardous chemicals in a biotechnology Lab

- Formaldehyde (carcinogen).
- Acrylamide (Neurotoxins).
- Acetonitrile (Nephrotoxins).
- Ethidium Bromide (mutagens).
- Formamide (Teratogens).
- Chloroform (Hepatotoxins).
- Phenol, strong acids and bases (Corrosives).

Handling Biological hazards

As all well aware, biotechnology laboratories are routinely doing experiments with many biological organisms and few of them are harmful to the user as well as to the environment. Bacteria, fungi and viruses isolated in a biotechnological laboratory include both harmless and harmful microorganisms and many of its toxicological and pathogenic properties are unknown to us. Hence as a general rule, extreme precautions are to be followed while handling microorganisms. Stringent protocols have to be designed especially in Indian Scenario as we do not have generalised pattern in connection with the microbial safety to be followed by all laboratories. The recombinant DNA molecules produced in biotechnology laboratories are to be considered as a biohazard as it could bring about dangerous changes in the host. Same is the case with animal and tissue cultured organisms as they can make an impact in the environment. General precautions such as wearing PPE, washing hands, wearing gloves *etc.* described above for chemicals are to be followed for handling live organisms as well. One has to be more careful and must avoid touching face wearing gloves and keep away all personal belongings in the designated area. Following are the essential precautions to be followed while dealing with live organisms.

- Avoid mouth pipetting and use mechanical devices.
- Splashes and aerosol generation is to be kept to minimum as majority of the incidents of infection is through inhalation.
- Always try to ensure the shaking or mixing in closed containers to avoid the release of aerosols.
- Disinfect the glassware and workbench after each work session.
- Specific microbial disinfectants like formalin are to be used along with common bleaching powder or ethanol.
- Use laminar flow hood safety cabinet.

Handling of Radioactive materials

It is quite common nowadays to use radioactive isotopes in biotechnology laboratories working with protocols in genomics and proteomics though non isotopic labelling methods are coming up for labelling experiments. Radioactive safety measures are generally followed by all the laboratories owing to awareness of health hazard associated. Laboratory must have the services of a trained person in radioactive safety as a mandatory requirement. Specific precautions to be practiced while dealing with radioactive materials are as follows.

- Earmark certain specific area displayed with radioactive safety symbols to carry out radioactive labelling experiments.
- Always stick to the guidelines prescribed by the suppliers (BARC) in handling the material.
- Work behind radioactive shields.
- Radioactive detectors are to be used to ensure the area is free from radiations after each work session.
- The wastes including the tissue papers are to be stored separately for disposal.

Disposal of biological, chemical and radioactive waste

One of the most sensitive and complex area with lots of challenges involved is the perfect and speedy disposal of wastes generated from the biotechnology laboratory. Methodologies to be followed depend on the characteristic feature of the waste to be disposed.

Chemicals wastes have to be neutralised before disposal through proper treatment. Buffers, acids and reagents have to be properly diluted before disposal. The real challenge in a biotechnology laboratory is the management of the disposal of biological wastes. Wastes from microbial cultures and other biological samples have to be collected in autoclave bags made of high melting point plastic so that they can be killed at high temperature and pressure before disposal. Students have to brief about the proper waste management and disposal before starting each session which involve lengthy experiments like tissue culture and cell line maintenance. Radioactive waste materials including the laboratory wares and filter papers used have to be collected in separate containers following the guidelines. Usually the radioactive wastes are disposed by separate department like health physics department upon receiving the same from laboratories.

Summary of Good Laboratory Practices

In order to solve the problems faced by a researcher, he/she should be aware about the role of the reagent in isolating macromolecules having different physical and chemical properties. You should be able to anticipate the molecular interaction between different buffers while using simultaneously.

Quality of the Reagents

Always try to use good quality enzymes and reagents in molecular biology and even if substitute go for a higher grade than a lower grade.

Quality of the Water

Try to ensure that you are using good quality water suiting to the requirements in molecular biology. Water prepared through reverse osmosis is the best quality preferred nowadays over distilled and deionized.

Storage life

Try to dispose buffers and other reagents beyond storage life and always check the microbial contamination within the stored reagents and buffers. Media and other buffers should be chemically as well

as microbiologically sterile to avoid contamination and precipitation.

Suggested readings

Molecular Biology Problem Solver, edited by Alan S. Gerstein
ISBN 0-471-37972-7.

Principles of Isolation, Purification and Analysis of Nucleic Acids

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Introduction

Advanced Biotechnological research is largely depended on the genome analysis and recombinant DNA technology. Good quality nucleic acid is an essential prerequisite for consistent results in most of the downstream applications in the genome analysis and recombinant DNA technology. The general principle underlying the isolation of nucleic acids is common with few modifications depending on the type of nucleic acids being isolated. The type of the nucleic acid intending to isolate is to be made free from the other biological macromolecules and cell debris. This is achieved by properly lysing the cell wall or cell membrane as the case may be and by selectively denaturing the other macromolecules like proteins. Nucleic acids thus recovered in its native form is to be purified by removing the very closely associated molecules. The finely purified molecule is precipitated by alcohol and suspended in sterile buffer or distilled water. Finally, the qualitative integrity of the isolated nucleic acids is to be checked by agarose gel electrophoresis and ethidium bromide staining before proceeding with the further downstream applications. Quantitative estimation of nucleic acids are carried out by spectrophotometric and fluorimetric methods.

The types of nucleic acids usually isolated on a routine basis are:

- Total genomic DNA
- Total RNA
- Plasmid DNA & Mitochondrial DNA

Total Genomic DNA

Breaking of the bacterial and plant cell walls as well as solubilizing the cell membrane of animal tissue are to be carefully carried out under optimum

conditions. Even the rapid stirring of solution can break high molecular weight DNA into shorter fragments. Vigorous shaking will cause nicks and even cut open the covalently closed circular structures of plasmid and mitochondrial DNA. If physical disruption is necessary as is the case with certain types of tissues, it should be kept to the minimum, and should involve cutting or squashing of cells, rather than the use of shear forces. Ultra sonic sounds are used to disrupt the tough cell wall of certain bacteria. Care has to be taken to prevent degradation of DNA by deoxyribonucleases. These enzymes are found in most cells, and may also be present in dust which could contaminate laboratory glass wares. Hence all the glass wares, plastic wares and the homogenizing buffer are to be made sterile by autoclaving. This enzyme activity can be inhibited by using EDTA in buffers which will chelate the Mg^{++} ions needed for DNase activity. Cell disruption and most of the subsequent steps should be performed at 4°C. The cell wall could be lysed enzymatically as well. The bacterial cell wall is usually lysed by the enzyme Lysozyme. The cell membranes on the other hand are solubilised by including suitable detergent in the homogenizing buffer. Upon lysis the nucleic acids will be released into the cytoplasm and now the target molecule, DNA, is to be made free from RNA and other associated proteins. The RNA molecules can be selectively denatured by enzymatic treatment with RNase. Prior to its use, the RNase is to be heat treated to inactivate any DNase contaminants. RNase is relatively stable to heat as a result of its disulphide bonds, which ensure rapid renaturation of the molecule on cooling. The other major contaminant, protein, is removed by enzymatic

treatment with proteinase K followed by shaking with water saturated phenol or with phenol-chloroform mixture, either of which will denature proteins but not nucleic acids. Centrifugation of the emulsion formed by this mixing produces a lower, organic phase, separated from the upper, aqueous phase by an interface of denatured protein. It is advisable to use cut micro tips while proceeding through these steps. The aqueous solution is recovered and deproteinised repeatedly until no material is seen at the interface. Finally the deproteinised DNA preparation is mixed with two volumes of absolute ethanol, and allowed to precipitate out of solution in a freezer. After centrifugation, the DNA pellet is redissolved in a buffer containing EDTA for protection against DNases, and this solution can be stored at 4°C for at least a month. DNA solutions can be stored frozen, but repeated freezing and thawing tends to damage long molecules by shearing and hence the DNA preparations in frequent use are normally stored at 4°C.

Plasmid & Mitochondrial DNA

The principle of isolation of plasmid and mitochondrial DNA is based on the structural characteristics. Plasmids are double stranded, Covalently Closed Circular (CCC) or super coiled structures. Similarly mt.DNA is also having the same structural characteristics and hence almost the same isolation procedure can be adapted. Bacterial cell wall is to be broken by enzymatic treatment (lysozyme) in a suitable buffer with a suitable metallic chelator like EDTA before initiating the isolation process. The tissue for the mt.DNA isolation is to be thoroughly homogenized under ice cold conditions.

The classical method is to isolate the plasmid and mitochondrial DNA by Caesium chloride density gradient ultra-centrifugation in the presence of ethidium bromide. Ethidium bromide causes unwinding of DNA as it binds to it, simultaneously producing a decrease in its buoyant density. Since the super coiled plasmid and mt.DNA can unwind to only a very limited extent, it will not bind as much ethidium bromide as with the linear and open circle forms of DNA in the presence of saturating levels of

ethidium bromide. Because of this density difference, plasmid and mt.DNA can be separated from other DNA by ultra-centrifugation.

Another method which is relatively fast is based on alkaline lysis. In this method the property of super coiled DNA to remain intact at pH between 12 and 12.5 is exploited for the isolation. At this pH selective denaturation of linear DNA will occur whereas the super coiled DNA will remain intact. Further reduction of the pH to acidic condition will enhance the formation of a complex network of proteins and linear DNA and the resultant supernatant after centrifugation will contain the intact plasmid or mt.DNA. This can be purified and precipitated as in DNA isolation procedures. For mitochondrial DNA, this method works well with fresh tissues with minimum nicks.

Mitochondrial DNA can also be isolated by differential centrifugation technique. This involves the selective isolation of the mitochondria which is further lysed with suitable detergents to release the mt.DNA. This will be further purified and precipitated by conventional means.

RNA

RNA molecules are relatively short, and therefore less affected by shearing. RNA is, however very vulnerable to digestion by RNases which are present abundantly even on fingers. These enzymes are stable and generally require no co-factors. Hence gloves should be worn, and a strong detergent should be included in the isolation medium to denature any RNases immediately. The solutions used are to be treated with nuclease inhibitors like Diethyl pyrocarbonate (DEPC). Care should be taken while using DEPC as it is a suspected carcinogen. Glass wares should be baked at 300°C for 4 to 5 hours as autoclaving alone may not be sufficient to fully inactivate RNases. The plastic ware can be rinsed with chloroform. Tissue homogenization is to be carried out under ice cold conditions with all the precautions detailed above. As in the case of DNA, RNA is to be made free from DNA and proteins. Proteins are denatured by proteinase K treatment followed by phenol chloroform extraction. This is followed by the ethanol precipitation of

RNA in the presence of sodium acetate or sodium chloride. The overnight precipitated pellet is washed with 70% ethanol to remove the salts and finally dissolved in DEPC treated water. Contaminating DNA can be removed by treatment with RNases free DNase. The RNase can be inactivated by RNAsin or vanadylribonucleoside complex.

Commercially available kits

Several readymade kits are available commercially and many laboratories are depending on such products for the isolation of nucleic acids. In most of these kits, the nucleic acids are either trapped by ultra-filtration membranes or allowed to bind with certain resins which have affinity towards nucleic acids. The advantage with these kits is that the process is very fast and devoid of using corrosive organic chemicals like phenol. The main disadvantage is that they are quite expensive and hence unaffordable to many laboratories. Hence it is advisable to use alternative non organic protocols, for DNA isolation, based on the use of high concentration of salts for removing proteins in place of phenol, which are easy to perform in the laboratories especially while isolating from

liquid connective tissues like blood, haemolymph etc. Meanwhile, the commercial kits are effectively used for the isolation of total RNA and mRNA as the manual isolation is a sensitive process with increased chances of degradation.

Quantitative Estimation of nucleic acids

DNA and RNA can be spectrophotometrically estimated by taking optical density (OD) at 260nm, 1 O.D corresponds to 50 micro gram of DNA and 40 micro gram of RNA. Purity of the DNA can also checked spectrophotometrically by taking O.D at 260 & 280nms. The ratio of 260 and 280 will result a value of 1.8 with pure nucleic acid preparations.

Suggested readings

- Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). Molecular cloning. Cold Spring Harbor, New York.
- Old, R.W. and Primrose, S.B. (1985). Principles of Gene manipulation, 3rd edition. Blackwell Scientific Publications, Oxford.
- Walker, J. M. and Gaastra, W. (Eds) (1983). Techniques in Molecular biology. Croom Helm, London.

Polymerase Chain Reaction and its various modifications

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Introduction

Polymerase Chain Reaction or PCR is a molecular technique which allows in vitro synthesis of billions of copies of a target DNA fragment within hours using a simple enzymatic reaction. This is achieved by using a pair oligonucleotide primers that hybridize (anneal) to the complementary sequences on the opposite strands of the target DNA, at positions flanking the region to be amplified. New strands are made through the simultaneous extension of both the primers by addition of nucleotides to the primers by the enzyme DNA polymerase. A repetitive series of cycles involving template denaturation, primer annealing and extension of the annealed primers by the enzyme DNA polymerase results in the exponential accumulation of the DNA whose termini are defined by the 5' ends of the primers. Since the primer extension products synthesized in one cycle can serve a template for the next, the number of target DNA copies approximately doubles at every cycle. Thus 20 cycles of PCR can yield about a million-fold amplification. The method is simple, as the PCR can be performed in a single tube. It can be performed on relatively crude DNA containing samples. These factors have made the PCR an attractive method for amplification of specific sequences. This method is extremely rapid; it takes only 3 hours to amplify a known sequence of interest. PCR generates sufficient copy numbers of target DNA sequences for their routine visualization through standard procedures such as electrophoresis followed by staining with ethidium bromide. The PCR products may be sequenced to determine the exact sequence of the nucleotides within the amplified product. As a result, the PCR permits routine analysis of DNA from single egg and larvae, and from non-invasively

secured tissues such as fin clips and scales. Even partially degraded DNA from poorly preserved sources can be analyzed if sufficiently small PCR products are identified.

Discovery of PCR

The concept of PCR was first conceived by Dr. Kary Mullis in 1983, first reported in 1985, while working at the Cetus Corporation in Emeryville, CA, along with other researchers at Cetus Corporation (Molecular Station, 2006). Kary Mullis discovered that by harnessing one component of molecular reproduction technology, ie a basic principle of replicating a piece of DNA using two primers, a target DNA of interest could be amplified exponentially. This DNA amplification procedure was an in vitro process (meaning in a test-tube). The first ever PCR product was the 110 base pair DNA fragment of a cloned segment of the human beta-globulin gene at the company labs, being the beginning of PCR as a basic technique in molecular biology (Mullis *et al.*, 1986, Mullis and Faloona 1987). Dr. Mullis was awarded the Nobel Prize in Chemistry in 1993 for his development of the Polymerase Chain Reaction (PCR), a central technique in biochemistry and molecular biology. Dr. Mullis subsequently was awarded the Japan Prize that same year.

Materials and reagents for PCR

The components required for the PCR are the template (the DNA to be amplified), a pair of primers, thermostable polymerase, the four types of de-oxynucleotide triphosphates (dATP, dCTP,

dGTP and dTTP) and appropriate reaction buffer containing magnesium ions (KCl, Tris-HCl (pH 8.4), MgCl₂ and gelatin). They are assembled in a tube and the amplification reaction is carried out by manipulating the temperature within the reaction tube, in cyclic manner, using a thermal cycler. For any given pair of primers, the optimal concentrations of all the above ingredients and parameters have to be standardized. Even though there is no single set of conditions and concentrations that will be optimal for all reactions, the parameters outlined below defines a common starting point from where modifications can be attempted.

Target DNA (Template): An advantage of PCR is that it can amplify relatively impure DNA or DNA from blood spots, archival material and ancient DNA. Concentration of template DNA also affects the degree of amplification. The sample DNA generally contains 10² to 10⁵ copies of template. Too high or too low concentration will result in poor amplification. Therefore, it is useful to optimize the template concentration in a PCR reaction to obtain maximum product. While typically DNA quantity is measured in ng, the relevant unit is actually moles, *i.e.*, how many copies of the sequence that will anneal with the primers are present. Thus, the amount of DNA in ng that is needed to add is a function of its complexity. In theory, a single molecule of DNA can be used in PCR but normally between 1000 and 100,000 molecules for eukaryotic nuclear DNA are used. The nucleotide composition of target DNA also affects the PCR amplification. Extremely GC rich DNA strands are difficult to separate. Addition of denaturing agents like formamide or DMSO can help to overcome the problem.

Primers: Primers are the most important components of PCR, and the success of a PCR largely depends on the primers. Primers are short, single stranded DNA molecules which will bind (anneal) to either ends of region to be amplified (one on each strand) and serve as the starting point for attaching nucleotides on its 3' end, leading to the building of a new complementary nucleic acid strand. Primers are generally made in pairs, called "forward" and "reverse". These primers are complimentary to the regions flanking the DNA segment to be amplified such that they can be

extended toward one another with DNA polymerase, forming new DNA molecules. The most important property of a primer is its sequence specificity, which determines what nucleic acid sequence it can bind to, how well it will bind, and how well it will serve as a site for extension of new nucleic acid molecules. Generally, a "specific" primer is designed to target a DNA sequence in a closely related group of organisms, while not matching organisms outside that group. "Universal" primers are designed to target DNA sequences shared by any species that contains the sequence of interest. Thus, care should be taken while designing the primers for a particular experiment. Oligonucleotide primers in the range of 18 to 30 bases are generally used for the PCR. Though there are no set rules that will ensure the synthesis of an effective primer pair, the following guidelines are useful.

- Wherever possible, select primers with a random base distribution and with a GC content similar to that of the fragment being amplified. Avoid primers with stretches of polypurines, polypyrimidines or other unusual sequences.
- Check the primers against each other for sequence complementarity. Use primers with low complementarity to each other. Avoid primers with 3' end overlaps in particular. This will reduce incidence of "primer dimers". Most primers are generally 18 to 30 bases in length and the optimal length to be used in an amplification will vary. Longer primer may be synthesized but are seldom necessary. If shorter primers or degenerate primers are used, the thermal profile should be modified considering the lower stability of the primed target. However, the 3' end of the primer should match the template exactly. Generally, concentrations ranging from 10 to 50 p moles of each primer should be used.

The optimum length of a primer depends upon its (A+T) content, and the T_m of its partner. A prime consideration is that the primers should be complex enough so that the likelihood of annealing to sequences other than the chosen target is very low. For example, there is a 1/4 chance (4-1) of finding an A, G, C or T in any given DNA sequence; there is a 1/16 chance (4-2) of finding any dinucleotide sequence (eg. AG); a 1/256 chance of finding a given 4-base sequence. Thus, a given sixteen base sequence

will statistically be present only once in every 416 bases (= 4 294 967 296, or 4 billion): this is about the size of the human or maize genome, and 1000x greater than the genome size of *E. coli*. Thus, the association of a greater than 17-base oligonucleotide with its target sequence is an extremely sequence-specific process. Generally, 17-mer or longer primers are routinely used for amplification from genomic DNA of animals and plants. Long primers will result in mismatch pairing and non-specific priming even at high annealing temperatures

Melting temperature (T_m) of primers: The annealing temperature is dependent on the T_m of primer. Annealing temperature can be the T_m value calculated using the following formulae:

$$(1) T_m = [(number\ of\ A+T\ residues) \times 2^{\circ}C] + [(number\ of\ G+C\ residues) \times 4^{\circ}C]$$

This formula was determined originally from oligonucleotide hybridization assays, which were performed in 1 M NaCl, and appears to be accurate in lower salt conditions only for primers less than or about 20 nucleotides in length.

$$(2) T_m p = 22 + 1.46 ([2 \times (G+C)] + (A+T))$$

This formula is reportedly useful for primers of 20-35 bases in length. The calculated annealing temperature is only a reference temperature from which to initiate experiments. The actual annealing temperature may be 3-12°C higher than the calculated T_m. The actual annealing temperature condition should be determined empirically. The optimum annealing temperature which gives the best PCR product should be used.

Deoxynucleotide triphosphate: The dNTPs are the building blocks of DNA. Once the primer binds to its target site, synthesis of the complementary strand of DNA takes place through primer extension by linking of nucleotide to its 3' end with the help of *Taq* DNA polymerase. Precursor dNTPs can be obtained as a neutralized solution, which are stable at -20°C for months. The deoxynucleotide triphosphate (dATP, dCTP, dGTP and dTTP) is generally used at concentrations of 200 mM (0.2mM) each. Higher concentrations may

lead to mis-incorporations. Low dNTP concentration reduces mispriming at non-target sites. The lowest dNTP concentration appropriate for the length and composition of the target must be standardized. As a thumb rule, 20mM of each dNTP in a 100 ml reaction is sufficient to synthesize 10 p Mol of a 400 bp sequence. In the standard reaction, all four triphosphates are added to a final concentration of 0.8mM; this leaves 0.7 mM of the original 1.5mM MgCl₂ not complexed with dNTP. Therefore, if dNTP concentration is changed significantly, a compensatory change in MgCl₂ may be necessary.

***Taq* DNA polymerase:** The discovery of thermostable DNA polymerase has revolutionized the PCR technology. They are obtained from organisms that thrive in extreme temperatures and have an optimum activity at 72° C. It is able to withstand the denaturing conditions (over 90°C) required during PCR cycling. Thus, unlike thermo-labile polymerase, with *Taq* polymerase there is no need for extra addition of enzymes during cycling process where strands separation required heating to over 90°C. There are now a plethora of commercially available enzymes to choose from that differ in their thermal stability, processivity, and fidelity. The choice of the DNA polymerase employed by PCR is determined by the goals of the experiment. The most commonly used thermostable polymerase is *Taq* DNA polymerase isolated from the bacterium *Thermus aquaticus* which inhabit the hot springs with extremely high temperatures. Isolation of the DNA polymerase from this bacterium yielded a PCR polymerase that was not rapidly inactivated at high temperatures. In 1986, Dr. David Gelfand and Ms. Susanne Stoffel of Cetus Corporation purified such a thermostable DNA polymerase, referred to as native *Taq* (*Thermophilus aquaticus* in short), from the organism *Thermus aquaticus*. *Taq* polymerase was shown to work successfully in PCR, enabling the process to be performed much more easily. Today, almost all PCR is done using recombinant *Taq*, a cloned version of the enzyme, as it is less expensive to manufacture than the native form of the enzyme (Roche Diagnostics, 2007). *Taq* was the first polymerase that was able to withstand the denaturing conditions (over 90°C) required during PCR cycling. *Taq* has an enzymatic half life

at 95°C of about 40 min. *Taq* DNA polymerase is unique in that it produces PCR products with A (Adenine) overhangs. This was found to be quite useful, and was exploited to produce TA Cloning and TOPO cloning. One of *Taq* polymerases' major disadvantages is its low replication fidelity. As *Taq* does not have 3' to 5' exonuclease proofreading mechanism to replace an accidental mismatch in the newly synthesized DNA strand, *Taq* produces more errors than proofreading polymerases, such as *Pfu* (Roche Diagnostics, 2007) *Pyrococcus furiosus*, where it functions in vivo to replicate the organism's DNA. *Pfu*'s have superior thermostability and 'proofreading' properties compared to other thermostable polymerases. Unlike *Taq* DNA polymerase, *Pfu* DNA polymerase possesses 3' to 5' exonuclease proofreading activity and corrects nucleotide-misincorporation errors. Thus *Pfu* DNA polymerase-generated PCR fragments will have fewer errors than *Taq*-generated PCR inserts. It also results in blunt-ended PCR products.

The required concentration of *Taq* DNA polymerase is between 1 and 2.5 units per 100 ml reaction when other parameters are optimum. When optimizing a PCR, enzyme concentration ranging from 0.5 to 5 units/ 100ml are tried and resultant products are visualized by agarose gel electrophoresis. If the enzyme concentration is too high, non-specific background products may accumulate and if too low, an insufficient amount of desired product is made.

The Reaction Buffer: The PCR buffer contains KCl, Tris HCl (pH 8.4), MgCl₂ and gelatin. The components

Table 1. Thermostable DNA polymerases

DNA Polymerase	Source
<i>Taq</i>	<i>Thermus aquaticus</i>
Amplitaq®	<i>T. aquaticus</i>
Amplitaq (Stoffel fragment)®	<i>T. aquaticus</i>
Hot <i>Tub</i> ™	<i>Thermus flavis</i>
Pyrostate™	<i>T. flavis</i>
Vent™	<i>Thermococcus litoralis</i>
Deep Vent™	<i>Pyrococcus</i> GB-D
<i>Tth</i>	<i>Thermus thermophilus</i>
<i>Pfu</i>	<i>Pyrococcus furiosus</i>
ULTma™	<i>Thermotoga maritima</i>

of PCR buffer, particularly the concentration of MgCl₂ have a profound effect on the specificity and yield of an amplification product. Success of PCR is dependent on MgCl₂ concentration in the reaction to a great extent. Mg²⁺ ions form a soluble complex with dNTPs which is essential for dNTP incorporation, stimulate polymerase activity and increase the T_m (melting temperature) of primer / template interaction (i.e. it serves to stabilize the duplex interaction). Concentration of about 1.0 to 1.5 mM is usually optimal (when 200uM each of dNTPs are used). Excess of Mg²⁺ will result in the accumulation of non-specific amplification products and insufficient Mg²⁺ will reduce the yield. Optimization by titration of MgCl₂ concentration is recommended to establish an optimum concentration for a particular reaction.

Several buffer formulations have been published & a consensus is emerging. The recommended PCR buffer should contain 10mM Tris-HCl (pH 8.4) also. KCl up to 50mM can be included in the reaction mixture to facilitate primer annealing. Excess KCl inhibits *Taq* polymerase activity. Gelatin or bovine serum albumin (100 mg/ml) and nonionic detergents such as Tween- 20 and NP40 (0.05–0.1%) are included to help stabilize the enzyme. The nonionic detergents can be replaced by 0.1% Triton X-100, but some detergent is essential.

Thermal Cycles for PCR

Amplification of a target DNA is achieved by repeated cycles of denaturation, primer annealing and extension. These events are controlled by manipulation of temperature. The above three major steps in a PCR are repeated for 35 to 40 cycles. This is done using an automated thermal cycler, which can heat and cool the tubes with the reaction mixture in a very short time.

Denaturation: Double stranded DNA used for the PCR is separated into single strands in the initial denaturation step. Typical denaturation temperature is 94°C for 15 to 60 seconds. Higher temperatures e.g. 97°C may be necessary for G + C rich targets. Denaturation steps that are too long or too high lead to unnecessary loss of enzyme activity. Denaturation of nucleic acid (NA) is carried out to make it

single-stranded for the purpose of annealing with primers. It is done by heating it to a point above the "melting temperature" of the double or partially double stranded form, and then flash-cooling it: this ensures the "denatured" or separated strands do not re-anneal. Additionally, if the NA is heated in buffers of ionic strength lower than 150 mM NaCl, the melting temperature is generally less than 100°C—which is why PCR works with denaturing temperatures of 91-97°C. The main reason of importance of denaturing temperature and time in relation to number of cycles is because *Taq* polymerase has a half-life of 30 min at 95°C. This half-life supports not more than about 30 amplification cycles. However, it is possible to reduce the denaturation temperature after about 10 rounds of amplification, as the mean length of target DNA is decreased. "Time at temperature" is the main reason for denaturation / loss of activity of *Taq*. Thus, with reduction of time, increases the number of cycles are possible, whether the temperature is reduced or not. It is possible, for short template sequences, to reduce this to 30 sec or less.

Primer annealing: At temperatures ranging from 47°C to 62°C, the primers anneal to its complimentary region on the template. The complimentary sequences will form hydrogen bonds between their complimentary bases (G to C, and A to T or U) and form a stable double stranded, anti-parallel molecule. During PCR, the primers are moving around, caused by the Brownian motion in the reaction mix. Hydrogen bonds are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bond lasts a little bit longer (primer that fit exactly) and on that little piece of doubling stranded DNA (template and primer), the polymerase can attach and starts copying the template. Once there are a few bases built in, the hydrogen bond is so strong between the template and the primer that it does not break any more. This is usually performed at temperatures between 47°C and 65°C for 30 to 60 seconds.

The temperature and length of time required for primer annealing depends upon the base composition, length and concentration of the primers. As a rule of the thumb, annealing temperature (T_a) of 5°C below the lowest melting temperature (T_m) of the

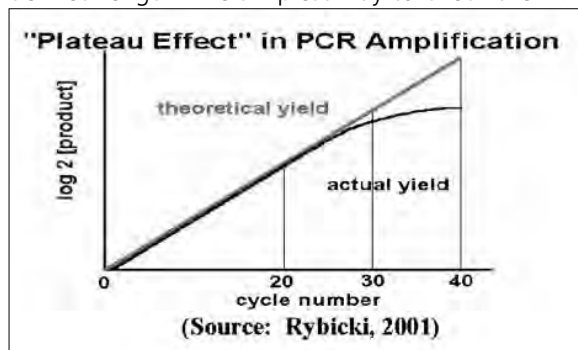
amplification pair of primers can be attempted. The annealing temperature chosen for a PCR depends directly on length and composition of the primer(s). Annealing temperature in the range of 55 to 65°C generally yield the best results. At the optimal primer concentration annealing will require only a few seconds. Increasing the annealing temperature enhances discrimination against incorrectly annealed primers and reduces mis-extension of incorrect nucleotides at the 3' end of the primers. Therefore, stringent annealing temperature, especially during initial few cycles will help to increase specificity.

Primer Extension: The DNA polymerase works ideally at temperature 72°C. The annealed primers, to which a few bases have been added, have a stronger attraction to the template, created by hydrogen bonds, than the forces breaking these attractions. Primers that are on positions with no exact match get loose again (because of the higher temperature) and do not give an extension of the fragment. The nucleotides (complementary to the template) are linked to the primer on the 3' side by the polymerase, from 5' to 3', reading the template from 3' to 5' side and bases are added complimentary to the template. Extension time depends on the length and concentration of the target sequence and upon the temperature. Primer extensions are usually performed at 72°C. The rate of nucleotide incorporation at 72°C varies from 35 to 100 nucleotides per second depending upon the buffer, pH, salt concentration and the nature of the DNA template. The length of the elongation step (30 seconds to three minutes) is determined by the speed of the enzyme, its ability to continue moving down the template DNA and the length of the DNA segment to be amplified. At around 70°C, the polymerase activity is optimal, and primer extension occurs at upto 100 bases/sec. A general guideline is 1 minute / kb of product length. An extension time of one minute at 72°C is considered sufficient for products up to 2 Kb size. Longer products require longer times: approximately 3 min for 3kb and longer products.

Cycling could include an initial denaturation at 94°C and a final extension at 72°C for 5 min. At the end reactions are stopped by chilling at 4°C or by addition of EDTA at 10mM.

Cycle number: The optimum number of amplification cycles necessary to produce a band visible on a gel depends largely on the starting concentration of the target DNA when other parameters are optimal. Because both strands are copied during PCR, there is exponential increase of the number of copies of the gene. For example if the PCR is initiated with one copy of the gene, after one cycle there will be 2 copies, after two cycles there will be 4 copies, three cycles will result in 8 copies and so on. Too many cycles may increase the amount and complexity of non-specific background products. Too few cycles give low product yield. Innis and Gelfand (1990) recommend from 40–45 cycles to amplify 50 target molecules, and 25–30 to amplify 3×10^5 molecules to the same concentration. This non-proportionality is due to a so-called *plateau effect* (Rybicki, 2001), which is the attenuation in the exponential rate of product accumulation in late stages of a PCR, when product reaches 0.3–1.0 nM. This may be caused by degradation of reactants (dNTPs, enzyme); reactant depletion (primers, dNTPs—former a problem with short products, latter for long products); end-product inhibition (pyrophosphate formation); competition for reactants by non-specific products; competition for primer binding by re-annealing of concentrated (10nM) product (Innis and Gelfand, 1990).

Detection and analysis of PCR product: The PCR product will be DNA fragments (amplicons) of defined length. The simplest way to check the PCR



product is to load a portion of it into an agarose gel containing ethidium bromide along with molecular weight markers and carry out an electrophoresis. The DNA fragments generated by the PCR should be readily visible over an ultraviolet transilluminator. Hybridizing the PCR product with suitable DNA probe

is also in practice for conformation.

Types / Variants of PCR

PCR has been adapted to fit many different applications and to achieve amplification of other molecules of interest like RNA. Hence, there are many different types and each one is unique to the application for which it is designed. The common types of PCR are: conventional/basic PCR, multiplex PCR, reverse transcription (RT)-PCR, nested PCR, real-time PCR and random primed PCR. And there are many other types for specific purposes.

Conventional/ Basic PCR: Conventional PCR uses a thermostable DNA polymerase to amplify a region of the DNA defined at each end by a specific primer. The exponential replication of the same target sequence produces enough DNA product or amplicons for use in subsequent analyses. PCR typically consists of three basic steps, as mentioned earlier.

Multiplex PCR: Multiplex PCR is a modification of conventional PCR in which two or more different PCR products are amplified simultaneously within the same reaction. This type of PCR consists of the same steps as conventional PCR, except that multiple sets of primers are used, each one priming a PCR product. Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction, and amplicon sizes, *i.e.*, their base pair length, should be different enough to form distinct bands when visualized by gel electrophoresis. The advantage is that it requires less time and effort in amplifying multiple target templates or regions than individual reactions and may be a useful screening assay. However, significant optimization is required to obtain all of the products with equal efficiency and sensitivity. By simultaneously amplifying more than one locus in the same reaction, multiplex PCR is becoming a rapid and convenient screening assay in both the clinical and the research laboratory. Since its first description in 1988 (Chamberlain *et al.*, 1988), this method has been successfully applied in many areas of DNA testing, including analyses of deletions, mutations and polymorphisms, or quantitative assays and reverse transcription PCR.

Nested PCR: Nested PCR is a very specific PCR amplification and is a variation of the conventional PCR, in that two pairs (instead of one pair) of PCR primers are used to amplify a fragment. The first PCR utilizes a pair of primers flanking the gene in question while the second PCR uses another pair of primers having complementarity to an internal segment of the gene, which was amplified in the first PCR. The fragment produced by the first reaction is used as the template for the second PCR. The second set of primers called nested primers (as they lie / are nested within the first fragment) is specific to the DNA sequence found within the initial PCR product. The use of a second amplification step with the “nested” primer set results in a reduced background due to the nested primers’ additional specificity to the region. The amount of product produced, which is shorter than the first one, is increased as a result of the second round of amplification. Carrying out nested PCR can further enhance the reliability of the PCR. Therefore, when information on the sequence of specific genes is available, amplification and visualization of that gene using a nested PCR could be carried out for confirmation.

Reverse Transcription Polymerase Chain Reaction (RT-PCR): This is based on the processes of reverse transcription and polymerase chain reaction. RT-PCR is a two-step process. The first step consists of the formation of complementary or copy DNA (cDNA) from RNA (generally mRNA). This is followed by the second step which is a conventional PCR using the cDNA as the template. The first step referred to as the “first strand reaction” uses enzyme reverse transcriptase for the production of the cDNA from the RNA. In the second step, the cDNA sequence is amplified by using primers specific to it. The RT-PCR forms a high sensitivity detection technique, where low copy number or less abundant RNA molecules can be detected. It is also used to clone mRNA sequences in the form of complementary DNA, allowing cDNA libraries to be created which contain all sequences of all the genes expressed in a cell. It allows the creation of cDNA constructs for the gene expression studies.

Real Time PCR: Real-time PCR is different from other PCR as it quantifies the initial amount of the template instead of detecting the amount of final amplified product (Freeman *et al.*, 1999; Raeymaekers, 2000). Real Time PCR is characterized by the point in time

during cycling when amplification of the PCR product of interest is first detected rather than the amount of the PCR product which has accumulated at the end point. Real Time PCR does this by using fluorescent dyes such as Sybr Green, or fluorophore-containing DNA probes such as *Taq Man* which get incorporated into each of the new strand, and monitoring the amount of fluorescence emitted during the PCR. This acts as an indicator of the amount of PCR amplification that occurs during each PCR cycle. Thus, in Real Time PCR machines, one can visually see the progress of the reaction in “real time”. Quantification using real-time PCR can be ‘relative or absolute.

Random /Arbitrary primed PCR: Random primed PCR conceived by Williams *et al.* (1990) is unique in that only single short primer (usually 10 bases long) is used instead of the primer pair in the conventional PCR. Prior knowledge of the sequence of the target DNA is not required and primer with any sequence can be employed. The underlying theory in AP-PCR is that the primer may find complimentary sequence at different locations on the two DNA strands used as template, and amplify the intervening regions at low PCR stringency conditions (36 – 40°C).

This is used to generate Random amplified polymorphic DNA (RAPD) profile which is increasingly being used as a method for the DNA finger printing and genetic characterization where prior knowledge of the sequence of the target DNA is not required. RAPD is used as a marker system, where sequence of the target DNA is not known. This is a rapid technique and can be useful for species/strain identification. Genomic variations between and within species could be identified as the difference in the molecular size and number of DNA fragments amplified. The PCR products variations shall be resolved by agarose gel electrophoresis.

Modifications of PCR for specific purposes

There are several modifications developed from the basic method to improve performance and specificity. Some of these commonly used in laboratories are:

Degenerate PCR: Degenerate PCR is in most

respects identical to ordinary PCR, but with one major difference. It is in a situation where the sequence of the gene to be amplified is not known, insert “wobbles” in the PCR primers are inserted. So, instead of using specific PCR primers with a given sequence, mixed PCR primers are used. For example, when a protein motif is back-translated to the corresponding nucleotide motif (Protein → Sequence), there will be more than one codon coding for particular amino acid, due to degeneracy of genetic code. Thus there will be more than one nucleotide sequence deciphered for a particular protein sequence.

Asymmetric PCR: Asymmetric PCR is used to preferentially amplify one strand of the original DNA more than the other. It finds use in some types of sequencing and hybridization probing where having only one of the two complementary strands is required. PCR is carried out as usual, but with a great excess of the primers for the chosen strand. Due to the slow (arithmetic) amplification later in the reaction after the limiting primer has been used up, extra cycles of PCR are required. A recent modification known as Linear-After-The-Exponential-PCR (LATE-PCR), uses a limiting primer with a higher melting temperature than the excess primer to maintain reaction efficiency as the limiting primer concentration decreases mid-reaction.

Thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR): Thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) is a fast and efficient method initially developed to amplify unknown sequences adjacent to known insertion sites in *Arabidopsis*. Nested, insertion-specific primers are used together with arbitrary degenerate primers (AD primers), which are designed to differ in their annealing temperatures. Alternating cycles of high and low annealing temperature yield specific products bordered by an insertion-specific primer on one side and an AD primer on the other. Further specificity is obtained through subsequent rounds of TAIL-PCR, using nested insertion-specific primers. The increasing availability of whole genome sequences renders TAIL-PCR an attractive tool to easily identify insertion sites in large genome tagging populations through the direct sequencing of TAIL-PCR products. For large-scale functional genomics approaches, it is desirable to obtain flanking sequences for each individual in

the population in a fast and cost-effective manner.

Hot Start PCR: This technique performed manually by heating the reaction components to the DNA melting temperature (*i.e.* 95 °C) before adding the polymerase. In this way, non-specific amplification at lower temperatures is prevented. Specialized enzyme systems have been developed that inhibit the polymerase’s activity at ambient temperature, either by the binding of an antibody or by the presence of covalently bound inhibitors that only dissociate at high-temperature. At the desired elongation temperature the polymerase is instantly activated. Hot-start/cold-finish PCR is achieved with these type of hybrid polymerases that are inactive at ambient temperature and are instantly activated at elongation temperature. Inhibition of the polymerase activity of the enzymes at lower temperatures during PCR reaction is achieved by chemical modifications or wax-barrier methods or by a *Taq*-directed antibody. By limiting polymerase activity prior to PCR cycling, non-specific amplification during the initial set up stages are reduced and the yield of desired PCR product is increased.

Touchdown PCR: Non-specific primer binding obscures polymerase chain reaction results, as the non-specific sequences to which primers anneal in early steps of amplification will “swamp out” any specific sequences because of the exponential nature of polymerase amplification. Touchdown PCR or touchdown style polymerase chain reaction is a variant of PCR that aims to reduce non-specific background by gradually lowering the annealing temperature as PCR cycling progresses. The annealing temperature at the initial cycles is usually a few degrees (3-5 °C) above the T_m of the primers used, while at the later cycles, it is decreased in increments for every subsequent set of cycles (the number of individual cycles and increments of temperature decrease is chosen by the experimenter) a few degrees (3-5 °C) below the primer T_m . The higher temperatures give greater specificity for primer binding, and the lower temperatures permit more efficient amplification from the specific products formed during the initial cycles.

Inverse PCR: Inverse PCR (IPCR), variant of PCR, was first described by Ochman *et al.* (1988). It is used

when only one internal sequence of the target DNA is known. It is therefore very useful in identifying flanking DNA sequences of genomic inserts. The inverse PCR method includes a series of digestions (DNA being cut by a restriction endonuclease). This cut results in a known sequence at either end of DNA of unknown sequence. The restriction fragment is self-ligated upon itself to form a circle and the inverse PCR uses standard polymerase chain reaction, however it has the primers oriented in the reverse direction of the usual orientation. Thus the template for the reverse primers for the inverse PCR is a restriction fragment that has been self-ligated upon itself to form a circle. Applications of Inverse PCR in molecular biology include the amplification and identification of sequences flanking transposable elements, and the identification of genomic inserts.

Long PCR: Long PCR is used when large segments of DNA (frequently over 10 kb) is to be amplified. For the accuracy of PCR, special mixtures of proficient polymerases such as *Pfu* are often used, which possesses 3' to 5' exonuclease activity or proofreading activity. The efficiency drastically declines when incorrect bases are incorporated. The 3' to 5' exonuclease activity removes these mis-incorporated bases and makes the further reaction proceed smoothly. Therefore, the amplification of long DNA fragments can be achieved. Long PCR is often used to clone larger genes or large segments of DNA which standard PCR cannot.

Gradient PCR: When a set of PCRs are run with different annealing temperatures all in the same block of the thermal cycler, simultaneously it is referred to as Gradient PCR. This is generally carried out for standardizing PCR conditions for heterologous primers. In many models of thermal cyclers, blocks are available with gradient annealing temperatures. This saves time and multiple blocks are not needed.

AFLP PCR: Amplified Fragment Length Polymorphism PCR, also called AFLP PCR was originally described by Zabeau and Vos, 1993. AFLP is a highly sensitive PCR-based method for detecting polymorphisms in DNA. AFLP can be also used for genotyping individuals for a large number of loci using a minimal number of PCR reactions.

Alu PCR: PCR using a primer that anneals to Alu repeats to amplify DNA located between two oppositely oriented Alu sequences. Used as a method of obtaining a fingerprint of bands from an uncharacterized human DNA.

Colony PCR: Colony PCR is mostly used after a transformation of bacterial (*E. coli*) or yeast clones to screen colonies for the desired (correct) ligation or plasmid products. Primers which generate a PCR product of known size are used. The colonies which give rise to an amplification product of the expected size are likely to contain the correct DNA sequence. Selected colonies of bacteria or yeast are picked inserted into the PCR master mix or pre-inserted into autoclaved water. PCR is then conducted to determine if the colony contains the DNA fragment or plasmid of interest.

In Situ PCR: In Situ PCR (ISH) is a polymerase chain reaction that actually takes place inside the cell fixed on slide. In situ PCR amplification can be performed on fixed tissue or cells. ISH applies the methodology of the nucleic acid hybridization technique to the cellular level. Combining cytochemistry and immunocytochemistry, it allows the identification of cellular markers to be identified and further permits the localization of to cell specific sequences within cell populations, such as tissues and blood samples.

Single Cell PCR: The advent of the polymerase chain reaction (PCR) has revolutionized the way in which molecular biologists view their task at hand, for it is now possible to amplify and examine minute quantities of rare genetic material: the limit of this exploration being the single cell. It is especially in the field of prenatal diagnostics that this ability has been readily seized upon, as it has opened up the prospect of preimplantation genetic analysis and the use of fetal cells enriched from the blood of pregnant women for the assessment of single-gene Mendelian disorders. However, apart from diagnostic applications, single-cell PCR has proven to be of enormous use to basic scientists, addressing diverse immunological, neurological and developmental questions, where both the genome but also messenger RNA expression patterns were examined. Furthermore, recent advances, such as optimized

whole genome amplification (WGA) procedures, single-cell complementary DNA arrays and perhaps even single-cell comparative genomic hybridization will ensure that the genetic analysis of single cells will become common practice, thereby opening up new possibilities for diagnosis and research.

Digital PCR: Digital PCR represents an example of the power of PCR and provides unprecedented opportunities for molecular genetic analysis in cancer. The technique is to amplify a single DNA template from minimally diluted samples, therefore generating amplicons that are exclusively derived from one template and can be detected with different fluorophores or sequencing to discriminate different alleles (e.g., wild type vs. mutant or paternal vs. maternal alleles). Thus, digital PCR transforms the exponential, analog signals obtained from conventional PCR to linear, digital signals, allowing statistical analysis of the PCR product. Digital PCR has been applied in quantification of mutant alleles and detection of allelic imbalance in clinical specimens, providing a promising molecular diagnostic tool for cancer detection.

Single nucleotide polymorphism PCR (SNP PCR): SNP PCR involves real-time PCR using single nucleotide polymorphisms (SNPs) as markers. It is a very sensitive and accurate method to quantify the percentage of recipient and donor cells to monitor the effect of stem cell transplantation (SCT) and sequential adoptive immunotherapy by donor lymphocyte infusions (DLI).

Assembly PCR: Assembly PCR is the artificial synthesis of long DNA sequences by performing PCR on a pool of long oligonucleotides with short overlapping segments. The oligonucleotides alternate between sense and antisense directions, and the overlapping segments determine the order of the PCR fragments thereby selectively producing the final long DNA product.

Helicase-dependent amplification: This technique is similar to traditional PCR, but uses a constant temperature rather than cycling through denaturation and annealing/extension cycles. DNA Helicase, an enzyme that unwinds DNA, is used in place of thermal denaturation.

Intersequence-specific (ISSR) PCR: A PCR method for DNA fingerprinting that amplifies regions between some simple sequence repeats to produce a unique fingerprint of amplified fragment lengths.

Ligation-mediated PCR: Ligation-mediated PCR uses small DNA oligonucleotide 'linkers' (or adaptors) that are first ligated to fragments of the target DNA of interest. Multiple primers that anneal to the linker sequences are then used to amplify the target fragments. This method is deployed for DNA sequencing, genome walking and DNA fingerprinting.

Methylation-specific PCR (MSP): The MSP method was developed by Stephen Baylin and Jim Herman at the Johns Hopkins School of Medicine, and is used to detect methylation of CpG islands in genomic DNA. DNA is first treated with sodium bisulfite, which converts unmethylated cytosine bases to uracil, which is recognized by PCR primers as thymine. Two PCR reactions are then carried out on the modified DNA, using primer sets identical except at any CpG islands within the primer sequences. At these points, one primer set recognizes DNA with cytosines to amplify methylated DNA, and one set recognizes DNA with uracil or thymine to amplify unmethylated DNA. MSP using qPCR can also be performed to obtain quantitative rather than qualitative information about methylation.

Semi-quantitative PCR

This technique allows an approximation to the relative amount of nucleic acids present in a sample. cDNA is obtained by RT-PCR when sample is RNA. They are then amplified along with the internal controls (that are used as markers). The markers commonly used are Apo A1 and β -actin. Amplification product is separated by electrophoresis. Agarose gel is photographed after ethidium bromide staining, and optical density is calculated by a densitometer. The disadvantage of the technique is possibility of nonspecific hybridizations, generating unsatisfactory results. Control of specificity is performed using highly specific probes for hybridization

Designing of primers

A primer is a short oligonucleotide (preferably 18-22 bases) which is the reverse complement of a region of the target DNA template. It would anneal to the DNA strand to facilitate the amplification of the targeted DNA sequence. There will be two primers namely, forward and reverse primers, for amplifying both the strands of target DNA template. Proper primer design is important for applications in PCR. Optimal primer sequence with required concentration is essential for achieving maximal specificity and efficiency of PCR. The manual selection of optimal PCR primer set is tedious process and not efficient. Thus, various bioinformatics tools are available for selection of appropriate primer pairs from a template sequence. Parameters like Specificity, stability, compatibility are to be considered for good primer designing.

Types of Primer

Based on the application, primers are classified into different categories.

- **Universal primers:** Primers to amplify specific DNA sequence/Gene/Gene product which is conserved in nature. These primers amplify specific sequence across different species in a group.
- **Guessmer:** Primers are used to amplify the particular sequence back translated from amino acid.
- **Oligo d (T):** Oligo dT is the classic primer mix used to prime synthesis of the first strand of cDNA; generally used for reverse transcription PCR.
- **Degenerate primers:** The mixture of primer used to amplify the same gene from different organisms, which are probably similar but not identical. The other use for degenerate primers is when primer design is based on protein sequence.
- **Specific primer:** Primers are designed specifically for a particular gene in a particular species.

Parameters for consideration

Efficacy and sensitivity of PCR largely depend on the efficiency of primers. A poorly designed primer can lead to failure of PCR due to nonspecific amplification and/or primer-dimer formation,

which can become competitive enough to suppress product formation. The sequences of the primers used for PCR amplification can have a major effect on the specificity and sensitivity of the reaction. When choosing appropriate primer pair for PCR, the following guidelines should be considered:

Specificity

Primer specificity is dependent on primer length, GC content, internal stability and unique sequence of the primer.

- **Uniqueness:** The primers primer sequence should be unique in the template DNA which means there should be only one target site present in the target template DNA.
- **Primer Length:** The length of the primers need to be between 15 and 30 base pairs so that they are long enough for adequate specificity and short enough for them to anneal to the DNA template.
- **Internal stability:** Internal stability is calculated with entropy values (ΔG) of neighbor nucleotides. To minimize false priming, it is critical that the stability at 5' end be high and the stability at 3' end be relatively low. The optimal internal stability is $\Delta G \sim 8.5$ Kcal/mol.
- **GC Content:** The GC content of the primer sequence should be relatively high as it has a direct relationship with the T_m . There should be a base composition of GC of about 50%-60%. The 3' end of the primer should finish with at least one G or C to promote efficiency in annealing due to the stronger bonding.

Stability

The stability of primer is mainly dependent on GC clamp, Annealing Temperature, melting temperature. Stretches of A and T are also to be avoided as these will open up stretches of the primer-template complex. G or C is desirable at the 3' end. This GC clamp reduces spurious secondary bands.

- **Melting temperature(T_m):** T_m is the temperature at which one-half of a particular DNA duplex will dissociate and become single strand DNA. The optimal melting temperature for primers generally

lies in the range of 52- 58°C. The stability of a primer template DNA duplex can be measured by its T_m . The melting temperature of nucleic acid duplex increases as the length and GC content increases. A simple formula for calculation of the (T_m) is:

$$T_m = 2(A+T) + 4(G+C)$$

This formula will be accurate in lower salt conditions only for primers less than or about 20 nucleotides in length.

Formula for primers of 20-35 bases in length is as below.

$$T_m = 22 + 1.46 ([2 \times (G+C)] + (A+T))$$

The calculated annealing temperature is only a reference temperature from which to initiate experiments. The actual annealing temperature may be 3-12°C higher than the calculated T_m . The actual annealing temperature condition should be determined empirically. The optimum annealing temperature which gives the best PCR product should be used.

- **Annealing temperature (T_a):** The annealing temperature (T_a) chosen for a PCR depends directly on length and composition of the primer(s). Generally, an annealing temperature about 5°C below the lowest T_m of the pair of primers is used. Too high T_a will produce insufficient primer-template hybridization resulting in low PCR product yield. Too low T_a may possibly lead to non-specific products caused by a high number of base pair mismatches.
- **GC clamp:** The presence of G or C bases within the last five bases from the 3' end of primers (GC clamp) helps promote specific binding at the 3' end due to the stronger bonding of G and C bases. More than 3 G's or C's should be avoided in the last 5 bases at the 3' end of the primer.

Compatibility

The compatibility of primer is dependent on following factors.

- **Primer Pair Matching:** Primers work in pairs – forward primer and reverse primer. Since they are used in the same PCR reaction, it shall be ensured that the PCR condition is suitable for both of them.

One critical feature is their annealing temperatures, which shall be compatible with each other. The maximum difference allowed is 3°C. Closer the T_a of the primer pairs; better the annealing.

- **3'-end Sequence:** It is well established that the 3' terminal position in PCR primers is essential for the control of miss-priming. Primers should be "stickier" on their 5' end than on their 3' ends. A highly "sticky" 3' end, as indicated by a high GC content, could potentially anneal at multiple sites on the template DNA. Optimal GC clamp reduces spurious secondary bands.
- **Secondary structure:** Presence of secondary structures of the primer, produced by intermolecular or intramolecular interactions can greatly reduce the availability of primers to the reaction which lead to poor or no yield of the product. Generally three different secondary structures are encountered in primers.
 - Hairpins loop formed by intramolecular interaction within the primer and should be avoided. (Optimally a 3' end hairpin with a ΔG of -2 kcal/mol and an internal hairpin with a ΔG of -3 kcal/mol is tolerated generally.)
 - Primer self-dimer is formed by intermolecular interactions between the two (same sense) primers. When primers form intermolecular dimers much more readily than hybridizing to target DNA, they reduce the product yield. (Optimally a 3' end self dimer with a ΔG of -5 kcal/mol and an internal self dimer with a ΔG of -6 kcal/mol is tolerated generally.)
 - Primer cross dimers are formed by intermolecular interaction between sense and antisense primers, where they are homologous. (Optimally a 3' end cross dimer with a ΔG of -5 kcal/mol and an internal cross dimer with a ΔG of -6 kcal/mol is tolerated generally.)

Other Recommendation

The concentration of primer in amplification reaction should be between 0.1 and 0.5 μM . The forward primer and the reverse primer should be between 300 and 2,000 base pairs apart. Primers designed for a sequence must not amplify other genes in the mixture. If possible, a computer search should be conducted against the vector and insert DNA sequences to verify

that the primer and especially the 8-10 bases of its 3' end are unique.

Tools for Primer Designing

The use of software in biological applications has given a new dimension the field of bioinformatics. There are a numerous web based resources and different programs available for primer designing. There are number of simple standalone programs as well as complex integrated networked versions of the commercial software available. Some important tools are doPrimer, primer 3, web primer, primo pro 3.4, GAP, PCR primer design, the primer generator etc, these all are web- based resources and very essential for molecular biologist. There are different software freely and commercially available for PC installation like PrimerSelect, Primer 3, Primer Premier, NetPrimer, GenomePRIDE 1.0, OLIGO 6, Primer Designer 4, GPRIME, Primer Designer, Primer Premier, Primer Design, Gene Runner, Primer BLAST etc.

In conclusion, the heart of PCR lies in the proper designing of primers. Several basic parameters including the length of the primer, %GC content and the 3' sequence, T_m value need to be optimized for successful PCR. Certain of these parameters can be easily by hand optimized while others are best done with available primer designing tools. The increasing use of information from the internet and the sequences held in gene databases are practical starting points when designing primers and reaction conditions for the PCR. It is also possible to include

more than one set of primers in a PCR for getting desired results.

PCR applications

PCR has transformed the way that most studies requiring the manipulation of DNA fragments and DNA cloning may be performed as a result of the simplicity and usefulness of PCR. Cell-free DNA amplification by PCR is able to simplify many of the standard procedures for DNA cloning, DNA analysis, and the modification of DNA. Previous molecular biology techniques for isolating a specific piece of DNA had relied on gene cloning, which is a tedious and slower procedure. An alternative to cloning, PCR, can be used to directly amplify rare specific DNA sequences in a complex mixture when the ends of the sequence are known. This method of amplifying rare sequences from a mixture has numerous applications in basic research, human genetics testing and forensics. Some of the PCR applications include site-specific mutagenesis studies, amplification and detection of DNA in situ from cells for rapid diagnosis, genomic subtraction, analysis of protein functions and intermolecular assembly, DNA fingerprinting (RAPD/AFLP/VNTR) for evaluation of genetic heterogeneity & relationship, paternity verification, forensic application, generation of single chain antibody fragments for immunology, sensitive disease diagnosis, cDNA synthesis from RNA for cDNA library construction, production of clones for sequencing, molecular epidemiology, molecular taxonomy and many more.

Quantitative Genetics

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The study on genetics of quantitative characters in a population is called as quantitative genetics. The aim of quantitative genetics is to bring about desirable change in the quantitative traits. The change in any character is possible only if variation exists in those characters that are genetically determined and are inherited. Any measurable or observable property of living individual that exhibit inherited differences among the individuals of a population is called a character or trait. Genetically determined character is called an inherited character and those inherited characters with variation among individuals of a population are called as heritable traits.

Classification of characters

Qualitative characters

The characters governed by single or few pairs of genes (Oligogenes) with major effect and least affected by environment are called as qualitative characters. The qualitative characters follow discontinuous variation among individuals of a population. The qualitative traits are influenced by few genes called as major genes and express mendelian ratios. Color pattern in different species of farm animals and presence or absence of body parts are few examples.

Quantitative characters

The characters governed by many genes with minor effect of each gene (poly genes or minor genes) and the effects of whom are modified to a greater extent by environment are called as quantitative characters or polygenic traits. Since the phenotypic expression of these traits is affected by many genes and environment, they are also called as multifactorial traits. The quantitative character shows continuous

variation wherein the phenotypic values of different individuals of a population form a continuous graded series of different phenotypes. Body weight, fleece production are few examples for quantitative traits.

Genetic basis of a character

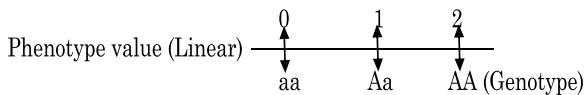
The development of any trait and its final expression in the form of a phenotype is totally under the control of genes. However, the genes don't produce the characters directly but through some biochemical reactions that in turn lead to the development of a character or trait. The phenotype so expressed is the joint product of gene and the environment. The phenotype (P) due to gene effect is called as the genotypic value (G). The environment modifies the genotypic value before its final expression as phenotype. This deviation of the P from G due to environmental effects is called as environmental deviation (E). Thus the two components of phenotypic value are genotypic value, G and environmental effects, E and denoted as $P = G + E$. Thus any change in genotype or environment results in a change of phenotype. This interaction of genes with the external environment like climate, nutrition, management etc. or the differential response of different genotypes in different environments is called as genotype–environment interaction, IGE.

Gene action and its types

The quantitative traits are under the control of polygenes and shows different types of gene action in modifying the expression of a character. The effect of a gene in modifying the expression of a trait or phenotype depends upon the presence or absence of another gene in the same or different locus. This forms the basis for variation in gene expression dividing into additive and non-additive gene action.

Additive gene action (AGA)

A gene may have an independent effect irrespective of the presence or absence of other genes in the same or different locus in the same or different chromosome. This type of gene action which has the increasing or decreasing effect of a gene on the phenotypic value is called the additive gene action. When the gene action is additive, there is no dominance among the alleles. The complete additive gene action produces a linear phenotype.



A – Contributes one unit to the phenotype.
a – Contributes zero units to the phenotype.

Non-additive gene action (NAGA)

In this type of gene action, the genes in addition to its individual effect, produces a different type of action when it combines with another gene. This interaction of genes is non-additive and is called as non-additive gene action. The interaction occurs between alleles of the same locus (intra allelic interaction) or between alleles of different locus (inter allelic interaction). Thus there are different types of NAGA namely dominance, incomplete dominance, over dominance and epistasis. The increased vigour observed when animals of different genetic background are mated as in case of cross breeding is called as heterosis, a non-additive gene effect.

The phenotypic value is the joint effect of genotype and the environment. *i.e.*

$$P = G + E$$

Where

P = Phenotypic value
G = Genotypic value (gene effect)
E = Environmental effect

But there exists a differential response of different genotypes in different environment. This forms the component of genotype-environment interaction, IGE. Therefore the phenotypic value is made up of the following components.

$$P = G + E + IGE$$

The genotypic component, G can be further partitioned into additive, dominance and epistasis effect.

$$G = A + D + I$$

Where

A – Additive gene action or breeding value
D – Dominance effect
E – Epistasis effect

The major component of the genotypic value is the additive effect of genes or the individual effect of genes. This portion of the genotypic value (additive genetic value or the breeding value) is a fixed effect and transmitted to the progeny as such without any change. The breeding value is thus defined as the sum of average effects of the genes carried by an individual.

The components of variance and its significance

The population mean does not reveal whether the phenotypic values recorded on different individuals of a population are similar or vary from the mean of the population. The individuals differ in their phenotypic values for quantitative traits. The differences in the phenotypic values of a character among individuals of a population are called as variation. The variation is thus the mean of the squares of the deviated values from the mean and denoted as

$$V = \sum \frac{(X_i - X)^2}{N}$$

Where

X_i is the numerical value of variable X
X is the mean of variable X
N is the number of observations

The components of the phenotypic variance are same as the components of the phenotypic value. Thus the partitioning of the phenotypic variance is analogous to partitioning of phenotypic value as

$$P = G + E + IGE \text{ and } VP = VG + VE + Cov_{GE}$$

Where

VP is the observed phenotypic variance

VG is the genotypic variance

VE is the environmental variance

CovGE is the interaction between genotypic values and environmental deviation.

The genotypic variance is further partitioned into

$$V_G = V_A + V_D + V_I$$

Where

V_A is the additive genetic variance

V_D is the dominance variance

V_I is the interaction variance

The partitioning of variance is done by analysis of variance. The splitting of variance helps us to understand the effect of different factors in causing variation in a trait and the percentage contribution of different factors to the total phenotypic variance. Thus the knowledge on variance helps us to determine the genetic properties of a population and to study the inheritance of quantitative trait. The variation is thus an important component which helps the breeder to bring about genetic improvement in the population. Any genetic improvement program would be effective only if variation exists among the individuals of a population. The whole of the variation is not transmitted to the next generation. It's the genetic part which is heritable. Even in the genetic variance, only the additive genetic component responds to the selection process and gets transmitted from parents to the offspring. Thus the partitioning of phenotypic variance throws light on the role of heredity and environment in influencing a trait. This degree of genetic determination in modifying the phenotypic value is called as the heritability.

Heritability

Heritability is defined as the ratio of genetic variance to the total phenotypic variance. When only the additive genetic variance is considered in the total genetic part, it is called as heritability in narrow sense (h^2). When the total genetic variance is considered,

it's called as heritability in broad sense (H^2).

$$h^2 = V_A/V_P$$

$$H^2 = V_G/V_P$$

Heritability measures the strength of relationship between the performance (phenotype) and breeding value of an individual for a particular trait. It is a measure of parent's superiority that is being transmitted to the progeny. The heritability values range from 0 to 1 depending upon the magnitude of phenotypic variability due to genetic effects. Thus heritability is a measure of population and not an individual. Heritability, an important genetic parameter plays a vital role while formulating genetic improvement programs. It provides the degree of genetic determination of a trait, amount of parent's superiority transmitted to the progeny, accuracy of selection, relation between the phenotypic value and breeding value and mode of gene action.

If the heritability value is 1, it means that the differences in phenotypic values are entirely due to effects of genes and thus $V_G = V_P$. If heritability value is 0, then the differences in phenotypic values are completely due to environmental factors and thus $V_E = V_P$ as in case of identical twins and pure lines. If a trait is highly heritable, then individual selection will bring about genetic improvement in a particular trait in progeny generation and indicates the additive gene action. In case of low heritable traits, non-additive gene action plays a major role in determining the phenotypic differences of a trait.

The resemblance among relatives (due to sharing of common genes) forms the basis for different methods of estimation of heritability like

Regression of offspring on parent.

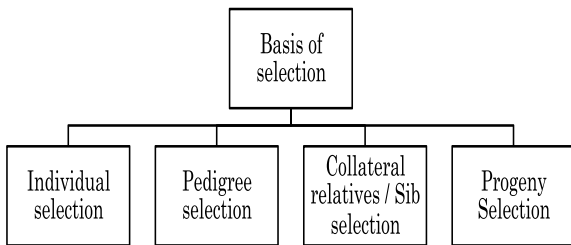
- Correlation between offspring and parent.
- Half sibs and full sib's intra class correlation method.
- Apparent and realized heritability.

Selection of superior animals

The foremost step in a genetic improvement program is to decide the characters that are to be improved. These are called as selection objectives. The characters that are to be improved vary between different classes of animal. The final aim in any selection program is to maximize the genetic gain and in turn response to selection.

Basis of selection

Different criteria's are to be used while estimating the breeding value of an individual. These criteria's are called as basis of selection.



Individual selection

The fundamental unit of any genetic improvement program is the animal itself and the animal's own phenotypic value of a trait is considered to estimate the breeding value of that trait in that individual. This is called as individual selection as animal's own performance is used to estimate its breeding value. Individual selection minimizes the environmental deviations as the individuals to be selected are tested under similar environmental conditions. This method can be applied for traits with high heritability and can't be used for sex limited traits. When an animal has to be selected for different traits at a time, then it is called as multi trait selection.

The different aids used for multi trait selection are tandem method, independent culling level and selection indices. In case of tandem selection, genetic improvement is practiced for one trait at a time till satisfactory progress is achieved. Then the second trait is selected and so on. But the genetic

progress achieved is less efficient and takes a long time. In case of independent culling level, selection is made simultaneously for all the characters but independently. Minimum standards are fixed for each trait and animals below the standard are culled irrespective for its superiority in other characters. In the case of selection index method, each trait is weighed by a score and the scores for individual characters are summed up to get a total score for each animal. Based on the total score, animals with high scores are selected and those with low scores are culled from the population. Discriminate function is used for discriminating the desirable genotypes from the undesirable ones. The overall genetic worth of an individual using the selection index method is

$$H = \sum a_i G_i (i=1 \text{ to } n)$$

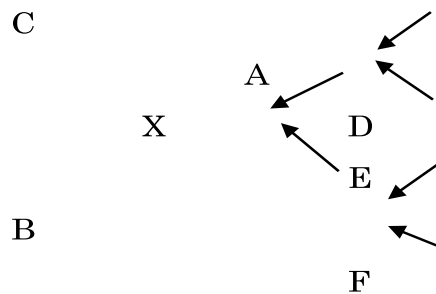
Where

a_i is the relative economic value of the trait

G_i is the additive genetic value

Pedigree selection

The list of record of ancestors like parents, grandparents and great grandparents is called as pedigree. The estimation of breeding value of an individual and further selection of that animal based on the performance of its relatives/ancestors is called as pedigree selection. The basis of pedigree selection is transfer of genes from ancestors to the next generation. The pedigree selection helps to select animals at early age and also for sex limited traits. The main disadvantage is that animals of similar pedigree would be culled without giving due credit to the individual merit.



Collateral relatives or family selection

The selection of an individual based on the performance of its collateral relatives is called as family selection. The collateral relatives may be full sibs, half sibs, cousins etc. The relatives must be closely related to the individual as in case of sibs. If the individual's own performance is included in estimating the family mean, it is called as family selection. If the individual records are excluded in estimating the family mean, it is called as sib selection. The sib selection is practiced for slaughter traits, sex limited traits and low heritable traits.

Progeny selection

The selection of an individual based on the performance of its progeny is called as progeny selection. The progeny of an individual is the index of parent's genetic worth as the progeny inherits half of genes from each parent. The progeny selection method is used for low heritable traits, sex limited traits and proves whether the male is free from any recessive lethal effects. This method can also be used for traits that can't be measured during the life time of an individual like carcass traits. As many progenies as possible should be considered for selecting an individual as the segregation may cause the progenies to receive good or bad genes from parents and environment is not same for all progenies of single parent. The accuracy of selection increases with increase in number of progenies.

Mating Systems

The genetic relationships between the mates are also important in bringing about genetic progress using different selection methods. These are called as mating systems. The different types of mating systems present in front of an animal breeder along with different selection tools are

Inbreeding

- Close inbreeding like selfing, parent-offspring and sib mating

- Line breeding.

Outbreeding

- Within species like out crossing, top crossing and pure breeding.
- Between species like inbred crossing, strain crossing, criss crossing, rotational crossing, grading up and species hybridization.

Inbreeding

The mating of very closely related individuals than the average members of the breed is called as inbreeding and the mates should have one or more common ancestors in the immediate 4-6 generations. Inbreeding increases the homozygosity by decreasing the percentage of heterozygotes without disturbing the gene frequency. The inbreeding increases the frequency of recessive genes in the population. This mating system fixes characters in an inbred population which may be either favourable or unfavourable. Inbreeding thus changes the genetic structure of the population by changing the genotypic frequencies. Due to inbreeding depression, the performance of the individual is greatly affected in terms of its vigour, growth and reproduction. Inbred animals are more susceptible to change in environmental conditions. Inbreeding is mainly followed to develop inbred lines, elimination of undesirable genes from the population, production of distinct families and to increase the prepotency of an individual.

Outbreeding

The mating of unrelated individuals is called as outbreeding. If the mating occurs between individuals of the same breed it is called as out crossing, between individuals of different breeds it is called as cross breeding and between species it is called as species hybridization. This is more popular in case of highly domesticated farm animals and uncommon in case of aquaculture species. Outbreeding is mainly practiced to increase the heterozygosity thereby increasing the performance of the individual called as hybrid vigour or heterosis. This increase in vigour is mainly due to non-additive gene action.

Selective breeding programme in aquaculture species

In contrast to livestock, only very few genetic improvement programs have been carried out in aquaculture species worldwide (Nile Tilapia in Asia, Atlantic salmon in Norway). The genetic improvement programs can be applied to variety of aquaculture and mariculture species to develop improved breeds to have a cost effective farming practices. The most important traits in case of aquaculture species that could be effectively improved through selective breeding are the growth rate, disease resistance, meat yield and survival rate.

In India, for the first time, genetic selection program was carried out to improve the growth performance or body weight of Indian major carps (*Labeo rohita*) by Central Institute of Freshwater Aquaculture, Bhubaneswar in collaboration with Institute of Aquaculture Research (AKVAFORSK), Norway. The main objective of this breeding program was to develop a national breeding plan and dissemination of improved variety of rohu to fish farmers for quality seed production. The improved variety named as Jayanti Rohu was produced using a nested design program (2 males nested within a male or vice versa). It was found that the maximum genetic gain per generation in case of rohu selection program after four generations of selection was 17%. The program started in 1992 and the first genetically improved variety Jayanti Rohu was released in 1997.

In CMFRI, bidirectional selection, individual/mass selection program was carried out for naupliar length in *Artemia franciscana* by taking two sub-populations namely small naupliar size (SNS) and big naupliar size (BNS) lines with the view of developing two divergent strains. In case of SNS, reduce naupliar size was the trait of selection and in case of BNS, bigger naupliar size was the trait of selection. It was found that the phenotypic responses in both the lines were substantial. CMFRI has also studied the performance of triploid edible oysters in terms of meat yield, quality and growth by chromosomal manipulation techniques.

Selective breeding program was also carried out in tilapia, one of the most famed aquaculture species of the world known as Genetic Improvement of Farmed Tilapia (GIFT) by world fish centre, ICLARM in collaboration with NFFTRC, AKVAFORSK and FAC. The main objective of the study was to develop a synthetic base population and to estimate the phenotypic and genotypic parameters in tilapia. Selective breeding programs were also carried out in Atlantic salmon by the Norwegians for different traits like weight at slaughter, age of sexual maturation, flesh colour, total fat content, and amount of fat tissues.

Conclusion

Selection and genetic improvement programs are important tools for increasing the aquaculture production in proportion to the growing food demand of the world. The scope exists for standardization and optimization of breeding programs in combination with the use of advanced molecular tools like implementation of marker assisted selection programs. The decision on using a selection program depends upon the variation existing in the phenotypic performance of different characters in a species. With proper investment of time, money, labour and long term planning, genetic improvement programs are going to be the future for improving the performance of different species to meet the demands of the population explosion.

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Marker Assisted Selection

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The majority of economically important traits in livestock is complex and has continuously distributed phenotypes, which are influenced by polygenes dispersed across the genome. The molecular architecture of complex quantitative traits gives new insights on understanding of the molecular physiology of the phenotypes and yield sophisticated selection strategies for an effective marker assisted breeding. The economically important characters of central importance such as food production, body weight, and health in terms of disease resistance are under the control of multiple genes plus the environment and are known as quantitative traits. The regions within the genomes that contain genes associated with a quantitative trait are known as quantitative trait loci (QTLs). The benefits of molecular markers linked to genes transformed the genetic selection program from phenotype based to genotype based. Since the identification of QTLs based on conventional phenotypic methods is not possible, the development of molecular markers leads to the characterization of QTLs and in turn the quantitative traits.

The selection is a process of giving preference to certain individuals (differential reproduction) in a population to reproduce and produce the next generation. Genetic selection therefore changes the frequency of genes and genotypes without creating any new genes. Selection is carried out to improve one or more desirable traits/characters in the population of a species. Genetic improvement of any population of a species involves periodic evaluation, selection and culling of animals. Selection of animals is an important aspect of genetic improvement program, because the selected parents will decide the genetic makeup of the progeny and hence their phenotypic performance.

The traditional genetic selection programmes involve the selection of animals based on the observable phenotypes since the genes contributing to the phenotype was unknown. The two stages of selection process are the phenotypic selection (selection based on phenotypic performance) and genotypic selection (selection based on breeding value). Thus there exist three types of selection criteria's namely

- Individual selection (animals own performance is considered as a measure of its genetic merit).
- Collateral relatives (performance records of ancestors and collateral relatives like family selection, within family selection, sib selection).
- Progeny selection (selection of an individual based on its progeny performance).

With the advent of genetic markers in 1980's, the association of certain genes with our traits of importance is established paving way for selection of animals based on animals genotype rather than its phenotype alone. Thus the process of selection of animals based on the information available on DNA is called as Marker Assisted Selection (MAS). The MAS is an indirect selection process wherein the trait of interest is selected based on a marker linked to the concerned trait.

Basis of marker assisted selection

Every individual in this world are made up of cells that are in turn programmed by genetic material called DNA. The DNA consists of four different nitrogenous bases namely, adenine, guanine, thymine and cytosine. Genes which are made up of a portion of DNA sequence code for proteins. More than 95% of the DNA sequence doesn't code for proteins and are called as non-coding sequences. The DNA is in

turn packed up into sets of chromosomes and the entire set is called the genome. Any gene is made up of two alleles, one from each parent (present over a pair of chromosomes) in a diploid individual. The genetic information present on a gene is involved in the synthesis of proteins and polypeptides which interact among themselves and form the phenotype.

The genetic makeup of an individual is called a genotype. The genes are made up of a sequence of nucleotides which varies between individuals. The alleles which are end product of nucleotide differences may affect the amino acid sequence of the proteins there by creating either additions or deletions and finally altering the phenotype of an individual. The individual animals harboring alleles that codes for quality proteins are selected to reproduce and propagate their germplasm to the next generation based on genetic markers linked to these alleles. MAS necessitate the need for known association between genetic markers and phenotype considered for selection. MAS have the potential to accelerate the genetic potential by identifying animals carrying desirable alleles for a given trait at an earlier age. MAS could also be practiced at the embryo stage in animals, thereby helping the breeder from maintaining the animals for a longer duration.

Genetic markers

The genetic markers are varying lengths of DNA sequences which helps to identify genes or short regions on a chromosomal DNA having a major effect on trait of a population of animals. The genetic markers are tightly linked to a gene of interest and tags for a particular gene. The genetic markers are very useful in breeding programmes as it helps the breeders in screening the segment of chromosomes inherited between generations. If a particular segment contains a gene responsible for variation between animals, then that segment could be used for association studies between the particular segment received by the animal and its performance. If a particular genetic marker has no direct role in effecting the performance of a trait, then it can be used to mark a chromosome segment harboring gene(s) which has a major effect on the performance trait. These segments are called a

QTL's which contribute to variation in growth and production performance of animals.

Efficiency of MAS

- Marker assisted selection is applicable when heritability of the target trait is low because the production traits of economic importance are identified not by its phenotype but by the genetic markers. Traditional selection methods fail to significantly improve traits that are having low heritability values. If the additive genetic variance component of the total variance explained by the genetic marker exceeds the heritability of the character, then selection using the genetic marker loci is more efficient than selection on the individual phenotype. But on the other hand, if the heritability of a trait is high, it becomes easier to select superior animals based on performance or phenotypic records.
- Marker assisted selection can also be applied for sex limited traits. In traditional selection methods, artificial selection can be applied only in sex where the trait is expressed. But when genetic markers are used, the molecular score so generated can be used for selection of opposite sex also wherein the trait is not expressed.
- Marker assisted selection can be used for selection of immature animals. Traditional selection methods based on phenotype can't be practiced for traits like age at sexual maturity, age at first spawning, number of eggs spawned etc. until animal attains sexual maturity. The genetic markers can be applied for selection of these traits even before the animal expresses the above said traits.
- Marker assisted selection can be practiced for traits which are very difficult to measure like feed conversion efficiency or disease resistance.
- Marker assisted selection can be used for traits that cannot be measured during the lifespan of the animal.
- Marker assisted selection can be practiced for species that is not fully domesticated. The situation is very specific to aquaculture/mariculture species that are very difficult to domesticate. In this situation, if genetic markers are available for desired traits, MAS could be practiced in every generation to decide the parents carrying beneficial alleles.

Marker assisted selection strategy

The MAS strategy of animals depends on the following factors.

- Precise identification of breeding objectives.
- The status and selection of genetic markers.
- Quantum of genetic markers to be used for selection.
- Identification of QTL's for the traits of interest.
- Genetic marker analysis.
- Relative efficiency of MAS to traditional selective breeding programmes.
- Economic viability of MAS.

Precise identification of breeding objectives

The identification of breeding objectives depends upon the nature of traits, genetic variation present in the traits, number of traits to be chosen for the selection process and accuracy of selection process. With the help of MAS, single locus affecting a quantitative trait could be identified using genetic markers so as to increase the genetic progress that could be achieved.

Status and selection of genetic markers

Number of genetic markers is available for different species of animals. The strategy should be to select the suitable marker according to our selection programmes. The strategy in selecting a marker is based on the assumption that a linkage disequilibrium exists between the genetic marker and QTL and there should be a tight linkage between genetic markers and QTL.

Molecular Markers

The advent of molecular markers over the last 2 decades has revolutionized the research in biological sciences and offered the opportunities of using the genomic variation of major genes for the genetic improvement of livestock. DNA based molecular markers are becoming versatile tools and are of great importance in various fields like embryology, physiology, genetic engineering and are constantly modified to increase their utility

and automate them for improving genomic analysis. The high polymorphic genetic markers also reveal the genetic variation existing in quantitative traits and help identify the genes that contribute to variation. Molecular markers are also widely used in transgenesis and marker assisted selection.

Types of Molecular Markers

Genetic marker can be defined as any stable and inherited variation that can be measured or detected by a suitable method and can be used subsequently to detect the presence of a specific genotype or phenotype other than itself, which otherwise is non-measurable or very difficult to detect. The markers revealing variations at the level of DNA are called as molecular markers. Molecular markers are usually repetitive sequences found in DNA, and the interval of occurrence of these repetitive sequences varies from individual to individual giving rise to polymorphisms. The macromolecules like proteins, deoxyribonucleic acid and biochemical constituents constitute different classes of molecular markers.

Based on their method of detection, they are classified as hybridization based and PCR based markers. The analysis of hybridization-based markers includes the traditional RFLP analysis and DNA fingerprinting. The PCR based markers include Cleaved Amplified Polymorphic Sequence (CAPS), Random Amplified Polymorphic DNA (RAPD) and Microsatellites. Depending on the size of motif, they can be called as minisatellites comprising of 10 to 60 base pairs or microsatellites composed of 1 to 5 base pairs. Allele Specific Oligonucleotide (ASO) and Single nucleotide polymorphisms form a new group of markers.

Markers are of two different kinds, direct and indirect. Specific loci or markers may be part of the gene and directly influence a quantitative trait. The indirect marker loci has no direct effect on the character of interest and could be utilized in selection by means of its associations or linkage disequilibria between alleles at the marker loci and quantitative trait loci

Genetic markers used in MAS			
Marker	Nature of marker	Type of Marker	Reference
Restriction Fragment Length Polymorphism (RFLP)	Co-dominant	I & II	Botstein <i>et al.</i> , 1980
Random Amplified Polymorphic DNA (RAPD)	Dominant	II	Welsh and McClelland 1990; Williams <i>et al.</i> , 1990
Amplified Fragment Length Polymorphism (AFLP)	Dominant	II	Vos <i>et al.</i> , 1995
Microsatellites or Simple Sequence Repeats (SSR)	Co-dominant	II > I	Tautz, 1989

Quantum of genetic markers to be used for selection

The number of genetic markers needed for selection depends upon the length of the marker and length of the genome (measured in centimorgan cM units). A cM is 1/100th of a morgan (1cM = 1/100M = 106 bp, basic unit of linkage map) and is equivalent to 106 bp. Since there exists variability in the length of the genome and marker present on the genome, a number of evenly spaced markers should be considered for the selection programme. Lande and Thompson (1990) gave a simple formula to arrive at minimum number of molecular markers (N) required for the likely detection of associations with important QTLs which is calculated as,

$$N = 2TL + C$$

Where, T = Number of generations since last hybridization event in the population

L = Map length in Morgans

C = Haploid chromosome number

Identification of QTLs for the traits of interest

The most important step in framing the strategy for MAS of animals is identifying as many QTLs as possible with regard to our trait of interest. The success in

identifying the QTL depends upon the utilization of F2 and back crossed animals obtained from the parents with divergence for the traits of interest. The resources required for identification of QTL are polymorphic DNA markers, high resolution linkage maps and mapping populations that are segregating for the polymorphic markers. The principles involved in the process of QTL mapping and MAS are as follows.

Linkage disequilibrium

Linkage disequilibria between loci are produced by three factors, hybridization, random genetic drift and epistatic selection. In a large population created by hybridization between genetically differentiated groups, after T generations of random mating, substantial linkage disequilibria are likely to be maintained between selectively neutral loci with recombination rates, $r < 1/T$. In a randomly mating population of effective size N_e , genetic drift is expected to produce substantial associations between polymorphic loci with recombination rates $r < 1/(4N_e)$. In domesticated populations, except those of very small size, the number of generations since the last hybridization event usually will be smaller than four times the effective population size *i.e.* $T < 4N_e$. Therefore, hybridization is more powerful mechanism for generating useful linkage disequilibria than random genetic drift.

A major limitation with these associations is that the linkage disequilibrium diminishes over generations by recombination during meiosis. Therefore it is mandatory to identify such indirect markers that are tightly linked to QTL or closely placed along with QTL on the chromosome so that the association is not disturbed by recombination.

Linkage maps

A linkage map of a species has molecular markers placed in an order along the length of the chromosomes in the genome of that species. Linkage map indicates the position and relative genetic distances between markers along the chromosomes. The crossing over is a random event and hence has equal probability of occurring at any position along the length of the paired chromosomes during

meiosis. Therefore, the markers that are closely placed are less likely to be separated during meiosis than distantly placed markers. Further, the frequency with which the markers are separated during meiosis (recombination frequency) is a direct estimate of the distance between markers. Recombination frequencies for different pairs of markers could be utilized to construct a map showing relative distances between markers on the chromosome. Recombination frequencies between any pair of markers range from 0 % (complete linkage) to 50 % (no linkage).

The genotype information of polymorphic markers in mapping is subjected to linkage analysis for the construction of linkage map. Popular software packages used for construction of linkage maps in aquaculture species include CARTHAGENE, CRIMAP, JOINMAP, LINKMFEX, MAPCHART, MAPINSECT 1.0 (<http://www.dpw.wau.nl/pv/pub/>), MAPMAKER and MAPMANAGER. Linkage between markers is calculated as odds ratio *i.e.* the ratio of linkage versus no linkage and is expressed as logarithm of odds (LOD) value or LOD score. Generally a pair of markers with a LOD score of >3 would be utilized for the construction of linkage map. A LOD score of 3 between markers indicates that presence of linkage between markers is 1000 times more likely than no linkage.

Large family sizes are required for detecting QTL of small effects. When the QTL explains 10 percent of the phenotypic variance in the target trait, the optimum family size appears to be 50 individuals per family for a QTL mapping experiment in outbred populations. When the total population size (individuals from all families) reaches 1000 individuals, family sizes of 25/50/100 give similar power of detecting a QTL that is explaining 10 % of phenotypic variance.

The basic principle involved in QTL mapping is detecting an association between phenotype of the trait and genotype of markers. Phenotypic records are available for the individuals in mapping population for the trait of interest. A polymorphic marker locus has 2 or more alleles in the population, though only 2 alleles are present in any diploid individual.

A mapping population can be divided into different genotypic groups on the basis of the genotype at the marker locus. A significant difference between phenotypic means of the groups for the trait of interest indicates that either marker itself is the QTL for the trait of interest or marker is linked to the QTL controlling the trait of interest. It should be noted that QTLs can only be detected for traits that segregate between the parents used to construct the mapping population.

The three different methods used in identifying QTLs are single marker analysis, simple interval mapping and composite interval mapping. Single marker considers single marker locus at a time. Simple interval mapping considers a pair of adjacently placed linked markers at a time and try to find out whether QTL exists or not in the interval between marker pair. Composite interval mapping combines simple interval mapping with linear regression and considers additional genetic markers along with adjacent pair of linked markers.

Markers identified should be validated for their reliability/repeatability in different environments in predicting the phenotype in different populations to avoid false positive associations between markers and the trait of interest. Markers that could not be validated can't be used for marker assisted breeding program.

Genetic marker analysis

The following points are to be considered before implementing the MAS such as identification of bred specific genetic marker, linkage status of QTL with the marker, estimation of gene frequency in different generations and number of generation taken to identify the animals with QTL. Thus 2 strategies exist for the MAS namely marker linked with QTL and marker unlinked with QTL.

Relative efficiency of MAS to traditional selective breeding programmes:

The MAS should be evaluated for its efficiency in order to compare its performance with conventional

selective breeding programmes. MAS is highly effective for traits with low heritability, sex limited traits, immature traits and population size.

Economic viability of MAS

The viability of MAS depends upon its cost effectiveness. Even though the cost involved in genotyping several locus per animal per generation is enormous, the amount spent on maintaining the animal till later age is relatively higher when compared to screening the animals at an early age. This in turn helps the breeder to select the animals at young age and reduce the amount spent on feeding and maintaining the animals till it attains maturity and then select the animal for its performance. Marker assisted selection is advantageous compared to conventional breeding methods as phenotypic screening is expensive, difficulty encountered in detecting presence of multiple alleles related to a single trait based on phenotype and selection for alleles of desirable traits at an early age even before the trait is expressed.

Relevance of MAS to traditional breeding programmes

Marker assisted selection allows for the accurate selection of animals with specific DNA markers

found to be associated with complex quantitative traits. The quantitative traits are under the control of several genes (polygenes) before it interacts with an environment and gets expressed as a phenotype. Therefore, breeding values estimated using phenotypic records for the trait should be utilized for making selection decisions as they consider all the genes that contribute to a given trait and marker assisted selection only complements and cannot act as replacement for traditional selection methods.

Conclusions

Most of the economically important traits are quantitative, polygenic and influenced by environment there by making genetic improvement of these traits more laborious and time consuming. The traits with low heritability such disease resistances which are difficult to measure are important potential targets for MAS. Marker assisted selection is likely to accelerate genetic progress in some traits better than others. Hence, target traits for MAS should be selected wisely.

Marker assisted breeding should not be considered as replacement for traditional selection methods. MAS only complement the selection process based on phenotypic records. The traits with high heritability can be selected based on breeding values or phenotypic performance. The decision on selecting an animal

Table 1. List of QTLs identified in aquaculture species.

Species	QTL for traits	QTL detecting method	Reference
Tilapia			
<i>O. mossambicus X O. aureus</i>	Cold tolerance & fish size	ANOVA, regression interval mapping	Cnaani <i>et al.</i> , 2003
Arctic charr			
<i>Salvelinus alpinus</i>	Body weight, condition factor, age at sexual maturation	Single marker and interval mapping	Moghadam <i>et al.</i> , 2007
Kuruma shrimp			
<i>Marsupenaeus japonicus</i>	Growth traits	Interval and composite interval mapping	Li <i>et al.</i> , 2006
Rainbow trout			
<i>Oncorhynchus mykiss</i>	Resistance to IPNV	c2 test and interval mapping	Ozaki <i>et al.</i> , 2001
<i>Oncorhynchus mykiss</i>	Meristic traits	Composite interval mapping	Nichols <i>et al.</i> , 2004
European seabass			
<i>Dicentrarchus labrax</i>	Morphometric traits	Half-sib interval mapping	Chatziplis <i>et al.</i> , 2007
Rainbow trout			
<i>Oncorhynchus mykiss</i>	Pyloric caeca number	Composite interval mapping	Zimmerman <i>et al.</i> , 2005
Tilapia			
<i>O. mossambicus X O. aureus</i>	Stress response, body weight & sex determination	Genome scan using ANOVA	Cnaani <i>et al.</i> , 2004

should take into consideration both the breeding value and marker information rather either alone. The weightage to be given to the marker information during the process of selection poses a big challenge to the breeder.

In case of aquaculture species, the quantum of information available on markers and QTLs are very less indicating the need for more genetic improvement programs. The construction of high resolution linkage maps, QTL detection programs is the need of hour. The whole process of identifying QTLs has become easy due to technological advancements. The breeder should consider the incorporation of QTL information while framing genetic improvement programs.

Even though there is a strong belief on the influence of QTLs for the phenotypic expression of economically important metric traits, its impact on the breeding programs and commercial applications in plants, livestock, forestry and fishery is yet to be proven. The breeder should rationalize the genetic gain made by MAS and compare it with the conventional breeding methods with regard to its cost effectiveness and economic returns. The traditional breeding methods still holds good for improving several traits. Though the animals harbor better genes for production traits, the interaction between the environment and the genotype plays a major role in the final performance of the animal. Therefore, efficient management of animals is must in framing genetic improvement programs for selection of superior animals.

Suggested readings

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Cryopreservation of fish spermatozoa and its Application in Aquaculture and Conservation

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Introduction

Cryopreservation is a branch of cryobiology which relates to the long-term preservation and storage of biological material at very low temperature, usually at -196°C , the temperature of liquid nitrogen. It is based on the principle that very low temperatures immobilize the physiological and biochemical activities of cells, thereby making it possible to keep them viable for very long period in a state known as suspended animation. Cryopreservation of fish sperm is one method successfully adopted from animal husbandry by the aquaculture industry. Cryopreservation overcomes the problem of males maturing before females, allows selective breeding and stock improvement and enables the conservation of genomes. The basic technique of cryopreservation involves collection of fish gametes in which specific diluent (extender) with cryoprotectant (such as dimethyl sulfoxide, glycerol, ethylene glycol and methanol) is added. After a period of specific equilibration, it is frozen rapidly and stored in liquid nitrogen. After thawing, the milt can be activated for use in fertilizing the eggs. In teleosts, spermatozoa are held within the testis in an immotile state by the chemical composition of seminal plasma. Under natural conditions, motility is initiated when the semen or milt is diluted with water on release during spawning. During cryopreservation, the sperms are to be retained in inactive condition and it is the extender that keeps the sperm in live but in immotile state so that ideal dilution of cryoprotectant can be made to mix with the milt. Cryopreservation allows virtually indefinite storage of biological material without deterioration over a time scale of at least several thousands of years (Mazur,

1985), but probably much longer. In India, NBFGR is the primary organization in India carrying out fish sperm cryopreservation for long term gene banking.

Cryopreservation of spermatozoa

The first success in preserving fish sperm at low temperature was reported by Blaxter (1953) who fertilized herring (*Clupea harengus*) eggs with frozen – thawed semen. Major efforts have been made during the past 20 years to effectively freeze salmonid sperm (Stoss, 1983) and now sperm from several species of fish have been frozen. The spermatozoa of several economically important species have been cryopreserved in the recent past which includes rainbow trout, Atlantic salmon, Tilapias, several cyprinids including Common, Chinese and Indian carps. Some economically important marine species for which spermatozoa have been frozen include Asian sea bass, Atlantic halibut, milk fish, black porgy, bluefin tuna, various catfishes, Indian marine fishes. Majority of the works on cryopreservation of fish semen concerns cultivated species or species of commercial interest. Considering the needs to establish gene-banks for endangered fish species and to avoid loss of genetic variability – there is still a need for investigation in the field of sperm preservation.

Extenders

Undiluted gametes are not suitable for freezing and they must be diluted with a suitable extender. An extender is a solution consisting of inorganic and organic chemicals

resembling that of blood or seminal plasma in which the viability of spermatozoa can be maintained during *in vitro* storage. Extender also helps to reduce the toxicity of the cryoprotectant used in cryopreservation. The efficacy of cryopreservation is greatly enhanced if the prefrozen milt is diluted with a suitable extender. Various extenders, containing KCl, NaCl, glucose, sodium citrate, Ringer's solution, cow serum and milk fish serum were used to preserve fish sperm in liquid nitrogen (-196°C). The sperm of the Indian carp, *Labeo rohita* were first preserved in liquid nitrogen using extender comprised of NaCl – 730 mg, NaHCO₃ – 500 mg, Fructose – 500 mg, Vegetable lecithin – 750 mg, Mannitol – 500 mg, Distilled water – 100 ml.

Cryoprotectant

Cryoprotectants are added to extenders to minimize the stress on cells during cooling and freezing. Glycerol, DMSO and methanol are the most widely used cryoprotectants for preserving teleost spermatozoa. The optimum concentration used for cryopreservation may vary species to species. However, the degree of success has been achieved with 10-15% glycerol, DMSO or methanol for many species. The optimum cryoprotectant concentration for a given protocol may also depend on the equilibration period that is the time allowed for cryoprotectant penetration into cells. There are two kinds of cryoprotectants namely permeating and non-permeating cryoprotectants. The permeating cryoprotectants include DMSO, glycerol, methanol, ethylene glycol etc. The non-permeating cryoprotectant includes egg yolk, milk and some other protein (Soyabean protein, Promine D and bovine serum album). A non-permeating cryoprotectant is often used in conjunction with a permeating cryoprotectant.

Dilution ratios

Various milt to diluent ratios have been tested. It is 1:1 to 1:9 in salmonids, 1:3 in rainbow trout and 1:20 in *O. niloticus*. In Indian cyprinids and catfishes, 1:3 and 1:4 ratios were found to be ideal.

Motility

The spermatozoa of most teleost fish species are immotile in testes and the genital tract and are activated

only after release into the external medium for a short period of motility. The initiation of motility of fish sperm is essentially a dilution effect upon expulsion of semen into the surrounding water. Spermatozoa motility in fish is usually estimated by an arbitrary scale of intensity ranging from 0 to 5, by the duration of motility for a given intensity, or by a combination of these two parameters. Sperm motility is one of the most important parameters of sperm quality and is usually expressed in duration of sperm movement and percentage of motile sperm immediately after activation.

Procedure of cryopreservation

Milt of the fish has to be collected in a dry and clean box and to be kept on ice till it used. The extender has to be prepared afresh every time and the cryoprotectant (@10% of the extender) to be mixed just before filling the straw. The milt extender ration should be kept 1:3. Just after mixing the milt with the extender, cryoprotectant to be added and then straws to be filled. and sealed by poly vinyl alcohol (PVA) powder and keep on ice for 10 minutes for bringing down the temperature to 0°C. After that straws should be transferred to liquid nitrogen vapour phase (~90°C) for 10 minutes and then to be immersed in liquid nitrogen for achieving the desired temperature (-196°C). The filled straw then can be stored in liquid nitrogen. The motility of the cryopreserved milt can be checked after thawing straws using an activator solution. After confirming the motility of the cryopreserved milt, it can be used for fertility experiments. Once fertility trials become successful, then cryopreservation of milt is complete. From the experiments one can find out best combination of extender and cryoprotectant and this can be used for long term cryopreservation fish milt.

Technical problems in fish sperm cryopreservation

The fish sperm cells are small, have no acrosome and are available in enormous quantities is semen for experimentation. Hence, recovering motile, fertile frozen thawed fish spermatozoa is not a problem. Yet, the published information reveals difficulties in developing a reliable and reproducible protocol. The problem is in the enormous diversity of fish spermatozoan physiology. Although all the species

studied are external fertilizers, the behaviour of the adults at spawning and the conditions in which sperm and egg must meet are very different. The two extremes are represented by salmonids spawning in freshwater, where sperm is deposited as close as possible to the eggs and swim for 30 seconds at most and marine species like herring where spermatozoa are released in a diffuse cloud over the spawning grounds and remain motile for hours. In species where spermatozoa swim for long periods in nature, cryopreservation is generally easier. Tilapias are a good example. They can spawn in fresh to full seawater and their spermatozoa will swim for hours in a saline solution. The real challenge facing cryobiologists interested in freezing fish spermatozoa is not recovering enough viable sperm cells to fertilize small numbers of eggs in the laboratory but in making the technique practical in the field. The cryopreservation strategy and technique used at NBFGR is simple, does not need any electrically operated equipment. This provides easy adaptability, customization in difficult remote locations and successful application has been proved in species of various taxonomic groups.

Constraint in milt cryopreservation of fishes is the need for development of species-specific protocol, unlike higher animals. Limited success in cryopreserving fish oocyte and embryo is due to their multi-compartmental biological systems, big issues such as chilling sensitivity, low membrane permeability and their large size which require extensive screening of cryoprotectants, studies on tolerance to chilling, determination of the appropriate rate of freezing and rate of thawing. Partial success achieved in cryopreservation of pluripotent blastomeres and embryonic cells in few fish species, however, cryotoxicity and chilling sensitivity are still major problems.

Application of cryopreservation of spermatozoa

Cryopreservation of fish sperm can be successfully used as a fishery management tool. It provides some means of control relative to mating and reproduction including artificial propagation with individuals of same species from the distant locations or of different mature season or developing a profitable new hybrid.

Sperm from males in the wild can be cryopreserved for the optimal mature season of hatchery stock so that genes from the fish in the field can be transferred into the hatchery population. Cryopreservation of fish sperms can be used to guarantee the specific fish stocks and to ensure against the loss of specific genes caused by natural, over-fishing or pollution causing disasters. It can be used in the hatchery management to solve the problem of having a disproportion between the males and the females. Induction of gynogenesis becomes highly feasible if the irradiated sperms are cryopreserved in advance. Functions of sperms and gene bank can be further facilitated by utilising this technique.

In this aspect, cryopreservation of gametes, germ cells and embryos are useful for genetic resource conservation and also for genetic improvement in aquaculture. Globally, milt cryopreservation has been successfully accomplished for more than 200 fish species with production of normal and viable offsprings mainly, salmon, trout, tilapia, sturgeon, Chinese and Indian major carps. Attempts have also been made to cryopreserve milt in more than 40 marine fish species of aquaculture importance mainly, grouper, cod, haddock, sea bream, sea bass, eel, striped bass, flounder etc. Successful preservation of spermatophores in crustaceans was also developed in *Macrobrachium rosenbergii*, lobsters; crabs, and shrimp *Penaeus monodon*. In India, milt cryopreservation protocols have been developed in more than 30 species, mainly carps and catfishes.

Conclusion

Storage of fish spermatozoa, eggs and embryos without loss of viability is of considerable value in aquaculture and conservation. The fish sperm cryopreservation needs development of species-specific protocols. Such protocols are developed through experimental standardization of various parameters, after the captive breeding protocol is developed. This becomes a bottleneck due to protracted breeding season and low domestication of most of the aquatic species, especially marine fishes. Nevertheless, in all such cases, time available in a year for conducting experiment is small and determined by breeding cycle of the species. In view

of the constraint, it is essential that candidate species for sperm cryopreservation are prioritized. In artificial propagation, sperm cryopreservation protocol can be an asset where such milt related problems exist. Cryopreserved sperm was also used to retrieve the whole species and clones. Sperm retrieved from fish, stored at -18°C has been used to produce androgenetic fish and interspecific androgenetic cloning, as a mode for restoration of species. Though milt cryopreservation is successful in many fish species fish gamete cryopreservation still faces an important challenge in the form of long-term storage of finfish eggs and embryos. Owing to large size, large amount of yolk and tough chorion or zona radiata with a low permeability coefficient, egg and embryo cryopreservation of teleosts and crustacea have not met with success anywhere in the world so far. The fundamental problem of sufficient dehydration during cooling due to the relatively large size (1-6 mm) of fish eggs and the presence of membrane of different water permeability has not been overcome. Cryopreservation or long term storage of fish eggs or embryos would be beneficial to the aquaculture industry. It would

provide a method of retaining specific genetic lines of fish without the expense of maintaining brood stock populations and would provide a secondary source of a genetic line in case of brood stock loss or would allow the preservation of endangered genetic line in the wild populations.

Fish milt cryopreservation efforts may be prioritized on endangered, commercially important and endemic species from various aquatic habitats and by establishing cryobanks. For large-scale use of cryopreserved fish sperm in aquaculture programmes, uninterrupted supply of liquid nitrogen in areas where aquaculture activities are centred is a pre-requisite. Sensitive and reliable tools for assessment of frozen-thawed milt quantity, physiological changes during long-time storage, DNA damage during cryopreservation and characterizing cryoinjury especially in sperm DNA are also essential. Standardization of cryopreservation protocol for pluripotent fish blastomeres needs to be researched in detail for providing optimal and uniform results.

Cytogenetics and its applications in fishes

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In brief, fish cytogenetics may be defined as a part of genetic study that concerned with the study of the structure and function of the chromosomes including analysis chromosomes, utilizing the G banding and or other cytogenetic banding techniques, as well as fluorescent *in situ* hybridization (FISH).

The cytogenetic study involved good quality metaphase chromosomes preparation from any tissue that contains good number of dividing cells at metaphase stage by arresting them with colchicine spindle fiber inhibitor at an extremely low concentration. Followed by hypotonic treatment, fixation, and dropping the cells onto microscope slides for chromosome spreading. The chromosomal staining is necessary because the colourless chromosomes are difficult to distinguish from equally colourless cytoplasm. The chromosome staining is performed with Giemsa. The active components of Giemsa are eosin Y and methylene blue, which form a magenta compound after binding with DNA. Giemsa has no specificity for any particular base in DNA and in normal circumstances, it stains chromosomes uniformly. The chromosomes are observed after staining under the microscope. A 10-X objective is generally required to screen the slide to find the metaphase plates and for photography, 100-X oil immersion objective is used.

In case of fishes mitotic chromosomes usually studied from the organelles that has rapidly dividing cells (*in vivo*) such as kidney and gills which are good source of dividing cells and the cells are arrested at metaphase stage using colchicine. The tissues, which do not divide rapidly like peripheral blood cells are cultured *in-situ* to induce division followed by *in-situ* colchicination. Chromosomes can also be prepared by giving colchicine treatment to the tissue *in-situ* especially

when the specimen is very large and from freshly dead fish especially under field conditions. the detailed process of chromosome preparation from fish is as described below.

Chromosome preparation

A. Detail procedure for chromosome preparation from kidney / gill tissues of fishes:

- Collect healthy fish specimens (preferably weighing 20-50 g).
- Inject 0.05% colchicine intramuscularly @ 1 ml per 100 g of body weight.
- Allow specimens to swim for 1- 2 h in a bucket after injection.
- Anaesthetize fish specimen with ethylene glycol, dissect out the kidney / gill tissues in a Petri dish, and cut into small pieces.
- Homogenize tissues in 6-8 ml hypotonic solution (0.56% KCl) in glass tissue grinder to make cell suspension.
- Pour the cell suspension in 15 ml centrifuge tube and keep it at room temperature for 20-25 min for cell swelling.
- Stop the hypotonic action KCl by adding 1-2 ml freshly prepared chilled Carnoy's fixative (Methanol: Acetic acid in 3:1 ratio) gradually. Mix it gently with pasture pipette
- Centrifuge cell suspension at 1200-1500 rpm for 10 min at room temperature to get cell pellet at the bottom.
- Remove supernatant with a pipette leaving approximately 2-3 ml of supernatant. Add 6-8 ml freshly prepared chilled fixative slowly.

- Keep the tube in refrigerator for 1-2 h for thorough fixation.
- Mix the contents and centrifuge cell suspension at 1200-1500 rpm for 10 min at room temperature.
- Remove the supernatant without disturbing cell pellet at the bottom and add fresh fixative.
- Repeat steps 12 -13 three times, till clear transparent cell suspension is obtained.
- Take small quantity of cell suspension in a pasture pipette and drop it onto grease free, pre-cleaned glass slide from 1.5-2.0 feet height.
- Allow the slide to air / flame dry.
- Keep the slide 1-3 days for ageing in dust free place.
- Stain it with 4-6% Giemsa in phosphate buffer (pH 6.8) for 15-20 min.
- Wash with DD water thoroughly.
- Air-dry and store the slides in a slide box.
- Observe metaphase spreads in bright field microscope to ascertain the quality of staining.
- Make the slides permanent by mounting with DPX mountant.
- Screen the slides for good spreads and take photographs of metaphase spreads under oil immersion objective (100X).
- For karyotype preparation, cut individual chromosomes from the photo prints. Group the chromosomes into four categories—metacentric (m), sub metacentric (sm), sub telocentric (st) and telocentric (t).
- Paste the chromosomes on ivory sheet in decreasing order of size within the group (align centromeres of all chromosomes in each row).
- Photograph the karyotype, which can be used as base line data for detection of chromosome aberrations.
- Karyotype can be also made from digital images of chromosomes acquired from digital camera with the help of suitable software.

Note: Unused cell suspension can be stored (in 2.5 ml eppendorf vials) for further use in refrigerator up to six months and at -20°C for years together without marked deterioration in quality.

B. *In-vitro* chromosome preparation:

- Take kidney tissue from live/ freshly dead fish.
- Homogenize the tissue to prepare cell suspension

- in 8 ml of RPMI 1640 culture medium.
- Add 50 μ l of 0.05% of colchicine.
- Incubate the cell suspension in BOD incubator for 30-50 min at 27-32°C.
- Centrifuge the cell suspension at 1200 rpm for 10 min. decant the supernatant.
- Add 8 ml hypotonic solution (0.56% KCl) to the cell pellet.
- Incubate the cells in this solution for 20-25 min at room temperature.
- Follow the steps 7-26 described in 'A'

Analysis of chromosomes

The chromosomes are observed under microscope after staining with suitable dye under the microscope. A 10-X objective is generally required to screen the slide to find the metaphase plates, which are often scarce among a large number of non-dividing nuclei. For photography, 100-X oil immersion objective is used and very good quality images/ photographs are required for measurements and further analysis. Ideally, metaphase spread should not contain any overlapping chromosomes. In case of overlaps, more images of the metaphase will be needed to prepare the Karyotype.

A chromosome in mitotic metaphase has two distinguishing features: its length and a transverse constriction that marks the position of the centromere. From the chromosome length and centromere position, three parameters are calculated: the *centromeric index*, the *arm ratio* and the *relative length*. The first two factors, *i.e.* centromere index and arm ratio, describe about the chromosome itself and the relative length denotes about the size of the chromosome in relation to other chromosomes in the complement. *Centromere index* is defined as length of shorter arm of the chromosome divided by the length of whole chromosome and multiplied by 100. *Arm ratio* is defined as the length of longer arm of the chromosome divided by the length of shorter one. It is, therefore, always ≥ 1 . The *Relative length* of a chromosome is defined as the length of the particular chromosome divided by the total length of all the chromosomes in the haploid set including the one being measured and multiplied by 100.

The first step for characterization of a complete chromosome set is by karyotyping. The karyotype of a chromosome complement is prepared from the cells exhibiting the complete somatic chromosome number and characteristic chromosome morphology. The homologous chromosomes were paired based on their length, morphology and position of centromere. The chromosome pairs were arranged in decreasing order of morphology and size in the karyotype. In fishes, the chromosome classification on the basis of arm ratio, as proposed by Levan *et al.*, (1964), has been widely used, which is described below:

Table 1. Chromosome Classification by Arm Ratio

Centromeric position	Arm Ratio	Chromosome type/ Symbol
Median	1.0-1.7	Metacentric (m)
Sub-median	1.71-3.00	Submetacentric (sm)
Sub-terminal	3.01-7.00	Subtelocentric (st)
Terminal	> 7.00	Telocentric (t)

Once the karyotype is prepared, a diagrammatic representation of the chromosome (ideogram) can be prepared on the basis of relative length of chromosomes.

Chromosome banding

Karyotyping using conventional 'Geimsa' staining of chromosomes does not give greater insight into the structure and organization of chromosomes. Further, in many fish species the chromosomal morphology is more or less similar making it difficult to identify and pair the homologous chromosomes. Unlike humans G- banding is not successful due to poor compartmentalization of genome into AT- & CG-rich isochores, however, NOR staining, Chromomycin A₃ C-banding has been widely used for genetic characterization of fishes. These techniques have been optimized in our laboratory and the protocol of the same is given below.

Silver nitrate staining (AgNO₃):

Staining of Nucleolar Organizer Regions (NORs) is performed according to the method of Howell and Black (1980) with minor modifications. For this, AgNO₃

(50%) solution and developing solution (2% Gelatin + 1.0% Formic acid) are prepared. Before staining, the slide is dried at 40°C for 4-5 h or overnight at room temperature. Place 8 drops of AgNO₃ and 4 drops of developing solution on a slide and mix the two solutions thoroughly and cover with a cover slip. Incubate the slide at 50°C for 3-5 min, till the solution become golden yellow in color. Rinse the slide with the double distilled water (DDW) and air-dry the slide. Mount the slide with DPX mountant and observe the chromosomes under microscope. The pattern of NOR for the species is usually determined by studying a minimum of 20 metaphase spreads per specimen.

Chromosome staining with Chromomycin A₃ (CMA₃):

For CMA₃ staining with DAPI, the mixture solution containing CMA₃ (0.5 mg CMA₃ in 1 ml DDW) and buffer (McIlvaine in DDW in 1:1 ratio, adjust pH at 7, added MgCl₂ to make the 2.5 mM conc. of MgCl₂) in 1:9 ratio is placed properly on the aged slide and covered with cover slip. Keep the slide in dark for 1 h and then wash in DDW. Place the DAPI mixture (2 mg/ml of DAPI in 1 ml of McIlvaine buffer, adjust pH at 7) on the slide and cover with cover slip and keep the slide in dark for 30 minute. Wash the slide with McIlvaine buffer and add antifade solution followed by covering with cover slip. The slide is observed under microscope in the dark and bands are determined in each species by studying a minimum of 20 metaphase spreads per specimen per species.

C-banding:

The C-bands are the regions of constitutive heterochromatin, which are predominantly contains transcriptionally inactive, highly repetitive DNA sequences. The use of C-banding techniques in fish has made it possible to correctly identify homologous chromosomes by revealing the distribution of constitutive heterochromatin and also to determine its role in karyotype evolution and speciation.

Constitutive heterochromatin banding is performed using Barium hydroxide-Saline-Giemsa (BSG) technique of Sumner (1990). The aged air-dried slide is treated with 0.2N HCl for 1 h at room temperature

followed by rinsing with DDW. The slide is placed in coplin jar containing 5% aqueous solution of barium hydroxide at 50°C for 35 min followed by rinsing with DDW. The slide is then incubated for 1 h at 60°C in 2X SSC (0.3M Sodium chloride; 0.03M Sodium citrate) followed by a rinse with DDW. The slide is then stained with 4-5% Giemsa in Phosphate buffer (pH 6.8) for 30 min. The slide is then dried and observed under microscope. C-band pattern is determined in each species by studying a minimum of 20 metaphase spreads per specimen per species.

Molecular cytogenetics:

Molecular cytogenetic techniques focus on specific chromosomes, chromosome regions, and unique DNA sequences or genes. In general, it involves the use of a series of techniques referred to as *in situ* hybridization (ISH) that has given researchers a powerful look into the cell and its genetic content. The principle behind ISH is the specific annealing of a labeled nucleic acid probe to complementary sequences in fixed cells/tissues, followed by visualization of one or more specific regions of the genome. The ISH technique was initially introduced by Gall & Pardue in 1969 to localize nucleic acids in individual cells. During the time, the *in situ* hybridization tool was restricted to highly repetitive DNA sequences using radioactive labeled probes visualized by autoradiography. The uses of radioisotopes had many disadvantages and were replaced by non-radioactive detection methods. In 1982, Langer-Safer group at Yale University published a non-radioactive procedure that was convenient and polynucleotide specific. The most commonly used reporter molecules are haptens, such as biotin and digoxigenin, which can be incorporated easily in the probe DNA. The tagged probes are then detected with labeled antibodies against the specific tag or with a labeled avidin molecule, as in the case of biotin. The biotin and digoxigenin have partly been replaced by directly fluorochrome-conjugated nucleotides that simplified the laboratory protocol, but the basic principle of FISH has essentially remained the same.

Fluorescent labeled probes allow researchers to selectively label a single gene, entire chromosomes or whole genome and then visualize it using fluorescence microscopy. Here, we are discussing few molecular

cytogenetic techniques like fluorescence *in situ* hybridization (FISH), Primed *in-situ* labeling, Fiber-FISH

Comparative genome hybridization and Spectral karyotyping.

Fluorescence *in-situ* hybridization (FISH):

The FISH technique is based on the discovery that labeled ribosomal DNA hybridizes to acrocentric chromosomes. It involves a fluorescently labeled DNA probe being hybridized to genomic DNA sequences, and can be used to physically map the specific site on a chromosome. Initially, radioactive isotopes were used for this technique, however, the use of fluorochrome is safer that requires a shorter reaction time and can give rise to different colors.

a. Chromosome preparation:

Metaphase chromosome spreads are prepared using colchicine to arrest the dividing cells during mitosis. Hypotonic solution (0.075M potassium chloride) and fixative (methanol and acetic acid in 3:1 ratio) are then applied sequentially for cell preparation. Finally, the chromosome suspension is dropped onto glass slides and chemical aging of slide in 2X SSC is done. Before hybridization, metaphase chromosomes and interphase nuclei are pretreated enzymatically (*i.e.* 0.005% pepsin) to enhance their accessibility to the probe and to reduce the amount of cytoplasm.

b. Probe labeling:

For probe labeling, pure DNA preparation is required and a fluorochrome must be incorporated into the DNA probe. DNA probes can be labeled by enzymatic procedures such as nick translation, random priming or by the polymerase chain reaction (PCR). For nick translation, 3'-5' exonuclease and 5'-3' polymerase activity of DNA Polymerase I is exploited to cause a single strand nick and subsequently, a specific fluorescently labeled nucleotide is incorporated into the nicked strand using non-nicked strand as a template. Feinberg and Vogelstein in 1983 introduced

probe labeling by random priming which depends on the ability of the Klenow fragment of DNA polymerase to copy single-stranded DNA templates primed with random hexa-nucleotide mixtures. Labeling by PCR involves a standard PCR reaction with labeled nucleotide in addition to unlabeled nucleotides.

c. Hybridization and Detection:

Following the pre-treatments, hybridization is carried out under optimal conditions for the annealing of probe to the specific target nucleic acid. The previously prepared probe and cot-1 or salmon sperm DNA are mixed, denatured and applied to the preheated slides for hybridization. Hybridization between probe and target DNA takes place during an incubation period of ~16–48 h at 37°C. This incubation time can vary depending on the probes used.

Detection of the probe permits the visualization of target DNA sequences. Detection starts with post-hybridization washes of the slide in formamide and SSC (sodium chloride, sodium citrate) salt solutions to remove any excess probe that is non-specifically bound. However, a detection step is not required for all DNA probes. In a direct-label reaction, a fluorochrome conjugated nucleotide [e.g. 2'-deoxyuridine 5'- triphosphate (dUTP) conjugated to fluorescein-isothiocyanate (FITC)] is incorporated into the strands. In an indirect-label reaction, reporter molecules such as biotin or digoxigenin are incorporated into the DNA. Such indirect labels require a detection step in which the reporter molecule or hapten is labeled with an agent such as avidin or antidigoxin that is conjugated to a fluorochrome. The detection methods for biotinylated probes employ avidin–fluorochrome conjugates, whereas those for digoxigenin-labeled probes employ antidigoxin–fluorochrome conjugates.

d. Chromosome counterstaining:

In addition to immunochemistry of signal detection, the DNA of the chromosomes or nucleoli must be counterstained for visualization. Typical fluorochromes used in conjunction with FISH include Hoescht 33258, DAPI and Propidium Iodide (PI). It may be necessary to vary the concentration of counter-stain in order to optimize signal detection.

e. FISH microscopy:

FISH signals are visualized by fluorescence microscopy using a light source that illuminates the fluorescently labeled probe. A variety of specific filter sets are used to separate the different fluorochromes and to visualize the fluorescence signal of the probe. Digital imaging systems, such as a CCD camera, to capture the image and quantify fluorescent signals are needed. Resultant images are analyzed on commercially available systems (e.g. from Vysis, Leica Microsystems or Applied Spectral Imaging).

Types of FISH probe

a. Gene-specific probes:

Gene-specific probes target specific nucleic acid sequences within the chromosome. Examples of such probes include bacterial artificial chromosome (BAC) and yeast artificial chromosome (YAC) probes and cosmids. Gene-specific probes are useful for mapping genes on chromosomes. These probes have proven useful particularly in the study of micro deletion, syndromes, where the absence of a gene often goes undetected by conventional banding methods.

b. Repetitive-sequence probes:

Repetitive-sequence probes bind to regions that are rich in repetitive base-pair sequences. Examples of such probes include centromeric and telomeric probes. Centromeres frequently contain AT- rich tandem repeats, whereas telomeres are recognized by the short repetitive sequence, *i.e.* TTAGGG. Centromeric probes have applications in the identification of marker chromosomes and numerical chromosome abnormalities in interphase nuclei and when specimens are sex mismatched. Telomeric and subtelomere specific probes are commonly used to identify cryptic chromosomal translocations such as those occurring in cases of unknown mental retardation.

c. Chromosome painting probe:

Chromosome-painting probes contain sequences that are specific to either a single chromosome (*i.e.* whole-chromosome-painting probe) or an arm of a

chromosome (*i.e.* chromosome arm-painting probe). After hybridization, one or more chromosomes of interest are 'lit up' in different colors, which are dependent on type of particular fluorochromes used. This technique is particularly useful for identifying chromosome arms that are involved in translocations, as well as for marker chromosomes and ring chromosomes.

d. Whole-genomic DNA probe:

Whole-genomic DNA probes are used for the FISH-based technique CGH. They can be used to detect genomic imbalances in tumor genomes by combining tumor and normal DNA to analyze gains and losses.

Primed *in-situ* Hybridization

Primed *in-situ* Hybridization (PRINS) technique is based on *in-situ* synthesis of non-radio isotopic hybridization probe. Unlabeled synthetic oligonucleotide primers anneal to fixed chromosome preparation in sequence specific fashion. This DNA then serve as a primer for chain elongation *in-situ*, catalyzed by DNA polymerase I. Fluorescently labeled nucleotides are used as a substrate for chain elongation. This procedure has been used for cytogenetic localization of high copy number, chromosome-specific, tandem repeat sequence including centromeric, telocentric and satellite sequences.

Fiber-FISH

High- molecular weight genomic DNA or individual DNA molecules from large-insert DNA clones can be spread on glass slides for FISH analysis. DNA prepared from bacterial artificial chromosome (BAC) or tissues extend approximately 2.5-3.5 kb/ μm on slides. Thus, the fiber-FISH method provides fine-mapping resolution of up to a few kilo-bases.

Comparative genomic hybridization

Comparative genomic hybridization (CGH) involves the differential labeling of test and reference DNA to measure genetic imbalances in entire genome. It is based on quantitative two-colour FISH. It has become

an invaluable technique for studying chromosomal aberrations that occur in solid tumors and other malignancies. A major advantage of the CGH technique is that only DNA from the tumor samples is needed for analysis, which avoids the often-difficult preparation of tumor metaphase chromosomes that may have poor morphology and resolution. Instead, karyotypically normal metaphase chromosomes are used to detect tumor associated chromosomal gains and losses. Another advantage of CGH is that formalin-fixed tissue sections can be used.

Spectral Karyotyping

Spectral karyotyping (SKY) is a FISH-based molecular cytogenetic technique that allows color karyotyping of two species chromosomes. Whereas FISH limits analysis to specific chromosomes or regions of chromosomes, and CGH visualizes only those changes that result in variations in copy number. The SKY permits the visualization of all chromosomes at one time, 'painting' each pair of chromosomes in different fluorescent color. Before the development of this technique, molecular cytogeneticists analyzed chromosomes with staining techniques that produced a black-and-white banding pattern. However, the identification of all chromosomal aberrations in a complex karyotype was often not possible from such patterns. SKY has been highly effective in deciphering many complex karyotypic rearrangements.

Application of FISH in fish genetics:

The probes for centromeric repetitive DNAs are developed and the repeats have been mapped to chromosomes of some of the fish species, namely zebrafish, puffer fish, tilapia, rainbow trout, lake trout and other chars. Most of the fish genomes have additional families of tandemly repetitive sequences that do not map to the centromeres but are localized in heterochromatic blocks in the genome. In zebrafish, there is a family of GC-rich repetitive DNA found at paracentric location on the long arms of about one third of the chromosome pairs. In puffer fish, a 10 bp repeat is found at short arms of most of the subtelocentric chromosomes. In atlantic salmon, three minisatellite sequences have been mapped using

FISH technique. Probes of 18S and/ or 28S rDNA have been used to localize the major ribosomal RNA cistron in many fish species. Probes of 5S rDNA also have been localized in zebrafish, puffer fish, atlantic salmon, brown trout, chinook, coho, rainbow trout and *Salvelinus* spp. Probes specific to histone genes are mapped in Atlantic salmon, brown trout, rainbow trout, *Coregonus lavertus* etc. In mice and humans, FISH experiments have shown that certain transposable elements accumulate preferentially on the sex chromosomes, possibly as a result of reduced crossing over. CILINE2 sequence is found preferentially on chromosomes 1 in tilapia, and this has now been shown to be the sex chromosome pair.

Mapping of polymorphic microsatellite markers would be especially useful for fish genetics. Since fish chromosomes are smaller than mammalian chromosome, fewer polymorphic loci would need to be mapped to have informative markers flanking most genes of interest. Many phenotypic traits of importance to aquaculture such as disease resistance, rapid growth, fat content of flesh, early or delayed maturity etc. may be the result of one or two major genes. The genes controlling these traits could be mapped using informative microsatellite loci. Once the chromosomal regions are identified, the genes could be isolated by positional cloning or by micro dissection.

To localize single-copy genes on chromosomes, clones from large insert libraries such as cosmids, PACs, BACs or YACs are usually required. In platy-fish and medaka, cosmid clones containing sex linked single copy genes were used as probes in FISH to identify the sex chromosomes. Moreover, sex-chromosome specific paint probes have been prepared for lake trout and tilapia. Both Yp and Yq probes exist for lake trout. Several chromosome or chromosome

arm specific probes have been made for rainbow trout. Another use of paint probes is to identify intraspecific deletions and translocations. The work on exploring the possible application of autosome-specific chromosome paint probes is undertaken in zebrafish. Species-specific probes are useful for quick identification of immature and related species and for detection of chromosomes in inter-specific diploid and triploid hybrids.

Application of cytogenetics in fishes

Cytogenetics is an exciting, dynamic field of study which analyzes the number and structure of human and animals chromosomes. Changes that affect the number and/or structure of the chromosomes can cause problems with growth, development, and how the body functions. Chromosomal abnormalities can happen when egg and sperm cells are being made, during early fetal development, or after birth in any cell in the body. Changes to chromosome structure can disrupt genes, causing the proteins made from disrupted genes to be missing or faulty. Depending on size, location, and timing, structural changes in chromosomes can lead to genetic defects, syndromes, some chromosomal changes may have no effect on individual's performance/ health. Cytogenetic analyses are commonly performed to determine if an individual is at risk for common aneuploidies (syndromes caused by having extra or missing chromosomes), syndromes caused by structural abnormalities (like unbalanced translocations or inversions), or to determine if extra or missing genetic material is present through cytogenetic microarray testing. In genetic research, chromosome analysis of embryonic stem cells and induced pluripotent stem cells to ensure the genetic integrity of these cell lines.

Molecular Taxonomy

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The field of biology that deals with the theory and practice of classification of organism is called as taxonomy. The term Taxonomy was proposed by de Candolle in 1813 which means law of arrangements (taxis- arrangement or order; nomos- law). It is the science of biological classification. It consists of three separate but interrelated parts *i.e.* 1) classification, in which we arrange organisms into groups or taxa based on their mutual similarity or evolutionary relatedness, 2) Naming or nomenclature and 3) identification which involves process of determining a particular isolate belonging to a recognized taxon.

Taxonomy helps us to organize huge amounts of knowledge about organisms because all members of a particular group share many characteristics. It places them in useful groups with precise names so that researchers can work with them and communicate efficiently. Initially morphological features formed the basis of identification but then other features were also taken into consideration for this purpose. Study of taxonomic features allows the construction of databases that can be used for a further rapid identification of organisms.

Advances in molecular techniques have helped to establish a new area in taxonomy research called molecular taxonomy, studying genetic relationship of different taxonomic categories using molecular markers. The information encrypted within these molecular data can dramatically improve the taxonomy studies. This made the taxonomists realize significance of molecular data and made them understand that other traditional methods are although important but molecular evidences could be final or confirmatory evidences.

Various molecular markers, mitochondrial DNA or nuclear DNA markers such as microsatellites, internal

transcribed sequences, ESTs, RAPDs are now being used. The choice of these markers for particular applications is not straightforward one and is often based on the prior experience of the investigators. It is also important to keep in mind that there is no perfect method and the choice of a particular technique is often a compromise that depends on several reasons, including: the resources of the laboratory, financial constraints, available expertise, time limitations and, more importantly, the research question pursued. All points should be scrutinized carefully to avoid an inappropriate choice. It has therefore become crucial that researchers have a basic understanding of molecular tools. The molecular techniques now have become less time consuming, molecular markers combined with new statistical developments enable finding out differences and similarities between individuals, and the population of origin of single fish, resulting in many new research possibilities and applications.

“Pre-DNA World” in taxonomy research

Conventional method using the morphological and anatomical features of an individual is still the most applied process in both identification and taxonomy. Although useful in several cases, the use of anatomical characters for species identification procedures has several disadvantages. First, there is a much morphological plasticity between organisms of the same species. The use of morphology is also complicated by the existence of sibling species which can lead to mistaken identifications if a few morphological features are considered in the analysis.

Finally, most morphology-based approaches cannot be applied in cases where there is just a small amount

of biological material available for examination. For instance, forensic laboratories often have to deal with low quantities of biological material, most of the times degraded after a prolonged exposition to harsh environmental conditions. Besides a reliable diagnostic procedure can be time-consuming and need expert taxonomist support. With the convergence of new ideas from genetics and biochemistry and new technological developments, the field of species identification started to rely on information from the molecular components of cell. The first molecular methods successfully employed were based on the analysis of proteins: protein electrophoresis, allozyme analysis, immunological reactions, etc. Although it had its own advantages, several features are known to limit the use of proteins: its rapid degradation in samples under stress conditions, the risk of cross-reactions with proteins from closely related species, the differential expression of proteins in specific tissues etc.

“Post-DNA World” in taxonomy research

A molecular marker is a DNA sequence used to “mark” or track a particular location (locus) on a particular chromosome, *i.e.* marker gene. It is a gene with a known location or clear phenotypic expression that is detected by analytical methods or an identifiable DNA sequence that helps the study of inheritance of a trait or a gene.

Over the past years, application of molecular tools has increased dramatically because of the advances in PCR, DNA sequencing, data analysis and it has been possible to apply in several areas such as species identification, population genetics, brood stock development, fish health management, transgenic, genetic diversity, conservation and genomics. Today, there are two general classes of genetic markers that are routinely used in population genetic and phylogenetic studies: (1) nuclear DNA and (2) extra nuclear DNA (mtDNA, cpDNA etc.) markers.

Major characteristic of the DNA molecule that makes it a useful tool for molecular species identification are that it is stable and can be easily isolated from minimum source material and can be stored efficiently for a very long-time. Methods for DNA cloning, sequencing

and hybridization developed in the 1970s and DNA amplification and automated sequencing during 1980s led to developing various classes of DNA markers. Later, with the advent of the PCR many different techniques emerged, ranging from sequencing of the DNA of interest to methods analyzing length polymorphisms, such as microsatellites.

DNA based tools used for Molecular taxonomy

DNA hybridization:

The hybridisation of complementary DNA oligonucleotides is a basic principle of molecular biology used in various methods with possible applications in species identification. Some of the early assays were based on solid-phase hybridizations conducted on nitrocellulose or nylon membranes between whole genomic or synthetic DNA probes of known origin and DNA extracted from the target sample. The probe was usually labelled with fluorescent or radioactive molecules. A positive hybridisation signals the presence of biological material from the species used to make the probe. A widely known DNA hybridisation-based approach is the fluorescence in situ hybridisation (FISH) technique. This technique uses fluorescently labelled probes to detect nucleic acid sequences in whole cells, allowing the direct detection of organisms

Restriction Fragment Length Polymorphisms (RFLPs)

The RFLP analysis is used for the detection of interspecies variation at the DNA sequence level. These restriction enzymes cleave the DNA molecule at specific recognition sites resulting in a set of fragments with different lengths that could be separated according to their molecular size by conventional gel electrophoresis. The distinctive RFLP profile of each species is the result of the unique genomic distribution of recognition sites and the distance between them. RFLP assays usually do not require any sophisticated equipment and no prior sequence information about the species. With the advent of the polymerase chain reaction (PCR)

technique RFLP analysis (known as PCR-RFLP) has become routinely used for species detection. Most PCR-RFLP approaches focus on mtDNA cytochrome b or ribosomal RNA (rRNA) genes. A major disadvantage of the RFLP technique is the possible existence of intraspecies mutations at restriction sites that can lead to false results due to the gain or loss of restriction fragments.

Amplified Fragment Length Polymorphisms (AFLPs)

The AFLP method combines the reproducibility of restriction fragment analysis with the power of PCR. It is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA. The method usually works by digesting a small amount of purified genomic DNA with two or more restriction enzymes. Double-stranded oligonucleotide adapters are ligated to the sticky ends of DNA fragments. The ligated DNA fragments are then amplified by PCR using primers complementary to the adapter and restriction site sequence. The AFLP technique allows the simultaneous screening of different loci randomly scattered throughout the genome. However, it is technically demanding, labour consuming and interpreting results may need automated computer analysis and still the results could produce non-specific outcomes.

Random Amplified Polymorphic DNA (RAPD)

RAPD profiles are generated by the random PCR amplification of DNA segments using short primers of arbitrary nucleotide sequence of usually 10 nucleotides long. These primers hybridize with different genomic regions at low annealing temperatures. Each species is identified by a specific banding pattern in an electrophoretic gel or similar technique resulting from the different genomic location of primer-binding sites. The RAPD method does not require prior sequence information for PCR primer design but is extremely dependent on variations in laboratorial conditions, needing carefully developed laboratory protocols to be reproducible and often overlapping results leading to confusion.

Conventional PCR using species specific primers

A conventional PCR based method consists of the design of PCR primers that will only produce an amplification product in the presence of DNA from the target species. The process of designing species-specific primers is now straightforward due to the vast number of genomic sequences available and software programs that assists in primer designing.

The specificity and sensitivity can be enhanced by performing a nested PCR, in which the target region is first amplified with an outer primer pair followed by a second amplification using an internal primer pair. The chance of amplifying unspecific genomic regions is reduced with nested PCR as compared to conventional PCR since undesired sequences amplified in the first round of PCR are not likely to contain a sequence to which the primers for the second amplification reaction will bind, increasing the specificity dramatically.

Real-time PCR

Real-time PCR is used for the detection of a specific DNA sequence in a sample by measuring the accumulation of amplified products during the PCR using fluorescent technology. An important benefit of this method is the ability to quantify the starting amount of a specific DNA sequence in the sample (this approach is also known as quantitative PCR).

The real-time PCR has the advantage over conventional PCR-based identification systems of working without post-PCR handling, with a minimised risk of carryover contamination in the laboratory. It also offers an increased sensitivity by allowing discriminating false PCR amplifications from non-target DNAs and is a relatively fast genotyping method, with some platforms affording high throughput automation.

Sequencing of PCR products

The DNA sequencing analysis is currently the most used method for molecular species identification. The advent of rapid and cost-effective PCR-linked DNA sequence analysis has circumvented the need

for screening of genomic libraries and cloning of DNA fragments. The identification is achieved by comparing the sequence of a genomic region found in the target sample with a comprehensive reference database. Ideally, the structure of the DNA region to be analysed must consist of a variable sequence (informative enough to discriminate species) flanked by highly conserved regions (ideal to design universal PCR primers that amplify in a large number of species).

In order to attain a correct identification it is crucial to consult a reliable database, namely one that guarantees that (a) the reference specimen was correctly identified by a taxonomic expert or by other molecular methods, (b) the same sequences were obtained in independent studies, preferentially from the full distribution range of the species and that (c) most related species have distinct DNA profiles. A common way to assign a particular sequence to its species of origin is to perform a BLAST search on the vast GenBank sequence database. However, care must be taken when assigning the questioned sequence to the species with the highest similarity, because several gaps and false sequences are known to be present in these databases. Moreover, this approach does not provide any information and can lead to false identifications if the target sample belongs to a previously uncharacterized species.

Although different regions have been targeted for species identification procedures, most studies rely on sequence information from nuclear ribosomal RNA genes and mtDNA regions. The list of studies using mtDNA cytochrome b gene for species identification is extensive. This gene shows a high level of similarity with species limits and can be amplified in several vertebrate species under standard conditions by using a single pair of universal primers.

DNA barcoding

A DNA barcoding system for all animal species has been proposed based on 650 to 750 bp of the mtDNA cytochrome c oxidase (COI) gene. The use of a single gene in delineating and identifying species and the extent of separation between intra- and interspecies variations have been of a concern for many. The

targeting of only a single DNA region could be problematic since a failure in the amplification of that region due to, for instance, the occurrence of a polymorphism in a primer binding region, may originate a false or null result. This problem can be overcome by using degenerate primers that pinpoint at polymorphic areas in the primer binding sites, in cases where these variants have been previously identified. Moreover, DNA sequencing methods do not allow the discrimination and identification of biological material from different species mixed in a same sample, unless fragments are cloned before sequencing to separate each molecule of DNA.

A solution to overcome some limitations of single-gene approaches is the use of multi gene sequence analysis, a method based on the sequencing of multiple protein-coding genes. A phylogenetic approach could be used to identify the species of origin of an individual by constructing a phylogenetic tree with concatenated sequences of multiple genes. The phylogenetic inference can be established by the clustering pattern of the species of interest with related species. An obvious limitation to the use of this approach is the necessity of sequencing several genomic regions, a fact that can be cumbersome with sophisticated labour, money and expensive labs.

DNA microarrays or DNA chips

DNA microarray consists of small glass microscope slides, silicon chips or nylon membranes with a large number of immobilized DNA fragments arranged in a regular pattern. A DNA microarray provides a medium for matching a reporter probe of known sequence against the DNA extracted from the target sample of unknown origin.

A DNA microarray built with species-specific DNA sequences can be used for identifications purposes. For instance, the DNA extracted from the target sample could be labelled with a specific fluorescent molecule and hybridized to the microarray DNA. A positive hybridization is detected with appropriate fluorescence scanning/imaging equipment. Advances in printing technology have enabled the production of microarrays containing hundreds of thousands of probes revealing the

potential to achieve sensitive and high-throughput species identifications. DNA microarrays need specialized robotics and imaging equipment that are not available in most laboratories. Advanced bioinformatics tools are also necessary to reduce the complex data into useful information.

Applications of molecular taxonomy in evolutionary studies

Analyzing and comparing the genetic material of different species is an important method for studying species evolution and also for identifying species/sub-species/strains/stocks etc. Molecular tools are used to make comparisons between the numbers, locations and sequences of genes in different organisms which reveal the evolutionary relationship between different species and also to identify the taxonomic position of new or unidentified species. Application of bioinformatics tools are central and made these tasks more simple and accurate.

Applications of molecular taxonomy in Population genetics and conservation of species

Population genetics is concerned with the analysis of demographic and evolutionary factors

affecting the genetic composition of a population. To understand the patterns of natural genetic diversity and basic genetic structure of isolated populations within the species is a basic need for developing scientific management strategies for conservation. Mitochondrial DNA (mtDNA analysis), microsatellites, allozymes, RFLP, RAPD and AFLP markers are the popular genetic markers employed in population genetic studies. The data generated employing these genetic markers are converted into numerical data matrix for using in different genetic data analysis software / bioinformatics tools to estimate different population genetic parameters. Based on these analysis and observations, a conservation policy based on MUs (Management Units) can be framed.

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An overview of the basic concepts and principles of Population Genetics

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Population genetics is a branch of the evolutionary biology that tries to determine the level and distribution of genetic polymorphism in natural populations and also to detect the changes in genetic composition that results from the operation of evolutionary forces (mutation, migration, selection and drift). It was developed as a theoretical discipline by R. A. Fisher (a British statistician), J. B. S. Haldane (a British Geneticist) and Sewall Wright (an American geneticist). This discipline focuses on the Mendelian population which is a group of interbreeding individuals who share a common set of genes (called the gene pool), to understand the genetics of evolutionary processes. An understanding of the genetic structure of a population is the key to our understanding of the importance of the genetic resources and the importance of genes for the conservation of species and biodiversity. In this chapter, basic concepts of population genetics are discussed first in order to have a better understanding of the principles and applications that are detailed in the preceding sessions.

Main ideas of population genetics

Genetic variation describes differences between the DNA sequences of individual genomes. Each individual has two nuclear genomes (a paternal genome and a maternal genome). A genetic **locus** is a DNA region having a unique chromosomal location. It is a sequence of DNA that may or may not code for a protein. A gene is defined as a sequence of DNA that codes for a protein.

Alleles are alternative forms of a particular

sequence, frequency of which is the proportion of chromosomes of that type in the population. A locus is **monomorphic** if there is only one allele in the population and **polymorphic** if there are two or more alleles in the population at appreciable frequencies. At any genetic locus the maternal and paternal alleles normally have identical or slightly different DNA sequences. If the two alleles are identical, they are **homozygotes** and if different termed as **heterozygotes**.

Every individual has both a phenotype and a genotype. The genotype is the specific set of genes carried by the individual. The phenotype is the set of characteristics (e.g., morphological, physiological, behavioral) expressed by the individual. The phenotype is produced by the genotype in interaction with environment. **Genotypes** are formed due to pairing of genes (alleles) during union of gametes for zygote formation. It is the set of alleles an organism carries at one or more loci. If there are n alternative alleles, there will be $n(n+1)/2$ possible genotypes. The genotypes of the parents are broken down and from the genes transmitted in the gametes a new set of genotypes is constituted in the progeny. Consequently, only alleles have continuity over time while the genotypes do not, and the gene pool evolves through changes in the frequencies of alleles.

The fundamental quantities in population genetics are **frequencies** of genes (alleles) and genotypes by which the genetic structure of populations is expressed. A frequency is a proportion or a percent. It always ranges between 0 and 1. **Genotypic frequency** is the number of individuals with one particular genotype divided by the total number

of individuals in the population. This is done for each of the genotype at the locus of interest. The sum of the genotypic frequencies should be 1. The distribution of genes in different individuals of the population is the **allelic (gene) frequencies**. The gene pool can be described with fewer parameters when allelic frequencies are used. Allelic frequency is the ratio of number of copies of a given allele in a population to sum of all alleles in the population. Allelic frequencies may be calculated from observed numbers of different genotypes at a particular locus, or from the genotypic frequencies. The frequencies of two alleles are commonly symbolized as p and q where $q = 1 - p$. A third allele is symbolized as r .

For example, for the two alleles A_1 & A_2 in the autosomal locus A , allelic frequency p & q respectively can be calculated as follows:

$p = (\text{frequency of the } A_1A_1 \text{ homozygote}) + (1/2 \times \text{frequency of the } A_1A_2 \text{ heterozygote calculated as } 2pq)$

$q = (\text{frequency of the } A_2A_2 \text{ homozygote}) + (1/2 \times \text{frequency of the } A_1A_2 \text{ heterozygote calculated as } 2pq)$

There are several basic evolutionary processes that act on a population and cause genetic variation. The first and the most fundamental is **mutation** which is defined as any heritable change in the genetic material. Mutation is the ultimate source of all genetic variation without which evolution cannot be materialized. **Recombination** is the secondary source of genetic variation which can create new combination of alleles, but not new alleles. New recombination of alleles can lead to new phenotypes upon which natural selection can act. **Natural selection** means that individuals with heritable favorable variations survive and reproduce at a higher rate than other individuals in the population. It operates through differences in the fertility of parents that is controlled by genes. **Fitness** is a concept related to selection. It is the number of offspring that an individual leaves during its lifetime or it is the lifetime reproductive output of the individual. The average fitness of all individuals in the population is called **population fitness** or mean fitness.

Another important evolutionary process is the **genetic drift**. Random change in allelic frequency from one generation to the next due to repeated

random sampling of gametes from a population is called genetic drift or simply drift. It is also called the *Sewall Wright Effect* in honor of the population geneticist who championed its importance in the 1930's. Genetic drift will cause isolated populations to diverge from one another.

Movement of genes takes place only when organisms or gametes migrate and contribute their genes to the gene pool of the recipient population. This process is referred to as **gene flow**. Gene flow is a source of genetic variation which introduces new alleles to the population and spreads unique alleles to other populations, long-term effect of which is the opposite of genetic drift. Through exchange of genes, different populations remain similar, and thus **migration** is a homogenizing force that tends to prevent populations from accumulating genetic differences among them.

Random mating or panmixia means that any individual has an equal chance of mating with any other individual in the population. There should be no special tendency for mated individuals to be alike in genotype or to be related to each other by ancestry. **Non-random mating** occurs when mating individuals are genetically related to one another or are phenotypically similar to each other than two individuals chosen at random.

Population genetics deals with how the evolutionary forces distribute the DNA polymorphism in biological populations. For evolution of a species to occur the gene frequencies of that population must undergo change and the evolutionary processes described above play a major role in it.

The Hardy-Weinberg law

Hardy-Weinberg law, formulated in 1908, is a mathematical model and is the fundamental principle in population genetics because it offers a simple explanation for how the Mendelian principles that result from meiosis and sexual reproduction influence allelic and genotypic frequencies of a population. The assumptions or conditions that must be present for the law to apply are (1) an infinitely large (2) randomly mating population (3) free from mutation (4) migration and (5) natural selection.

The law states that “In a large random-mating population with no selection, mutation or migration, the gene frequencies and the genotype frequencies are constant from generation to generation”. If the conditions of the Hardy-Weinberg law are met, the population will be in genetic equilibrium, and two results are expected. First, the frequencies of the alleles will not change from one generation to the next, and therefore the gene pool is not evolving at this locus. Second, the frequency of any genotype in the population after one generation of random mating is the product of the parental allelic frequencies. These two conclusions have been demonstrated experimentally to be valid and form the basis upon which all further population and evolutionary genetics research is based. When the genotypes are in the proportions, the population is said to be in Hardy-Weinberg equilibrium. Genotypic frequencies after one generation of random mating are given by terms in the expansion of $(p + q)^2 = p^2 + 2pq + q^2$ where p and q represent allele frequencies. An important use of the H-W law is that it provides a mechanism for determining the genotypic frequencies from the allelic frequencies when the population is in genetic equilibrium.

Testing for Hardy-Weinberg proportions

The H-W law can be used as a null model to which the genetic structure of any particular population can be compared and it provides a way of evaluating which assumptions are being violated when the expected theoretical distribution of genotypes does not match an empirically determined distribution. From allelic frequencies, we can calculate the expected genotypic frequencies and compare these frequencies with the actual observed frequencies of the genotypes using a chi-square test. The chi-square test gives us the probability that the difference between what we observed and what we expect under H-W law is due to chance. (0.05, 0.10, etc.)

Factors affecting genetic structure of populations

In contrast to idealized populations at Hardy-Weinberg equilibrium, real stocks and populations experience

changes in allelic and genotypic frequencies. Two types of processes cause these changes: dispersive processes (inbreeding and genetic drift) and systematic processes (mutation, migration and selection). Dispersive processes cause changes in allelic and genotypic frequencies that are random in amount and direction of change. Systematic processes cause changes that are consistent and predictable. The allelic and genotypic frequencies of any population are due to the combined effects of inbreeding, genetic drift, mutation, migration, and selection.

Mutation

Usually converts one allelic form of a gene to another. A_1 to A_2 is called a forward mutation; A_2 to A_1 is called a reverse mutation. The rate of mutation is generally low, but varies among loci and among species. Mutations can change the frequencies of alleles. Mutation provides the raw genetic material for evolution. Most mutations will be detrimental and will be eliminated from the population.

Migration

Migration has the potential to disrupt H-W equilibrium and may influence the evolution of allelic frequencies within populations. Migration among populations tends to increase the effective population size (N_e) of the populations and divergence among populations.

Natural selection

Mutation, migration, and genetic drift all influence the pattern and process of adaptation, but adaptation arises chiefly from natural selection. Natural selection is the dominant force in the evolution of many traits and has shaped much of the phenotypic variation observed in nature. Selection in natural populations can be a) Directional selection b) Stabilizing selection or c) Disruptive selection

Fitness and the coefficient of selection.

We measure natural selection by assessing reproduction. It is measured in terms of **fitness** which is defined as the relative reproductive ability

of a genotype or contribution of offspring of an individual to the next generation. Often symbolized as W , and is also called the adaptive value or selective value of a genotype. **Selection coefficient**, s , is a measure of the relative intensity of selection against a genotype and $s = 1 - W$. The coefficient measures the selective advantage of the fitter genotype, or the intensity of selection against the less fit genotype.

The effect of selection on allelic frequencies depends not only on the intensity of selection but also on initial gene frequency. Mutation can continuously reproduce the allele lost by selection.

Genetic drift

In small populations, chance deviations from expected ratios can cause changes in allelic frequency (genetic drift). When the sample is small, the sampling error can be large. All genetic drift arises from such sampling error. Based on population size we can make predictions about the magnitude of drift, direction of which is unpredictable. If the sexes are equal and all individuals have an equal probability of producing offspring, the effective population size is the effective number of breeding individuals.

When males and females are not present in equal numbers the effective population size (N_e) is

$$N_e = 4 \times N_f \times N_m / (N_f + N_m)$$

Other factors such as differential production of offspring, fluctuating population size, and overlapping generations can further reduce the effective population size. The amount of variation among populations resulting from genetic drift is measured by the variance of allelic frequency: $sp^2 = pq/2N_e$

There are several ways in which genetic drift via sampling error occurs in natural populations.

- Population size remains continuously small over many generations
- Founder effect—occurs when a population is initially established by a small number of breeding individuals. Although the population may subsequently grow in size and later consist

of a large number of individuals, the gene pool of the population is derived from the genes present in the original founders (which may have been determined by chance). This has a profound effect on the gene pool in subsequent generations.

- Bottleneck effect—a form of genetic drift that occurs when a population is drastically reduced in size. Some genes may be lost from the gene pool as a result of chance. This can be considered as a form of founder effect, since the population is refounded by those few individuals that survive the reduction.

The genetic drift causes a) change in allelic frequencies of a population over time and cause fixation and extinction alleles b) Reduction in genetic variation within populations c) Differentiation between sub-populations and d) Increased homozygosity.

Nonrandom mating

In order for the H-W principle to hold, individuals in a population must mate at random. But many populations do not mate randomly for some traits, and when nonrandom mating occurs, the genotypes will not exist in H-W equilibrium.

In the broad sense, there are three kinds of nonrandom mating described as follows. **Assortative mating** occurs when two mating individuals are phenotypically more alike than two individuals chosen at random. **Disassortative mating** occurs when mates are phenotypically less alike than two individuals chosen at random. Neither affects the allelic frequencies, but both may affect the genotypic frequencies if the phenotypes are genetically determined. **Inbreeding** is mating between individuals that are related to each other by ancestry. Relatively small amounts of inbreeding can cause tremendous damage to the reproductive potential and productivity of a fish stock.

Inbreeding

Inbreeding is an exception to random mating that increases the number of homozygous individuals above their expectation described by the Hardy-Weinberg principle. It is the mating of consanguineous individuals (individuals having a common ancestor)

which may have alleles that are identical by descent. Two alleles are identical by descent if they have originated from the replication of one single gene in the previous generation. Inbreeding is always defined relative to some reference population in which all individuals are assumed to be unrelated. It is often measured as the **coefficient of inbreeding** (F) which is the probability that two alleles in an individual are identical by descent and can be calculated from pedigrees. It can also be defined as the proportionate reduction in heterozygosity compared to a reference population.

$F = \frac{\text{Expected heterozygosity} - \text{Observed heterozygosity}}{\text{Expected heterozygosity}}$

In a finite population, the average inbreeding coefficient increases each generation, the rate of which depends on population size. Self-fertilization decreases heterozygosity by half each generation and in random mating, $F=0$

Inbreeding depression occurs when inbred individuals have lower fitness than non-inbred individuals because of the increased homozygosity with harmful alleles in double doses and lower frequencies of heterozygotes. Outbreeding is the opposite of inbreeding; *i.e.* mating between individuals less related than the average in the population. The outbred population can have higher fitness than any of the involved inbred populations because of “hybrid vigour”, but **outbreeding depression** can also occur if matings occur between substantially diverged populations.

Population subdivision

If subpopulations are completely isolated and differ in allele frequencies, but are sampled without distinguishing between them, there will be a perceived deficiency of heterozygotes in the sample, even though there is no such deficiency in any subpopulation. This is called **Wahlund effect**.

In a subdivided population, the overall deviation from H-W expected heterozygosity has two components: the deviation due to factors acting within subpopulations, and the deviation due to subdivision (the Wahlund effect). Wright (1951) defined three F coefficients

that describe these divisions *viz.* F_{IS} , F_{IT} & F_{ST} which are related by the expression

$$(1 - F_{IS})(1 - F_{ST}) = 1 - F_{IT}$$

F_{IS} is a statistic used to estimate the deviations of genotype frequencies from the H-W model within subpopulations which make up the total population. F_{IT} is a statistic used to estimate deviations from the H-W model within the total population. F_{ST} is an index genetic differentiation used to describe the degree to which a total population is genetically divided into subpopulations and can vary between 0-1. Nei (1973) developed a similar statistic to F_{ST} called the coefficient of genetic differentiation G_{ST} which is based on the heterozygosity values across local and total population. F_{ST} and G_{ST} are valuable measures of population subdivision.

Genetic variation in natural populations

One of the most significant questions addressed in population genetics is how much genetic variation exists within natural populations. Natural populations show substantial variation for quantitative traits which are measured on a continuous scale. Phenotypic values of quantitative traits are almost jointly determined by the effects of many genes and environmental effects.

Genetic variation is important for several reasons:

- It determines the potential for evolutionary change and adaptation.
- The amount of variation also provides us with important clues about the relative importance of various evolutionary processes, since some processes increase variation and others decrease it.
- The manner in which new species arise may depend upon the amount of genetic variation harbored within populations.
- Evolution by natural selection depends on the existence of genetic variation within a population.

Variation that is not subject to natural selection is useful in DNA fingerprinting, conservation biology, and empirical population genetics. Genetic variation can be quantified by estimating genotype and allele frequencies and also by considering the proportion

of polymorphic loci in a population (P) or by the observed heterozygosity. The genetic variation can be measured both at protein and DNA levels.

Measuring genetic variation with protein electrophoresis

Protein electrophoresis is a biochemical technique that separates proteins with different molecular structures which can be used to quickly determine the genotypes of many individuals at many loci. Most species possess large amounts of genetic variation in their proteins. The technique misses a large portion of the genetic variation that is present, because only genetic variants that cause a change in the movement of the protein on a gel will be observed.

The amount of genetic variation is measured in this via two parameters

- The proportion of polymorphic loci (P) is calculated by dividing the number of polymorphic loci by the total number of loci examined
- Average observed heterozygosity which is defined as the proportion of heterozygotes, averaged over all loci.

Measuring genetic variation via DNA sequence variation

Molecular markers provide useful tools for studying mutation rates, population sizes, natural selection, population structure, and other important ideas in evolution and conservation biology. Techniques in molecular biology provide a means to examine directly nucleotide sequence differences in the DNA. The genetic diversity between and within populations displayed by molecular markers receive extensive interest due to the usefulness of this information in breeding and conservation programs.

These techniques fall into two main kinds: Direct methods using DNA sequencing technology, and indirect methods using restriction enzyme analysis and other molecular techniques to infer variation in DNA sequence.

DNA sequencing has revealed high levels of variation in nucleotide sequences. Single nucleotide differences

(SNPs) are the most common kind of variation at the DNA level. Newer techniques like DNA microarrays or gene chips allow rapid automated detection of SNPs. The variation at DNA level can be quantified by calculating the nucleotide polymorphism (the proportion of nucleotide sites that are polymorphic in a sample) and nucleotide diversity in the population.

Indirect estimates of DNA variation are cheaper and faster than DNA sequencing and are useful for preliminary screening of a large number of individuals. Restriction fragment length polymorphisms (RFLPs), Amplified Fragment Length Polymorphisms (AFLPs), Random Amplified Polymorphic DNA (RAPD), Single Strand Conformation Polymorphisms (SSCPs) etc. come under this.

Certain kinds of satellite DNA in eukaryotic chromosomes are dispersed more or less randomly throughout the genome. Each cluster of repeats is considered a locus and the number of repeats at a particular locus is variable. Such loci are called the VNTR loci for the variable number of tandem repeats. There are two kinds of VNTR loci depending on the length of repeat sequence *viz.* Minisatellite loci and microsatellite loci, both of which are useful genetic markers. Microsatellite loci are highly variable and can be analyzed locus by locus, hence more powerful for detection of genetic variation at population level. Variation in length of DNA sequences is also common and is frequently due to insertion or removal of transposable elements.

Applications of population genetics in fisheries

Fishes are major food source harvested from wild populations. Due to increasing levels of exploitation, current fishery yields have reached or exceeded sustainable limits. Management efforts have focused on the estimation of sustainable yields and the maintenance of sufficient stocks. Little consideration was given to how fishing may affect genetic diversity and selection pressures in the exploited stocks. The foundation for understanding of genetic effects caused by harvesting comes from models in evolutionary biology and population genetics. The high exploitation rate may affect fish species in different ways such as a)

extinction of reproductively isolated populations due to local overfishing, b) irreversible genetic changes in exploited populations due to selective removal of fishes with certain characteristics and c) reduced genetic variability as well as increased rates of genetic drift and inbreeding as a result of reduction in the effective size of a stock.

An important prerequisite for elaborating management strategies in fisheries is identifying the number of reproductively distinct populations, the presence of which can be confirmed by genetic assessments. Significant differences in allele frequencies will be detected when populations are isolated for a sufficient number of generations. Measuring genetic diversity in wild fish populations or aquaculture stocks is essential. Non-interbreeding populations have to be identified to assess the gene flow between different genetic stocks, and to monitor temporal changes in the gene pools. Population size is extremely important in evaluating conservation priorities for a species. Small populations are at risk of going extinct because of demographic stochasticity and genetic drift.

Population genetics has a role in the management of hatchery populations also. Genetic changes to hatchery stocks can occur through selection, drift, or stock transfers. Inbreeding is a major problem in hatchery stocks. It is influenced by a number of factors like the number of breeding individuals in the population, sex ratio, variation in the reproductive success of individual spawners, and effective

population size during previous generations. The effective size of hatchery populations should be as large as possible in order to minimize loss of genetic diversity. It is suggested that N_e of 500-1000 should be maintained to prevent inbreeding and genetic drift related problems and to maintain the genetic variance in fish populations that are used for stocking programmes. Yet, N_e has to be customized at the level of farmers as it may not always be possible to maintain the recommended numbers.

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Protein Isolation and purification by different chromatographic techniques.

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Introduction

Isolation and purification of proteins from crude mixture is an imperative step in the identification and analysis of proteins. Selective methods to isolate individual proteins from a mixture, usually exploit their very specific properties such as binding specificity or biochemical functions. Proteomic studies have revealed that most effective proteome analysis uses a combination of separation and identification techniques. The purification methods are chosen depending on the percentage of purity required and the end use of the protein.

Chromatography has been used for centuries as a means of separation and, over time, has developed into a sophisticated analytical technique. It is a central technology in many fields of applied science such as the synthesis of drugs and pharmaceuticals, purification of the products of organic synthesis, as well as food science, clinical chemistry and forensic science. Due to their high resolving power, different chromatography techniques have become dominant for protein purification. Any separation technique that distributes the components of a mixture between two phases, a fixed stationary phase and a free mobile phase, is known as chromatography. In proteomics, liquid chromatography is more often used than other chromatography formats because of its versatility and its compatibility with MS. In Liquid chromatography, the stationary phase is a porous matrix, usually in the form of packed beads that are supported on some form of column. The mobile phase, a solvent containing dissolved proteins or peptides flows through the column under gravity or is forced through under high pressure. The rate, at which the protein

mixture flows through the column, depends on its affinity for the matrix, and matrices with different chemical and physical properties can be used to separate proteins and peptides according to different selective principles. The main LC techniques used in protein purification are ion exchange, size exclusion, affinity, reversed-phase – HPLC, Hydrophobic interaction Chromatography etc.

Ion Exchange Chromatography

The basic principle of ion exchange chromatography involves the reversible ionic interaction between the surface charge of the proteins and the oppositely charged groups on the surface of the ion exchange adsorbent. Proteins can be positively or negatively charged depending on its isoelectric point and the pH of the surrounding medium.

Stationary phase: Consist of a column packed with ion exchange resins which bind to proteins of opposite charge. Cation exchangers are negatively charged and hence bind to positive charges on the surface of protein. Eg: Sulfopropyl-Sephadex (Strong Cation exchanger), Carboxy Methyl-Cellulose (Weak Cation exchanger). Anion exchangers are positively charged and bind to the negative surface charges on a protein. Eg. Quaternary Amine-Sephadex (Strong anion exchanger), Diethyl amino ethyl-Sephadex (Weak anion exchanger).
Mobile Phase: The mobile phase consists of a buffer, the pH and conductivity of which are of utmost importance. A buffer which gives low conductivity and with pH that gives an optimal charge to the protein is chosen.
Elution Strategy:

Proteins of the same charge as that of the resin will pass through the column while the oppositely charged resins while bind to the column.

Change in pH: The elution buffer will have varying pH which will create a pH gradient in the column and the proteins elute in the order of their isoelectric point (at which pH, the net charge on the protein becomes zero).

Change in Ionic strength: The elution buffer will have varying ionic strength and the relative affinity of the protein to the resin will decrease with increasing ionic strength. Loosely bound molecules will elute at lower ionic strength while strongly bound molecules require higher ionic strength of the buffer to elute out.

Size Exclusion Chromatography

Size exclusion chromatography or gel filtration is a profiling technique which is used to separate proteins according to their size. The separation process depends on the different ability of various proteins to enter all, some or none of the channels in the porous beads. Molecules running through a SEC column have to solve a maze which becomes more complex the smaller the molecule is, as the small molecules have more potential channels that they can access. Larger molecules on the other hand, are for steric reasons excluded from the channels, and pass quickly between the beads. The detour through the channels will thus retard smaller molecules in comparison to larger proteins which elutes first.

The pore size of the gel can be adjusted to exclude all molecules above a certain size. All molecules larger than the pore size are completely excluded from the pore channels and thereby unretained and elute together. The size of the pores in the matrix will also determine the rate of molecules that can enter the pores. This technique is ideal for final polishing steps in purification when sample volumes have been reduced. Samples are eluted isocratically (single buffer, no gradient). Buffer conditions are varied to suit the sample type or requirements for further purification, analysis or storage step, since buffer composition does not directly affect resolution. Proteins are collected in

the purified in the chosen buffer, in the purified form.

Stationary phase: The matrices used in SEC are often composed of natural polymers such as agarose or dextran but may also be composed of synthetic polymers such as polyacrylamide. Gels may be formed from these polymers by cross-linking to form a three-dimensional network. Different pore sizes can be obtained by slightly differing amounts of cross-linking. The degree of crosslinking will define the pore size

Mobile Phase: In contrast to other types of media the selectivity of a SEC matrix is not adjustable by changing the composition of the mobile phase. Optimally there is no adsorption involved, and the mobile phase should be considered as a carrier phase and not one which has a large effect on the chromatography. However, the sample may require a buffer solution with a well-defined pH and ionic composition chosen to preserve the structure and biological activity of the substances of interest.

Elution Strategy: Since molecules are not adsorbed but only retarded on a SEC column the proteins are eluted isocratically and will elute in order with the largest first. A single buffer is used, and hence no gradient pumping systems are needed.

Affinity Chromatography

Affinity chromatography partitions proteins or peptides on the basis of their specific, ligand binding affinity. It is based on the reversible interaction between a protein or a group of proteins and a specific ligand attached to the chromatographic matrix. AC offers high selectivity, hence high resolution, and usually high capacity for the protein of interest. The target protein is specifically and reversibly bound by a complementary binding substance (ligand). The sample is applied under conditions that favour specific binding to the ligand. Unbound material is washed away, and the bound target protein is recovered by changing conditions to those favouring desorption. Ligand-protein interaction is often based on a combination of electrostatic and hydrophobic interactions and hydrogen bonds. Agents that weaken the interaction such as a competitive ligand or changes in pH, ionic strength or polarity are used for desorption

of the bound protein. Samples are concentrated during binding and protein is collected in purified, concentrated form. The ideal stationary phase consist of a gel matrix possessing suitable chemical groups (linkers) that can be covalently coupled to ligand molecules and provide a relatively large surface area for attachment. The ligands can be extremely selective and bind to only a single or very small number of proteins e.g. Antibodies, protein receptors, steroid hormones, vitamins etc. Some ligands are less selective and bind to a group of closely related compounds. Immobilized metal affinity chromatography or IMAC is a type of affinity chromatography where the solid phase contains positively charged metal ions such as Fe^{2+} , Ni^{2+} etc. This can be used to selectively isolate phosphoproteins/peptides, proteins with oligo histidine tags and other negatively charged proteins. Affinity chromatography finds numerous applications such as purification of antibodies (immunoaffinity), glyco-conjugates, DNA binding proteins, enzymes, isolation of nucleic acids etc.

Hydrophobic Interaction Chromatography (HIC)

HIC separates proteins with differences in hydrophobicity. The separation is based on the reversible interaction between a protein and the hydrophobic surface of the chromatographic medium. The proteins are separated according to the differences in the amount of exposed hydrophobic amino acids. To facilitate hydrophobic interaction, the protein mixture is loaded onto the column in a buffer with high salt concentration. Samples in high ionic strength solution (e.g. 1.5 M ammonium sulphate) bind as they are loaded onto the column. Conditions are then allowed so that the bound substances are eluted differentially. Elution is usually performed by decreases in salt concentration. Changes are made in

step wise or decreasing salt gradient. Most commonly samples are eluted with decreasing concentration of ammonium sulphate. Target proteins are concentrated during binding and collected in purified, concentrated form. The most widely used ligands in HIC are linear chain alkanes, with or without terminal amino groups. Aryl groups such as phenyl are also used. The strength of the interaction between the ligand and the protein increases with increase in the number of carbon atoms in the chain and ideally, 4-10 carbon atoms are most suitable for separation.

Reverse Phase Chromatography (RPC)

Reverse phase chromatography separates proteins and peptides with differing hydrophobicity based on the reversible interaction with the hydrophobic surface of a chromatographic medium. The technique is closely related to hydrophobic interaction chromatography. In RPC, the stationary phase is more highly substituted with hydrophobic ligands than in those used for HIC and hence the hydrophobic interaction between the protein and the ligand is much stronger. It is therefore possible to adsorb proteins even in pure water. However, the very strong interaction requires the use of organic solvents and other additives (ion pairing agents) for elution. Elution is usually performed by increase in organic solvent concentration, most commonly acetonitrile. RPC is often used in final polishing steps and is ideal for analytical separations such as peptide mapping. RPC is not recommended for protein purification, if recovery of activity and tertiary structure are required.

Suggested readings

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Genes as Molecular Guardians in Environment Management and Aquaculture

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Introduction

It is a universal phenomenon that all living beings across the species and taxa have more or less a similar system to regulate and protect its own machinery and thereby making the species more sustainable. Such systems play a vital role in the survival of living organism amidst the challenging situations which is imposed by environment in which they live. Environment is prone to routine pressures from various sources such as climatic changes, impact of human civilization etc. It is quite obvious that these pressures will be directly transferred to living organisms living in the particular environment. The intensity or depth of these challenges are more in aquatic species as the developmental activity like industrialization leading to and the continuous discharge of various organic and inorganic waste and other industrial sewage into the aquatic medium are on the increase. Further, the phenomenon of global warming also has got a direct impact in aquatic environment, and the rising concern associated with global climate change has regained the interest in the physiological mechanism that animals use to tolerate extreme heat and adapt to thermal changes in their natural environment. One could see a regular interplay between the genes and the changes takes place in the environment.

Marine habitat as a model of gene environment interaction

Marine bivalves are regularly exposed to varying physico-chemical conditions on a day to day or

seasonal scale. Among these bivalves the intertidal molluscs represented by oysters and mussels are regularly undergoing regimes of immersion and emersion and thereby exposed to abiotic stresses. Stress can be defined as a condition which disturbs the dynamic equilibrium or homeostasis of an organism by the action of intrinsic or extrinsic forces usually referred as stressors. The various abiotic stressors encountered by bivalves both in the wild as well as in the aquaculture include thermal variations, oxidative fluctuations and salinity changes. The bivalves are also prone to biotic stressors like microbial infection and other parasitic attack. The increasing contamination of bodies of natural freshwater and marine ecosystem around the world by anthropogenic substances is another category of environmental stressor. The composite threats of all these factors are more relevant in this era of global warming causing chronic stress on aquatic organisms. The tolerance to varying temperature is an indication of population response to climate changes which will ultimately determines the speciation of the region. Living organisms manage to overcome these harsh environmental challenges through the regulating the expression of certain genes playing vital roles in stress management.

Multigene families linked to stress management and bio monitoring

Latest findings based on the transcriptome sequencing reveal that certain families of genes are readily

expressed in response to environmental challenges. Among them the prominent ones are heat shock family genes, cytochrome P450 multigene families and anti-oxidant enzyme genes. Among the stress related genes, heat shock protein (HSP) genes are much studied across different flora and fauna. They also represent the evolutionarily conserved molecular chaperones with prominent roles in managing all sorts of abiotic and biotic stress. The presence of both constitutive and induced isoforms indicates the importance of these proteins in bivalve life. Heat shock protein family members help the inter tidal bivalves in making themselves 'prepared for stress' amidst the much challenging environment. The role of these multigene family members in thermo tolerance is well documented among bivalves. Latest report of HSP gene sequence divergence and synonymous single nucleotide polymorphism (SNPs) suggest the potential use of these genes in population genomics as well. The studies co relating the presence of HSP and aging in bivalves has opened up a new way of assessing the process of aging.

The HSPs play a major role in host-parasite interactions and this has received considerable attention of researchers as it is relevant from both clinical and biological perspectives. HSPs of invading parasites elicit immune response within host and can be used as potential source for generating vaccines. Owing to the responsiveness to diverse forms of stress, heat shock response as detected through HSP gene expression provide widespread application in bio monitoring and environmental toxicology. HSPs are believed to play a major role in managing global warming. Global warming is a slow process and HSPs can evolve more efficiently and offer better protection to the individuals. Individuals with mutant forms or over expression of HSPs points towards the probable natural selection and open up scope for selective breeding as most characters of economic importance and evolutionary significance are inherited quantitatively (Quantitative trait loci, QTL).

Role of HSPs in controlling diseases in aquaculture

HSPs also play an important role in activating the innate system of immunity. The role of stress protein

like HSPs in promoting immune responses to protect the cytoplasmic components from biotic stressors such as bacterial challenge is documented in recent years. The reports on the role of heat shock proteins in disease control in aquatic organisms are coming up fast. The increased expression of heat shock protein genes are reported in fishes and shellfishes infected with viruses and bacteria like white spot syndrome virus (WSSV), Noda viruses, *V. harvei*, *V. anguillarum*, *V. parahaemolyticus*, *V. alginolyticus*, *Renibacterium salmoninarum* etc.

A new approach to induce the expression of HSPs in animals to manage disease and other stressors are gaining attention. Biochemical inducers of plant origin are found to be an effective non-stress inducer to enhance the HSP production. Administering Feed containing HSP enriched bacteria is a new approach of delivering HSPs for protection against diseases in aquaculture. HSPs are also used to raise vaccines to prevent diseases in fishes using pathogen derived HSPs as antigens. Such vaccines are found to be giving long lasting protection and suggested as a promising alternative to antibiotics in aquaculture. The American oyster *Crassostrea virginica* infected with the parasite *Perkinus marinus* found to be surviving well after a sub lethal thermal shock than the control group. All these findings suggest that there is a clear case for exploring HSPs further and applying its potentials in management of different kinds of abiotic and biotic stressors in aquaculture.

Aquatic Oxidative stress and role of antioxidant genes

Oxygen is a vital factor for the existence of life and it is an important element for all flora and fauna of terrestrial and aquatic origin. Marine organisms, especially bivalves like mussels and oysters are sedentary and sessile growing attached on a suitable substratum. They primarily live in the inter tidal zone within aquatic habitat. Owing to the characteristics of the habitat, these bivalves are constantly exposed to changing levels of oxygen, temperature and salinity. Reactive oxygen species (ROS) are produced in abundance when the animals are exposed to oxygen related stress such as hypoxia.

ROS molecules such as super oxide anion radical (O_2^-), hydroxyl radical (HO) and hydrogen peroxide (H_2O_2) used during oxidation can destroy tissues and cells. Prolonged hypoxia can result in mass mortality in oysters and a control system is essential to survive the situation.

Hence suitable physiological mechanisms mediated through biochemical pathways are essential to maintain the cellular homeostasis. Oxidative reactions are a part of aerobic life and as a consequence results in the production of reactive oxygen species within the cell. Accumulation of ROS can cause structural damage to organic macromolecules like protein, carbohydrate and lipids. Such oxidative damage resulting from reactive oxygen species (ROS) is called as Oxidative stress. The aquatic organisms are usually prone to more oxidative stress owing to the chronic exposure to xenobiotic pollutants resulting in more ROS). Oxidative stress causes imbalance between exogenous and endogenous reactive oxygen species which result a decline in cellular defense. The increase in ROS results the slowdown of the cell's antioxidant defense system and consequently free radicals generated are allowed to disrupt cellular functions, causing damage to DNA with DNA lesions, denaturing the integrity of cellular membranes by lipid peroxidation, modifying proteins by protein peroxidation. The ROS defense system comprise of enzymatic antioxidants and non-enzymatic antioxidants like vitamin E, ascorbate, β -carotene, and urate. Antioxidant enzymes play a pivotal role in maintenance of homeostasis within the cells and in cell mediated antioxidant defense by removing ROS. Among the antioxidant enzymes, Super oxide dismutase (SOD) is the key defense molecule which fight ROS followed by others such catalase (CAT), glutathione transferase (GST) and glutathione peroxidase (GPX) playing vital roles in the detoxification pathways.

Super oxide dismutase (SOD) removes the super oxide free radicals through the process of dismutation by converting them into hydrogen peroxide and this in turn is further reduced to water and oxygen by the action of CAT and GPX. Among them SOD is the leading enzyme readily responding to stress. SODs (EC 1.15.1.1) are categorized based on the

metal content. Two isoforms are normally present in most of the organisms. They are the extracellular and intracellular Cu/ Zn SOD present in the extra cellular matrix and the intra cellular space respectively within the tissues. The intra cellular Cu/ Zn SOD is also found in inter membrane space of mitochondria and nuclei. Recent literature states that the intracellular Cu/ Zn SOD also perform certain key functions other than dismutation in molluscs. It prevents the formation of toxic compound peroxyxynitrite by stimulating to produce nitric oxide and superoxide molecules. The up regulated expression levels of SODs as revealed through transcriptomic studies in *Haliotis discus discus* and *Crassostrea gigas*, *Crassostrea madrasensis* exposed to heavy metals and thermal stress or hydrocarbon are reported. Moreover, elevated level of expression of SOD gene after *Vibrio anguillarum* challenge point towards its involvement in immune function. The potential benefits of these genes are widely explored in bio monitoring and in selective breeding programs. The SOD, CAT and GPX are being used as a biomarker to monitor the level of mercury, lead and copper as the reduction of these enzymes are noticed in the bivalve *Chlamys farreri* undergoing lipid peroxidation on exposure to lead.

Stress adaptations as revealed through oyster genome sequencing

The latest report based on the findings of the sequencing of whole oyster genome along with selected transcriptomes have revolutionized the hitherto understanding about the factors behind defense and stress adaptations in Pacific oyster *Crassostrea gigas*. As reported in the paper published in the prestigious journal NATURE (SEP 2012), the Pacific oyster genome contains eighty eight heat shock protein 70 (HSP70) genes with potentials roles in protecting cells from thermal and other stressors. This huge number of HSP70 genes is a record compared to the seventeen in human beings. Further, the report reveals the expansion of genes like superoxide dismutase, cytochrome P450 and inhibitor of apoptosis proteins (IAPs) in oyster.

Conclusion

From the above descriptions, it is evident that there exists an array of genes regularly in interaction with the changes in the surrounding habitat. Selection of individuals with better presence or expression of these as brood stock using these genes as biomarkers would be helpful in raising offspring with better survivability and performance in aquaculture. Hence tapping the potentials of this genomic approach would be a better strategy to make the culture practices more sustainable and profitable.

Suggested readings

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Molecular Systematics

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Systematics is the scientific study of the diversity and kinds of organisms and their relationships based on which biological evolution and diversity is assessed. It comprises of classification of organisms into groups based on phenetic or phylogenetic characters, naming of organisms and final identification organisms and placing them into any of the previously described groups. Systematics also provides clues to the evolutionary biology of an organism and it is another way of studying the evolutionary patterns on a temporal scale. Molecules carry information regarding the ancestry and history of an organism and hence inferring the biological relationships based on molecular systematics provides coherent answers to many biological questions. The ease of generating sequence data for many independent data sets and relating the phenotyping variations with sequence divergence is the key to taxonomic inferences. The use of molecular data along with conventional taxonomy has become a scientific practice for the past few decades after the emergence of sequencing technologies and this is termed as integrative taxonomy.

Inferring Phylogenetic information

Inferring phylogenetic information is the key to molecular systematics. The individuals within and between populations are genetically related directly or indirectly sharing common ancestors. The degree of genetic divergence provides insights into the extent of relationships among and between individuals in populations. The process of phylogenetic estimation begins with the collection of sequence data which are homologous. The sequence data could be generated by collecting the organisms and sequencing a particular gene or it could be retrieved from databases

like NCBI, GenBank. The next step is to align sequences and identify mutations like insertions and deletions. Wherever needed, gaps should be inserted into the sequences to increase their similarity. There are many algorithms to align multiple sequences and these algorithms tries to minimize the total effect of all the possible changes in pair wise sequence comparisons. When some sequences are ambiguously aligned, they should be excluded from the data set. A phylogenetic tree can be constructed using sequence data and the tree could be rooted using outgroup sequences from distantly related species so as to get more insights into the evolutionary relationships. The choice of outgroup is very important as it will affect the topology of the tree. In addition to the sequence data, a model of sequence evolution must also be selected as the methods in molecular phylogenetics are based on a series of assumptions about substitution process.

Methods for phylogenetic inference

There are four methods that have been widely used for phylogenetic inference; maximum parsimony, neighbor-joining, maximum likelihood and Bayesian inference.

Maximum parsimony

It is one of the earliest inference methods used to infer phylogeny. Maximum parsimony method uses character states as compared to distance methods and it is based on the optimality criterion which is the rule to decide the best alternative tree; it selects the tree or trees which require the fewest character state changes and thus attempt to minimize homoplasy. The length of an unrooted tree is directly calculated using Fitch's algorithm which moves through the tree

assigning one or more character states to each of the internal nodes. The tree space (theoretically possible tree topologies for a given number of taxa) is usually searched using heuristic searches or exact searches. Exact searches examine all possible trees (exhaustive searches) or parts and thus guarantee that the tree found is optimal whereas heuristic searches do not search all possible trees. The advantage of this analysis is that it is very fast for the analysis of large data sets which contain many sequences and it gives robust estimates when the branches of the tree are short. But maximum parsimony performs poorly when the rates of evolution vary substantially.

Neighbour-joining and minimum evolution

It is based on pair-wise distance methods on the assumption that dissimilarity between any sequences is directly related to their phylogenetic relationship. This dissimilarity arises from the number of changes along the branches or in other way the evolutionary distance. Distance methods are clustering methods like neighbor-joining or optimality methods like minimum evolution. In neighbor joining methods, DNA or amino acid sequences are converted into a distance matrix which is then used to construct a phylogenetic tree. Optimality methods evaluate the score of a tree based on the squared deviation of the pair wise observed distances between each pair of taxa, estimated from the data matrix and the distance separating those taxa on the tree. In the minimum evolution, the sum of branch lengths optimized according to the least-square criterion is the optimality criterion. The advantage of distance methods is that they are comparatively fast compared to all the other methods and they perform well when divergence between sequences is low. But there occurs loss of information when the sequences are converted to distances and it is difficult to obtain reliable estimates when the sequences are highly divergent.

Maximum likelihood

Maximum likelihood is one of the standard methods of statistics and it has also been applied to phylogenetics. The likelihood of a phylogenetic tree is the probability of observing the data given

the tree and the model of evolution. This also uses an optimality method. The best tree is the one that renders the observed sequences most likely to have evolved under the expected evolutionary model. The tree space is usually explored using heuristic searches and the advantage of likelihood is that it allows the inference of phylogenetic trees using complex models of sequence evolution. Maximum likelihood based methods are the most robust way for estimating molecular phylogenies and understanding sequence evolution. But the main disadvantage is that the results are highly dependent on the model of sequence evolution used and the method is computationally demanding when the number of sequences is large.

Bayesian inference

It is the most recent phylogenetic inference method. Bayesian statistics is closely related to maximum likelihood method; the optimal hypothesis is the one which maximize the posterior probability. Bayes' theorem states that the posterior probability for a hypothesis is proportional to the likelihood multiplied by the prior probability of that hypothesis. Bayesian analysis allows complex models of sequence evolution to be applied to for the whole sequence data set and for different partitions of it. It involves specifying a model along with a prior distribution and then integrating the product of these quantities over all parameter values that are possible so that the posterior probability values of each tree could be determined. But the likelihood functions for phylogenetic models are too complex to integrate analytically and hence Bayesian approaches depend on Markov chain Monte Carlo (MCMC) procedures. This algorithm functions by sampling trees from the distribution of posterior probabilities. Unlike maximum likelihood which looks for the single most likely tree, Bayesian MCMC searches for the "best set of trees" in the tree space. Bayesian analysis given a robust approximation of the evolutionary tree and hence it is based on powerful statistical foundation as in maximum likelihood method. The disadvantage of Bayesian methods is that prior distributions for the parameters must be specified and it is also difficult to determine if the MCMC approximation has run for sufficient number of cycles.

Statistical support for phylogenetic trees

Parametric bootstrapping uses a Monte Carlo simulation to generate the data. A simulation is carried out using replicate data sets of the same size as original according to the null hypothesis being tested. Likelihoods according to both the null and alternative hypotheses are estimated for each replicate data set and the LRT (Likelihood Ratio Test) statistic is derived and a significance test is implemented based on simulated values. Parametric bootstrap is computationally very demanding when the data sets are large. Several non-parametric likelihood based tests are also used which determine whether the difference in fit of two or more alternative tree topologies to the data is significantly higher than expected under the null hypothesis of random sampling error. The most widely used methods are the Kishino-Hasegawa, the Shimodaira-Hasegawa and the unbiased tests. Non-parametric tests appear to be conservative and parametric tests appear to be liberal in empirical comparisons. But generally, to assess branch support for phylogenetic trees non-parametric bootstrapping is used.

Molecular markers used for molecular systematic studies

To infer the information required for phylogenetic analysis, several molecular markers can be used. Molecular markers can be characterized as Type I and Type II markers; Type I markers are associated with genes of known function and type II markers are associated with genes of unknown function. Allozyme markers are type I markers as the proteins they encode are associated with some functions. Microsatellites and other neutral markers are type II markers unless they are associated with genes of some known function.

Allozymes

Allozymes are codominant markers having been expressed in a heterozygous individual in a Mendelian way. Thus allozyme analysis provides us with data on single locus genetic variation which can answer many questions about fish and fish populations. To

detect allozyme variation, the first step is to extract allozymes from tissues using specific protocols. Then the variation is detected through electrophoresis in an acrylamide or cellulose acetate gel. Individuals that are homozygous show a single band whereas heterozygous individuals show two bands. Allozymes are one of the most studied forms of molecular variation due to their simplicity, low cost and the requirement of little specialized equipment. Any kind of soluble protein is suitable for allozyme analysis. A large number of loci can be screened at a time. The limitations of this technique include requirement of a large amount of tissue and consequently this method could not be applied when the organisms are small (for eg; larval forms). The tissue sampling method is invasive and so the fish needs to be sacrificed and the tissue needs to be stored cryogenically. A point mutation in a nucleotide sequence may not result in a change in amino acid at all and thus could not be detected by protein electrophoresis. In addition to that, a change in DNA that results in a change in amino acid will not result in the overall charge of the protein and therefore is not detected. In spite of their limitations, the use of allozyme analysis has been widespread in fisheries mainly in fish systematics, population structure, conservation genetics, mixed stock fishery analysis and forensic analysis.

Mitochondrial DNA markers

Mitochondrial DNA is non-nuclear DNA in the cell having located in within organelles in the cytoplasm called mitochondria. Mitochondrial DNA is maternally inherited with a haploid genome. The entire genome undergoes transcription as one single unit. They are not subjected to recombination and thus they are homologous markers. They are selectively neutral occurring in multiple copies in each cell. Mitochondrial DNA is physically separate from the rest of cell's DNA and so it is relatively easy to isolate from any tissue or blood sample. Due to the maternal inheritance of mitochondrial DNA, the effective population size is smaller than nuclear DNA and so mitochondrial DNA variation is more sensitive to population bottlenecks and hybridizations. The differences in the nucleotide sequence of DNA molecule in the mitochondria can be determined directly or indirectly by several methods. Many population genetic studies have employed RFLP

(Restriction Fragment Length Polymorphism) analysis of mitochondrial DNA for understanding population genetic variation either by digesting the whole purified mtDNA with restriction endonucleases or by DNA sequencing of small segments of mtDNA molecule obtained by PCR amplification. These techniques with increased resolution and maximum information have made mtDNA analysis very popular.

The newly emerged sequencing technologies have enabled direct sequencing of mitochondrial genes and several sets of universal primers have been developed from conserved sequence regions. Slow evolving gene regions are being used for inter species comparisons and fast evolving gene regions for population comparisons. The only non coding region of mtDNA is D-loop region and this region is fast evolving and mostly used for population comparisons. The cytochrome b and ND-1 and ND-5/6 gene regions are also being used widely. Mitochondrial Cytochrome C Oxidase I gene has been identified as the universal barcode for species level identification due to its conserved nature across a wide range of taxa. DNA barcodes are segments of approximately 600 base pairs of the mitochondrial COI gene which is a fast, efficient and inexpensive technique helpful in cataloguing the biodiversity. During the last two decades, mitochondrial DNA genes have found widespread application fish taxonomy, biology and population genetics.

Arbitrary Nuclear DNA markers

Arbitrary markers are used when we target a segment of DNA of unknown function. The widely used methods of amplifying unknown regions are RAPD (Random Amplified Polymorphic DNA) and AFLP (Amplified Fragment Length Polymorphism) DNA. RAPD uses an arbitrary primer which can amplify anonymous loci. It is fast, cheap and shows very high amount of polymorphism and this marker does not require knowledge of the genetic makeup of the organism. The major drawback with RAPD markers is the lack of reproducibility and repeatability and the large number of products generated. RAPD is a dominant marker and so homozygous and heterozygous states cannot be differentiated and these patterns are sensitive to

slight changes in amplification conditions. Amplified Fragment Length Polymorphism (AFLP) markers combine the benefits of both RFLP and RAPD. The total genomic DNA is digested using two restriction enzymes. Double-stranded nucleotide adapters are ligated to the ends of DNA fragments to serve as primer binding sites for PCR amplification. Primers complementary to the adapter and restriction site sequence, with additional nucleotides at the 3'-end, are used as selective agents to amplify a subset of ligated fragments. The presence or absence of DNA fragments are detected on polyacrylamide gels and thus polymorphisms are studied.

Specific Nuclear DNA markers

Variable Number of Tandem Repeat is a segment of DNA that is repeated tens or even hundreds to thousands of times in nuclear genome of eukaryotes. They repeat in tandem; vary in number in different loci and differently in individuals. There are two main classes of repetitive and highly polymorphic DNA; minisatellite DNA referring to genetic loci with repeats of length 9-65 bp and microsatellite DNA with repeats of 2-8 bp (1-6) long. Microsatellites are much more numerous in the genome of vertebrates than mini satellites. They are widely used in population genetics of fishes and aquatic invertebrates. Minisatellites can be classified into multilocus and single-locus minisatellites. Multilocus minisatellites are composed of tandem repeats of 9-65 base pair and have a total length ranging from 0.1 to 7kb. Minisatellite loci are used mainly in parentage analysis. They are less useful for population genetic analysis unless we use large sample sizes. The complexity of mutation processes undergone by minisatellite loci is also a limitation. Due to the difficulties in the interpretation of multilocus fingerprints, the research work were concentrated on single locus minisatellite probes and this procedure required reasonable quantities of high-quality DNA. These single locus minisatellite probes have been very useful and successful in detecting genetic variations within and between populations. It has also been used in fisheries for forensics, parentage, genetic identity, estimating mating success and confirming gynogenesis.

Microsatellites

A microsatellite is a simple DNA sequence which is repeated several times across various points in the DNA of an organism. These repeats are highly variable and these loci can be used as markers. Microsatellite occur once in every 10 kbp while minisatellite loci occur once in every 1500 kbp in fishes and due this, microsatellites are more useful in genome mapping and population genetics studies. They are highly variable, non-coding and selectively neutral and the basic assumption while using microsatellite loci is that the predicted amount of sequence divergence between units of interest is directly related to length of time since separation. Microsatellites are codominant markers which are inherited in a Mendelian fashion and they are highly evolving with 10^{-3} - 10^{-4} mutation/generation. The high levels of polymorphism shown by microsatellites have made them one of the most popular genetic markers. Cross amplification with primers developed in closely related species is also possible which minimizes the cost associated with detecting microsatellite sequences in a different species. The analysis of microsatellite loci involves DNA extraction, amplification of the microsatellite loci using specific primers in a PCR machine and examination of the bands using poly acrylamide gel electrophoresis. The recent introduction of automated genotyping machines has made the analysis of size polymorphisms of microsatellite loci with automated genotyping using labeled primers. The use of large number of samples and loci is now possible due to automated genotyping which has increased precision and speed with microsatellite analysis. The constraints of using microsatellite markers are the presence of null alleles and presence of stutter bands. Null alleles are found when mutations occur at primer binding sites of microsatellite locus. The presence of null alleles reduce accuracy especially in parentage or relatedness analysis and assignment tests and the best option is to discard loci showing null alleles. Stutter bands occur when a ladder of bands differing between 1-2 bp is seen and these occur due to slipped strands impairing during PCR or incomplete denaturation of amplification products. Tri-nucleotide and tetra nucleotide repeats usually do not show significant

amounts of stuttering. Microsatellite markers are used in fisheries and aquaculture for phylogenetics and phylogeography, population genetic structure, biodiversity conservation, stocking impacts and hybridization. It is also being increasingly used for forensic identification of individuals, genome mapping and determination of kinship and behavioral patterns.

Single Nucleotide Polymorphisms

Single nucleotide polymorphisms arise due to single nucleotide substitutions (transitions/transversions) or single nucleotide insertions/deletions. These point mutations give rise to different alleles with alternative bases at a particular nucleotide position. SNPs are the most abundant polymorphisms in the genome (coding and non-coding) of any organism. These single nucleotide variants can be detected using PCR, microchip arrays or fluorescence technology. They are considered as next generation markers in fisheries and can be employed for population genetics studies, genomics studies and for detection of diseases.

DNA microarrays or DNA chips

DNA microarray consists of small glass microscope slides, silicon chip or nylon membranes with many immobilized DNA fragments arranged in a standard pattern. A DNA microarray can be utilized as a medium for matching a reporter probe of known sequence against the DNA isolated from the target sample which is of unknown origin. Species-specific DNA sequences could be incorporated to a DNA microarray and this could be used for identification purposes. DNA extracted from a target sample should be labeled with a specific fluorescent molecule and hybridized to the microarray DNA. When the hybridization is positive a fluorescent signal is detected with appropriate fluorescence scanning/imaging equipment.

Expressed Sequence Tags (ESTs)

ESTs are single-pass sequences which were generated from random sequencing of cDNA clones. ESTs can be used to identify genes and analyze their expression by

means of expression analysis. Fast and reliable analysis can be made for the genes expressed in particular tissue types under specific physiological conditions or developmental stages. Differentially expressed genes could be identified using cDNA microarrays in a systematic way. ESTs are most valuable for linkage mapping.

Future of Molecular Systematics

With the advent of Next generation sequencing, enormous amounts of sequence information are being generated for a large number of taxa. So the challenge will be in handling the very large data sets and then integrating different levels of genomic information.

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The science of 'omics' – Genomics, Proteomics and Metabolomics

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Introduction

In Biological science there are more than 1000 "omics" field. Different views on the origin of the word "ome" exists, the claim that it originated from the Sanskrit word OM is one among them. Just as the Sanskrit word OM stands for completeness and fullness, the different "omics" fields in biology adopts a holistic approach in its study, for eg. genomics is the study of the complete genome of an organism, whereas proteomics relates to universal detection of all the proteins present in the cell at a particular point of time, and metabolomics studies all the metabolites present in the cell at a particular point of time.

Genomics

Genomics is the study of the genome of an organism, including the interaction of the genes with each other and with the environment. Genome broadly refers to the total amount of DNA that is present in a single cell of an organism, including its genes *i.e.* the whole genetic information encoded in the DNA. Genes are the units of heredity, which encodes the information to carry out all cellular processes. The term "genomics" was coined by mouse geneticist, Thomas H. Roderick in 1986 and has swiftly developed over the years into a new discipline. Genomics include the mapping, sequencing and functional characterization of genome using recombinant DNA technology, DNA sequencing and bioinformatics tools.

DNA was isolated as early as in 1896; however, the first genome was isolated only a century later. Early genomics began in the 1970's, but it was the techniques developed by group of

scientists in the 1950's that laid the foundation for genomics research. Development of techniques for radiolabelling of biological molecules and discovery of the double helical structure of DNA by Watson and Crick in 1953 propelled more studies on genome. These discoveries led to the understanding of processes such as DNA replication, gene expression and protein synthesis. Later, technological advances such as the polymerase chain reaction (PCR) developed in the 1980's, allowed DNA amplification from small amounts of starting material. Automated DNA sequencing technologies enabled fast genomic sequencing of DNA. PCR and DNA sequencing can be thought of as the prime building stones of the field of genomics.

The first genome was sequenced in the 1970's by Fredrick Sanger and was that of a virus and mitochondrion. Sanger also developed a technique for DNA sequencing, known as the "Sangers Sequencing", for which he received the Nobel Prize in 1980. Since then, whole genome of several organisms has been sequenced. Walter Fries in 1972 sequenced the first gene and ultimately the first whole genome sequence of bacteriophage MS2, a RNA virus. *Haemophilus influenzae* is the first free living organism to have its genome sequenced and *Ceanorhabd tiselegans* genome is the first multicellular organism genome which has been completely sequenced. In the years that followed, genomes of various organism have been sequenced including humans at a very rapid pace. Presently, whole genome sequences are available for over 2700 viruses, 1000 bacteria and archae, and 36 eukaryotes, with new sequences added almost every day.

The field of genomics can be sub classified into (a) *structural genomics* involving the construction of genomic sequence data, genomic maps and gene discovery (b) *Functional Genomics* encompassing the study of the function of the genes, its regulation, gene products etc. and (c) *comparative genomics* which is used to compare gene sequences to understand the functional or evolutionary relationships.

The fundamental technologies used in genomics include PCR, microarray analysis, and DNA sequencing. PCR revolutionised the study of DNA to the extent that it is used every day in identification of microbes, disease diagnosis, bar coding, etc. The basic methodology of PCR involves three important steps: (i) Denaturation : where the template DNA is denatured into its single strand components as a result of the destruction of hydrogen bonds which holds the two strands together. It is often carried out at 94°C (ii) Annealing: here the primers, which are short stretches of DNA, attach to complementary sequences in the template strand. Annealing occurs at lower temperatures in the range of 50°C to 60°C (iii) Extension: DNA synthesis by DNA polymerases now begins at a temperature of 72°C, which is the optimum temperature for most DNA polymerases. The cycle of denaturation-Annealing-Extension is repeated to obtain multiple copies of the same product.

DNA Microarray is a technique that allows large scale genomic analysis. DNA Microarray analysis is a hybridization based technology which enables to investigate and address issues which were previously impossible. It is mainly used to study gene expression and a typical a microarray uses DNA as the hybridization probes and thousands of such probes or spots maybe present in a single chip, which enables the large scale analysis of multiple samples. These probes are immobilized on a solid support (a microscope glass slides or silicon chips or nylon membrane). The mRNA in the tissue is isolated and is converted to cDNA using fluorescently labelled nucleotides. The cDNA is then incubated in the microarray and allowed to hybridise. The unbound particles are then washed off. Only those target sequences which are attached strongly to the probes remain in the chip and these fluorescently labelled target sequences generate a signal which

can then be visualised. Total strength of the signal emitted from a spot depends upon the amount of target sample binding to the probes present on that spot. Microarray technique uses relative quantitation in which the intensity of a feature is under different conditions, and the identity of the feature is known by its position.

Microarray analysis is mostly used in functional genomics for studying gene expression, in detection of single nucleotide polymorphisms and also in comparative genomics where it is used to study the increase or decrease in chromosomal fragments.

DNA sequencing is the process of determining the exact sequence of order of nucleotides within a DNA molecule. The first major sequencing technology to be developed was the "dideoxy method" or the "chain termination" method or the "sangers sequencing" method. Here, the technique uses DNA polymerases to synthesis DNA strands and the main feature of the technique is the inclusion of dideoxy nucleotides. Dideoxy nucleotides lack the 3' oh group to form the phosphodiester group and hence when a dideoxy nucleotide is added to the growing chain, it gets terminated. Four different dideoxy nucleotides are used (ddA,C,T,G) so that the synthesis is not always terminated at the same nucleotide and the reactions are carried in four sets with each set having a different dideoxynucleotide. This results in a set of fragments of varying lengths which are then analysed by gel electrophoresis and the sequence is determined by analysing the distribution of the fragments in the gel. Sanger sequencing methods are used even today for genomic sequencing, however its cost and larger time required for sequencing limits in applications.

Pyrosequencing is another sequencing technology used. This is a single nucleotide addition mechanism which makes use of a DNA polymerase and a chemiluminiscent enzyme. The process involves synthesis a complementary strand to the template, one base at a time and detecting which base was added based on the fluorescent signal. After the fluorescent detection, synthesis is resumed. The light emitted is recorded as pyrogram which corresponds to the order of the nucleotides added which in turn gives the sequence of the sequence of template DNA. Pyrosequencing have become highly

automated and capable of handling large amount of data and in less time.

Shot gun sequencing techniques are used in the sequencing of long DNA strands. The longer sequencing subdivided into smaller groups and the small fragments are sequenced using the chain termination method. Several rounds of such sequencing are carried out using different fragments and the overlapping fragments are then assembled into one long sequence with the aid of computer programs. As the size of the molecule to be sequenced increases, shot gun analysis becomes more complex and leads to more errors, especially in repetitive regions. This is solved by the development of a genome map. A genome map shows the exact position of genes in a chromosome and the relative distance between genes. Once a genome map has been developed, sequencing can be carried out by short gun method using the landmarks or distinctive features on the genome map as reference.

Genomic mapping can be divided into genetic mapping and physical mapping. Genetic mapping makes use of genetic techniques to construct maps showing the position of genes and other sequence features on the genome. It makes use of DNA markers such as Restriction Fragment Length Polymorphisms, Single Nucleotide Polymorphisms, Simple sequence length polymorphisms etc. These are DNA markers which do not contain any genetic information. However, maps based on genetic methods alone are rarely sufficient in aiding the sequencing projects because of their lower resolution and limited accuracy. Thus, physical maps are developed with techniques such as (i) restriction mapping: which locates the relative position of DNA based on the recognition sequences for restriction endonucleases (ii) Fluorescent in situ Hybridization: in which marker locations are identified using a fluorescent hybridizing probe containing the marker of intact chromosomes or (iii) sequenced tagged site (STS) mapping, in which the position of short fragments of DNA are mapped by examining collection of genomic DNA fragments by PCR and or hybridization analysis.

As the scope of genomics grew over the years, the field of bio-informatics developed and evolved in

larger capacities. Bio informatics has mainly three aims:—to organise the existing data in a way which allows easy access of existing data and submission of new entries as they are produced. Secondly, it aims in the development of tools and resources for the analysis for data generated. The third aim is to generate results using tools from these data in a biologically meaningful manner. The main driving force behind bioinformatics is the search for similarities between different biomolecules. Apart from enabling systematic organisation of data in databases, bio informatics tools helps in the prediction of secondary structure (Expasy), construction of phylogenetic tree (clustal W), develop antibody, annotating whole genomes, management of microarray data (Array Track) etc.

Table 1. List of databases in use

Database	URL
Protein sequence (primary) SWISS-PROT PIR-International	www.expasy.ch/sprot/sprot-top.html www.mips.biochem.mpg.de/proj/protseqdb
Protein sequence (composite) OWL NRDB	www.bioinf.man.ac.uk/dbbrowser/OWL www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein
Protein sequence (secondary) PROSITE PRINTS Pfam	www.expasy.ch/prosite www.bioinf.man.ac.uk/dbbrowser/PRINTS/PRINTS.html www.sanger.ac.uk/Pfam/
Macromolecular structures Protein Data Bank (PDB) Nucleic Acids Database (NDB) HIV Protease Database Rel.iBase PDBsum CATH SCOP FSSP	www.rcsb.org/pdb ndbserver.rutgers.edu/ www.ncifcrf.gov/CRYSDb/NEW_DATABASE www2.ebi.ac.uk:8081/home.html www.biochem.ucl.ac.uk/bsm/pdbsum www.biochem.ucl.ac.uk/bsm/cath scop.mrc-lmb.cam.ac.uk/scop www2.embl-ebi.ac.uk/dali/fssp
Nucleotide sequences GenBank EMBL DDBJ	www.ncbi.nlm.nih.gov/Genbank www.ebi.ac.uk/embl www.ddbj.nig.ac.jp
Genome sequences Entrez genomes GeneCensus COGs	www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Genome bioinfo.mbb.yale.edu/genome www.ncbi.nlm.nih.gov/COG
Integrated databases InterPro Sequence retrieval system (SRS) Entrez	www.ebi.ac.uk/interpro www.expasy.ch/strs5 www.ncbi.nlm.nih.gov/Entrez

(Source: Luscombe N. M., Greenbaum. D., Gerstein.M.2001. Review Paper: what is bioinformatics? An introduction and overview. Year book of medical informatics)

Applications

- Drug development, diagnostics and prognostic development.
- Genotyping to predict patient susceptibilities to diseases.
- Molecular medicine.
- Microbial Genomics.
- Bioarcheology, Anthropology and Evolution studies.

- Personalised health care based on an individual's genomic features.
- DNA identification/Forensics.
- Agriculture, Plant breeding.
- Livestock breeding.
- Metagenomics

Proteomics

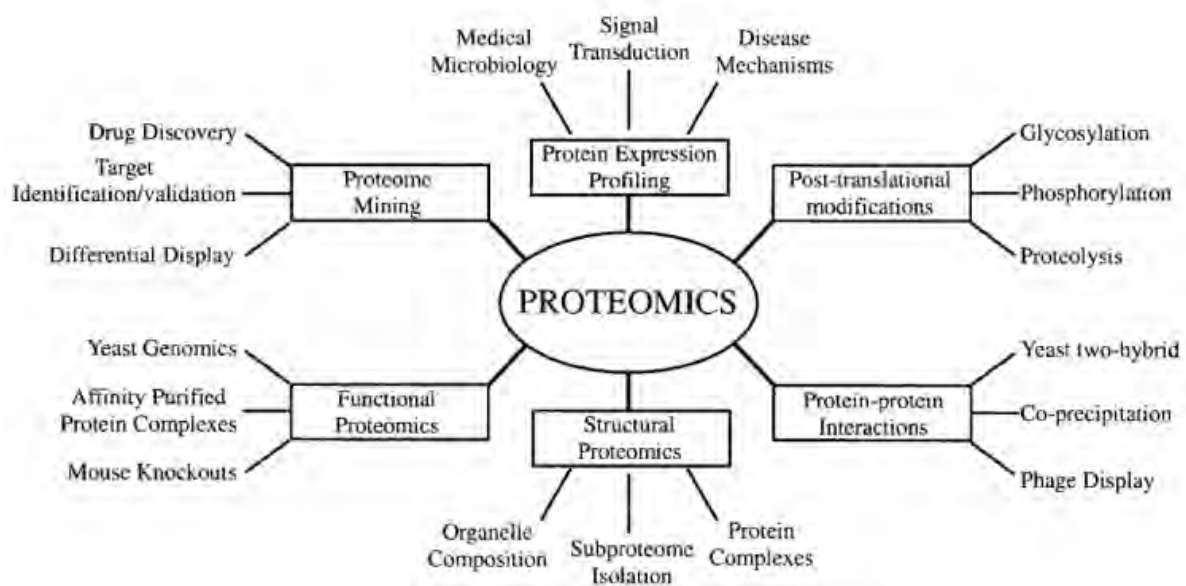
The term proteomics and proteome was introduced in 1995 by Maurice Wilkins & colleagues to emulate the concept of genomics and genome. It can be defined as the "large-scale characterization of the entire protein complement of a cell line, tissue, or organism". Proteome refers to the protein complement of the genome and proteomics, its study. A proteome indicates the quantitative protein expression profile of a cell, an organism or a tissue under any defined condition. However, the concept of proteome is not as literal as it may seem. It essentially deals with understanding the multiprotein systems; the interplay of multiple, distinct proteins and their functions as part of a larger complex network or system.

Essentially, genomics and proteomics are interlinked and synergistic and yet, they are remarkably different. Genomics has several fundamental restrictions, since the sequence of the gene does

not contain sufficient information to understand the gene products. There is very little correlation between gene expression and protein function. The time point of gene expression varies from that of the protein product, and the activity displayed by the protein will become unpredictable by the gene. Post transcriptional splicing and RNA recombination leads to various protein products and thus will multiply the number of components and functions. Also, the rate of synthesis and degradation of nucleotides and proteins vary and their individual stability gives rise to discrepancies in their correlation. In addition, proteins undergo various post translational modifications like addition of functional groups such as sugar or phosphate. A premature protein is enzymatically cleaved to become active or intra-or inter molecular interactions give rise to functional oligomeric proteins. All these modifications give rise to the complexity of the protein products. The different types of biological information cannot be hence correlated or linked quantitatively. The study of genomics and proteomics are different entities, thus, becomes imperative.

Proteome Analysis

Measuring the protein expression or protein analysis poses many analytical challenges. Analysis of



Types of proteomics and their applications to biology

gene expression is made possible by microarrays which rely on two essential components: PCR and hybridization of oligonucleotides to complementary sequences. Such tools are not available with protein analysis. Also, since proteins are post translationally modified, multiple protein products will be present for a particular gene and differentiating between these products adds to the challenge. However, despite of all these challenges characterizing the proteome and its components is achievable. This was made possible with the development of techniques which provide sensitive and specific means to identifying and characterising proteins. The combination of sequential methods exploiting different properties can provide high resolution analysis of very complex mixtures. The present analytical strategies can reach different level of resolution, depending on platform used.

2D gel electrophoresis and multidimensional liquid chromatography are the two methods that are predominantly used in protein separation. The two techniques used differ in their sensitivities and high throughput possibilities. The 2D PAGE or the two dimensional poly acrylamide gel electrophoresis was developed by Patrick H.O'Farell by combining two electrophoretic techniques: SDS PAGE and Isoelectric focussing. The method uses two non-related physical properties sequentially. In the first dimension, proteins are separated owing to their migration in an immobilized pH gradient. This method is also called as iso-eletric focussing. It is an electrophoretic method, where protein molecules move under the influence of electric current. Proteins being amphoteric molecules, presents a negative or positive charge in their ionised groups depending on the pH of their surrounding medium. When the electric current is applied, the proteins migrate till it reaches the pH where its net charge equals zero (known as pI of Protien). At this point protein migration cease, and the proteins get focussed. At this point, the second dimension is applied. The proteins are now separated according to their mass. The IEF strips are equilibrated with SDS detergent which imparts a negative charge to the protein. An electric current is applied and the protein moves based on their charge under the influence of the current. The separated proteins appear as bands

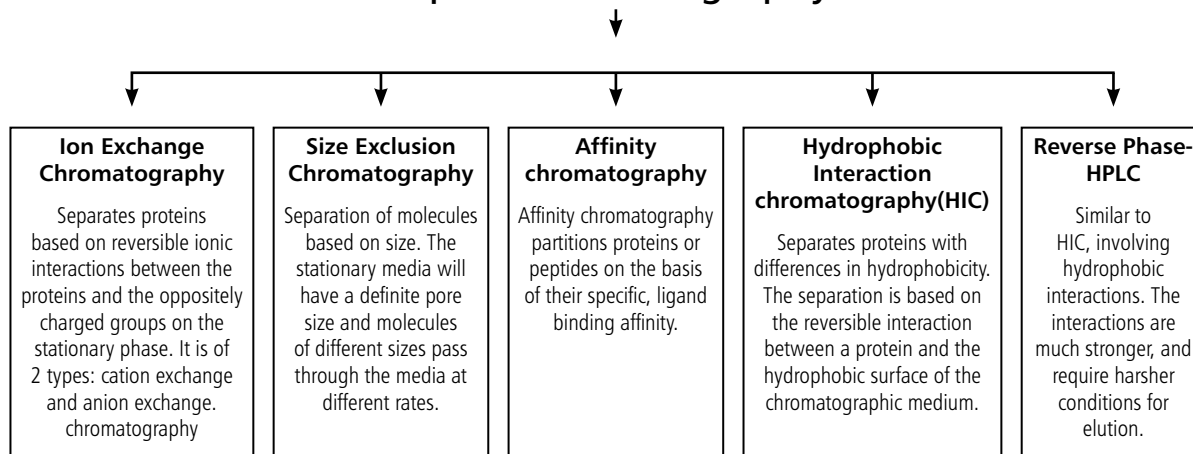
and can be visualized with naked eye using non fluorescent dyes such as silver nitrate or coomassie blue or fluorescent dyes. A protein profile map can be drawn by image analysis, displaying and matching the spot pattern on computer screen. This can be then be compared with related proteome. The spots obtained by 2D-PAGE can also be used for protein identification through Mass spectrometry. Although, there are several gel free strategies available today for protein identification, 2D PAGE remains a popular method.

Chromatography is any separation technique that distributes the components of a mixture between two phases, a fixed stationary phase and a free mobile phase. In proteomics, liquid chromatography is more often used than other chromatography formats because of its versatility and its compatibility with MS. In Liquid chromatography, the stationary phase is a porous matrix, usually in the form of packed beads that are supported on some form of column. The mobile phase, a solvent containing dissolved proteins or peptides, flows through the column under gravity or is forced through under high pressure. The rate, at which the protein mixture flows through the column, depends on its affinity for the matrix, and matrices with different chemical and physical properties can be used to separate proteins and peptides according to different selective principles. The different types of liquid chromatography used in proteomics are below:

Liquid Chromatography

Protein subsequent to purification can be used for acquisition of protein structure information and protein identification. One of the earliest methods used for protein identification was microsequencing by Edman chemistry (known as Edman Sequencing) to obtain N-terminal amino acid sequences. Although the use of Edman sequencing is waning in the field of proteomics, it is still a very useful tool particularly so because it helps to obtain the N-terminal sequence of a protein (if possible) to determine its true start. It involves labelling the N-terminal amino acid sequence of a protein or peptide with Phenyl isothiocyanate. Mild acid hydrolysis then results in the cleavage of the peptide bond immediately

Liquid Chromatography



adjacent to the modified residue, but leaves rest of the residue intact. The terminal amino acid (or rather its phenyl hydrodantoin residue) can then be identified by chromatography and the procedure is repeated on the next residue and next, thus building up a longer sequence.

Mass spectrometry has become an indispensable tool in proteomics for acquiring the protein sequence information. Mass Spectrometer is instrument that can measure the mass/charge ration of ions in vacuum. From these data, molecular masses can be determined with high accuracy, allowing the composition of a given sample or analyte to be determined. In proteomics two types of analysis can be carried out: (i) *Analysis of intact peptide ions*: this allows the mass of intact peptide ions to be calculated, and these masses can be used to identify proteins in sample by database searching (ii) *Analysis of fragmented ions*: This enables the mass of peptide fragments to be determined which can be used in correlative database searching to derive *de novo* sequences. Mass spectrometers have three main components: a source of ions, a mass analyser and an ion detector. The ionization source converts the analyte into gaseous phase ions in vacuum, ions are then accelerated in an electric field towards the analyser. The analyser separates the ions based on their m/z ratios and the ions finally hit the detector, which record the impact of individual ions. MS in proteomics can be categorised into two based on the mode of ionisation as: MALDI-TOF MS (matrix assisted laser desorption ionisation-time of flight MS) and ESI-MS (Electrospray Ionisation MS). ESI MS is mainly linked to liquid chromatographic

interaction while MALDI-TOF is more adapted to high throughput approaches and is therefore suited for large scale proteomics. The mass analysers used in proteomics are of four type, viz., triple quadruple (TQ), time of flight (TOF), ion trap and Fourier transform ion cyclotron resonance (FT-ICS) analysers. MS instrumentation can provide mainly three types of highly useful analysis in proteomics:—(i) MS can provide accurate molecular mass measurements of intact proteins (ii) definitive identification of target proteins by digesting the protein with enzymes to obtain peptides and comparing the peptide mass against databases and (iii) sequence information of the protein using tandem mass spectrometry.

Proteins have evolved under selective pressure to carry out different functions which depends on the way in which proteins interact with other molecules. This interaction ultimately depends on the three dimensional structure of the protein – its overall shape, presence of clefts, cavities complementary to particular ligand/substrate, distribution of charges internally and on the surface, positioning of key amino acids etc. Thus, structural analysis of a protein is the key to understanding its biological function. The two major techniques that can be used for this purpose are X-ray crystallography (XRC) and Nuclear magnetic resonance (NMR) spectroscopy. X ray crystallography exploits the fact that X rays are scattered or diffracted in a predictable manner when they pass through a protein crystal. Protein molecules, when arranged regularly in a crystal, diffract X-rays and the nature of diffraction depends on the nature of electrons that are present in each atom and the organization of atoms in space.

The purified protein sample at high concentration is crystallised and the crystals are then exposed to an x ray beam. The resulting diffraction patterns can then be processed, initially to generate information about the crystal packing symmetry and the size of the repeating unit that forms the crystal. This information is obtained from the pattern of the diffraction spots. The intensities of the spots are further analysed to determine the "structure factors" from which a map of the electron density can be calculated. Various additional methods are thereafter used to improve the quality of this map until it is of sufficient clarity to permit the building of the molecular structure using the protein sequence. The resulting structure is further refined to fit the map more accurately and to adopt a thermodynamically favoured conformation.

Nuclear magnetic resonance is a phenomenon that occurs because certain atomic nuclei have magnetic properties. These properties are exploited to obtain chemical information in NMR spectroscopy. NMR spectroscopy is used to determine the structure of proteins in solution in size range between 5 and 25 KDa.

Although the technology for solving protein structures has advanced significantly, it still remains a labour-intensive and expensive process. An alternative, although somewhat less accurate, method for obtaining structural information is to predict protein structures using bioinformatics tools. Secondary structure of proteins can be predicted using three state predictions because residues in a protein can be present in any of the three states, which are alpha helix, beta sheets and coil. Early methods for three state predictions were based on the statistical likelihood of particular amino acid appearing in a given type of structure. For example, some amino acids such as glutamate, have helical propensity and hence more likely to occur in alpha helices than anywhere else in the protein. While other residues such as valine have strand propensity *i.e.* they are abundant in beta sheets and strands. Currently, there are sophisticated structural prediction algorithms such as PSI- PRED which uses multiple alignments and also incorporate evolutionary and structural information from databases to increase the accuracy of prediction.

Tertiary structure of a protein can be predicted with reasonable accuracy if the structure of a closely related protein is available and can be used as a template. This approach is known as comparative modelling or homology modelling and generally works well if the two sequences show greater than 30 % identity over 80 or more residues. The first step is to find suitable templates, which is achieved by searching for homologous protein sequences and identifying those with solved structures. When a suitable template has been found, the sequence of the template protein(s) and query protein are aligned using an algorithm such as clustal W. Different softwares use slightly different methods, a common strategy is to identify residues in the template that are part of the proteins structural core and those forming the surface loops. The main disadvantage of comparative modelling methods is that only structures with suitable templates can be modified. In contrast, *ab initio* methods can predict protein tertiary structures from first principles *i.e.* in the absence of any structural information. A typical procedure would involve defining a mathematical representation of polypeptide chain and the surrounding solvent, define an energy function that accurately represents the physicochemical properties of proteins and use algorithm to search for the chain conformation which possesses the minimum free energy. *Ab initio* methods are often not used for polypeptides greater than 200 residues for the fact that larger polypeptide chains can fold into potentially infinite number of different structures.

Integration of all the above techniques encompasses the current technologies used in proteomics. The large scale analysis of proteins has generated huge amounts of data due to the new technologies in proteomics. Protein sequences database play a vital role as a central resource for storing the data generated. Several large scale protein databases act as repositories of protein information. Some of them are GenPept, NCBI Entrez protein, RefSeq, Swissprot, PIR-PSP (Protein information resource-proteins sequence database), TrEMBL etc.

Applications of proteomics

Protein Mining: It is the identification of all (or as many as possible) the proteins present in a sample. Mining enables the cataloguing of the

proteome and reduces the dependence on gene expression patterns for identifying composition of a proteome.

Protein Expression profiling : This involves the identification of proteins in a particular sample as a function of the particular state of the organism or the cell (eg. Disease state, developmental state, differentiation state etc.). Protein expression profiles are used in cancer research, for example to identify tumour subtypes and to obtain a more reliable classification.

Protein Network Mapping: Network mapping is the study of how proteins interact with each other in a living system. All proteins work in close association with each other. Complex network of protein interactions can be characterised through methods such as affinity capture techniques. It offers the ability to study the status of all the components in a biochemical pathway and is one of the most ambitious and powerful potential future applications of proteomics.

Mapping of protein Modifications: Here, the main task includes the identifying the mechanism of protein modification. A variety of analytical techniques are available with which it is possible to study and identify modified proteins and also the nature of modifications.

Metabolomics

Metabolomics is the systematic study of the unique chemical fingerprints that specific cellular processes leave behind. In simple terms, it is the overall study of metabolic expression at any given point in time. Metabolomics is a rapidly emerging field and it combines several strategies to identify and quantify cellular metabolites using sophisticated analytical technologies with the application of statistical and multi-variant methods for information extraction and data interpretation.

Metabolites are the smallest chemical components which are present in any cell at any time and are known to “act as spoken language, broadcasting signals from the genetic architecture and the

environment”. They are the intermediate and end product of any metabolism and provide information on the physiology of the cell. Metabolites, unlike genes and proteins are not subject to regulation and post translational modifications, and hence serve as a direct signature of the biochemical activity. Therefore, they are also easier to correlate with the phenotype of an organism. In this context, metabolomics or metabolic profiling has become a powerful strategy in biochemical profiling of cells for various applications such as disease diagnosis, therapeutic purposes etc.

“Metabolome” refers to the complete repertoire of small molecules in cells, tissues, organs, and biological fluids. The term metabolome was coined in synergy with proteome & transcriptome and like both; metabolome is dynamic and changing every second. It includes products or metabolites from a large network of metabolic reactions where the product of one reaction serves as the input for other chemical reactions. Hence, it is the combination of metabolites, rather than individual metabolites, which are of biological relevance.

The concept of metabolic profiling existed as early as in the 1940’s. The metabolic profiling of saliva and urine were first performed by Roger Williams, who identified the difference in patterns of metabolic profiles for patients associated with Schizophrenia. The term metabolic profiling was coined in 1971 by Horning, who used Gas chromatography- Mass spectrometry to measure the components of urine and tissue extracts quantitatively. By the 1980’s, NMR technologies which could identify metabolites in unmodified biological samples were developed. NMR based metabolomics took shape in 1984 by the research works done by Jeremy Nicholson who used HNMR to diagnose Diabetes Mellitus. Metabolomics since then has come a long way and in 2005, the first metabolomics database called as the METLIN was developed to characterise the human metabolites. With over 60,000 deposits, METLIN is currently the largest repository of data in metabolomics.

Metabolome analysis

Metabolomic assessment can be done using cells, fluids or tissues. Biofluids are the easiest samples to

work with and include serum, plasma, urine, ascitic fluid, saliva, bronchial washes, prostatic secretions or fecal water. The Initial step in metabolome analysis involves freezing of all metabolic processes within the cell using physical agents such as liquid nitrogen. Following which the metabolites are extracted by alkali, acid or ethanol extraction. The extracted sample is then analysed for profiling. Subsequent to extraction, the sample is then separated using chromatographic techniques. Since the physico-chemical nature of the different metabolites in a metabolome is different and metabolites in tissues or body fluids are present in a broad range of concentration, no single analytical method is capable of analyzing all the metabolites. Hence, multiplexed methods of separation are used for separation and extraction. This also increases the number of compounds that are detected. For example, extracting the same cell with aqueous and organic solvent enhances the number of hydrophilic and hydrophobic compound detected. Similarly, using two separation techniques like reverse phase chromatography and hydrophilic interaction chromatography increases the variety of metabolites purified. Reverse phase chromatography and hydrophilic interaction chromatography purifies hydrophobic and hydrophilic compounds respectively.

Subsequent to purification, the sample is analysed, commonly using mass spectrometry techniques. Quadruple TOF MS are often used due to its increased sensitivity and reliability, but other TOF and ion trap instruments are also used. MS technologies using MALDI are also used in metabolome analysis but it results in significant background at lower molecular weights which complicates the analysis. Other surface based technologies for MS analysis of metabolome include secondary ion mass spectrometry (SIMS), Desorption electrospray ionisation (DESI) etc. NMR techniques are also employed for metabolic profiling and are the only detection technique which does not rely on the separation of analytes. NMR enables the detection of all types of metabolites and is highly reproducible and simple, but is relatively insensitive when compared to mass spectrometry.

The metabolite peaks obtained using mass spectrometry is then analysed using bioinformatics

tools. Metabolome software such as XCRS enables the users to upload data, perform data processing and browse results within a web based interface.

The metabolomics software does not produce any metabolite identification. Instead, it searches for peaks which vary in groups of sample and the peaks of interest are selected and generate putative matches from selected databases by comparing the m/z ratio of the selected peaks. This must be now confirmed by additional MS/MS and comparing the MS/MS fragmentation data and retention time with standard compounds. Even though metabolite databases have increased considerably over the last decade, a large number of metabolite features detected from biological samples do not return any matches. Identification of these unknown features requires denovo characterization using traditional methods. Thus, it is important to note that identification of all metabolite features detected by LC/MS is not completely possible.

Metabolome study or analysis can be carried out in two approaches:-targeted approach and the Non-targeted approach.

Targeted approach

This approach to metabolomics is when there is a specific biochemical question or a hypothesis to be answered or studied like for instance, studying the effect of a drug. This is the most developed approach in metabolomics that is used to measure the concentration of a limited number of chemically related metabolites like amino acids or hormones and often used in the pharmacokinetic analysis of drug metabolism, measuring the influence of a drug or genetic modifications of a specific enzyme. Triple quadrupole-MS methods are often used in targeted analysis since these methods are quantitatively reliable and offer the absolute quantification of even low concentration of metabolites. It is a well established approach in the diagnosis of inherited metabolic disorders.

Non-targeted Approach

Non targeted approach is global in scope and is used for simultaneous measurement of as many metabolites

as possible in a biological sample irrespective of its chemical class. It can be carried out using either NMR or MS technologies. Liquid chromatography coupled with Mass spectrometry is the most common method of choice for metabolic profiling and can be used in the detection of almost all analytes. Unlike targeted approach, non targeted approach generates vast amount of data which are complex and large to analyse. Introduction of metabolome software such as MathDAMP, MetAlign, MZmine and XCMS has made the analysis of untargeted metabolome data easier. Untargeted metabolomics approach has the potential to provide greater insights into many fundamental biological processes. It helps reveal many previously uncharacterised metabolites with respect to their structure and function.

Applications

Metabolomics was originally proposed as a method of functional genomics, but now its applications extend well beyond that. It is used for comparing mutants, assessing responses to environmental stress, studying global effects of genetic manipulation, comparing different growth stages, toxicology, drug discovery, nutrition, cancer, Diabetes and natural product discovery. Some of the important functions are described below:

- **Toxicology:** This type of metabolic profiling enables understands the physiological changes in response to a toxic insult of a chemical or a mixture of chemicals. It can be used as a biomarker of hepatic, renal and lung toxicity by analyzing various metabolites such as glucose, lactate, lipoproteins and amino acids which may either increase or decrease thus providing for a recognizable pattern associated with an organ dysfunction. Though much of the data have not been validated and some overlap exists between various toxins, the pattern, temporal rate of change and extent of change in metabolites can still provide toxicity assessments which can be used for preclinical drug
- screening and for following a patient clinically to monitor target organ effects.
- **Therapeutic Metabolomics:** Most of the drugs act at the level of metabolites and metabolomics is a more direct measure of the action of a drug. Thus, measuring the biochemical status using metabolomics helps in understanding how the disease manifests, how drugs work and also in identifying responders and non responders to treatment. It also involves identifying altered metabolic pathways in diseases that represent novel drug targets. Example: In cancer cells with isocitrate dehydrogenase mutation, an increased level of metabolite 2-hydroxyglutarate was observed. The result suggests the inhibition of 2-hydroxyglutarate as a therapeutic approach to the cancer.
- **Biomarker discovery for disease identification:** Here, metabolite profiling is used to generate quantitative data of metabolites from control populations and test population with disease. The data is then analysed to and determine which metabolites are discriminatory for the disease and which of these could be used in predictive medicine. Characterization of function of unknown gene and proteins by screening for metabolites that accumulate after gene metabolism or enzyme inhibition etc. Also in the determination of phenotype as a result of genetic manipulation such as gene deletion or insertion.
- **Metabolic foot printing:** Metabolic foot printing refers to the extracellular metabolite or exometabolome characterization. The footprint of the extracellular medium consists of the medium components (less the substrate uptake) and the secreted metabolites. This is applicable only in the case of cell culture medium and is useful in studying the behaviour and responses of the cultured cells. This has potential clinical applications especially in IVF treatments.

Functional Genomics

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Introduction

The sequence information comprising of billions of bases do not communicate the role of the genes and how they are influencing the formation of organism, aging, biological pathways and what changes in them lead to diseases etc. Functional genomics comes into action to answer the above queries and it provides information about the role of different genes in comprising the function of cells and organism. The science of functional genomics helps the researchers to find out the gene expression and its regulation and its impact on complex production traits at the genome level. The goal of functional genomics could also be described as to find out the biological factors behind a phenotype and its expression in different situations or environment. With the advancement of sequencing techniques, researchers can directly go into the sequence variant lines and sub types and thereby to identify the polymorphic forms such single nucleotide polymorphisms (SNPs) and their impact in regulating the expression of certain genes. Functional genomics provides a reliable platform to understand gene environment interaction at the genome level and thereby to design strategies in breeding and production. Further, the elements such as non-coding RNAs (ncRNAs) and introns conventionally believed as non-functional are getting into limelight with the advancement in sequencing technologies. Many such hitherto unrevealed entities are coming up and even now the real challenge in functional genomics is not in obtaining new data but in analysing it. A series of techniques are available and also emerging in functional genomics with specific focus on their application to enhance production in agricultural, livestock and aquaculture systems.

Functional genomic approaches

The targets in functional genomics is achieved through the integration of different branches such as transcriptomics, proteomics, metabolomics, nutrigenomics and epigenetics. Transcriptomics deals with the quantification gene expression at the transcript level. Proteomics focus on the expression of specific proteins and their structures. Metabolomics explains various metabolites produced in cell during various phases in cell cycle. Interactomics is another branch with applications in agriculture as they focus on the host pathogen interaction. Another area namely epigenetics studies the non-inheritable changes taking place at the genome and its impact in phenotype. Nutrigenomics deals with the how diets are affecting gene expression. All these branches are working together to meet the challenges in food security and to ensure sustainable production. Further it is anticipated in long term that the results of functional genomics are properly integrated with quantitative genetics and ultimately expected to contribute improved agricultural productivity.

Single and multiple gene expression profiling methods

Analysis of the single gene expression is normally carried out by a variety of techniques which include Northern Blotting, Ribonuclease Protection Assay (RPA), Reverse transcription PCR and quantitative real time PCR. Expression profiling of more number of known mRNA is possible by procedures like Differential Display (DD), Representational difference analysis (RDA) and suppression subtractive hybridization (SSH). As the technological advancement

increases, following techniques based on sequencing the transcriptome is getting popular.

Transcriptomic techniques

Transcriptome constitute the whole population of RNA such as m RNA, r RNA, micro RNA and other regulatory RNAs and the study of this contribute the field of transcriptomics. The popular techniques in transcriptome studies are Expressed sequence Tag(EST), microarray, and serial analysis gene expression (SAGE). Many candidate genes of commercial importance which could be used in breeding programmes are being isolated. Further the evolution of inflammatory response markers through transcriptomic studies helped to detect the nutritional additives negatively regulating the growth of farmed animals like Atlantic salmon.

EST and Microarray analysis

Genomics involve the generation of DNA sequences and DNA markers, genetic linkage, physical maps and QTL mapping, gene discovery and the genetic control of complex traits and metabolic pathways. There exist three levels of genomic branches namely structural genomics, functional genomics and comparative genomics. Essential steps or targets of functional genomics involves the gene expression profiling through monitoring RNA and protein expressions following proteomic and transcriptomic tools and also find out mutant forms of gene. It co relates the gene function and gene trait relationships through gene expression analysis with the advanced procedures like DNA microarray. Expressed Sequence Tags (ESTs) are generated by creating libraries of mRNAs represented as different clones. The sequences from ESTs are properly clustered to create EST assemblies which form the basis to develop microarray probes and also to generate data for gene identification. National Center for Biotechnology information (NCBI) keeps ESTs numbering into millions and serve as a major database support.

Serial analysis of gene expression (SAGE)

SAGE works on the principle that each RNA molecule could be identified through a unique short sequence

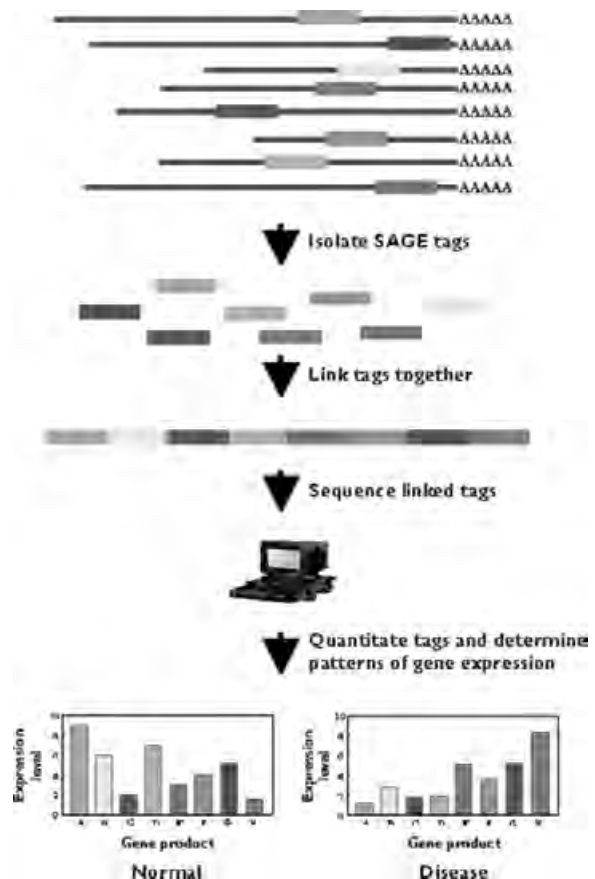
tag of 10-14 bp in length. In this technique C-DNA is synthesized from mRNA through reverse transcription and the unique identifying sequence is extracted, ligated into a concatemer and the quantification of the same done through sequencing. This sequencing based technique is useful in developing bio markers and also enable the identification of differentially expressed genes not revealed through Microarray.

Massively Parallel signature sequencing (MPSS)

In this process, the cDNA produced from experimental RNAs are tagged and attached to microbeads for flow cell sorting to quantify RNAs. This is useful in studying gene expression of species with little knowledge on sequences like pacific oyster.

Transcriptome sequencing (RNA-Seq)

This is the novel and emerging technology based on next-generation sequencing enable the researcher to simultaneously identify the novel regions of the



sequences generated as well as the quantification of the transcript and thereby measures relative abundance. These advantages make it more suitable than microarray and the use of this technique is getting popular.

Functional genes related growth studied in fishes

Specific genes associated with growth related traits in fishes involve in regulating the growth of fishes in wild as well as in culture. Their expression pattern is also depending on the environmental parameters. Hence specific strategies are essential to study the genes controlling the harvest traits living in diverse habitats. In many cases, the polymorphism of these genes negatively or positively correlates the growth of the fishes. In addition to the growth hormone gene (GH) genes like myostatin (MSTN), a member of the transforming growth factor- β superfamily and the insulin-like growth factor 1 (IGF1) are two important genes which influence the growth.

IGF

IGF1 and 2 along with few binding proteins and receptors work together under the stimulation by growth hormone to development and normal growth. Polymorphism within this highly conserved IGF gene. Genetic variations were detected in the IGF1 gene in Artic charr and siniperid species where they are found to be associated with growth traits. Single nucleotide polymorphisms (SNPs) regulating growth trait positively were also detected in farmed atlantic salmon, making them suitable for developing markers for marker assisted selection in aquaculture industry.

Myostatin (MSTN)

Myostatin is a member of TGF- β superfamily that

inhibits or negatively regulates muscle growth in animals and it is conserved in vertebrates. Interestingly a mutant or polymorphic form of the gene has gained attention as the phenotypic growth is enhanced to a great percentage in muscle growth. Hence the much focus is given to this gene in farmed animals. Recent years have witnessed research findings from fishes almost in the same line as in livestock animals. This has made the research groups to initiate work in detecting myostatin polymorphisms from wild to be used in marker assisted selection of the brood stock.

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121 AGCCGAGACATCGTGAAGCAGCTCCTGCCCAAAGCGC
    S R D I V K Q L L P K A
181 CAGTACGACGTACTGGGAGATGACAACAGGGATGTGG
    Q Y D V L G D D N R D V
241 GCCATCACGGAGACGATAATGATGATGGCCACTGAAC
    A I T E T I M M M A T E
301 TGGGGAACCAAAGTGCCTGCTTTTTTCTCTTTTACACAA
    W G T K V L L F L F Y T
361 CCGGGCGCAGCTCTGGGTGTATCTGCGCGCTGCGGAG
    P G A A L G V S A R C G G G
421 GATCTCCCGCCTCATGCCGGTTCACAGACGGGAACAGG
    D L P P H A G H R R E Q
481 GATCGACGTGAACGCCGGGGTCAGCTCTTGGCAAAGT
    D R R E R R G Q L L A K

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Myostatin sequences of *Lutjanus argentimaculatus*

Many results co relate the presence of SNPs within the exon of the gene and incidence of higher growth in many fishes including sea bass, Atlantic salmon, common carp etc.

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Population Genomics of Fishes

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Fishes are the most successful vertebrates in the planet with around 28,600 extant species compared to 4,629 species in mammals and 9,946 species in birds. They live in wide range of habitats from below freezing to more than 40°C and from freshwater to hyper saline waters and exhibit different life history and reproductive strategies. They have varied patterns of biological interactions with respect to prey, predator and parasites. This enormous diversity and divergence makes them excellent models for genomic investigations on adaptation, selection and consequent evolution. In spite of their importance and diversity, genome level investigations on fishes are still in infancy and most of the studies were focusing on a few model species. Genomic studies on fishes will provide vital and revolutionary insights into ecological speciation and adaptation.

Population genomics and population genetics

Over the last few decades, population genetics has evolved from a theoretical science to a fully developed empirical science with the extensive use of neutral markers like microsatellites and mitochondrial DNA markers to infer genetic stock structure information. Thus with the tools of population genetics, inferences regarding genetic drift, gene flow and inbreeding could be made with reasonable precision and this has improved our understanding regarding evolutionary processes shaping population structure in varied oceanic habitats. With the advent of next generation sequencing techniques, population genetics is moving towards more inclusive population genomics where both neutral and non-neutral regions could be studied simultaneously by whole genome scans with increased speed and efficiency. Population genetic statistics like Wright's F statistics which were considered as point

estimates could now be evaluated and assessed as continuous distributions across the genome.

Population genomic approaches are based on information from many genome wide markers in contrast to population genetics approaches where only a few markers are employed. In addition, neutral and non-neutral markers are employed simultaneously so as to gain information regarding genic and non-genic regions. When both neutral and non-neutral markers are used, the effects due to selection, mutation and recombination on populations (locus specific effects) could be differentiated from the effects due to genetic drift, inbreeding and gene flow (genome wide effects). When neutral markers alone are used, information about adaptive evolution is missing whilst adaptive divergence plays a major part in evolution of resident populations with improved fitness for that particular environment. Knowledge about locally adapted populations also aid in suggesting conservation measures aimed conserving genetic diversity over ecological time scales. It is also important to understand climate mediated micro-evolution and information about selection and divergence is crucial to predict climate related habitat shifts and movements of marine fishes.

Finding out the footprints of selection on a particular trait at genome level is not an easy task as several genes are involved in determining the characteristics of an adaptive trait and consequent variations in phenotype. In this context, the relevance of population genomics is more emphasized as population genomics aims to gain insights into genomic variations in several functional genes linked to adaptation and selection within and between populations in space and time. Expressed Sequence

Tags sequencing (EST), transcriptome sequencing and complete genome sequencing are some of the methods used to infer information about functional gene modifications linked to differing environmental conditions.

Approaches in population genomics

Candidate Gene Approach

Candidate genes are functional genes having a known function which has a major influence on an adaptive trait. They can be genes linked to variations in structure of fishes or genes related to some physiological processes and they are directly or indirectly linked to phenotypic variation. The information regarding functional gene variations can be derived from sequence information or allele frequency based tests. Sequence based data is more informative to understand the action of evolutionary forces. The candidate gene approach is relatively faster with the help of comparative genomic approaches which enable efficient characterization and comparison of different genes and it does not require any controlled experimental set up as far as a direct linking with phenotypic variation is not desired. Variations across populations could be efficiently detected and the influence of selective pressures in relation to environmental fluctuations could easily be deduced. The only challenge with candidate gene approach is to identify the correct candidate gene related to the trait under study and the need for undertaking time consuming sequencing.

Genome Scan Approach

Genome scan approach is based on the principle of genetic hitchhiking whereby identification of regions or loci showing high amount of structuring due to selection is studied. Selective forces acting on such loci may either due to balancing selection or directional selection. This requires large numbers of genetic markers which are widespread in the genome which can be random markers like microsatellites, SNPs or AFLPs or functional markers

like genes linked to ESTs. Genes linked to ESTs could be detected through transcriptome sequencing and genome scan approaches help to detect loci under selection.

Quantitative trait locus, admixture and association mapping

QTL mapping is based on the information from linkage maps constructed using a dense set of markers which provides information regarding the genetic basis underlying phenotypic traits through family crosses under controlled laboratory conditions. QTL information could be compared among closely related species which helps to minimize cost. QTL studies require controlled experimental conditions and so species amenable to aquaculture will be the best candidates for quantitative trait loci related investigations.

Association mapping depends on the information regarding linkage disequilibrium between large number of genetic markers and we try to link linkage disequilibrium information with phenotypic variations. Admixture mapping is used to detect natural events of admixture like intra-specific hybrid zones and the information regarding linkage disequilibrium in wild populations is utilized for this purpose.

Seascape genomics approaches

In Seascape or landscape genomics, information obtained by the use of genetic markers is combined with geographic information so as to derive geographic patterns of genetic differentiation. Both neutral and non-neutral markers are employed in sea scape genomics so that information regarding selection could be deduced whenever there is divergence in non-neutral markers based on geographic location. The presence of genetic divergence in non-neutral markers based on geographic locations is indicative of locally adapted populations.

Transcriptomics approaches

Transcriptomic approaches are based on the data regarding differential gene expression patterns and mass scale RNA sequencing enables identification of differential gene expression patterns in real

time and this method can be applied to all species due to the ease of transcription profiling and annotation simultaneously. Microarray are used to measure differential expression of hundreds or thousands of genes and real time PCR is used to measure differential gene expression of one or a few candidate genes. Real time PCR follows the principles of normal PCR and the product is measured in real time by tagging the reaction mixture with fluorescent dyes and measuring the fluorescence after each PCR cycle which corresponds to expression levels of a gene. But with real time PCR studies, only a small number of genes within a genome could be studied. DNA microarrays are designed to investigate gene expression of thousands of genes at once. Microarray are thousands of single stranded DNA spots attached to surfaces like glass slides and each spot corresponds to a gene. To measure the gene expression, fluorescently tagged cDNA is washed over the slide, so that complementary strands hybridize on to the array and fluoresce. The intensity of each spot is proportional to the expression of that gene. Gene expression studies also require controlled experimental conditions so as to relate gene expression data with adaptive variation.

Present status of fish genomics research

Marine fishes are highly diverse and heterogeneous possessing large effective population sizes and high amounts of gene flow. The high gene flow is attributed to pelagic larval phase and consequent dispersal of many pelagic and demersal fish resources. Extensive studies on population genetic structure of many fish groups like scombrids (eg: mackerel, tuna and bonito), clupeids (eg: herring, anchovy and sardine), pleuronectids (eg: plaices, soles and flounders) and gadids (eg: cod, hake and haddock) have been carried out with an aim to find out genetic stock structure and define management units. Most of the studies have concluded weak genetic structuring and high levels of intra-population diversity values. But, in spite of their large effective population sizes, it is possible to have locally adapted populations as many studies have pointed

out substantial divergence in morphological and physiological characteristics.

Genomic resources are available for some of the model species like zebra fish, *Danio rerio*; Japanese puffer fish, *Takifugu rubripes*; medaka, *Oryzias latipes*; Spotted green puffer fish, *Tetraodon nigroviridis* and three-spined stickleback, *Gasterosteus aculeatus*. There are large scale genome sequencing projects for some of the species like flat fish (PLEUROGENE) and Atlantic cod. In addition to that, linkage maps, BAC libraries and microarrays are being developed worldwide for many species which are relevant to aquaculture. With the level of genomic information available for species like European Seabass, Japanese flounder and gilt head sea bream, it is now possible to link phenotypic variations and genome level alterations.

EST information is also increasing rapidly for many fish species like Three spined stickle back (*Gasterosteus aculeatus*), Atlantic cod (*Gadus morhua*), Killifish (*Fundulus heteroclitus*), Gilt head seabream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*), Antarctic tooth fish (*Dissostichus mawsoni*), Piked dog fish (*Squalus acanthias*), Little skate (*Leucoraja erinacea*), Japanese pufferfish (*Takifugu rubripes*), Copper rock fish (*Sebastes caurinus*), Atlantic halibut (*Hippoglossus hippoglossus*), Turbot (*Scophthalmus maximus*), Grass rock fish (*Sebastes rastrelliger*), Senegalese sole (*Solea senegalensis*), Pacific electric ray (*Torpedo californica*), Blue fin tuna (*Thunnus thynnus*), Japanese flounder (*Paralichthys olivaceus*), Barramundi (*Lates calcarifer*), Striped seabream (*Lithognathus mormyrus*), Sheepshead minnow (*Cyprinodon variegates*), European flounder (*Platycthis flesus*), Long-jawed mudsucker (*Gillichthys mirabilis*), Tongue sole (*Cynoglossus semilaevis*) and Large yellow croaker (*Pseudosciaena crocea*). In the dbEST database of NCBI, over one million teleost EST sequences are currently listed.

Several candidate genes have been studied in marine fishes to understand their divergence and selection. Ectodysplasin (EDA) gene in stickle back, Lactate dehydrogenase B (Ldh-B) in killifish and Pantophysin

(Pan I) in Atlantic cod are some of the well studied functional genes in marine fishes. Haemoglobin polymorphisms have been studied in Atlantic cod which showed clear genetic differentiation across populations. Pantophysin gene also showed signals of adaptive differentiation showing diversifying selection. Heat shock cognate gene Hsc70 has been studied in European flounder (*Platichthys flesus*) and several insertion/deletion polymorphisms have been detected.

Studies based on genome scans have been conducted in three-spined stickle back to derive insights to their adaptation to freshwater and marine environments. EST-based microsatellites and EDA gene linked indels were studied understand divergence in different ecotypes. A mass scale genome scan study was conducted in Atlantic Cod identifying many outlier loci potentially subject to selection. The study also proved that even if geographical differentiation was limited, adaptive population divergence may be possible in fishes.

QTL analysis had been carried out in few fishes like three spined stickleback, European seabass, Barramundi and Japanese flounder. QTL analysis in three spined stickleback could identify a dominant gene, Ectodysplasin which determines lateral armour plate pattern and number. Another gene, involved in pelvic skeleton formation, Pitx1 locus was also mapped in stickle backs using QTL analysis. QTLs linked with size and growth has been identified in both European seabass and barramundi and another QTL linked with disease resistance had been detected in Japanese flounder.

Large – insert libraries (BAC; Bacterial Artificial Chromosome, YAC; Yeast Artificial Chromosome and fosmid; A low copy vector which uses *Escherichia coli* F-factor origin for replication) construction is another method which is a prelude to full genome sequencing and this aims at producing multiple copies of large segments of DNA of the desired organism through cloning. These libraries also serve as tools for genetic mapping, marker assisted selection and positional cloning of QTLs for traits that are of interest.

Population transcriptomics studies had been

undertaken to identify variations in gene expression patterns in killifish distributed across steep thermal gradients on the east coast of North America. There were differing patterns of gene expression in Lactate dehydrogenase (Ldh-B) gene for fish kept in controlled conditions. Similar gene expression studies were also conducted in killi fish to understand differential gene expression patterns in response to chemical pollution. In European flounder, a microarray was developed for ecotoxicology studies and to understand differences in gene expression between populations from saline and brackish water environments.

Prospects of population genomics of fishes

Fishes live in a dynamic environment where they face many physical, chemical and biological challenges. Adjusting to such varying environmental conditions require altering their phenotypes, moving to more favorable locations or by adapting genetically to altered conditions. Temporal patterns in changes in effective population size, migration patterns and gene flow are to be studied in detail so as to gain more insights into adapted genotypes and selection patterns. Population genomic approaches have a great potential to elucidate patterns of natural selection in the wild and information from population genomics could be translated to management measures for conservation of marine diversity. The advent of Next Generation Sequencing technologies has revolutionized the field of population genomics expanding the knowledge base with respect to role and importance of functional genes. More and more non-model species are to be focused upon so as to aid in comparisons and future conservation decisions.

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Next Generation Sequencing and RAD sequencing

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DNA sequence information is fundamental to any research as it helps to decode the mysteries of life and this knowledge has transformed the way we perceive natural science for the last two decades. DNA sequence analysis has broad range of applications in population genetics, molecular breeding, disease resistance investigations, health care and comparative evolutionary studies. DNA sequence development and detection has undergone revolutionary changes during the past 30 years moving from first generation to third generation technologies. First draft human genome was completed in 2003 using the Sanger sequencing method and from then onwards genomics has progressed exponentially with modern techniques making it fast, efficient and cheap. The availability of draft human genome has provided momentum to a new era of genomic and personalized medicine.

First Generation Sequencing Technologies

Sanger sequencing was introduced by Frederick Sanger in 1977 and this sequencing technology was based on chain-termination method. This technique has undergone improvement over years and Applied Biosystems Company introduced the first automatic sequencing machine (AB370) in 1987 based on capillary electrophoresis which made sequencing faster and accurate. AB 370 was capable of detecting 96 bases at a time, and the read length could reach 600 bases. After improvisations, it is now possible to analyze a read length of up to 900-1000 bases. In high-throughput sanger sequencing machines, DNA to be sequenced is prepared by any one of the two approaches; for shot gun de novo sequencing, randomly fragmented DNA is cloned into a high-

copy number plasmid, which is consequently used to transform *Escherichia coli*; or in the second approach, PCR amplification of the target DNA is carried out using primers that flank the target. Thus using either of the approaches an amplified template is produced. Sequencing reactions take place in a "cycle sequencing" mode with cycles of template denaturation, primer annealing and primer extension and the primer used is complementary to the sequences flanking the region of interest. Every step of primer extension is terminated stochastically by incorporation of fluorescently labeled dideoxy nucleotides (ddNTPs). The resulting mixture contains end-labelled extension products and the label on the terminating ddNTP of any given fragment corresponds to the nucleotide identity of its terminal position. Consequently, the sequence is determined by capillary based polymer gel by high-resolution electrophoretic separation of the fragments which are single stranded and end-labeled. The fluorescent fragments of discrete length are laser excited as they exit the capillary and with a four colour detection of emission spectra which provides the read out that is represented in a Sanger sequencing trace. A Software is capable of translating these traces into DNA sequence along with error probabilities for each base call. A limited level of parallelization is possible with simultaneous electrophoresis in 96 or 384 independent capillaries.

Second Generation/ Next Generation Sequencing Technologies

Second Generation Sequencing makes use of many alternative strategies in sequencing and they are

1) micro-electrophoretic methods 2) sequencing by hybridization 3) real time observation of single molecules and 4) cyclic array sequencing. 454 Genome Sequencers by Roche Applied Science uses cyclic array sequencing. It is also called pyrosequencing. Illumina uses reversible terminator sequencing, ABI/Solid uses sequencing by ligation technique and Helicos uses single-molecule sequencing.

Sequencing in Roche 454 platform/ pyrosequencing

The 454 system was first next generation sequencing platform which was available as a commercial product. DNA libraries can be constructed by any method which gives rise to a mixture of short, adaptor-flanked fragments. Clonal amplification of target sequence is achieved by emulsion PCR and the amplicons are captured to the surface of 28 μ m beads. The emulsion is broken to fragments and the beads are treated with denaturants to remove untethered strands which are then subjected to a hybridization-based enrichment for amplicon bearing beads. The hybridization reaction with a sequencing primer is carried out using a universal adapter at the position and orientation that is immediately adjacent to the start of unknown sequence.

Sequencing reactions are carried out using pyrosequencing method. The amplicon-bearing beads are pre-incubated with the enzyme *Bacillus stearothermophilus* (Bst) polymerase along with single-stranded binding protein which is then deposited to a microfabricated array of picolitre scale wells (the dimensions are such that only one bead will fit per well) to make this biochemistry compatible with array-based sequencing. Smaller beads bearing immobilized enzymes (ATP sulfurylase and luciferase) required for pyrosequencing are also added. While sequencing, one side of the array functions as a flow cell for introducing and removing sequencing reagents and the other side is bonded to a fiber-optic bundle for CCD (charge coupled device) based signal detection. During the course of sequencing, a single species of unlabeled nucleotide is introduced at each of several hundred cycles. When there is an incorporation event in templates, pyrophosphate is released. With the help of enzymes ATP sulfurylase and luciferase, the incorporation events

generate a burst of light and this is detected by the CCD corresponding to the array coordinates of specific wells. When multiple cycles are performed, (eg: A-G-C-T.....) the incorporation events reveals the sequence of templates represented by individual beads. Greater read-length is possible with 454 sequencing. The 454 FLX instrument is capable of producing approximately 4,00,000 reads per instrument-run with lengths of 200-300bp. The presence of homopolymers (consecutive instances of the same base such as AAA or GGG) may increase the rate of error. The absence of a terminating moiety which will prevent multiple consecutive incorporations at a given cycle prevents inference regarding length of homopolymers and this inference has to be made from signal intensity. This increases the error rate and the dominant error type in 454 platforms is insertion-deletion.

Illumina Genome Analyzer

This also commonly referred to as "Solexa" and four companies were merged together; Solexa (Essex, UK), Lynx Therapeutics (Hayward, CA, USA), Manteia Predictive Medicine (Coonsville, Switzerland) and Illumina. Any method can be used for the production of libraries which gives rise to a mixture of adapter-flanked fragments up to several hundred base pairs in length. A bridge PCR is used to generate amplified sequencing features. In this method, forward and reverse PCR primers are secured to a solid substrate by a flexible linker and all the amplicons arising from any template molecule during the process of amplification will be immobilized and clustered to a single physical location on the array. The bridge PCR relies on alternating cycles of extension with Bst polymerase and denaturation with formamide. The clusters that are produced consist of approximately 1000 clonal amplicons. Several million clusters can be amplified to specific locations within each of eight independent "lanes" which are on a single flow-cell (such that eight independent libraries can be sequenced in parallel during the same instrument run). Once the cluster generation is completed, the amplicons are linearized or single stranded and a sequencing primer is hybridized to a universal sequence flanking the region of interest. Every cycle of sequence interrogation is composed of single base extension with a modified DNA polymerase and a mixture of four nucleotides. The nucleotides are modified in two ways;

reversible terminators in which a chemically cleavable moiety at the 3' hydroxyl position allows only a single base incorporation at each cycle and one out of four fluorescent labels which are chemically cleavable corresponds to the identity of each nucleotide. Read-lengths of upto 36bp are currently possible and longer reads incur higher error rate. The common error type is substitution than insertions and deletions.

AB SOLiD

Libraries can be constructed by any method which may give rise to a mix of short, adaptor flanked fragments. Clonal sequencing by emulsion PCR and capturing of amplicons to the surface of 1 μ m paramagnetic beads are carried out. The emulsion is broken and the beads bearing products of amplification are selectively recovered and immobilized to a solid planar substrate which generates a dense, disordered array. Sequencing by synthesis is carried out by a DNA ligase than a polymerase. A universal primer which is complementary to adapter sequence is hybridized to the array of amplicon bearing beads and each cycle of sequencing involves the ligation of a degenerate population of fluorescently labeled octamers. The mixture of octamers is structured in such a way that the identity of specific positions within the octamer (base 5) correlate with the identity of the fluorescent label. When octamer ligation is carried out progressively, every fifth base is sequenced. Subsequent steps could identify different base positions and the sequencing could be completed.

HeliScope

This also depends on cyclic interrogation of a dense array of sequencing features. No clonal amplification is required here. A highly sensitive fluorescence detection system is used to directly interrogate single DNA molecules through sequencing by synthesis. Template libraries prepared by random fragmentation and poly A – tailing (without PCR amplification) are captured by hybridization to surface bound poly-T oligomers to produce a disordered array of primed single molecular sequencing templates. At each cycle, DNA polymerase and a single species of fluorescently labeled nucleotide are added, which results in template dependent extension of surface-captured primer-template duplexes. When acquisition

of images tiling the full array is completed, chemical cleavage and release of fluorescent label permits subsequent cycle of extension and imaging. Thus several hundred cycles of single-base extension (A, G, C, T, A, G, C, T) will yield average read-lengths of 25bp or greater. The dominant error type is deletion.

Third Generation Sequencing Technologies

Third generation sequencing technologies are characterized by new chemistry, less operation time, desktop design and lower operation cost. The major third generation sequencers are; Pacific Biosciences real time single molecule sequencing (PacBioRS), Complete Genomics combined pre anchor hybridization and ligation (cPAL) and Ion Torrent of Life Technologies, Inc.

PacBioRS is a real time single molecule-single polymerase sequencing platform which will be able to produce 1000bp read. The chip consist of zero-mode wave guided (ZMW) nano structures with holes of size 100nm and inside this hole, DNA polymerase performs sequencing by synthesis with the help of phospholinked nucleotides labeled with fluorophores introduced sequentially. With the help of this instrument, it is also possible to monitor the kinetics of nucleotide incorporation and thus epigenetic information may also be extracted in future.

Complete Genomics uses a combined approach of probe-anchor hybridization and ligation (cPAL) sequencing and they claim the highest throughput among third generation sequencers. The method is based on rolling circle amplification of small DNA sequences into so-called nanoballs. Nucleotide sequence is determined by unchained sequencing by ligation method. In this approach, many DNA nanoballs could be sequenced per run and with low costs.

Ion Torrent technology (Life Technologies Inc.) is considered currently as one of the most versatile and low cost method and it is being supplied as a personal genomic machine (PGM) as benchtop instrument to laboratories involved in research and medicine. The technology is based on proton release during incorporation of each nucleotide by DNA polymerase.

Applications, Limitations and Future of NGS methods

Next Generation Sequencing methods are fast, efficient and cheap and offer immense possibilities and applications in human and animal medicine, ecological and evolutionary studies. The major problems with NGS data is the many number of short reads generated which has to be assembled and annotated using reference sequences. Longer reads may have more error reads towards the end. Repetitive sequences may be difficult to read and end up in more errors. In spite of all these problems, NGS technologies find varied applications in human medicine mainly in diagnostics, prognostics and therapeutics. It also finds applications in whole genome sequencing, targeted sequencing of exomes or selected genes related to a specific disorder or category of disease, epigenetic mapping, transcriptome sequencing and microbial population sequencing.

Restriction Site Associated DNA (RAD) sequencing

In RAD sequencing, two techniques in molecular biology are used in combination with Illumina sequencing. Restriction enzymes are used to cut DNA into fragments and molecular identifiers (MID) are used to relate sequence reads to specific individuals. DNA from one individual is fragmented with specific restriction enzyme which produces a set of sticky ended fragments. The sticky ended fragments are ligated to a P1 adapter which also contains a matching sticky end and a molecular identifier. The restriction fragments from different number of individuals which are tagged are pooled and then randomly sheared to produce fragments with an average length of a few hundred base pairs. The fragments which are sheared are then ligated to a second P2 adapter and amplified using PCR with P1 and P2 primers. The P2 adapter is characterized by a divergent "Y" structure which will not bind to P2 primer unless it is completely amplified by the P1 adapter. Thus it is ensured that all the amplified fragments have the P1 adapter and MID, the partial restriction site, the flanking sequence consisting of a few hundred bases and a P2 adapter. These fragments which are ready for sequencing are selected for size (approximately 200-500 base fragments) and the RADSeq library is sequenced on

an Illumina platform. Sequence is then generated from the molecular identifier in the P1 adapter and across the restriction enzyme site which generates a data set of RAD tags which is derived from a reduced part of the original genome.

RAD sequencing has many advantages as compared to whole genome sequencing as regions of interest can be selected and sequenced. Since it is one of a number of reduced representation methods which is sampling only a shared set of sites across the genome in several individuals, population level sequencing could be carried out at a fraction of the cost required for whole genome sequencing. RAD-seq finds many applications for fine scale linkage mapping, phylogenetics and phylogeography, genome scaffolding and population genetics. It has recently been applied in many species like salmon, cutthroat and rainbow trout for deducing inferences regarding population genetic structure.

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Software Packages used in Population Genetics

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Many software packages are freely available for population genetics analysis. Software packages for aligning sequences, construction of phylogenetic trees using different methods, analysis of genetic differentiation, analysis of historical demography, constructing network diagrams, analyzing genetic population structure and a number other analyses are available as freely downloadable files. The major software packages used for population genetic analyses are listed below.

Bioedit

Bio Edit is a user-friendly biological sequence alignment editor and analysis program and is an important bioinformatics tool for molecular biologists. It was developed initially as a graphical biological sequence alignment editor written for Windows only. The purpose of this program is to provide a useful molecular biology tool which can be started up and used easily. However, in the last few years it has been improved dramatically to integrate many other features and functions such as several modes of hand alignment, plasmid drawing and annotation, restriction mapping and much more.

BioEdit can accept a wide variety of formats that is commonly used with other bioinformatics application. This allows the swapping of Data files between BioEdit and other programs. It contains many features for sequence alignments like split window view, user defined color, information based shading and auto integration with other programs such as Clustal W and Blast. Most of sequence alignment programs do not have many other functions or useful tools for molecular biologist comparing to BioEdit.

BioEdit (Current version 7.2.5 Last updated 12/11/2013) can be downloaded from the following Server: <http://www.mbio.ncsu.edu/BioEdit/bioedit.html>

Mega (Molecular Evolutionary Genetics Analysis)

The Molecular Evolutionary Genetics Analysis software is a multi platform desktop application designed for comparative analysis of homologous gene sequences either from multi-gene families or from different species with a special emphasis on inferring evolutionary relationships and patterns of DNA and protein evolution. In addition to the tools for statistical analysis of data, MEGA provides many convenient facilities for the assembly of sequence data sets from files or web-based repositories, and it includes tools for visual presentation of the results obtained in the form of interactive phylogenetic trees and evolutionary distance matrices.

The Molecular Evolutionary Genetics Analysis (MEGA) software aims to serve both of these purposes in inferring evolutionary relationships of homologous sequences, exploring basic statistical properties of genes and estimating neutral and selective evolutionary divergence among sequences.

MEGA is an integrated workbench for biologists for mining data from the web, conducting automatic and manual sequence alignment, inferring phylogenetic trees, estimating rates of molecular evolution, testing evolutionary hypotheses and generating publication

quality displays and descriptions. MEGA is used by biologists in a large number of laboratories for reconstructing the evolutionary histories of species and inferring the extent and nature of the selective forces shaping the evolution of genes and species.

MEGA is distributed in two editions: a graphical user interface (GUI) edition with visual tools for exploration of data and analysis results and a command line edition (MEGA-CC), which is optimized for iterative and integrated pipeline analyses. Both GUI and command-line versions of MEGA6 (current version) can be downloaded from www.megasoftware.net free of charge.

Genepop

GENEPOP is a population genetics software package. The basic tools of this program are designed for multi-allelic markers like microsatellite, short tandem repeat-STR etc. It is used for descriptive statistics, estimates of genetic differentiation (traditional & microsat specific estimates of genetic differentiation), Hardy-Weinberg equilibrium (pairwise comparisons of single locus and global tests), geographic vs. genetic distance.

Three major tasks can be performed in Genepop. 1) It computes exact tests: for Hardy-Weinberg equilibrium, for population differentiation and for genotypic disequilibrium among pairs of loci. 2) computes estimates of classical population parameters, such as F_{st} and other correlations, allele frequencies, etc. and 3) converts the input Genepop file to formats used by other programs, like Biosys, Fstat and Linkdos.

The latest version of Genepop (4.2) is now available from <http://kimura.univ-montp2.fr/~rousset/Genepop.htm>. Genepop 4.2 runs under Windows, and can also be compiled to run under Unix or Linux. The Genepop programs is a simple command-line executable files, where the settings of the computations can often be specified on a command line. Even though being less user friendly than graphical packages, command-line programs have the advantage that they can be easily launched in parallel on computer clusters and can be used to automatically analyze a large number of input files, like those resulting from large genomic studies or simulations.

A web-based front-end is available for teaching purposes and for those who, for some reason, cannot run the Genepop on their local PC or Mac.

GenAlEx

GenAlEx 6.5 is used for a range of population genetic analysis with a wide range of molecular markers within the Microsoft Excel environment on both PC and Macintosh computers. It has a user friendly interface with rich graphical outputs for data exploration and publication, tools for data manipulation and export options to many other software packages. GenAlEx offers analysis of codominant, haploid and binary genetic loci and DNA sequences. Both frequency-based (F -statistics, heterozygosity, HWE, population assignment, relatedness) and distance-based (AMOVA, PCoA, Mantel tests, multivariate spatial autocorrelation) analyses are provided. In GenAlEx 6.5 there are new features including calculation of new estimators of population structure: $G'ST$, $G''ST$, Jost's D_{ST} , and $F'ST$ via AMOVA, Shannon Information analysis, linkage disequilibrium analysis for biallelic data, and heterogeneity tests for spatial autocorrelation analysis. Direct data export is provided to more than 30 other software packages, and indirectly via common formats to many more packages.

DnaSP

DNA sequence polymorphism (DnaSP) is an interactive computer program for the analysis of DNA polymorphism from nucleotide sequence data. It calculates several measures of DNA sequence variation within and between populations; linkage disequilibrium, recombination, gene flow and gene conversion parameters. In addition, DnaSP performs some neutrality tests. DnaSP is written in Visual Basic v. 6.0 (Microsoft) and DnaSP version 5.10.1 is freely downloadable from <http://www.ub.edu/dnaspl/>

DnaSP can automatically read the following types of data file formats: FASTA, MEGA, NBRF/PIR, NEXUS, PHYLIP and HapMap3 Phased Haplotypes. In all cases one or more homologous nucleotide sequences should be included in just one file (ASCII file). The sequences must be aligned (i. e. the sequences must have the same length).

Various analysis options are included in the software such as DNA Polymorphism analysis, GC content at non-coding and coding positions, Haplotype/ Nucleotide Diversity and Divergence. There are options to find out the number of Segregating Sites (S), total number of mutations (Eta), the number of haplotypes, Haplotype (gene) diversity and its sampling variance, Nucleotide diversity, Pi (π), The average number of nucleotide differences (k), Nucleotide divergence with Jukes and Cantor, Theta (per gene or per site) from Eta (η) or from S and ZnS statistic, Neutrality tests such as Tajima's D with its statistical significance, Fu and Li's D, Fu's Fs etc. This software also estimates the raggedness statistic (r) which quantifies the smoothness of the observed pairwise differences distribution. More powerful statistics for detecting population expansion such as the Fu's Fs Test and the Ramos-Onsins and Rozas's R2 can also be carried out using the software.

Arlequin

The free population genetic software Arlequin provides the user with quite a large set of basic methods and statistical tests, in order to extract information on genetic and demographic features of a collection of population samples. The latest version Arlequin ver 3.5.2.1 can be downloaded in Windows XP/Vista/7 based systems with a minimum of 256 MB RAM from <http://cmpg.unibe.ch/software/arlequin35/Arl35Downloads.html>

Arlequin can handle several types of data either in haplotypic or genotypic form. The basic data types are DNA sequences, RFLP data, Microsatellite data, Standard data and Allele frequency data. The analyses Arlequin can perform on the data fall into two main categories: intrapopulation and inter-population methods. In the first category statistical information is extracted independently from each population, whereas in the second category, samples are compared to each other. Intra-population methods include standard diversity indices like the number of polymorphic sites, gene diversity, molecular diversity indices like nucleotide diversity, different estimators of the population parameter θ , mismatch distribution of the number of pairwise differences between haplotypes, Haplotype frequency estimation

present in the population by maximum likelihood methods, linkage disequilibrium which is a test of non-random association of alleles at different loci., Hardy-Weinberg equilibrium which is the test of non-random association of alleles within diploid individuals, Tajima's neutrality test, Fu's Fs neutrality test, Ewens-Watterson neutrality test, Chakraborty's amalgamation test and Minimum Spanning Network (MSN) analyses. Inter-population methods include searching for shared haplotypes between populations, different hierarchical Analyses of Molecular Variance (AMOVA) to evaluate the amount of population genetic structure, FST based pairwise genetic distances for short divergence time, exact test of population differentiation, assignment of individual genotypes to particular populations according to estimated allele frequencies, detection of loci under selection from F-statistics and mantel test to test for the presence of isolation-by-distance.

Network

Network is a free phylogenetic network software available from fluxusengineering.com. It is used to reconstruct phylogenetic networks and trees, infer ancestral types and potential variants and evolutionary branching patterns. The Network programs have user-friendly graphical interfaces, allowing users to easily choose the types of analysis and computation parameters to be performed. The basic algorithms of this program are designed for non-recombining biomolecules, thus it can be effectively used for mtDNA, Y-STR, amino acid, RNA, viral DNA, bacterial DNA, some non-recombining autosomal DNA, and also for non-biomolecule data such as linguistic data.

The Network software was developed to reconstruct all possible least complex phylogenetic trees (all maximum parsimony or MP trees) from a given data set. The data can be DNA sequences or nucleotide sequences, amino acid sequences or protein sequences, STR (short tandem repeat) or microsatellite data, language/linguistic or manuscript data.

Two independent network-building options are included for arriving at optimal networks; the Reduced Median networks or RM network algorithm for binary data and the Median Joining networks

or MJ network algorithm for all types of data. MJ networks are commonly used to visualize relationships of closely related mitochondrial or nuclear haplotypes, for which traditional phylogenetic methods yield multiple possible trees. For interpretation of complex and large data a neat skeleton phylogeny can be constructed using Star Contraction pre-processing. Age estimation for ancestral nodes or branching points is also possible in Network software.

Current Version is Network 4.6.1.3., released on 24 December 2014, and it is available from www.fluxusengineering.com. Network runs on Windows 7, Vista, XP, 2000, many Linux versions with WINE Windows emulation, and Macs with Parallels Desktop Windows, or VirtualBox with Windows. Network can run from memory stick no Windows registry entries and no Windows Administrator required for installation. Network software package includes a data editor and a graphics program. FASTA files can be imported and prepared for Network using Fluxus' DNA Alignment software. Higher-quality graphics of Network's results files can be prepared using Fluxus' Network Publisher software.

Mr Bayes

MrBayes (Ronquist and Huelsenbeck 2003) is a program for doing Bayesian phylogenetic analysis. The posterior probability distribution is sampled using Markov Chain Monte Carlo (MCMC) techniques. Metropolis-coupling is used to accelerate convergence in MrBayes. In parallel with regular "cold" chain, three heated chains are also run in parallel. The heated chains will sample from distributions obtained by raising the posterior probability with some factor smaller than 1, which result in flattening ("melting") of the peaks in the landscape defined by the posterior distribution. At specified intervals, the parameter values (the locations in the landscape) are swapped between cold and heated chains, making it possible for the cold chain to escape local peaks. This comes at the cost of increased computational complexity.

The last official release of the program is version 3.1.2. The most recent development version of the program

(version 3.2) includes a number of significant new features, such as check-pointing, strict and relaxed clock models, and functionality for dating. Version 3.2 can be compiled from source code freely available on SourceForge (see instructions below).

MrBayes version 3.1.2 is available for Windows, OSX, and Linux. All versions use a command-line interface and the program looks virtually the same on all platforms.

MrBayes 3 is a program for Bayesian inference and model choice across a large space of phylogenetic and evolutionary models. The program has a command-line interface and should run on a variety of computer platforms, including large computer clusters and multicore machines. Depending on the settings, MrBayes analyses may demand a lot on the machines, both in terms of memory and processor speed. Many users therefore run more challenging analyses on dedicated computing machines or clusters. Several computing centers around the globe provide web access to such services.

MrBayes 3 is distributed without charge by download from the MrBayes web site, <http://mrbayes.net>.

WSTRUCTURE is freely downloadable software which uses a model based clustering method to infer genetic population structure. It uses multi-locus genotype data for investigations on genetic population structure with a Bayesian approach (MCMC: Markov Chain Monte Carlo). It can be used for knowing the presence of distinct populations, assigning individuals to populations, studying hybrid zones, identifying migrants and admixed individuals, and estimating population allele frequencies. Results based on most of the commonly used genetic markers like SNPS, microsatellites, RFLPs and AFLPs could be analyzed using this software. This software could also be used to compute the proportion of the genome of an individual originating from each inferred population (quantitative clustering method).

It can be downloaded from: http://pritch.bsd.uchicago.edu/software/structure2_1.html.

General Methods of Tissue Culture

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Introduction

Tissue culture is a technique that requires a high level of expertise, utmost care during the preparatory and execution phases and a sound knowledge on the cytology of the culture material being studied. There are certain basic principles and methods that need to be followed in order to set up a tissue culture facility and to establish successful cultures.

Laboratory Space and Equipment

The space allocated for a tissue culture laboratory should ideally consist of two rooms, a preparation room opening into a smaller, aseptic culture room which should be dedicated exclusively to tissue culture activities. A sliding door should be used to separate the preparation room from the culture room, in order to reduce the draft of air that would enter during the opening and closing motions of a regular door. The preparation room is used for all preliminary activities which do not require a sterile environment, including washing/drying and sterilization of glassware, weighing of chemicals, preparation of reagents etc. The culture room should house a culture table, a closed cupboard for storing sterile supplies and all the necessary cell culture equipment, such as the laminar flow cabinet, CO₂ incubator and microscopes. This room should be kept sterile, and its walls and floors should be smooth and clean. Entry into this area should be restricted in order to minimize chances of contamination of cultures. If possible, a dressing room through which the culture room can be entered can also be added. The culture room should be fitted with ultra violet lamps, which should be turned on for about half an hour before commencement of a culture

experiment, in order to control air-borne bacteria in the room. They should not, however, be used for long durations when culture work is in progress, because UV light may have a deleterious effect on the tissues being cultured.

Basic Equipment

- **Autoclave:** Used to sterilize glassware, stainless steel surgical instruments, heat-resistant plastic lab ware, certain salt solutions etc. Wasted dishes and other disposable material that come in contact with pathogens must also be autoclaved before they are discarded. The material to be autoclaved is usually subjected to high pressure saturated steam at 121°C for 15 min. Purified water should always be used in the autoclave to minimize contamination of material being sterilized due to condensed water.
- **Laminar-flow cabinet:** Used during media preparation and culture handling to reduce contamination from airborne particles by continuous displacement of air that passes through a HEPA (high efficiency particle air) filter. It is more common to use a flow cabinet in which air flows from the inside to the outside. However, when working with hazardous organisms, it is preferable to use a vertical flow cabinet (biology safety cabinet), where the aerosols generated in the hood are filtered out before being released into the surrounding environment. The flow cabinets are equipped with a short-wave UV light that must be turned on for 10-20 minutes before use to sterilize the surfaces of cabinet. The cabinet should be kept clean and clutter-free. All surfaces need to be wiped with ethanol before and after each use.

- **CO₂ Incubator:** Used to provide a temperature controlled atmosphere of high humidity and increased CO₂ tension for optimal cell growth. A mixture of air with 5-10% CO₂ and saturated water vapor is generally used to keep the pH of the culture medium constant in mammalian cell culture. Invertebrate cells, on the other hand are in general insensitive to changes in pH of culture media, and hence a CO₂ incubator is not a strict requirement for their culture.
- **Inverted microscope:** Used to examine cultured cells in flasks and multi-well plates. The inverted microscope should be equipped with a bright field and phase-contrast optic system with 4x, 10x, 20x, and 40x objectives, and digital imaging and processing facilities. If fluorescent microscopy is required, an epifluorescent attachment should be included.
- **Refrigerator and freezer (–20°C):** Used to preserve culture media, salt solutions, sera, amino acid solutions, hormones, vitamins etc.
- **Centrifuge:** Used to spin down cells in a cell suspension at low speed during washing, harvesting or subculturing of cells from cultures.
- **Osmometer:** Used to measure the osmolality of culture media prepared in the laboratory.
- **Filtration system:** Used to sterilize heat labile culture media, enzymes, supplements etc. that cannot be autoclaved. A basic filter system is comprised of a filter holder set, a membrane filter having a pore size of 0.22 μm and an aspiration pump (peristaltic or vacuum).

Minor equipment

In addition to these major equipment, a hot air oven is required for drying/sterilizing glassware, surgical instruments etc., a water purification system for the supply of purified water and an electronic balance, pH meter, water bath and magnetic stirrer for preparation of reagents and culture media. A hemocytometer, electronic cell counter or flow cytometer is required to make accurate counts of cells in cultures.

Other requirements include culture dishes, centrifuge tubes, disposable Pasteur pipettes, variable volume automatic micropipettes, disposable/autoclavable pipette tips etc.

Culture System

Culture vessels must have a biologically inert, optically transparent surface. Sterile, disposable polystyrene plastic dishes are preferred over glass because attachment of cells and tissue fragments is poor on glass. The different types of culture vessels include petri dishes, multi-well plates (6, 12, 24, 48 and 96-well configurations) and screw cap flasks (T-25, T-75, T-150). Culture flasks with vented caps containing filters are also available, where gas exchange can be carried out without having to loosen the cap. The inner surface of the culture flasks is coated with substrates such as collagen, poly L-lysine, fibronectin etc. to increase adhesiveness, for closed and stationary cultures. For large-scale culture of suspended cells, a spinner bottle and magnetic stirrer, or an Erlenmeyer flask placed on a rotary shaker are commonly used. Culture of substrate-dependent cells usually requires the use of roller bottles.

Aseptic Measures

Aseptic technique and the proper use of laboratory equipment are very essential when working with cell cultures. It is mandatory to use sterile equipment and reagents, and disinfect hands, reagent bottles and work surfaces with 70% alcohol before beginning culture work. Glass pipettes and necks of bottles should be flamed before and after use. Screw caps should be placed open side down on the surface of a clean bench. Glass or disposable plastic fast-flow pipettes should preferably be used to increase speed of dispensing solutions. Bulbs or electric pipette controllers should be used to dispense reagents since mouth pipetting could be hazardous to the operator or lead to contamination. Pipettes with cotton plugs at the top should be used. Care should be taken not to mix liquids by rapidly pipetting up and down or releasing the contents of a pipette from a height into the receiving vessel. All liquid waste should be treated with bleach or similar detergents before disposal.

Conditions for Culture

An *in vitro* culture should recreate the physical, nutritional, and hormonal environment of the cell *in*

vivo. The physicochemical properties that are required include controlling the temperature, pH, osmolality, and gaseous environment.

Temperature

The incubation temperature will depend on the type of cells being cultured. While mammalian cells require a temperature of 37°C, invertebrate cells require a lower temperature range of 15°C-30°C. Most mammalian cell cultures are grown in incubators that are set at 37°C.

pH

Regulation of extracellular and intracellular pH is very essential for survival of mammalian cells. The pH is important for maintaining appropriate ion balance, as well as for maintaining the optimal function of cellular enzymes. Minor changes in pH can also alter cell metabolism and induce the production of heat-shock proteins, which can ultimately lead to apoptosis. Most media aim to maintain a pH between 7.0 and 7.4. Different cell types may however have varying ranges of optimal pH. Cells usually tolerate slow changes in pH as compared to rapid changes. Insect cells in culture are insensitive to wide variations in pH. The pH of media are regulated through a variety of buffering systems.

Osmolality

The osmolality of a culture medium is mainly determined by the dissolved salts and glucose, although amino acids may also contribute significantly. Most of the commercial media used for mammalian cell culture are formulated to have a final osmolality of around 300 mOsm. Significant alterations in osmolality will affect cell growth and function. The optimal osmolality for growth of most cells has been determined to be around 290 to 310 mOsm. Extreme changes in osmolality will result in loss of membrane integrity leading to explosion or collapse of cells.

CO₂, Oxygen, and other Gases

The buffering capacity of the medium is increased

by bicarbonate (NaHCO₃). The CO₂/HCO₃ balance is maintained by gaseous CO₂, which requires the use of gassed incubators to maintain an atmosphere of 5-10% CO₂ in air.

Light

Light can have an adverse effect on cells by inducing production of toxic compounds in some media. In such cases cells should be cultured in the dark and exposed to room light as little as possible.

Cell Culture Media

Culture medium is the most important factor affecting the success of a cell culture. The main component of all media being water, it is recommended that the water being used for media preparation is ultrapure. Culture media are of two types.

- **Natural media:**Media containing natural sources of nutrients such as biological fluids (serum, amniotic fluid, pleural fluid, insect hemolymph etc.) or tissue extracts (extracts from liver, leukocytes, embryo, bone marrow etc.) as the main components.
- **Synthetic media:**Media which are artificially prepared by the addition of various organic and inorganic nutrients, salts, vitamins, serum, carbohydrates etc. Synthetic media can further be categorized into two types, serum containing media and serum-free media.

Serum containing media are considered advantageous for the culture of most types of cells and are supplemented with 5-20% serum. Serum is a complex mixture of essential growth factors (platelet-derived growth factor, epidermal growth factor, fibroblast growth factor, endothelial growth factor, etc.), hormones, attachment and spreading factors (fibronectin, serum spreading factor etc.), binding and transport proteins, protease inhibitors and trace elements. It has the capacity to bind and neutralize toxins such as heavy metals and reactive organic components, and also increases the buffering capacity of a medium. However, the use of serum in culture media also has certain disadvantages. Firstly, it is not chemically defined and therefore its composition is

highly variable. Since serum components are affected by factors such as age, health and nutrition of the donor animals, the quality of serum varies from batch to batch, which makes it necessary to test every new batch for the required quality standards. Serum can also be a source of contamination by mycoplasma, viruses etc. Fetal calf/bovine serum (FCS/FBS) is the most frequently used serum.

Serum-free media are chemically defined media where the chemical composition and the concentration of every component are known. These media are consistent from batch to batch and are optimized for growth of specific cell types and product synthesis. Development of serum-free media for the culture of mammalian cells *in vitro* has shown great progress over the last two decades. However, they have not yet been able to replace conventional serum based formulations, mainly due to the high cost involved and the need for maintaining specificity in formulation for each cell type. Cell lines usually require the use of expensive serum-free media specific to a particular cell type. A number of serum-free medium formulations have been described for mammalian and insect cell lines as well as for primary cultures.

Nutritional requirements: The basic components of cell culture media are inorganic salts, sugars, amino acids, vitamins, lipids, proteins and peptides, hormones, vertebrate sera, invertebrate hemolymph, tissue extracts and growth factors. Basic salts contained in cell culture media include NaCl, KCl, CaCl₂, and MgCl₂. Inorganic salts are important to adjust the osmotic and ionic balance of the cells and maintain their membrane potential. Carbohydrates are an important energy source, and are mainly used in the form of glucose. Amino acids serve as a source of nitrogen. Media also contain lipids, mostly in the form of a mixture of fatty acids, or in some cases as more complex lipids such as cholesterol. Some media formulations (eg. medium 199) contain detergents such as Tween 80 to help emulsify the lipids. Vitamins such as niacin, folic acid, riboflavin, inositol and thiamine are commonly incorporated in many media to augment continued cell replication. Macromolecules such as thymidine, adenosine, and hypoxanthine are also included in specific media to improve the growth of some cells.

Certain media require additional supplements such as collagen and fibronectin, hormones such as estrogen, and growth factors such as epidermal growth factor and nerve growth factor, to promote cell attachment and proliferation.

The choice of a cell culture medium depends on the type of cells being cultured and is an extremely important factor that affects the success of cell cultures. Different cell types have highly specific growth requirements, and the most suitable medium for each cell type needs to be determined experimentally. Commonly used basal media include Eagle Minimal Essential Medium (MEM), Dulbecco's Modified Eagle Medium (DMEM), RPMI 1640, Ham F10, M199 and L-15, which contain a mixture of amino acids, glucose, salts, vitamins, and other nutrients. Basal media usually need to be supplemented with serum, L glutamine, and antibiotics/fungicides just prior to use.

Use of antibiotics in cultures

Antibiotics are routinely incorporated in cell culture media in order to avoid contamination due to bacteria and fungi. However, it is important to keep the concentration of antibiotics to a minimum amount that is necessary to prevent microbial growth, since higher concentrations can have deleterious effects on cell viability. Their continuous use can lead to the development of antibiotic resistant strains, persistence of low-level contamination and hide the presence of mycoplasma infections and other cryptic

Table 1. Commonly used antibiotics and fungicides for animal cell culture

	Concentration	Effective against
Penicillin	50–100 U/ml	Gram-positive bacteria
Streptomycin	50–100 µg/ml	Gram-negative bacteria
Kanamycin	100 µg/ml	Gram-positive and gram-negative bacteria; mycoplasma
Gentamycin	5–50 µg/ml	Gram-positive and gram-negative bacteria; mycoplasma
Amphotericin B	0.25–2.5 µg/ml	Yeasts and molds
Nystatin	100 U/ml	Yeasts and molds

contaminants. The commonly used antibiotics include penicillin, streptomycin and gentamycin.

Selection of Animals

Successful cell cultures can be obtained by using animals that are young, actively growing and healthy. In the case of aquatic invertebrate cell culture, the animals should be acclimatized to the laboratory conditions for at least a week before cell culturing is initiated, and starved for a day before they are used for the experiments.

Types of Cultures

Explant culture: In explant culture, a small fragment of tissue is allowed to adhere to an appropriate substrate, either spontaneously or aided by mechanical means, and cultured in a nutrient-rich medium. Cell migration is promoted following attachment to give rise to an outgrowth of cells. The tissue is usually cut with a scalpel into slices of 0.5–1.0 mm thickness. One of the main advantages of this method is that some aspects of the tissue's architecture can be preserved within the explant.

Dissociated Cell culture: Cell culture refers to cultures derived from dissociated cells obtained either mechanically or enzymatically from the original tissue. The dispersed cells form a cell suspension which may then be cultured as a monolayer on a solid substrate, or as a suspension in the culture medium. Tissue disaggregation is capable of generating larger cultures more rapidly than explant culture, but explant culture may be preferable where only small fragments of tissue are available or where the cells are too fragile to survive after disaggregation.

Organ culture refers to the culture of the whole organ or part of the organ in a way that allows differentiation and preservation of architecture of the tissue. The tissue is usually cultured at the liquid-gas interface on a grid or gel.

Maintenance of Cultures

Cultures should be examined daily and a tissue culture log should be maintained. Observations on

morphology, color of the medium, density of cultured cells, dates on which cells were subcultured etc. must be recorded.

Growth Patterns

Depending on the cell type, seeding density and media components used, cells in culture initially go through a lag phase, followed by an exponential growth phase where the cells have the highest metabolic activity. The cells finally enter into a stationary phase where they cease to proliferate and cultures become confluent (completely covering the surface of the culture vessel).

Harvesting and Sub-culturing

Cells are usually harvested when they are in a semi-confluent state and are still in log phase. Adherent cell cultures die if they are left in a confluent state for too long and therefore need to be routinely passaged, where a fraction of the cells are transferred to a new culture vessel. Suspension cells exhaust their culture medium once cell density becomes very high, hence these cultures also need to be passaged regularly. Most cells are passaged (or at least fed) three times a week. Suspension cultures are fed by dilution into fresh medium. Adherent cultures that do not need to be divided can be fed by just replacing the old medium with fresh medium.

Several methods can be used to remove semi-confluent cells from the growth surface in adherent cultures so that they can be passaged or sub-cultured:

Mechanical: A rubber cell scraper can be used to physically detach the cells from the growth surface. Although this method is quick and easy, it can damage cell and may cause cell death. This method can mainly be used for harvesting cells for extract preparation etc., where cell viability is not very important.

Proteolytic enzymes: Trypsin, collagenase, pronase or dispase are usually used in combination with EDTA to detach adherent cells from the surface of a cell culture vessel. Although this

method is fast and reliable, it can damage the cell surface by digesting exposed cell surface proteins. Termination of proteolysis can be quickly achieved by the addition of complete medium containing serum.

EDTA: EDTA alone can also be used to detach cells and seems to be gentler on the cells than trypsin.

Cryopreservation

Cell lines can be preserved by freezing to maintain stocks without aging and to protect them from contamination. Factors favoring good survival after freezing and thawing are: (i) High cell density at freezing (1×10^6 – 1×10^7 cells/ml). (ii) Presence of a cryopreservative, such as glycerol or dimethyl sulfoxide (DMSO) at 5–10%. (iii) Slow freezing at $1^\circ\text{C}/\text{min}$, down to -70°C and then rapid transfer to a liquid nitrogen freezer. (iv) Rapid thawing. (v) Slow dilution with medium to dilute out the preservative without causing osmotic damage to cells. (vi) Reseeding at 2- to 5-fold the normal seeding concentration. (vii) Changing medium the following day to remove preservative.

Cell Culture Contamination

Contamination of cultures is one of the most common problems encountered during cell culture activities. Contaminants can be of two types: chemical contaminants which include impurities in media, sera and water, and biological contaminants such as bacteria, molds, yeasts, viruses and mycoplasma, and cross contamination by other cell lines.

Bacterial contamination: Bacterial contamination is a very common occurrence in cell cultures and can be detected by visual inspection in the initial stages of contamination itself. Cultures appear cloudy/turbid and sometimes have a thin film on the surface. A sudden lowering of pH of the culture medium is also observed.

Mold, yeast and virus contamination: Molds have multicellular filaments (hyphae) which form a connected network called a colony or mycelium. The pH of the medium increases rapidly in the

later stages of contamination, and the culture becomes turbid. Yeast contamination can also be visually detected by the presence of turbidity in cultures. Viruses are microscopic in nature and can be detected only by electron microscopy, immunostaining, ELISA assays, or PCR.

Mycoplasma contamination: Mycoplasma are extremely small sized bacteria which are difficult to detect until their densities increase to such an extent that they cause deterioration of cell cultures. Mycoplasma contamination can be detected only by periodical testing of cell cultures using fluorescent stains, ELISA, immunostaining, PCR, autoradiography or microbiological assays.

Cross-contamination: Although cross-contamination is not encountered as frequently as microbial contamination, it can be a serious problem in the culture of cell lines. Cross-contamination can be avoided making periodic checks on the characteristics of the cell lines and also by practicing good aseptic techniques for culture.

Safety Considerations

- Appropriate personal protective gear (aprons, caps, masks, gloves etc.) must be worn always. Gloves must be changed when contaminated, and used gloves disposed of with other contaminated laboratory waste.
- Separate footwear must be used in the culture room.
- Movement of personnel in and out of the culture room must be minimized.
- Hands must be washed after working with potentially hazardous materials and before leaving the laboratory.
- Talking must be avoided when culture activities are in progress.
- Eating, drinking, or storing food for human consumption in the laboratory must be prohibited.
- Contaminated sharp objects (needles, scalpels, pipettes, broken glassware etc.) must be disposed of properly in separate biohazard waste bins.
- Care must be taken to minimize the creation of aerosols and/or splashes.

- All work surfaces must be decontaminated with an appropriate disinfectant before and after the experiments, and immediately after any spill or splash of potentially infectious material.
- Laboratory equipment must be cleaned routinely, even if it is not contaminated. Culture room must be routinely decontaminated using a UV lamp.
- All potentially infectious materials must be autoclaved and decontaminated before disposal.
- All liquid waste after each experiment must be treated with bleach before disposal.

Suggested readings

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In vitro Culture of Finfish Cells – Principle and its Applications

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Introduction

Vertebrate cell cultures are *in vitro* models. The term *in vitro* refers to keeping entities of an organism outside the living body in an artificial environment, in contrast to *in vivo*, *i.e.* in the organisms. Primary cultures start from cells, tissues or organs taken directly from organisms. If a primary culture can be divided into new culture vessels and successfully propagated, it becomes a cell line. A cell line may be propagated a limited number of times, in which case it is finite, or indefinitely, in which case it becomes an immortal or continuous cell line.

Although animal cell culture was first successfully undertaken by Ross Harrison in 1907, it was not until the late 1940's to early 1950's that several developments occurred that made cell culture widely available as a tool for scientists. First, there was the development of antibiotics that made it easier to avoid many of the contamination problems that plagued earlier cell culture attempts. Second was the development of the techniques, such as the use of trypsin to remove cells from culture vessels, necessary to obtain continuously growing cell lines (such as HeLa cells). Third, scientists were able to develop standardized, chemically defined culture media that made it far easier to grow cells. These three areas combined to allow many more scientists to use cell, tissue and organ culture in their research. During the 1960's and 1970's, commercialization of this technology had further impact on cell culture that continues to this day. The overall result of these and other continuing technological developments has been a widespread

increase in the number of laboratories and industries using cell culture today.

Any interaction of a toxic substance with an organism is initiated at the cellular level. From cells, alterations can translate to changes in tissue, organ function and finally impact on whole organism. Based on the central role of cells in the expression of toxicity, several cell lines or *in vitro* models have received regulatory acceptance by the organization as alternative to whole animal tests in health sciences. Besides their potential to replace or reduce animals in toxicity tests, cell lines have several advantages compared to whole animal tests. Large numbers of potentially toxic substances can be screened rather quickly in multiple-well plates, which can be analysed rapidly. As well, cells can help identify the mechanisms underlying a toxic response. If, for a particular purpose, a suitable continuous cell line can be used, a donor animal is never again needed. Based on these reasons, the role of cell lines is expected to significantly increase.

Fish cell line

Fish cell lines have been useful in many areas of research. Originally developed to support the growth of fish viruses for studies in aquatic animal viral diseases, fish cell lines have grown tremendously in number covering a wide variety of species and tissues of origin and an array of applications. Fish immunology, physiology, genetics and development, toxicology, ecotoxicology, endocrinology, biomedical research, disease control, biotechnology and aquaculture are some of the areas in which fish cell lines have made significant contributions.

Fish cell lines being of poikilothermic origin, grow well at room temperatures without the need of thermo regulated incubators, furthermore, an amino acid-rich nutrient medium such as Leibovitz- 15 that does not require CO₂ buffering has been successfully used with fish cell lines, thus CO₂ incubators are not necessary and cells can be grown conveniently in any undisturbed areas. Additionally, because of lower metabolic rates than eurythermic cells, fish cells can be maintained with little care for long periods of time.

In 1962, the first teleost cell line was reported in the literature. The first continuous fish cell culture (RTG-2) was originated over 20 year ago from rainbow trout gonad tissue. Subsequently, many other cell lines of poikilothermic origin have been developed. Most fish cell lines have been established and utilized for isolating, identifying, and studying viruses that cause economically important diseases. Consequently, the majority of these cell lines originate from species that are artificially propagated to some extent. Moreover, most such fish cell lines have been developed in North America or Europe. Nowadays, more fish cell lines are available from fishes indigenous to Asia, since aquaculture and fish farming are pursued on a large scale in this part of the world.

Wolf & Mann (1962) enumerated 61 cell lines originating from 36 fish species, representing 17 families. These cell lines were chiefly used for viral diagnostic purposes, and many had not been well characterized or previously reported. Fryer and Lannan (1993) have compiled a new listing of the fish cell lines reported in the literature that have been at least partially characterized. Recently Lakra *et al.* (2010) made a comprehensive review on the characterized fish cell lines of both freshwater and salt water that have been developed after 1993.

Most fish cell lines originate from normal tissues, and embryos or normal fins are most frequently listed as the source of the tissue used in the primary culture. However, few cell lines were initiated from fish tumors, and in some cases, these cells remained tumorigenic *in vivo* following repeated *in vitro* passage. Traditionally, the chief uses of these cell lines were for detection and study of fish viruses and for diagnosis of the diseases caused by these agents. Today, fish cell cultures are

increasingly utilized in research unrelated to disease, and with the recent identification of rickettsial fish pathogens, the diagnostic role of cultured fish cells has also expanded. Along with the multiplying uses of fish cell culture is a concomitant increase in the need for guidelines for the health and maintenance of fish cell lines.

Fish Cell Culture characteristics

The physiology and the blood plasma constituents of teleost fishes are very much like those of terrestrial vertebrates; therefore the methodology for culture of cells and tissues is also similar. Most fish cell lines are readily propagated *in vitro* using unmodified media and techniques developed for mammalian cells, with appropriate adjustment of incubation temperatures to reflect the temperature range normal for the donor fish species. Also, osmolarity of the media must be adjusted upward for fishes of marine origin. Most important, fish tissue culture often requires less time for preparation and maintenance. Mammalian cell culture techniques need only be modified to reflect the lower incubation temperature requirements and the slower replication rates of the poikilothermic cells.

Appropriate incubation temperatures for cultured fish cells correspond to the normal temperature range of the fish species from which the cell line is derived. For lines from coldwater fishes, incubation temperatures range from 4-24°C with an optimal range of 15-21°C. For lines from warm water fishes, incubation temperatures range from 15-37°C, and the range of optimal incubation temperatures is 25-35°C.

Cell Culture Systems

Once in culture, cells exhibit a wide range of behaviors, characteristics and shapes. Some of the more common ones are described below. Two basic culture systems are used for growing cells. These are based primarily upon the ability of the cells to either grow attached to a glass or treated plastic substrate (Monolayer Culture Systems) or floating free in the culture medium (Suspension Culture Systems).

Types of Cells

Cultured cells are usually described based on their morphology (shape and appearance) or their functional characteristics.

There are three basic morphologies:

- **Epithelial-like:** cells that are attached to a substrate and appear flattened and polygonal in shape.
- **Lymphoblast-like:** cells that do not attach normally to a substrate but remain in suspension with a spherical shape.
- **Fibroblast-like:** cells that are attached to a substrate and appear elongated and bipolar, frequently forming swirls in heavy cultures.
- **Endothelial cells:** Endothelial cells are very flat, have a central nucleus, are about 1-2 μm thick and some 10-20 μm in diameter.
- **Other types:** Macrophages, neuronal cells, melanocytes, etc.

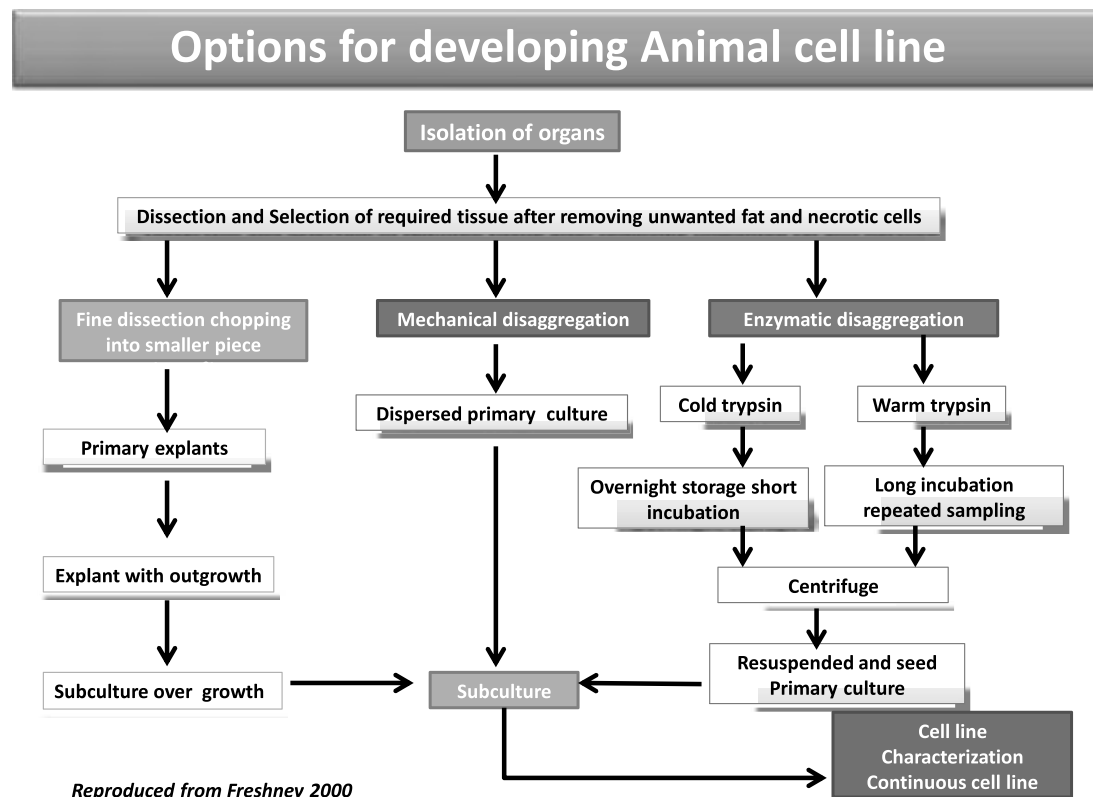
It is important to remember that the culture conditions play an important role in determining shape and that many cell cultures are capable of exhibiting multiple morphologies.

Development of cell line

Primary Culture

There are several ways with which monolayer cultures of fish cells may be initiated. This is a quick method that employs multiple explants of tissues of either fresh water or marine fish as the simplest way to produce monolayer cultures. When cells are surgically removed from an organism and placed into a suitable culture environment, they will attach, divide and grow. This is called a Primary culture. There are two basic methods for doing this. First, for Explant Cultures, small pieces of tissue are attached to a glass or treated plastic culture vessel and bathed in culture medium. After a few days, individual cells will move from the tissue explant out onto the culture vessel surface or substrate where they will begin to divide and grow.

The second, more widely used method, speeds up this process by adding digesting (proteolytic) enzymes, such as trypsin or collagenase, to the tissue fragments to dissolve the cement holding the cells together. This creates a suspension of single cells that are then placed into



culture vessels containing culture medium and allowed to grow and divide. This trypsinization method describes warm (1-2 hrs at 37°C) and cold (4 - 6°C overnight (16 hr) trypsinization of fish tissues which yields cultivable cells and small aggregates of cells for monolayer cultures. The disaggregated cells obtained by this procedure generally yield more uniform monolayer more quickly than do cultures initiated with minced tissues alone. This method is called Enzymatic dissociation.

Before starting the preparation of primary culture, food should be withheld from donor fish for a day or more before use. Healthy specimens free of external lesions are preferred; otherwise there is risk of encountering systemically disseminated bacteria. Cells and tissues should be cultured at a temperature similar to the environmental temperature preferred by the donor species. Extended exposure of tissues from cold-water fishes such as salmon and trout to 30°C can be lethal. In contrast, many and perhaps most fish tissues remain viable even if held for a day or two on ice or at 4°C to 6°C. Internal tissues may be safely removed after thorough topical disinfection; this is conveniently done by total immersion of the fish for several minutes. A solution of liquid household bleach having 500 ppm available chlorine, or a 1:1000 dilution of a quaternary ammonium compound may be used. Excess disinfectant should be rinsed off with chlorinated tap water or sterile water and the surgical site sponged with 70% isopropanol or ethanol. External tissues such as those of fins, gills, corneas and barbels are severely damaged by such disinfection. Consequently, such tissues should be excised first and decontaminated separately. Immersion for 1 hr in a solution containing 500 IU polymyxin B, 500 µg neomycin and 40 IU bacitracin is suggested, for these are bactericidal antibiotics.

Subculturing

When the cells in the primary culture vessel have grown and filled up all of the available culture substrate (called monolayer) they must be subcultured to give them room for continued growth. This is usually done by removing them as gently as possible from the substrate with enzymes. These are similar to the enzymes used in obtaining the primary culture and are used to break the protein bonds attaching the

cells to the substrate. Some cell lines can be harvested by gently scraping the cells off the bottom of the culture vessel. Once released, the cell suspension can then be subdivided and placed into new culture

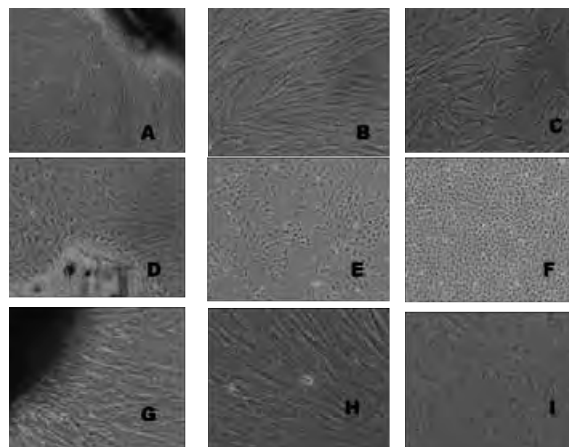


Fig. 1. Phase contrast photomicrographs of rohu cells derived from Heart - (A, B, C), Fin - (D, E, F) Swim bladder - (G, H, I). (A, D, G)-Primary culture on Day 5 following seeding tissue explant E, H) - subcultured at Passage 10; (C,F,I) - subcultured at Passage 30.

vessels. Once a surplus of cells is available, they can be treated with suitable cryoprotective agents, such as dimethylsulfoxide (DMSO) or glycerol, carefully frozen and then stored at cryogenic temperatures (below -130°C) until they are needed.

Development of continuous cell lines

Some cell lines may give rise to continuous cell lines. The ability of a cell line to grow continuously probably reflects its capacity for genetic variation, allowing subsequent selection. Genetic variation often involves the deletion or mutation of the p53 gene, which would normally arrest cell cycle progression, if DNA were to become mutated, and over expression of the telomerase gene. Possibly the condition that predisposes most to the development of a continuous cell line is inherent genetic variation, so it is not surprising to find genetic instability perpetuated in continuous cell lines. The alteration in a culture that give rise to a continuous cell line is communally called in vitro transformation and may occur spontaneously or be chemically or virally induced. immortalization means the acquisition of an infinite life span and transformation implies an alteration in growth characteristics (anchorage independence, loss

of contact inhibition and density limitation of growth) that will often, but not necessarily correlate with tumorigenicity

Many (if not most) normal cells do not give rise to continuous cell lines. Normal human fibroblasts remain euploid throughout their life span and at crisis will stop dividing (around 50 generations), although may be viable for 18 months. Human glia cells and chick fibroblasts behave similarly. Epidermal cells, on the other hand, have shown gradually increasing life span with improvements in culture techniques and may yet be shown capable of giving rise to continuous growth. Continuous cell line of lymphoblastoid cells is also possible by transformation with Epstein-Barr virus.

Table 1. Properties of finite and continuous cell line

Properties	Finite cell line	Continuous cell line
Ploidy	Euploid, eiploid	Aneuploid, heteroploidy
Transformation	Normal	Immortal, growth control altered and tumorigenic
Anchorage dependence	Yes	No
Contact inhibition	Yes	No
Density limitation of cell proliferation	Yes	No
Mode of growth	Monolayer	Monolayer or suspension
Maintenance	Cyclic	Steady phase possible
Serum requirement	High	Low
Cloning efficiency	Low	High
Markers	Tissue specific	Chromosomal, enzymic, antigenic
Growth rate	Slow	Rapid
Yield	Low	High
Control parameters	Generation number, tissues specific markers	Stain characteristics

Stem cell cultures

Stem cells have the remarkable potential to develop into many different cell types in the body during early life and growth. Stem cells are distinguished from other cell types by two important characteristics. First, they are unspecialized cells capable of renewing

themselves through cell division, sometimes after long periods of inactivity. Second, under certain physiologic or experimental conditions, they can be induced to become tissue- or organ-specific cells with special functions. In some organs, such as the gut and bone marrow, stem cells regularly divide to repair and replace worn out or damaged tissues. In other organs, however, such as the pancreas and the heart, stem cells only divide under special conditions.

Until recently, scientists primarily worked with two kinds of stem cells from animals and humans: embryonic stem cells and non-embryonic “somatic” or “adult” stem cells. Scientists discovered ways to derive embryonic stem cells from early mouse embryos nearly 30 years ago, in 1981. In 2006, researchers made another breakthrough by identifying conditions that would allow some specialized adult cells to be “reprogrammed” genetically to assume a stem cell-like state. This new type of stem cell, called induced pluripotent stem cells (iPSCs).

Stem cells are important for living organisms for many reasons. In the 3- to 5-day-old embryo, called a blastocyst, the inner cells give rise to the entire body of the organism, including all of the many specialized cell types and organs such as the heart, lung, skin, sperm, eggs and other tissues. In some adult tissues, such as bone marrow, muscle, and brain, discrete populations of adult stem cells generate replacements for cells that are lost through normal wear and tear, injury, or disease.

Given their unique regenerative abilities, stem cells offer new potentials for treating diseases such as diabetes and heart disease. Laboratory studies of stem cells enable scientists to learn about the cells’ essential properties and what makes them different from specialized cell types. Scientists are already using stem cells in the laboratory to screen new drugs and to develop model systems to study normal growth and identify the causes of birth defects.

Stem cell research is one of the most fascinating areas of contemporary biology, but, as with many expanding fields of scientific inquiry, research on stem cells raises scientific questions as rapidly as it generates new discoveries.

Characterization of Cell Lines

In contrast to mammalian cells, are easier to maintain and manipulate, and unlike primary cultures, produce highly reproducible results. This ease of handling and simpler growth requirements makes cross-contamination of cell lines a more likely possibility, Proper characterization and identification of the cell lines are hence critical for scientific usage.

Authentication of a cell line is the sum of the process by which a line's identity is verified and shown to be free of contamination from other cell lines and microbes. Tests used to authenticate cell cultures include iso-enzyme analysis, antigenic markers, karyotyping/ cytogenetic analysis and more recently molecular techniques of DNA profiling. Whilst most of the techniques above are generalized tests and are applicable to all cell lines additional specific tests may also be required to confirm the presence of a product or antigen of interest.

Cell line contamination

Cell line contamination is a major drawback of main cell banks of the world and it has cost of losing important biological products or valuable research. The causative agents are different chemicals, invertebrates, bacteria, fungi, parasites, viral species and even other cell lines. Bacteria, fungi, parasite, viruses, invertebrates and mycoplasmas are main causative agents of cell line contamination.

The bacterial and fungal (including molds and yeast) contamination of cell lines (except mycoplasmas) can be readily detected, as these organisms cause increased turbidity, shift in media pH (change in medium color) and cell destruction. Some reports have indicated that putative pathogens such as nanobacteria also will not be detected by this method.

In the case of mycoplasmas their cell line contamination is always undetected for many passages. They can proliferate within the cell, tolerate antibiotics and their growth always does not have any obvious microbial evidence like turbidity and pH changes or cytopathic

effect. Their contamination also spreads quickly to the other cell lines

Sources of contamination

Another approach to cell culture contamination is sources of contamination. The sources of microbial culture contamination are different and may be grouped under four subjects.

Contaminated cells, which are used as the primary starting material for cell culture.

Glassware or apparatus, including storage bottles and pipettes

Culture media (serum, basal cultural media containing heat-sensitive essential amino acids and vitamins, enzymes like trypsin, pronase and collagenase, and basic salt solutions).

Airborne modes which can occur anytime the culture vessel is opened or contact is made with culture fluid through a defective culture vessel, stopper, or poor technique

Cross-Contamination and Misidentification

The problem of intraspecies and interspecies cross-contamination among cell lines has been recognized for half a century. For those scientists working on cell lines derived themselves or received from a colleague, basic authentication tests such as STR profiling, isoenzyme analysis, and contamination tests are readily available and should be routinely used. Transferring cell lines to colleagues should be avoided, or when it does occur, accompanied with comprehensive documentation verifying the integrity of the material or tests need to be repeated. Although cross-contamination of fish cells with other cell types has not been widely reported, conveyed the identification of a cell line dubbed Clone 1A believed to be derived from rainbow trout as being CHSE-214, a cell line derived from Chinook salmon embryos. Accordingly, awareness of good laboratory practices and careful vigilance with fish cell cultures as detailed by Lannan should be followed to avoid confusion of cell lines.

Applications of cell culture

Fish cell lines have been useful in many areas of research. Originally developed to support the growth of fish viruses for studies in aquatic animal viral diseases, fish cell lines have grown tremendously in number covering a wide variety of species and tissues of origin and an array of applications. Fish immunology, physiology, genetics and development, toxicology, ecotoxicology, endocrinology, biomedical research, disease control, biotechnology and aquaculture are some of the areas in which fish cell lines have made significant contributions

Toxicity Testing

Cultured cells are widely used alone or in conjunction with animal tests to study the effects of new drugs, cosmetics and chemicals on survival and growth in a wide variety of cell types. Especially important are liver- and kidney-derived cell cultures.

Cancer Research

Since both normal cells and cancer cells can be grown in culture, the basic differences between them can be closely studied. Since, the normal cultured cells could be induced into cancer cells, the mechanisms that cause the change can be studied. Cultured cancer cells also serve as a test system to determine suitable drugs and methods for selectively destroying types of cancer.

Virology

One of the earliest and major uses of cell culture is the replication of viruses in cell cultures (in place of animals) for use in vaccine production. Cell cultures are also widely used in the clinical detection and isolation of viruses, as well as basic research into how they grow and infect organisms.

Cell-Based Manufacturing

Cultured cells can be used to produce many important products, like viral vaccines, genetically engineered

protein of medicinal and commercial value and replacement of tissues and organs.

Genetic Counseling

Amniocentesis, a diagnostic technique that enables doctors to remove and culture fetal cells from pregnant women, has given doctors an important tool for the early diagnosis of fetal disorders. These cells can then be examined for abnormalities in their chromosomes and genes using karyotyping, chromosome painting and other molecular techniques.

Genetic Engineering

The ability to transfect or reprogram cultured cells with new genetic material (DNA and genes) has provided a major tool to molecular biologists wishing to study the cellular effects of the expression of these genes (new proteins).

Gene Therapy

The ability to genetically engineer cells has also led to their use for gene therapy. Cells can be removed from a patient lacking a functional gene and the missing or damaged gene can then be replaced. The cells can be grown for a while in culture and then replaced into the patient. An alternative approach is to place the missing gene into a viral vector and then “infect” the patient with the virus in the hope that the missing gene will then be expressed in the patient’s cells.

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Methods for examination of Cell Culture

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The cell cultures have to be evaluated for their growth properties and reproducibility of culture conditions. The general health of cell culture depends upon four important characteristics namely morphology, growth rate, plating efficiency and expression of functions. The morphology of cells changes with the culture conditions but it is very difficult to characterize or quantify the morphology based on microscopic examination. The growth rate of cells is determined by counting the number of cells which again vary with the culture conditions. Plating efficiency is done to test the growth rate and survivability of cells by placing few cells on to media and testing the number and size of cell colonies formed. The specialized functions of cells are evaluated using biochemical and immunological assays.

Gross examination

Before subjecting the cultures for assays, the culture plates must be looked at morphologically for any observations. The change of color of medium (Purple coloration/yellow coloration) indicates that the pH of the medium has changed either due to problem with CO₂ supply or acidity of the medium. The plates also should be checked for cloudiness or turbidity, floating of debris or any other contamination in the medium. All the above indicate the overall healthiness of the cell cultures.

Microscopy

The most important step in evaluating a cell culture is the microscopic examination. The cultures should be magnified to more than 100 folds before making quantitative measurements. The changes in cell shape, cell size and cell to cell

associations can be noted down. The images of all these changes can be recorded using a camera attached with the microscope. The different types of microscope that can be used for evaluating a cell culture are.

Phase contrast microscopy

The differences in phases occur by placing a phase annulus in the objective and mounting a fixed annular phase ring in the condenser system. This configuration creates a ring of light in the rear focal plane of the objective, where 75% of the central beam is absorbed. The refracted beam of light is brought into focus at the eyepiece, where it produces a bright image. Since the beam intensity is significantly reduced in the rear focal plane of the objective, a contrasting dark background is produced for the bright image, called as "phase contrast." The inverted phase contrast microscope gives much information when viewing unstained living tissue.

Fluorescence microscopy

The phase contrast microscope is fixed with epifluorescent objective to form a fluorescent illumination. The cells are labeled with fluorescent dyes like Fluo-3, Fluo-4, Rhod-2, Calcium Green, and Calcium Orange for assessing cell viability, function and proliferation.

Confocal microscopy

Confocal microscopy is used in the scanning imaging mode for three dimensional imaging of specimens. This helps in studying the cell's physiology, cell motility, and three dimensional structures.

Scanning Electron Microscopy (SEM)

The SEM helps in looking at the surface of cells at higher magnifications. The cells that are coated with a reflective metal such as gold are bombarded with an electron beam and the image thus obtained in the microscope is created from the reflected electrons. The cell surface features like cilia, cell shape and cell to cell association can be seen by scanning electron microscopy.

Transmission Electron Microscopy (TEM)

The TEM visualizes sub-cellular components such as Mitochondria, Golgi vesicles and other organelles; even individual macromolecules such as DNA can be observed under TEM.

Cell Counting

The growth of cells can be assessed under a microscope with regard to its stage of culture. But still the cells must be counted during the course of an experiment for proper analysis which in turn decides the reproducibility of a culture and also further subcultures. The different methods used for counting cultures are

- Direct methods like Hemocytometer, flow cytometry
- Indirect methods like staining with crystal violet and MTT.

Hemocytometer: In this method, the cells in suspension are placed in an optically flat chamber. Since the area bounded by the lines and depth of the chamber is known, it helps us in counting the number of cells in a particular volume of suspension and in turn the number or concentration of cells in overall culture.

$$\text{Concentration of cells} = \frac{(\text{Number of cells counted})}{(\text{Volume counted})}$$

i.e., Concentration of cells = Number of cells x 10⁴/ml

Flow Cytometry: It's a laser based technology for detection of cell concentration by allowing the cell suspension to pass through an electronic beam. The cells are passed as single file through the flow cytometer, the laser hits the cells, light is scattered and the photomultiplier measures the light intensity and the sensor measures the scatter pattern. Based on the nature and type of cells, the computer categorizes the cells and its quantity. Flow cytometry helps in quantifying the cells, categorizing cells and also separate subpopulations of cells.

MTT assay

This assay measures the cell viability and cytotoxicity. The cells are stained with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide). The assay is based on the principle of conversion of MTT into formazan crystals by living cells. This method is easy-to-use, safe, high reproducible and widely used in both cell viability and cytotoxicity tests.

Trypan blue assay

Trypan blue is widely used for staining dead cells. In this method, the cells are mixed with 0.4% trypan blue dye and centrifuged. The supernatant is discarded and the cell suspension in pellet form is loaded on to a hemocytometer chamber and viewed under a microscope. The live cells have intact cell membrane and so exclude the dye from entering into the cell. In contrast, the dead cells due to the loss of membrane integrity take up the dye readily and get stained in blue color. Thus dead cells are seen as blue in color and the viability is determined by counting the unstained cells with a microscope. However, this method can't differentiate the healthy cells from that of live cells losing functions.

Neutral red assay

The neutral red assay provides a quantitative estimation of the number of viable cells in a culture. It is one of the most used cytotoxicity tests based on the ability of viable cells to incorporate and bind the neutral red dye in the lysosomes.

LDH assay

The assay gives us an indication of the number of dead cells based on the estimation of lactate dehydrogenase released by the dead cells into the medium. Since LDH is a stable enzyme, its release into the medium indicates the presence of dead tissues or cell damage.

Contamination and its detection

The detection of contamination and subsequent eradication from the cell culture system poses a major challenge for the researchers dealing with cell cultures. The contamination may influence all the parameters that are to be studied during the course of a cell culture experiment. Even if one well is contaminated, it is better to discard the plates safely without retaining other wells for further experiments. Contaminants like mold can be seen with naked eye while others like bacteria and yeast are too small to be seen except under high power microscope. The contamination turns the medium cloudy and changes the pH of the medium. The contaminated cultures should be viewed under microscope and if confirmed, should be discarded immediately in proper sealed bags after autoclaving.

Mycoplasma

In comparison to bacteria and yeast, mycoplasma contaminants grow very slowly. They don't destroy or kill the cells immediately. They alter the function and metabolism of the cell culture thereby causing chromosomal aberrations, cytopathology, slow down growth rate of cells, cell transformation and alteration of membrane permeability, interfere with nucleic acid metabolism and change cell behavior. The different assays used for the detection of mycoplasma contamination are fluorescent staining, culture, DNA probes and co-cultivation. Molecular tools like hybridization of probes and PCR have been widely used for the easy detection of mycoplasma. They can also be detected using high power microscopy or SEM.

Bacteria and Fungi

The change in PH of the medium and subsequent

turbidity indicates the presence of bacterial contamination. The cells may die subsequently. The fungal/mold contamination is indicated by the presence of puffy milky white clumps or mat like colonies. Under microscopic examination, fungal filaments are seen in the cultures. The yeast contamination is indicated by budding ovoid structures in the cell cultures on microscopic examination. There should be routine examination of the cell culture plates for the presence of bacterial and fungal contamination. The media routinely used to test bacterial and fungal contamination are blood agar, thioglycollate broth, tryptic soy broth, Sabouraud broth, and nutrient broth.

Insects and parasites also contaminate the cell culture system. The viral contamination is generally not a common feature in cell cultures. The use of infected bovine serum may pose threat in the form of viral contamination in cell cultures. If present, it is very difficult to detect except by immune staining, ELISA and PCR.

Immunohistochemistry

Immunohistochemistry is used to visualize the localization of a specific protein or antigen in cells by use of a specific primary antibody that binds to it. It provides a rapid method for demonstrating the presence of an antigen in a cell culture system. With advances in antibody labeling and cell staining techniques, immunohistochemistry can be a powerful technique for the detection of surface and intracellular antigens. Fluorochrome labeled antibodies is used for detecting sub-cellular components at high magnification. The sensitivity of detecting the antigens is increased by the use of enzyme labeled antibodies.

Karyotyping

The characterization of a cell line is very important to establish its future value. The analysis of chromosome known as Karyotyping is one of the conventional methods utilized for characterization of cell lines. Chromosome banding and chromosome painting are generally used to distinguish individual chromosomes by using specific molecular probes.

Isoenzyme analysis

The analysis of isozymes is one of the qualitative methods to differentiate cell strains based on enzyme protein polymorphisms. The enzymes are separated either by chromatography or electrophoresis and the distribution patterns of the enzymes are very characteristic of a particular tissue or cell line.

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Tissue Culture—Marine Invertebrates

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Introduction

In biological and medical research, cell cultures are valuable tools as it replaces the need for using live animals in experiments by providing an alternate method. The culture of animal cells and tissues has undergone a major development from being a purely experimental procedure to become an accepted technological component of many aspects of biological research and commercial exploitation. Remarkable progress has been achieved in medical and biological sciences in cellular biology, genetics and pathology by *in-vitro* investigations. Even though the use of tissue culture in invertebrate physiology and pathology has been popular in recent years, only few studies have been reported in marine invertebrates in countries like Japan, USA, Canada, France and China. Marine invertebrates encompassing 20 different phyla is the richest source of varied cells and tissue types. Cell culture works are reported mainly in Porifera, Cnidaria, Crustacea, Mollusca, Echinodermata and Urochordata (Rinkevich, 2005) and no single marine invertebrate cell line has been reported to date.

Porifera

Cell culture of sponges has been selected as a model system for various cellular aspects. Large scale production of novel highly potent pharmacologically significant bioactive compounds can be obtained from *in-vitro* culture of sponges. Embryos and larvae from sponge have been evolved as a new source for cell culture (continuous cell lines). In comparison with the adult sponge cells, sponge embryos and larvae are more adaptable as it has high content of stem cells and also shows resistance to infections (De Caralt, 2007). Marine sponges are the sources of metabolites including cytotoxic and anticancer compounds. Bioactive

compounds from marine sponges have anti-oxidant, radical scavenging, anti-bacterial anti-inflammatory properties (Perdicaris, 2013). The cell cultures from sponges are getting great importance in producing new medical drugs. Sponge cell culture is still in its primary stage and facing problems for maintaining long term cultures. Use of antibiotics for maintaining the axenic condition in sponge cell culture has inhibitive effect on cell growth and contamination is the great problem without antibiotics. Single cells apparently do not have the potency to produce secondary metabolites and primorph model is found as a suitable system for the synthesis of bioactive compounds *in vitro*. Improved media and improved culture conditions in this group should prospect the long term cultures (Rinkevich, 1999, 2005).

Cnidaria

Cnidarians are the more primitive of the animal phyla and includes organisms such as jellyfish, sea anemones, corals and hydras. Primary cultures were also made from the neurons which are disassociated from the nerve rings of the jellyfish, *Polyorchis penicillatus* (Rinkevich, 1999). Symbiotic relationship between many cnidarians and unicellular algae was studied through *in-vitro* cnidarians culture. Tissues of the striated muscle and endoderm of the jellyfish have also been used as the substratum for primary cell cultures. *Anemonia viridis*, a temperate symbiotic sea anemone which belongs to the cnidaria was used as an experimental tool to study the molecular and cellular events involved in the rupture of symbiosis between animal cells and their microalgae. To study this molecular events primary culture of *A. viridis* from its tentacles tissue was carried out. (Verdier, 2013). Corals are the most basic cnidarians and culturing their cells *in-vitro* helps in the production of extra-cellular matrix

and precipitation of calcium carbonate (Helman, 2007). *In vitro* crystallization of aragonite in coral cell cultures was proved as an innovative approach to investigate reef-building coral calcification at the cellular level in the cell cultures of a hard coral, *Pocillopora damicornis* (Rinkevich, 2005).

Echinodermata

The primary cultures from echinoderms were mainly performed on sea urchin cells, from ovaries of the edible sea urchin *Paracentrotus lividus*. Primary cell cultures from starfish, *Marthasterias glacialis* with tissue samples taken from tube feet and body wall also reported (Bavington *et al.* 2000). Coelomocytes from the sea cucumber *Holothuria tubulosa* and star fish, *Asterias rubens* were used as a cellular model for toxicity testing and biomonitoring (Ronning, 2006). Primary cell cultures of echinoderms were used as an ideal system to explore the cellular, biochemical, and genomic aspects of echinoderm regenerative properties (Bello *et al.*, 2015).

Urochordata

Cell cultures of tunicates, a primitive group of phylum Chordata is a powerful tool for studying the phylogeny of different biological characters such as developmental and cellular biology, immunology and genetics. Hemocytes and the pharyngeal explants from the colonial tunicates were the major cell type studied from the tunicate *Styela clava*. Proliferative activity of pharyngeal hemocytes and differentiation state of circulatory hemocytes was studied by *in-vitro* culture. Proliferation activity of the pharyngeal cells of tunicates was enhanced in *in-vitro* primary cell culture after stimulating these cells by Interleukin 1 like molecule extracted from the tunicates. Cells from the pallial buds of the colonial tunicate *Botryllus schlosseri* was used as a source in epithelial cell culture (Rinkevich, 1999, Rabinowitz and Rinkevich, 2003). Kawamura *et al.*, 2006 established *in vitro* culture of mesenchymal lineage cells from the colonial tunicate *Botryllus primigenus*.

Crustacea

Attempts were made to develop cell lines from crustaceans mainly with *penaeid* species. *P. monodon*

and *P. japonicas* were used widely in cell culture techniques. The embryonic, ovary and larval tissues were selected for cell culture as these cells are mitotically active and resistant to contamination than adult tissues. Cell cultures derived from crustaceans are widely used in studying pathogens, especially viral pathogens. Therefore it is necessary that the cell line should be capable of supporting viral growth in *in-vitro* conditions (Rinkevich, 2005). Lymphoid organ is mostly selected as a tissue source as it is the target organ for many viral infections. Tissues from heart, gill, nerve, gut, testis, eye, epidermis have also been used as a source for primary cell cultures. The development of cell line from crustaceans will help in understanding the viruses that attack them and in developing control measures. Primary hemocyte culture from the *Penaeus monodon* a marine crustacean helps in assessing the cytotoxicity and genotoxicity of the environmental pollutants such as cadmium chloride and mercuric chloride (Jose, *et al.* 2011).

Mollusca

Molluscs are the largest marine phylum which includes snails, mussels, limpets, oysters, clams etc; Molluscan cells can be derived from any tissue and can be cultured in *in-vitro* conditions. As for now only one molluscan cell line developed is the Bge cell line (embryonic) which is isolated from a fresh water snail, *Biomphalaria glabrata* Say (Hansen, 1976). The early developing embryos and the larval stages are the most suitable source of primary cell culture. Hemocytes in molluscs exist as different cell types and express different functions like phagocytosis, cell signaling pathways etc., Due to its defensive phagocytic property, it is easier to develop cultures with limited contamination than other molluscan tissues. Circulatory hemocytes are terminally differentiated and short lived. However, long term cultures from marine clams were reported from neoplastic and cancerous hemocytes. Hemocyte cultures play a crucial role in investigating pathogens like *Perkinsus marinus* and immune function of commercially important bivalves. Neuronal cells of molluscs are used as models for studying the complexity of memory and other behavioural capacities of higher organisms. Primary cell cultures of neurons were derived from molluscan species of the genus *Aplysia*, *Lymnaea*, *Helisoma* and *Helix*. Primary cell cultures were also obtained from

tissues derived from organs such as heart, mantle, gill, digestive gland and gonads. Cardiomyocyte cultures of oysters are used as model systems for electrophysiological studies, molecular genetic studies and environmental toxicological monitoring. Tissues from cardiac muscle of surf clam *Spisula solidissima* have also been used in growing mono layer cell lines for long periods. As gill and digestive glands have direct contact with the natural environment, primary cell cultures of these tissues are considered as a suitable system for biomonitoring of different chemical contaminants in aquatic systems (Yoshino, 2013). *In-vitro* pearl production was successful through the culture of mantle tissue from the abalone *Haliotis varia* Linnaeus (Suja and Dharmaraj, 2005). Mechanism of cell proliferation, pearl sac formation, crystal secretion and nacre formation was revealed by the mantle tissue culture of the pearl oyster, *Pinctada fucata*, abalone *Haliotis varia* and other molluscs (Dharmaraj and Suja 2006; Awaji and Machi, 2011). Primary cell culture derived from molluscs was proved to be a suitable tool for *in-vitro* studies of bio-mineralization (Bordenave, 2010). *In-vitro* synthesis of proteoglycans and collagen was possible by culturing the mantle cells from nacreous mollusc *Haliotis tuberculata*. Through this model molluscan extra-cellular matrix was studied well (Rinkevich, 2005). In the last decade *in vitro* pearl sac formation and nacre biomineralization created great interest in the cell culture of molluscs.

Culture Conditions

Culture medium is the vital factor to control the growth of culture. Basic cell culture media should provide an optimal pH value, specific osmolality, nutrients such as vitamins and other trace elements. There are several types of media which are enriched in various supplements to support the growth of marine invertebrates cell cultures. The basis of cell culture media is the balanced salt solution and it should be sterile. Sea water, Medium 199, L-15, Ham's F-12, RPMI 1640, EMEM, and Iscove's MDM etc. are the common media available in markets which can be procured directly from companies or can be prepared as per compositions. Growth supplements like tissue extracts, FCS (Foetal calf Serum), Lactalbumin hydrolysate, growth factors (EGF) vitamins, carbohydrates, lipids, amino acids, salts,

concanavalin A are incorporated to the culture medium according to the requirement. Antibiotics such as kanamycin, streptomycin, rifampicin, gentamicin, nystatin, amphotericin, penicillin, ampicillin are added in the culture media to prevent contamination. Sterile vessels can be selected according to the requirement of cell growth. Petri plates, T25 flasks, 6, 12, 24 and 96 well plates are also available and are used for different cell culture studies.

Cell cultures are incubated inside a CO₂ incubator for regulating CO₂ concentration. The marine invertebrates require lower temperature at 20-25°C than mammalian cell types at 37°C. Some media requires no additional CO₂, if it is cultured in sealed vessels. Cryopreservation is mainly used to conserve various tissues such as embryo, larvae for a long time. Cryopreservation was basically developed for vertebrate cells. But few studies reported successful cryopreservation in marine invertebrates also. Cell cultures obtained from the tissues of marine invertebrates can be frozen upto -196°C. Dimethyl sulphoxide (DMSO) and trehalose are commonly used as cryoprotectants (Odintsova, 2001).

Obstacles in Development of Cell Cultures

Organisms in the marine environment are exposed to natural microbial flora and fauna of the habitat. The successful development of cell cultures depends on axenic conditions. Contamination is the major problem for long term cultures of marine invertebrates. The control of microbial contaminants with the antibiotics will adversely affect the growth of primary cells. The common type of contamination in many cultures is the appearance of thraustochytrids. The standardization of optimum dose of antibiotics in culture is a vital step for the success of culture. Marine invertebrate organisms comprise of different cells and tissues and each cell type require different culture conditions. Hence components of culture media should be formulated according to the cell types with optimum pH, and osmotic conditions, and this is detrimental to cell proliferation. Limited knowledge of cell adherence preferences, optimum concentration of inorganic and organic components of media, toxic

compounds released during explant preparation and culture are some of the factors for the failures of cell growth in marine invertebrates. Use of trypsin and other enzymes for harvesting and subculturing of cells will also affect the viability of cells. Better understanding of secondary metabolites during cell growth can answer the problems in cell viability for long term maintenance.

Conclusion

Marine invertebrate tissue culture has its application in various fields such as molecular biology, medicine, pharmaceutical industries, cosmetics production, detection of marine pollutants, pearl production mechanisms etc. Numerous studies on marine invertebrates primary cell culture indicate that we still need to establish standard protocols for the production of secondary cell metabolites and cell immortalization (Rinkevich, 2005). The increasing demand of biomaterials from marine invertebrates fascinated new researchers into this challenging field. The development of cell cultures from economically important species will be a powerful tool for disease diagnosis, biomonitoring, drugs and novel biomaterials. Development of enhanced culture media with growth additives and factors will lead to a better insight in the development of cell cultures from marine invertebrates. Introduction of novel genomic and proteomic tools will help in developing continuous cell lines using standard procedures. Control of contamination through advanced laboratory protocols and combination of new antibiotics will definitely give better growth and survival of cultures. Modifications from the existing protocols with modern techniques will certainly give promising results in this field.

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Molecular markers in Population Genetics

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Genetic variation describes naturally occurring genetic differences among individuals of the same species. Natural populations show variation at all levels, from gross morphology to DNA sequences. They differ in their morphology, their microscopic structure, their chromosomes, the amino acid sequences of their proteins, and in their DNA sequences. Population genetics is defined as the science of how genetic variation is distributed among species, populations and individuals. Population is a sub-species category, formed by a group of conspecific individuals as a breeding unit sharing a particular habitat at a certain time. The stock concept subdivided species to closed populations or 'stocks' that maintain themselves in a given area and are reproductively isolated from other such populations.

The logical and practical reasons for identifying population units are that such units may have their own characteristics of recruitment, growth, natural mortality, migration, behaviour etc. Assessment using ecological parameters, tagging, parasite distribution, physiological, behavioral, morphometric, meristic, calcareous, biochemical and cytogenetic characters identify stock structure and genetic variation in a population. Different methodologies evaluating genetic variation begin with the very traditional comparative examination of morphology to the most innovative DNA-based genetic markers. The inefficiency of conventional morphometric measurements led to the development of 'truss network analysis', where the shape of the body forms of fish or shellfish is also taken into account along with the size. In the mid-fifties, protein electrophoresis and histochemical staining methods gained advantage over morphological studies. Analysis of allozyme loci remained one of the most popular approaches in examining population genetics and stock structure questions in fishes till the advent of the PCR. The

development of DNA-based genetic markers has had a revolutionary impact on animal genetics. A number of different techniques have emerged, ranging from sequencing of the DNA of interest to methods analyzing length polymorphisms, such as microsatellites. It is theoretically possible to observe and exploit genetic variation in the entire genome of organisms with DNA markers.

Genetic markers can be categorized based on their mode of transmission and evolutionary dynamics into (1) protein markers such as allozymes and (2) DNA markers such as (2.1) mitochondrial DNA and (2.2) nuclear DNA markers like randomly amplified polymorphic DNA (RAPDs) and variable number of tandem repeats (VNTRs) loci such as minisatellites and microsatellites.

Principles and categorization of molecular markers

All organisms are subject to mutations as a result of normal cellular operations or interactions with the environment, leading to genetic variation (polymorphism). In conjunction with selection and genetic drift, there arises genetic variation within and among individuals, species, and higher order taxonomic groups. For this variation to be useful to geneticists, it must be (1) heritable and (2) discernable to the researcher, whether as a recognizable phenotypic variation or as a genetic mutation distinguishable through molecular techniques. At the DNA level, types of genetic variation include: base substitutions, commonly referred to as single nucleotide polymorphisms (SNPs), insertions or deletions of nucleotide sequences (indels) within a

locus, inversion of a segment of DNA within a locus, and rearrangement of DNA segments around a locus of interest. DNA marker technology can be applied to reveal these mutations. Large deletions and insertions (indels) cause shifts in the sizes of DNA fragments produced upon digestion by restriction enzymes, and are among the easiest type of mutations to detect, mainly by electrophoresis of the fragments on an agarose gel; smaller indels require DNA sequencing or more elaborate electrophoretic techniques to determine smaller changes in size. Inversions and rearrangements that involve restriction sites can be easy to detect because they disrupt the ability of a restriction enzyme to cut DNA at a given site and thus can produce relatively large changes in DNA fragment sizes. Point mutations are more difficult to detect because they do not cause changes in fragment sizes. Several marker types are highly popular in genetics. In the past, allozyme and mtDNA (restriction fragment length polymorphism (RFLP)) markers have been popular genetics research. More recent genetic markers employed in fisheries and aquaculture are mitochondrial DNA sequence information, randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellite, single nucleotide polymorphism (SNP), and expressed sequence tag (EST) markers.

Molecular markers are classified into two categories: type I are markers associated with genes of known function, while type II markers are associated with anonymous genomic segments. RFLP, allozyme and EST markers are type I markers because they were associated with known genes or transcripts of genes. RAPD, AFLP and Microsatellite markers are type II markers because these are amplified from anonymous genomic regions via the polymerase chain reaction (PCR). SNP markers are mostly type II markers unless they are developed from expressed sequences (eSNP or cSNP). Indels are becoming more widely used as markers since they often are discovered during genomic or transcriptomic sequencing projects; they can be either type I or type II markers depending on whether they are located in genes. In addition to their functions as markers in population studies, type I markers are becoming very important in studies of genetic linkage and QTL mapping. Type I markers have utility in studies of comparative genomics,

genome evolution, candidate gene identification etc. Type II markers are considered to be non-coding and therefore selectively neutral. Such markers have found widespread use in population genetic studies whose characterizations of genetic diversity and divergence within and among populations are based on assumptions of Hardy–Weinberg equilibrium and selective neutrality of the markers employed.

Protein Markers

Allozymes

Isozymes are functionally similar and separable forms of enzymes encoded by one or more loci. Isozyme products of different alleles at the same locus are termed as allozymes. They inherit co-dominantly so that genetic interpretation (genotype) of the phenotype is facilitated because all products are normally visible and not masked by dominance of one over another. Allozyme electrophoresis has been used in defining genetic markers for stock identification on the basis of differences in allelic frequencies between stocks in many species. Using allozyme markers, it is possible to determine whether a population is a random mating one with equilibrium genotypes frequencies or sample comprise of an assembly of genetically distinct units. Their allele frequencies primarily respond to mutation, gene flow and drift. One of the limitations of enzyme variants as genetic markers is the low level polymorphism observed in some species and populations. The extensive allozymes studies undertaken on fish stocks have not only proven valuable for estimating population divergence, but also have focussed attention on the underlying evolutionary forces that promote differentiation.

DNA markers

Based on the source of DNA, markers can be classified into (1) mitochondrial (2) nuclear and (3) chloroplast DNA markers.

Mitochondrial DNA (mt DNA)

The mitochondrial genome is a small and double stranded circular DNA molecule. It is haploid, maternally transmitted, non-recombinant and has

high mutation rate compared to nuclear DNA. These factors in combination reduce the effective population size for mt DNA to one fourth in comparison to single copy nuclear DNA and making it a powerful tool for elucidating population structures and estimating phylogenetic relationships.

The vertebrate mitochondrial genome is composed of about 15 to 20 kb in different organisms, coding for 40 genes responsible for 2 ribosomal RNAs, 22 transfer RNAs, and 13 proteins essential in respiration. It also has a non-coding region (+ 1000bp) responsible for replication, known as the "control region" or "d-loop", that evolves 4–5 times faster than the entire mtDNA molecule which itself evolves 5 to 10 times faster than nuclear DNA mainly because the mitochondria do not have repair enzymes for errors in the replication, nor for the damages of the DNA. Partial sequences of mitochondrial DNA genes especially 16S rRNA and Cytochrome c Oxidase I and Cytochrome b has proved suitable to resolve the phylogenetic relationships. There are some disadvantages also with this marker like; maternal inheritance does not provide information about males in populations, which may display different dispersal behavior to females and its haploid nature is also an issue as no inferences about the neutrality and equilibrium of populations, as well as other aspects based on allelic frequencies can be addressed.

Beyond discriminating closely related species, mt DNA has been used to explore intra-specific genetic polymorphism. Fast evolving ATPase and COI gene sequence information is useful in assessing population structure of the same species.

Nuclear DNA Markers

Random Amplified Polymorphic DNA (RAPD): The principle behind Randomly amplified polymorphic DNA (RAPD) analysis is that at low annealing temperatures or high magnesium concentrations, a primer is likely to find many sequences within the template DNA to which it can anneal. Depending on the length and complexity of genome of an organism, there can be numerous pairs of these sequences and they will be arranged inversely to and within about two kilobases of each

other. Considering this, PCR will amplify many random fragments that can vary in sizes when different species, subspecies, populations or individuals are analysed and this will constitute the basis of identification.

RAPD analysis has several advantages. These include relatively shorter time (1-2 days) required to complete analysis after standardization; no need of prior information on the genome of an organism; availability of series of primers for analysis; minimal operational cost requirement; relatively smaller amount ($\gg 20$ ng) of high molecular weight DNA; simpler protocol and involvement of non-invasive sampling for tissue analysis. However, the application and interpretation of RAPD – PCR in population genetics is not without technical problems and practical limitations. The main negative aspect of this technique is the necessity of extensive standardization to obtain reproducible results. In addition, most of the RAPD polymorphisms segregate as dominant markers and individuals carrying two copies of an allele cannot be distinguished from individuals carrying one copy of an allele. The limited sample size in each population and the specific RAPD primers utilized can also have an influence over the results. Even then RAPD technique is used in microbes, plants and animals for resolving taxonomic ambiguities and stock identification.

RAPD-PCR technique can also generate species-specific, sex-specific and population specific fragments. These fragments are useful in developing specific "Sequence Characterized Amplified Region (SCAR) Markers". For this, SCAR primers need to be synthesized from specific RAPD fragments. Usually, fragments above 1000 bp and less than 300 bp are not considered to develop SCAR markers owing to difficulties arising from co-migration and the lesser possibility of designing suitable primers from smaller fragments. The identified fragments are excised from the gel, purified and sequenced; and based on the sequence information, suitable SCAR primers are synthesized. These primers will amplify only specific fragments that are useful in settling taxonomic disputes and identifying sex or distinct populations. However, to identify specific RAPD fragments, screening of large number of samples and RAPD primers are required.

Microsatellites: Microsatellites—also referred to as short tandem repeats (STRs) or simple sequence repeats (SSRs), discovered in the early 1980s. Microsatellites are repeated DNA sequences having a unit length of 1-6 base pairs tandemly repeated minimum 6 times usually; maximum several times at each locus. Depending on the purity and complexity of the motifs, SSRs can be perfect (single motif in an uninterrupted array) or imperfect (single motif interrupted) or compound (two or more motifs in interrupted or uninterrupted arrays). Based on the number of base pairs in a repeat unit, microsatellites can be again classified into mono (e.g. C or A), di (e.g. CA), tri (e.g. CCA), tetra (e.g. GATA) repeat unit microsatellites. The most common ones are dinucleotide repeats.

Microsatellites represent ideal molecular markers because they have multiple alleles that with highly polymorphic among individuals and are highly abundant (once every 10 kbp in fish species) and dispersed evenly throughout eukaryotic genomes. Microsatellites are neutral and inherited in Mendelian fashion. High variability, ease and accuracy of assay make microsatellites the best for high-resolution population analysis. While genes offer functional sequences, microsatellites offer highly polymorphic sequences. Length and point mutations are the mutational events proposed to occur in microsatellites. Length mutations, *i.e.* copy (or repeat) number mutations lead to new allelic variants. Compared to other genomic markers, microsatellites are highly reproducible and easily transferable between laboratories. The main limitation of SSR markers is primer design for each new species demands considerable time, effort and cost. The approaches available to develop microsatellite markers are: 1) Survey of GenBank and EMBL databases 2) screening of genomic or cDNA libraries 3) use of primers already developed in related species 4) next-generation sequencing (NGS).

Microsatellites are also becoming increasingly popular in forensic identification of individuals, and determination of parentage and relatedness, genome mapping, gene flow and effective population size analysis. Microsatellites too have some negatives like appearance of stutter bands, presence of null alleles (existing alleles that are not observed using standard

assays); homoplasmy; and need for very high sample size if more alleles are noticed. Also, microsatellite flanking regions (MFRs) sometimes contain length mutations which may produce identical length variants that could compromise microsatellite population level studies.

EST-SSR: Expressed sequence tags (ESTs) are single-pass sequences generated from random sequencing of cDNA clones. ESTs are type I molecular markers with known functions which provide gene candidates for production traits and are important components in genome mapping projects. The vast EST data collections available from genomic libraries and random genomic sequences are a valuable source of gene-based SSR markers for population genetic analyses. EST-SSRs have been found to be significantly more transferable across taxonomic boundaries and less polymorphic than traditional 'anonymous' SSRs.

Single nucleotide polymorphism (SNP): Single nucleotide polymorphisms or SNPs (pronounced "snips") are DNA sequence variations that occur when a single nucleotide (A, T, C, or G) in the genome sequence is altered between individuals. SNPs are again becoming a focal point in molecular marker development since they are the most abundant polymorphism which can be used for population genetic studies. SNPs can occur in both coding (gene) and noncoding regions of the genome. Theoretically, a SNP within a locus can produce as many as four alleles, each containing one of four bases at the SNP site: A, T, C, and G. Practically, however, most SNPs are usually restricted to one of two alleles (most often either the two pyrimidines C/ T or the two purines A/G) and have been regarded as bi-allelic. Obviously, their PIC is not as high as multi-allele microsatellites, but this shortcoming is balanced by their great abundance. SNP markers are inherited as co-dominant markers. If SNPs change either the function of a gene or its expression, and the change provides greater fitness for a population (*i.e.*, a higher capacity to survive and/or reproduce in a given environment), the change will be favored by natural selection. Therefore, SNPs can be the basis of evolutionary change. The physical location of SNPs will be determined in a similar way to micro-satellite markers. SNPs will, at least initially, be selected based on how informative they might be as genetic markers. SNPs in low proportions (< 10 %

will be less informative than SNPs at higher frequency (30-50 %) in a given population.

Suggested readings

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Recombinant DNA technology and Molecular Cloning

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Introduction

The basis of most molecular biology technologies is the gene and to facilitate the study of genes, they have to be isolated and amplified. One method of isolation and amplification of a gene of interest is to clone the gene by inserting it into another DNA molecule that serves as a vehicle or vector that can be replicated in living cells. When these two DNAs of different origin are combined, the result is a recombinant DNA molecule. The recombinant DNA molecule is placed in a host cell, either prokaryotic or eukaryotic and the host cells are then replicated (producing a clone), and the vector with its foreign piece of DNA also replicates. The foreign DNA thus becomes amplified in number, and following its amplification can be purified for future applications.

Molecular cloning

The basic procedure of molecular cloning involves a series of steps. First, the DNA fragments to be cloned are generated by using PCR and restriction digestion. Second, the fragments produced by digestion with restriction enzymes are ligated to other DNA molecules that serve as vectors. Vectors can replicate autonomously (independent of host genome replication) in host cells and facilitate the manipulation of the newly created recombinant DNA molecule. Third, the recombinant DNA molecule is transferred to a host cell. Within this cell, the recombinant DNA molecule replicates, producing dozens of identical copies known as clones. As the host cells replicate, the recombinant DNA is passed on to all progeny cells, creating a population of identical cells, all carrying the cloned sequence.

Finally, the cloned DNA segments can be recovered from the host cell, purified, and utilized for various applications.

Sources of DNA for cloning

The cloning will work for any random piece of DNA. But since the goal of many cloning experiments is to obtain a sequence of DNA that directs the production of a specific protein, we need to first consider where to obtain such DNA. Sources of DNA for cloning into vectors may be DNA fragments representing a specific gene or portion of a gene, or may be sequences of the entire genome of an organism, depending on the end goal of the researcher. Typical “inserts” include genomic DNA, cDNA, polymerase chain reaction (PCR) products, and chemically synthesized oligonucleotides. When previously isolated clones are transferred into a different vector for other applications, this is called “subcloning.”

Vector DNA

Cloning vectors are carrier DNA molecules. Four important features of all cloning vectors are that they: (i) can independently replicate themselves and the foreign DNA segments they carry; (ii) contain a number of unique restriction endonuclease cleavage sites that are present only once in the vector; (iii) carry a selectable marker (usually in the form of antibiotic resistance genes or genes for enzymes missing in the host cell) to distinguish host cells that carry vectors from host cells that do not contain a vector; and (iv) are relatively easy to recover from the host cell. There are many possible choices of vector depending on the

purpose of cloning. The greatest variety of cloning vectors has been developed for use in the bacterial host *E. coli*. Thus, the first practical skill generally required by a molecular biologist is the ability to grow pure cultures of bacteria.

Choice of vector is dependent on insert size and application

The classic cloning vectors are plasmids, phages, and cosmids, which are limited to the size insert they can accommodate, taking up to 10, 20, and 45 kb, respectively. In some cases genes are often greater than 100 kb in size, so there were limitations in cloning complete gene sequences. To circumvent this new generation of artificial chromosome vectors like bacterial artificial chromosomes (BACs), yeast artificial chromosomes (YACs), and mammalian artificial chromosomes (MACs) where engineered more recently have problem by mimicking the properties of host cell chromosomes.

Plasmid DNA as a vector

Plasmids are naturally occurring extrachromosomal double-stranded circular DNA molecules that carry an origin of replication and replicate autonomously within bacterial cells. The plasmid vector pBR322, constructed in 1974, was one of the first genetically engineered plasmids to be used in recombinant DNA. These early vectors were often of low copy number, meaning that they replicate to yield only one or two copies in each cell. pUC18 is a derivative of pBR322. This is a "high copy number" plasmid (> 500 copies per bacterial cell).

Plasmid vectors are modified to contain a specific antibiotic resistance gene and a multiple cloning site (also called the polylinker region) which has a number of unique target sites for restriction endonucleases. Cutting the circular plasmid vector with one of these enzymes results in a single cut, creating a linear plasmid. A foreign DNA molecule, referred to as the "insert," cut with the same enzyme, can then be joined to the vector in a ligation reaction. Ligations of the insert to

vector are not 100% productive, because the two ends of a plasmid vector can be readily ligated together, which is called self-ligation. The degree of self-ligation can be reduced by treatment of the vector with the enzyme phosphatase, which removes the terminal 5'-phosphate. When the 5'-phosphate is removed from the plasmid it cannot be recircularized by ligase, since there is nothing with which to make a phosphodiester bond. But, if the vector is joined with a foreign insert, the 5'-phosphate is provided by the foreign DNA. Another strategy involves using two different restriction endonuclease cutting sites with noncomplementary sticky ends. This inhibits self-ligation and promotes annealing of the foreign DNA in the desired orientation within the vector.

Cutting and joining of DNA

Two major categories of enzymes are important tools in the isolation of DNA and the preparation of recombinant DNA: restriction endonucleases and DNA ligases. Restriction endonucleases recognize a specific, rather short, nucleotide sequence on a double-stranded DNA molecule, called a restriction site, and cleave the DNA at this recognition site or elsewhere, depending on the type of enzyme. DNA ligase joins two pieces of DNA by forming phosphodiester bonds.

Transformation: transfer of recombinant plasmid DNA to a bacterial host

The ligation reaction mixture with 'vector' and 'insert' DNA is introduced into bacterial cells in a process called transformation. The traditional method is to incubate the cells in a concentrated calcium salt solution to make their membranes leaky. The permeable "competent" cells are then mixed with DNA to allow entry of the DNA into the bacterial cell. Alternatively, a process called electroporation is also used that drives DNA into cells by a strong electric current.

Since bacterial species use a restriction-modification system to degrade foreign DNA lacking the

appropriate methylation pattern, including plasmids, the molecular biologists have circumvented this defense system by using mutant strains of bacteria, deficient for both restriction and modification, such as the common lab strain *E. coli* DH5 α .

Successfully transformed bacteria will carry recombinant plasmid DNA. Multiplication of the plasmid DNA occurs within each transformed bacterium. A single bacterial cell placed on a solid surface (agar plate) containing nutrients can multiply to form a visible colony made of millions of identical cells. As the host cell divides, the plasmid vectors are passed on to progeny, where they continue to replicate. Numerous cell divisions of a single transformed bacteria result in a clone of cells (visible as a bacterial colony) from a single parental cell. This step is where “cloning” got its name. The cloned DNA can then be isolated from the clone of bacterial cells.

Recombinant selection & Screening

The transformation process generates a mixed population of transformed and non-transformed host cells. As we are interested only in transformed host cells it becomes necessary to filter them out. This is exactly what is done in the selection process. There are many existing selection strategies and the strategy depends on the particular vector.

In most cases the vector carries a selectable marker gene for resistance to an antibiotic. If the plasmid vector is introduced into a plasmid free antibiotic sensitive bacterial cell, the cell becomes resistant to antibiotic. Non-transformed host cells contain no recombinant plasmid, therefore they will not be antibiotic-resistant, and their growth will be inhibited on agar containing antibiotic. Transformed bacterial cells may contain either

nonrecombinant (selfligated vector only) or recombinant (vector containing foreign DNA insert). Both types of transformed bacterial cells will be antibiotic resistant. To distinguish non-recombinant from recombinant transformants various screening techniques like blue-white screening, colony PCR, colony hybridization etc. can be used.

Amplification and purification of recombinant plasmid DNA

When a positive colony containing recombinant plasmid DNA is transferred aseptically to liquid growth medium, the cells will continue to multiply exponentially. Within a day or two, a culture containing trillions of identical cells can be harvested. The final step in molecular cloning is the recovery of the cloned DNA.

Plasmid DNA can be isolated by alkaline lysis method and further purified from crude cell lysates by chromatography using silica gel or anion exchange resins that preferentially bind nucleic acids under appropriate conditions and allow for the removal of proteins and polysaccharides. The purified plasmid DNA can then be eluted and recovered by ethanol precipitation in the presence of monovalent cations. Ethanol precipitation of plasmid DNA from aqueous solutions yields a clear pellet that can be easily dissolved in an appropriate buffered solution. Further screening can be done by restriction endonuclease digest to confirm the presence and orientation of the insert and by DNA sequencing.

Suggested readings

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Marine Microbiology

Fish Health Management

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Aquaculture is one of the fastest growing food producing sectors in the world and reduced growth in agriculture and animal husbandry sectors has shifted the focus of attention to aquaculture. As aquaculture production expands, diversifies and becomes more intensive, the risk and effects associated with disease outbreaks and pathogen spread are also enhanced. The growth, economic viability and sustainability of aquaculture primarily depend on the successful prevention/control of diseases. Unlike in land based farming systems, disease problems in aqua farming are complicated due to the multi-dimensional nature of culture system where dynamic interactions between hosts, pathogens and environment take place.

Mariculture – Indian scenario

The strength of Indian aquaculture lies in (a) large water bodies suitable for aquaculture, (b) tropical Climate, (c) species diversity and (d) availability of cheap labour. While the weakness includes (a) unregulated development, (b) lack of regulations (c) disease problems and (d) lack of scientific approaches. It is estimated that about 5 million tones of aquatic animal products can be produced annually through aquaculture, in India. Shrimp farming still dominates Indian aquaculture scene. The accumulated losses due to white spot syndrome virus (WSSV) alone in India, during the past decade is about Rs. 3000 crores. In this context, finfish and shellfish mariculture as an alternative for shrimp farming has gained importance. The research efforts by CMFRI in the development of mariculture technologies of the candidate species such as bivalves, swimming crabs, sand lobsters, finfishes and marine ornamentals have shown good success. Farming of the green mussel and edible oyster has become a popular livelihood activity by the self-

help groups along the costal belt of Kerala, and the farming area is growing every year.

Diseases

Disease is an abnormal condition characterized by a gradual degeneration of the animals' ability to maintain normal physiological state due to various factors adversely affecting its wellbeing. Absence of clinical signs or obvious diseases does not indicate that pathogens or possibilities of diseases do not exist. Domestication and intensive rearing practices always carry risks of enhanced incidence of infection and potential for disease. Creation of Intensive rearing systems aiming for higher production and profits, without proper planning and management, always create stress and pave way for infections and diseases.

One of the most important factors in dealing with the disease is INFORMATION. Knowledge is required on the potential disease causing pathogens, role of environmental factors, health status of the host, diagnostics and therapeutic options. The four K's essential for scientific aquaculture health management are:

- Knowledge about the disease process
- Knowledge about the pathogen
- Knowledge about the host and
- Knowledge about the environment.

Information forms the key element in deciding upon the best means of dealing with a disease or formulating a health management strategy. Hence the best approach in collecting the information should be proactive, rather than waiting for a disease outbreak to happen. One single piece of information, that the disease is caused by a viral pathogen and there is no cure, would save the farmer from spending large

amount of money for 'bogus' cure, and also from additional losses due to delayed harvesting.

Genesis of disease

Diseases can be caused by a variety of factors, the most important being pathogens. Other factors include stress, environmental/water quality, physical agents, nutritional imbalance, toxins etc. or a combination of these. Thus a 'disease condition' is actually a complex situation resulting from the interaction/modification of the primary disease condition by various biotic and abiotic factors.

Stress and disease development

Role of stress in predisposing fish/shrimp to infections is widely recognized and many of the routine aquaculture practices are known to induce stress. Stress is a non-specific response and involves a series of changes in the animal in trying to adapt to the changed situation. Adaptive responses of the animal if extended beyond the normal range, disturbs the normal functions, and these series of changes termed "stress response" tries to help the animal to restore normal homeostasis. This process has both advantages and disadvantages. During stress, in an effort to mobilize additional energy to regain the internal homeostasis, hypothalamus pituitary- inter-renal axis (HPI axis) gets stimulated leading to an increased output of stress hormones called corticosteroids. However, these stress hormones are basically immunosuppressive in nature and reduces the efficiency of both non-specific and specific immune system increasing the susceptibility to disease. Common husbandry practices like handling, netting, transportation and the normal features associated with intensive culture systems like suspended solids, low oxygen, high organic matter, overcrowding, high ammonia, etc. can elevate the level of corticosteroids in the blood. Similarly, many of the pollutants at very low levels can also stress the fish and make them relatively more susceptible to infection. Most of the stressors encountered in intensive culture systems are of chronic nature and can keep the level of corticosteroids above basal levels for longer duration.

Disease process

A pathogen can cause a clinical disease only when it

can establish on/in the host, proliferate, overcome the non-specific and/or specific defense barriers of the host, produce the pathogenic factors, cause cellular and tissue damage, produce significant pathological changes, impair the function of the target tissue/organ and cause mortality. The sequence of events in an acute infection is as follows.

- Contact with the pathogen
- Infiltration into the body
- Development / proliferation–incubation (usually short in fishes)
- Spreads throughout the body/accumulate in target organ
- Symptoms appear
- Mortality

In the case of chronic infections the pattern of development is

- Slow
- May or may not show pathology / symptoms
- Remain in the body and serve as reservoir / carrier

All infections need not result in disease manifestation. The sequence of disease development will depend on the nature of the pathogen, environmental factors, size of the host, pathogen load or intensity per unit area or unit weight of the host and their interactions. The complexity of the situation makes health management a difficult proposition.

Common pathogens encountered

Bacterial Diseases: Fish are susceptible to a wide variety of bacteria (mostly opportunistic) in the environment and many of these become pathogenic when fishes are physiologically unbalanced, nutritionally deficient, or in the presence of other stressors. Bacteria are known to cause infections / diseases in shellfish farming also.

Viral Diseases: Viruses are obligatory intracellular parasites requiring a living cell to replicate. Outcome of diseases due to virus infection is complex and depends on several factors including the immune status of individuals and infectious dose of virus. Mortality rates may vary and in some cases, the

pathogen remains at a low level of infection serving as reservoirs/carriers which are difficult to detect. Viral diseases have been a major cause of diseases and subsequent economic loss.

Fungal Diseases: Generally fungal diseases can be external or systemic and are difficult to cure. Except a few, they are generally considered less important pathogens of fishes.

Parasitic Diseases: Parasitic diseases in fishes range from extremely pathogenic ones to those, which are practically harmless. Many of the protozoan parasites are important pathogens of fishes while metazoan parasites except a few are generally less pathogenic in fishes.

Non-infectious Diseases: Feed-derived wastes also affect the culture environment through direct

Disease Diagnosis in Aquaculture

Diagnosis forms the first step in any disease control programme, which determines the ultimate success or failure of the programme. Once a disease is suspected, the next step is to draw a diagnostic procedure, to identify the root cause. The diagnostic procedure may include a single test or a combination of tests. In the case of routine pathogen watch or health monitoring, a set of selected diagnostic tests are performed to cover the potential pathogens. The approach generally followed is location specific and problem specific, where the first consideration is the availability of the diagnostic facility and expertise and there is no hard and fast method, which can be applied for all cases.

Table 1. Diagnostic methods

History	History of disease at facility or region, facility design, source of seed, type of feed used, environmental conditions etc.
Gross clinical signs	Lesions visible, behavior, abnormal growth, feeding or food conversion efficiency, etc.
Direct microscopy	Bright-field, phase contrast, or dark field examination of stained or unstained tissue smears, whole-mounts, etc. of diseased or abnormal specimens.
Histopathology	Routine histological or histochemical analysis of tissue sections
Electron microscopy	Ultrastructural examination of tissue sections, negatively stained virus preparations, or sample surfaces
Culture and bio chemical studies	Routine culture and isolation of bacterial isolates and identification using biochemical reactions
Enhancement	Rearing samples of the appropriate life stages under controlled conditions to enhance expression of latent or low level infections
Bioassay	Exposure to potential pathogens
Serological methods	Use of specific antibodies as diagnostic reagents in immunoblot, agglutination, ELISA, IFAT, or other tests.
Tissue culture	In vitro culture of pathogens in cell lines
DNA based Diagnostics	PCR, nested PCR, Multiplex, real time PCR

pollution, which in turn affects the culture organisms. Uneaten feeds, faeces and metabolic wastes contribute to nutrient and particulate loading of the water and substrate which in turn induce stress, reduce growth and increase their vulnerability to diseases. Improper diets can negatively influence the health by inducing nutrient deficiencies, imbalances or toxicoses. An impaired nutritional status contributes to defective host resistance. Malnourished fish may harbor latent infections, and certain physiological conditions and environmental stress may predispose them to infection.

Once the right diagnostic picture along with the water and soil parameters are available, control measures with respect to the causative factor(s) can be initiated.

Treatment

Treatment or therapy is intended to restore the normal health of the diseased or infected animal. Drugs can be given oral, intramuscular, intraperitoneal, intravenous or topically as baths or dips. Selection of the proper route depends on the environmental situation, the species and condition of the animal, and the drug

being delivered. Unlike the land-based animal rearing systems, where the diseased animals can be identified and treated individually, the scope for disease control in aquaculture through detection and treatment is very limited, mainly due to the co-existence of the pathogen and host in the aquatic rearing system. In the case of mariculture, where the extent of the water bodies are without boundaries, the scope of control over the host, pathogen and environment is all the more difficult than in the case of inshore/inland aquaculture.

Chemotherapy is not advised in culture systems and should be used only as a last resort since the use of antibiotics can lead to residues in tissues as well as development of antibiotic resistant microbes in the environment, which in turn can create other public health issues. The fish is constantly bathed in potential pathogens, *viz.*, parasites, bacteria, fungi and viruses. Separating the infected or diseased animals from the population and subjecting them to individual treatment regimes is impractical. Hence, disease treatment becomes a difficult proposition in aquaculture, and prevention remains the only natural choice and chemotherapy, if at all required, should be practiced judiciously and restricted to broodstock alone. Though vaccines can play an important role in controlling/preventing bacterial diseases, in many cases especially with viral pathogens, protection is found to be for short periods with variable results. In the absence of successful drug or vaccine, an integrated management approach to tackle the diseases with respect to animal, environment and pathogen using diagnostics as a functional tool is required.

Aquaculture Health Management

The management practices that are designed to prevent the occurrence of disease in a culture system are termed as AQUACULTURE HEALTH MANAGEMENT. This holistic approach using diagnostics along with farm management, can avoid the introduction of the pathogens into the system. The success of this approach mostly depends on the right choice and use of diagnostics along with other farm management measures, to keep both the animal and its environment in a healthy condition. Different components *viz.*,

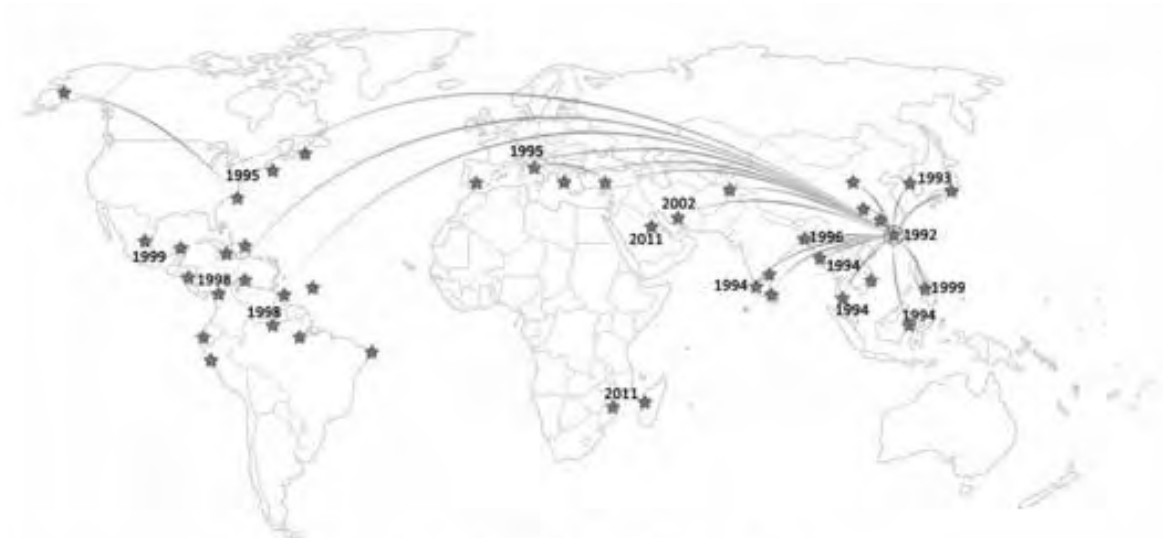
biosecurity, quarantine, screening of broodstock and larvae/fingerlings, Specific Pathogen Free (SPF) animals, pond and water quality management etc. are involved.

Translocation/introduction of aquatic animals has been frequently identified as an event that precedes major outbreaks of new/emerging diseases in a region or species. The commercial/ economic reasons for species introductions in aquaculture include (a) cost-efficient species in terms of production costs to output revenues, (b) high growth potential, (c) resistance to environmental stressors and pathogens, (d) good market opportunities, (e) pre-existing knowledge of rearing methodologies/ technologies etc. The potential sources of introduction include live fish, eggs, larvae, contaminated water, wrappings or packaging etc. Factors like pathogenicity, host-pathogen interactions, vectors, climatic conditions, susceptibility and resistance of the hosts etc. play an important role in deciding/modifying the outcome of pathogen introductions. Open aquatic farming systems favouring easy dispersal of the pathogens along with their ability for long-term survival outside the host further complicates the issue.

WSSV – an example

Outbreaks of WSSV, the most virulent virus known to affect cultured shrimps were first reported in *Penaeus japonicus* in Taiwan and China in 1992. In 1993, it has spread to other species of shrimp and resulted in outbreaks in Japan and Korea. In 1994, it was reported from Thailand, India and Malaysia and by 1996, has spread over the entire Asian continent. In 1995, it was also reported in the USA, entered the central and South Americas in 1998 and Mexico in 1999. Entered Europe during 1995-2001, Iran in 2002 and Saudi Arabia and Mozambique in 2011 (WAHID, 2012). Currently, WSSV is known to be present in all shrimp-growing regions except Australia. The practice of moving grossly normal brood stock and post larvae (PL) freely amongst countries was probably the most rapid and effective means of its spread throughout Asia (Flegel, 2006).

Similarly, Furunculosis in European trout, Whirling disease in US, Crayfish Plague in Europe, viral nervous necrosis (VNN) in marine fish, and many molluscan diseases are typical examples. Epizootic Ulcerative



Spread of WSSV during 1992 to 2011

Syndrome (EUS) epidemic caused by the fungus, *Aphanomyces invadans* in Asian freshwater and estuarine fishes has spread throughout Asia, Australia and has even reached the African continent.

Emerging diseases

Emerging disease problems, particularly in developing countries, are often slow to be recognized. The recent outbreaks of Koi herpes virus (KHV), in the neighbouring South-East Asian countries is a cause of worry for India. Methods for detecting, reporting and responding much more quickly to such emerging diseases should be developed. The design and implementation of effective disease surveillance programs, early warning and reporting systems and contingency plans for dealing with serious disease outbreaks will help in reducing the social, economic and biological impacts of disease.

Health management

Fish health management primarily constitutes two aspects, the farm health management and the fish health management. Successful integration of these two aspects only can deliver a disease free environment. Farm health management constitutes the maintenance of (a) good soil quality (b) good water quality (c) good farm productivity (d) feed management and (e) maintenance of proper farm quarantine to prevent horizontal transmission of disease causing

pathogens. Fish health management deals with (a) proper animal quarantine b) screening of Broodstock and larvae/ fingerlings and (c) crop health monitoring and pathogen watch. Effective implementation of all the above three aspects of fish health management depends entirely on the early and accurate diagnosis of the disease causing agents.

Applying quarantine and biosecurity principles helps to prevent the entry of disease causing pathogens and thereby avoid serious problems, mainly related to infectious diseases. Bio-security can be defined as a set of standard scientific measures, adopted to exclude pathogens from the country (national level bio-security) and from culture environment and host (farm level biosecurity) and, more broadly, to limit pathogen establishment and spread. Biosecurity can be more easily implemented in small, intensive, and controlled farming systems than in outdoor and large-scale operations. Biosecurity principles serve as the cornerstone for implementing the NACA and OIE guidelines for aquatic animal health management. Quarantine helps in the (a) evaluation of the health condition of the new fish (b) reduction of disease transmission risk to pre-existing fish (c) gradual acclimatization of the new fish and (d) convenient administration of drugs. Avoidance or at least minimizing the introduction of known infectious pathogens is also important. Preventative treatments ("prophylactic" treatments) can be helpful in removing initial loads of external parasites.

Aquatic animal health Management at national level

Legislation has an important role in enhancing responses to aquatic animal health emergencies. It should enable and guide those involved in fish health related activities and should clearly define the duties of various authorities involved at the national, provincial and district levels and promote effective coordination, power-sharing and communication between all those involved. Each country should develop a national strategy/plan which includes short, medium and long-term action plans, with the following components in place

- Competent Authority
- Legislative support
- National advisory committee
- National list of diseases
- National surveillance system
- Disease reporting
- Emergency preparedness and contingency planning
- Quarantine and health certification
- Import risk analysis
- Zoning

The plan should be based on the various guidance tools (Standards and agreements like OIE Aquatic Code, relevant technical guidelines, SOPs and BMPs) and implementation tools (risk analysis, diagnostics, quarantine & health certification, surveillance & disease reporting, contingency plans and disease control strategies. Strong national coordination, good leadership, involvement of stakeholders and appropriate monitoring and review systems are essential for its successful implementation (Subasinghe & Bondad-Reantaso 2006).

Regional co-operation

Many countries in a region can share common social,

economic, industrial, environmental, biological and geographical characteristics, and in this situation a regionally adopted health management programme is considered a practical approach. An Asia-Pacific Regional Strategy better known as "Asia Regional Technical Guidelines on Health Management for the Responsible Movement of Live Aquatic Animals" has been developed through an FAO/NACA initiative involving the participation and agreement of 21 regional countries (FAO/NACA. 2001; Subasinghe and Bondad-Reantaso, 2008).

Conclusion

The key elements of an ideal health management system can be summarized as:

- Control over the fish/animal stocks at hatchery / farm levels
- Identify excludable disease/pathogens of concern
- Prophylaxis /Vaccination
- Diagnostics for the detection of pathogens of concern
- Adequate environmental control to prevent the introduction of pathogens of concern (specific pathogen free stock)
- Biosecurity & Quarantine
- Routine management/husbandry practices to ensure pathogen exclusion (sterilization of influent water, pathogen free feed, prevention of pathogen transfer through men, material and vectors)
- Disinfection, treatment and pathogen eradication methods to contain and eradicate disease outbreaks due to pathogens of concern

Thus in mariculture, development of species specific and location specific health management models with broader management approach for the control of farm/cage level environmental deterioration, pathogens (Virus, bacteria, parasites and fungi) introduction and disease outbreaks is imperative to ensure the sustainability and economic viability.

Livestock Disease Surveillance

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Every country in the world has some sort of animal disease surveillance system. Surveillance is needed to understand the health status of the animals in the country, so that problems can be identified and actions can be taken. However, different countries have very different surveillance needs and surveillance capabilities: a wealthy country with few diseases that depends on exports of animals and animal products will have sophisticated surveillance systems to protect trade. A poor country with uncontrolled land borders with multiple other countries that have regular outbreaks of epidemic diseases will be unable to maintain sophisticated surveillance systems and will aim primarily at minimizing the impact of major animal diseases (Cameron, 2012). Thus, world trade in animals and animal products has expanded dramatically over the last decade and will continue to increase. Political changes in the world and world trade agreements opened new markets for a wide variety of livestock products. However, the increased

potential for international trade places additional constraints on both importing and exporting countries with regard to disease surveillance and the monitoring of animal health.

The four major purposes of surveillance are

1. Demonstration of freedom from disease
2. Early detection of disease
3. Measuring the quantum of disease
4. Finding cases of disease.

The underlying principle is that surveillance is an essential, potentially complex health management tool.

Disease monitoring and surveillance

Disease surveillance and monitoring are often used interchangeably; however, many definitions are

Table 1 Definitions of disease monitoring and surveillance (adapted from Christensen J. 2001)

Textbooks	Monitoring	Surveillance
Martin <i>et al.</i> 1987 (page 259)	Animal disease monitoring describes the ongoing efforts directed at assessing the health and disease status of a given population	The term "disease surveillance" is used to describe a more active system and implies that some form of directed action will be taken if the data indicate a disease level above a certain threshold.
Thrusfield, 1995 (page 22)	Monitoring is the making of routine observations on health, productivity and environmental factors and the recording and transmission of these observations.	Surveillance is a more intensive form of data recording than monitoring.
(page 358 and 360)	The routine collection of information on disease, productivity, and other characteristics possibly related to them in a population.	An intensive form of monitoring (q.v.), designed so that action can be taken to improve the health status of a population, and therefore frequently used in disease control campaigns.
Noordhuizen <i>et al.</i> , 1997 (page 379)	Monitoring refers to a continuous, dynamic process of collecting data about health and disease and their determinants in a given animal population over a defined time period (descriptive epidemiology).	Surveillance refers to a specific extension of monitoring where obtained information is utilised and measures are taken if certain threshold values related to disease status have been passed. It, therefore, is part of disease control programmes.

provided by various authors as shown at Table 1.

The World Organization for Animal Health, OIE (Office International des Epizooties) defines surveillance as “Means the continuous investigation of a given population to detect the occurrence of disease for control purposes, which may involve testing of a part of the population, while Monitoring constitutes on-going programmes directed at the detection of changes in the prevalence of disease in a given population and in its environment”. In other word, It is an observational method based on continuous recording to follow health status or risk factors in a defined population, and particularly to detect the appearance of pathological processes and study their development over time and in space, with a view to adopting appropriate control measures. To simplify further, Surveillance implies anxiously waiting for a disease that we hope will never appear.

The Centers for Disease Control and Prevention (CDC) USA, has promoted the following definition.

The epidemiological surveillance is the ongoing systematic collection, analysis, and interpretation of outcome using the specific data essential to the planning, implementation and evaluation of public health practice, closely integrated with the timely dissemination of these data to those who need to know.

Objectives of Surveillance (Doherr and Audige 2001, and Dufour and Hendrikx, 2009)

- To detect the appearance of an exotic disease in a given region, so as to enable its early control;
- To determine the true importance of a disease (incidence, prevalence, economic losses, etc.) and follow the course of the situation, so that a decision as to appropriate control (or not) can be taken.
- To enable the establishment action priorities.
- To evaluate results of control programmes by monitoring decline of the disease.
- Monitoring of risk factors and other information related to monitoring and surveillance activities.
- Meeting a strategic goal of most veterinary regulatory agencies

To attain the above mentioned objectives surveillance has three requirements viz.,

1. **Early warning** :To improve the awareness and knowledge of the distribution of disease or infection and which might permit forecasting the further evolution of an outbreak.
2. **Early reaction**: rapid and effective containment of, and leading to, the elimination of a disease outbreak, thus preventing it from turning into a serious epidemic.
3. **Coordination**: For global eradication of an identified animal disease such as transboundary disease.

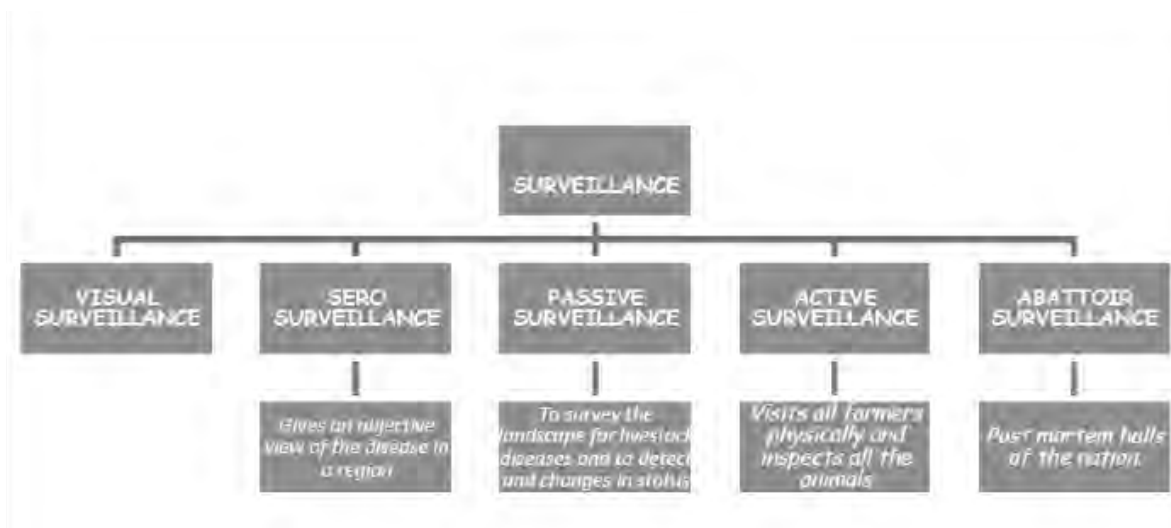


Fig. 1. Types of Surveillances

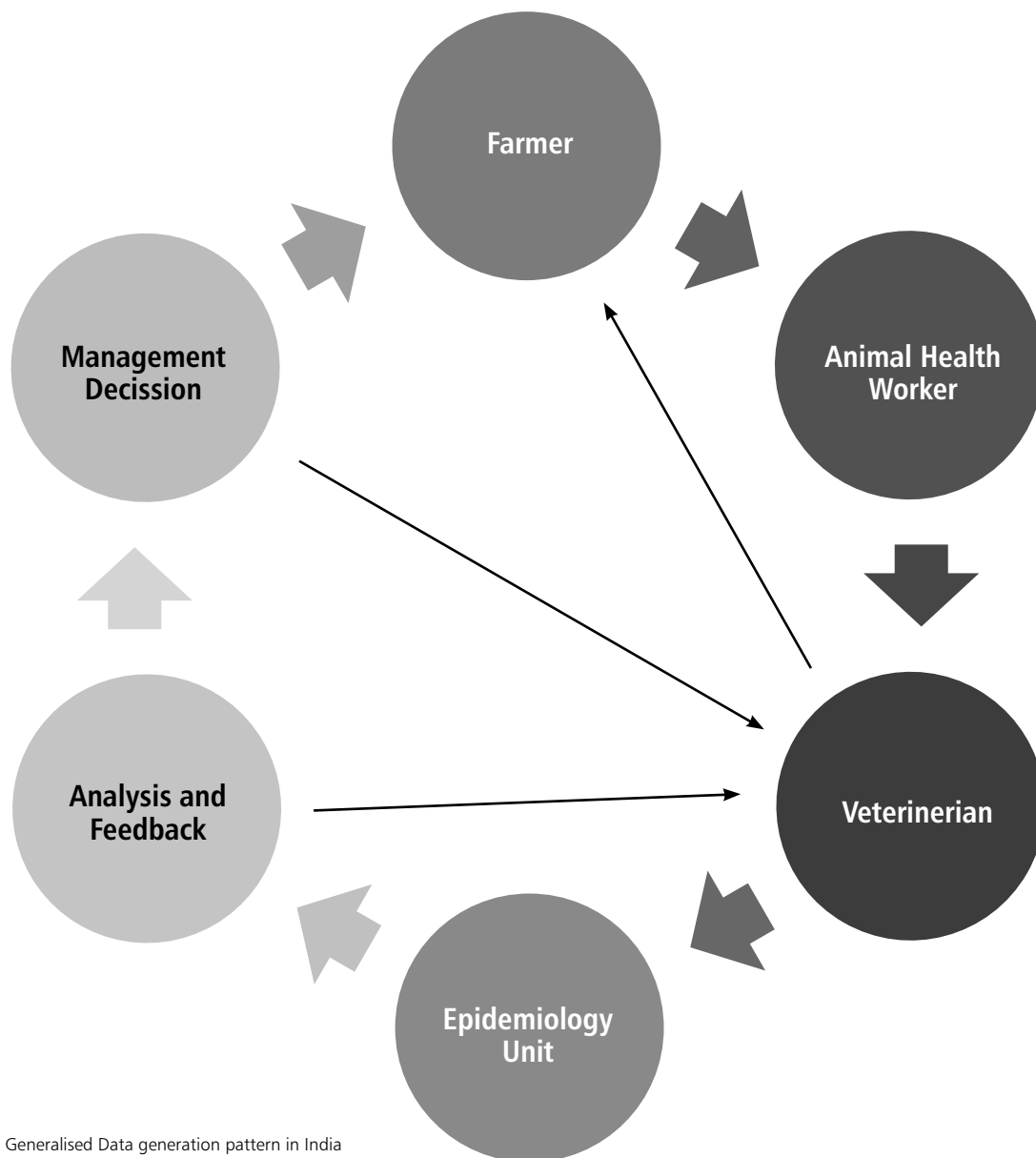


Fig. 2. Generalised Data generation pattern in India

Surveillance is a manpower intensive activity and substantial cost is involved. Large number of personnel is required to gather data, compile and collate, analyse the data, and inform the authority for appropriate activities. Thus, early warning or information helps in early reaction. These together improve the control/ eradication programmes by better coordination. Hence, surveillance activities pose a challenge of amalgamating and coordinating various agencies involved to carry out one activity.

Based on the type of surveillance activity carried out, surveillance can be broadly classified as in Fig.1.

Surveillance programs may be developed at a number of different levels viz.,

Farm or village level: which include monitoring of economically significant variables to the individual.

Region or state: this may involve testing to establish freedom from particular diseases which may give individuals collective financial advantages over competitors, such as brucellosis freedom, or FMD free areas,

National: such programs are usually very costly if active. This is specially required for prioritizing the

livestock diseases. This knowledge will in turn help in assessing the vaccine requirements, formulating control / eradication strategies and the knowledge of economic impact the disease causes. This is carried out involving multiple agencies, both governmental and non-governmental. A few of them may be taken up to assess the Transboundary animal diseases (TAD) together by neighbouring countries.

The data for surveillance programs may be generated by a number of methods which include clinical evaluations, laboratory reports, slaughter inspection data, screening tests, or owner reports.(Fig. 2)

Types of Surveillance

Passive surveillance

This is the routine surveillance conducted to understand the extent of livestock diseases and detect the changes in status over a specified area. The area could be region or country. Passive surveillance is an important key element in early warning of a disease. The word "Passive" entail characterization of technique and not a sign of lowered importance of the work. Passive surveillance through a disease reporting system is the main method of collecting information on livestock diseases currently used in most countries. Passive disease surveillance is collection of data on disease incidents from sources from farmers, field veterinary officers, based on the clinical samples submitted to laboratories and their results. Thus, Passive surveillance is a system wherein the reports gathered from various sources are collected and compiled.

The disadvantages of passive surveillance are that they are not able to provide information on the total amount of disease, cannot provide representative information on the level of disease in the population, or the geographical pattern of disease. Passive disease reports are not reliable enough to be used to calculate rates or proportions. They will not necessarily inform about the absence of the disease in a country. The results of passive surveillance cannot be used to demonstrate the disease status to trading partners.

The advantages of passive surveillance include

identification of most common diseases in the country, locations from where they are reported frequently and species of livestock involved.

Active surveillance

Active surveillance is defined as any activity that is frequent, intensive and aims at establishing the presence or absence of a specific disease. (FAO: Manual of livestock disease surveillance and information systems). In active surveillance, usually the veterinary authorities make active efforts to collect the information needed. Since the information is collected by technical / trained personnel, the information will be of appropriate quality.

The data can be used for calculations of incidence / prevalence rates and proportions.

The advantages of active surveillance are that it identifies high risk pockets and their epidemiological assessment, close integration of field activity with laboratory investigations, trade benefits for commercial livestock industry, establishment of rapid communication systems, associated risk factors and socio-economic data. Active surveillance strengthen the passive surveillance programme like targeted serological surveys.

The disadvantages are cost involved, time consuming and requires trained manpower.

Serological surveillance

Serosurveillance is an important component of any comprehensive surveillance system for vaccine preventable diseases. It is the gold standard for measuring immunity in a population, thereby complementing traditional disease surveillance methods. Serological surveys should be carefully designed to yield statistically valid information on the disease status of animal populations. Serosurveillance is essential to declare a country free from a particular disease. The advantage of this method is that large number of samples can be screened at a time. The major disadvantage is that it needs large resources from collection of sera to its despatch to the diagnostic laboratory. The sterility of the sample is to be maintained so also the cold

chain. It is not possible to differentiate the vaccinal antibodies to antibodies developed due to infection unless sophisticated tests are employed. This may lead to misinterpretation.

Following types of surveillance are less used or used for a limited objective.

Early warning surveillance (epidemiological watch, epidemiovigilance)

Surveillance of health indicators and diseases in defined populations in order to increase the likelihood of timely detection of undefined (new) or unexpected (exotic or reemerging) threats. These are surveillance systems which aim to achieve the early detection of these threats.

Indicator-based surveillance

Traditional communicable disease surveillance which relies on the collection of data about the occurrence of pre-defined diseases or conditions using agreed case definitions; these data are analysed to produce indicators that point towards the existence of a threat.

Indicator-based surveillance may be hazard-specific or general and includes the use of clinical or other data for syndromic surveillance.

Hazard-specific surveillance

Surveillance that is focused on one or more pre-defined hazards (disease, condition, biological, chemical or physical agent, or event) often using specific diagnostic tests (e.g. molecular diagnostic methods).

Syndromic surveillance

Surveillance that uses health-related information (clinical signs or other data) that may precede or substitute for formal diagnosis; this information may be used to indicate a sufficient probability of a change in the health of the population to deserve further investigation or to enable a timely assessment of the impact of health threats which may require action. This type of surveillance is not usually focused on a particular hazard and can be used to detect a variety of diseases or pathogens including new (emerging) diseases so is particularly applicable for early warning surveillance.

Event-based (media-based) surveillance

Surveillance that complements indicator based surveillance by continuously scanning the Internet and other communication media to detect certain information that may lead to the recognition of emerging threats. It uses unstructured data which then need to be studied and verified and which cannot be summarized as an indicator.

Sentinel Surveillance

A sentinel is one who stands guard to warn when something happens. Sentinel herds act as indicators for the rest of the population to warn that disease is present. In this type of surveillance, certain herds are maintained as sentinel herds and are observed for any sign, either clinical or serological, for the presence of disease(s) that are known to be absent in that particular country/ locality. If the herds show any sign then it is considered as the disease is being introduced in the country/ locality.

Aggregation Points (Abattoirs, Markets, Watering Points, Dip Tanks)

Abattoir surveillance and surveillance at other aggregation points is commonly used as a form of either active or passive surveillance. This section deals primarily with abattoir surveillance, as animal inspection is normally carried out for public health reasons, providing potentially valuable passive animal disease surveillance data. Other animal aggregation points such as markets or dip tanks act as convenient and practical sites for active surveillance. Instead of conducting a representative survey, examination and specimen collection can be done at the one location. This makes the surveillance faster and less expensive, but the population under surveillance is no longer completely representative of the overall population. Any possible biases must be taken into consideration when interpreting the results.

Abattoir surveillance

The primary advantages of abattoir surveillance are that it

- is inexpensive. Animals are being processed and inspected for other purposes, so the costs are primarily only related to data capture and any

- laboratory tests performed
- is able to cover a very large number of animals
- allows easy collection of diagnostic specimens, such as blood or tissue samples, for laboratory testing
- provides a relatively constant supply of surveillance data
- enables data to be collected from a relatively small number of abattoirs locations, which slaughter animals from a large number of farms or villages (thereby decreasing the data collection costs).

Syndromic Surveillance

Various forms of syndromic surveillance have been used for many years. However recent interest from the field of human surveillance has led to a great deal of interest and research in the area.

A syndrome is defined as a collection of signs that indicate the presence of a disease. Syndromic surveillance is therefore concerned not with the detection and reporting of disease, but of the signs and groups of signs that are associated with disease. These signs may be clinical signs (such as fever, lameness, diarrhoea), or less traditional signs. For instance, a decrease in the feed consumption at the pen level in a piggery may be considered as a sign of disease; an increase in antibiotic feed additive sales from a supplier may be another.

Syndromic surveillance involves the identification of specific signs or groups of signs, and analysis of the patterns of these signs, in space and time. The purpose is not to diagnose a specific disease, but to detect abnormal patterns of signs that may be due to one of a large number of diseases. When an abnormal pattern is detected, a disease investigation follows, in order to diagnose the actual cause of the disease.

Why do we need Surveillance?

The main reasons for requirement of disease surveillance in any country are as follows

1. Early detection or early warning of a disease, either endemic, or emerging or exotic. It is more

- of necessity in the case of emerging and exotic.
2. To prioritize the diseases for control/ eradication for policy decisions and to take up research projects
3. To declare a region or nation free from disease
4. To ascertain the baseline level of any disease in an country
5. To describe any changes in the health of livestock population
6. To understand any threat to the health of livestock population which may lead to change in the population structure
7. To estimate the economic losses due to diseases and also the gain in applying the control measures. To study the cost effectiveness of the control / eradication programme

Surveillance will help the policy makers to formulate strategies for the following:

- Management of outbreaks
- Informing trade
- Prioritisation
- Informing control

Disease surveillance: Indian Scenario

Currently in India, the main method of collecting information on livestock diseases is through a passive disease reporting system. When an animal is noticed sick, the owner may contact the veterinary authorities, who may then either submits a disease report, or sends a specimen to a diagnostic laboratory. As such, in this system, the information is not collected about all cases or all the diseases, and the populations at risk are not recorded. Hence, this system of passive monitoring is being practiced because the veterinary services in the country have the primary objective of providing free treatment to the ailing animals.

Reportedly only the occurrence of 10-20 percent of actual outbreaks is only recorded. These reports collected and compiled by the animal husbandry department of the state governments are further get compiled at the national level by Department of Animal Husbandry Dairying and Fisheries Ministry of Agriculture, Government of India, which publishes these as Animal disease surveillance Bulletins.

Although not comprehensive, these reports/bulletins never the less provide the following information based on clinical diagnosis.

- Which of the diseases are present in the country, and where located
- Provides information to respond to the disease outbreaks
- Meet the basic disease reporting requirements of OIE

Finally, for any disease monitoring and surveillance programme to be successful, availability of good diagnostics is a prerequisite. Choice of diagnostic test depends on the disease, its prevalence, no of samples to be screened purpose of survey etc. Currently, arrays of diagnostic tests starting from conventional tests to molecular one are being used for disease surveillance and monitoring.

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Disease Diagnostic Techniques in Aquaculture

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Introduction

Aquaculture, the fastest growing food producing sector in the world and also a significant source of livelihood to millions of people world over, is constrained by the limitations imposed by several infectious diseases. Aquaculture in India, which is almost synonymous with shrimp culture, has been witnessing rampant disease outbreaks which led to catastrophic mortalities. Since the early Nineties, Indian shrimp aquaculture has been plagued by various diseases, among which white spot disease (WSD) caused by white spot syndrome virus (WSSV) is the most dreaded one. The disease continues to cause heavy mortality resulting in huge economic loss to the farmers and valuable foreign exchange for the country. According to Stentiford (2011), WSSV has become an endemic barrier to production in all known production zones through its global distribution with traded crustaceans. According to Lightner *et al.*, 2012, global production loss due to WSD was predicted to be \$ 8 bn. However, they have stated that the actual losses could be as high as \$ 15 bn. The example of WSSV shows how impactful a disease could be in aquaculture. Recognising the negative impact of diseases, disease diagnosis in aquaculture and their prevention and control or in other words aquatic animal health management have become the central theme of aquaculture research for many years. Besides developing vaccines, therapeutics and other strategies to manage diseases, developing and applying various rapid and sensitive diagnostic techniques to aquatic animal pathogens have been the corner stone of health management. This article describes the evolution of the diagnostic procedures in aquaculture with

emphasis on molecular diagnostics, especially PCR-based diagnosis.

Disease Diagnosis in Aquaculture

Control and prevention of diseases in aquaculture is a function of management, and diagnosis of diseases forms the fundamental step in the health management programme. To diagnose a disease is to recognise the occurrence of an abnormality and to identify the disease causing agent. In aquaculture, failure in accurately diagnosing diseases would lead not only to large-scale mortality but also to indiscriminate use of drugs and chemicals thereby causing environmental contamination, drug residual effect and often occurrence of drug-resistant pathogens. Therefore, accuracy/specificity forms one of the fundamental factors in disease diagnosis. It is quite obvious from the literature on aquatic animal diseases that diagnosis of aquatic animal pathogens has evolved over a period of time and has advanced from microscopic characterisation and morphological description of pathogens to molecular characterisation and probe-based diagnosis.

Traditional diagnostic methods for pathogens such as viruses, parasites, bacteria and fungi include microscopic characterisation and morphological description using light microscopy, histopathology and electron microscopy. In the traditional diagnostic methods, history and gross signs of the disease, examination of wet mount preparations and tissue impression smears also play important roles. Histopathology is an important diagnostic tool

for tentative as well as confirmatory diagnosis of most of the infections in aquatic animals. Besides, histological technique is an essential component of diagnostic protocols for infectious diseases using gene probes and immunohistochemistry. Although histopathology supported by parasitological, bacteriological and virological methods is a widely used proven technology, the major disadvantages of the technique are that it requires high level of expertise and is often time-consuming. Besides, the technique is not very specific and cannot be employed if the pathogen is present in very low number. As a next level, however, antibody-based diagnosis, both polyclonal and monoclonal, on different formats such as ELISA, dot-blot and lateral flow chromatographic assay, is being widely used. Several rapid methods have been developed for the detection of pathogens in fish, shellfish, molluscs and their environment though immuno- and molecular diagnostics (Adams *et al.*, 2008).

As the diagnostic techniques evolved over the years, application of modern biotechnology found a prominent place in characterising many pathogens at molecular level and developing molecular probes for the detection of many pathogens. Thus, an array of rapid and sensitive genomic probes using non-radioactive labels which could be used either in a dot-blot or *in situ* hybridization format are available for various aquatic animal pathogens. Simultaneously, the development of highly sensitive DNA amplification method based on polymerase chain reaction (PCR) has resulted in tremendous improvement in the diagnosis of diseases in aquaculture. These tests are finding increasing application in routine screening of broodstock as well as larval stages, epidemiological studies, development of specific-pathogen-free stock and many other areas of aquaculture. However, although various PCR tests have been used and can provide sensitive and accurate diagnosis, risk of misdiagnosis (false positive and false negative) is considerable in these tests. As an improved version of the conventional PCR, over the years, real-time PCR is increasingly being used in pathogen detection and quantification. Real-time PCR has the advantage of being simple, sensitive, highly reproducible and amenable to high throughput screening. The test also facilitate in accurately quantifying infection level.

Consequently, real-time PCR assays employing either SYBR-green or Taqman probe fluorescence detection system have been developed to detect and quantify several aquatic animal pathogens.

Antibody-based Diagnostic Techniques

As mentioned earlier, conventional diagnostic methods rely solely on the microscopic examination and its visual recognition of pathogens, which requires a great deal of experience with the organisms that often change so dramatically in their morphology during the course of their development. Microscopic examination of gross pathology is also quite challenging as many pathogens can produce similar pathology. Therefore, in the evolution of disease diagnostic procedures in aquaculture, antibody-based (protein-based) immunodiagnosis plays a significant role. This method has the advantage over other traditional methods in that it can detect sub-clinical/latent/carrier state of infection and can also discriminate the antigenic differences. This technique is relatively rapid and more specific and sensitive. Further refinement of conventional immunodiagnostic techniques has resulted in the development of monoclonal antibody-based techniques and this has increased the accuracy of detection and has allowed studying the pathogenesis of diseases (Bartholomew, 1989). Nevertheless, the specificity of antibodies also limits their usefulness because major antigens are not conserved among life-stages of certain pathogens (Bartholomew *et al.*, 1995). Many routine immunodiagnostic tools have been developed for a variety of pathogens infecting fish and shellfish such as agglutination (slide/latex); fluorescent antibody test (FAT/IFAT); immunohistochemistry (IHC); enzyme linked immunosorbent assay (ELISA); and dot-blot (dot-blot/dip-stick/western blot) (Adams 1999; Adams 2004; Adams *et al.*, 1995, Mialhe *et al.*, 1995). Further, immunochromatography/lateral flow method was reported to have great scope in the detection of aquatic animal pathogens (Adams and Thompson, 2006), and accordingly many tests have been reported (Pantoja and Lightner, 2001; Flegel, 2006; Sithigorngul *et al.*, 2006, 2007, 2011; Chaivisuthangkura *et al.*, 2013). Further, monoclonal-antibody-based assays have been developed for

various aquatic animal pathogens, especially shrimp viruses (Sithigorngul *et al.*, 2000, 2002; Poulos *et al.*, 2001; Liu *et al.*, 2002; Chaivisuthangkura *et al.*, 2010; Siriwattanarat *et al.*, 2013; Chaivisuthangkura *et al.*, 2014). However, certain inherent limitations of protein-based diagnostics prompted the researchers to look for more exquisitely sensitive and specific detection tools.

Molecular Diagnosis

Molecular diagnosis works on the fundamental understanding that every properly classified species of organism has some unique molecular sequences/signatures that distinguish it from other species. And, each organism's genetic composition is essentially a finger-print that can be used for its identification (Tenover, 1988). Molecular diagnostic procedures have been available since the 1970's, when researchers first began using cloned DNA probes to detect viral nucleic acids. However, Mosely *et al.* (1980) first used DNA probes for enterotoxigenic *E. coli*. Since then, large number of DNA probes to detect various pathogens have been developed.

Nucleic acid probes are fragments of DNA labeled in some fashion that can seek out and bind with high specificity to stretches of DNA or RNA that have complementary sequences (hybridization reaction). These probes may be either RNA strands directed toward DNA targets or labeled DNA sequences directed towards RNA targets. Initially, molecular diagnostic methods were not widely accepted because these tests used radioisotope detection methods. Radioisotope detection methods have been employed for detection of aquatic animal pathogens (Bruce *et al.*, 1993), however, they are more expensive and labour-intensive when compared to traditional antibody detection methods. However, in recent years non-radioactive probes have become increasingly popular and with high specificity probes labeled with markers such as biotin and digoxigenin are available. These probes have advantages over the radioactive-labeled probes in that they are safer and have longer shelf-lives. Nucleic acid hybridization reaction consists of four components; the probe, the target DNA/RNA (in the sample), the reporter molecule (the label on the probe) and the hybridisation method. Hybridisation can be performed

in solutions or on solid support (dot-blot) or even on sections of tissues fixed on slide (*in-situ* hybridisation). *In-situ* hybridisation has the advantage in that non-specific tissue effects which may result in false-positive diagnosis in dot-blot assay can be distinguished from specific histological lesions (Lightner, 1996). Many reports are available on the *in-situ* hybridation probe-based detection of viral pathogens of shrimp (Wongteerasupaya *et al.*, 1996; Tang and Lightner, 1999; Pantoja and Lightner, 2001; Soowannayan *et al.*, 2003; Flegel, 2006). Although *in situ* hybridization assays have advantages in that it can detect the pathogens as well as it can be used to demonstrate the tissue tropism and sequential progression of infection. However, the major disadvantage of the ISH is its low sensitivity compared to PCR.

Polymerase Chain Reaction (PCR)

In order to refine the sensitivity of molecular diagnostic tests to detect the pathogens in very low numbers it was imperative to find out an amplification method. Accordingly, Saiki *et al.*, (1985, 1988) and Mullis and Faloona (1987) developed a simple method for making multiple copies of a DNA sequence. PCR is a technique of *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. In other words, it is a simple rapid and sensitive method for selectively amplifying defined sequences/regions of DNA/RNA from an initial complex source of nucleic acid. Copying of a specific genetic target is accomplished in a 3 step reaction: (i) Denaturing the target DNA at elevated temperature (90-95 °C) (ii) Cooling the reaction to promote annealing of oligonucleotide primers that are complementary to either strand of target DNA (ii) Extending the bound primers by DNA polymerase action resulting in the replication of target sequence. The reaction is repeated in successive cycles of heating and cooling, referred to as thermal cycles. Each cycle doubles the input target sequence. As the newly synthesized DNA copies can also serve as template in subsequent replication, after a series of cycles an exponential amplification can be achieved. This final PCR product can be characterised in many ways and the most common is to determine the size of the amplified fragments using gel electrophoresis.

PCR diagnosis is the most widely used diagnostic tool in aquatic animal pathogen detection. It forms one of the crucial components of screening of shrimp broodstock and larvae for viral pathogens. Several PCR-based assays have been reported for a variety of shrimp viruses (Wongteerasupaya *et al.*, 1997; Belcher and Young, 1998; Cowley *et al.*, 2004; Lo *et al.*, 1996b; Pantoja and Lightner, 2000; Anonymous, 2014; Nunan *et al.*, 1998; Sritunyalucksana *et al.*, 2006).

PCR Methodologies

There are a variety of procedural variations of a standard PCR available, and these are developed for specific purposes. However, the present article discusses only about a few such methodologies, which are commonly employed in the detection of shrimp viruses. These are nested and semi-nested PCR, multiplex PCR and reverse transcriptase PCR or RT-PCR.

Nested and semi-nested PCR

This is based on employing an additional set of primers to increase the efficiency of the standard one-step PCR. After the initial amplification of the target sequence, an aliquot of the primary PCR product is subjected to a second round of amplification using another set of primers designed to amplify an internal part of the amplified product. Primary product can also be amplified using a semi-nested format in which one of the primers of primary PCR and a second primer complementary to an internal region are used. Both these methods give additional specificity and increased sensitivity to the PCR and accordingly many PCR tests have been reported for the sensitive detection of shrimp viruses (Lo *et al.*, 1996; Kimura *et al.*, 1996; Kiatpathomchai *et al.*, 2001).

Multiplex PCR

Multiplex PCR is employed in simultaneously amplifying more than one segment of target DNA in the same reaction. This is achieved by designing primers, which can amplify different regions of the same template DNA (a particular virus) or primers that can amplify two entirely different DNA templates (two distinct viruses). However, to amplify, the primers should be designed in such a way that their T_m should be similar

and the intended amplified product may be of similar length. If there is a significant difference between the amplicon lengths, there is a possibility that shorter product will have more amplified products at the expense of the longer product. Multiplex PCR assays have been developed for the simultaneous detection of many shrimp viruses (Tsai *et al.*, 2002; Xie *et al.*, 2007; Khawsak *et al.*, 2008; Sibonga *et al.*, 2013).

Reverse transcriptase PCR or RT-PCR

If the target sequence to be detected is RNA such as RNA viruses of shrimp (YHV, GAV, MoV, TSV), the conventional PCR step would precede a reverse transcription (RT) step by which RNA is enzymatically converted to complementary DNA (cDNA). An oligo deoxynucleotide primer hybridizes to mRNA and is extended by an RNA-dependent DNA polymerase. The newly synthesized single-stranded cDNA can be amplified using specific primers in a conventional PCR. The RT-step can be carried out using either an oligo (dT) primer or random hexamer or a gene-specific primer. The choice of primer depends on the specific application. Reports on the RT-PCR detection of RNA viruses of shrimp include Wongteerasupaya *et al.*, 1997; Nunan *et al.*, 1998; Cowley *et al.*, 2004, 2005).

Advantages and disadvantages of PCR

In comparison, PCR has the advantage over other methods that use nucleic acid probes. PCR is more sensitive in that on an average less than 10 target molecules are sufficient to get a positive result, whereas in the case of most of the probes, sensitivity is approximately 10^4 to 10^5 molecules of the target. Presently, PCR methods are available for a variety of pathogens. Apart from the sensitivity and rapidity of diagnosis, principal advantage of molecular diagnostic methods is in the detection of non-culturable agents. Further, DNA amplification can assist in detecting the pathogens that are present in low numbers and also in handling tiny volume of specimen. It can also be used to detect latent infection and thereby identifying the reservoir hosts of infection that is significant in epizootiology, besides it can be used to differentiate antigenically similar pathogens.

These methods are cost-intensive procedures. The methods do not provide an accurate quantitative assessment of infection level. Further, the test will not give any indication of whether the pathogen present in the hosts is replicating/live or dead. Therefore, the carrier status of the host and the viability of the pathogen cannot be assessed by this method. Since the molecular tools are based on specific genetic sequence, any change in the genome of the pathogen through mutation might render the assays non-functional. As PCR is a very sensitive tool, it is prone to contamination. The contamination which occurs during PCR processing will lead to false-positive results. PCR method may also lead to false negative results due to either a wrong selection of tissue for nucleic acid extraction or faulty processing leading to low/no quantity or low quality of nucleic acid or due to low level of pathogens present in the host. Like any other molecular methods, PCR also will have difficulty in detecting new pathogens as the exclusive use of the technique would overlook such infections.

Loop Mediated Isothermal Amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method that amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions (Natomi *et al.*, 2000). When combined with reverse transcription, this method can also amplify RNA sequences with high efficiency. The method relies on auto-cycling strand displacement DNA synthesis using a DNA polymerase with a high strand displacement activity and a set of four specially designed primers. These four primers, termed as inner and outer primers, recognise six distinct sequences of the target DNA, which improves the specificity of the reaction. The reaction is carried out at isothermal condition, as the denaturation of strands takes place by strand displacement. LAMP technique has been employed in the detection of many shrimp viruses including RT-LAMP for RNA viruses (Kono *et al.*, 2004; Mekata *et al.*, 2006; He *et al.*, 2010; Nicolasora *et al.*, 2014).

In the initial stages of LAMP reaction, all the four primers are involved, however, only the inner primers

are used in the later cycling reaction for strand displacement DNA synthesis. The LAMP reaction is initiated by an inner primer containing sequences of sense and anti-sense strands of the target DNA. This is followed by the release of a single-stranded DNA through the priming by an outer primer. This single-stranded DNA will serve as a template for DNA synthesis primed by the second inner and outer primers that can hybridize at the other end of the target. This process will result in the formation of a stem-loop DNA structure. In the subsequent step of LAMP cycling, one inner primer will hybridise to the loop on the product and initiate strand displacement DNA synthesis which will result in the original stem-loop DNA and a new stem-loop. Cycling continues for a period of approximately 1 h and results in the accumulation of 10^{10} copies of the target. The final products of the reaction are stem-loop DNA with several inverted repeats of the target and cauliflower-like structures with multiple loops.

End products of LAMP reaction can be visualised through several methods. The most common method of visualization is by agarose gel electrophoresis. Similar to PCR detection, agarose gel is stained with intercalating dyes such as ethidium bromide or SYBR Green I. On the agarose gel, LAMP product will appear as smear at the top and bands at the base of the gel, as the end products of LAMP consist of stem-loop DNA and cauliflower-like structures with multiple loops of various lengths. Since, one of the characteristics of the LAMP reaction is its ability to synthesise extremely large amount of DNA, addition of intercalating dye, SYBR Green I, into the reaction tube itself would help in visualising the product under a UV- transilluminator (Natomi *et al.*, 2000). This method is useful in the field-level application. Another method is also based on the accumulation of large amount of pyrophosphate ion byproduct of the reaction, which will yield white precipitate of magnesium pyrophosphate in the reaction mixture. Hence, detection of presence or absence of white precipitate will provide an easy distinction of whether the target DNA is amplified during the reaction. Further, since the increase in turbidity of the reaction mixture according to the production of the precipitate, correlates to the

amount of target DNA synthesized, a colorimetric estimation of the turbidity in real-time is also being used as an efficient method of visualizing the amplified product.

Advantages and disadvantages of LAMP

LAMP amplifies the target DNA under isothermal amplification with high efficiency (end product generated is 10^{10} compared to 10^7 produced in a PCR reaction); The detection limit of LAMP is comparable to PCR; No significant influence of the co-presence of non-target DNA; LAMP allows simple, easy and selective detection; LAMP is highly specific for the target sequence, as it employs four primers targeting multiple sequences; LAMP is simple and easy to perform, as it requires (after appropriate primers are prepared) only a regular laboratory water bath or heat block for the reaction; By incorporating reverse transcription, LAMP can be used for amplifying RNA as well. LAMP reaction is vulnerable to contamination because amplification of the target DNA is very high at the final stage. Further, multiplexing of the reaction is not possible with LAMP.

Real-time PCR

Molecular diagnosis, especially PCR-based diagnosis has made tremendous impact in the field of aquatic animal health management. However, PCR has advanced from the endpoint detection of conventional PCR to the detection of PCR amplification during the early phases (exponential phase) of the reaction and following the entire amplification in real-time. Development of robust and high throughput quantification technique, based on real-time PCR, which detects even a single copy of the genome, has been reported for many aquatic viruses. And, measuring the kinetics of the reaction in the early phases of PCR provides a clear advantage over the conventional PCR. There are mainly two different chemistries involved in the real-time assay; SYBR green (A highly specific double-stranded DNA minor groove-binding dye which can detect all double-stranded DNA, not single-stranded and primers) and TaqMan probe-based assays (A fluorogenic 5' exonuclease assay). Quantification of pathogen by real-time PCR

is based on the theory that there is a quantitative relationship between the input target DNA and the amount of PCR product generated at any given cycle number. Real-time PCR detects the accumulation of amplicon at every cycle and the data are then measured at the exponential phase of the PCR.

Advantages and disadvantages of real-time PCR

Real-time PCR technology has enormous advantages in the detection and quantification of pathogens owing to its increased sensitivity, reproducibility, and quantitative accuracy, apart from the decreased hands-on-time and less chances of contamination. The major advantage of this procedure is that it gives quantitative data on starting copy number and has the sensitivity limit of single copy of the genome. Further, it is amenable to high throughput and turnaround. In the diagnostic format, the greatest advantage of real-time PCR is that as there is no post-PCR manipulation involved in the visualisation of the result, cross-contamination and false-positive result can be minimised. The assay is the key tool used in gene expression studies. In short, real-time PCR, due to its increased sensitivity, reproducibility, and quantitative accuracy, apart from the decreased hands-on-time and less chances of contamination, is a very important tool in the health management in aquaculture. Accordingly, real-time PCR assays have been developed and applied for detection and quantification of large number of shrimp viruses (Dhar *et al.*, 2001, 2002; Tang and Lightner, 2002, 2004; Jang *et al.*, 2009; Yan *et al.*, 2010; Yadav *et al.*, 2015).

Conclusion

Diagnostic techniques used in aquatic animal pathogen detection have advanced tremendously over the last three decades. Molecular methods such as conventional PCR, dot-blot hybridisation, *in situ* hybridisation, LAMP and real-time PCR have now been developed for a wide range of pathogens. Further, these techniques have been adopted and widely used routinely in hatchery, farms and large number diagnostic laboratories because of their exquisite sensitivity and specificity. New advanced variants of the technologies are also being reported regularly. However, PCR has been considered as the

gold standard and it is the key diagnostic tool widely employed today.

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Diseases in Fish Hatcheries

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Introduction

Sustainable aquaculture production can only occur when fish are healthy and free from disease. Fish disease management is a combination of preventing the onset of disease and measures to reduce losses from disease when it occurs. Fish cultured in hatcheries or floating cages become particularly susceptible to disease when various environmental parameters such as temperature, salinity, dissolved oxygen and suspended particles fluctuate suddenly or widely, or following rough, although often unavoidable, handling operation. Once conditions suitable for pathological changes develop, progress to disease in the warm water environment is rapid. Early detection of behavioral changes and clinical signs in the cultured fish are critical for proper diagnosis of the disease.

Disease rarely results from simple contact between the fish and a potential pathogen. Environmental problems, such as poor water quality, or other stressors often contribute to the outbreak of disease. The cobia, *Rachycentron canadum*, is distributed worldwide in tropical and subtropical water. Cobia, Pompano and Grouper culture offers great possibilities in aquaculture because of its fast growth rate and commercial interest. In India, the first sea cage farming trial with hatchery produced fingerlings of cobia was carried out during 2010, and the first success in breeding and seed production of silver pompano was achieved during in 2011 at the Regional Centre of Central Marine Fisheries Research Institute (CMFRI), Mandapam, Tamil Nadu.

Types of Fish Diseases

There are two broad categories of disease that affect

fish, infectious and non-infectious diseases. Infectious diseases are caused by pathogenic organisms present in the environment or carried by other fish. In contrast, non-infectious diseases are caused by environmental problems, nutritional deficiencies, or genetic anomalies; they are not contagious and usually cannot be cured by medications.

Non-infectious diseases:

Non-infectious diseases can be broadly categorized as environmental, nutritional, or genetic.

- A hygienic fish culture environment is essential to the health and productivity of farming operations. The reasons for this include:
- Disease risks are increased in poor and polluted environments.
- Quality of the product depends on clean and healthy environments.

Infectious diseases

- Infectious diseases are broadly categorized as parasitic, bacterial, viral, or fungal diseases.

Predisposing Factors

- Fish stocks living under stressful conditions become less able to defend against a pathogen and hence will become sick more readily. Fish that are well cared for generally do not become sick even in the presence of a pathogen. The most common error in fish husbandry is overstocking. This leads to problems such as:
- Fish to fish aggression
- Increased fish and feed wastes

- Ease of disease spread,
- Increased concentration of pathogens
- Resultant poor water quality
- High fish density, stress, and ease of transmission increase susceptibility of the fish population to diseases and parasites.
- In marine hatcheries diseases present in wild fish can infect cultured fish and spread rapidly through the population.

Stress

Fish in husbandry are exposed to a multitude of

Common Disease and Disorders Encountered in the Hatchery

i) Diseases of Cobia (*Rachycentron canadum*)

S.No	Bacterial disease	Causative organism
1	Pasteurellosis	<i>Photobacterium damsella</i> sub sp <i>piscicida</i>
2	Streptococcosis	<i>S. iniae</i>
3	Vibriosis	<i>V. anguillarum</i> , <i>V. alginolyticus</i>
4	Mycobacterium infection	MY. Sp, <i>Aeromonas hydrophila</i>
5	Viral disease Lymphocystis	Irido virus
6	Amyloodiniosis	<i>Amyloodinium ocellatum</i>

ii) Common Diseases of Pompano (*Trachinotus blochii*)

S.No	Disease	Causative agent
1	White spot disease	Ciliate protozoan, Cryptocaryon irritans
2	Cardiac myxosporidiosis	Myxosporidian protozoan, <i>Henneguya</i> sp
3	Monogenetic trematode infestation	Bicotylophora trachinoti- gills Benedenia sp- body
4	Parasitic dermatitis (infestation)	Sea lice (<i>Calligus elongatus</i>)
5	Amyloodiniosis	<i>Amyloodinium ocellatum</i>

iii) Common Diseases of Ornamental fishes

S.No	Disease	Causative agent
1	Red pest, Fin Rot	Gram negative bacteria
2	Fish tuberculosis	<i>Mycobacterium</i> sps
3	External Gas Bubble disease	Commonly caused by excess gas in the system, brought about by super-saturation of gas in high pressure water mains
4	Amyloodiniosis	<i>Amyloodinium ocellatum</i>

stressors. Among the important stress-inducing factors are those with strong psychological components that cause fright, excitement and discomfort. Stress can be induced by such activities as handling, transport and weighing. Moreover, crowding at high densities also produces a variety of stimuli that cause stress. Outbreaks of diseases are associated with depressed oxygen levels. Predisposing risk factors include also overcrowding, organic pollution and hypoxia.

Common Disease in Hatchery

Fish bacterial infections can occur as a bacteremia, which implies the presence of bacterial organisms in the bloodstream without clinical signs. Others occur as a septicemia, which indicates that bacteria and toxins are actually present in the circulatory system and usually precipitate disease and clinical signs. Inflammation, hemorrhage and necrosis are clinical signs associated with septicemia

i) Vibriosis

Vibriosis, a disease caused by numerous species of vibrio, is a primary disease of marine fish in salt & brackish waters and creates huge economic loss in the mariculture industry, affects large number of fish and shellfish species, both cultured and feral. *Vibrio alginolyticus* is a Gram-negative facultative anaerobic bacterium, which was formerly regarded as an opportunistic pathogen causing vibriosis in marine fish and shellfish. Vibriosis characterized mainly by the hemorrhage erosion of gill lamellae, fluid accumulation in the perinoteal cavity and hemorrhagic septicemia. The clinical signs were skin discoloration, red necrotic lesions in the abdominal muscles erythema at the base of the fins, vent and in the mouth, abdominal distention, and exophthalmia.

Treatment

- Oxytetracycline @ 100mg/kg biomass/day USDA approved drug in aquaculture.
- Agrimin(Virbac, Mumbai) forte as mineral mixture and immune modulator filled in the empty capsules and given along with the feed.

Common Parasitic Diseases in Hatchery

i) Amyloodiniosis

Amyloodiniosis is otherwise called as the Marine Velvet disease. The dinoflagellate *Amyloodinium ocellatum* is one of the most important pathogenic ectoparasite affecting the cultured marine and brackish water fish, causing *Amyloodiniosis*. There are different stages in the life cycle of *Amyloodinium*. Trophont, Tomont, and Dinospores are the 3 stages in the life of *Amyloodinium*. In the Trophont stage it feeds as a parasite. It attaches itself to the fish with the help of rhizoids and feeds on the host. As it grows it comes to the next stage of life called Tomont at which its size will be around 350 micrometers. At this stage it disengages from its host and starts reproduction. Reproduction is by repeatedly dividing itself until there are 256 offsprings. To complete this stage it needs around 3 days after which it hatches tiny dinospores and these dinospores start infecting the host for about 15 days after which they go to the next stage in their lifecycle.

Amyloodiniosis was recorded in Pompano brood stock, Cobia fingerlings and some ornamental fishes. The pathogen was identified as *Amyloodinium ocellatum*. The infective stage of dinospores was located in gill surfaces. The infection has finally resulted in acute mortality of the fishes.

Treatment

- Only the free-swimming dinoflagellate form of the organism (the dinospore) is susceptible to treatment. The encysted form is not susceptible to any treatment.
- Fresh water dip for 2-3m. This fresh water dip will remove the *Amyloodinium* from the fishes which are in the stage of dinospores. You need several fresh water dip treatment to remove other stages of *Amyloodinium*.
- The most common treatment is the use of copper in water. Water that has free copper at the level of 0.2 mg/l is used in treating fish that are affected by *Amyloodinium*. Ionic copper level of between 0.15 and 0.2 parts per million for a minimum of 14 days

- Formalin dip 200ppm for 10-15 min
- Removing the fish to a separate tank and allowing the tank to run fish-free for a month is probably necessary. This will allow the organism to run through its life cycle and die out due to the lack of a host.

ii) Caligus infestation

Caligus (Müller, 1785) is the largest genus of parasitic copepods, containing more than 250 species. Sea lice are marine ectoparasites that feed on the mucus, epidermal tissue and blood of host marine fish. The affected fishes showed frequent surfacing and anorexia. Sea lice caused physical and enzymatic damage at the sites of attachment and resulting in abrasion like lesions that varying in their sizes about 1mm to 3mm in dia. The skin surface also showed moderate to severe ulcers measuring that measuring 2mm to 4mm in dia.

Treatment

- Fresh water dipping, RO water dipping, formalin dip treatment and decreasing salinity leads to detachment of the parasite.
- Ulcerated skin treated with Povidone Iodine ointment or solution as antiseptic to enhance the healing process.

Fish Health Management

Fish health management is a term used in aquaculture to describe management practices which are designed to prevent fish disease. Successful fish health management begins with prevention of disease rather than treatment. Prevention of fish disease is accomplished through good water quality management, nutrition, and sanitation. The hatchery management practices that help prevent the introduction and spread of disease. Fish diseases caused by parasites, bacteria or viruses can be spread from tank to tank or from hatchery to hatchery by the transfer of infected fish and by animals, people, equipment and water contaminated by contact with infected fish or fish pathogens. To prevent the introduction of new diseases onto a fish hatchery, there should be no

contact between the fish on the farm/cage and any potential disease carriers.

Hatchery equipment should be cleaned and disinfected before each use. Workers should disinfect clothing, boots and other gear before having contact with healthy fish. Sanitation is particularly important in preventing the spread of disease between tanks or hatchery when sick fish are present.

Potential sources of disease agents:

- Fish – carrier or diseased, wild or farmed;
- Equipment – nets, boats, diving gear, and feeding materials

Disinfection of equipment

Well operated fish hatchery requires a tank or facility to permit regular disinfection of equipment such as dipnets, siphons, etc. Tanks also require regular disinfection to avoid disease outbreaks. Chlorine can be used to disinfect tanks and equipment, formalin can also be used and mentioned, ultra violet light sterilizers are required to kill bacteria and micro organisms that could affect the fish.

Aims of disinfection:

- Prevent disease incursions in biosecurity programs
- Routine hygiene measures to reduce build-up of disease agents on hatchery
- Eradicate disease agents from outbreaks

Quarantine

Quarantine is must for the newly arrived or wild brood stock fishes as well as the fishes shifting from cage to hatchery. Wild fishes may act as the carrier for the many of the viral, bacterial and parasitic diseases. Salinity reduction and adaptation of the fishes to the moderate salinity or even fresh water dipping may dislodge the protozoa or ectoparasites from the skin or mucous. Along with the freshwater dip some USFDA approved disinfectant like povidone iodine, formaldehyde also can be used as disinfectant.

No fish or water should be allowed to escape

from quarantine facilities. The equipment used in quarantine facilities should not be moved to non-quarantined areas until it has been disinfected. A quarantine period must last until the fish are exposed to the full range of seasonal water temperatures at which any disease of concern can be detected. If possible, new fish should be quarantined in the isolated place as far as practical from the rest of the hatchery.

Equipment

For the best results in killing pathogens, you must clean, disinfect and dry equipment before it is used elsewhere on or off the hatchery. This is especially critical for equipment that has been used to handle, harvest or transport sick fish. Equipment must be thoroughly scrubbed clean with a brush and detergent and then rinsed to remove any dirt and detergent residue. Then an appropriate disinfectant should be applied and left on the equipment long enough to kill disease organisms. Rinsing after disinfection ensures that no residues are left behind. Drying equipment in the sun will destroy bacteria or viruses that may have survived.

Table 2. Methods of cleaning and disinfecting

S.No	Item	Method of cleaning
1	Plastic buckets, Rubber boots, wet suits	Scrub clean with detergent, Povidone iodine 100ppm solution
2	Netting (Dip nets)	clean with detergent, 100 to 150 ppm Povidone iodine, Rinse and dry 50ppm formalin- Rinse and dry
3	Transport tanks	Scrub and clean with iodine 200 to 215ppm. Rinse and dry
4	Tank bottom, water	Drain out all water. Remove all fish and vegetations. Add hydrated lime(.....)
5	Infected fish	Remove the dead fish. Use USFDA approved drug Oxytetracyclin @ 7-10mg/kg .Body biomass. Used in the feed with binder.

Feed storage

A system of feed management needs to be put in place to ensure feeding of older feed stocks first, to minimise wastage or feeding of expired feeds. Fish feeds generally contain a high percentage of unsaturated fatty acids (up to 40%) which may become rancid (oxidised) with storage or exposure to high temperature. Feed may also become mouldy from

storage in high humidity. Rancid fats or mouldy feed can cause disease in fish, and even if not contributing to overt clinical disease, it can affect growth. The high protein content feed able to ferment or develop toxin if not storing in the ideal temperature. The store place should keep it dry without moisture content. Feeding should be done in the early morning at the rate of 5 to 8% of the body weight, twice in a day.

The feed return should take immediately once after the fish feed as maximum as. The high content of the protein content of the feed leads to formation of invisible.

Treatment

A good practice is to maintain only those chemicals that do have specific approval for aquaculture uses at the production facility. The presence of non-approved chemicals at an aquaculture facility may imply their use to an inspector even if they are never used. Regulations concerning approved chemicals for use in aquaculture are continuously being updated.

All chemicals have precautions and considerations associated with their use. If an aquaculturist has no experience with a particular chemical, a small group of fish should be treated first, as a test before the entire lot is treated, to avoid potentially heavy losses due to toxicity associated with overtreatment. Extreme caution should be practiced when applying any chemical treatment. Indiscriminate use of antibiotics leads to drug resistance and loss of immunity to the cultured fishes

- **Terramycin** is an antibiotic used to treat systemic (internal) bacterial infections. It is approved by the U. S. Food and Drug Administration (FDA) for the treatment of sensitive bacteria of the genera *Aeromonas*, *Pseudomonas*, *vibriosis*, and *Hemophilus* in cobia Pompano and Grouper. It is used as a feed additive at a rate of 2.5 grams of drug (active ingredient)/100 pounds of fish weight/day for 5 days. A 21-day withdrawal period is required before the fish may be slaughtered and used for human consumption.
- **Copper Sulfate** (CuSO_4) is used to treat a variety of external parasites of fish. It is also an effective and

approved algicide, and can kill fish if used improperly. The relationship between toxicity of copper sulfate and alkalinity is very important (alkalinity is the total concentration of alkaline substances in the water expressed as equivalent calcium carbonate). In water with an alkalinity less than 50 milligrams per liter (mg/L), copper sulphate can be very toxic to fish and should not be used unless a bioassay has been run in the water first with a limited number of the fish to be treated. Since copper sulfate is an algicide, consideration must be given to dissolved oxygen in a tank to be treated. If a tank already has low dissolved oxygen, an alternate treatment should be used. Copper sulfate will only aggravate low dissolved oxygen problems by killing the primary source of oxygen (the algae) and by adding a large biological oxygen demand in the form of dead and decomposing algae.

- **Formalin** is approved for use in the treatment of several external parasites. It is commonly used as an indefinite pond treatment at 15 milligrams per liter (mg/L). Formalin will remove 1 mg/L dissolved oxygen for every 5 mg/L of formalin used as a treatment. Therefore, if dissolved oxygen in a pond is low, aeration must be provided or a different treatment should be used. Formalin must be stored at temperatures above 40° F because it will form very toxic paraformaldehyde at low temperatures.
- **Potassium Permanganate** (KMnO_4) is approved for use in Aquaculture as an oxidizer and detoxifier. It has been used effectively against a number of external disease organisms of fish. The normal treatment is 2-8 milligrams per liter (mg/L). Ideally, one would like to maintain a "wine red" color in the water for a 12 hour period to ensure an effective treatment. A preliminary test can be performed with a small volume of culture water to determine the appropriate dose for the system.

Farm hygiene is vital to maintaining fish health.

It involves routine activities carried out by the farmer to ensure the following:

- Cleaning of utensils and equipment used to handle

or feed fish.

- Water quality testing and correction of poor water quality includes the following:
- Measure dissolved oxygen and water
- Chemistry values e.g. salinity, temperature, pH, ammonia, nitrite and nitrates.
- Measure bacterial counts e.g. *Vibrio* spp. counts of the water
- Aeration to maintain dissolved oxygen
- Cleaning of the farm seabed and fallowing or rotation of sites Minimising organic pollution from fish wastes and feed wastes.

Preventive measures

- Preventing the introduction of pathogens
- Maintenance of good water quality
- Avoidance or reduction of environmental stressors
- Adequate nutrition
- Isolation of cultured animals from feral stocks
- Immunization

Three steps to solve a disease problem

- Determining that a problem exists.
- Identifying the cause of the disease or source of the distress
- Successfully curing the fish and eliminating the disease or cause of distress.

Summary

Arriving at an accurate and correct diagnosis has wide implications on disease management. Parasites have different life cycles, bacteria have different susceptibility to drugs and depending on the impact of these disease agents on the fish tissues, different management strategies may be necessary.

Immune incompetency can be age related or in fish after periods of stressful events, e.g. transport, handling, temperature extremes;

Chronic exposure to seemingly benign environment stress or infectious agents can deplete the fish immune system;

Culture conditions can cause disease agents to build up, e.g. high stocking density, low water exchange rates, increased total bacterial loads; and

Feeding regime can cause organic wastes build-up in culture system, e.g. tank/pond bottoms, sea-cage bottoms

While some of the factors that predispose to disease cannot be controlled, the disease may be managed by altering management.

Disease may be precipitated by stressful events. Understanding effects of disease on fish hosts helps with treatment & control.

Vaccination may be necessary in preventing diseases predisposed by unavoidable management procedures such as transport, handling and grading. Studies have shown that high parasite loads can lead to vaccination failures. Vaccination programs must be carried out with management of other potential pathogens.

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Autopsy Procedure in Fish

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Introduction

An autopsy can be defined as an examination of a body of the fish after death in order to determine the cause of death or the changes produced by the disease. Examination of an ailing fish before death due to disease is termed as necropsy. The necropsy would help the fish pathologists to determine the causes of morbidity and mortality in fish. It may be noted that unlike terrestrial animals, majority of disease of fish are related closely to water quality (including water source, ammonia levels, nitrite, nitrate, pH, temperature, dissolved oxygen, hardness, alkalinity, salinity and presence of heavy metals and other toxins) and management issues (history of water exchanges, cleaning, filtration backwash, etc, disinfection, and fish quarantine before introduction of new stocks, feeding with live feeds, trash fish, compound feed, feed storage). Hence, an accurate information pertaining to water quality and husbandry practices are critical prior to necropsy.

Materials required for autopsy examination of fish:

1. Compound microscope, dissecting microscope and magnifying lamp
2. Latex gloves
3. Scissors (blunt tips and with pointed tips)
4. Scalpel with blade
5. Rat toothed forceps
6. Autopsy tray or Petri-dish (in case of juveniles)
7. Microscopic slides with cover glasses
8. Syringe with hypodermic needle
9. Culture swabs for microbiology
10. Microbiological media (Tryptose soya broth with 2% NaCl)
11. Sterile loops
12. Ten percent neutral buffered formalin for histopathology

Autopsy procedure:

1. For fish necropsy, an ailing fish or fish which is just dead is advisable because decomposition after death results in anatomical changes not due to disease. Before the autopsy, fish should be examined for the presence of behavioural abnormalities like flashing, spiral swimming, finrot, etc. and external abnormalities like presence of body ulcers, frayed fins, discoloration of the body, abscesses, abrasions, fungus on the body surface, excess mucus on gills and body surface, protozoan or metazoan parasites, malformations, exophthalmia, spinal deformities, etc.
2. When external lesions like ulcers or abrasions are present, inoculations should be made into suitable media supplemented with sodium chloride, wherever necessary.
3. Peripheral blood smear should be made by collecting a drop of blood on a microscopic slide by cutting the caudal peduncle. The blood smear may be stained with suitable stains like any of the Romanowsky stains which are neutral stains consisting of a mixture of oxidized methylene blue and Eosin Y.
4. In order to conduct the post mortem examinations, fish should be placed on their right side. By using a scalpel blade, skin scrapings and gill impression wet mounts can be taken on a microscopic slide and examined under the microscope after placing few drops of PBS for the presence of parasites. Gill impressions should be examined immediately since bronchial epithelium gets deteriorated due to post mortem changes which may lead to post mortem artefact.
5. The body surface of the fish may be disinfected by pouring 70% ethanol. The tools used for autopsy may also be disinfected in a beaker containing

- absolute ethanol.
6. Lift the operculum and examine gills. Observe the gills and record the colour, presence of parasites, whitish nodules, excess mucus, haemorrhages,
 7. Approach the abdominal cavity by making an incision from the anal opening upto pectoral fin. The skin flap may be pulled with the help of a pair of scissors thus exposing the internal organs like liver, stomach and intestines, kidney and air bladder. While doing so, make sure that the air bladder remains inflated and alimentary tract remains intact.
 8. The viscera and adjacent organs (heart, kidney, air bladder, liver, kidney, pyloric caecae and GI tract) may be examined carefully for the presence of abnormalities like discoloration, enlargement, haemorrhage, ascitis, cysts, parasites, neoplastic growth, etc. Record the colour of the internal organs, haemorrhages, enlargement of spleen if any, presence of white spots or whitish nodules on liver, spleen and kidney, hardening of the gall bladder, presence of fluid in the peritoneal cavity and its colour and consistency. Examine the swim bladder for thickening, haemorrhages, necrosis, and parasites.
 9. If bacterial isolations are to be made, the tissue can be charred with a heated scalpel and a sterile bacteriological loop may be pierced into the charred area and inoculated on to suitable media.
 10. If viral isolations are to be made, the tissues can be placed in a suitable tissue culture media and refrigerated.
 11. Squash of lower intestine should be made on microscopic slide and examined for type of food and pathogens like hexamita.
 12. In case nervous signs are reported, small portion of the brain and optic nerve should be placed in "RNA later" for molecular studies. Small pieces of tissues may also be preserved in 95% ethanol for molecular studies.
 13. Representative samples of all organs should be placed in 10% buffered formalin for histopathology.

Bacterial Diseases of Marine Fish and Shellfish

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The demand for mariculture of high value fishes in sea cages has been rapidly increasing in the recent years. Most prominent farmed fish species across the globe include Atlantic salmon, sea bass, sea bream, turbot, mullet and tuna. In India, after the successful breeding and seed production, demand for cage farming of cobia is also increasing. Other than cobia, Asian seabass is also regarded as a potential marine fish species for sea cage farming in India.

Increased intensification of mariculture production involving high density culture in low volume invite the risk of diseases associated with rearing stress and fish welfare. According to the Code of Conduct for Responsible Fisheries of the Food and Agriculture Organization (FAO), farming implies some sort of intervention in the rearing process to enhance production, such as regular stocking, feeding, protection from predators, etc. Rearing of high value fish in high density is also allied with manifestation of altered host-parasite interactions resulting from hosts being reared in new geographic locations. The altered host-parasite association may also result from aboriginal hosts being reared in diverse environmental conditions (Kent, 2000). In case of marine cage culture, interactions between wild and cultured fish populations are of greatest disquiet for both aquaculturists and environmentalists.

Diseases occur due to intricate interactions between host, pathogen and environment (Snieszko, 1974). Olivier (2002) suggested presence of pathogen in both fish and environment (water), presence of susceptible host, viability of the pathogen (number and longevity) in the environment, and viable infection route as essential pre-requisites for a disease to spread

from either cultured fish to wild fish or vice-versa.

Diseases of farmed fish occurring due to bacterial pathology have been one of the major important limiting factors in mariculture practices. As regards to the diseases caused by bacteria in marine fish farming, although many pathogenic bacteria have been described in the majority of the existing taxonomic groups, fairly small numbers are responsible for major economic losses in the culture system globally. Majority of the bacterial pathogens described in the culture systems are normally present in wild fish populations. However, in natural environments, in the absence of stressful conditions, they seldom cause mortality due to disease outbreak. High stocking density and poor environmental parameters act as stressful situations for the occurrence of diseases in culture system.

Vibriosis

Vibriosis is one of the most prevalent bacterial diseases of cultured finfish caused by bacteria belonging to the genus *Vibrio*. *Vibrios* are gram negative bacteria, ubiquitous to marine and estuarine environment as well as marine fish farms. There are around 34 recognized species within the genus *Vibrio*. Within the family *Vibrionaceae*, the species causing the most economically serious diseases in marine fish and shrimp culture include *Vibrio anguillarum*, *V. alginolyticus*, *V. harveyi*, *V. parahaemolyticus* and *V. splendidus*. Other species like *V. ponticus* and *V. ordalii* are also seldom reported to cause mortalities in fish culture system. Vibriosis caused by *V. anguillarum* is a major problem in seabass farming. However, *V. alginolyticus* has been suggested to be a pathogen of humans and several marine fish species like seabream and

Asian seabass. This species has been reported to be the causal agent of outbreak of vibriosis in grouper. An outbreak of Vibriosis caused by *V. alginolyticus* in Asian seabass cultured in marine cages in Indian has been reported (Sharma *et al.*, 2013). The outbreak of the disease in this case was associated with increased water temperature. Isolation of *V. ordalii*, which has been established to accommodate strains formerly classified as *V. anguillarum* biotype 2 (Schieve and Crosa, 1981), has been reported predominantly from North America, Japan and Australia affecting salmonid fishes. Mutharia *et al.* (2002) found that cross-reactions subsist between *V. ordalii* and *V. anguillarum* serotype O₂ using polyclonal antisera, but immunoblot analysis with absorbed antisera reveal that the LPS of both species do not have identical antigenic properties. Most of the pathogenic Vibrios are normal inhabitants of sea water and sediment. Hence, these organisms are opportunistic in nature causing disease when fish are subjected to stress.

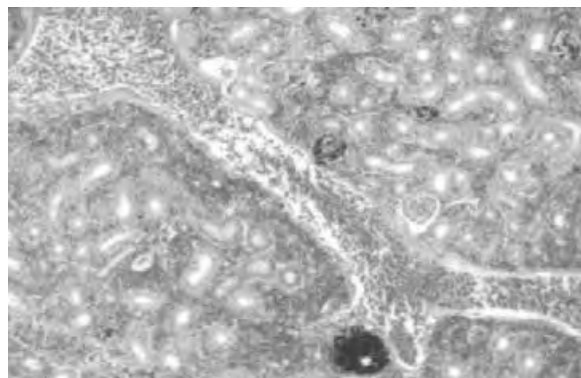
Vibrio harveyi, another member of the family Vibrionaceae, is a luminous marine bacterium which is a normal microflora of warm marine environment, body of fish and shellfish, light-emitting organs of marine fish and cephalopods, and intestinal microflora of marine vertebrates and invertebrates. The organism is predominantly responsible for the occurrence of luminous vibriosis, which affects a wide range of marine invertebrates, especially penaeid shrimp and phyllosoma larvae of the rock lobster resulting in severe economic losses. Pathological manifestations of vibriosis caused by *V. harveyi* in the cage farmed mangrove red snappers associated with increased water temperature and handling stress is also reported (Sharma *et al.*, 2014).

Pathology: In case of vibriosis, the pathogen may enter the host orally, through skin lesion and gill surface consequent to wound caused by ectoparasites and protozoa. Fish affected by classical vibriosis show typical signs of a generalized haemorrhagic septicaemia with the presence of haemorrhagic lesions at the base of fins, ulcerations on the body surface, especially in chronic cases, exophthalmia and corneal opacity (Fig. 1-A). Ailing fish are often anorexic with pale gills due to anaemia arising from haemorrhages.



Microscopic lesions in case of vibriosis also reflect the haemorrhagic nature of the disease. Histologically, bacteria invading the dermis, subcutaneous adipose tissue, and the underlying musculature are evident. Affected tissues are necrotic and heavily infiltrated by granulocytes. Gill filaments and lamellae are also infiltrated by neutrophils with haemorrhage. Liver shows hypertrophy of the bile ducts, necrosis, haemorrhage and congestion. In myocardium, loss of cross striations and infiltration of polymorphonuclear cells into the endocardium is noticed. Kidneys reveal characteristic lesions of acute glomerulonephritis with increased expression of melano-macrophage centres (Fig. 1-B). Gastric mucosa contains engorged capillaries and loss of tubular glands. Extensive tissue lesions in vibriosis are primarily due to the release of proteinases and other extra-cellular enzymes produced by the bacteria.

Diagnosis: Vibrios are gram negative rods characteristically curved or comma shaped. This morphological appearance may not be always observed when organisms are selected for gram staining from solid media. Specific media like Thiosulfate-citrate-bile salts-sucrose agar (TCBS) agar may be used for selective growth of Vibrios. Species



level identification can be done by biochemical tests, PCR using specific primers and 16S rDNA amplification using universal primers and sequencing.

Treatment and prevention: Even though *Vibrios* are susceptible to majority of broad-spectrum antibiotics, limitations exist based on the farming system. Since *Vibrios* are opportunistic pathogens, vibriosis can be best managed by proper husbandry practices. Handling, transportation, overcrowding, low dissolved oxygen and increased water temperature make the farmed fish susceptible to vibriosis. Periodical enumeration of the bacterial load of water and sediment would help in preventing outbreaks.

Due to diversity of *Vibrios* and their serovars, the advancement in vaccine development against vibriosis has been dawdling, and commercial vaccine is not currently available. However, attempts have been made to vaccinate fish against different *Vibrio* spp. using oral, killed and sub-unit vaccines. In case of oral vaccination, the vaccine is either mixed with the feed, top dressed on the feed, or bio-encapsulated. Bio-encapsulation is used when fish fry are to be vaccinated. In case of bio-encapsulation, live feed, such as artemia nauplii, copepods or rotifers incubated in a suspension of vaccine are fed to the fish fry. Oral vaccination causes no stress to the fish. However, they have a very short term stability once mixed with the feed. More recently, the outer membrane proteins molecules are used for development of subunit vaccines due to the exposed epitopes on the bacterial surface and conserved nature in different serovars. It has also been demonstrated that outer membrane proteins molecules like OMP -K acts as protective antigen against fish vibriosis caused by *V. alginolyticus*.

Photobacteriosis

Photobacteriosis, also known as fish pasteurellosis, is caused by the halophilic bacteria *Photobacterium damsela* subsp. piscicida. Gauthier *et al.* (1995) included the fish pathogen *Pasteurella piscicida* in the species *P. damsela* according to phylogenetic analyses of 16S rDNA sequences and DNADNA relatedness, and named as *Photobacterium damsela* subsp. piscicida. Pasteurellosis has been a serious disease in Japan affecting the aquaculture

production considerably. The disease is characterized by the presence of whitish nodules on liver, spleen and kidney. Severe mortalities occur in pasteurellosis when water temperature is above 18– 20°C. Below this temperature, fish can harbour the pathogen for prolonged periods without causing clinical infection. This disease was first discovered in natural populations of white perch (*Morone americanus*) and striped bass. The disease affects various species of fishes like yellow tail juveniles (*Seriola quinqueradiata*), ayu (*Plecoglossus altivelis*), black seabream (*Mylio macrocephalus*), red seabream (*Acanthopagrus schlegeli*), oval file fish (*Navodan modestus*) and red grouper (*Epinephelus okaara*).

Pathology: Fish pasteurellosis is a septicaemic disease and manifests as an acute or chronic form. Pale gills, dark pigmentation and presence of petechial haemorrhages on the body surface and fin base are normally observed in acute form. Enlarged spleen and mottled liver are seen internally. In case of chronic form, nodules resembling tubercles are seen in spleen and kidney.

Another disease caused by *Photobacterium damsela* subsp. damsela is also responsible for mortality in many cultured marine fish species. This disease has been reported from India in cage farmed cobia. The lesions are haemorrhagic in nature resembling the lesions found in vibriosis. The pathological manifestations in both the infections are primarily due to the extra cellular products (ECP) secreted by the bacteria. Both pathogens are normal inhabitants of marine environment.

Disease transmission in case of photobacteriosis occurs through direct contact and ingestion. The bacteria are unable to survive in fresh or brackish water. Predisposing factors for the outbreak normally include rise in water temperature.

Diagnosis: The pathogen can be isolated and cultured on marine agar and ordinary media supplemented with sodium chloride. The organism can be confirmed by biochemical tests, 16S rDNA sequencing and slide agglutination. *Photobacterium damsela* subsp. piscicida has to be differentiated from *Photobacterium damsela* subsp. damsela using

a multiplex PCR that combines specific primers for 16S rRNA and urease genes (Osorio *et al.*, 2000).

Treatment and control: Several commercial vaccines against *P. damsela* subsp. *piscicida* are available, wherever the disease is more prevalent. Efficacy of these vaccines depends on the species of fish, fish size, etc. Since outbreak of pasteurellosis normally occurs during larval stages to fingerling stage, a vaccination programme involving dip immunisation during the larval stage with a booster dose when fish reaches a size of 1–2 g is advocated.

Flexibacteriosis

Flexibacteriosis, also known as gliding bacterial disease of sea fish, eroded mouth syndrome, and black patch necrosis, is a disease of marine fish caused by *Tenacibaculum maritimum* (formerly *Flexibacter maritimus*). *T. maritimum* exists exclusively in the marine environment. The disease is normally distributed in cultured and wild fish in North America, Australia, Europe and Japan. Turbot, sole, seabream, seabass, red seabream and black seabream are the cultured fishes affected by *T. maritimum*. The disease occurs in a more severe form in young fish when compared to adults. The severity of the disease increases with increase in water temperature. The predisposing factors in a farming system include injuries and skin abrasions. Stress induced by husbandry practices like inferior water quality, increased water temperature, handling etc., may also predispose the fish to flexibacteriosis.

Pathology: Affected fish are weak, develop pinkish ulcers on the skin with loss of scales. Affected fish also have eroded and haemorrhagic mouth and frayed fins with tail rot. White necrotic lesions develop on gills of the affected fish. Microscopically, long, thin basophilic bacteria can be seen in sections taken from skin and muscle lesions. Colonization of the bacteria on the scale pockets, loss of scales and dermatitis are also noticed. Congestion and haemorrhage in the superficial dermis is also evident histologically.

Diagnosis: The initial presumptive diagnosis of marine flexibacteriosis include microscopic observation of accumulations of long, thin, rod shaped flexing bacteria in wet mounts or Gram-negative stained

preparations obtained from gills or lesions. Phase contrast microscopy is advantageous in bacterial identification. The bacteria cannot be easily isolated on conventional media. This pathogen grows only in specific media since it needs an absolute requirement of seawater as well as low concentration of nutrients. Some of the specific media devised for the isolation of this pathogen include Flexibacter Maritimus Medium (FMM). FMM has been regarded as most effective media. The bacteria can be grown at 13 to 34°C. Colonies are rhizoid with uneven edges. A nested PCR which is rapid and sensitive was also developed for an accurate diagnosis (Cepeda *et al.*, 2003).

Treatment and prevention: Hydrogen peroxide used at a dose of 240 ppm would be beneficial in treating the infection. Many of the antibiotics are also effective in treating the fish infected with *T. maritimum*. However, they have to be used with caution depending on the type of antibiotic, farming system and environmental factors. A formalin killed vaccine was also tried in Japanese flounder to reduce the effects of infection.

Streptococcosis

Streptococcosis is a re-emerging disease of both fresh and marine fish caused by gram positive bacteria characterized by central nervous system damage followed by exophthalmia and meningoencephalitis. Streptococcosis is also known as “pop-eye”, since one of the most characteristic clinical sign found in this disease is the accumulation of muco-purulent exudates around the eyes. Streptococcosis, a problem in both farmed and wild marine fish, has been reported from USA, Japan, and Spain. In Japan and Spain, this disease forms a major limiting factor for the marine fish production of yellowtail turbot. Fish of all the size are susceptible. The sea water and sediment harbours the pathogen which can be isolated from these sources round the year. It has been reported that this pathogen can survive in frozen products for at least 6 months. Warm water streptococcosis which occurs when water temperatures are above 15°C is normally caused by *L. garvieae*, *S. iniae*, *S. agalactiae* and *S. parauberis*. Cold water streptococcosis seen when water temperatures are below 15°C is generally caused by *L. piscium* and *V. salmoninarum*. Pathogens responsible for warm water

streptococcosis are of zoonotic importance since they can cause disease in humans. Horizontal transmission can occur through injuries and abrasions.

Streptococci capable of causing disease in marine fish falls into three categories: alpha-haemolytic, beta-haemolytic, and non-haemolytic. The majority of disease epizootics are generally caused by streptococci belonging to alpha-haemolytic group. Among these fish streptococci, *L. garvieae*, *S. iniae* and *S. parauberis* can be regarded as the main aetiological agents causing diseases in marine aquaculture.

L. garvieae infects marine fish like yellowtail in Japan. *S. iniae* is an important fish pathogen causing disease and mortality in many cultured fish species in both tropical and sub tropical environments. *S. iniae* is the main aetiological agent of streptococcosis in tilapia in USA and rainbow trout in Israel. However, *S. iniae* was also isolated from marine fish including yellowtail, flounder, European seabass, and Asian seabass. There have been no reports from India. *S. parauberis* is reported to be endemic to cultured turbot.

Pathology: The lesions caused by different streptococcal species in diverse host species are similar. The lesions are of general septicemic in nature affecting liver, spleen, eye, brain and kidney. The eyes show severe exophthalmia with granulomatous inflammation. Granulomas with the presence of bacteria may also be seen in pericardium, alimentary tract, peritoneum, brain, ovary, testes and spleen. The most significant clinical signs are exophthalmia, distended abdomen, haemorrhages in the eyes, opercula, fin base, ulceration of the body surface and darkening of the skin. Internally, the abdominal cavity is filled with variable amounts of purulent exudate. A yellowish exudate often covering the peritoneum and the pericardium may be seen. Yellowish exudates may also be seen in cranial cavity. Haemorrhages are found on all visceral organs.

Diagnosis: Fish streptococci can be generally isolated from internal like spleen, liver and brain using brain heart infusion agar or tryptone soya agar supplemented with 1% yeast extract or 0.5% glucose, and growth is enhanced on blood agar. The incubation period is 24 days at 25-30°C. The

isolates are then characterized either biochemically or serologically. Significant characteristics are spherical or ovoid colony morphology and formation of pairs or chains. All strains of streptococci are Gram positive, oxidase and catalase negative, non-motile and non-sporulating. Slide agglutination and immunofluorescent techniques are widely used for diagnosis of streptococcosis. PCR based diagnosis including 16S rRNA amplification and sequencing can also be employed.

Treatment and prevention: Good husbandry practises including avoiding over-crowding, excess feeding, handling and the timely removal of diseased or dead fish would help to minimise the economic losses due to streptococcosis. Apart from this, vaccination, use of chemotherapeutics and immunostimulants has also been tried. Many of the isolated strains were sensitive to antibiotics like erythromycin, tetracycline, ampicillin and doxycycline. Attempts have been made to develop vaccines against streptococcosis in fish. Intra-peritoneal route was found to be most effective. -1-3-glucans used as immunostimulants is also found to be effective.

Mycobacteriosis (Fish tuberculosis)

Mycobacteriosis is a chronic wasting granulomatous disease of fish caused primarily by *Mycobacterium marinum*. Some species of mycobacterium are found in soil and water. Mycobacteriosis was earlier known as fish tuberculosis or piscine tuberculosis because the causative pathogen is taxonomically similar to *Mycobacterium tuberculosis*, which caused tuberculosis in humans. But other than acid-fast staining characteristics and taxonomic position, the organisms causing tuberculosis in fish and human are dissimilar. In cultured fish, mycobacteriosis was reported in salmon, pejerrey, snakehead fish, turbot, tilapia, European seabass and red drum. Mycobacteriosis caused by *M. marinum* represents a significant threat especially for seabass cultured on the Mediterranean and the Red Sea coast of Israel. Mycobacteriosis is regarded as a serious threat to the turbot culture in Europe. In general mycobacteriosis is more dangerous to marine fishes when compared to fresh water fishes.

Incidence of mycobacteriosis is higher in aquarium fish since these fish are kept for long time and also mycobacteriosis being a chronic disease.

Ingestion, trans-ovarian transmission and direct contact from water or infected fish have been suggested as the possible routes of transmission in case of mycobacteriosis. Also, high density of fish in an intensive culture system would be a responsible for an outbreak of mycobacteriosis due to increased opportunity for transmission through the water column, faecal products or cannibalism (Hedrick *et al.* 1987).

Pathology: Clinical signs include emaciation, stunted growth, exophthalmia and slowed swimming. Internal lesions in mycobacteriosis vary depending on the fish species involved but typically consists of greyish-white granulomas in the spleen, kidney and liver. Microscopically, these typical granulomas consist of central area of necrosis surrounded by macrophages, epithelioid cells and fibrous connective tissue. Granulomas are generally seen in spleen, liver and kidney during initial stages of the disease, but later may spread to all internal organs in more advanced cases. Externally, loss of scales and haemorrhages extending to the musculature is seen in advanced cases.

Diagnosis: Presumptive diagnosis can be made by clinical signs, gross and microscopic pathology. However, these are many a times are inconsistent and hence definitive diagnosis is not possible base on pathology. Smears from spleen and kidney can be stained with Ziehl-Neelsen stains so as to identify the acid-fast short bacilli. Isolation on specific media can also be helpful. Methods presently under research genetic techniques, high-performance liquid chromatography (HPLC), and capillary gas chromatography for fatty-acid methyl-ester (FAME) analysis.

Treatment and prevention: Since no approved drugs or anti-mycobacterial agents are available, depopulation and proper disinfection is the most commonly adapted policy in case of culture conditions. However, disinfection may not be always successful due to the resistance acquired by the pathogen to many disinfectants (Jacobs *et al.*, 2009).

Shrimp vibriosis

Shrimp vibriosis also known as sea gull syndrome, is caused by numerous etiological agents like *V. harveyi*, *V. vulnificus*, *V. parahaemolyticus* and *V. alginolyticus* causing severe economic losses in shrimp hatcheries and post larvae rearing ponds. Infections of the exoskeleton extend into digestive tract, including the hepatopancreas, and finally septicaemia occurs leading to severe mortality. These bacteria are Gram-negative, motile, rod-shaped bacteria that require supplementation of sodium chloride in the media for their growth. Among the *Vibrio* spp. which cause disease in shrimp, *V. harveyi*, is one of the primary etiologic agents of that causes mass mortalities in *Penaeus monodon* larval rearing ponds. Epizootics of vibriosis occur in all life stages of the shrimp, but are more common and lethal in shrimp hatcheries. Most of the *Vibrio* spp. form part of the natural microflora of wild and cultured shrimps and cause disease when natural defence mechanisms are suppressed due to various stress factors like inferior water quality, deteriorating environmental factors and overcrowding.

Pathology: Most of the *Vibrio* spp. form part of the natural microflora of wild and cultured shrimps and cause disease when natural defence mechanisms are suppressed due to various stress factors like inferior water quality, deteriorating environmental factors and overcrowding. Clinical signs of shrimp vibriosis include high mortality, shrimp congregating in surface of pond edge and presence of luminescence which can be appreciated in darkness. Gross lesions include melanosis of the shell which appear as black spots on the cuticle and delayed clotting. Post-larvae infected with *Vibrio* spp. have cloudy hepatopancreas. Gills are mostly brown in colour. Histopathologically, atrophy of the hepatopancreas with multifocal necrosis associated with haemocytic infiltration is evident. Presence of localized haemocytic nodules in the lymphoid organ, heart and connective tissues of the gills, hepatopancreas, antennal gland, telson and muscle are also observed. Vibriosis in *P. monodon* is also associated with the appearance of "spheroids" in the lymphoid organ. Large numbers of gram negative

bacteria are present in the hemolymph. Adult shrimps infected with vibriosis are hypoxic, show reddening of the body surface, reduced feed intake and move slowly mostly at the edges and surface of pond. Some of the *Vibrio* spp. also causes red-leg disease which is characterised by red discolouration of the pleopods and gills associated with mortality up to 95% during summer months when the water temperature is high.

Diagnosis: *Vibrio* infection can be readily diagnosed based on clinical signs and demonstration of rod-shaped *Vibrio* bacteria in lesions, nodules or haemolymph. Haemolymph may be inoculated on TCBS agar plate. Luminescent colonies may be observed after 6 to 12 hr when inoculated onto tryptone soya agar.

Treatment and prevention: Luminescent vibriosis is normally prevented in the hatcheries using appropriate chemicals so that bacterial load of the rearing units or the incoming water can be reduced. In shrimp hatcheries, washing eggs with iodine and formaldehyde and preventing contamination of eggs by excreta is advocated. In case of pond culture, increase in daily water exchanges and reduction in pond biomass by partial harvesting would help in reducing the mortality in case of an outbreak. Probiotics may also be administered through water or feeds.

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Viral Diseases of Marine Fish and Shellfish

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Viruses are microorganisms that can replicate only inside the living cells of other organisms. Viruses can infect all types of life forms including animals, plants and microorganisms, including bacteria and archaea. Virus particles or virions normally consist of three components: i) genetic material made up of either DNA or RNA which carries the genetic information; ii) a coat protein that protects these genetic materials; iii) a lipid envelope that surrounds the protein coat which may be absent in some viruses. The shape of viruses may be helical or icosahedral or more complex structures.

Viruses do not multiply through cell division. They normally make use of the host cell genetic material and metabolic pathways to produce multiple copies of themselves. The life cycle of viruses differs greatly between species but there are six basic stages in their life cycle which include attachment, penetration, uncoating, replication, assembly and release.

The aquaculture as an industry has expanded globally with an increase in both productions in terms of biomass and also in number of fish species being cultured. Intensification of aquaculture operations globally has provided new opportunities for the transmission of fish viruses and hence the occurrence of diseases caused by viruses forms a major limiting factor for the sustainable aquaculture production.

Fish viruses have been the subject of research interest in the past two decades. Compared to diseases caused by fresh water fish viruses, there have not been extensive studies on marine fish viruses. Establishment of various fish cell lines lead to path breaking research in fish virology in the recent years. Major viral groups under which fish viruses can be classified include herpes virus, iridovirus, rhabdo virus, reo virus, noda

virus and calci virus. However, the most lethal viral disease causing enormous loss to finfish farming is the disease caused by betanodavirus. As for as shrimp farming is concerned, the major viral diseases of *P. monodon* include white spot syndrome and yellow head disease that can also cause serious mortalities in *P. vannamei* farming.

Viral nervous necrosis

Betanodavirus is one of the genera making up the family Nodaviridae which is the etiological agent of viral nervous necrosis (VNN) also known as encephalomyelitis and vacuolating encephalopathy and retinopathy. This virus has remained as a major threat for the establishment and expansion of Asian seabass (*Lates calcarifer*) and striped jack (*Pseudocaranx dentex*). The disease was first documented in 1990 in hatchery-reared Japanese parrotfish (*Oplegnathus fasciatus*) in Japan and Asian sea bass in Australia. Later, it was reported in turbot (*Scophthalmus maximus*), European sea bass (*Dicentrarchus labrax*), redspotted grouper (*Epinephelus akaara*), striped jack (*Pseudocaranx dentex*) and more recently in cultured warm-water and cold-water marine fish species throughout the world (Munday *et al.*, 2002). In India, mortality caused by betanodavirus infection in hatchery produced larvae of Asian seabass was first reported by Azad *et al.* (2005). An Indian strain of betanodavirus belonging to RGNNV group was isolated from Asian seabass juveniles reared in a brackish water farm in Bhimavaram in Andhra Pradesh in 2012. Outbreak of mortality due to nodavirus infection in Asian seabass juveniles cultured in fresh water cages in the south west coast of India has also been reported. Mortality of Asian seabass juveniles cultured in indoor cement tanks as well as open sea cages and in cobia cultured

in cages in India associated with RGNNV was also recorded (unpublished report).

Betanodaviruses can infect fish species belonging to tropical, sub-tropical, or temperate waters. These viruses can multiply at an optimum temperature depending on the strain of the virus. For RGNNV, the optimum temperature requirement is 25–30°C while for SJNNV, it is 20–25°C.

Mostly, betanodaviruses are a concern in marine fish species. The species susceptible cobia, sea bass, seabream, bluefin tuna, grouper, halibut, surgeonfish, lined surgeonfish and tiger puffer. The freshwater fish species susceptible to betanodaviruses include tilapia and the guppy.

Pathology: In farming system stress factors like high density, transportation, high temperature can act as predisposing factors making the fish susceptible to VNN. Although young fishes are more susceptible, older fishes may also get infected especially when water temperature is high.

During the acute stage of the disease, when the mortality is very high, especially in juveniles, there would be no gross lesions on the body surface or gills. However, affected juveniles and older fish show an abnormal swimming behavior such as spiral, whirling, floating with inflation of swim bladder, or laying down at rest, circling on their own axis. This erratic swimming behaviour may not be noticed in infected fish larvae. Grossly, the brain is oedematous and in many cases severely congested (Fig. 2-A).

Microscopically, lesions are characterized by severe vacuolation and necrosis of the central nervous system (Fig.2-B). In general, the anterior brain is more severely affected when compared to the posterior part of the brain and spinal cord. Larvae of the fish are more severely affected by betanodaviruses than juveniles. The most characteristic lesion in the fish larvae is the presence of vacuoles in the grey matter of the brain which are intracytoplasmic. Basophilic, intra cytoplasmic inclusions have been reported in brain cells of Asian seabass.

Lesions in the retina of the infected fish have also

been described in all Species. The lesions include vacuolation of the cellular components of the retina especially the bipolar and ganglionic nuclear layers.

Under transmission electron microscopy, fish betanodaviruses appear icosahedral, non-enveloped with a mean diameter of about 25 nm. The virions may be membrane bound by endoplasmic reticulum or are free in the cytoplasm and may present as paracrystalline arrays. Cells containing virions normally include neurones, astrocytes, oligodendrocytes and microglia cells.

Diagnosis: The diagnostic methods for fish nodaviruses have been extensively studied. According to the Munday *et al.* (2002), VNN can be diagnosed by:

1. Demonstration of characteristic vacuolar lesions in the brain or retina by light microscopy.
2. Detection of virions and viral antigens by electron microscopy and serology
3. Detection of viral nucleotides by molecular techniques including RT PCR, RT Nested PCR, cloning and sequencing by designing the primers for the strain of interest.
4. Tissue culture of virus in a suitable cell line.

Treatment and prevention: Betanodaviruses are highly resistant to various environmental conditions and they can survive for a long time in sea water. The disease can also be reproduced by simple co-habitation of the healthy fish with infected fish. Control measures are including imposing strict bio-security to exclude the virus from the farm premises. The broodstocks should be tested for the presence of viruses in the gonadal tissues and VNN specific antibodies in serum and any positive fish should be culled at first.

White spot syndrome

More than 1100 viruses of invertebrates have been reported so far. Most important groups of viruses reported in Crustacea include Reoviridae, Picornaviridae, Parvoviridae, Togaviridae, Baculoviridae, Paramyxoviridae, Rhabdoviridae and Iridoviridae.

White spot syndrome (WSS) is a viral infection

of penaeid shrimp caused by a double stranded DNA virus belonging genus *Whispovirus* within the *Nimaviridae* family which is a most devastating virus of cultured shrimp characterized by severe mortality and appearance of white spots on the carapace of the infected fish. All decapod crustaceans including prawns, lobsters and crabs from marine, brackish or freshwater environments are susceptible to infection. WSSV is the largest DNA virus of which whole genome sequencing has been done.

WSSV virions are ovoid or ellipsoid to bacilliform in shape and measure 80–120 nm in diameter and 250–380 nm in length. Most noteworthy feature is the presence of flagella like extension at one end of the virion. Under laboratory conditions, WSS virus is viable for at least 30 days at 30°C in seawater and the virus is viable in ponds for at least 3–4 days.

The virus is normally transmitted by horizontal transmission through water and feed infected. Vertical transmission is also possible brooders to offspring.

Pathology: Shrimp with acute WSS show a rapid reduction in food consumption, lethargic movements and have a loose cuticle with the presence of white spots which measure 0.5 to 2.0 mm in diameter. The targets organs for the virus are the cells of ectodermal and mesodermal origin, including those of the epidermis, gills, foregut, hindgut, antennal gland, lymphoid organ, muscle, eye-stalk, heart, gonads, haematopoietic cells and cells associated with the nervous system. Hence the death is normally due to multi-organ dysfunction. These white spots are apparent on the inside surface of the carapace. The white spots signify abnormal deposits of calcium salts by the cuticular epidermis. Shrimp showing these signs show high mortality, sometimes up to 100%, in 3 to 10 days of the onset of clinical signs. In a typical shrimp farm, WSSV infected shrimp gather near the pond edge and display clinical signs 1 or 2 days before the first mortalities occur.

White spot disease is histopathologically characterized by the presence of widespread and severe nuclear hypertrophy, chromatin margination and eosinophilic to basophilic large intranuclear inclusions with focal necrosis in most tissues of ectodermal and

mesodermal origin including gills, haemocytes and haematopoietic tissue, lymphoid organ, connective tissues, subcuticular epidermis, stomach, foregut and hindgut epithelium, heart, striated muscle, midgut and ovary walls, antennal gland and the nervous tissues. These inclusion bodies are strikingly distinct and bigger than the Cowdry A-type inclusions which are seen in infectious hypodermal and haematopoietic necrosis virus infection.

Diagnosis: The disease can be diagnosed and confirmed by using DNA probes, PCR using WSSV primers, nested PCR, monoclonal antibody based kits which can be used at field level.

Treatment and prevention: Even though research results are available on immunity against WSSV in shrimp injected with inactivated WSSV virions or recombinant structural protein, or by using RNA interference (RNAi), or by administering orally bacterially expressed VP28 dsRNA, there are still no field data for either the vaccination or the RNAi approach.

Yellow Head Disease

Yellow head diseases is a viral disease of shrimp caused by most virulent shrimp virus, yellow head virus genotype-1 characterized by yellowish discolouration of the cephalothorax and mass mortality. Yellow head virus (YHV) can remain viable in aerated seawater for up to 72 hours. YHV is an enveloped, rod-shaped, ssRNA virus with a helical nucleocapsid and prominent glycoprotein projections on the virion surface.

Yellow head disease (YHD) outbreaks have been reported in the black tiger prawn (*P. monodon*) and the white Pacific shrimp (*P. vannamei*) and pacific blue prawn (*P. stylirostris*) and few other shrimp species. *P. monodon* which are beyond PL15 are susceptible to YHV. Like WSSV, YHV also targets tissues of ectodermal and mesodermal origin including lymphoid organ, haemocytes, haematopoietic tissue, gill lamellae and spongy connective tissue of the subcutis, gut, antennal gland, gonads, nerve tracts and ganglia. YHV infection can be transmitted horizontally by injection, ingestion of infected tissue, or by co-habitation of healthy shrimp with infected shrimp. Homologous

genetic recombination is also an attribute of the yellow head virus complex. The prevalence and geographic distribution of these recombinant viruses indicate that the reason of swiftly increasing genetic diversity of the virus is primarily due to international trade in live shrimp.

Pathology: YHV genotype-1 can cause upto 100% mortality in *P. monodon* within 3–5 days of the first appearance of clinical signs. Stress induced by sudden changes in pH or dissolved oxygen levels can be the predisposing factor for an outbreak of the disease in shrimp farms. Moribund shrimp may congregate at pond edges near the surface. In shrimp farms, infection can result in mass mortality especially in early to late juvenile stages. Clinical symptoms of the disease may include cessation of feeding, aggregation of infected shrimp at pond edges and bleached appearance. Moribund shrimp may exhibit a bleached overall appearance and a yellowish discoloration of the cephalothorax. This is caused by the underlying yellow hepatopancreas, which may be remarkably soft when compared with the brown hepatopancreas of

a healthy shrimp. Unusually high feeding activity by the shrimp followed by a sudden cessation of feeding may occur within 2 to 4 days of the appearance of gross clinical signs of disease and mortality.

Microscopically moderate to large numbers of deeply basophilic, evenly stained, spherical, intra-cytoplasmic inclusions of approximately 2 μm in diameter can be seen in tissues of the lymphoid organ, stomach, and gills.

Diagnosis: YHD can be diagnosed by gross and microscopic lesions and RT-PCR using YHV specific primers.

Suggested readings

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Microbiological Staining Techniques

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Etiological agents of bacterial diseases of marine fish and shellfish can be identified by various microbiological methods. The samples should always be taken from the fish under aseptic condition and they are first inoculated onto non-specific media, then the various microbial diagnostic methods are used. The commonly used microbiological diagnostic tools include:

- Staining
- Motility test
- Culturing
- Biochemical test

Staining

Different staining methods are in use to identify various bacterial pathogens. Gram staining is the most important and most commonly used method.

Gram stain

The Gram stain classifies bacteria according to whether they retain crystal violet stain (gram-positive-blue) or not (gram-negative-red). If the bacteria can retain crystal violet, then they are classified as Gram +ve and take purple colour. If they do not retain crystal violet, then they are classified as Gram -ve and appear pink. The morphology of the bacteria, whether they are rod shaped or cocci or cocco-bacillary and arrangements of bacterial cells in the form of clumps, diploids or chains can also be ascertained by Gram staining.

Principle

The basic principle of Gram staining is the properties of cell walls of some bacteria to retain the crystal violet dye. The cell walls of Gram positive bacteria have a large amount of peptidoglycan and lower lipid content. The cell wall of Gram negative bacteria has

less peptidoglycan and they have lipopolysaccharide containing endotoxin.

Preparation of reagents

- Primary Stain : Crystal Violet

Solution A:

- Crystal violet : 2g
- Ethanol, 95% : 20 ml

Solution B

- Ammonium oxalate : 0.8 g
- Distilled water : 80 ml

Mix both A and B solutions to obtain working crystal violet reagent.

Mordant: Gram's Iodine

- Iodine : 1.0 g
- Potassium iodide : 2.0 g
- Distilled water : 300 ml
- Decolourizing agent : Ethanol, 95%
- Counter stain : Safranin

Stock solution

- Safranin O : 2.5 g
- 95% Ethanol : 95 ml

Working Solution:

- Stock Solution : 10 ml
- Distilled water W : 90 ml

Procedure

1. Air-dry and heat-fix the smear.
2. Pour crystal violet and wait for 1 min.
3. Wash slide gently using tap water for 2 seconds
4. Flood slide with the mordant and wait for 1 min
5. Wash slide gently with tap water for 2 seconds.

- Flood slide with decolorizing agent. Wait for about 15 seconds until no more decolorizing agent run off from slide
- Flood slide with safranin and wait for 1 min. Wash slide gently with tap water until no colour appears in the effluent and dry the slide. Observe under oil immersion

Interpretation

Gram negative: pink/red and gram positive: blue/purple

Wright's stain

These stains are used for detection of parasites in blood, phagocytes and tissue cells, intracellular inclusions formed by viruses and also some intracellular bacteria.

Procedure

Make thin smears across a sterile slide by means of a second slide or cover glass. Air dry.

Staining:

Place 1.0 ml of the Wright Stain Solution upon the smear (1 min). Add 2.0 ml distilled water (2 min). Rinse stained smear with water or the Phosphate buffer pH 6.5 until the edges show faintly pinkish-red. Blot dry very carefully. Stain may be adjusted by further dilution or in the timing of either before or after dilution in the above procedure.

Interpretation:

Red blood cells: red to pink

Neutrophils: dark purple nuclei, pale pink cytoplasm, reddish-lilac small granules

Eosinophils: blue nuclei, pale pink cytoplasm, red to orange-red large granules

Basophils: purple to dark blue nucleus

Lymphocytes: deep bluish purple nuclei

Platelets: violet to purple granules

Motility (Hanging Drop Method)

Procedure

- Place a small drop of bacterial culture in a broth at the center of a coverslip
- Place a small drop of water at each corner of the coverslip
- Invert a cavity slide with a central depression over the coverslip
- The coverslip will stick to the slide and when the slide is inverted the drop of bacterial culture will be suspended in the well
- Examine microscopically for motile organisms

Culturing bacteria pathogen in selective medium

Some bacteria have the ability to grow in selective media which can be used to identify the pathogen. One of the selective media is TCBS on which only vibrio can grow. General media is one on which any aerobic bacteria can grow. For e.g., TSA or nutrient agar. In a general purpose media, bacterial cultures produce colonies. The form, elevation and margin of the colonies can also be used to identify the bacteria (Fig.1)

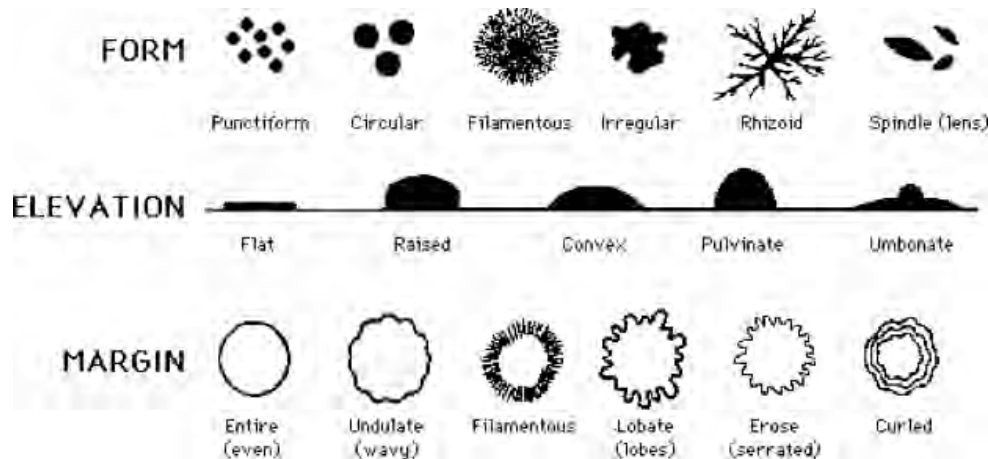


Fig.1. Bacterial colony characteristics

Histopathology

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Histology is the microscopic examination and study of biological cells/ tissues while the term Histopathology refers to the study of diseased cells/ tissues. It helps to understand the fine changes or abnormalities in cells/tissues caused by pathogens/ diseases. Histopathology involves a series of procedures starting from fixation.

The primary objective of fixation is to preserve the morphology of the tissues in a condition as close as possible to that existing during life and hence proper fixation is fundamental to all satisfactory histological preparations. Unless fixation is carried out in a proper way or if it is delayed, a tissue can be irreversibly damaged and any effort to rectify in the later stages will not help. The moment any tissue is deprived of its blood supply, autolysis sets in, leading to tissue digestion by intracellular enzymes released following cell death, and subsequent bacterial decomposition or putrefaction. Fixation aims to arrest these degenerative processes with minimal damage to the tissue architecture. Loss and diffusion of soluble substances in the cells/tissues can be avoided by precipitation or coagulation or by cross-linking them to other insoluble structural components. Fixation also help in protecting the tissues from the harmful effects of tissue processing including dehydration and infiltration, at the same time retaining the reactivity to stains and other reagents. Normally the process of fixation will produce a number of changes to the tissues including shrinkage, swelling and hardening of various components. Fixation in 10% buffered formalin causes slight swelling initially, but subsequently during processing this end up to about 20%–30% shrinkage in volume. The choice of fixative depends on the precise requirement, because the fixative used can influence the degree to which individual elements will stain with various histochemical and immuno-histochemical reagents.

Compared to that of homeotherms the rate of autolysis of fish tissues is rapid and hence, must be fixed immediately to prevent any degenerative changes. For satisfactory histological preparations, only freshly killed or moribund fish should be considered.

Types of fixation

Various method are employed to achieve fixation, the most common processes are chemical or physical means. Physical methods include heating, micro-waving and cryo-preservation (freeze drying). However, heat fixation is mainly used to fix smears of micro organisms and microwave fixation, which is another form of heat fixation, is widely practiced. Cryo-preservation is not used routinely for diagnostic tissue preparations.

Microwave fixation can be used for tissue fixation and heat generated during the process is responsible for tissue fixation. Apart from increasing diffusion rates heat will increase molecular kinetics and speed up chemical reactions. Fresh tissue, in saline or other isotonic medium, can be irradiated to produce primary fixation or alternatively, specimens can be initially placed in buffered formalin or other fixative and later microwaved to assist the fixative action of the fixing agent (microwave-assisted fixation). Microwave-assisted fixation is more commonly used than primary microwave fixation. Microwaves have little effect on tissue beyond a depth of 4 mm and hence for primary microwave fixation tissue slices should not exceed 3 mm in thickness and they should immediately be sliced to 2 mm and placed in 70% ethanol after microwaving. For best results in microwave assisted fixation 2 mm thick slices should be prepared from tissues initially fixed in formalin prior to microwave treatment.

Chemical fixation is carried out by immersing the specimen in the fixative (immersion fixation) or, by perfusing the vascular system with fixative (perfusion fixation). In some cases, fixatives such as paraformaldehyde and osmium tetroxide can also be used to vapour-fix freeze-dried tissues. Usually fixative solutions may contain a single fixative agent dissolved in a solvent such as water or alcohol or a buffer solution to stabilize pH. There is no ideal fixative and hence, a mix of fixative solutions containing different fixing agents in combination is used, so that the deficiency in one can be compensated by the addition of others. The process of fixation is in fact “a complex series of chemical events” the various tissue elements (peptides and proteins, lipids and phospholipids (membranes), carbohydrates and carbohydrate complexes, various types of RNA and DNA) will chemically react with the fixative, get ‘stabilised’ by cross-linking, while other unfixed components may get trapped within the matrix formed by various fixed elements.

Generally fixatives can be classified into “coagulant” or “non-coagulant” based on their effect on soluble proteins in solution. Coagulant fixatives produce a permeable meshwork of protein strands whereas non-coagulant fixatives which are additive in nature, form extensive cross-links producing a less permeable gel. The two major mechanisms underlying the fixation of proteins and protein complexes are denaturation and addition and cross-link formation.

In denaturation, dehydrating agents like alcohols or acetone remove and replace free water in cells/tissues thereby altering the tertiary structure of proteins by destabilizing hydrophobic bonding. Hydrophobic areas, on the inside of protein molecules, are relieved from the repulsion of water and occupy a greater area. In hydrophilic areas, hydrogen bonds which bind water molecules are also destabilized. These changes in the conformation of protein molecules leads to changes in their solubility, thereby rendering water soluble proteins insoluble, a change that can be reversed if returned to an aqueous environment.

In the case of Addition and cross-link formation, the non-coagulant fixing agents chemically react with proteins and other cell/tissue components and forms inter-molecular and intra-molecular cross-links. Most

of these agents are highly reactive and bind to a variety of chemical groups in tissues, often affecting/modifying the charge at the site of attachment, thereby affecting the subsequent staining characteristics of the particular protein as well as altering its molecular conformation and solubility. For example, tissue fixed with formaldehyde stains poorly with eosin because formaldehyde reacts extensively with amino groups to form methylene bridges and thus these groups are no longer available to bind negatively charged dye molecules such as those of eosin. The ability to form cross-links can vary considerably. For example glutaraldehyde is more efficient in forming cross-links and hence preferred for electron microscopy. It also explains why glutaraldehyde-fixed tissues stain poorly with conventional dye-staining methods.

Factors influencing chemical fixation

Various factors are known to influence the rate and effectiveness of tissue fixation.

Temperature: Increase in temperature will increase the rate of diffusion of the fixative into the tissue and enhance the rate of chemical reaction between the fixative and tissue elements. At the same time, this can also adversely affect fixation by increasing the rate of tissue degeneration in unfixed areas of the specimen. Fixation for light microscopy is usually carried out at room temperature. Microwave fixation may involve the use of higher temperatures, up to 65°C, but for relatively short periods.

Time: During fixation the fixative has to penetrate the specimen by diffusion and react with the tissues. Both diffusion time and reaction time depend on the particular reagent used and the optimum time will vary depending on the fixative used. Incomplete fixation can lead to poor quality sections showing tissue distortion and poor quality staining because poorly fixed tissue does not process well.

Penetration rate: The penetration rate of a fixing agent depends on its diffusion characteristics and varies from agent to agent. It can be expressed as $d = K\sqrt{t}$, where d is the depth of penetration, K is the coefficient of diffusion (specific for each fixative),

and t is the time. In simple terms, the coefficient of diffusion (K) is the distance traversed by the fixative in millimeters in one hour. For 10% formalin $K = 0.78$. This means that formalin should not be expected to penetrate more than 1 mm in an hour.

Specimen size: Good fixation depends on the dimensions of the specimen/tissue. A specimen should not be more than 4 mm thick, the most ideal thickness for fixation & processing is 3 mm.

Volume ratio: It is important to have an excess volume of fixative in relation to the total volume of tissue because with additive fixatives the effective concentration of reagent is depleted as fixation proceeds and in a small total volume this could have an effect on fixation quality. The minimum advised ratio of fixative to tissue is 20:1.

pH: Though the pH of a fixative may not affect the quality of preservation in light microscopy as most of the formulations have low pH, especially with fixatives containing acetic or picric acids. However pH can be important in the case of formaldehyde solutions, where breakdown of formaldehyde to form formic acid produces an acidic solution which in turn reacts with hemoglobin to produce an artefact pigment (acid formaldehyde hematin). The most popular formaldehyde solution in use today is therefore buffered to pH 6.8 – 7.2 for this reason.

Osmolality: The osmotic effects exerted by the fixative are more visible at the ultrastructural level, because the phospholipid membranes get easily damaged by excessively hypotonic/hypertonic solutions. Generally the osmolality of the vehicle (buffer) is more and it is always better to adjust it to that of tissue fluid (eg. formalin in isotonic saline). To avoid the damage to tissues caused by non-isotonic fluids such as water before actual fixation starts, it is better to keep them moist with gauze soaked in isotonic saline for a short time, but never immerse in saline for extended periods.

Fixing agents

There are a number of reagents that are regularly used to fix tissues.

Formaldehyde: Formaldehyde (CH_2O) is the gaseous aldehyde, dissolved in water to saturation (at 37% – 40% w/v) and this generally referred to as “formalin”. For fixation, one part formalin is usually diluted with nine parts of water or buffer. This produces a 10% formalin solution which contains about 4% formaldehyde w/v, an optimal concentration for fixation. To avoid the formation of paraformaldehyde (highly polymerised form of formaldehyde which gets deposited as a white precipitate in concentrated formaldehyde solutions), small amount of methanol (up to 15%) is added to proprietary solutions. Unbuffered formalin will slowly oxidize to formic acid, lowering the pH and react with hemoglobin in tissues producing acid formaldehyde hematin a brown-black granular artefact pigment which is deposited in blood-rich tissues. This often gets confused with micro organisms or other pathological pigments. For this reason 10% formalin solutions are usually buffered to pH 6.8 – 7.2.

Formaldehyde reacts with the side-chains of proteins to form reactive hydroxy-methyl groups. It can penetrate nuclear proteins and nucleic acids stabilizing the nucleic acid-protein shell and modifying the nucleotides by reacting with free amino groups. Formaldehyde can also react with some groups in unsaturated lipids particularly if calcium ions are present, but does not react with carbohydrates. Though washing can reverse some of these reactions, cross-linkages remain. The ability to preserve the peptides of cellular proteins has made formaldehyde the best general purpose fixative.

Glutaraldehyde: or glutaric dialdehyde ($\text{CHO}(\text{CH}_2)_3\text{CHO}$) is regarded as a bifunctional aldehyde, possessing aldehyde groups at either end of the molecule and react with the same chemical groups as formaldehyde does. Glutaraldehyde fixed tissues will be more extensively cross-linked (irreversibly), though this may adversely affect certain immunohistochemical staining, it provides excellent ultrastructural preservation and hence considered as the best primary fixative for electron microscopy. The rate of penetration is rather slow and tissue thickness should be less than 1 mm for satisfactory fixation. For electron microscopy glutaraldehyde is available in sealed ampoules and can be added to a suitable buffer at pH 7.2 – 7.4 (usually

cadodylate, phosphate or maleate) to produce a 3% glutaraldehyde concentration for use.

Other commonly used fixatives include

Mercuric chloride: Mercuric chloride (HgCl_2) is a powerful protein coagulant, react with amines, amides, amino acids and sulphhydryl groups, producing cross-links. It reacts with phosphate residues of nucleic acids and effectively fixes nucleoproteins. Disadvantages include corrosive nature, toxicity and formation of greenish-brown artefact in tissues. Treatment with Lugol's iodine during processing or sections prior to staining, followed by treatment with sodium thiosulphate is widely used to clear this.

Zinc salts: Zinc sulphate (ZnSO_4) and zinc chloride (ZnCl_2) are used to replace mercuric chloride in many formulated fixatives. Zinc enhances fixation and staining, particularly of nuclei, as in the case with mercuric chloride, are less toxic than mercury salts.

Picric acid: Picric acid or trinitro phenol ($\text{C}_6\text{H}_2(\text{NO}_2)_3\text{OH}$) Picric acid is a coagulant fixative which changes the charges on the ionisable side chains of proteins and disrupts electrostatic and hydrogen bonds and is always used in combination with other agents. It imparts a yellow colour to tissues during fixation and has to be washed from tissues with 70% ethanol before processing and residues can alter the staining characteristics of the tissues.

Potassium dichromate: Potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$) acts as a coagulant at $\text{pH} < 3.4 - 3.8$, and is a component of several compound fixatives.

Ethanol and methanol: Ethanol ($\text{CH}_3\text{CH}_2\text{OH}$) and methanol (CH_3OH) are coagulants that denature proteins, they replace water in the tissues by disrupting hydrophobic and hydrogen bonding thereby altering their tertiary structure and their solubility in water. Fixation starts at a concentration of 50 – 60% for ethanol and $>80\%$ for methanol. Methanol is commonly used to fix blood films and 95% ethanol is used as a fixative for cytology smears but combinations of these with other agents are widely used.

Acetone: Acetone (CH_3COCH_3) though resemble alcohol in its activity, is generally used as a fixative and dehydrant for tissue processing, particularly of small specimens. Generally not used in tissue processors because it may affect seals and other components of the equipment.

Acetic acid: Acetic acid (CH_3COOH) is coagulant that reacts with nucleic acids but generally does not fix proteins. Hence is incorporated in various compound fixatives to preserve nucleic acids and to counter the shrinkage caused by other ingredients such as ethanol. Though penetration is rapid, it can lyse red blood cells.

Histopathology procedures for finfishes: The most widely used fixatives are formaldehyde and Bouin's fixative. Small fish/fry can be directly dropped into the fixative while in larger samples, incisions should be made into the abdomen so that the fixative reaches the internal organs/viscera. It is always advisable to make incisions in the tissue (4 mm size) and transfer into a vial of fixative (20 times the volume of tissue)

Histopathology procedures for Bivalves: Bivalves less than 6 cm in length (shucked) can be fixed whole, by dropping into preservative. Animals must be shucked cleanly from the shell by severing adductor muscles prior to fixation. For perfect fixation, larger bivalves require 3 incisions (anterior, mid, posterior) made across the surface of the animal about mid-way through the tissues. Never cut completely through the animal so that tissues do not get mixed. Common fixatives used for bivalves are Helly's fixative, Bouin's fixative or Davidson's fixative. Small bivalves can either be embedded whole or cut longitudinally on the median axis and both tissue halves placed face down within a cassette.

Bivalve Larvae: Are fixed in a test tube of Helly's fixative and centrifuged @ 1,500 rpm for 10 minutes. Discard supernatant and embed the larvae in an agar plug. Remove the plug from the test tube for dehydration and embedding in wax in the usual manner (trim if necessary).

Histology of Shrimp: The chitinous exoskeleton of shrimp prevents adequate penetration of fixatives.

Hence, the fixative must be injected into the internal areas of each animal before dropping the whole shrimp into the fixative. The fixative is injected using a 10-ml syringe with an appropriately sized needle. Subsequently, the cuticle is slit from the last (6th) abdominal segment to the base of the rostrum. The incision in the cephalothoracic region should be lateral to the dorsal midline and that in the abdominal region should be mid-lateral. This will help to break the cuticle and allow sufficient fixative penetration. After injection and body incisions, the animal can be dropped whole into the fixative and after 48 hrs the animal is transferred to 70% ethyl alcohol. Commonly used fixatives for shellfish: Davidson's fixative, Buffered formalin & Helly's Fixative.

Getting best results in fixation

The key to good fixation are:

Fresh tissue

- Fix as soon as possible, the moment cells are deprived of blood supply, degeneration starts.
- If unable to fix immediately better refrigerate, but never freeze the tissues.
- Do not allow specimens to dry, desiccation/shrinkage of surfaces may result in permanent damage.
- Distortion/other mechanical damage will result in altered morphology, making interpretation difficult
- Precise labelling is absolutely essential.

Proper penetration of fixative

- Always place specimens in containers with fixative so that good penetration from all sides is assured. Cavities/hollow organs should be slit open/exposed to allow access of fixative.
- Fixation by perfusion of vascular system of whole organs or small experimental animals may produce uniform fixation.
- Thickness of specimen/tissue should not exceed 4mm
- Gentle agitation/swirling of the specimen during its first few minutes in fixative will help better penetration.
- Volume of fixative should be in excess (20:1 at least).

- Allow sufficient time for fixation.

Right choice of a correctly formulated fixative

- Always use quality reagents.
- Unstable fixatives should be made up from stock solutions immediately before use.
- Never reuse fixatives
- Avoid metal lids and containers.

The fixed tissues are washed, dehydrated in ethanol series, cleared, embedded in paraffin and sectioned at 5-7 μm thickness. The sections are then stained with Haematoxylin & Eosin and observed under the microscope.

Staining

Hematoxylin and Eosin (H&E) is the most widely used stain in routine histology and histopathology studies. Hematoxylin is a natural dye obtained from the heartwood of *Haematoxylon campechianum*, a tree commonly found in Central America. In its pure form haematoxylin is a colourless or slightly beige powder which cannot stain and the active staining agent is an oxidation product, haematein (usually at an acid pH). Haematein can be produced naturally through exposure to air and sunlight or UV light (a process referred to as 'ripening') but is a time consuming process. The use of a chemical oxidants hastens the process and various agents like potassium permanganate, iodine, sodium iodate, sodium periodate, potassium periodate, hydrogen peroxide or mercuric oxide etc are used. Mayer's and Ehrlich's haematoxylin use sodium iodate, whereas Harris' haematoxylin relies upon both vigorous boiling and addition of mercuric oxide to generate haematein. The advantage of chemical oxidation is—a working dye can be produced immediately, but care should be taken to avoid over-oxidizing the haematoxylin.

Though the actual staining is done by haematein, a mordant for haematoxylin is added to stain tissues effectively. Various metal salts are used as mordants with haematoxylin, but those with aluminium, iron or tungsten are better. A combination of mordant and dye is known as a 'lake' and the haematoxylin-

mordant lakes are often positively charged, behaving as cationic dyes at low pH. Haematoxylin is the most widely used natural dye in histology/histopathology and among the various hematoxylin preparations used in histology, Gill's hematoxylin, Harris's hematoxylin and Mayer's hematoxylin are the most popular. Hematoxylin acts like a basic dye with a purplish blue colour. It stains acidic, or basophilic, structure including the cell nucleus (which contains DNA and nucleoprotein), and organelles that contain RNA such as ribosomes and the rough endoplasmic reticulum. In a clinical histology laboratory, all specimens are initially stained with H&E and special or advanced staining is carried out if required.

Haematoxylins can be used as either progressive or regressive stains. Solutions such as Mayer's, and Gill's, will not overstain and can be used progressively. Progressive reactions provide a mechanism for highly selective staining but often require extended reaction times to maximize dye-tissue binding and produce sections of high contrast. On the other hand, regressive techniques are designed to overstain and the solution will stain a range of tissue components including nuclei. The overstained sections are differentiated carefully using a weak acid solution (normally prepared in 70% ethanol to improve control) to achieve acceptable level of nuclear staining. Staining with Ehrlich's & Harris haematoxylin is done using regressive staining.

Eosin is an acidic dye that is typically reddish or pink and which requires an acidic environment to work. In solution the dye molecule is negatively charged and stains basic, or acidophilic, structures which include the cytoplasm, cell walls, and extracellular fibres.

Staining procedure:

Dewax and rehydrate slides

a) Immerse slides in the following sequence of baths:

- Xylene I–10 minutes
- Xylene II–10 minutes
- Xylene III–10 minutes
- 100% ethanol–2 minutes

- 95% ethanol–2 minutes
- 50% ethanol–2 minutes
- Water–2 minutes

Staining

- Immerse slides in Hematoxylin solution for 10–20 minutes.
- Rinse 2 minutes in water or Scott's Tapwater substitute.
- Differentiate with 2–5 dips in 70% ethanol containing 0.1% HCl.
- Immerse for 2 minutes in Scott's Tapwater substitute.
- Stain in 1% aqueous Eosin with 0.1 acetic acid for 10 minutes.

Dehydrate, clear and mount

a) Immerse slides in the following sequence of baths:

- 50% ethanol–2 minutes
- 95% ethanol–2 minutes
- 100% ethanol–2 minutes
- Xylene I–10 minutes
- Xylene II–10 minutes
- b) Mount coverslip with DPX

Results

- Nuclei–blue.
- Other tissue components–shades of red and pink.

H&E stains almost all the cell structures including cytoplasm, nucleus and organelles as well as extracellular components and can reveal changes in the general organization of the tissues/cells and abnormalities or specific indicators/clues of diseases/pathogens thereby aiding diagnosis. Thus H&E staining provides a clear basic picture of the tissue and hence forms a critical part of histopathology. Various other advanced staining procedures/techniques are widely employed in histopathology to stain particular tissues/structures depending on the specific requirement. In clinical histology, all specimens are initially stained with H&E and special or advanced staining is carried out only if additional information/details are required to get more details to clear any ambiguities/doubts.

Electron Microscopy

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Microscopy is the science of visualizing objects that cannot be seen with the naked eye. Optical or light microscopy involves passing visible light through a series of lenses to allow a magnified view of the sample. Light microscopy has advanced much since its discovery and has attained the limit of resolution imposed by the nature of light in the past century itself. The major limitation of light microscopy is that, diffraction limits its resolution to approximately 0.2 micrometres, providing a useful magnification of ~1000x which is good for obtaining information up to cellular level. When it comes to the study of diseases, understanding the pathogen and the pathogenesis at cellular levels are very important and for this far higher resolutions are necessary. Electron Microscopes are instruments that use a beam of highly energetic electrons to examine objects on a very fine scale and function exactly like their optical counterparts. With its very high resolving and magnifying powers, electron microscopy allows visualizing structures within individual cells and can identify cell types/organelles/pathogens which are far beyond the reach of light microscopy. Modern electron microscopes are capable of providing magnifications up to 1200000 X with a resolving power of less than 0.1 nm. Transmission electron microscopy (TEM) can reveal ultra structural details at cellular levels, whereas Scanning electron microscopy (SEM) can show the morphology of minute structures/organisms in its three-dimensional state. Combining the TEM and SEM, it has become possible to study and classify the viruses and virus like organisms.

Commonly employed methods for disease diagnosis include histology, serology, microbiology, molecular diagnostics and electron microscopy and each method has its own advantages and disadvantages.

Histology uses light microscopy and is still an invaluable tool in disease diagnosis, the advantage being – its less sophisticated nature and simplicity. However, in many cases it cannot provide enough evidence to make a confirmatory diagnosis. In the case of viral infections, one can observe CPEs, lesions or inclusions, which are only suggestive of a specific viral infection through histopathology. Moreover, due to the limited magnification and resolution, ultra structural/sub cellular changes and minute pathogens/stages cannot be observed. Whereas TEM can clearly provide information on the morphology of pathogens, sub cellular changes / particles / structures etc.

Sero-diagnostic methods play an important role in disease diagnosis, especially in field conditions and serology still remains the mainstay of viral diagnosis. The tests are generally based on specific antibodies (immunoprobes) and can detect sub clinical / latent / carrier states of infection. However, the draw backs of serological tests are (a) highly variable sensitivity & specificity (b) many viruses often produce clinical disease before the appearance of antibodies (c) less useful in the case of latent viruses (d) antigenic cross-reactivity between related viruses may lead to false positive results and (e) less effective in invertebrates which does not produce antibodies.

Microbiological methods are widely used for the diagnosis of bacterial infections and involve culture, isolation and identification of the pathogens. But the procedure is tedious and time consuming and may even take weeks in some cases. In the case of non-culturable organisms/pathogens, microbiological methods may fail to provide conclusive results.

Molecular biology tools involve the detection of genetic material of pathogens using molecular probes

and is presently considered as the most important tool in disease diagnosis. Advantages of molecular tools include (a) extremely high sensitivity (b) easy to set up and (c) fast turnaround time. Disadvantages are (a) expensive (b) extremely liable to contamination (c) high degree of operator skill required (d) quantitative assay difficult and (e) difficulty in interpreting positive results, especially with latent viruses and (f) though they are more sensitive, are only capable of identifying the presence of previously identified agents and thus fail to identify new/emerging pathogens.

Electron microscopy can be an important adjunct to conventional culture and serologic techniques in diagnosing viral diseases. Though detection of viruses by E M requires relatively large numbers of virions, and cannot provide information on the specific serotypes within a virus family, it has the distinct advantage of being simple and rapid. Some viruses do not grow in tissue culture or grow only after special manipulation, and may not survive if transportation conditions to the lab are not optimal. Naturally, culturing would miss these agents. Additionally, a wide variety of agents can be visualized by E M; because specific reagents such as antibodies, antigens, or nucleic acid and protein probes are not required, one is not limited to the availability of these reagents, and prior knowledge of the virus identity for reagent selection is not required. Diagnostic electron microscopy has two advantages over enzyme-linked immunosorbent assay and nucleic acid amplification tests. After a simple and fast negative stain preparation, the undirected, "open view" of electron microscopy allows rapid morphologic identification and differential diagnosis of different agents contained in the specimen.

The biggest advantage of electron microscopy lies in the fact that it provides direct visual evidence of various pathogens/biological processes, while most of the other techniques are indirect and in some instances non-specific. Electron microscopic diagnosis is uniquely suited for rapid identification of infectious agents. A specimen can be ready for examination and an experienced virologist or technologist can identify, by electron microscopy, a viral pathogen morphologically within 10 minutes of arrival in the electron microscopy laboratory. Once the histopathological observations using light

microscopy provides primary information on the target tissues, electron microscopy can be employed to visualize the pathogens and study its morphology. Electron microscopy can also provide information on the ultrastructural modifications/changes at sub-cellular levels caused by the pathogen. So compared to other diagnostic methods, E M benefits from an "open view", it also reveals double infections and the presence of agents that might not otherwise have been considered. Finally, since the test entails the visualization of the virus itself, rather than a color change or agglutination reaction, false positive tests resulting from cross-reactions of reagents with similar materials are not likely. Hence electron microscope can be considered as the ultimate tool in identifying the etiology of emerging diseases.

Two types of preparations are primarily used for routine EM virus identification, negative staining and thin sectioning, although specialized research techniques such as specific antibody aggregation or labeling with electron-dense tags, *in situ* labeling, cryomicroscopy, and high-voltage microscopy have also been used to classify viruses and describe virus-host relationships. With the simple negative staining preparation available, E M allows the rapid and direct detection of an etiological agent on a sample from a patient, or from diagnostic cell cultures.

Negative staining of liquid samples is fairly rapid, and can provide an answer within a few minutes to a couple of hours. It enables the examiner to view cell particles and organelles in isolation. The isolated cell/particle is placed in a "puddle" of staining material, usually uranyl acetate or phosphotungstic acid, and is then supported on a thin, plastic/formvar film. The stain molecules deposit into surface crevices in the specimen during the drying process and typically produce a "ghost" image in which the specimen appears light against a dark background.

Sensitivity and specificity of E M may be further enhanced by immuno electron microscopy, which includes classical immune electron microscopy and solid phase immuno electron microscopy. In classical immuno electron microscopy, the sample is treated with specific anti-sera before being subjected to electron microscopy. The viral particles present will

be agglutinated and thus congregate together by the antibody, making them easily visible. In solid phase immuno electron microscopy the grid is coated with specific anti-sera. The virus particles present in the sample will be absorbed onto the grid by the antibody thus enhancing the visibility under the microscope.

Advantages: The most important among the benefits offered by the electron microscope is undoubtedly the very high resolution. Since timely and accurate diagnosis forms the first step in the health management of farmed fishes and shellfishes, the right diagnosis defines the very success of disease control. Though E M has an important role in the diagnosis of viral infections, it is equally useful in the diagnosis and understanding the pathogens as well as the pathological changes caused by various other pathogenic organisms. As a confirmatory diagnostic method for many of the existing and emerging diseases, especially of viral origin, electron microscopy still remains an indispensable tool in the field of disease investigation and control. To exploit the full potential of diagnostic electron microscopy, it should be quality controlled, applied as a frontline method, and be coordinated and run in parallel with other diagnostic techniques.

Disadvantages: However, the disadvantages of E M in the diagnosis of infections are (a) detection of viruses by electron microscopy requires relatively large numbers of virus particles (b) possibility of false negatives, if concentration is very low (c) provides no information regarding specific serotypes within a virus family and (d) factors like high cost of operation and infrastructure, need for skilled technical personnel, laborious and time-consuming procedures, expertise needed for interpretation etc. restricts the use of electron microscopy as a routine diagnostic tool.

Specimen processing

Sampling: Soon after the death of the organism, post mortem changes sets in, making the tissue unsuitable for ultra-structure studies. Hence for electron microscopy, always live animals are preferred. The animals are sacrificed, the desired tissues/samples dissected out and immediately placed in cold fixative or perfusion carried out to ensure uniform fixation of

tissues in the case of small animals. The desired size of the tissue to achieve proper fixation is about 1 mm. Very small animals and larvae less than 2 mm size are fixed whole in ice-cold fixative in live condition. The sample vial should be labelled properly.

Fixatives: Fixatives help to preserve the structures in the living cell and prevent changes induced by autolysis. There is no single ideal fixative and so a combination of fixatives is preferred depending on the type and nature of the tissues. In electron microscopy, 2 to 4 % Glutaraldehyde is used as the primary fixative which is excellent in fixing nucleic acids, nuclear proteins and carbohydrates but not lipids. Poor contrast and slow penetration are the limiting factors of glutaraldehyde fixative. Osmium tetroxide is used for secondary fixation. It acts as both fixative as well as stain, fixes nucleic acids, carbohydrates and lipids and provides contrast and fast penetration. The combination of glutaraldehyde and Osmium tetroxide as primary and secondary fixatives, gives the desired results in contrast and resolution. Fixatives are prepared in a suitable buffer for two reasons, to maintain the pH (7.2 to 7.4) and to maintain the osmolality, in order to minimize the swelling or shrinkage of the tissues which may otherwise lead to artifacts. The most commonly used buffer is Sodium Cacodylate buffer, while phosphate buffer is also used.

Primary fixation: Tissues are fixed in 2 to 4% Glutaraldehyde in 0.1 M Cacodylate buffer (in the case of marine species, 3 to 5% NaCl or sucrose can be added to the fixative). For proper penetration of the fixative, the tissues should not exceed 1 mm in size. Fixation is carried out for 4 to 6 hrs (varies depending on the nature of the tissues), at 4°C. After fixation, the fixative is drained and tissues washed thrice (15 mts each) with buffer. In case of larger tissues, further trimming is done if required and washed with fresh buffer.

Secondary Fixation or Post fixation: For secondary or post fixation, the washed tissues are transferred to 1% Osmium tetroxide (OsO_4) in 0.1 M cacodylate buffer, kept for 1- 2 hrs at 4°C (above 4°C, OsO_4 disintegrates). OsO_4 treatment turns the tissues black. OsO_4 is drained and tissues washed two to three times

with buffer, for 15 min each, or until free of a black precipitate formed from excess OsO_4 . Samples can be stored in buffer under refrigeration until further processing is desired. (Since OsO_4 is highly toxic, care must be taken while handling. Always use gloves and carry out all operations under a hood).

Dehydration: Dehydration is done through graded alcohol or acetone series to remove the water from the tissues.

Dehydration can be done as follows

30% Acetone	two changes, 15 mts each at 4°C
50% Acetone	two changes, 15 mts each at 4°C
70% Acetone	two changes, 15min each at 4°C, (can be stored in 70% acetone indefinitely)
80% Acetone	two changes, 15 mts each at 4°C
90% Acetone	two changes, 15 mts each at 4°C
95% Acetone	two changes, 15min each, 4°C
100% Acetone	two changes, 15 min each, 4°C
100% Acetone	two changes, 30 mts each at room temperature
Propylene oxide	two changes of 15 min each at room temperature

* Analar grade acetone is used to ensure proper dehydration

Infiltration and Embedding: Fixed and dehydrated tissues are infiltrated with liquid plastic resins and then cast into blocks. The purpose of embedding is to allow future ultra thin sectioning of the material. Commercially available Plastic resins like Epon or Spurr are used for embedding. The media is mixed as per the instructions, under a fume hood. Prepare fresh media 2 to 3 hrs. prior to use, as it will absorb water vapor from surroundings and the components will begin to polymerize. Mixture of embedding medium (Spurr's medium) and acetone is prepared in various grades (mix. A – medium and acetone in the ratio 1: 3, mix. B – medium and acetone in the ratio 2 : 2 and mix. C – medium and acetone in the ratio 3 : 1) and the tissue kept in each for 1 to 2 hrs each or as specified (period varies with the medium used) for infiltration. For embedding, medium is prepared as instructed, poured into readymade moulds made of plastic or silicon rubber and infiltrated tissues transferred to it, taking care not to trap any air bubbles. The moulds are then kept in an incubator at 70°C for 12 to 24 hrs.

Each tissue with reference to the experimental

objective requires an evaluation of the methods, subjected to a careful examination of pertinent literature. There is no schedule that will work for all tissues and conditions.

Trimming: The resin blocks are trimmed to remove the unwanted areas using a glass knife fitted to an Ultramicrotome.

Sectioning and staining: To achieve high resolution for electron microscopy, the sections should be very thin (60 nm) and are prepared using an ultramicrotome. The resin blocks are trimmed using a glass knife. Standard procedures are followed for obtaining semi-thin and ultra-thin sections for light and electron microscopy respectively. Semi-thin sections are first taken, stained with methylene blue and observed under a light microscope for determining the area for ultra-thin sectioning. The blocks are again trimmed and ultra-thin sections taken. These sections are floated on distilled water, stretched to remove the wrinkles and collected over the matt/dull surface of the copper/nickel grid.

Staining: Double staining with Uranyl acetate and Lead citrate is employed for routine electron microscopy studies. The sections are first stained with Uranyl acetate. A drop of Uranyl acetate (saturated solution in 50% ethanol) is taken on a clean glass slide and the grid with the section side down is kept on to the stain drop and is covered with any opaque object to ensure darkness to carry out the staining effectively. After 10–15 mts, the grid is taken out and washed 3–4 times in double distilled water (ensuring that the sections are not washed away) and dried with a filter paper. The grids are then stained with Lead citrate for 1–4 mts., washed well and dried. In the case of particulate specimen, the specimen is taken on formvar-coated grids, subjected to negative staining using 1-3% Phosphotungstic acid and dried.

Observation and photography: The grid carrying the stained section is loaded into the electron microscope, the image observed and recorded on photographic plates/film or digitally. In order to study and interpret EM results one has to have a thorough knowledge about the ultrastructure of the normal cells and the pathogen.

Scanning Electron Microscopy: The scanning electron microscope, like the TEM consists of an electron optical column, a vacuum system and electronics and works under the same principle as that of the TEM. The electron gun produces an extremely fine beam of electrons, which are focused into a fine spot less than 4 nm on the specimen and scanned in a rectangular raster over the specimen. The secondary electrons produced by the interaction of the electron beam with the specimen surface as well as the backscattered primary electrons (depending upon the topography) are detected using a suitable sensor/detector. The signals from the detector are electronically amplified to modulate the brightness of a Cathode Ray Tube (CRT) so as to produce an image, which can be recorded photographically.

Specimen preparation for S E M: The specimen is first fixed with glutaraldehyde as in the case of TEM and washed well in buffer. Post fixation with OsO_4 is optional. The specimen is subjected to dehydration using ascending grades of acetone as in TEM processing. The dehydrated specimen for SEM has to be dried without causing any shrinkage. Except in the case of fine particulate specimen, critical point drying or freeze drying is usually preferred for drying

SEM samples. The dried specimen is then coated with a thin conductive metal film (Gold, Palladium etc.) using an ion coater to prevent charging artifacts and to stabilize the specimen mechanically. Variable pressure SEM's can operate without high vacuum thus avoiding the time consuming specimen preparation techniques as well as reduce specimen damage caused during coating.

Benefits: The most important among the benefits offered by the electron microscope is undoubtedly the very high resolution (as low as 0.1 nm) and magnification (up to 12, 00,000 X) in TEM and 0.4 nm resolution with a magnification of up to 800,000X in SEM. Since timely and accurate diagnosis forms the first step in the health management of farmed fishes and shellfishes, the right diagnosis defines the very success of disease control. Factors like high cost of operation and infrastructure, need for skilled technical personnel, laborious and time-consuming procedures, thorough knowledge needed for interpretation etc. restricts the use of electron microscopy as a routine diagnostic tool, but as a confirmatory diagnostic method for many of the existing and emerging diseases, especially of viral origin, electron microscopy still remains an indispensable tool in the field of disease investigation and control.

Fish Immunological Techniques

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Introduction

The vertebrate immune system is considered to be the strategically advanced protective system which is capable of responding to many of the infective challenges that arise in the body and its environment. Such immune response from the immune system follows two phases. The first and foremost phase is the recognition of the pathogen or other foreign material and second phase involves the effort of the body to eliminate the disturbance. An immune response from the body falls into two categories; the innate immunity and the adaptive or acquired immunity. The innate immunity forms the first line of defence against any infectious agent. The adaptive immunity is the specific counterpart which becomes more and more effective over several days after initial activation. The most important cell types in the acquired immunity are B and T lymphocytes. The B lymphocytes produce antibodies on antigenic stimulation. An antigen is any foreign substance capable of producing an immune response leading to the production of antibodies which is specific to particular antigen. Immunologists developed a number of techniques based on the selective reversible and covalent binding of antibody to specific antigen. Such immunological techniques forms integral to many clinical, pharmaceutical and basic scientific investigations. The widely used immunological techniques, their principles and application are described in this chapter.

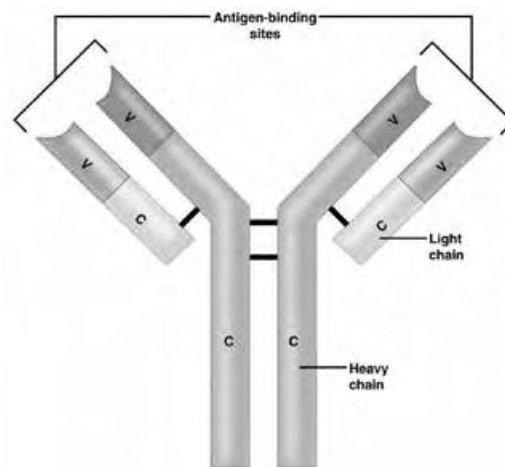
Characteristics of immunological techniques:

- Simple, rapid and robust
- Highly sensitive
- Easily automated – Applicable to regular clinical laboratories

- Does not require extensive sample preparation
- Do not require expensive instrumentation
- Mostly based on simple photo-fluorimetric detection
- Measurements may be either qualitative/quantitative

Methods of Analysis:

All immunochemical methods are based on a highly specific and sensitive reaction between an antigen and an antibody. Structurally, antibodies are often visualized as Y-shaped molecules, each containing 4 polypeptides – 2 identical polypeptide units called heavy chains and another 2 called light chains. It has a domain called Fab, the site where it binds to

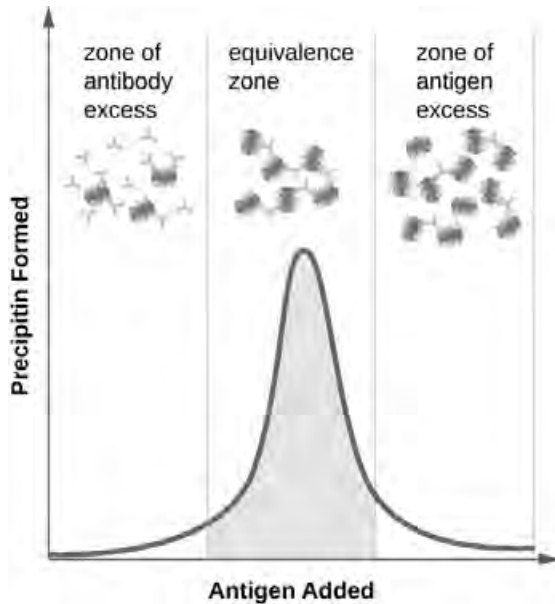


Uroteuthis (Photololigo) duvauceli
an antigen.

The region of an antigen binding to an antibody is called an **epitope**. The measure of the strength of the binding is called **affinity**, and it is usually expressed in terms of the concentration of an

antibody-antigen complex measured at equilibrium. It is measured by quantitative precipitin curve (basis for many immunochemical techniques) proposed by Heidelberger and Kendall in 1935.

Quantitative precipitin curve: It describes the relationship between the antigen concentration and the amount of precipitate for a constant quantity of



an antibody. Three zones can be distinguished from the precipitin curve:

- I. Antibody excess zone – first phase where less antigen is present in sample
- II. Equivalence zone – both antigen and antibody are cross-linked forming precipitate; no free antigen or antibody is present
- III. Antigen excess zone – amount of precipitate reduces due to high antigen concentration

The precipitin curve forms the basis of most of the immunological techniques that can be performed in laboratories

Types of Antibody Used

The antibodies used for the methods are produced by various ways:

1. Monoclonal antibody – products of single clone of plasma cells by B lymphocytes; mostly prepared in laboratory. They are directed against single epitope – identical copies with same structure and antigen specificity. They have excellent specificity but poor ability to precipitate antigen.
2. Polyclonal antibody – they are conventional, *i.e.*, produced by immunization of animals with antigen. Thus antibody consists of mixture of monoclonal antibodies having specificity for complex antigens. Sometimes monoclonal is called as “monovalent” and polyclonal as “polyvalent” which indicates the antigen specificity.

Applications of immunological tests

- Antigen – Antibody interactions: Where the high specificity of the antibody is used to identify, isolate or quantify the antigen
- To identify the cell populations: Cell populations are characterised by their surface markers, using techniques of immunofluorescence and immunohistochemistry
- To isolate cell populations: Also done by the surface markers of the cell, using fluorescence activated cell sorting, panning and density dependant centrifugations
- Principal assay for lymphocyte functions: Assays for antibody/cytokine production, proliferation in response to antigen or by cytotoxicity

Techniques based on antigen-antibody interactions:

These methods exploit the property of antibody – antigen complex and use antibodies as reagent to detect and quantify antigens. This method is subdivided to two steps

Particle method: Can be divided into two categories

Precipitation of the large immune complexes:

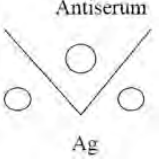

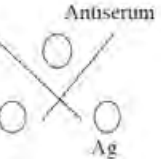
The reaction which occurs when specific antibody combines with soluble antigen is known as the

precipitation reaction. This reaction requires that the antigen contain multiple binding sites (epitopes) for the antibody or anti-sera used. This allows for cross-linking to occur with the formation of large macromolecular species. These large complexes become insoluble and subsequently precipitate. The precipitate may be read visually in a gel such as in immunofixation electrophoresis or may be measured by an increase in light scatter in a nephelometer. Some common techniques where precipitation reaction is being used are described below:

Immunodiffusion

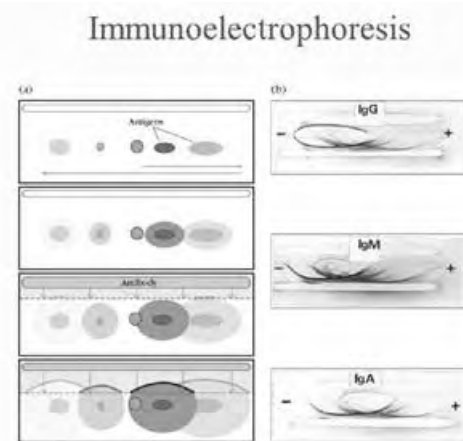
Immunodiffusion in gel encompasses a number of techniques that are used for the analysis of antigen antibody interactions. Immunodiffusion in gel is classified as single diffusion and double diffusion. In Ouchterlony double diffusion, both antigen and antibody are allowed to diffuse into the gel. This assay is frequently used for comparing different antigen preparations. In this test, different antigen preparations, each containing single antigenic species are allowed to diffuse from separate wells against the antiserum. Depending on the similarity between the antigens, different geometrical patterns are produced between the antigen and antiserum wells. The patterns of lines form can be interpreted to determine whether the antigens are same or different.

Different patterns of lines obtained on Ouchterlony double diffusion

	<p>Pattern of identity: A The antibodies in the antiserum react with both the antigen resulting in a smooth line of precipitate. The antibodies cannot distinguish between the two antigens. <i>i.e.</i>) the two antigens are immunologically identical.</p>
	<p>Pattern of identity: B In the 'pattern of partial identity', the antibodies in the antiserum react more with one of the antigens than the other. The 'spur' is thought to result from the determinants present in one antigen but lacking in the other antigen.</p>
	<p>Pattern of identity: C In the 'pattern of non-identity', none of the antibodies in the antiserum react with antigenic determinants that may be present in both the antigen <i>i.e.</i> the two antigens are immunologically unrelated as far as that antiserum is concerned.</p>

Immuno-electrophoresis

Immuno-electrophoresis combines two techniques; electrophoresis and immunodiffusion. It is a simple, quick and reproducible method for determining the concentration of antigen (Ag) in an unknown sample. Various concentrations of antigen are located side by side in small circular wells along the edge of an agarose gel that contains the specific antibody (Ab). On electrophoresis, the antigen begins to migrate towards the anode and interacts with antibody molecules to form a soluble antigen-antibody complex. However, as the samples electrophorese farther through the gel, more antibody molecules are encountered that interact with the antigen and when the "equivalence point" is reached. The Ag-Ab complex precipitates in the form of a rocket shape. Higher the amount of antigen loaded in the well, farther the antigen will travel through the gel. Hence, with increasing antigen concentration, a series of rockets of increasing heights are seen that is proportional to amount of antigen in the well. Therefore, a direct measurement of the height of rocket will reflect upon the antigen concentration.



A standard graph of antigen concentration versus peak height is then constructed and from the peak height of the unknown sample, concentration of antigen is determined.

Agglutinations

The reaction which occurs when antibodies react with particulate antigen is known as an agglutination reaction. Agglutination reactions may be designed with antigen or antibody bound to any large particle. Materials may include starch particles, synthetic

latex beads or red cells. The common thread in all types of agglutination systems is that the reaction is read visibly by the clumping of the particles when antibody crosslink with antigen.

Label methods

Involves the use of a label on either the antibody or antigen to identify antigen antibody complex formation

Radioimmuno assay (RIA)

Radioimmunoassays (RIAs) use antibodies to detect and quantitative the amount of antigen (analyte) in a sample. These assays are typically very sensitive and specific. It is possible to detect as low as a few picograms of analyte in the experimental tube when using antibodies of high affinity ($K_d = 10^{-8} - 10^{-11} \text{ M}$). The basic principle of radioimmunoassay is competitive binding, where a radioactive antigen («tracer») competes with a non-radioactive antigen for a fixed number of antibody or receptor binding sites. When unlabeled antigen from standards or samples and a fixed amount of tracer (labeled antigen) are allowed to react with a constant and limiting amount of antibody, decreasing amounts of tracer are bound to the antibody as the amount of unlabeled antigen is increased. Results obtained for the standards are used to construct a standard

(dose-response) curve from which the unknowns are calculated by interpolation.

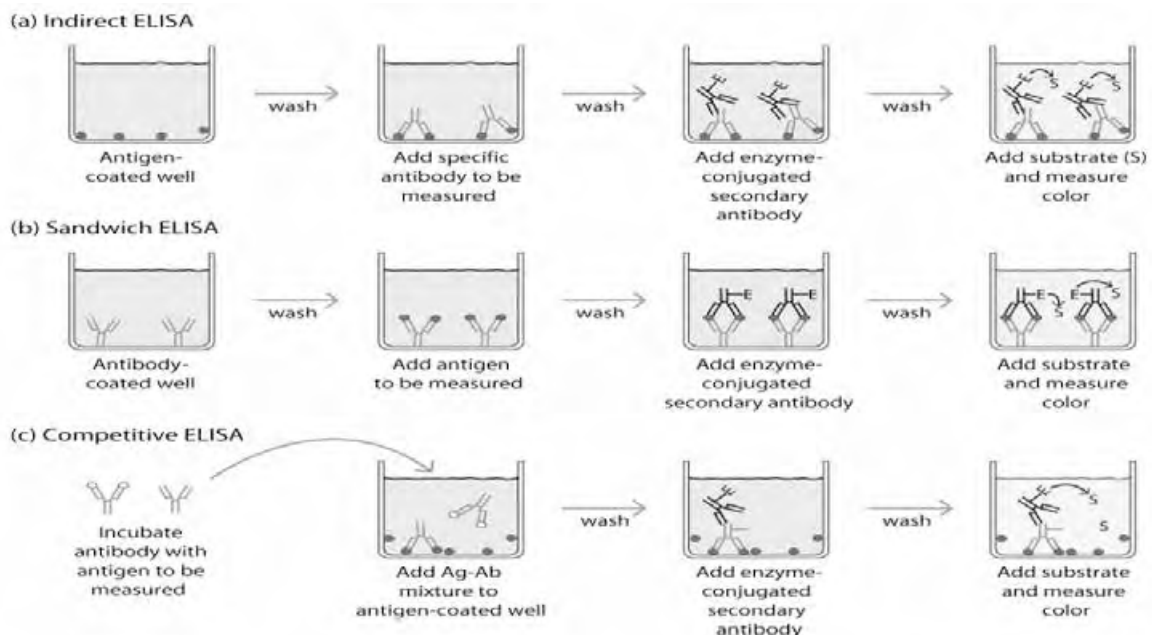
ELISA

Is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample. The technique is divided into;

Competitive ELISA: The labelled antigen competes for primary antibody binding sites with the sample antigen (unlabeled). The more antigen in the sample, the less labelled antigen is retained in the well and the weaker the signal)

Sandwich ELISA (also called direct ELISA): The ELISA plate is coated with Antibody to detect specific antigen. Prepare a surface to which a known quantity of capture antibody is bound and block any non specific binding sites on the surface. On applying the antigen-containing sample to the plate followed by a chemical which is converted by the enzyme into a coloured product, it will develop a colour. The absorbency of the plate wells is measured to determine the presence and quantity of antigen.

Indirect ELISA: In this technique, an antigen is passively adsorbed to solid phase by incubation and antibodies are added and incubated which are specific

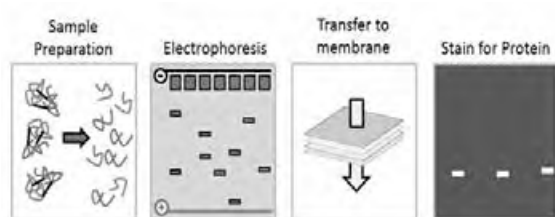


and will bind to antigen on the solid phase. Excess antibodies or nonbinding components are washed away after incubation phase. A secondary antibody labelled with enzyme (conjugate) directed against the particular species in which the original antibodies were produced (anti-species) were allowed to bind to any antibodies which are attached to antigen. Excess conjugate is washed away after a period of incubation. Substrate/ chromophore are added to this and a colour develops as a result of enzyme present. After a period of incubation the colour development is stopped and read by spectrophotometer.

After reading the results the standard curve is drawn where the concentration is blotted on the X-axis and the absorbance on the Y-axis. This standard curve is used to determine the unknown concentration of each sample by finding the opposite concentration to the absorbance.

Western blot

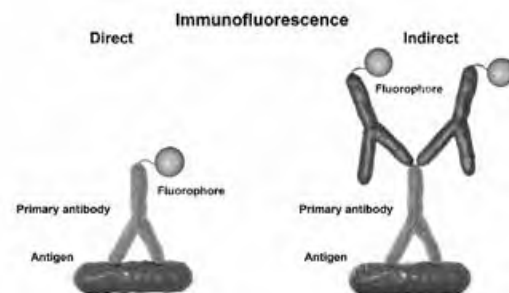
Western blotting, also known as immunoblotting or protein blotting is a core technique in cell and molecular biology. In most basic terms, it is used to detect the presence of a specific protein in a complex mixture extracted from cells. The western blotting procedure relies upon three key elements to accomplish this task: the separation of protein mixtures by size using gel electrophoresis; the efficient transfer of separated proteins to a solid support; and the specific detection of a target protein by appropriately matched antibodies. Once detected, the target protein will be visualized as a band on a blotting membrane, X-ray film, or an imaging system.



Immunofluorescence

In IF techniques, antibodies are chemically conjugated to fluorescent dyes such as fluorescein isothiocyanate

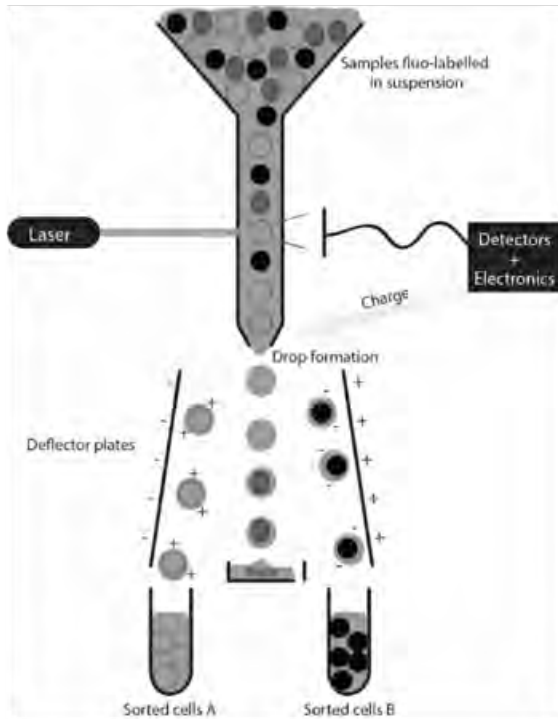
(FITC) or tetramethyl rhodamine isothiocyanate (TRITC). These labeled antibodies bind (directly or indirectly) to the antigen of interest which allows for antigen detection through fluorescence techniques. The fluorescence can then be quantified using a flow cytometer, array scanner or automated imaging instrument, or visualized using fluorescence or confocal microscopy.



The two main methods of immunofluorescent labeling are direct and indirect. Less frequently used is direct immunofluorescence whereby the antibody against the molecule of interest is chemically conjugated to a fluorescent dye. In indirect immunofluorescence, the antibody specific for the molecule of interest (called the primary antibody) is unlabeled, and a second anti-immunoglobulin antibody directed toward the constant portion of the first antibody (called the secondary antibody) is tagged with the fluorescent dye.

FACS

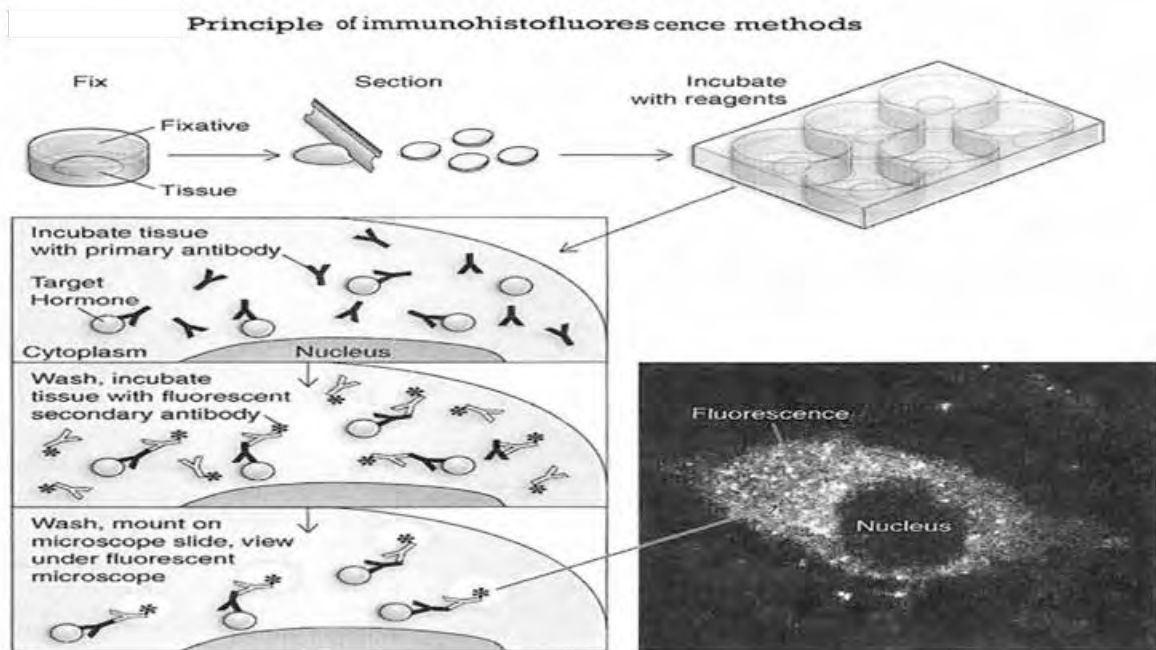
The traditional method for sorting cells is a fluorescence-activated cell sorting (FACS) machine. A suspension of cells containing varying amount of the protein of interest are placed into a flask. Antibodies that bond with the specific protein are coated with a fluorescent dye. Therefore, cells that contain the protein will also hold the fluorescent dye. A nozzle at the end of the flask is set to vibrate, which forms drops that contain only one cell at a time. The cells pass through the focus of a laser, operating at the wavelength of absorption for the fluorescent dye that is being used. Therefore, cells that hold the protein of interest will fluoresce. Traditionally, the fluorescence will be measured by a photomultiplier tube whose signal is being monitored by a computer. The



side of the plates will collect the cells. Therefore, the population has been sorted based upon the presence of the specific protein.

Immunohistochemistry

Immunohistochemistry (IHC) combines anatomical, immunological and biochemical techniques to identify discrete tissue components by the interaction of target antigens with specific antibodies tagged with a visible label. IHC makes it possible to visualize the distribution and localization of specific cellular components within cells and in the proper tissue context. Immunohistochemistry is a technique that uses antibodies (matching molecules) that can seek out, identify and attach themselves to these markers on cells. The antibodies themselves can be seen under the microscope, which helps the technician make precise identification.

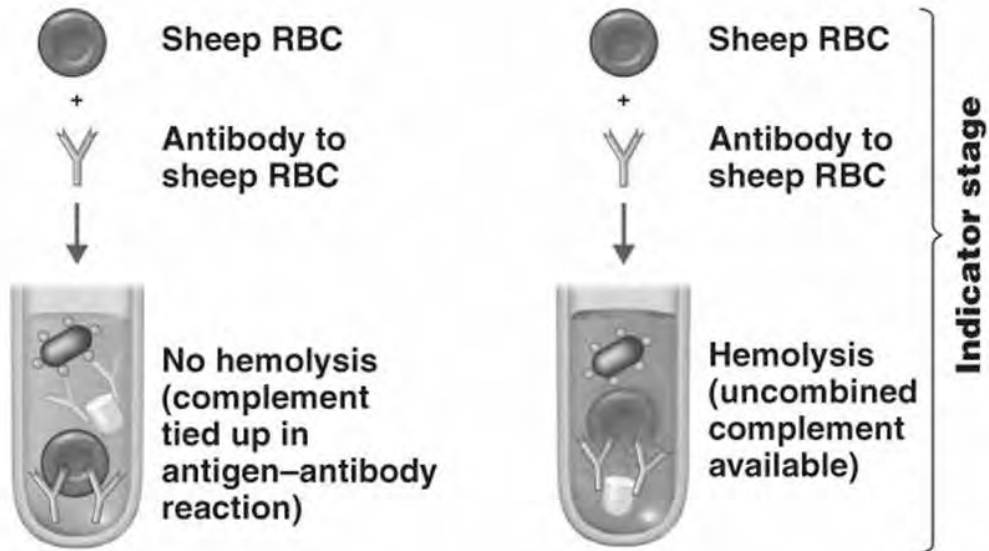


computer controls an electric wire which generates an electric charge in the cell. If the cell fluoresces (it holds the protein), it will be positively charged. Otherwise, it will be negatively charged. All of the cells then pass through two charged plates. The positively charged cells will be forced towards the negative plate and the negatively charged cells will be forced towards the positive plate. Two collection tubes placed at either

Compliment fixation test

A known volume of antigen is mixed with the test serum to be assayed for antibody and Ag/Ab complexes are allowed to form. At the same time a control tube in which no Ag is added is also prepared. If no Ag/Ab complexes are present in the tube none of the complement will be fixed. However, if Ag/

Complement Fixation Test



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Figure 18.10

Ab complexes are present, they will fix complement and thereby reduce the amount of complement in the tube. After allowing for complement fixation by any Ag/Ab complexes, a standard amount of red blood cells, which have been pre-coated with anti-erythrocyte antibodies, is added to the first tube. The amount of antibody-coated RBC is predetermined to be just enough to completely use up all the complement initially added if it were still there. If

all the complement was still present (*i.e.* no Ag/Ab complexes formed between the Ag and Ab in question), all the RBC will be lysed by the complement.

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Hybridoma Technology and its Use in Disease Diagnosis and Therapy

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Introduction

In a landmark discovery, Kohler and Milstein (1975) developed a technique that allows the growth of clonal population of cells secreting antibodies with a defined specificity. In this technique an antibody-secreting cell, isolated from an immunized animal is fused with a myeloma cell, a type of B-cell tumor. These hybrid cells or hybridomas can be maintained *in vitro* and will continue to secrete antibodies with defined specificity. Antibodies that are produced by hybridomas are known as monoclonal antibodies.

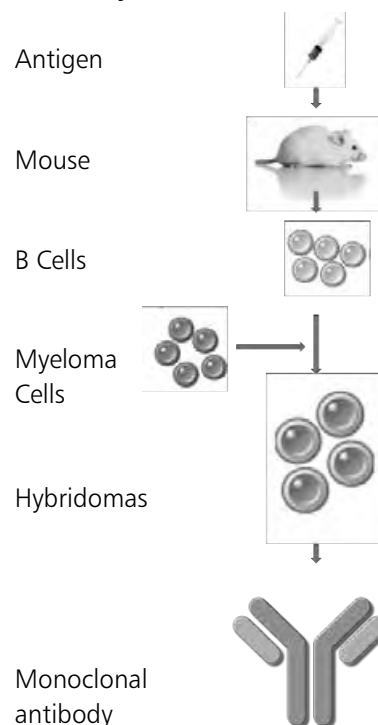
Stages of Hybridoma production

For the production of monoclonal antibodies, animals are injected with an antigen preparation, and once a good humoral response has appeared in the immunized animal, an appropriate screening procedure is developed. The sera from the test bleeds are used to develop and validate the screening procedure. For fusion, antibody-secreting cells are prepared from the immunized animal, mixed with the myeloma cells and fused in the presence of a suitable fusogen. Mostly PEG (polyethylene glycol) is used for this purpose. After the fusion, cells are diluted in selective medium (HAT: hypoxanthene, aminopterin and thymidine medium) which allows only the growth of fused myeloma-antibody secreting cells. Now hybridomas are tested and cells from positive wells are grown and then single cell cloned. These single-cell cloned hybridoma produces antibodies that are termed as monoclonal antibodies.

Applications

The usefulness of monoclonal antibodies stem from three characteristics their specificity of binding, their homogeneity and their ability to be produced in unlimited quantities. The production of monoclonal antibodies allows the isolation of reagents with a unique, chosen specificity. Because all of the antibodies produced by descendants of one hybridoma cell are identical, monoclonal antibodies are powerful reagents for testing for the presence of a desired epitope. These characteristics make them attractive for using them in diagnosis as well as therapeutic agents in various diseases.

Hybridomas Technology



Diagnostic uses

Antibodies produced in the mouse or rat are most commonly used for diagnostic purposes as they are more readily produced. The major advantage of monoclonal antibodies over conventional sera in diagnostic uses is their high specificity, which enhances the accuracy and speed of diagnosis and their ready availability for an infinite period at standard titer thus antibodies to common serum analytes such as protein hormones or alphafoetoprotein are already commercially marketed and are slowly replacing conventional sera. A very large number of monoclonal antibodies have been produced to a wide range of viruses such as influenza (Gerhard *et al.* 1981), hepatitis (Shih, Wands *et al.* 1981), polio (Ferguson *et al.* 1982), Epstein-Barr virus (Hoffman *et al.* 1980) and rabies (Wiktor and Koprowski, 1978). Their high specificity has led to accurate identification between similar strains of virus such as Simplex types I and II. They are also used for early diagnosis of the IgM production in affected patients.

The considerable potential of monoclonal antibodies in the study of parasitic diseases such as malaria, leishmaniasis and schistosomiasis has already been widely exploited. Monoclonal antibodies for immunological tissue typing are also produced by several laboratories.

Tumor diagnosis is undoubtedly the field in which there has been most interest in monoclonal antibodies at the present time. Efforts are on the way to produce monoclonal antibodies, which react solely with tumor associated antigens and can consequently be used as wide ranging diagnostic tools. However antibodies to tissue or cell type specific antigens are already generated and these have great potential in the detection of tumors and their metastases.

Characterization of viruses using Monoclonal antibodies (Mabs)

Using Mab technology, it is possible to produce a large amount of homogenous antibody against many antigenic epitopes on a virus. A single Mab

therefore can provide information on protein relatedness, structure, function, synthesis, processing, cellular or tissue distribution and on association between molecules.

The procedure for production of monoclonal antibody is now well established. The advantages of mabs are that they are homogenous and they

recognise one antigenic determinant which is not possible with conventional polyclonal sera e.g. detection of different strains of viruses. Mabs can also reveal relationships between viruses at structural and biological levels. They can be used after preliminary tests for reactivity with individual viral peptides such as in Western blotting and virus neutralisation etc.

In order to obtain mab the virus antigen employed for the induction should be free from extraneous proteins. Characterization of viruses with mabs is done through analysis of individual proteins. Active areas of the molecule, its synthesis and processing can be monitored by mabs. Mabs distinguish between related proteins and can be used to provide a genetic marker in recombinant experiments.

Therapeutic uses

Due to various problems that have been encountered in the production of human monoclonal antibodies, majority of the monoclonal antibodies used for therapeutic applications, to date, have been raised in mouse. Murine antibodies have the obvious disadvantage of being foreign to the human system and likely therefore to lose their efficacy on continual application as a host response is mounted. However, the treatment of bone marrow allogenic or autologous transplants is one such area in which monoclonal antibodies have been shown to be of value in either reducing graft versus host disease or in removing leukemia cells from autografts. Orthoclone (marketed by Janssen Pharmaceutica) is such a murine monoclonal antibody which reverses graft rejection by blocking the function of CD3 molecule (mw 20,000 dalton), found on the membrane of human T-cells that has been associated in *vitro* with the antigen recognition structure of T-cells and is essential for signal transduction.

Human monoclonal antibodies are likely to prove of considerable value in immunosuppression in heart and kidney transplant recipients as well as in the prevention of both graft versus host and host versus graft disease in bone marrow transplantation. Despite facing various problems, some laboratories have succeeded in producing human monoclonal antibody. For example HERCEPTIN (marketed by Genetec Inc) is a recombinant DNA-derived humanized monoclonal antibody that selectively binds with high affinity to the extracellular domain of the human epidermal growth factor receptor 2 protein, HER2. The antibody is an IgG1 kappa that contains human framework regions with the complementary-determining regions of a

murine antibody (4D5) that binds to HER2. In case of human breast carcinoma, it inhibits the proliferation of human tumor cells that over express HER2. In the long term there may be possibility of specific immune therapy for autoimmune disease such as the use of anti-idiotypic antibodies.

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Antibiotic Susceptibility Test -Applications in Fisheries Science

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Introduction

Antibiotic susceptibility test (ABST) or antibiogram is an *in-vitro* test which determines the susceptibility of a microbe against different antibiotic agents. The tests are performed under standardized conditions to get reproducible results. Clinical signs produced by the organism in a disease sometimes helps in identifying the microorganism, but it is not always possible to determine reliably which antibiotic is to be used for treatment, as different strains of the same bacterial species differ substantially in antibiogram. In medical and veterinary field, antibiotic susceptibility pattern of the causative agent helps the clinician in selecting the most effective antibiotic which has least toxicity to host. Susceptibility testing of individual isolates is very important in some species that possess acquired resistance mechanisms (eg: members of the *Enterobacteriaceae*, *Pseudomonas* sp., *Staphylococcus* sp., *Enterococcus* sp. and *Streptococcus pneumoniae*). However, results of antimicrobial susceptibility test should be combined with clinical information and experience while selecting the most appropriate antibiotic for the treatment.

Applications in fisheries science

ABST helps in selecting an antibiotic that will successfully and economically treat an infection during a disease outbreak in aquaculture systems: Antibiotics can be used for the treatment of infection in case of ornamental and pet fish. Similarly, antibiotics can be used for treatment of high value

fish and during severe outbreak in commercial fish farms but only after the isolation into contained environment preferably, in a quarantine/hospital tank. Legalities must also be considered when selecting antibiotics for food fish, as they have fewer options than ornamental fish. The antibiotic treated fish should be released into routine farms or will be used for consumption only after the prescribed withdrawal period. Thus, ABST results of pathogen will help in selecting the most suitable antibiotic for treating an infection. However, only approved antibiotics for fish have to be used for treatment in aquacultures systems. Affected fish should not be treated with antibiotics before taking the samples for analysis as it may result in poor culture yields. While waiting for the culture results, the fish health specialist may suggest a broad-spectrum antibiotic that can be used until culture and sensitivity tests have been completed. However, ability of antibiotics to eliminate a fish disease, depends on whether the disease actually has a bacterial component, whether the proper dosage and treatment intervals being used, and whether the other contributing stress factors (poor water quality, drastic temperature change, nutrition, and handling or transport) are removed or reduced etc.

As indiscriminate use of antibiotics without knowing the susceptible pattern will lead to the emergence of antibiotic resistant strains, doing treatment after knowing the ABST pattern, can help to control the use of antibiotics in fisheries field. In an attempt to avoid the emergence of antibiotic resistant pathogens, some farmers are rotating the antibiotics they use. However, the best solution is to positively identify the bacteria and their antibiogram pattern, by running culture

and sensitivity tests, and thereby avoid unnecessary, costly, and potentially harmful treatments.

The occurrence of antibiotic resistant bacteria is increasing in aquatic and marine environments. Thus, results of ABST pattern of bacteria in water basins, sediments and bivalves can be used to investigate the occurrence and distribution of antibiotic resistant bacteria in various aquatic environments.

The anthropogenic impacts on coastal areas through inflow of domestic effluents can also be evaluated by measuring the occurrence of bacterial resistance to antimicrobial agents.

METHODS OF ANTIBIOGRAM: There are different methods for performing the antibiotic susceptibility test for a microbe.

Tube dilution method (Macrobroth dilution method): This is one of the earliest antimicrobial susceptibility testing methods. This procedure involves preparing two-fold dilutions of antibiotics in a broth medium dispensed in test tubes. The antibiotic containing tubes are then inoculated with a standardized bacterial suspension. Following the overnight incubation, the tubes are examined for visible bacterial growth as indicated by turbidity. The lowest concentration of antibiotic that prevents bacterial growth represents the minimal inhibitory concentration (MIC). The precision of this method is considered to be only plus or minus 1 two-fold concentration, due to errors during the manually preparation of serial dilutions of the antibiotics. The advantage of this technique is the generation of a quantitative result. The principal disadvantages of this method are, it is tedious, manual task of preparing the antibiotic solutions for each test, the possibility of errors in preparation of the antibiotic dilutions during each test and the requirement of larger amount of reagents and space for each test.

Microdilution method: The miniaturization and mechanization by use of disposable, plastic microdilution trays has made the broth dilution test as practical and popular. Microdilution test uses about 0.05 to 0.1 ml total broth volume. Using a

single tray containing 96 wells 12 antibiotics can be tested in a range of 8 two-fold dilutions. Frozen or dried microdilution panels can be purchased from various commercial suppliers (outside India). The cost of such preprepared ABST panels vary from approximately \$10 to \$22 each. The advantages of this procedure include generation of MICs, convenience of having preprepared panels and, economy of reagents and space due to the miniaturization of the test. However, there is some inflexibility of antibiotic selections available in standard commercial panels.

Disc diffusion method: Due to the convenience, efficiency and cost, the agar disc diffusion test is probably the most widely used method for ABST. This method is also known as Kirby–Bauer method of disc diffusion.

Materials required

- Sterile Mueller-Hinton agar (MHA) plate
- Sterile cotton swabs
- Antibiotic discs
- 4-6 h old culture of test organism

Procedure

Adjust the turbidity of the culture to 0.5 McFarland opacity tube ($1-2 \times 10^8$ CFU/ml) using sterile saline.

Then, a sterile swab is dipped into the turbidity adjusted culture, squeezed the excess fluid against the side of the tube and inoculated into MHA plate so that a complete lawn is formed on the agar surface. Dip the sterile swab in the turbidity adjusted culture, squeeze the excess fluid against the side of the tube and inoculate into MHA plate so that a complete lawn is formed on the agar surface. Let the plate stand for 10 min to imbibe the broth inoculum.

Select the required antibiotic discs and place it at equidistance using a sterile forceps in such a way that the zone of inhibition of two antibiotics shall not overlap each other. Incubate at suitable temperature (optimum temperature for that pathogen) for overnight.

Measure the zone of inhibition of bacterial growth

around each disc and consult the interpretive chart to determine whether the organism is resistant (R), fully susceptible (S) or intermediately susceptible (I).

Interpretation of Susceptibility Test Results

If the bacterial isolate is susceptible to a particular antibiotic, a clear area of “no growth” can be observed around that particular disc. The zone around an antibiotic disc that has no growth is referred to as the zone of inhibition and this approximates the minimum antibiotic concentration sufficient to prevent the growth of that isolate. This zone is then measured in mm and compared to a standard interpretation chart and the isolate is then categorized as susceptible, intermediately susceptible or resistant. The zone diameters of each drug are interpreted using the criteria published by the Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards or NCCLS) or those included in the US Food and Drug Administration (FDA). The results of the disc diffusion test are “qualitative,” in that a category of susceptibility (ie, susceptible, intermediate, or resistant) is derived from the test rather than an MIC. However, some commercially available zone reader systems claim to calculate an approximate MIC with some organisms and antibiotics, by comparing zone sizes with standard curves of that species and drug stored in an algorithm. Generally, reporting the ABST result as susceptible, intermediate, or resistant provides the clinician with the information necessary to select appropriate therapy. It is important that the tables used for susceptibility test interpretations represent the most current criteria. Indeed, the CLSI documents are reviewed and updated frequently, usually once per year. Use of old or outdated information from the original editions of FDA-approved drug labels or older CLSI tables could represent a serious shortcoming in the reporting of patients’ results.

Advantages of the disc method

- The test does not require any special equipment
- The results that can be easily interpreted by all clinicians

- Allows flexibility in selection of discs for testing
- It is the least costly of all susceptibility methods (approximately \$2.50–\$5 per test for materials)

Disadvantages

- Lack of mechanization or automation of the test
- Some fastidious bacteria cannot be accurately tested by this method

E-TEST: E-test (AB Biodisk, Solna, Sweden) is a commercially available method that utilizes plastic strips that are impregnated on the underside with a dried antibiotic concentration gradient. The concentration gradients are marked on the upper surface with a concentration scale. As many as 5 or 6 strips can be placed in a radial fashion on the surface of a 150-mm agar plate that has been inoculated with a standardized organism suspension like that used for a disc diffusion test. After overnight incubation, the tests are read by viewing the strips from the top of the plate. The MIC is determined by the intersection of the lower part of the ellipse shaped growth inhibition area with the test strip. This method provides a convenient quantitative test of antibiotic resistance. However, a separate strip is needed for each antibiotic, and therefore the cost of this method can be high. The gradient diffusion method has intrinsic flexibility for selecting the drugs. E test strips cost approximately \$2–\$3 each. Hence, this method is best suited to situations in which MIC for only 1 or 2 drugs is needed.

Mechanism-specific tests: Resistance may also be established through tests that directly detect the presence of a particular resistance mechanism. For example, beta lactamase detection can be accomplished using an assay such as the chromogenic cephalosporinase test (Cefinase disk by BD Microbiology Systems, Cockeysville, MD and BBL DrySlide Nitrocefina, Becton Dickinson, Sparks, MD). Another example is detection for chloramphenicol modifying enzyme chloramphenicol acetyltransferase (CAT) utilizing commercial colorimetric assays such as a CAT reagent kit (Remel, Lenexa, Kansas).

Genotypic methods: Since resistance traits are genetically encoded, we can sometimes test for

the specific genes that confer antibiotic resistance. However, although nucleic acid-based detections systems are generally rapid and sensitive, it is important to remember that the presence of a resistance gene does not necessarily equate to treatment failure, because resistance is also dependent on the mode and level of expression of these genes. Some of the most common molecular techniques utilized for antimicrobial resistance detection are PCR, Modifications of PCR and DNA hybridization.

Current Test Methods and Future Directions

The antimicrobial susceptibility testing methods described here provide reliable results when used

according to the procedures defined by CLSI or by the manufacturers of the commercial products. However, there is a need for development of new automated instruments that could provide faster results and also save money by virtue of lower reagent costs and reduced labor requirements. To accomplish this, it will likely be necessary to explore different methodologic approaches for detection of bacterial growth. The direct detection of resistance genes by polymerase chain reaction or similar techniques has limited utility, because only a few resistance genes are firmly associated with phenotypic resistance. Thus, it seems likely that phenotypic measures of the level of susceptibility of bacterial isolates to antimicrobial agents will continue to be relevant for years to come.

Immunization of Fish: A Tool for Aquaculture Health Management

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Introduction

Aquaculture is the fastest emerging global food industry among all other food animal producing sectors. Nevertheless, the economical losses imposed by high mortality rates due to incidences and outbreaks of infectious diseases form a major challenge to develop productive, feasible and sustainable aquaculture systems. Numerous reasons including intensive aquaculture practices, introduction of new fish species into farming practices, increased trade in ornamental fish and increased interaction between wild fish and farmed fish have altogether caused increased economic losses, due to infectious diseases in the aquaculture systems. Literature proposed that among the aetiological agents responsible for periodical disease outbreaks in fish, 54.9% are caused by bacteria, 22.6% by viruses, 3.1% by mycotic agents and 19.4% are by parasitic agents (Dhar *et al.*, 2014). The Office International des Epizooties (OIE)/World Organization for Animal Health has listed that viral diseases including epizootic hematopoietic necrosis, koi herpesvirus disease, red sea bream iridovirus disease, infectious hematopoietic necrosis virus, infectious salmon anemia virus, spring viremia of carp, and viral hemorrhagic septicemia as the agents causing major catastrophe for large scale aquaculture industry.

Therefore, prevention and control of fish diseases has got high priority in aquaculture industry. The farming practices of fish (in both freshwater and seawater aquaculture) in developed countries have already demonstrated that effective disease management is the key to profitability in commercial aquaculture systems. On contrast, unlike treating human or other animal diseases, only few drugs are available for

treating diseases in fish. Feeding infected fish with antibiotic-medicated food is a universal practice followed by many aquaculture farmers for health management. However, this is usually costly, and may be ineffective as sick fish may be anorectic. More significantly, frequent use of antimicrobial compounds may lead to the development of antimicrobial resistance, posing serious challenges to health and national security and thus, cannot be encouraged. Therefore, immunization of fish is becoming an increasingly important part of aquaculture as it is a cost effective method for controlling different threatening diseases.

Overview of fish immune system: Fish immunology has a more recent history than human and veterinary immunology. It is still a young and maturing science but amazing advances can be made in future. Fish are reported to have a functional immune system similar to mammals comprising of two arms of immunity such as innate and adaptive immunity. The major difference from other vertebrates is that their immune response is temperature dependent. The innate arms of fish immunity which does not require any previous exposure to the particular agent includes physical barriers (skin and mucus layers), specialized cells (macrophages and natural killer cells and particular soluble molecules such as complement and interferon. The mucosa-associated lymphoid tissues are subdivided into gut, skin and gill. Recent studies have also confirmed the presence of functional homologues of mammalian cytokines in fish.

In adaptive immunity, fish above the level of the Agnatha display typical vertebrate adaptive immune responses characterized by presence of

immunoglobulins, T-cell receptors, cytokines, and major histocompatibility complex molecules. However, the immune system of fish is quite different in its efficiency and complexity from that of higher vertebrates. Acquired immunity in fish includes both humoral and cell mediated response. The primary and secondary lymphoid organs in mammals are present in fish, except lymphatic nodules and bone marrow.

The anterior portion of kidney is most likely the source of histocompatibility complex. In teleost fish, progenitor T-cells migrates from the kidney to the thymus for T-cell maturation and for self, non-self-recognition. B-lymphocytes originate and mature within the kidney. B-cells of fish produce antibody when stimulated.

Routes of immunization for fish: For vaccination in developed countries, fish are usually transported in pipes from the rearing tanks to an anaesthetic bath and the anaesthetized fish are then immunized. Vaccines are usually administered to fish by any of the three methods: injection (intraperitoneal/intramuscular), immersion (short bath/long bath/spray vaccination), or oral administration. All these methods have their own advantages and disadvantages with respect to the level of protection, side effects, practicality and cost-efficiency.

Advantages and disadvantages of immersion vaccination: Advantages include: suitable for mass vaccination and for all sizes of fish, reduced stress for fish during immunization procedures, lower labour costs and less risk to vaccination team. Major disadvantages are large amount of vaccine are required and there will be only a low level protection. Duration of immune-protection is also short.

Advantages and disadvantages of injection: This is the most common vaccine delivery method in fish as it is highly efficient in generating both humoral and cellular immune response. However, it is unsuitable for small fish and needs sophisticated machinery or large skilled workforce. It also causes significant handling stress so that risk of post vaccination mortality and local reactions are more.

Advantages and disadvantages of oral

Vaccination: In this method vaccine is mixed with feed. It is the easiest method for mass vaccination of all sizes of fish and it saves labour and avoids stress. However, large quantities of antigen are required. Moreover, immune protection is generally weak and of short duration

Currently, it is widely accepted that only injection and immersion routes give enough protection to be used as the primary route of fish immunization in commercial production. The less immune response in oral vaccination can be improved by protecting the antigens from digestion and decomposition, during the passage through gut. Some promising results have been obtained using microencapsulation of antigens. From the economic point of view, oral vaccination will be the ideal route to be employed in fish vaccination program which requires one or more booster immunizations.

Current status of fish vaccines: As per the latest review, now, vaccines are accessible in many countries for more than 17 fish species which can induce protection for more than 22 different kinds of bacterial diseases as well as 8 viral diseases (Dadar *et al.*, 2017). The range of bacterial infections for which vaccines are commercially available now comprises classical vibriosis (*V. anguillarum* and *V. ordalii*), cold-water vibriosis (*V. salmonicida*), furunculosis (*Aeromonas salmonicida* subsp. *salmonicida*), yersiniosis (*Y. ruckeri*), pasteurellosis (*Photobacterium damselae* supsp. *piscicida*), edwardsiellosis (*E. ictaluri*), winter ulcer (*Moritella viscosa*), and streptococcosis/lactococcosis (*S. iniae*, *L. garviae*). The viral diseases for which commercial vaccines are available include Koi herpesvirus, Iridovirus, red sea bream iridiovirus, salmon alphaviruses, infectious hematopoietic necrosis virus, spring viremia of carp virus, infectious salmon anemia virus and infectious pancreatic necrosis virus.

The major producers of fish vaccines are Intervet International, Novartis Animal Health, Schering-Plough Animal Health, Pharmaq and Bayer Animal Health. The key commercial markets are currently the salmon and trout industries in Northern Europe, Canada, USA, and Chile. Commercial vaccines are also available for catfish industry in USA and for European seabream,

seabass and tilapia. Some locally developed vaccines are also available in countries such as China, Japan, Russia, Spain and Germany (Somerset *et al.*, 2005).

Important considerations during fish immunization

- Species of fish to be immunized
- Size of fish at vaccination- Smaller the fish, more stress and higher risk of local reactions
- Temperature and salinity at the time of vaccination- Higher the temperature higher risk of local reactions
- Onset of immunity: It is temperature dependent
- Diseases to be controlled and when these diseases occur
- Adjuvants-Variou adjuvants are tried in fish in which oil based adjuvants give best protection
- Types of vaccines: Killed/Attenuated live/Gene deleted live/Toxoid/Subunit vaccines as DNA vaccine/recombinant vector vaccine/recombinant protein/conjugate/
- Stress: Stress caused by environments, crowding, handling and transport, can induce immune suppression and can be a limiting factors for vaccine efficacy
- Nutrition
- Cost benefit
- Farming technology

Limitations in fish vaccine development

As with all veterinary vaccines, cost effectiveness in the field is an essential limitation to commercial fish vaccine development. Ideal vaccines for aquaculture must be effective in preventing death, be inexpensive to produce and license, provide sustained immunity of long duration, stable, will not interfere the diagnosis and be easily administered. In case of fish they generally need a larger antigen dose compared to terrestrial animals so that cost effective vaccines are difficult to develop. In the past ten years, commercial vaccine products for fish have more often consisted of mixtures of multiple vaccines. Considering the fact that not all antigens stimulate protective immune response, that antigens vary in their immune-dominance relative to each other and

that the immune system of fish has a defined and limited capacity to respond to individual antigens, it becomes increasingly difficult to formulate these complex mixtures into effective commercial products. The other limitation is many fish species are too vulnerable to handle the stress induced during the vaccination so that oral vaccination should be considered in future as the most desirable method for immunizing fish. However, the limited protection by this route challenges the fish immunization. Another problem in fish immunization is that the major disease problems appear during the larval or fry stages during which the animal is small enough to be vaccinated or will not have functional immune system. The apparent lack of maternal immunity in fish limits the possibilities to protect offspring by parental vaccination.

Future prospects and conclusion

Vaccines have become a reputable, verified, and cost-effective method for reducing the occurrence of diseases and usage of antibiotics in fish production. However, to achieve further progress in fish vaccinology, an increased collaborative research between the microbiologist and vaccinologist is needed. The fish vaccination may be benefitted in future by increased knowledge of their immune system and pathogens, through the application of comparative genomic and transcriptome analysis. This would facilitate to open new generation vaccines in aquaculture systems. Improvement in oral immunization by better delivery systems may also benefit the future fish vaccinology. Development of novel non-mineral oil adjuvants lacking side effects, development of polyvalent vaccines and standardization of immunization calendar appropriate for each economically important fish species *etc.* can be possible novel approaches for fish vaccine development. Also, vaccines designed to augment cell mediated immunity must be targeted against viral fish diseases. Novel expression systems, improvements in adjuvants, better immunostimulants, progress in DNA vaccinology and development of passive immunization *etc.* may also benefit fish immunization strategies in future.

Polyphasic Taxonomy as a Consensus Methodology for Bacterial Identification

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Introduction

Microbial taxonomy or microbial systematics deals with the classification, identification and nomenclature of microorganisms. Microbial systematics is a vast intuitive science but has become increasingly objective due to the introduction of the new concepts and methods. Classification of microorganisms on the basis of traditional microbiological methods (morphological, physiological and biochemical) creates a blurred image about their taxonomic status and thus needs further clarification. It should be based on a more pragmatic approach of deploying a number of methods for the complete characterization of microbes. The new advancements, mainly in molecular systematics, stimulated the need to compare established and more recent approaches to microbial classification which helped the integrated use of genotypic and phenotypic information. Hence, the methods now employed for bacterial systematics include, the complete *16S rRNA* gene sequencing and its comparative analysis by phylogenetic trees, DNA-DNA hybridization studies with related organisms, analyses of molecular markers and signature pattern(s), biochemical assays, physiological and morphological tests. Collectively these genotypic, chemotaxonomic and phenotypic methods for determining taxonomic position of microbes constitute what is known as the 'polyphasic approach' for bacterial systematics. Polyphasic approach is a recent trend in microbial taxonomy, which provides natural and authentic system of classification of microbes. The term coined by Colwell in 1970, refers to the integration of genotypic, chemotypic and phenotypic information of a microbe in order to perform reliable grouping of the organism. This approach is currently the most

popular choice for classifying bacteria and several microbes, which were previously placed under invalid taxa have now been resolved into new genera and species. It helps to yield good quality of phenotypic and genotypic data which is required to obtain a better understanding of microbial diversity. This field is rapidly expanding and therefore it is imperative to update readers with the recent technical advances.

Identification of Bacteria

Morphological identification

Gram Staining: This technique is used to differentiate gram positive and gram negative bacteria. Gram positive bacteria stains as purple colour. Gram negative bacteria stains as pink color. This method helps to differentiate their shapes (rod, cocci, comma, etc.) and their arrangements (cluster, tetrad, etc.)

Cultural identification

Cultural characteristics of the isolates can be used to help identify the bacterial species. Colony characters such as colony colour, form, elevation and margin has to be noted.

Biochemical identification

The major tests followed in a routine microbiology lab include

a) KOH Test (String formation)

Based on the differences in the chemistry of the bacterial cell wall, the cell wall of gram negative

bacteria gets easily disrupted when exposed to dilute alkali solution resulting in the formation of string. Absence of string formation is observed for gram positive bacteria.

Procedure

A loopful of 24-hour-old growth from a colony of the organism was emulsify on the surface of a clean glass slide in a suspension of 3% potassium hydroxide. The suspension was mix continuously for 10 seconds after which the toothpick pulled from the suspension.

Interpretation

String formation:—gram negative bacteria (formation of a string within 10 sec of mixing the bacteria).
Absence of string formation:—gram positive bacteria.

b) Oxidation Fermentation (OF) reaction

Oxidative organisms can only metabolize glucose or other carbohydrates under aerobic conditions ie. oxygen is the ultimate hydrogen acceptor. Other organisms ferment glucose and the hydrogen acceptor is then another substrate. e.g., Sulphur. This fermentative process is independent of oxygen and cultures of organisms may be aerobic or anaerobic. The end product of metabolizing a carbohydrate is acid. The method described, is sometimes referred to as the Hugh and Leifson test, is a semi-solid medium in tubes, containing the carbohydrate under test (usually glucose) and a pH indicator

Procedure

- Heat two tubes of Hugh Leifson's medium in boiling water for 10 minutes to drive off the oxygen, cool and inoculate by stabbing with a straight wire.
- Incubate one tube aerobically and either incubate the second tube anaerobically or seal the surface with a layer of sterile liquid paraffin oil to create an anaerobic condition.
- Incubate at 37°C for 24-48 hours.

Interpretation

Oxidation: Acid in aerobic tube only (yellow colour in aerobic tube, green in anaerobic), Fermentation: Acid in both tubes (yellow colour), Neither fermentation nor Oxidation: No colour change.

c) Triple Sugar Iron Agar Test

TSI Agar is used for the determination of carbohydrate fermentation and hydrogen sulfide production in the identification of gram-negative bacilli. Carbohydrate fermentation is detected by the presence of gas and a visible colour change (from red to yellow) of the pH indicator, phenol red. The production of hydrogen sulfide is indicated by the presence of a precipitate that blackens the medium in the butt of the tube. To facilitate the detection of organisms that only ferment dextrose, the dextrose concentration is one-tenth the concentration of lactose or sucrose. The small amount of acid produced in the slant of the tube during dextrose fermentation oxidizes rapidly, causing to remain red or revert to an alkaline pH. In contrast, the acid reaction (yellow) is maintained in the butt of the tube because it is under lower oxygen tension.

Procedure

- Inoculate a slant of triple sugar iron agar slant by using a straight needle.
- First stab the butt down to the bottom, withdraw the needle & streak the surface of the slant.
- Read the results after incubation at 37°C for 18-24 h.

Interpretation

Yellow coloration of the slope:—Oxidative reaction;
Red coloration of the slope:—Alkaline reaction;
Yellow coloration throughout the tube or in the butt:—Fermentative reaction; Blackening of the butt:—Hydrogen sulphide production; Split or gas bubble in the butt:—Gas production

d) Oxidase Test

The oxidase test is based on the bacterial production of an intracellular oxidase enzyme. The oxidase reaction is due to the presence of a cytochrome oxidase system which activates the oxidation of reduced cytochrome by molecular oxygen. The cytochrome oxidase enzyme is able to oxidize the substrate tetramethyl-p-phenylenediamine dihydrochloride, forming a colored end product, indophenol. The dark- purple end product will be visible if a small amount of growth from a strain that produces the enzyme is rubbed on substrate-impregnated filter paper.

Procedure

- A filter paper moistened with 2-3 drops of Wurster's reagent (1% tetramethyl-p-phenylene diamine dihydrochloride (TPDD)).
- Pick a colony grown freshly on nutrient agar and make a compact smear on a filter paper
- Observe for a colour change to blue or purple within 10 secs (timing is critical).

Interpretation

Blue to purple colour formation within 10 to 30 sec is considered as positive reaction and no colour change is negative reaction

e) Catalase Test

The catalase test is used to detect the presence of catalase enzyme, by the decomposition of hydrogen peroxide to release oxygen and water. Hydrogen peroxide is produced by some bacteria as an oxidative end product of the aerobic break down of sugars. If allowed to accumulate it is highly toxic to bacteria and can result in cell death. Aerobes, facultative aerobes and microaerophilic organisms degraded this product using catalase enzyme.

Procedure

- Prepare a thick smear of the organism from a 24 hr culture on a clean slide
- A drop of hydrogen peroxide is placed on the slide
- Observe the immediate formation of gas bubbles.

Interpretation

Immediate formation of gas bubbles indicated the liberation of oxygen is the positive reaction.

f) Aminoacid decarboxylases test

The aminoacid decarboxylase test demonstrates the bacterial decarboxylation of amino acid lysine, arginine, and ornithine. In this test, the decarboxylation or the elimination of a molecule of carbon dioxide from the amino acid results in the formation of an amine with one carbon atom less. Alkaline degradation products are produced in the course of decarboxylation and subsequently color of the media changes to violet or purple. Bromocresol purple is the dye used in the test

Procedure

- Inoculate the test organisms in a required amino acid decarboxylation broth (Moller Decarboxylase Broth with corresponding amino acids)
- Incubate for 24 hours at 37°C and observe the color change of the medium.

Interpretation

Colour change of the medium to violet or colour is taken as positive result.

g) Indole production Test

This test is done to determine if bacteria can breakdown the amino acid tryptophan into indole, which is present in tryptone broth. This liberated indole reacts with Kovacs' reagent to produce red colour at the top of the medium.

Procedure

- Inoculate the culture in to tryptone broth and incubate for 24 to 48 hrs.
- After incubation add about 0.5 ml of Kovacs' reagent to each tube.
- Observe the colour of ring formed at the top of the medium.

Interpretation

A red coloured ring at the top of the medium is taken as positive result.

h) Methyl Red test

This is used to determine whether the bacteria can convert glucose to acidic products like lactate, acetate, and formate. The production of acid from glucose has lowered and held pH at about 4.2 or below and this is detected using methyl red indicator.

Procedure

- Inoculate pure culture to the MR VP broth and incubate for 24- 48 hours at 37°C.
- Add a few drops of methyl red indicator and observe the colour change.

Interpretation

A definite red colour of the medium can be considered as positive.

i) Voges-Proskauer Test

Some organisms, after producing acids from glucose, are capable of converting acids to acetylmethyl carbinol or 2,3butanediol, which are neutral substances. Aeration in the presence of alkali then converts the products to diacetyl, which in turn reacts with the peptone constituents producing a red colouration.

Procedure

- Inoculate pure culture to the MR VP broth and incubate for 24- 48 hours at 37°C.
- Add 4 drops of Barrits reagent A and 2 drops of Barrits reagent B to the broth & shake well.
- Observe the broth for colour change after 3 hours.

Interpretation

Red colour formation is positive result.

j) Citrate utilization

This test demonstrates the ability of the microbes to utilize citrate as a sole source of carbon and oxygen. Utilization of citrate and growth in citrate agar results in an alkaline reaction, which changes the colour of the medium provided. In this Simmon's citrate agar medium, bromothymol blue indicator is used which changes from green to bright blue on utilization of citrate.

Procedure

- Prepare Simmon's citrate agar medium in the form of agar slopes in the tubes.
- Inoculate the slope by streaking over the surface with a loopful of culture.
- Incubate for 24-48 hours and observe the result.

Interpretation

Colour change from green to bright blue indicates a positive result.

k) Nitrate reduction

The reduction of nitrates by some aerobic and anaerobic microorganisms occurs in the absence of molecular oxygen. Nitrate reduction can be determined by cultivation of organisms in nitrate

broth medium. The medium was basically a nutrient broth supplemented with 0.1% potassium nitrate as the nitrate substrate. Following incubation, the ability of organisms to reduce nitrate to nitrite is determined by the addition of two reagents. Solution A, which is sulfanilic acid, followed by solution B, which is alpha-naphthylamine. Following reduction, the addition of solution A & B will produce a cherry red colour.

Procedure

- Culture is inoculated into the autoclaved nitrate broth and incubate for 24 hours at 37°C.
- Add 2 drops of each nitrate solution A and nitrate solution B.
- Observe the colour change of the media.

Interpretation

Appearance of a blood red colour indicates the positive reaction. No characteristic colour change for negative reaction.

Molecular identification–16S rDNA Gene Amplification

Total genomic DNA was extracted from bacterial cultures grown in nutrient broth using phenol-chloroform extraction method. Purified colony was inoculated to 5 ml of LB broth, incubated overnight in a shaking incubator at 30°C. After the incubation, the cells were harvested by centrifugation at 10,000 rpm for 15 min. The pellet was re-suspended in 450 µl TEG buffer with lysozyme (5 mg/ml of TEG buffer) mixture and vortexed well. A volume of 50 µl of 10% SDS was added and mixed well. Sample was kept on ice for 10 min followed by incubation at room temperature for 15 min and then in water bath at 60°C for 10 min. To this, 10 µl of Proteinase-K was added, vortexed and incubated in water bath at 60°C for 1 h (until the proteins got digested). Equal amount of phenol-chloroform-isoamyl alcohol (25: 24: 1) was added, mixed gently by inverting and centrifuged at 10,000 rpm for 15 min at 4°C. The aqueous phase was collected in another micro-centrifuge tube without disturbing the interphase and lower phase. To the aqueous phase, 1/10th volume of 3 M sodium acetate was added followed by 2.5 times ice cold absolute ethanol and incubated at -20°C overnight so that the DNA got precipitated and was again centrifuged

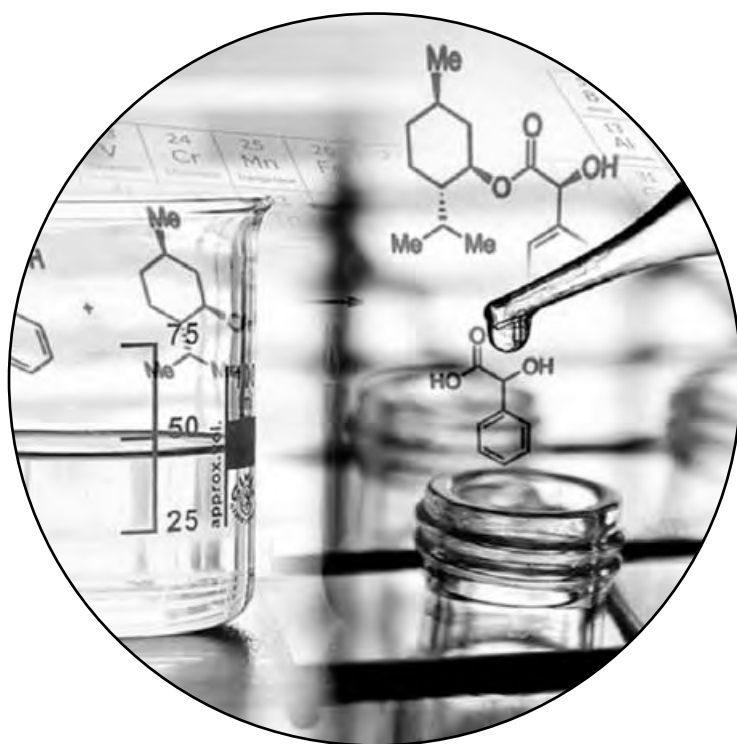
for 15 min at 10,000 rpm. The supernatant was discarded and the pellet was rinsed with 500 μ l 70% ethanol, centrifuged again at 10,000rpm for 15 min at 4°C and the tubes were air dried after discarding the supernatant. The DNA samples were then re-suspended in 30 μ l of DNA dissolving buffer (TE/elution buffer) and stored at -20°C. Further, purity of DNA was checked by agarose gel electrophoresis (AGE) and quantified using Biophotometer.

Amplification of *16S rRNA* gene

16S rRNA gene amplification was carried out using universal prokaryotic primers; NP1F 5'GAGTTTGATCCTGGCTCA-3' and NP1R 5'-ACGGCTACCTTGTTACGACTT-3'. Each polymerase chain reaction (PCR) mixture consisted of 1 μ l of template DNA, 2.5 μ l of 1X Taq buffer, 0.5 μ l of dNTP mix, 0.5 μ l of Forward and Reverse Primer and 1.25 U of DNA polymerase. The PCR programme of each for sample included initial denaturation at (95°C for 5 min) followed by 35 cycles of denaturation (95°C for 30 sec), annealing (58°C for 1 min) and extension (72°C for 1.30 min). Final extension was carried out at (68°C for 5 min). The PCR products were then characterized by submarine gel electrophoresis (1% agarose gel). The amplified products were purified and sequenced. The obtained sequences were then subjected to BLAST search (NCBI) and the bacteria were identified. The *16S rRNA* gene sequences were analysed and the relative phylogenetic positions were determined by searching GenBank database using BLASTn algorithm (Altschul *et al.* 1997).

Conclusion

In this regard, a polyphasic taxonomic approach is advantageous because it exploits simultaneously both conventional as well as molecular identification techniques. The major advantages of the polyphasic approach is that certain groups of bacteria as case studies to arrive at a consensus approach to microbial identification. Now-a-days there is an increasing access to microbial genomes due to the accumulation of numerous DNA sequences which ultimately leads to a higher level of accuracy and reliability of results. Though polyphasic taxonomy is a useful technique to meet the challenge of identifying any unknown strain, new mathematical and informative strategies should be developed for the possible development of a synthetic taxonomy. Thus, the conventional biochemical basis of identification practices cannot be replaced completely and the vast majority of the data should lead to a perfectly reliable and stable identification and classification system. Though many advanced techniques are available now-a-days for the identification of any unknown bacterium, a far less number of the total number of microbial species have been discovered and identified till now; many of them are yet to be cultured under laboratory conditions and some of them may possess certain unique characteristic features. Microbial taxonomy and biosystematics is a major modern discipline which needs further financial and intellectual support to determine its role in biotechnology, biodiversity, agriculture, medical science and environmental science.



Marine Chemistry

Laboratory- Safety and Hazards

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Introduction

A key element of planning an experiment is assessing the hazards and potential risks associated with the chemicals and laboratory operations to be used. The primary responsibility for proper hazard evaluations and risk assessments lies with the person performing the experiment. The actual evaluations and assessments may be performed by trained laboratory personnel, but these should be checked and authorized by the supervisor. The supervisor is also responsible for ensuring that everyone involved in an experiment and those nearby understand the evaluations and assessments. Some organizations have environmental health and safety offices, with industrial hygiene specialists to advise trained laboratory personnel and their supervisors in risk assessment. As part of a culture of safety, the supervisor and scholars in the laboratory must work cooperatively to create a safe environment and to ensure that hazards are appropriately identified and assessed prior to beginning work. All laboratory personnel should be familiar with and have ready access to their institution's chemical hygiene plan. In some laboratories, chemical hygiene plans include standard operating procedures for work with specific chemical substances, and the chemical hygiene plan may be sufficient as the primary source of information used for risk assessment and experiment planning. However, most chemical hygiene plans provide only general procedures for handling chemicals, and prudent experiment planning requires that laboratory personnel consult additional sources for information on the properties of the substances that will be encountered in the proposed experiment. Many laboratories require documentation of specific hazards and controls for a proposed experiment.

Material Safety Data Sheets (MSDSs)

Federal regulations (OSHA Hazard Communication Standard 1910.1200) require that manufacturers and distributors of hazardous chemicals provide users with material safety data sheets (MSDSs), which are designed to provide the information needed to protect users from any hazards that may be associated with the product (Globally Harmonized System for Hazard Communication). MSDSs have become the primary vehicle through which the potential hazards of materials obtained from commercial sources are communicated to trained laboratory personnel. Institutions are required by law (OSHA Hazard Communication Standard) to retain and make readily available the MSDSs provided by chemical suppliers.

OSHA recommends the general 16-part format created by the American National Standards Institute (ANSI Z400.1). The information typically found in an MSDS follows:

- Supplier (with address and phone number) and date MSDS was prepared or revised.
 1. Chemical.
 2. Physical and chemical properties.
 3. Physical hazards related to flammability, reactivity, and explosion hazards.
 4. Toxicity data.
 5. Health hazards-Acute and chronic health hazards together with the signs and symptoms of exposure.
 6. Storage and handling procedures.
 7. Emergency and first-aid procedures.
 8. Disposal considerations.
 9. Transportation information.

MSDSs remain the best single source of information for the purpose of evaluating the hazards and assessing the risks of chemical substances. However, laboratory personnel should recognize the limitations of MSDSs as applied to laboratory-scale operations. If MSDSs are not adequate, specific laboratory operating procedures should be available for the specific laboratory manipulations to be employed:

1. The quality of MSDSs produced by different chemical suppliers varies widely.
2. Unique morphology of solid hazardous chemicals may not be addressed in MSDSs; for example, an MSDS for nano-size titanium dioxide may not present the unique toxicity considerations for these ultra-fine particulates.
3. MSDSs must describe control measures and precautions for work on a variety of scales.
4. Many MSDSs comprehensively list all conceivable health hazards associated with a substance without differentiating which are most significant and which are most likely to actually be encountered. As a result, trained laboratory personnel may not distinguish highly hazardous materials from moderately hazardous and relatively harmless ones.

Globally Harmonized System (GHS) for Hazard Communication

The GHS of Classification and Labeling of Chemicals is an internationally recognized system for hazard classification and communication (available at <http://www.unece.org>.) It was developed with support from the International Labour Organization (ILO), the Organisation for Economic Co-operation and Development, and the United Nations Sub-Committee of Experts on the Transport of Dangerous Goods with the goal of standardizing hazard communication to improve the safety of international trade and commerce.

GHS recognizes 16 types of physical hazards, 10 types of health hazard, and an environmental hazard.

Physical hazards include

- explosives
- flammable gases

- flammable aerosols
- oxidizing gases
- gases under pressure
- flammable liquids
- flammable solids
- self-reactive substances
- pyrophoric liquids
- pyrophoric solids
- self-heating substances
- substances which, in contact with water, emit flammable gases
- oxidizing liquids
- oxidizing solids
- organic peroxides
- corrosive to metals

Health hazards include

- acute toxicity
- skin corrosion or irritation
- serious eye damage or eye irritation
- respiratory or skin sensitization
- germ cell mutagenicity
- carcinogenicity
- reproductive toxicology
- target organ systemic toxicity-single exposure
- target organ systemic toxicity-repeated exposure
- aspiration hazard

Environmental hazard includes

- Hazardous to the aquatic environment: acute aquatic toxicity or chronic aquatic toxicity with bioaccumulation potential rapid degradability.

Laboratory Chemical Safety Summaries (LCSSs)

Although MSDSs are invaluable resources, they suffer some limitations as applied to risk assessment in the specific context of the laboratory. LCSSs provide information on chemicals in the context of laboratory use. These documents are summaries and are not intended to be comprehensive or to fulfill the needs of all conceivable users of a chemical. LCSS gives essential information required to assess the risks associated with the use of a particular chemical in the laboratory. LCSSs also contain a concise critical discussion, presented in a style readily understandable to trained laboratory personnel, of

the toxicity, flammability, reactivity, and explosivity of the chemical; recommendations for the handling, storage, and disposal of the title substance; and first-aid and emergency response procedures.

Labels

Commercial suppliers are required by law (OSHA Hazard Communication Standard) to provide their chemicals in containers with precautionary labels. Labels usually present concise and non-technical summaries of the principal hazards associated with their contents. It is of note that precautionary labels do not replace MSDSs and LCSSs as the primary sources of information for risk assessment in the laboratory. However, labels serve as valuable reminders of the key hazards associated with the substance.

Additional Sources of Information

The resources described above provide the foundation for risk assessment of chemicals in the laboratory. Although MSDSs and LCSSs include information on toxic effects, in some situations laboratory personnel should seek additional more detailed information. This step is particularly important when laboratory personnel are planning to use chemicals that have a high degree of acute or chronic toxicity or when it is anticipated that work will be conducted with a particular toxic substance frequently or over an extended period of time.

The following annotated list provides references on the hazardous properties of chemicals and which are useful for assessing risks in the laboratory.

1. International Chemical Safety Cards from the International Programme on Chemical Safety (IPCS, 2009). The IPCS is a joint activity of the ILO, the United Nations Environment Programme, and the World Health Organization. The cards contain hazard and exposure information from recognized sources and undergo international peer review. They are available in 18 languages and can be found online through the NIOSH Web site, www.cdc.gov/niosh, or through the ILO Web site, www.ilo.org.
2. NIOSH Pocket Guide to Chemical Hazards (HHS/

CDC/NIOSH, 2007). This volume is updated regularly and is found on the NIOSH Web site (<http://www.cdc.gov/niosh>). These charts are quick guides to chemical properties, reactivities, exposure routes and limits, and first-aid measures.

3. A Comprehensive Guide to the Hazardous Properties of Chemical Substances, 3rd edition (Patnaik, 2007). This particularly valuable guide is written at a level appropriate for typical laboratory personnel. It covers more than 1,500 substances; sections in each entry include uses and exposure risk, physical properties, health hazards, exposure limits, fire and explosion hazards, and disposal or destruction. Entries are organized into chapters according to functional group classes, and each chapter begins with a general discussion of the properties and hazards of the class.
4. 2009 TLVs and BEIs: Based on the Documentation of the Threshold Limit Values for Chemical Substances and Physical Agents and Biological Exposure Indices. A booklet listing ACGIH threshold limit values (TLVs) and short-term exposure limits (STELs). These values are under continuous review, and this booklet is updated annually. The multivolume publication Documentation of the Threshold Limit Values and Biological Exposure Indices reviews the data that were used to establish the TLVs.
5. Fire Protection for Laboratories Using Chemicals (NFPA, 2004). This is the national fire safety code pertaining to laboratory use of chemicals. It describes the basic requirements for fire protection of life and property in the laboratory.
6. Fire Protection Guide to Hazardous Materials, 13th edition (NFPA, 2001). This resource contains hazard data on hundreds of chemicals and guidance on handling and storage of, and emergency procedures for those chemicals.
7. Hazardous Chemicals Handbook, 2nd edition (Carson and Mumford, 2002). This book is geared toward an industrial audience. It provides basic information about chemical hazards and synthesizes technical guidance from a number of authorities in chemical safety. The chapters are organized by hazard (e.g., "Toxic Chemicals," "Reactive Chemicals," and "Cryogens").

A number of Web-based resources also exist. Some of these are NIOSH Databases and Information Resources (www.cdc.gov/niosh) and TOXNET through the National Library of Medicine (NLM; www.nlm.nih.gov).

The National Library of Medicine Databases

The databases supplied by NLM are easy to use and free to access via the Web. TOXNET is an online collection of toxicological and environmental health databases. TOXLINE, for example, is an online database that accesses journals and other resources for current toxicological information on drugs and chemicals. It covers data published from 1900 to the present. Databases accessible through TOXNET include the Hazardous Substance Data Base (HSDB) Carcinogenic Potency Database (CPDB), the Developmental and Reproductive Toxicology Database (DART), the Genetic Toxicology Data Bank (GENE-TOX), the Integrated Risk Information System (IRIS), the Chemical Carcinogenesis Research Information System (CCRIS), and the International Toxicity Estimates for Risk (ITER). Other databases supplied by NLM that provide access to toxicological information are PubMed, which includes access to MEDLINE, PubChem, and ChemIDPlus. Free text searching is available on most of the databases. Another source of toxicity data is Chemical Abstracts Service (CAS). In addition to the NLM, several services provide CAS, including DIALOG, ORBIT, STN, and SciFinder. Searching procedures for CAS depend on the various services supplying the database. Additional information can be found on the CAS Web site, www.cas.org. Searching any database listed above is best done using the CAS registry number for the particular chemical.

Training

One important source of information for laboratory personnel is training sessions, and the critical place it holds in creating a safe environment should not be underestimated. Facts are only as useful as one's ability to interpret and apply them to a given problem, and training provides context for their use. Hands-on, scenario-based training is ideal because it provides the participants with the chance to practice activities

and behaviors in a safe way. Such training is especially useful for learning emergency response procedures. Another effective tool, particularly when trying to build awareness of a given safety concern, is case studies. Prior to beginning any laboratory activity, it is important to ensure that personnel have enough training to safely perform required tasks. If new equipment, materials, or techniques are to be used, a risk assessment should be performed, and any knowledge gaps should be filled before beginning work.

Toxic effects of laboratory chemicals

The chemicals encountered in the laboratory have a broad spectrum of physical, chemical, and toxicological properties and physiological effects. The risks associated with chemicals must be well understood prior to their use in an experiment. The risk of toxic effects is related to both the extent of exposure and the inherent toxicity of a chemical. As discussed in detail below, extent of exposure is determined by the dose, the duration and frequency of exposure, and the route of exposure. Exposure to even large doses of chemicals with little inherent toxicity, such as phosphate buffer, presents low risk. In contrast, even small quantities of chemicals with high inherent toxicity or corrosivity may cause significant adverse effects. The duration and frequency of exposure are also critical factors in determining whether a chemical will produce harmful effects. A single exposure to some chemicals is sufficient to produce an adverse health effect; for other chemicals repeated exposure is required to produce toxic effects. For most substances, the route of exposure (through the skin, the eyes, the gastrointestinal tract, or the respiratory tract) is also an important consideration in risk assessment. For chemicals that are systemic toxicants, the internal dose to the target organ is a critical factor. Exposure to acute toxicants can be guided by well-defined toxicity parameters based on animal studies and often human exposure from accidental poisoning. The analogous quantitative data needed to make decisions about the neurotoxicity and immunogenicity of various chemicals is often unavailable.

When considering possible toxicity hazards while planning an experiment, recognizing that the

combination of the toxic effects of two substances may be significantly greater than the toxic effect of either substance alone is important. Because most chemical reactions produce mixtures of substances with combined toxicities that have never been evaluated, it is prudent to assume that mixtures of different substances (*i.e.*, chemical reaction mixtures) will be more toxic than their most toxic ingredient. Furthermore, chemical reactions involving two or more substances may form reaction products that are significantly more toxic than the starting reactants. This possibility of generating toxic reaction products may not be anticipated by trained laboratory personnel in cases where the reactants are mixed unintentionally. For example, inadvertent mixing of formaldehyde (a common tissue fixative) and hydrogen chloride results in the generation of bis(chloromethyl)ether, a potent human carcinogen.

All laboratory personnel must understand certain basic principles of toxicology and recognize the major classes of toxic and corrosive chemicals. The next sections of this chapter summarize the key concepts involved in assessing the risks associated with the use of toxic chemicals in the laboratory.

Dose-Response Relationships

Toxicology is the study of the adverse effects of chemicals on living systems. The basic tenets of toxicology are that no substance is entirely safe and that all chemicals result in some toxic effects if a high enough amount (dose) of the substance comes in contact with a living system. For example, water, a vital substance for life, results in death if a sufficiently large amount (*i.e.*, gallons) is ingested at one time. On the other hand, sodium cyanide, a highly lethal chemical, produces no permanent (acute) effects if a living system is exposed to a sufficiently low dose. The single most important factor that determines whether a substance is harmful (or, conversely, safe) to an individual is the relationship between the amount (and concentration) of the chemical reaching the target organ, and the toxic effect it produces. For all chemicals, there is a range of concentrations that result in a graded effect between the extremes of no effect and death. In toxicology, this range is referred to as the dose-response relationship for the

chemical. The dose is the amount of the chemical and the response is the effect of the chemical. This relationship is unique for each chemical, although for similar types of chemicals, the dose-response relationships are often similar. Among the thousands of laboratory chemicals, a wide spectrum of doses exists that are required to produce toxic effects and even death. For most chemicals, a threshold dose has been established (by rule or by consensus) below which a chemical is not considered to be harmful to most individuals.

Some chemicals (e.g., dioxin) produce death in laboratory animals exposed to microgram doses and therefore are extremely toxic. Other substances, however, have no harmful effects following doses in excess of several grams. One way to evaluate the acute toxicity (*i.e.*, the toxicity occurring after a single exposure) of laboratory chemicals involves their lethal dose 50 (LD_{50}) or lethal concentration 50 (LC_{50}) value. The LD_{50} is defined as the amount of a chemical that when ingested, injected, or applied to the skin of a test animal under controlled laboratory conditions kills one-half (50%) of the animals. The LD_{50} is usually expressed in milligrams or grams per kilogram of body weight. For volatile chemicals (*i.e.*, chemicals with sufficient vapor pressure that inhalation is an important route of chemical entry into the body), the LC_{50} is often reported instead of the LD_{50} . The LC_{50} is the concentration of the chemical in air that will kill 50% of the test animals exposed to it. The LC_{50} is given in parts per million, milligrams per liter, or milligrams per cubic meter. Also reported are LC_{LO} and LD_{LO} values, which are defined as the lowest concentration or dose that causes the death of test animals. In general, the larger the LD_{50} or LC_{50} , the more chemical it takes to kill the test animals and, therefore, the lower the toxicity of the chemical. Although lethal dose values may vary among animal species and between animals and humans, chemicals that are highly toxic to animals are generally highly toxic to humans.

Assessing Risks of Exposure to Toxic Laboratory Chemicals

Exposure to a harmful chemical results in local toxic effects, systemic toxic effects, or both. Local effects

involve injury at the site of first contact; the eyes, the skin, the nose and lungs, and the digestive tract are typical sites of local reactions. Examples of local effects include (1) inhalation of hazardous materials causing toxic effects in the nose and lungs; (2) contact with harmful materials on the skin or eyes leading to effects ranging from mild irritation to severe tissue damage; and (3) ingestion of caustic substances causing burns and ulcers in the mouth, esophagus, stomach, and intestines. Systemic effects, by contrast, occur after the toxicant has been absorbed from the site of contact into the bloodstream and distributed throughout the body. Some chemicals produce adverse effects on all tissues of the body, but others tend to selectively injure a particular tissue or organ without affecting others. The affected organ (e.g., liver, lungs, kidney, and central nervous system) is referred to as the target organ of toxicity, although it is not necessarily the organ where the highest concentration of the chemical is found. Hundreds of systemic toxic effects of chemicals are known; they result from single (acute) exposures or from repeated or long-duration (chronic) exposures that become evident only after a long latency period.

Hazard Level	Toxicity Rating	Oral LD ₅₀ (rats, per kg)	Skin Contact LD ₅₀ (rabbits, per kg)	Inhalation LC ₅₀ (rats, ppm for 1 h)	Inhalation LC ₅₀ (rats, mg/m ³ for 1 h)
High	Highly toxic	< 50 mg	< 200 mg	< 200	< 2,000
Medium	Moderately toxic	50 to 500 mg	200 mg to 1 g	200 to 2,000	2,000 to 20,000
Low	Slightly toxic	500 mg to 5 g	1 to 5 g	2,000 to 20,000	20,000 to 200,000

Laboratory chemicals are grouped into several classes of toxic substances, and many chemicals display more than one type of toxicity. The first step in assessing the risks associated with a planned laboratory experiment involves identifying which chemicals in the proposed experiment are potentially hazardous substances. The term "health hazard" includes chemicals that are carcinogens, toxic or highly toxic agents, reproductive toxins, irritants, corrosives, sensitizers, hepatotoxins, nephrotoxins, neurotoxins, agents that act on the hematopoietic systems, and agents that damage the lungs, skin, eyes, or mucous membranes. The OSHA Laboratory Standard further requires that certain chemicals be identified as particularly hazardous substances (commonly known as PHSs) and handled using special additional procedures. PHSs include chemicals that are select carcinogens (those strongly

implicated as a potential cause of cancer in humans), reproductive toxins, and compounds with a high degree of acute toxicity. When working with these substances for the first time, it is prudent to consult with a safety professional prior to beginning work. This will provide a second set of trained eyes to review the safety protocols in place and will help ensure that any special emergency response requirements can be met in the event of exposure of personnel to the material or accidental release.

The following are the most common classes of toxic substances encountered in laboratories.

Acute Toxicants

Acute toxicity is the ability of a chemical to cause a harmful effect after a single exposure. Acutely toxic agents cause local toxic effects, systemic toxic effects, or both, and this class of toxicants includes corrosive chemicals, irritants, and allergens (sensitizers). In assessing the risks associated with acute toxicants, it is useful to classify a substance according to the acute toxicity hazard level as shown in the following table.

Acute Toxicity Hazard Level

Special attention is given to any substance classified according to the above criteria as having a high level of acute toxicity hazard. Chemicals with a high level of acute toxicity make up one of the categories of PHSs defined by the OSHA Laboratory Standard. The following table lists some of the most common chemicals with a high level of acute toxicity that are encountered in the laboratory.

Examples of Compounds with a High Level of Acute Toxicity	
Acrolein	Methyl fluorosulfonate
Arsine	Nickel carbonyl
Chlorine	Nitrogen dioxide

Diazomethane	Osmium tetroxide
Diborane (gas)	Ozone
Dimethyl mercury	Phosgene
Hydrogen cyanide	Sodium azide
Hydrogen fluoride	Sodium cyanide (and other cyanide salts)

Types of Toxins

Irritants, Corrosive Substances, Allergens, and Sensitizers

Lethal dose and other quantitative toxicological parameters generally provide little guidance in assessing the risks associated with corrosives, irritants, allergens, and sensitizers because these toxic substances exert their harmful effects locally. It would be very useful for the chemical research community if a quantitative measure for such effects were developed. When planning an experiment that involves corrosive substances, basic prudent handling practices should be reviewed to ensure that the skin, face, and eyes are protected adequately by the proper choice of corrosion-resistant gloves and protective clothing and eyewear, including, in some cases, face shields. Similarly, LD₅₀ and LC₅₀ data are not indicators of the irritant effects of chemicals, and therefore special attention should be paid to the identification of irritant chemicals by consulting LCSSs, MSDSs, and other sources of information. Allergens and sensitizers are another class of acute toxicants with effects that are not included in LD₅₀ or LC₅₀ data.

Asphyxiants

Asphyxiants are substances that interfere with the transport of an adequate supply of oxygen to vital organs of the body. The brain is the organ most easily affected by oxygen starvation, and exposure to asphyxiants leads to rapid collapse and death. Simple asphyxiants are substances that displace oxygen from the air being breathed to such an extent that adverse effects result. Acetylene, carbon dioxide, argon, helium, ethane, nitrogen, and methane are common asphyxiants. Certain other chemicals have the ability to combine with hemoglobin, thus reducing the capacity of the blood to transport oxygen. Carbon monoxide, hydrogen cyanide, and certain organic and

inorganic cyanides are examples of such substances.

Neurotoxins

Neurotoxic chemicals induce an adverse effect on the structure or function of the central or peripheral nervous system, which can be permanent or reversible. The detection of neurotoxic effects may require specialized laboratory techniques, but often they are inferred from behavior such as slurred speech and staggered gait. Many neurotoxins are chronically toxic substances with adverse effects that are not immediately apparent. Some chemical neurotoxins that may be found in the laboratory are mercury (inorganic and organic), organophosphate pesticides, carbon disulfide, xylene, trichloroethylene, and *n*-hexane.

Reproductive and Developmental Toxins

Reproductive toxins are defined by the OSHA Laboratory Standard as substances that cause chromosomal damage (mutagens) and substances with lethal or teratogenic (malformation) effects on fetuses. These substances have adverse effects on various aspects of reproduction, including fertility, gestation, lactation, and general reproductive performance, and can affect both men and women. Various reproductive hazards have been noted following exposure to halogenated hydrocarbons, nitro aromatics, arylamines, ethylene glycol derivatives, mercury, bromine, carbon disulfide, and other chemical reagents.

Carcinogens

A carcinogen is a substance capable of causing cancer. Cancer, in the simplest sense, is the uncontrolled growth of cells and can occur in any organ. The mechanism by which cancer develops is not well understood, but the current thinking is that some chemicals interact directly with DNA, the genetic material in all cells, to result in permanent alterations. Other chemical carcinogens modify DNA indirectly by changing the way cells grow. Carcinogens are chronically toxic substances; that is, they cause damage after repeated or long-duration exposure, and their effects may become evident only after a long latency period. Carcinogens are particularly insidious

toxins because they may have no immediate apparent harmful effects.

Flammable, reactive, and explosive hazards

In addition to the hazards due to the toxic effects of chemicals, hazards due to flammability, explosivity, and reactivity need to be considered in risk assessment. Reactive hazards arise when the release of energy from a chemical reaction occurs in quantities or at rates too great for the energy to be absorbed by the immediate environment of the reacting system, and material damage results.

The following outline provides a summary of the steps that laboratory personnel should use to assess the risks of managing physical hazards in the laboratory.

- Identify chemicals to be used and circumstances of use.
- Consult sources of information. Consult an up-to-date laboratory chemical safety summary, material safety data sheet, or NIOSH *Pocket Guide to Chemical Hazards* (HHS/CDC/NIOSH, 2007).
- Evaluate type of physical, flammable, explosive, or reactive hazard(s) posed by the chemicals.
- Evaluate the hazards posed by chemical changes over the course of the experiment.
- Evaluate type of physical hazard(s) posed by the equipment required.
- Select appropriate procedures to minimize risk.
- Prepare for contingencies. Be aware of institutional procedures in the event of emergencies and accidents.

Flammable Hazards

Flammable substances, those that readily catch fire and burn in air may be solid, liquid, or gaseous. The most common fire hazard in the laboratory is a flammable liquid or the vapor produced from such a liquid. An additional hazard is that compounds can enflame so rapidly that it produces an explosion. Proper use of substances that cause fire requires knowledge of their tendencies to vaporize, ignite, or burn under the variety of conditions in the laboratory.

Flammability Characteristics

Flash Point

The flash point is the lowest temperature at which a liquid has a sufficient vapor pressure to form an ignitable mixture with air near the surface of the liquid. Note that many common organic liquids have a flash point below room temperature: for example, acetone (-18°C), benzene (-11.1°C), diethyl ether (-45°C), and methyl alcohol (11.1°C). The degree of hazard

Table 1. NFPA Fire Hazard Ratings, Flash Points (FP), Boiling Points (bp), Ignition Temperatures, and Flammable Limits of Some Common Laboratory Chemicals

	NFPA Flammability Rating ^a	Flash Point (°C)	Boiling Point (°C)	Ignition Temperature (°C)	Flammable Limits (% by volume)	
					Lower	Upper
Acetaldehyde	4	-39	21	175	4	60
Acetic acid (glacial)	2	39	118	463	4	19.9
Acetone	3	-20	56	465	2.5	12.8
Acetonitrile	3	6	82	524	3	16
Carbon disulfide	4	-30	46	90	1.3	50
Cyclohexane	3	-20	82	245	1.3	8
Diethylamine	3	-23	57	312	1.8	10.1
Diethyl ether	4	-45	35	180	1.9	36
Dimethyl sulfoxide	2	95	189	215	2.6	42
Ethyl alcohol	3	13	78	363	3.3	19
Heptane	3	-4	98	204	1.05	6.7
Hexane	3	-22	69	225	1.1	7.5
Hydrogen	4		-252	500	4	75
Isopropyl alcohol	3	12	83	399	2	12.7 @ 200 (93)
Methyl alcohol	3	11	64	464	6	36
Methyl ethyl ketone	3	-9	80	404	1.4 @ 200 (93)	11.4 @ 200 (93)
Pentane	4	< -40	36	260	1.5	7.8
Styrene	3	31	146	490	0.9	6.8
Tetrahydrofuran	3	-14	66	321	2	11.8
Toluene	3	4	111	480	1.1	7.1
p-Xylene	3	25	138	528	1.1	7

^a 0, will not burn under typical fire conditions; 1, must be preheated to burn, liquids with FP ≥ 93.4°C (200°F); 2, ignitable when moderately heated, liquids with FP between 37.8°C (100°F) and 93.4°C (200°F); 3, ignitable at ambient temperature, liquids with FP < 22.8°C (73°F), bp ≥ 37.8°C (100°F) or FP between 22.8°C and 37.8°C (100°F); 4, extremely flammable, readily dispersed in air, and burns readily, liquids with FP < 22.8°C (73°F), bp < 37.8°C (100°F).

SOURCE: Adapted with permission from Fire Guide to Hazardous Materials (13th Edition), Copyright © 2001, National Fire Protection Association.

associated with a flammable liquid also depends on other properties, such as its ignition point and boiling point. At ambient pressure and temperature, an acetone spill produces a concentration as high as 23.7% acetone in air. Although it is not particularly toxic, with a flash point of -18°C and upper and lower flammable limits of 2.6% and 12.8% acetone in air, respectively clearly an acetone spill produces an extreme fire hazard.

Ignition Temperature

The ignition temperature (autoignition temperature) of a substance, whether solid, liquid, or gaseous, is the minimum temperature required to initiate or cause self-sustained combustion independent of the heat source. The lower the ignition temperature, the greater the potential for a fire started by typical laboratory equipment. For instance, carbon disulfide has an ignition temperature of 90°C , and it can be set off by a steam line or a glowing light bulb. Diethyl ether has an ignition temperature of 160°C and can be ignited by a hot plate.

Reactive Hazards

Water Reactives

Water-reactive materials are those that react violently with water. Alkali metals (e.g., lithium, sodium, and potassium), many organometallic compounds, and some hydrides react with water to produce heat and flammable hydrogen gas, which ignites or combines explosively with atmospheric oxygen. Some anhydrous metal halides (e.g., aluminum bromide), oxides (e.g., calcium oxide), and nonmetal oxides (e.g., sulfur trioxide), and halides (e.g., phosphorus pentachloride) react exothermically with water, resulting in a violent reaction if there is insufficient coolant water to dissipate the heat produced.

Pyrophorics

For pyrophoric materials, oxidation of the compound by oxygen or moisture in air proceeds so rapidly that ignition occurs. Many finely divided metals are pyrophoric, and their degree of reactivity depends on particle size, as well as factors such as the presence of

moisture and the thermodynamics of metal oxide or metal nitride formation. Other reducing agents, such as metal hydrides, alloys of reactive metals, low-valent metal salts, and iron sulfides, are also pyrophoric.

Explosive Hazards


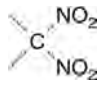
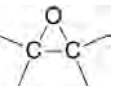

An explosive is any chemical compound or mechanical mixture that, when subjected to heat, impact, friction, detonation, or other suitable initiation, undergoes rapid chemical change, evolving large volumes of gases that exert pressure on the surrounding medium. Hydrogen and chlorine react explosively in the presence of light. Acids, bases, and other substances catalyze the explosive polymerization of acrolein, and many metal ions can catalyze the violent decomposition of hydrogen peroxide. Shock-sensitive materials include acetylides, azides, nitrogen triiodide, organic nitrates, nitro compounds, perchlorate salts (especially those of heavy metals such as ruthenium and osmium), many organic peroxides, and compounds containing diazo, halamine, nitroso, and ozonide functional groups.

The following table lists a number of explosive compounds. Some are set off by the action of a metal spatula on the solid; some are so sensitive that they are set off by the action of their own crystal formation. Diazomethane (CH_2N_2) and organic azides, for example, may decompose explosively when exposed to a ground glass joint or other sharp surfaces.

Functional Groups in Some Explosive Compounds

Azos, Peroxides, and Peroxidizables

Organic azo compounds and peroxides are among the most hazardous substances handled in the chemical laboratory but are also common reagents that often are used as free radical sources and oxidants. They are generally low-power explosives that are sensitive to shock, sparks, or other accidental ignition. They are far more shock sensitive than most primary explosives such as TNT. Inventories of these chemicals should be limited and subject to routine inspection. Liquids or

Structural Feature	Compound
$\text{— C} \equiv \text{C —}$	Acetylenic compounds
$\text{— C} \equiv \text{C — Metal}$	Metal acetylides
$\text{— C} \equiv \text{C — X}$	Haloacetylene derivatives
	Diazirines
>CN_2	Diazo compounds
>C—N=O	Nitroso compounds
>C—NO_2	Nitroalkanes, C-nitro and polynitroaryl compounds
	Polynitroalkyl compounds
>C—O—N=O	Acyl or alkyl nitrites
>C—O—NO_2	Acyl or alkyl nitrates
	1,2-Epoxides
>C=N—O—Metal	Metal fulminates or aci-nitro salts
	Fluorodinitromethyl compounds
>N—Metal	N-Metal derivatives
>N—N=O	N-Nitroso compounds
>N—NO_2	N-Nitro compounds
>C—N=N—C<	Azo compounds

Structural Feature	Compound
>C—N=N—O—C<	Arenediazoates
>C—N=N—S—C<	Arenediazo aryl sulfides
>C—N=N—O—N=N—C<	Bis-arenediazo oxides
>C—N=N—S—N=N—C<	Bis-arenediazo sulfides
>C—N=N—N—C< R	Triazenes (R = H, —CN, —OH, —NO)
>N=N—N=N<	High-nitrogen compounds, tetrazoles
>C—O—O—H	Alkylhydroperoxides
>C—CO—COOH	Peroxyacids
>C—O—O—C<	Peroxides (cyclic, diacyl, dialkyl)
>C—CO—COOR	Peroxyesters
$\text{N} \rightarrow \text{Cr—O}_2$	Aminechromium peroxocomplexes
—N_3	Azides (acyl, halogen, nonmetal, organic)
$\text{>C—N}_2^+\text{S}^-$	Diazoniumsulfides and derivatives, "xanthates"
$\text{N}^+ \text{—HZ}^-$	Hydrazinium salts, oxosalts of nitrogenous bases
$\text{—N}^+ \text{—OH Z}^-$	Hydroxylammonium salts
$\text{>C—N}_2^+\text{Z}^-$	Diazonium carboxylates or salts
—O—X	Alkyl perchlorates, chlorite salts, halogen oxides, hypohalites, perchloric acid, perchloryl compounds

SOURCE: Carson and Mumford (2002). Reprinted from Hazardous Chemicals Handbook (Second Edition), Carson, P. and Mumford, C. "Reactive Chemicals", p. 228, Copyright 2002, with permission from Elsevier.

solutions of these compounds should not be cooled to the point at which the material freezes or crystallizes from solution, however, because this significantly increases the risk of explosion. Refrigerators and freezers storing such compounds should have a backup power supply in the event of electricity loss. Users should be familiar with the hazards of these materials and trained in their proper handling.

Certain common laboratory chemicals form peroxides on exposure to oxygen in air (see the following table). Over time, some chemicals continue to build peroxides to potentially dangerous levels, whereas others accumulate a relatively low equilibrium concentration of peroxide, which becomes dangerous only after being concentrated by evaporation or distillation.

Classes of Chemicals That Can Form Peroxides

Class A: Chemicals that form explosive levels of peroxides without concentration	
Isopropyl ether	Sodium amide (sodamide)
Butadiene	Tetrafluoroethylene
Chlorobutadiene (chloroprene)	Divinyl acetylene
Potassium amide	Vinylidene chloride
Potassium metal	
Class B: These chemicals are a peroxide hazard on concentration (distillation/evaporation). A test for peroxide should be performed if concentration is intended or suspected.	
Acetal	Dioxane (p-dioxane)
Cumene	Ethylene glycol dimethyl ether (glyme)
Cyclohexene	
Cyclooctene	Furan
Cyclopentene	Methyl acetylene
Diaacetylene	Methyl cyclopentane
Dicyclopentadiene	Methyl-isobutyl ketone
Diethylene glycol dimethyl ether (diglyme)	Tetrahydrofuran
Diethyl ether	Tetrahydronaphthalene
	Vinyl ethers
Class C: Unsaturated monomers that may autopolymerize as a result of peroxide accumulation if inhibitors have been removed or are depleted	
Acrylic acid	Styrene
Butadiene	Vinyl acetate
Chlorotrifluoroethylene	Vinyl chloride
Ethyl acrylate	Vinyl pyridine
Methyl methacrylate	

* These lists are illustrative, not comprehensive. SOURCES: Jackson *et al.* (1970) and Kelly (1996).

Essentially all compounds containing C—H bonds pose the risk of peroxide formation if contaminated with various radical initiators, photosensitizers, or catalysts. For instance, secondary alcohols such as isopropanol form peroxides when exposed to normal fluorescent lighting and contaminated with photosensitizers, such as benzophenone. Acetaldehyde, under normal conditions, autoxidizes to form acetic acid.

Physical hazards

Compressed Gases

Compressed gases can expose the trained laboratory personnel to both mechanical and chemical hazards, depending on the gas. Hazards can result from the flammability, reactivity, or toxicity of the gas; from the possibility of asphyxiation; and from the gas compression itself, which could lead to a rupture of the tank or valve.

Nonflammable Cryogenics

Nonflammable cryogenics (chiefly liquid nitrogen) can cause tissue damage from extreme cold because of contact with either liquid or boil-off gases. In poorly ventilated areas, inhalation of gas due to boil off or spills can result in asphyxiation. Another hazard is explosion from liquid oxygen condensation in vacuum traps or from ice plug formation or lack of functioning vent valves in storage Dewars. Because 1 volume of liquid nitrogen at atmospheric pressure vaporizes to 694 volumes of nitrogen gas at 20°C, the warming of such a cryogenic liquid in a sealed container produces enormous pressure, which can rupture the vessel.

High-Pressure Reactions

Experiments that generate high pressures or are carried out at pressures above 1 atm can lead to explosion from equipment failure. For example, hydrogenation reactions are frequently carried out at elevated pressures, and a potential hazard is the formation of explosive O₂/H₂ mixtures and the reactivity/pyrophoricity of the catalyst. High pressures can also be associated with the use of supercritical fluids.

Vacuum Work

Precautions to be taken when working with vacuum lines and other glassware used at sub ambient pressure are mainly concerned with the substantial danger of injury in the event of glass breakage. The degree of hazard does not depend significantly on the magnitude of the vacuum because the external pressure leading to implosion is always 1 atmosphere. Thus, evacuated systems using aspirators merit as much respect as high-vacuum systems. Injury due to flying glass is not the only hazard in vacuum work. Additional dangers can result from the possible toxicity of the chemicals contained in the vacuum system, as well as from fire following breakage of a flask (e.g., of a solvent stored over sodium or potassium).

Ultraviolet, Visible, and Near-Infrared Radiation

Ultraviolet, visible, and infrared radiation from lamps and lasers in the laboratory can produce a number of hazards. Medium-pressure Hanovia 450 Hg lamps are commonly used for ultraviolet irradiation in photochemical experiments. Ultraviolet lights used in biosafety cabinets, as decontamination devices, or in light boxes to visualize DNA can cause serious skin and corneal burns. Powerful arc lamps can cause eye damage and blindness within seconds. When incorrectly used, the light from lasers poses a hazard to the eyes of the operators and other people present in the room and is also a potential fire hazard. Depending on the type of laser, the associated hazards can include mutagenic, carcinogenic, or otherwise toxic laser dyes and solvents; flammable solvents; ultraviolet or visible radiation from the pump lamps; and electric shock from lamp power supplies.

Electrical Hazards

The electrocution hazards of electrically powered instruments, tools, and other equipment are almost eliminated by taking reasonable precautions, and the presence of electrically powered equipment in the laboratory need not pose a significant risk. But, in the laboratory these safety features should not be defeated by thoughtless or ill-informed modification. Equipment malfunctions can lead to electrical fires. Some special concerns arise in

laboratory settings. The insulation on wires can be eroded by corrosive chemicals, organic solvent vapors, or ozone (from ultraviolet lights, copying machines, and so forth). Eroded insulation on electrical equipment in wet locations such as cold rooms or cooling baths must be repaired immediately. In addition, sparks from electrical equipment can serve as an ignition source in the presence of flammable vapor. Operation of certain equipment (e.g., electrophoresis equipment) may involve high voltages and stored electrical energy.

Magnetic Fields

Increasingly, instruments that generate large static magnetic fields (e.g., NMR spectrometers) are present in research laboratories. Such magnets typically have fields of 14,000 to 235,000 G (1.4 to 23.5 T), far above that of Earth's magnetic field, which is approximately 0.5 G. The magnitude of these large static magnetic fields falls off rapidly with distance. Many instruments now have internal shielding, which reduces the strength of the magnetic field outside of the instrument. Strong attraction occurs when the magnetic field is greater than 50 to 100 G and increases by the seventh power as the separation is reduced. However, this highly nonlinear falloff of magnetic field with distance results in an insidious hazard.

Ergonomic Hazards in the Laboratory

General workplace hazards also apply in the laboratory. For example, laboratory personnel are often involved in actions such as pipetting and computer work that can result in repetitive-motion injuries. Working at a bench or at a microscope without considering posture can result in back strain, and some instruments require additional in-room ventilation that may raise the background noise level to uncomfortable or hazardous levels. With these and other issues such as high or low room temperatures and exposure to vibrations, it is important to be aware of and to control such issues to reduce occupational injuries. For example, microscope users may find that using a camera to view images on a screen, rather than direct viewing through the eyepiece, reduces back and eye strain. The Centers for Disease Control

and Prevention (CDC) and the National Institutes of Health have information on their Web sites (www.cdc.gov and www.nih.gov, respectively) describing specific ergonomic concerns for laboratories and proposed solutions. The CDC provides a downloadable self-assessment form to aid in evaluating these hazards. NIOSH (www.cdc.gov/niosh) and OSHA (www.osha.gov) provide information about vibration, noise levels, and other workplace hazards.

Bio hazards

Biohazards are a concern in laboratories in which microorganisms, or material contaminated with them, are handled. Anyone who is likely to come in contact with blood or potentially infectious materials at work is covered under OSHA's Bloodborne Pathogen Standard. These hazards are usually present in clinical and infectious disease research laboratories but may also be present in any laboratory in which bodily fluids, tissues, or primary or immortalized cell lines of human or animal origin are handled. Biohazards are also present in any laboratory that uses microorganisms, including replication-

deficient viral vectors, for protein expression or other *in vitro* applications. Risk assessment for biological toxins is similar to that for chemical agents and is based primarily on the potency of the toxin, the amount used, and the procedures in which the toxin is used.

Suggested readings

OSHA Hazard Communication Standard 1910.1200

Dedicated website: <http://www.unece.org>

International Chemical Safety Cards from the International Programme on Chemical Safety IPCS, 2009

Dedicated website: www.cdc.gov/niosh

Dedicated website: www.ilo.org

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A Comprehensive Guide to the Hazardous Properties of Chemical Substances, 3rd edition (Patnaik, 2007).

Hazardous Chemicals Handbook, 2nd edition, Carson and Mumford, 2002

NIOSH Databases and Information Resources, www.cdc.gov/niosh TOXNET through the National Library of Medicine (NLM; dedicated website: www.nlm.nih.gov)

Dedicated website: www.cas.org

NIOSH Pocket Guide to Chemical Hazards (HHS/CDC/NIOSH, 2007)

Dedicated website: www.cdc.gov

Dedicated website: www.nih.gov

Dedicated website: www.cdc.gov/niosh

Dedicated website: www.osha.gov

General Biochemical Methodologies

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Introduction

All biochemistry experimental activities in a research laboratory are replete with techniques that must be carried out on an almost daily basis. This lecture outlines the theoretical and practical aspects of some of these general and routine procedures.

Keeping records and communicating experimental results

The biochemistry laboratory experience is not finished when you complete the experimental procedure and leave the laboratory. Reports are most easily prepared outside of the lab using notes taken in a laboratory notebook while the experiment is in progress. These notes usually include procedural details, preparation of all reagents and solutions, setup of equipment, collection of data, and your thought processes and observations during the experiment. Experiments are often complex and move rather quickly, and it would be impossible to write down your data and not a good practice to record results on scraps of paper or on paper towels that may easily become lost or destroyed. The lab notebook will also come in handy if you need to troubleshoot or repeat an experiment because of inconsistent results. Your instructor may have his or her own rules for preparation of the lab notebook, but here are some useful guidelines:

1. The notebook should be hardbound with ruled pages; writing should be done with pen. This provides a permanent, durable record and the potential for construction of tables, graphs, charts, etc. Number each page of the book.
2. Save the first few pages of the book for construction of a table of contents. Keep this

up-to-date by entering the name of each experiment and page number.

3. Use the right-hand pages only for writing your experimental notes. The left-hand pages may be used as scratch paper for your own personal notes, reminders, or calculations not appropriate for the main entry. Each entry for an experiment or project must begin with a title and date.

Using biochemical reagents and solutions

Water Purity

Water is the most common and widely-used substance in the biochemistry laboratory. Applications of water usage include: (1) solvent for preparing most buffer and reagent solutions; (2) column chromatography; (3) high-performance liquid chromatography; (4) tissue culture; and (5) washing glassware. Both the quality and quantity of water required must be considered for each lab application. Ordinary tap water is relatively abundant, but its quality is very low. It contains a variety of impurities including particulate matter (sand, silt, etc.); dissolved organics, inorganics, and gases; and microorganisms (bacteria, viruses, protozoa, and algae). In addition, the natural degradation of microorganisms leads to the presence of byproducts called pyrogens. Tap water should never be used for the preparation of reagent solutions or for any sensitive procedures. For most laboratory procedures, it is recommended that some form of purified water be used. There are five basic water purification technologies—distillation, ion-exchange, activated carbon adsorption, reverse osmosis, and membrane filtration. Most academic and industrial

research laboratories are equipped with “in-house” purified water, which typically is produced by a combination of the above purifying

processes and piped throughout all the labs in a building. The water quality necessary will depend on the solutions to be prepared and on the biochemical procedures to be investigated. For most procedures carried out in the biochemistry lab, water purified by ion-exchange, reverse osmosis, or distillation is usually acceptable.

Cleaning Laboratory Glassware

The results of your experimental work will depend, to a great extent, on the cleanliness of your equipment, especially glassware used for preparing and transferring solutions. There are at least two important reasons for this: (1) many of the chemicals and biochemicals will be used in milligram, microgram, or even nanogram amounts. Any contamination, whether on the inner walls of a beaker, in a pipet, or in a glass cuvette, could be a significant percentage of the total experimental sample; (2) many biochemicals and biochemical processes are sensitive to one or more of the following common contaminants: metal ions, detergents, and organic residues. In fact, the objective of many experiments is to investigate the effect of a metal ion, organic molecule, or other chemical agent on a biochemical process. Contaminated glassware will virtually ensure failure in these activities.

The preferred method for cleaning glassware is to begin with hot tap water. Rinse the glassware at least 10 times with this; then rinse 4–6 times with distilled or de-ionized water. Occasionally it is necessary to use a detergent for cleaning. Use a dilute detergent solution (0.5% in water) followed by 5–10 water rinses with distilled or de-ionized water. Dry equipment is required for most processes carried out in the biochemistry laboratory. When you needed dry glassware in the organic laboratory, you probably rinsed the glassware with acetone, which rapidly evaporated, leaving a dry surface. Unfortunately, this technique coats the surface with an organic residue consisting of nonvolatile contaminants found in the acetone. Because this residue could interfere with your

experiments, it is best to refrain from acetone washing. Glassware and plastic ware should be rinsed well with purified water and dried in an oven designated for glassware, not one used for drying chemicals. Never clean cuvettes or any optically polished glassware with ethanolic KOH or other strong base, as this will cause etching. All glass cuvettes should be cleaned carefully with hot tap water or 0.5% detergent solution, in a sonicator bath or in a cuvette washer, followed by thorough rinsing with purified water.

Concentrations and Calculations

The concentrations for solutions used in the biochemistry laboratory may be expressed in several different units. The most common units are:

Molarity (M): concentration based on the number of moles of solute per liter of solution. A 1 M solution of the amino acid alanine contains 1 mole, or 89.1 g, of alanine in a solution volume of 1 liter. In biochemistry, it is more common to use concentration ranges that are millimolar micromolar or nanomolar

Percent by weight (% wt/wt): concentration based on the number of grams of solute per 100 g of solution.

Preparing and Storing Solutions

In general, solid solutes should be weighed on weighing paper or plastic weighing boats, with the use of an electronic analytical or top-loading balance. Liquids are more conveniently dispensed by volumetric techniques; however, this assumes that the density is known. If a small amount of a liquid is to be weighed, it should be added to a tared flask by means of a disposable Pasteur pipet with a latex bulb. The hazardous properties of all materials should be known before use and the proper safety precautions obeyed. The storage conditions of reagents and solutions in the biochemistry lab are especially critical. Although some will remain stable indefinitely at room temperature, it is good practice to store all solutions in a closed container. Often it is necessary to store some solutions in a

refrigerator at this inhibits bacterial growth and slows decomposition of the reagents. Some solutions may require storage below 0°C. If these are aqueous solutions or others that will freeze, be sure there is room for expansion inside the container. Stored solutions must always have a label containing the name and concentration of the solution, the date prepared, and the name of the preparer. All stored containers, whether at room temperature, or below freezing, must be properly sealed. This reduces contamination by bacteria and vapors in the laboratory air (carbon dioxide, ammonia, HCl, etc.). Volumetric flasks, of course, have glass stoppers, but test tubes, Erlenmeyer flasks, bottles, and other containers should be sealed with screw caps, corks, or hydrocarbon foil (Parafilm). Remember that hydrocarbon foil, a wax, is dissolved by solutions containing nonpolar organic solvents like chloroform, diethyl ether, and acetone. Bottles of pure chemicals and reagents should also be properly stored. Many manufacturers now include the best storage conditions for a reagent on the label. The common conditions are: store at room temperature; store at store below or store in a desiccator at room temperature, or below. Many biochemical reagents form hydrates by taking up moisture from the air. If the water content of a reagent increases, the molecular weight and purity of the reagent change.

Quantitative transfer of liquids

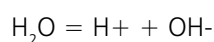
Practical biochemistry is highly reliant on analytical methods. Many analytical techniques must be mastered, but few are as important as the quantitative transfer of solutions. Some type of pipet will almost always be used in liquid transfer. The use of any pipet requires some means of drawing reagent into the pipet. Liquids should never be drawn into a pipet by mouth suction on the end of the pipet! Small latex bulbs are available for use with disposable pipets. For volumetric and graduated pipets, two types of bulbs are available. One type features a special conical fitting that accommodates common sizes of pipets. To use these, first place the pipet tip below the surface of the liquid. Squeeze the bulb with the left hand (if you are a right-handed pipettor) and then hold it tightly to the end of the pipet. Slowly release the pressure on the bulb to allow liquid to rise to 2 or 3 cm above

the top graduated mark. Then, remove the bulb and quickly grasp the pipet with your index finger over the top end of the pipet. The level of solution in the pipet will fall slightly, but should not fall below the top graduated mark. If it does fall too low, use the bulb to refill.

Special procedures are required for cleaning glass pipets. Immediately after use, every pipet should be placed, tip up, in a vertical cylinder containing warm tap water or a dilute detergent solution (less than 0.5%). The pipet must be completely covered with solution. This ensures that any reagent remaining in the pipet is forced out through the tip. If reagent solutions are allowed to dry inside a pipet, the tips can easily become clogged and are very difficult to open. After several pipets have accumulated in the water or detergent solution, the pipets should be transferred to a pipet rinser. Pipet rinsers continually cycle fresh water through the pipets. Immediately after detergent wash, tap water may be used to rinse the pipets, but distilled water should be used for the final rinse. Pipets may then be dried in an oven.

pH, buffers, electrodes, and biosensors

Most biological processes in the cell take place in a water-based environment. Water is an amphoteric substance; that is, it may serve as a proton donor (acid) or a proton acceptor (base). The following equation shows the ionic equilibrium of water.



In pure water, in other words, the pH or is 7.

[The pH scale runs from 0 (very acidic) to 14 (very basic)]. Acidic and basic molecules, when dissolved in water in a biological cell or test tube, react with either hydrogen ions or hydroxide ions to shift the equilibrium of the equation and result in a pH change of the solution. Biochemical processes occurring in cells and tissues depend on strict regulation of the hydrogen ion concentration. Natural acids and bases are often generated in cells by normal biological processes and they must be neutralized by buffers. Biological pH is maintained at a constant value by naturally-occurring

buffer systems such as phosphate. When biological processes are studied *in vitro*, artificial media must be prepared that mimic the cell's natural environment. Because of the dependence of biochemical reactions on pH, the accurate determination of hydrogen ion concentration has always been of major interest.

Measurement of pH

A pH measurement is usually taken by immersing a glass or plastic combination electrode into a solution and reading the pH directly from a meter. At one time, pH measurements required two electrodes, a pH-dependent glass electrode sensitive to ions and a pH-independent calomel reference electrode. The potential difference that develops between the two electrodes is measured as a voltage, as defined by the following equation.

$$V = E_{\text{const}} + (2.303 RT/F) * \text{pH}$$

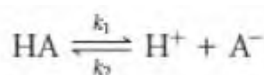
where

- V = voltage of the completed circuit
- Econstant = potential of reference electrode
- R = the gas constant
- T = the absolute temperature
- F = the Faraday constant

A pH meter is standardized with buffer solutions of known pH before a measurement of an unknown solution is taken. It should be noted from the above equation that the voltage depends on temperature. Hence, pH meters must have some means for temperature correction. Older instruments usually have a knob labeled "temperature control," which is adjusted by the user to the temperature of the measured solution. Newer pH meters automatically display a temperature-corrected pH value.

Biochemical Buffers

Buffer ions are used to maintain solutions at constant pH values. Weak acids and bases do not completely dissociate in solution, but exist as equilibrium mixtures as described in the following equation.



HA represents a weak acid and represents its conjugate base; represents the rate constant for dissociation of the acid and k2 the rate constant for association of the conjugate base and hydrogen ion.

$$K_a = \frac{k_1}{k_2} = \frac{[\text{H}^+][\text{A}^-]}{[\text{HA}]}$$

The equilibrium constant, for the weak acid HA is defined by Equation 3.4.

which can be rearranged to define the following equation,

$$[\text{H}^+] = \frac{K_a[\text{HA}]}{[\text{A}^-]}$$

The is often reported as pH, which is in a similar fashion, is represented by the following equation,

$$\text{pH} = \text{p}K_a + \log \frac{[\text{A}^-]}{[\text{HA}]}$$

This equation is termed as the Henderson-Hasselbalch equation, which defines the relationship between pH and the ratio of acid and conjugate base concentrations. The salt of the acid is also referred to as the proton acceptor (A) and the acid (HA) as the proton donor (D). The Henderson-Hasselbalch equation is of great value in buffer chemistry because it can be used to calculate the pH of a solution if the molar ratio of buffer ions and the of HA are known. Also, the molar ratio of HA to that is necessary to prepare a buffer solution at a specific pH can be calculated if the is known.

A solution containing both HA and has the capacity to resist changes in pH; *i.e.*, it acts as a buffer. If acid were added to the buffer solution, it would be neutralized by in solution:



Base added to the buffer solution would be neutralized by reaction with HA:



Biosensors

Electrodes or electrode-like devices are currently being developed for the specific measurement of physiologically important molecules such as urea, carbohydrates, enzymes, antibodies, and metabolic products. This type of device, now referred to as a biosensor, is an analytical tool or system consisting of an immobilized biological material (such as an enzyme, antibody, whole cell, organelle, or combinations thereof) in intimate contact with a suitable transducer device that will convert the biochemical signal into a quantifiable electric signal. The important components of a biosensor are:

1. A reaction center consisting of a membrane or gel containing the biochemical system to be studied,
2. A transducer,
3. An amplifier, and
4. A computer system for data acquisition and processing.

When biomolecules in the reaction center interact, a physicochemical change occurs. This change in the molecular system, which may be a modification of concentration, absorbance, mass, conductance, or redox state, is converted into an electrical signal by the transducer. The signal is then amplified and displayed on a computer screen. Each biosensor is specifically designed for a particular type of molecule or biological reaction.

Measurement of protein solutions

Biochemical research often requires the quantitative measurement of protein concentrations in solutions. Several techniques for such measurement have been developed; however, most have limitations because either they are not sensitive enough or they are based on reactions with specific amino acids in the protein. Since the amino acid content varies from protein to protein, no single assay will be suitable for all proteins. In this section, we discuss different assays that are widely used.

When substances containing two or more peptide bonds react with the biuret reagent, alkaline copper sulfate, a purple complex is formed. The colored

product is the result of coordination of peptide nitrogen atoms with the reagents, and the amount of product formed depends on the concentration of protein. In practice, a calibration curve must be prepared by using a standard protein solution. An aqueous solution of bovine serum albumin (BSA) is a commonly used standard. Various known amounts of this solution are treated with the biuret reagent, and the color is allowed to develop. Measurements of absorbance at 540 nm are made against a blank containing biuret reagent and buffer or water. The A₅₄₀ data are plotted versus protein concentration (mg/mL) or amount of protein (mg). Unknown protein samples are treated with biuret reagent and measured after color development. The protein concentration is determined from the standard curve. The biuret assay has several advantages, including speed, similar color development with different proteins, and few interfering substances. Its primary disadvantage is its lack of sensitivity.

The Lowry protein assay is one of the more sensitive assays and has been widely used. The principle behind color development is identical to that of the biuret assay except that a second reagent (Folin-Ciocalteu) is added to increase the amount of color development. Two reactions account for the intense blue color that develops: (1) the coordination of peptide bonds with alkaline copper (biuret reaction), and (2) the reduction of the Folin-Ciocalteu reagent (phosphomolybdate-phosphotungstate) of the biuret assay. A standard curve is prepared with bovine serum albumin or other pure protein, and the concentration of unknown protein solutions is determined from the graph. The obvious advantage of the Lowry assay is its sensitivity, which is up to 100 times greater than that of the biuret assay; however, more time is required for the Lowry assay.

Techniques for sample preparation

Dialysis

One of the oldest procedures applied to the purification and characterization of biomolecules is dialysis, an operation used to separate dissolved

molecules on the basis of their molecular size. The technique involves sealing an aqueous solution containing both macromolecules and small molecules in a porous membrane. The sealed membrane is placed in a large container of low-ionic-strength buffer. The membrane pores are too small to allow diffusion of macromolecules of molecular weight greater than about 10,000. Smaller molecules diffuse freely through the openings. The passage of smaller molecules continues until their concentrations inside the dialysis tubing and outside in the large volume of buffer are equal. Thus, the concentration of small molecules inside the membrane is reduced. Equilibrium is volume dependent and is reached after 4 to 6 hours. If the outside solution (dialysate) is replaced with fresh buffer after equilibrium is reached, the concentration of small molecules inside the membrane will be further reduced by continued dialysis. Dialysis membranes are available in a variety of materials and sizes. The most common materials are collodion, cellophane, and cellulose. Recent modifications in membrane construction make a range of pore sizes available. Spectrum Laboratories offers Spectra/Por membrane tubing with complete molecular weight cutoffs ranging from 100 to 300,000. Dialysis is most commonly used to remove salts and other small molecules from solutions of macromolecules. During the separation and purification of biomolecules, small molecules are added to selectively precipitate or dissolve the desired molecule. For example, proteins are often precipitated by addition of organic solvents or salts such as ammonium or sodium sulfate. Since the presence of organics or salts usually interferes with further purification and characterization of the molecule, they must be removed. Dialysis is a simple, inexpensive, and effective method for removing all small molecules, ionic or nonionic. Dialysis is also useful for removing small ions and molecules that are weakly bound to biomolecules.

Ultrafiltration

Although dialysis is still used occasionally as a purification tool, it has been largely replaced by ultrafiltration and gel filtration. The major disadvantage of dialysis that is overcome by the newer methods is that it may take several days of dialysis to attain a

suitable separation. The other methods require 1 to 2 hours or less. Ultrafiltration involves the separation of molecular species on the basis of size, shape, and/or charge. The solution to be separated is forced through a membrane by an external force. Membranes may be chosen for optimum flow rate, molecular specificity, and molecular weight cutoff. Two applications of membrane filtration are obvious: (1) desalting buffers or other solutions, and (2) clarification of turbid solutions by removal of micron- or submicron-sized particles. Ultrafiltration membranes have molecular weight cutoffs in the range of 100 to 1,000,000. They are usually composed of two layers: (1) a thin (0.1–0.5), surface, semipermeable membrane made from a variety of materials including cellulose acetate, nylon, and polyvinylidene, and (2) a thicker, inert, support base. These filters function by retaining particles on the surfaces, not within the base matrix. Membrane filters of these materials can be manufactured with a predetermined and accurately controlled pore size. These filters require suction, pressure, or centrifugal force for liquid flow. A typical flow rate for the commonly used membrane is 57 mLmin⁻¹ cm⁻² at 10 psi. Ultrafiltration devices are available for macroseparations (up to 50 L) or for microseparations (milli- to microliters).

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Introduction to Marine Bio-prospecting

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Introduction

Marine bioprospecting can be described as targeted and systematic search for bioactive compounds within marine organisms. This may include bacteria, fungi and viruses and larger organisms such as sea plants, shellfish and fish. The result of the bioprospecting could be a purified molecule that is produced biologically or synthetically, or the entire organism. The purpose of marine bioprospecting, from a business perspective, is to find the components and purified compounds that may be included as components in products or processes. Marine bioprospecting, therefore, is not an industry in the traditional sense, but it may procure different compounds that may be used in many different industries. Marine bioprospecting makes exploitation of bioactive compounds from marine organisms possible. Collection and extensive analysis/preparation of the collected material is necessary before the substance is suitable for further development to an end product or product component. The various phases within marine bioprospecting can be divided as development from collection of marine organisms, preparation, categorisation, storage and analysis to creation of a substance, *i.e.* marine genetic and biological material with the potential for further use.

Applications and importance

Humans have traditionally searched for natural bioactive substances that can be used in medicines and for other purposes. It has been asserted that over 65% of medicines used today are based on natural substances, most of which are derived from the biodiversity found on land. The biodiversity that can be found in the sea has not been explored

to the same extent despite the fact that the sea covers more than 70% of the Earth's surface. The evolution of the marine environment started several million years earlier than that of the land, and the marine biodiversity is thought to be greater than that found on land. Up to this point in time, little is known about the molecules and genetic properties of the marine species. This applies particularly to organisms from cold waters. Up to now, research has mainly concentrated on life forms from tropical and temperate regions. In the future, we will probably see a shift in focus to mapping biological material from northern waters. In addition, there is also increasing interest in life at sea, as products isolated from marine organisms tend to be more bioactive than equivalent bioactive compounds isolated from land. In relation to future economy related to biotechnology, also called "the bioeconomy", the OECD estimates that the industrial application of biotechnology will be of great significance until 2030. It is estimated that biotechnology-based output could account for approximately 35% of the output value of chemicals and other industrial products. Correspondingly this will be 80% for pharmaceutical and diagnostic products and 50% for agriculture/food. Within the biotechnology area generally, industrial processes could be the largest sector with 39%, while agriculture/food and health is estimated at 36% and 25% respectively.

Marine flora and fauna and their potential use

The great ocean forms more than 70% of the earth, though we know to some extent of the coastal and the upper layers of the ocean, the 80 percent of the ocean, which constitute 62 percent of the entire

earth's surface, are unknown. The marine environment provides a wide range of goods and services essential for human life. Other than food, marine ecosphere constitutes a vast reservoir of valuable compounds with wide range of bioactivities against several life-threatening diseases.

The "Marine Pharmacology Review 2003-2004" shows initial pharmacological results of 166 marine chemicals with

- About 67 marine organisms showing antibacterial, antifungal, antimalarial, antituberculosis or antiviral activities
- About 45 marine derived compounds reported to have significant effects on the cardiovascular, immune and nervous system as well as possessing anti-inflammatory effects.
- About 54 marine derived compounds, which act on a variety of molecular targets with a potential contribution to several pharmacological classes (Source: WWF).

There is a high degree of representation of terrestrial-derived bioproducts, and, therefore, the number of marine natural products that have found their way into pharmacies is thus far small. This has more to do with the relative infancy of marine bioprospecting

(than terrestrial bioprospecting). The natural products isolated from marine sources tend to be more highly bioactive than terrestrial counterparts because they have to retain their potency despite dilution in surrounding seawater to be effective in the "chemical warfare" that allows marine flora and fauna to ward off would-be predators and animals that might attempt to grow over and smother them. Despite lesser attention paid to marine natural products historically, there are notable marine-derived bio products that are commercially available and IP protected.

Bioactive Compounds from Marine Organisms

Ocean is a potential source of bioactive compounds, which does not have a significant history of use in traditional medicine as in the case of terrestrial plants (Kamboj, 1999). Previously, the research was focused mainly on terrestrial plants because Number of marine natural products: 1971- 2005 (Blunt, 2007) of their easier availability. The isolation of biologically unique molecules from marine organisms that are not found in terrestrial sources leads to a remarkable progress in marine bioprospecting. The boom of marine bioprospecting began in recent years and 18000 plus natural compounds from marine organisms have been

Diseases	Biomolecules/bioactive compounds	Marine source organisms
HIV	Cyclodiderniserinol trisulfate	Tunicate
	Lamellarin α -20-sulfate	Tunicate
Pain	Conotoxins	Gastropods
Cancer	Bryostatin	Bryozoan
	Didemnin-B	Tunicate
	Dolastatin 10	Sea hare
	Halichondrin B	Sponge
	Ecteinascidin 743	Tunicate
	Kahalalide F	Gastropod
Asthma	Aplidine	Tunicate
	Contignasterol	Sponge

(Modified after Kijjoa and Sawangwong 2004)

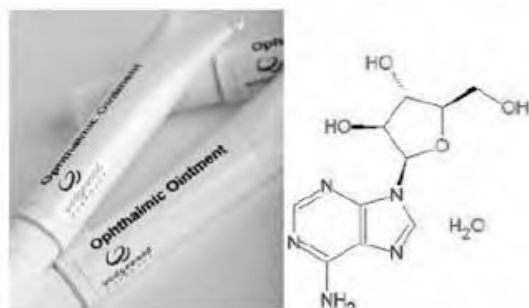
isolated as compared to 155000 terrestrial products (Blunt, 2004; Mayer *et al.*, 2007). Between 1969 and 1995, 63 marine substances were patented as antitumour agents, accounting for half the marine molecules patented for pharmaceutical purposes (MartínezPrat, 2002). There are a significant (and growing) number of marine-derived compounds with pharmaceutical potential in the pipeline. The accompanying table in the next page (modified from one included in Kijjoa and Sawangong, 2004) presents the marinederived potential therapeutic compounds used for drug discovery efforts. Many of these are still undergoing preclinical evaluation, but several others are currently being administered to patients as part of clinical trials

Marine-Derived Drugs

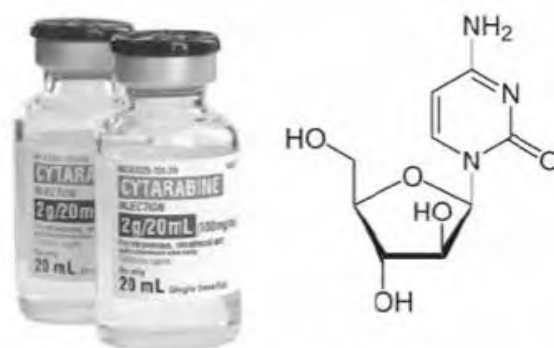
The first modern marine-derived drugs dated back more than 50 years. Werner Bergman extracted the novel compounds spongothymidine and spongouridine from the Caribbean sponge *Tethya crypta* in the early 1950s. These compounds were nucleosides similar to those forming the building blocks of nucleic acids (DNA and RNA). These natural nucleoside analogs were discovered to have unexpected antiviral properties.

The arabinoside Vidarabine® (ARA-A) and Cytarabine® (ARA-C) (two of the first ever discovered marine drugs) are the compounds extracted from the marine sponge *Tethya crypta*. Vidarabine is patented, and is commonly prescribed for viral infection as ophthalmic ointment, whereas patented Cytarabine® (ARA-C) is a chemotherapy drug. This medicine reduces the growth of cancer cells, and can suppress the immune system. Cytarabine® is sold under the trade name Cytosar-U® by Pharmacia & Upjohn. It was FDA-approved for the treatment of certain leukemias in 1969, making it the first such approved marine-derived drug for use in cancer chemotherapy.

Azidothymidine (or Zidovudine, AZT) is an antiretroviral drug used for the treatment of HIV/AIDS based on a group of compounds (arabinosides) extracted from the sponge *Tethya crypta* more than 40 years ago. AZT was the first approved treatment

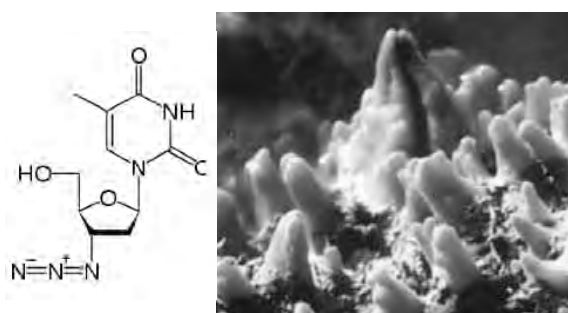


Vidarabine® (ARA-A)



Cytarabine® (ARA-C)

for HIV, sold under the names Retrovir. AZT use was a major breakthrough in AIDS therapy in the 1990s that significantly altered the course of the illness. This success story from marine ecosystem represents an annual market of about \$50 million. AZT works by inhibiting the action of reverse transcriptase (Mitsuya *et al.*, 1985; Yarchoan *et al.*, 1986; Mitsuya *et al.*, 1990).

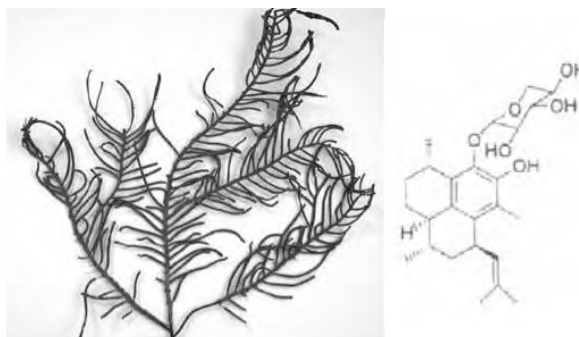


AZT (Zidovudine) was originally isolated from a marine sponge and manufactured under the trademark Retrovir® and was the first drug licensed for the treatment of HIV infection.

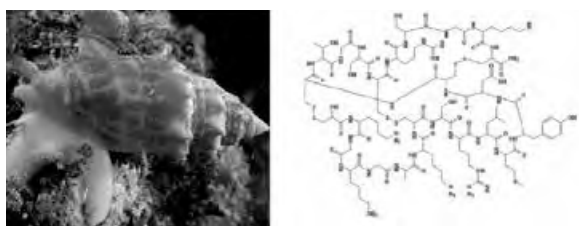
Anti-inflammatory and analgesic pseudo pterosins isolated from a Caribbean marine gorgonian

(*Pseudoterigorgia elisabethae*), which led to the development of bioproducts now used in Estee Lauder skin care and cosmetics lines and currently worth \$3-4 million a year. Pseudopterosins belong to a class of patented compounds known as tricyclic diterpene glycosides (Kijjoa and Sawangwong, 2004; Kohl and Kerr, 2003)

Ziconotide (trade name Prialt®) is a synthetic form of a compound extracted from the venom of predatory tropical cone snails (*Conus* spp). The conotoxins from the various species of cone snails alone represent more than 100 patents and patent applications. In December 2004, Prialt® was approved by the FDA (approval was granted to Irish pharmaceutical company Elan Corporation to market its product for pain management) as a treatment for severe cases of chronic pain in patients who require intrathecal analgesia and conditions such as cancer and AIDS.



Pseudopterosins have been originally isolated from marine soft coral species called a sea whip (*Pseudopterogorgia elisabethae*)



Cone snails are found in tropical seas, carnivorous molluscs known as cone snails sport venomous harpoons that can instantly paralyze small fish and other prey. The snails' venom contains hundreds of compounds, some of which chemists have used to create highly powerful, nonaddictive painkillers such as Ziconotide. Ziconotide (trade name Prialt®) is a synthetic form of a compound extracted from the

venom of predatory tropical cone snails (*Conus* spp).

Marine Natural Products

Natural products have long been used as foods, fragrances, pigments, insecticides, medicines, etc. Due to their easy accessibility, terrestrial plants have served as the major source of medicinally useful products, especially for traditional or folk medicine. About 25% of all pharmaceutical sales are drugs derived from plant natural products and an additional 12% are based on microbially produced natural products. The marine environment covers a wide thermal range (from the below freezing temperatures in Antarctic waters to about 350°C in deep hydrothermal vents), pressure range (1-1000 atm), nutrient range (oligotrophic to eutrophic) and it has extensive photic and non-photoc zones. This extensive variability has facilitated extensive speciation at all phylogenetic levels, from microorganisms to mammals. Despite the fact that the biodiversity in the marine environment far exceeds that of the terrestrial environment, research into the use of marine natural products as pharmaceutical agents is still in its infancy. This may be due to the lack of ethno-medical history and the difficulties involved in the collection of marine organisms. But with the development of new diving techniques, remote operated machines, etc., it is possible to collect marine samples and during the past decade, over 5000 novel compounds have been isolated from shallow waters to 900-m depths of the sea.

Conclusions

Ocean is a potential source of bioactive compounds, which does not have a significant history of use in traditional medicine as in the case of terrestrial plants (Kamboj, 1999). Previously, the research was focused mainly on terrestrial plants because Number of marine natural products: 1971- 2005 (Blunt, 2007) of their easier availability. The isolation of biologically unique molecules from marine organisms that are not found in terrestrial sources leads to a remarkable progress in marine bioprospecting. The boom of marine bioprospecting began in recent years and 18000 plus natural compounds from marine organisms have been isolated as compared to 155000 terrestrial products (Blunt, 2004; Mayer *et al.*, 2007). Between 1969

and 1995, 63 marine substances were patented as antitumour agents, accounting for half the marine molecules patented for pharmaceutical purposes (MartínezPrat, 2002). There are a significant (and growing) number of marine-derived compounds with pharmaceutical potential in the pipeline.

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Importance of Marine Organisms for Prospecting Bio-molecules

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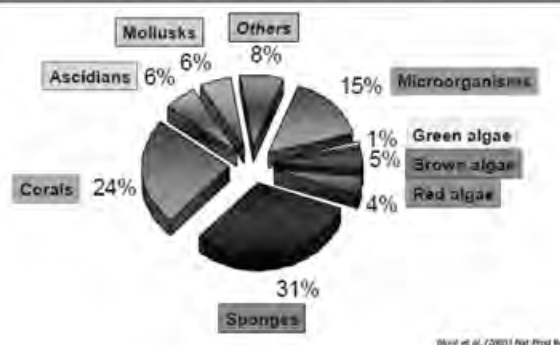
Introduction

The marine environment covers a wide thermal range (from the below freezing temperatures in Antarctic waters to about 350°C in deep hydrothermal vents), pressure range (1-1000 atm), nutrient range (oligotrophic to eutrophic) and it has extensive photic and non-photoc zones. This extensive variability has facilitated extensive speciation at all phylogenetic levels, from microorganisms to mammals. New metabolites from marine organisms have resulted in the isolation of more or less 10,000 metabolites, many of which are endowed with pharmacodynamic properties. Rich diversity of marine organisms represents an enormous resource for the discovery of potential compounds with valuable pharmaceutical and biomedical potential (Kamboj, 1999). These bioactive compounds belong to different chemical classes viz., terpenoids, steroids/sterol glycosides, phenolics, amino acids, fatty alcohol esters, glycolipids etc. Despite the fact that the biodiversity in the marine environment far exceeds that of the terrestrial environment, research into the use of marine natural products as pharmaceutical agents is still in its infancy. This may be due to the lack of ethno-medical history and the difficulties involved in the collection of marine organisms. But with the development of new diving techniques, remote operated machines, etc., it is possible to collect marine samples and during the past decade, over 5000 novel compounds have been isolated from shallow waters to 900-m depths of the sea.

Natural biologically active compounds from Marine Algae

The fact that microalgae/cyanobacteria in general and marine forms in particular are one of the richest

Sources of Bioactive Compounds from Marine environment



David et al., (2003) Nat Prod Res

Drug - Like Molecule

About 30 marine compounds in Human Clinical Trials
Cancer, Anti-inflammatory, Anti-infectives, Pain etc.

Bioactive Molecule

More than 18000 marine metabolites registered in MarineLit
(Marine Literature database, Vpc 14.3, June, 2007)

Current Commercial Marine Drugs

Medicine

Chronic pain (analgesic) Approved by FDA in 2004 Prialt® - Elan Pharm.		
Cancer (soft tissue Sarcoma) Positive Opinion by EMEA in 2007 Currently in Phase III ovarian cancer (LJ28746) Yondelis® - PharmaMar		
Antiinflammatory (skin care lotions) Patented by U. California 1989. Yearly income = 750,000\$ Resilience® - Estée Lauder		

sources of known and novel bioactive compounds including toxins with wide pharmaceutical applications is unquestionable. Among the five divisions of microalgae, studies of biomedical natural products have been concentrated on only

two divisions, *i.e.*, Cyanophyta (blue-green algae) and Pyrrophyta (dinoflagellates). Although several metabolites have been isolated from cyanophytes, most of them are isolated from fresh water species, which are cultured easily in comparison to marine organisms. Lyngbyatoxin-A and debromoaplysiatoxin are two highly inflammatory but structurally different metabolites isolated from toxic strains of alga *Lyngbya mausculata* collected in Hawaii, and anatoxin-a from *Anabaena ciecinalis*. Some of the marine cyanobacteria appear to be potential sources for large-scale production of vitamins of commercial interest such as vitamins of the B complex group and vitamin-E. The carotenoids and phycobiliprotein pigments of cyanobacteria have commercial value as natural food colouring agents, as feed additives, as enhancers of the color of egg yolks, to improve the health and fertility of cattle, as drugs and in the cosmetic industries. Some anti-HIV activity has been observed with the compounds extracted from *Lyngbya lagerhaimanii* and *Phormidium tenue*. More than 50% of the 100 isolates from marine sources are potentially exploitable bioactive substances. The substances tested for were either the ones that killed cancer cells by inducing apoptotic death.

Natural biologically active compounds from seaweeds

Seaweeds are abundant in the intertidal zones and in clear tropical waters. However, they have received comparatively less bioassay attention. Seaweeds, popularly known as green algae, are widely distributed in both inter-tidal and deep-water regions of the seas. These seaweeds are of immense pharmaceutical and agricultural value. A wide range of compounds, particularly terpenes, polyphenolic compounds and steroids, have been reported from various seaweeds (Blunt *et al.*, 2006), amongst which terpenoid compounds represent a major share. For example, *Caulerpa brownii* from Australia was reported to yield a number of bioactive novel diterpenoids and terpenoid esters (Handley & Blackman, 2005). Capisterones A and B are triterpene sulphate esters that were isolated from the tropical green alga, *Panicillus capitatus*, and were found to exhibit potent antifungal activity against the marine algal pathogen *Lindra thalassiae* (Puglisi *et al.*, 2004).

Monocyclic diterpenes have been purified from the Tasmanian green alga *Caulerpa trifaria* (Handley & Blackman, 2000). The green alga, *Caulerpa racemosa*, was reported to yield a bioactive sesquiterpene acid (Anjaneyulu *et al.*, 1991). Halitunal, a novel antiviral diterpene aldehyde has been isolated from the marine alga, *Halimeda tuna* (Koehn *et al.*, 1991). 2-Hydroxy-10-methylzeatin has been purified from seaweeds, NIO-143, and the absolute configuration of the said cytokinin has been determined by spectroscopic procedures (Farooqi *et al.*, 1990). Kahalalide F, a cytotoxic, antiviral and antifungal cyclic depsipeptide, was isolated from a Hawaiian species of *Bryopsis* sp. (Hamann & Scheuer, 1993). A method to purify labdane diterpenoids as major constituents of dichloromethane-soluble fraction green alga *Ulva fasciata* has been illustrated. Antimicrobial assay showed that the compounds labda-14-ene-3a,8a-diol (ULV2) and labda-14-ene-8a-hydroxy-3-one (ULV4) were inhibitory to the growth of *Vibrio parahaemolyticus* and *Vibrio alginolyticus* with minimum inhibitory concentrations of 30 µg/ml by ULV2, and 40 µg/ml by ULV4, respectively against the former and 30 µg/ml by ULV2, and 80 µg/ml by ULV4, respectively, against the latter (Chakraborty *et al.*, 2010). Two new guaiane sesquiterpene derivatives, guai-2-en-10a-ol (G1) and guai-2-en-10a-methanol (G2), were chromatographically purified as major constituents of the CHCl₃/CH₃OH (1:1, v/v) soluble fraction of *Ulva fasciata*. Acetylation of G2 furnished guai-2-en-10a-methyl methanoate (G3) with acetyl group at C₁₁ position. Compounds G2 and G3 exhibited significant inhibition to the growth of *Vibrio parahaemolyticus* with minimum inhibitory concentrations of 25 and 35 mg/mL, respectively (Chakraborty *et al.*, 2010). The antiinflammatory agent produced by *Ulva lactuca* was identified as 3-O-b-glucopyranosylstigmasta-5,25-diene (Awad, 2000). A survey of the metabolites of *U. lactuca* led to the proposal that 4-hydroxybenzoic acid is the most likely biosynthetic precursor of 2,4,6-tribromophenol, an antibacterial compound (Flodin & Whitfield, 1999). Two new antimicrobial terpenes, taxifolione and 7,7-didehydro-6-hydroxy-6,7-dihydrocaulerpenyne, were purified from *Caulerpa taxifolia*, a tropical green alga from Cap Martin, France (Guerriero *et al.*, 1993). *Neomeris annulata*, from Kwajalein Atoll, was reported to possess three brominated sesquiterpenes, shown

to deter fish feeding (Paul, Cronan Jr., & Cardellina II, 1993). A product containing anti-inflammatory and antioxidant principles from seaweeds (CadalmintTM GAe) for use against inflammatory disorders with an ecofriendly “green” technology has been developed by CMFRI. The product know-how has been patented (Chakraborty K *et al.* IP 2064/CHE/2010), and is under commercialisation. The active ingredients in CadalmintTM GAe also suppress the build-up of uric acid in hyperuricemic patients.

Bioactive metabolites from molluscs

More than 2600 scientific studies over the last 20 years testify to the important contribution of toxins extracted from marine molluscs to medicine and cellular biology. To date, only 100 out of a potential 50,000 toxins have been extracted and analyzed. The *Conus* species produce deadly nerve toxins. Some of the conotoxins block channels regulating the flow of potassium or sodium across the membranes of nerve or muscle cells; others bind to N-methyl-D-aspartate receptors to allow calcium ions into nerve cells; and some are specific antagonists of acetylcholine receptors responsible for muscle contraction. Thus, conotoxins are valuable probes in physiological and pharmacological studies. Bivalve molluscs and cephalopods are widely used in different parts of the world for bioprospecting, but only recently they have been recognized as potential sources for bioactive compounds. In the marine environment, where the animals are constantly exposed to the threat of biofouling, molluscs remain relatively free of biofouling, due to the ability of these sedentary organisms control fouling epibionts by effective antimicrobial mechanisms (Tincu & Taylor, 2004; Bansemir *et al.*, 2006; Mayer *et al.*, 2007). Preliminary studies indicated marine bivalves and cephalopods as rich sources of structurally diverse compounds with antibacterial potential (Chandran *et al.*, 2009). There is evidence that bivalve molluscs are useful in the treatment of inflammatory joint diseases (Couch *et al.*, 1982; Miller *et al.*, 1993). Nonsteroidal anti-inflammatory drugs (NSAIDs), *viz.*, aspirin and ibuprofen, are often used for inflammatory conditions. However, most of these medications can produce the unfortunate side effects, which may lead to stomach

ulcer if taken frequently. Therefore exploring the bivalve molluscs for their anti-inflammatory and antioxidant activities and development of product therefrom may significantly reduce adverse side effects resulting from taking NSAIDs. There are reports of dried flesh of the New Zealand mussel *Perna canaliculus* possessing polyunsaturated fatty acids (PUFAs) with possible anti-inflammatory effects (Croft, 1979; Zwar, 1994; Gibson & Gibson, 1981). The anti-inflammatory, antioxidant, and anti-prostaglandin activities were reported in green lipped mussels of New Zealand (Couch *et al.*, 1982; Miller *et al.*, 1993). Neosurugatoxin isolated from *Babylonia japonica* is useful in characterizing two classes of acetylcholine receptors. Dolastatin, a cytotoxic peptide from *Dolabella auricularia* is an antineoplastic substance. Ulapualide-A, a sponge-derived macrolide isolated from the nudibranch *Hexabranhus sanguineus* exhibits cytotoxic activity against L 1210 murine leukemia cells and antifungal activity, which exceeds that of clinically useful amphotericin-B. Chromodorolide-A isolated from *Chromodoris cavae* exhibits *in vitro* antimicrobial and cytotoxic activities. Onchidal from *Onchidella bieyi* is a useful probe for identifying the active site residues that contribute to binding and hydrolysis of acetyl cholinesterase. A team of researchers from the University of Melbourne extracted the conotoxin from a cone-shell snail. It not only inhibits pain as being 10,000 times more powerful than morphine, but also accelerates the recovery of injured nerves. The absolute stereochemistries of membranones A–C, -dihydropyrone-containing polypropionates isolated from the skin of the Mediterranean mollusc *Pleurobranchus membranaceus*, have been determined by stereocontrolled syntheses of the enantiomers. The first synthesis of siphonarins B has confirmed the absolute stereochemistry of the metabolite isolated from the molluscs *Siphonaria zelandica* and *S. atra*. Bursatellin-P, a 60-kDa protein was purified from the purple ink of the sea hare *Bursatella leachii*. The protein exhibited anti-HIV activity. The first total syntheses of aplyolides B–E, ichthyotoxic macrolides isolated from the skin of sea hare *Aplysia depilans*, have been reported confirming the absolute stereochemistry reported for the metabolites. Cephalopods, gastropods, and bivalve molluscs constitute a major share of marine fauna, and were reported to possess structurally diverse

anti-stress metabolites with respect to antibacterial, antioxidant, and anti-inflammatory properties (Chandran *et al.* 2009). A product (Cadalmi™ GME) developed by CMFRI containing 100% natural anti-inflammatory ingredients was prepared from green mussel *Perna viridis* to combat joint pain and inflammatory diseases (Chakraborty *et al.*, 2010a; Chakraborty *et al.* 2010b).

CMFRI develops new product Cadalmi™ Green Mussel Extract (GME) to combat arthritis

Cadalmi™ GME contains 100% natural marine bioactive anti-inflammatory ingredients extracted from green mussel *Perna viridis*. The product is effective to combat chronic joint pain, arthritis/ inflammatory diseases, and improves cardiovascular functioning. It is an effective green alternative to synthetic non steroidal anti-inflammatory drugs (viz., aspirin containing drugs having undesirable side effects). The active principle in Cadalmi™ GME effectively inhibits inflammatory cyclooxygenase-II and lipoxygenase-V, and its activity was found to be comparable to the drugs available in the market. Consuming Cadalmi™ GME will avoid unfortunate side effect of these synthetic non steroidal anti-inflammatory drugs. This product is a blend of nutraceutical and nutritional elements. Cadalmi™ GME is designed to find a unique way to prevent the degradation by air, moisture, heat and light and to maximize the activity. The product is free from deleterious trans fatty acids, free radicals/free radical adducts, and low molecular weight carbonyl compounds. This product is available as capsules and packaged in food grade polypropylene bottles. Cadalmi™ GME is an indigenous product, and is highly cost effective with that of the imported products available in the market. Efforts are underway to commercialize this product.



(Source : CMFRI, Cochin)

in the world and most of them live in marine waters. A range of bioactive metabolites has been found in about 11 sponge genera. Three of these genera (*Haliclona*, *Petrosia* and *Discodemia*) produce powerful anti-cancer, anti-inflammatory agents. The discovery of spongouridine, a potent tumor-inhibiting arabinosyl nucleoside in Caribbean sponge *Cryptotethia crypta*, focused attention on sponges as a source of biomedically important metabolites. The compound manoalide from a Pacific sponge has spawned more than 300 chemical analogs, with a significant number of these going on to clinical trials as anti-inflammatory agents. An aminoacridine alkaloid, dercitin, has been isolated from the deep-water sponge, *Dercitus* spp. that possesses cytotoxic activities in the low nanomolar concentration range and in animal studies, prolongs the life of mice-bearing ascitic P388 tumours, and is also active against B16 melanoma cells and small cell Lewis lung carcinoma. Halichondrin-B, a polyether macrolide from Japanese sponge *Theonella* spp., has generated much interest as a potential anticancer agent. The theopederins are structurally related to mycalamide-A from marine sponge, *Mycale* spp. collected in New Zealand and onnamide-A from marine sponge, *Theonella* spp. collected in Okinawa, which show *in vitro* cytotoxicity and *in vivo* antitumour activity in many leukemia and solid tumour model systems. Isoquinolinequinone metabolite criboastatin from the Indian Ocean sponge *Cribochalina* spp. shows selective activity against all nine human melanoma cells in National Critical Technologies (NCT) panel. Spongostatin, a macrocyclic lactone from the Indian Ocean collection of *Spongia* spp., is the most potent substance known against a subset of highly chemoresistant tumour types in the NCT tumour panel. Two new -pyrones (herbarin) along with a new phthalide, herbaric acid, were isolated from two cultured strains of the fungus *Cladosporium herbarum* isolated from the sponges *Aplysina aerophoba* and *Callyspongia aerizusa* collected in the French Mediterranean and in Indonesian waters, respectively.

THE ECONOMIC TIMES
Mussels: From delicacy to arthritis cure

Efforts to fight arthritis have finally taken us to what are best known for being mouth-watering delicacies: mussels.

A team of scientists at the Kochi-based federal government-run Central Marine Fisheries Research Institute (CMFRI) has developed a nutraceutical product which 4 days offers health benefits to arthritis patients and those suffering from joint pain.

According to the research institute, the product, made by including anti-inflammatory ingredients from green mussels, could be a substitute for the regular aspirin-containing anti-inflammatory arthritis drugs with undesirable side effects.

In a country where one out of six people are affected by arthritis, CMFRI has won a patent for the nutraceutical product, Cadalmi™ GME. The state-run organisation, currently busy with pre-commercialisation trials, will shortly "test the technology of making the mussel extract to enter products", says Dr. D. K. Vijayan, director, CMFRI, which falls under the Indian Council of Agricultural Research (ICAR).

The research institute will likely be the knowledge partner in an agreement through which multiple companies will get licenses to sell the anti-arthritis product, which is said to be effective enough not only to control chronic joint pain, arthritis/inflammatory diseases but also to improve cardiovascular functioning.

Though physicians in India don't prescribe, but only suggest nutraceutical products for arthritis, in the absence of any specific therapeutic drug for treating a disease as debilitating as arthritis, scientists at the research body expect its product, which will be available at regional medical stores across the country, to be a huge hit thanks to word-of-mouth and self-massage.

A mussel-based product, *Leucine*, manufactured by New Zealand-based firm Healthline, is already marketed in India by Delhi-based Pharma Healthcare for Rs 35 for a 200 mg tablet. An official at Pharma Healthcare's Delhi office was tight-lipped about the response from users to *Leucine* in its domestic sales.

Dr. B. Suresh Kumar, deputy director general (pharmas), ICMR, when contacted, said the product will, without doubt, serve as an "important substitute" and "will have competitive pricing" when compared with the imported product currently marketed in India.

Another source official at CMFRI, who asked not to be named, said Cadalmi™ GME could really be priced at around Rs 5 per 100 mg tablet. "As knowledge partner, we want to see that the pricing is normal and not exorbitant. We have done the calculation, including the scaling, investment, etc. The production cost would be around Rs 4.5 approximately, hence by pricing it at Rs 5 per tablet, there will be a decent margin."

Dr. K.K. Vijayan, geneticist and head, marine biotechnology division, CMFRI, said one of the essentials in the team that developed the mussel extract along with Dr. K. Raju Chakraborty and Dr. P. Vijayapada, says a patient has to take "a three-month course, one 100 mg tablet daily, to get good results". Without disclosing further details, he says the initial feed back for pre-commercialisation trials "is really impressive".

"We will subsequently go for international patents depending on the feedback from India," he says, adding that CMFRI's trial have shown that green mussel contains those major components that have been identified as being responsible for easing joint pain: the n-3 polyunsaturated fatty acids, glycosaminoglycans and phenolic flavonoid glycosides and (20-hydroxy) compounds such as terpenoids.

Vijayan says since the demand for green mussels in the country is yet to pick up, a product with an Cadalmi™ GME can help those involved in organized mussel farming sustain themselves or even hit pay dirt, considering the growing number of people in the country who need relief from joint pain through arthritis and other ailments. His experts say the efficacy of products such as these aren't proven yet.

Marine-based nutraceuticals Dr. Kapil Mohan says he usually advises Chondroitin for early-stage arthritis and surgery for later stages. "The efficacy of supplements like *Leucine* and Cadalmi™ GME is yet to be verified," he says.

Currently, a big chunk of organized mussel farming in India takes place in northern Kerala. Thanks to government help, it has seen a massive participation of women. Extending from Kuttanada to Kanyakulam islands, the region is one of India's largest "natural" farms, producing more than 20,000 tonnes of green mussels annually and transforming livelihoods. Similar efforts could be replicated elsewhere in the country's coastal areas where large populations of mussels are found, says Vijayan.

"It is one of the most environment-friendly, cost-effective and sustainable mode of producing valuable protein for human consumption. One just has to put these mussel seeds in a clean sea environment. Your role is just that of a custodian. They just thrive on plankton and sunlight," says Vijayan.

"But what could be a worry is the relatively low consumption of mussels in India," he notes. Of course, in northern Kerala, kullimammyala mussels or arakkattika (mussels stuffed with spices) is a very popular delicacy. And as many enjoy it with appam or dosa or puttu (staple cakes). Elsewhere in India, they are often served grilled or deep-fried. You could also bring your plate with some mussel pods from select restaurant stores.

But for a large majority of Indian, mussels, which reportedly have aphrodisiac properties, are still an occasional delicacy dish they have just heard of. Therefore, it makes some business sense that they are used for purposes other than eating.

Metabolites from Sponges

Approximately 10,000 sponges have been described

Polyunsaturated fatty acids from marine fish

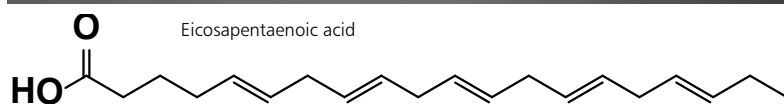
Long-chain polyunsaturated fatty acids (LC-PUFAs), viz., eicosapentaenoic acid (EPA, 20:5 n3),

docosahexaenoic acid (DHA, 22:6 n3) and linolenic acid (LA, 18:3 n3) are widely available in a large variety of marine organisms, like microalgae, polychaetes, fin fish and shellfish. These LC-PUFAs are recognised to have special pharmacological and physiological effects on human/animal health (Harris, 1989). The n3 and n6 long-chain polyunsaturated fatty acids (LCPUFAs), viz., eicosapentaenoic acid (EPA, 20:5n3), docosahexaenoic acid (DHA, 22:6n3), and arachidonic acid (AA, 20: 4n6), are essential fatty acids in the diet of animals and human beings, because they cannot synthesize it *de novo* from precursor molecules (Chakraborty *et al.*, 2010). Therefore they require greater concentrations of PUFAs for their growth, reproduction and survival (Cahu *et al.*, 1994). Diets deficient in these PUFAs particularly EPA have been found to have a negative effect on various vital biochemical and physiological pathways (Chakraborty *et al.*, 2009). The important natural sources of n3 LC-PUFAs are marine fish oils such as sardine, mackerel, cod, shark, and menhaden, which contain PUFA levels of about 30%. For this reason, marine fish oils are preferentially used as raw material to prepare n3 PUFA concentrates. Additionally, it was reported that n3 PUFAs were moderately absorbed by the intestine as triglycerides and most promptly absorbed when free fatty acids (FFA) were given orally. Therefore, it is convenient to prepare n3 concentrates as FFA after chemical hydrolysis of marine oils. Argentation silica gel chromatography of urea inclusion adducts from cod liver oil yielded highly pure DHA in the process, while for EPA, the recovery in the combined process was 29.6%. EPA and DHA have been concentrated from shark liver (*Isurus oxyrinchus*) in one single step, in which fish liver oil was simultaneously extracted, saponified, and concentrated. Additionally, the PUFA concentrate

was winterized to crystallize the remaining saturated fatty acids, resulting in a further increase in the concentration of DHA and EPA. The polyunsaturated fatty acids EPA and AA have been purified from the red microalga *Porphyridium cruentum* by the urea inclusion method followed by silica gel column chromatography of the urea concentrate. The unique substrate specificity of microbial lipases has been utilised for the enhancement of PUFA content in fish oils by several groups (Harris, 1989; Matori *et al.*, 1991). Isolates of *Pseudomonas fluorescens* have been found to produce enzymes active on lipolytic substrates under alkaline conditions (Kojima & Shimizu, 2003). Lipase genes from *Pseudomonas* have been cloned and expressed in *Escherichia coli*, due to their potential industrial applications (Kojima *et al.*, 2003). *P. fluorescens* SIK W1 was found to produce extremely heat-stable lipase that has high lipolytic activity for short- to medium-chain triacylglycerols (Chung *et al.*, 1991). An extracellular alkaline metalloproteinase with molecular weight of 74.8 kDa derived from cultures of *Bacillus licheniformis* MTCC 6824 was found to enrich D5 olefinic double bond fatty acids, viz., EPA and AA (Chakraborty *et al.*, 2008b).

Polyunsaturated Fatty Acid (PUFA) Enriched Formulation

A thermophilic and alkalophilic lipase from *Bacillus coagulans* BTS-3 was purified and biochemically characterised (Kumar *et al.*, 2005). A lipase produced by recombinant *B. licheniformis* was found to be stable at alkaline pH of 12.0 (Nthangeni *et al.*, 2001). These results are in contrast to thermotolerant lipases from *Bacillus thermoacetenuatus* and *Bacillus thermoleovorans*, which display maximum activity



Sardine oil used raw material to prepare PUFA concentrate



at pH 8.0 (Lee *et al.*, 1999; Rua *et al.*, 1997). *B. coagulans* NCIMB 9365 has been reported to possess an intracellular carboxylesterase (Molinari *et al.*, 1996). The substrate specificity of lipase has been utilised for the recovery of EPA (D5) and DHA (D4) from marine oils and α -linolenic acid (D6) from borage seed oil (Morioka *et al.*, 1987). DHA-rich triglycerides were prepared from fish oil with lipases obtained from *Candida cylindracea* and *Chromobacterium viscosum* (Tanaka *et al.*, 1992, 1994). Substrates containing D2–D7 isomers of 18:1 were resistant to pancreatic lipase-catalysed hydrolysis, resulting in higher concentrations of oleic acid, and the discrimination was the greatest for the D5 isomer (Heimermann *et al.*, 1973). An extracellular lipase purified from *Pseudomonas fluorescens* MTCC 2421 was used to enrich sardine oil triglycerides with eicosapentaenoic acid and linolenic acid to 35.28% and 8.25%, respectively (Chakraborty *et al.*, 2010). An extracellular lipase derived from *Bacillus circulans*, isolated from marine macroalga, *Turbinaria conoides*, was used to prepare n-3 polyunsaturated fatty acid (PUFA) concentrates from sardine oil triglycerides. The enzyme was purified 132-fold with specific activity of 386 LU/mg. The purified lipase was able to enrich sardine oil with $37.7 \pm 1.98\%$ 20:5n-3 and $5.11 \pm 0.14\%$ 18:3n-3 in the triglyceride fraction (Chakraborty *et al.*, 2010).

Marine Bacteria as a Source of marine natural products

It has been argued that because of the high dilution effect of seawater, marine-derived bioactive compounds may have evolved great potency. This theory was supported in 2004 with the report of a first-in-class antimicrobial compound from a marine isolate *Verrucosispora*. Renewed interest in marine microorganisms and their ability to produce antimicrobials has resulted in numerous reports of novel antimicrobial compounds. The period of antimicrobial drug discovery from the early 1940s to the 1960s is referred to as the Golden Age. During this time, the industrialization of penicillin production created the expertise and facilities to make significant quantities of antimicrobial compounds by fermentation. The clinical use of antibiotics heralded a health care miracle; deaths due to bacterial infections

were significantly reduced, resulting in increases in life expectancy. The majority of compounds that were discovered during this period were isolated from soil bacteria, most notably the filamentous *Actinobacteria*. Microorganisms are a prolific source of structurally diverse bioactive metabolites and have yielded some of the most important products of the pharmaceutical industry. Microbial secondary metabolites are now being used for applications other than antibacterial, antifungal and antiviral infections. It was during 1928s when Alexander Fleming (Fleming, 1929) began the microbial drug era when he discovered in a Petri dish seeded with *Staphylococcus aureus* that a compound (penicillin) produced by a fungus/mold killed the bacteria. Later, penicillin was isolated as a yellow powder and used as a potent antibacterial compound during the Second World War. Following this extraordinary discovery by Fleming, the antibiotics chloramphenicol and streptomycin, were isolated. Naturally occurring antibiotics are produced by fermentation, an old technique that can be traced back almost 8000 years. Owing to technical improvements in screening programs, and separation and isolation techniques, the number of natural compounds discovered exceeds 1 million (Ecker *et al.* 2005). Among them, 50–60% are produced by plants (alkaloids, flavonoids, terpenoids, steroids, carbohydrates, etc.) and 5% have a microbial origin. Of all the reported natural products, approximately 20–25% show biological activity, and of these approximately 10% have been obtained from microbes. Furthermore, from the 22 500 biologically active compounds that have been obtained so far from microbes, 45% are produced by bacteria or bacteria-like microbes, 38% by fungi and 17% by others (Berdy, 2005). The increasing role of microorganisms in the production of antibiotics and other drugs for treatment of serious diseases has been dramatic. However, the development of resistance in microbes to various life-threatening diseases and in aquaculture has become a major problem and requires renewed research effort to combat it. Antimicrobial development after the Golden Age was characterized by semi-synthetic modifications of compounds that were already clinically proven. The poor antimicrobial discovery rate from microbes, coupled with the availability of chemically synthesized small molecule libraries, led to the abandonment of

microbial screening programmes in the majority of pharmaceutical companies. To date, small chemical libraries have failed to deliver a new antimicrobial compound to the clinic, prompting many to speculate that the withdrawal of microbial screening was premature, exacerbating the threat of antibiotic resistant bacteria.

Microbial natural products

Microbial natural products that have reached the market without any chemical modifications are a testimony to the remarkable ability of microorganisms to produce drug-like small molecules. Although still in clinical trials, a feature example of this is salinosporamide A (NPI-0052), a novel anticancer agent found in the exploration of new marine environments (Fenical *et al.* 2009). In 2008, over 1000 marine natural products were reported (Blunt *et al.* 2010). However, out of the 19 microbial-derived drugs reported in 2008, no natural products from marine microbes were present, signifying the novelty of their systematic exploration (Ganesan, 2008). Currently, >30 compounds of marine microbial origin are in clinical or preclinical studies for the treatment of different types of cancer (Simmons *et al.* 2005), clearly demonstrating that potential of marine microorganisms as an essential resource in the discovery of new antibiotic leads. The evolution of marine microbial natural product collections and development of high-throughput screening methods have attracted researchers to the use of natural product libraries in drug discovery. These libraries include subsections of crude extracts, pre-fractionated extracts (automated HPLC-MS fractionation) and purified natural products. A research group in Ireland has developed a two-dimensional chromatographic strategy that includes a protocol to generate purified marine natural product libraries that are accurately characterized by mass spectroscopy during production to expedite dereplication of known compounds and identification of novel chemotypes. Although the biosynthetic and regulative crosstalk of secondary metabolite biosynthesis is complex within and between microorganisms, all levels can be influenced by imitating natural environmental changes. Development and testing of new culture media for the maximum expression of secondary

metabolites is important as chemical diversity in the construction process of an marine natural products library. An optimization of 'one strain, many active compounds' can be used together with 'fingerprint' methods (HPLC and nuclear magnetic resonance) including tandem analytical techniques such as MS/MS, GC-EI/MS, HPLC-SPE-NMR, LC-MS-MS and LC-NMR for the optimization/selection of culture media for high-throughput fermentation of novel strains. Tormo *et al.* (2003) developed a method for the selection of production media for bacterial strains based on their metabolite HPLC profiles, that yielded the highest metabolite diversity and least overlapping HPLC profiles were selected for large-scale fermentation. Targeted high-throughput screening methods are important for the speed and accuracy of identification of novel antimicrobials. From these evaluation models, many crude extracts or purified compounds were obtained as positive hits. In addition for evaluation purposes, it is worthy to note that these screening assays also provide mode of action hypothesis from the crude extracts.

Antibiotics from marine microbes

During recent decades, we have seen an increasing number of reports on the progressive development of bacterial resistance to almost all available antimicrobial agents. In the 1970s, the major problem was the multidrug resistance of Gram-negative bacteria, but later in the 1980s the Gram-positive bacteria became important, including methicillin-resistant staphylococci, penicillin-resistant pneumococci and vancomycin-resistant enterococci (Moellering, 1998). In the past, the solution to the problem has depended primarily on the development of novel antimicrobial agents. However, the number of new classes of antimicrobial agents being developed has decreased dramatically in recent years. The conventionally used antibiotics/drugs become resistant to most of the natural antimicrobial agents that have been developed over the past 50 years (Hancock, 2007) thereby limiting the effectiveness of current antimicrobial drugs. In 2004, more than 70% of pathogenic bacteria were estimated to be resistant to at least one of the currently available antibiotics (Katz *et al.* 2006). The

so-called 'superbugs' (organisms that are resistant to most of the clinically used antibiotics) are emerging at a rapid rate. *S. aureus*, which is resistant to methicillin, is responsible for many cases of infections each year (Balaban *et al.* 2005). The incidence of multidrug-resistant pathogenic bacteria is increasing. The Infectious Disease Society of America (IDSA) reported in 2004 that in US hospitals alone, around 2 million people acquire bacterial infections per year (dedicated website: <http://www.idsociety.org/Content.aspx>). There are also other examples of Gram-positive (*Enterococcus* and *Streptococcus*) and Gram-negative pathogens (*Klebsiella*, *Escherichia*, *Enterobacter*, *Serratia*, *Citrobacter*, *Salmonella* and *Pseudomonas*) (Cragg & Newman, 2001). Among them, *Pseudomonas aeruginosa* accounts for almost 80% of these opportunistic infections. They represent a serious problem in patients hospitalized with cancer, cystic fibrosis and burns, causing death in 50% of cases. Other infections caused by *Pseudomonas* spp include endocarditis, pneumonia and infections of the urinary tract, central nervous system, wounds, eyes, ears, skin and musculoskeletal system (Levin, & Bonten, 2004). New families of anti-infective compounds are needed to enter the marketplace at regular intervals to tackle the new diseases caused by evolving pathogens. At least 30 new diseases emerged in the 1980-2000s and they are growing. Emerging infectious organisms often encounter hosts with no prior exposure to them and thus represent a novel challenge to the host's immune system. Several viruses responsible for human epidemics have made a transition from animal host to humans and are now transmitted from human to human. HIV, responsible for the acquired immunodeficiency syndrome (AIDS) epidemic, is one example. Although it has not been proven, it is suspected that severe acute respiratory syndrome (SARS), caused by the SARS coronavirus, also evolved from a different species (Kremer *et al.* 2000). One additional reason for developing new antibiotics is related to their own toxicity. As with other therapeutic agents, the use of antibiotics may also cause side effects in patients. Some side effects are more severe and, depending on the antibiotic, may disrupt the hearing function (aminoglycosides), kidneys (aminoglycosides and polypeptides) or liver (rifampin). In recent times, several research groups are making concerted efforts to find novel antimicrobial

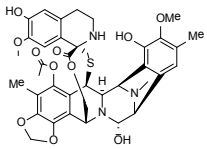
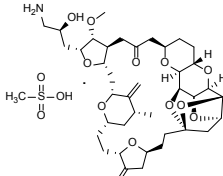
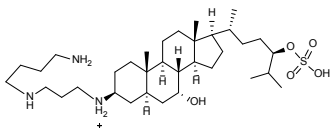
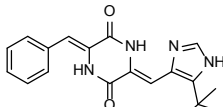
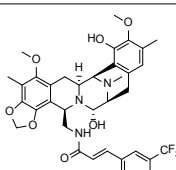
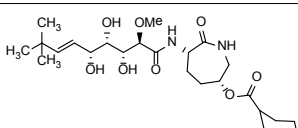
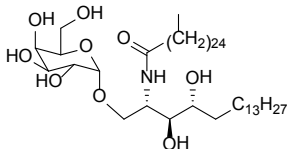
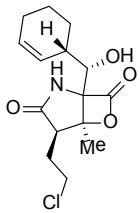
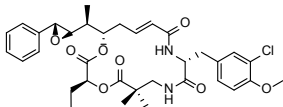
agents as a solution towards multiresistant antibiotic and drug molecules.

Aquaculture grade antimicrobial chemicals from marine microbes

Disease caused by bacterial pathogens has been widely recognized as a major cause of economic loss in many commercially cultured fish and shellfish species in India, with mortality of larval stages in hatcheries and the growing stages in different mariculture systems. Pathogenic vibrios are involved in significant mortalities in the larviculture and growout phases of famed finfish and shellfishes. In an attempt to control the proliferation of pathogenic vibrios, the prophylactic and therapeutic use of antibiotics has been practiced in commercial hatcheries, creating more serious problem of antibiotic resistance among the microflora in the environment. With safety concerns about synthetic antibiotics, and the antibiotic resistance problems, considerable interest has arisen in finding alternative natural sources (Gomez-Gil *et al.*, 2000). Screening and development of aquaculture-grade chemicals from bacterial flora could be a highly promising approach to produce these bioactive molecules. Members of the genus *Pseudomonas* and *Bacillus* either free living or associated with marine flora are common beneficial bacterial candidates, and are known to produce a wide range of secondary metabolites (Raaijmakers *et al.*, 1997; Alavandi *et al.*, 2004; Vijayan *et al.*, 2006) inhibiting a wide range of pathogenic bacteria (Rengpipat *et al.*, 1998). The metabolites 6-oxo-de-O-methylasiadiplodin, (E)-9-etheno-lasiadiplodin, lasiadiplodin, de-O-methylasiadiplodin, and 5-hydroxy-de-O-methylasiadiplodin, were isolated from the mycelium extracts of a microbe obtained from South China Sea (Yang *et al.*, 2006). Marine bacterial strain, *Pseudomonas* I-2, producing inhibitory compounds against shrimp pathogenic vibrios including *Vibrio harveyi*, *V. fluvialis*, *V. parahaemolyticus*, *V. damsela* and *V. vulnificus* was reported by Chaitanya *et al.* (2002) and Vijayan *et al.*, (2006). Bioactive compounds were isolated from a marine bacterium *Bacillus circulans* (Chakraborty *et al.*, 2010). Labda-14-ene-3a,8a-diol and labda-14-

ene-8a-hydroxy-3-one were found to be inhibitory to the growth of *Vibrio parahaemolyticus* with minimum inhibitory concentrations of 30-40 $\mu\text{g}/\text{mL}$ (Chakraborty *et al.*, 2010), and their structures

have been elucidated by ^1H NMR and ^{13}C NMR spectra, including 2D NMR. Several bacterial flora were isolated from marine ecosystem (*Bacillus subtilis*, *Bacillus amyloliquifaciens*, *Pseudomonas*

Compound Name	Structure	Chemical Class	Organism	Company	Status
Trabectedin (ET-743)		Alkaloid	Tunicate	PharmaMar	Approved
Eribulin Mesylate (E7389)		Macrolide	Sponge	Eisai Inc.	Phase III
Squalamine lactate		Amino-steroid	Shark	Genaera	Phase II
Plinabulin (NPI-2358)		Diketopiperazine	Fungus	Nereus Pharmaceuticals	Phase II
Zalypsis		Alkaloid	Nudi-branch	PharmaMar	Phase II
LAF389		Amino acid	Sponge	Novartis	Phase I
KRN7000		α -galactosyl ceramide	Sponge	Kirin	Phase I
Marizomib, Salinosporamide A; NPI-0052		Beta-lactone-gamma lactam	Bacterium	Nereus Pharmaceuticals	Phase I
LY355703, CRYPTO 52		Cryptophycin	Cyano bacterium	—	Preclinical

Clinical status of marine derived antitumor agents, their chemical class and mode of action

putida, and *Pseudomonas aeruginosa*) with potential activities (> 20 mm inhibition zone) against pathogenic *Vibrios* (Chakraborty *et al.*, 2010). The antibacterial component in the CHCl₃ fraction of *P. aeruginosa* was found to be N-substituted methyl octahydro-1-phenazinecarboxylate. The other important antibacterial molecules were found to be propyl 2-oxoacetate and phenethyl 2-oxoacetate. About 4530 bacterial isolates were purified from seaweeds and sediments, and 23 isolates (*B. subtilis* MTCC 10402, 10403 & 10407, *B. amyloliquifaciens* 10456, *P. putida* MTCC 10458, *P. aeruginosa* MTCC 10610) were found to be potential against pathogenic *Vibrios*. Pyocyanins, N-substituted phenazinecarboxylate, propyl/phenethyl 2-oxoacetates were the major antibacterial molecules in bacteria (Preetha *et al.* 2010).

Conclusions

“Poison kills the poison,” the famous proverb is the basis for researchers in finding the biomedical metabolites from living organisms. Sea has got plenty of metabolites and other resources in living or dead form. The main emphasis is given in the search of drugs for deadly human diseases as cancer and AIDS. Efforts of the researchers around the world are continuing their efforts in finding new molecules and drug candidates in the context of deadly diseases such as AIDS, cancer, cardiovascular disease, arthritis, diabetes, etc.

Suggested readings

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Marine Organisms –Treasure House of Valuable Products and their Chemical Perspectives

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Introduction

The oceans and sea constitute more than 70% of the earth's surface. The biologically diverse and ecologically complex marine kingdom comprising both flora and fauna may, therefore, be considered as the largest reservoir of natural products on earth. Marine ecosystem accommodates more than 80% of earth's phyla comprising 34 of the total 36 phyla, out of which 13 are exclusive of marine origin. Marine biota excluding some culinary organisms are the store house for compounds of pharmaceutical and pharmacological importance, biochemical molecular probes for pharmacological studies, models for drug development, anticancer agents, nutraceuticals, and other therapeutic importance. They are the facilitators for the stability of marine ecosystem. They include corals, sponges, micro and macro algae, coelenterates (soft and hard corals, gorgonians), bryozoans, molluscs, tunicates (ascidians), echinoderms, micro organisms and miscellaneous including soap fish, hydroid, annelid, sea grass, mangroves, etc. The compounds associated with them as secondary metabolites are now mostly attributed to the symbiotic bacteria, fungi, cyanobacteria housing in sponges, algae, corals etc. with compelling evidence. eg. bacteria, *Candidatus Endobugula sertula* of bryozoan, *Bugula neritina* for the production of bryostatin 1. Chemical symbiosis is for host organism's defense and food, shelter for the parasites. This chemical warfare has been occurring for thousands of years for their existence/survival with their hostile environment. Few of them find place for successful clinical trials

like ET 473, ziconotide, bryostatin A, etc. With the advancement in the instrumentation like 2D NMR, HRMS, etc. it is also now possible to elucidate the chemical structure of the compounds which are normally available in traces.

Secondary metabolites

The biosynthesis and breakdown of proteins, fats, nucleic acids and carbohydrates, which are essential to all living organisms, is known as primary metabolism with the compounds involved in the pathways known as "*primary metabolites*". The mechanism by which an organism biosynthesizes compounds called "*secondary metabolites*" (natural products) is often found to be unique to an organism or is an expression of the individuality of a species and is referred to as "*secondary metabolism*". Secondary metabolites are generally not essential for the growth, development or reproduction of an organism and are produced either as a result of the organism adapting to its surrounding environment or are produced to act as a possible defense mechanism against predators to assist in the survival of the organism.

Marine natural products (MNPs)

Marine natural products are compounds with novel structural features found typical of marine origin. Many are having definite biological activity with unique structural features mainly of chemical interest. Any report of the new product from marine organisms is followed by its synthesis reported by another group

elsewhere. The compounds are the result of either metabolism by organism itself or of the symbiotic microbiota present in inter or intra specific mode in the cells of the organisms. Some compounds are indeed specific to particular genus useful for chemotaxonomical classification.

The abundance of the source of MNPs from the organisms is approximately in the order:

Sponge > *Coelenterates* > *Microorganisms & Phytoplankton* > *Echinoderms = Tunicates* > *Red algae* > *Molluscs* ≥ *Brown algae* ≥ *Green algae* > *Bryozoans*

Since sponges cover most of the structurally featured compounds, the compounds retrieved from them have been taken for discussion. On the basis of novelty in the structures, they are classified under the major groups of organic compounds:

1. Terpenoids
2. Alkaloids
3. Heterocycles
4. Steroids
5. Polyacetylenes, peptides, polyethers, polyketides, macrolides and
6. Glycosides & nucleosides

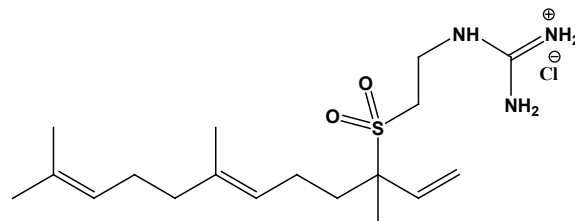
Terpenoids

Terpenes are the major class of compounds found among the sponge secondary metabolites. Original compounds, artifacts, analogues and functional derivatives are among the range of the terpenoids identified from sponge extracts. The terpenes isolated and characterized from sponges generally include sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterterpenes (C_{25}) and triterpenes (C_{30}), with functional groups comprising formamide, hydroquinone, epoxy, halogen substituted carbonimides, peroxides, isocyanate, furan, sulphate, keto, aldehyde, hydroxyl, acetoxy, aromatic, isonitrile, pyrrole, amino, guanidine, adenine, pyran, etc. Devoid of one or two carbons result in the norterpenes. Stereo isomers include either enantiomers or diastereoisomers. The compounds are cyclic and/or acyclic and rearranged form.

Sesquiterpenes

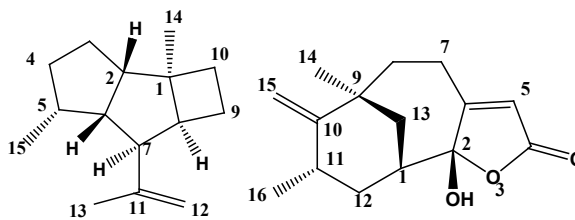
Agelasidine A

It is a sesquiterpene based taurocyamine derivative from a Pacific Sponge *Agelas* Sp.



Agelasidine A

The sesquiterpenes having unusual carbocyclic skeleton are present in *Cymbastela hooperi*. The compounds are $1R^*, 2S^*, 5R^*, 6R^*, 7S^*, 8R^*$ -1,5-dimethyl-7-(1'-methylene)-tricyclo [6.2.0.3] decane (kelsoene).

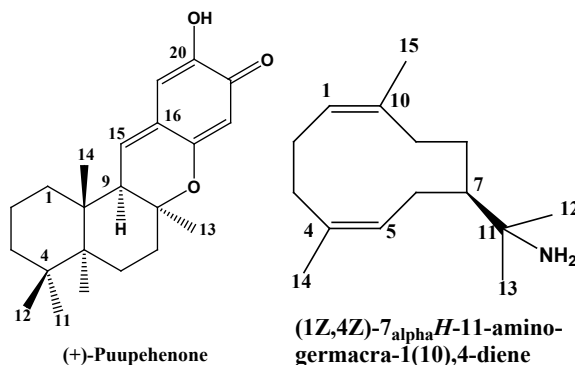


Kelsoene

Furanosquiterpene

Sesquiterpene with incorporated furanone moiety has been reported from sponge, *Dysidea herbacea*. Germacrane sesquiterpenes (1Z,4Z)-7RH-11-aminogermacra-1(10),4-diene.

The formation of the artifacts from the parent compound puupehenone by methanol adduct is discussed. *Hyrtios* sp. (Puupehenone Congeners)

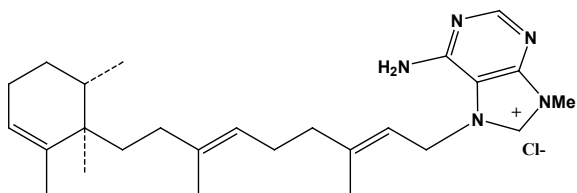


(+)-Puupehenone

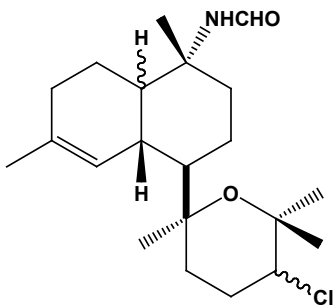
(1Z,4Z)-7 α -H-11-amino-germacra-1(10),4-diene

Diterpenes

They include agelaine and kalihinane.



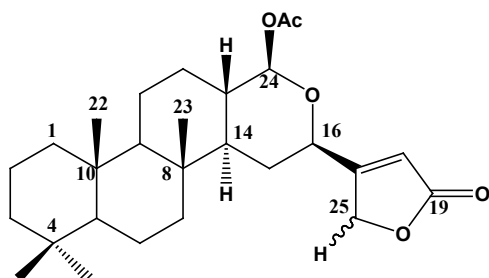
Agelaine A



Kalihinene X - 1 beta H, 14 alpha Cl
Kalihinene Y - 1 alpha H, 14 alpha Cl
Kalihinene Z - 1 alpha H, 14 alpha Cl

Sesterterpenes

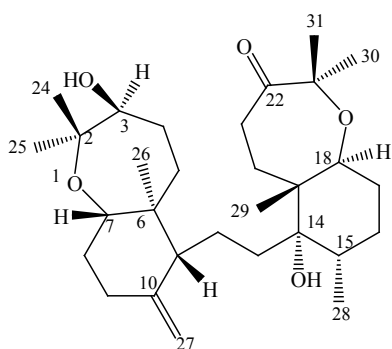
Cavernosolide, *Fasciospongia cavernosa*, 35



Cavernosolide

Triterpenes

Sodwanone K

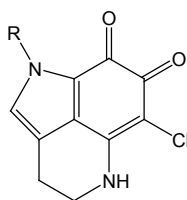


Sodwanone K

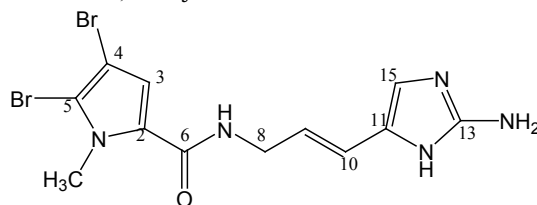
Alkaloids

Alkaloids are having unique structures different from that of terrestrial origins. They include derivatives having heterocyclic structural units of bromopyrrole, pyrroloquinoline, pyrroloiminoquinone, bromoindole, cyclic amine linked to a β -carboline, imidazole, oxazoles, tryptophan, tyrosine, guanidine, isoquinoline, pyridine, purine, etc

Pyrroloquinoline alkaloids, *Zyzzya fuliginosa*;
Sventrin, *Agelas sventres*

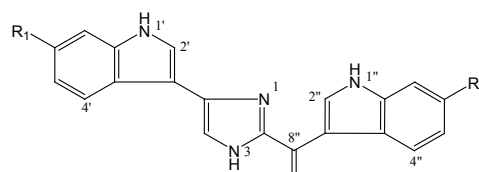


Batzelline D, R=H
Batzelline C, R=CH₃



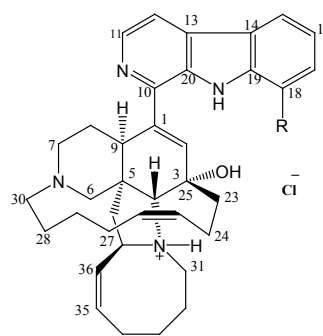
Sventrin

Bromoindoles



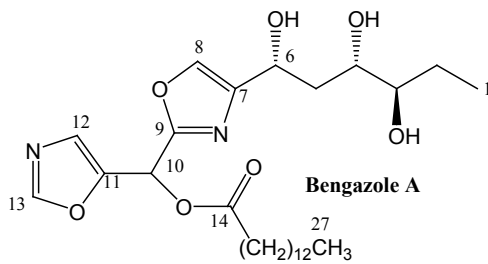
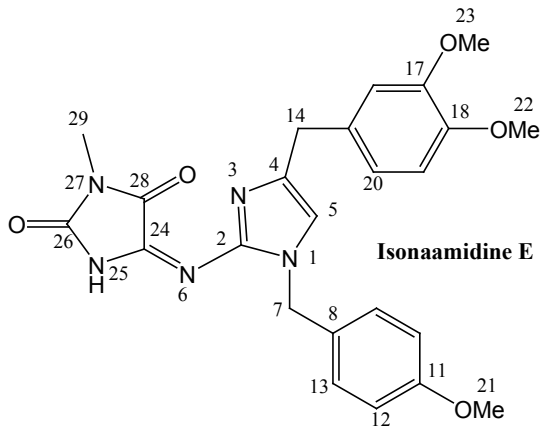
Topsisentin A, R₁=H, R₂=H
Topsisentin B1, R₁=H, R₂=OH
Topsisentin B2, R₁=Br, R₂=H

Cyclic amine linked to a β -carboline

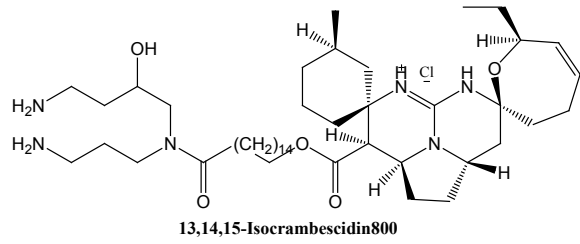


Manzamine A HCl, R=H
ent-18-Hydroxymanzamine A HCl, R=OH

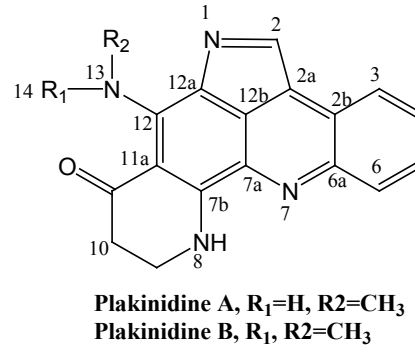
Imidazoles alkaloids



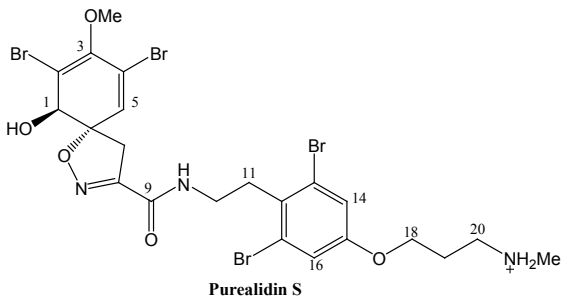
Guanidine alkaloids



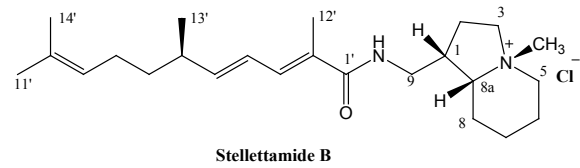
Sulfamate Indoles



Tryptophan and tyrosine alkaloids

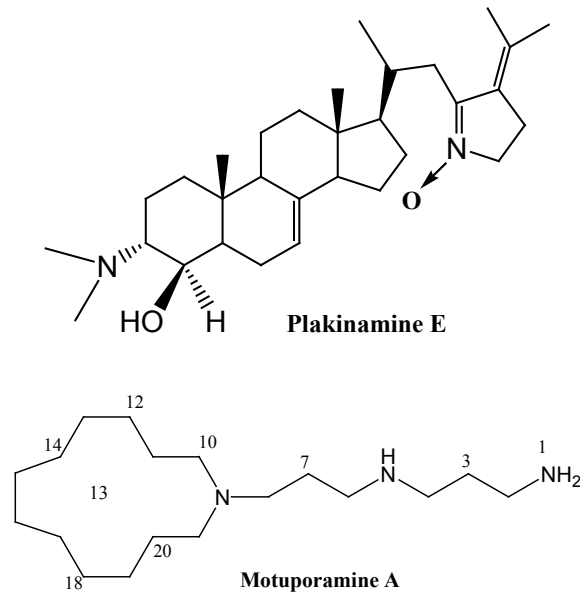
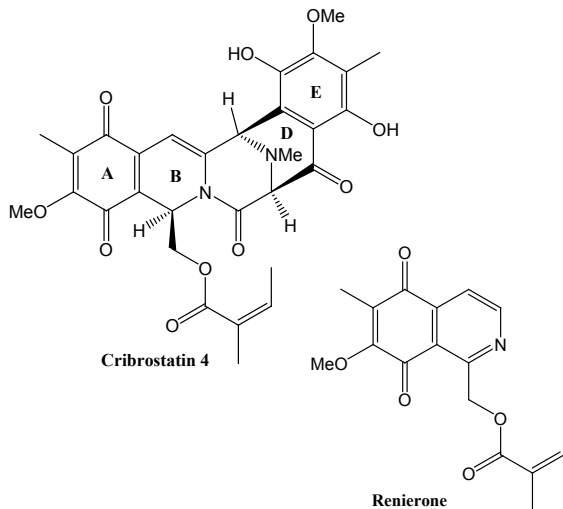


Indolizidine alkaloid



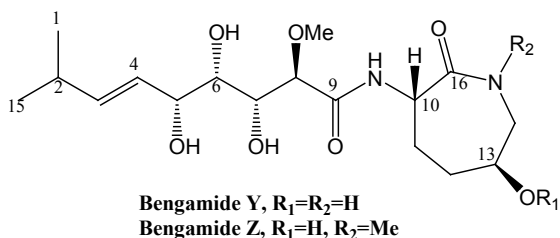
Steroidal alkaloids

Isoquinoline alkaloids

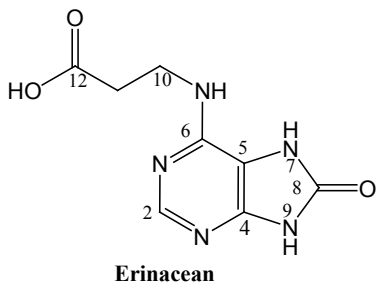


Heterocycles

Bengamides

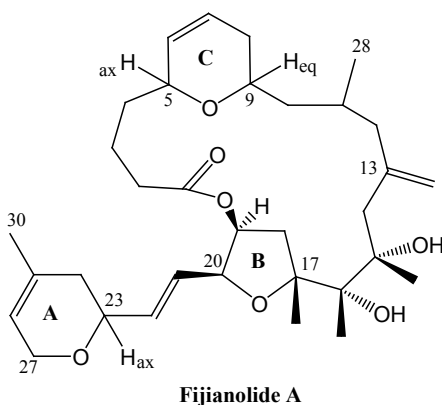


Purine and nucleoside metabolites

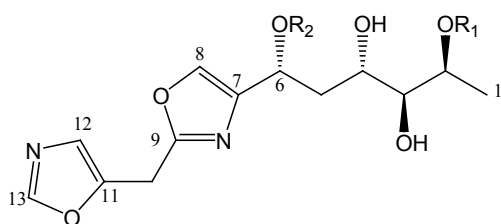


Heterocyclic macrocyclic lactones (Polyketide)

Fijianolides



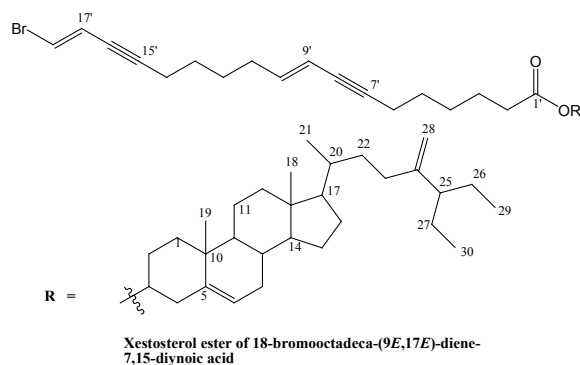
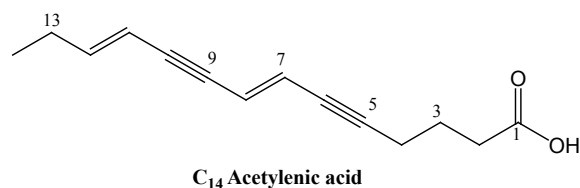
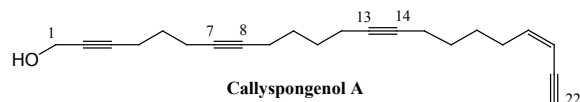
Bengazoles are homologous fatty acid esters of a heterocyclic nucleus comprised of a bis(oxazoly)-methanol further substituted with a hexanetetrol side chain.



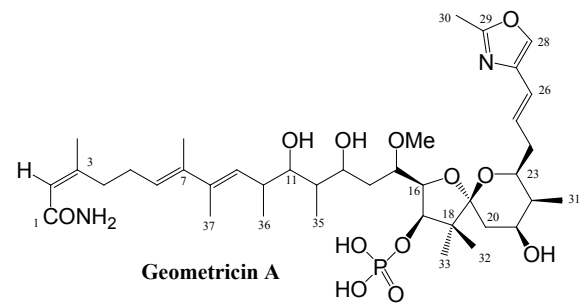
Bengazole 1, $R_1=CO(CH_2)_{14}CH_3, R_2=H$
Bengazole 2, $R_1=H, R_2=CO(CH_2)_{14}CH_3$
Bengazole 3, $R_1=CO(CH_2)_{12}CH(CH_3)_2, R_2=H$
Bengazole 4, $R_1=H, R_2=CO(CH_2)_{12}CH(CH_3)_2$
Bengazole 5, $R_1=CO(CH_2)_{13}CH_3, R_2=H$
Bengazole 6, $R_1=H, R_2=CO(CH_2)_{13}CH_3$

Polyacetylenes, polyethers, polyketides, peptides and macrolides

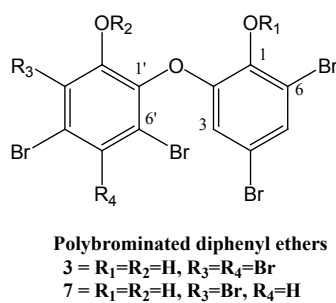
Acetylenic acids



Calculinamide related compounds: Geometricin

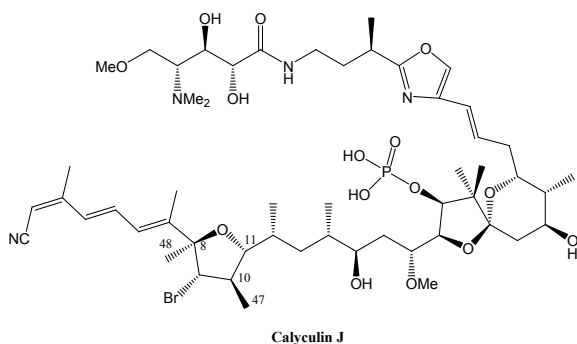


Polybrominated diphenyl ethers

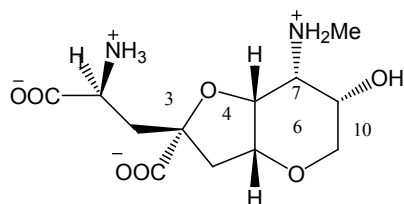


Calculins: Calculin J- It is a spiro ketal of an unprecedented skeleton bearing phosphate, oxazole,

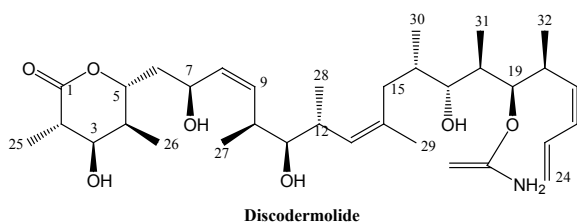
nitrile, and amide functionalities, *Discodermia calyx*; *Hamigera tarangaensis*[163]



Dysiherbaine: It is a *cis* fused hexahydrofuro[3,2-*b*]pyran ring substituted with a 3-[2-aminopropanoic acid] side chain, *Dysidea herbacea*

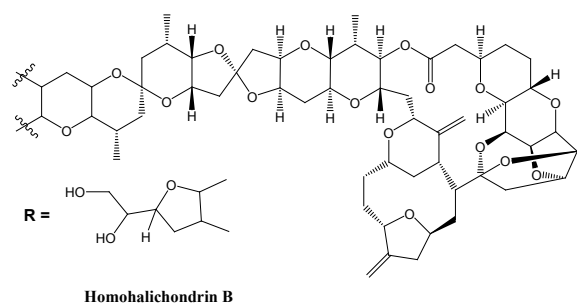


Discodermolide

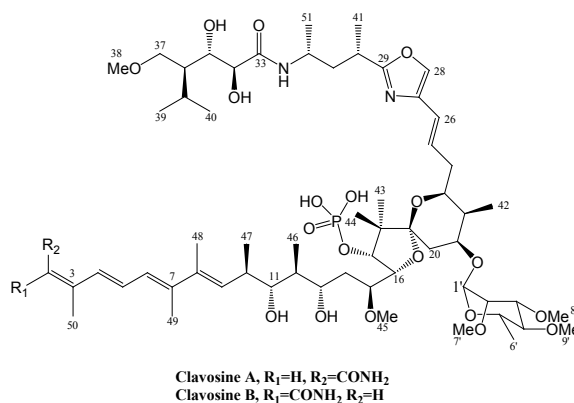


Polyether macrolide

Homohalichondrin B

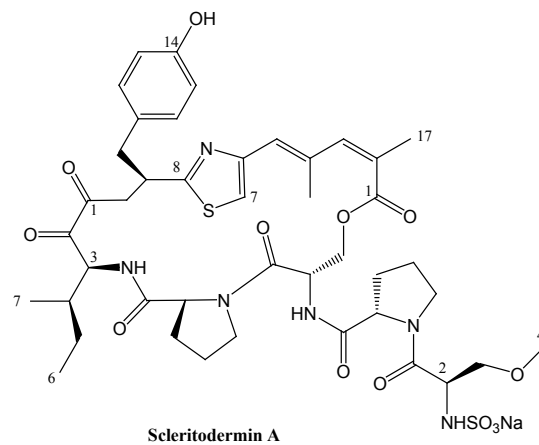


Clavosines A and B are closely related to calyculins and calyculinamides

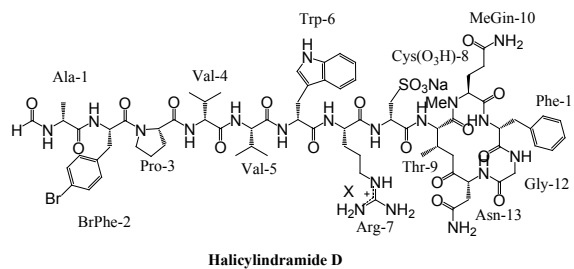


Macrocyclic lactone/lactams

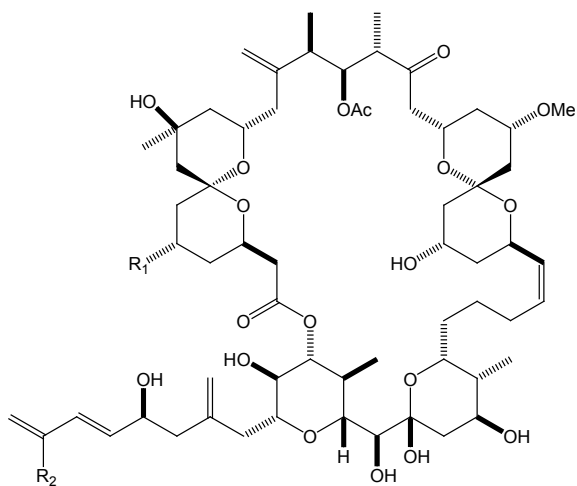
Bicyclic peptides



Homohalichondrin B

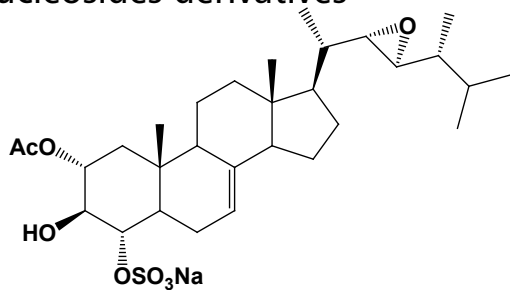


Macrolactone

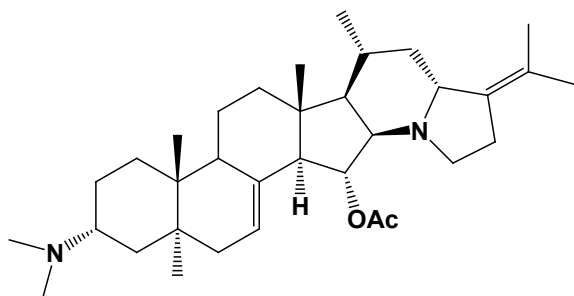


5-Desacetylaltohyrtin A, R1=OH, R2=Cl

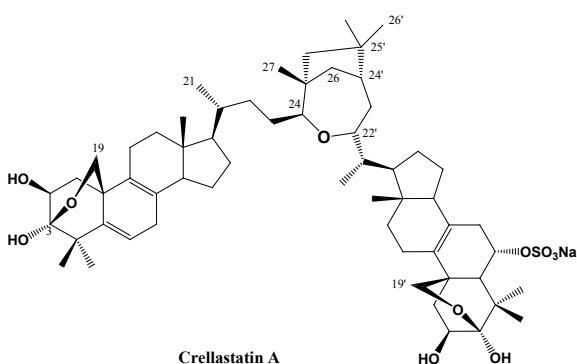
Sterols, glycosides and nucleosides derivatives



Acanthosterol sulfate J

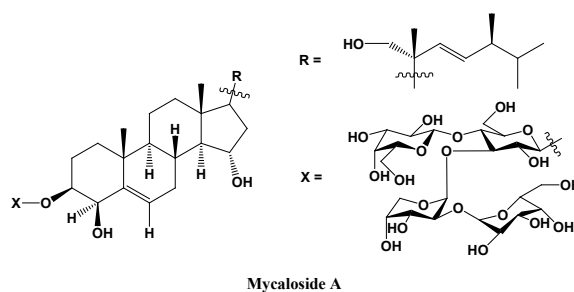


Plakinamine I



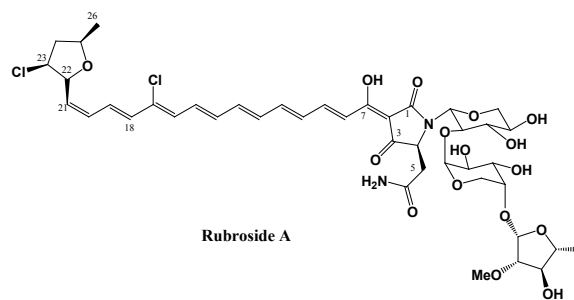
Crellastatin A

Steroidal oligoglycosides



Mycaloside A

Glycosides and nucleosides derivatives



Rubroside A

Marine algae

They include both micro and macro algae and are easily renewable product sources of the oceans. Unlike other organisms they are somewhat easily accessible for the harvest and product isolations. Major products are the sulphated polysaccharides in general and in particular are the agar, algin and carrageenan depending on their sources of red, brown and red respectively. In the oriental countries of Japan, China, Korea, etc. marine macro algae is the traditional staple food. Apart from the algal polysaccharides, minor products like, phenolics, phlorotannins, lipids, terpenoids, sterols, vitamins and minerals are also available. They have good medicinal properties as micro and macro nutrients essential for the balanced diet. They act as the natural antioxidants and antimicrobial compounds.

With the explorations of their utility, algal products

are increasingly felt applicable in various fields as, biofuels, cosmetics, nutraceuticals, biofertilizers, functional foods, antifouling compounds, etc. The polysaccharides are composed mainly of galactose, fucose, guluronic acid, mannuronic acid as major sugar units. With their extent and conformations they form different polysaccharides among the three algal groups.

Industrial important marine natural products (MNPs)

Cold active proteinase (psychrophilic enzyme) from Atlantic cod is temperature and acid sensitive making them use in food processing industries. They have higher catalytic activity at very low temperature. Products of aquaculture importance – useful in feed formulations as binder (agar), feed additives (micro nutrients) from algae, probiotic marine microbes for aquaculture, culture and isolation technologies of the nutraceuticals from micro algae, immunostimulants from marine sources for commercial important fish cultures.

Biotechnological tool for MNPs

There is a serious problem regarding yield of such promising compounds to complete the pharmacological trials, as most of the compounds are present in very low quantity. This supply problem can be ameliorated with the advent of modern biotechnological tools. Enzymes (proteins) are responsible for the production of these secondary metabolites and these are like cells' machines to use raw materials like amino acids and sugars to biosynthesize them. The responsible genes that encode the production of these compounds will be identified

to express these genes in a convenient and suitable host such as bacterium like *E. coli*. Sequencing these responsible genes of bacteria is indeed a challenging task. Inserting the gene cluster (~55,000 base pairs in the case of bryostatin) into a suitable bacterium, will trigger the synthesis of the proteins that in turn produce the desired compound. This approach will help save marine environment's depletion of marine biota by harvest for the want of required quantity of the product. Using DNA recombinant technology, marine drugs are being attempted in a convenient culture systems. This is because marine microbes have very huge genome with one billion base pairs per cell with two picogram (10^{-12}), which is obstacle for search of genes responsible for biosynthesis of these compounds. However researchers are unraveling the possibilities to simulate the functions by recombinant technology for a target molecules.

Conclusion

Marine organisms are the store house of marine micro biota to have unique molecules. The associated microbes are having prime role in the formation of these secondary metabolites. Modern techniques of biotechnology are increasingly felt essential for genetically modified organisms to get the desired product in bioprospecting the marine ecosystem.

Suggested readings

- Colegate, S.M.; Molyneux, R.J. 2008 *Bioactive Natural Products: Detection, Isolation and Structure Determination*; CRC Press: Boca Raton, FL, USA, pp. 421–437
- Antonov AS, Afiyatullova SS, Kalinovsky AI *et al.* 2003, Mycalosides B-I, Eight New Spermostatic Steroid Oligoglycosides from the Sponge *Mycale laxissima*. *J Nat Prod* 66:1082-1088
- Kim, S K and Chojnacka (Eds), K 2015 *Marine Algae Extracts– Processes, Products, and Applications Vol I & II*, Wiley-VCH Verlag GmbH & Co, KGaA

Classification of Organic Compounds with Reference to Natural Products

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Introduction

The aim of the classification of natural products was to record them depending on their medicinal value and other biological activities. They were classified into their structural groups with functional groups responsible for the bio activity. Though they fall into the organized literary structural groups of organic compounds, their medicinal and physical properties are taken into account for their classification. It is for the convenience to identify their chemical structural features and in turn correlate them to their bio activity. With the classification of organic compounds having identical structural features, documentation of a particular compound with respect to its chemistry and bio activity has become easy for further reference. Proper documentation of the identified compounds helps to dereplicate the known compounds in an extract during the exploration of an organism for a product of medicinal value. This dereplication is necessary to save time and money in rediscovering the known compounds from an extract.

IUPAC system of nomenclature and homologous series

In order to document the compounds with universal scientific names, chemists agree to common rule to adapt to avoid the controversy in naming an organic compound. In this way, the IUPAC nomenclature system is a set of logical rules devised and used by organic chemists to circumvent problems caused by arbitrary nomenclature. With these rules one can write a unique name for every possible compound or

isomer from a structural formula. A uniform variation of the molecular entity in a series of compounds is called homologous. Eg. Alkanes, the members have the uniform difference of $-CH_2$ and common formula of C_nH_{2n+2} . Likewise it is treated for alkenes, alkynes, alcohols, cyclic alkanes, with common formulae.

Classification of organic compounds

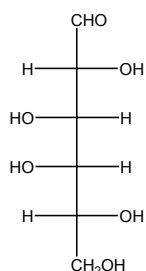
The compounds isolated from marine natural resources by bioprospecting generally fall under the categories of carbohydrates, terpenoids, carotenoids, polycyclic aromatic hydrocarbons (PAH), steroids, heterocyclic compounds, peptides and proteins, alkaloids, polysaccharides, anthocyanins, nucleic acids, vitamins, pigments, toxins and prostaglandins. Some groups like toxins and carbohydrates are special to marine resources with respect to their unique structural features specific to marine origin. Though each group has the spectrum of bioactivity, these bioactivities overlap among one another leaving the option to have the compound quantitatively for further utilization under renewable condition. So the quantitative availability of the product depends on the extent of the source to be renewed and/or cultured. In this way the product leads have the difficulty in reproducibility from the same species of the source. Some of the lead compounds under each group will be seen in the following sections.

Carbohydrates

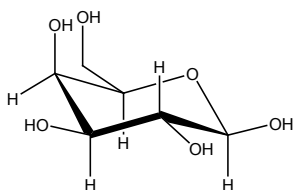
Carbohydrates are general term used to represent sugars of monomer, oligo and polysaccharides with

general formula $C_x(H_2O)_y$ with the equivalence of hydrogen and oxygen as that of water present in it along with carbon. But number of other structural units are found out from the natural sources, which may not conform to the general formula. eg. formaldehyde, acetic acid. Their names end with ose; carbohydrates with an aldehyde group are called aldoses and those with ketonic, ketoses. Molecules containing four carbons are tetrose, a pentose five, a hexose six, etc. The basic units by which the macro molecule are built, are the monomers. The macro molecules depending on the extent of multiplicity of the basic units are called oligosaccharides and polysaccharides. The deoxy representation of the sugar name means the replacement of a hydroxyl group by hydrogen.

The agar and carrageenan are the polysaccharides present in red seaweeds with galactan unit. The agarose is composed of agarobiose repeating disaccharide units alternating with 1, 3-linked- β -D-galactopyranose



Fischer projection

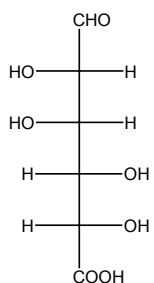


Beta-Pyranose form

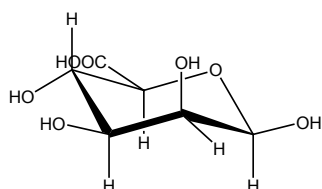
Galactose

and 1, 4-linked-3, 6-anhydro- α -L-galactopyranose.

Algin is an anionic polysaccharide found in brown seaweeds. It has mannuronic acid and guluronic acid as building blocks with 1 to 4 linked β -D-mannuronic

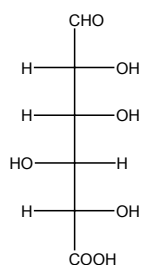


Fischer projection

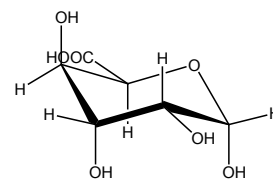


alpha-pyranose form

Mannuronic acid



Fischer projection



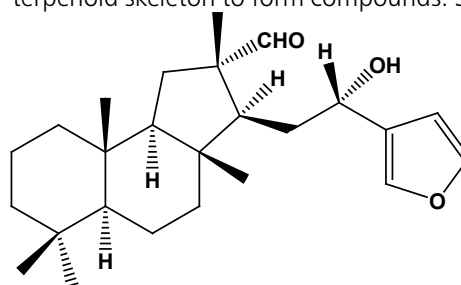
alpha-pyranose form

Guluronic acid

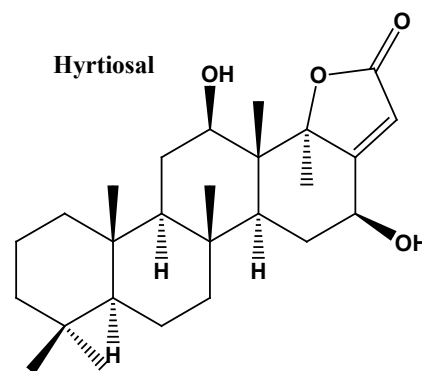
acid and α -L-guluronic acid.

Terpenoids

These are compounds formed generally by the basic unit of isoprene joined together in "head to tail" mode with the multiplicity of 5. Monoterpenoids are compounds having the formula of $C_{10}H_{16}$, diterpenoids of $C_{20}H_{32}$, sesterterpenoids of $C_{25}H_{40}$, triterpenoids of $C_{30}H_{48}$, tetraterpenoids of $C_{40}H_{64}$ (separate class of carotenoids) and polyterpenoids of general formula, $(C_5H_8)_n$. compounds of marine origin in this class of compounds limits to triterpenoids. They found mostly as oxygenated derivatives with alcohol, aldehyde, ketone functional groups. However in MNPs heterocycles like furan, quinines are also linked to the terpenoid skeleton to form compounds. Some of the

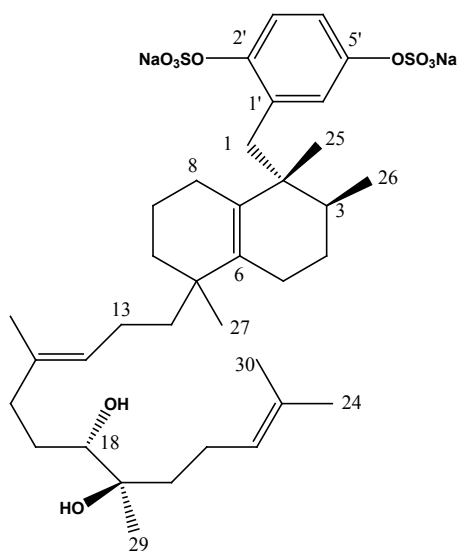


Hyrtiosal



Scalarane sesterterpene d

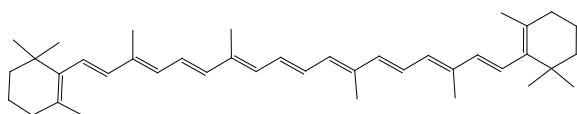
examples with good biological activities are:



Adociasulfate 3

Carotenoids

They are polyenes the molecular formula of $C_{40}H_{56}$. They are yellow or red pigments associated with chlorophyll. Hydrocarbon based compounds are

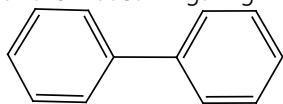


beta-Carotene

known as the carotenes and that of oxygenated derivatives as xanthophylls.

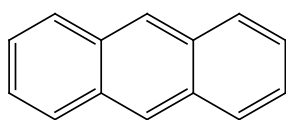
Polycyclic aromatic hydrocarbons (PAH)

They may be of two types with the isolated rings and of fused rings. Eg. Biphenyl, anthracene. They

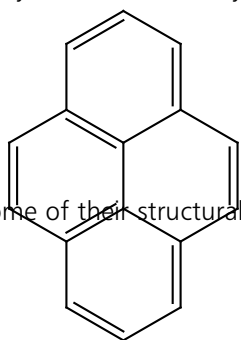


Biphenyl

primarily occur in coal tar. Some of their structural



Anthracene

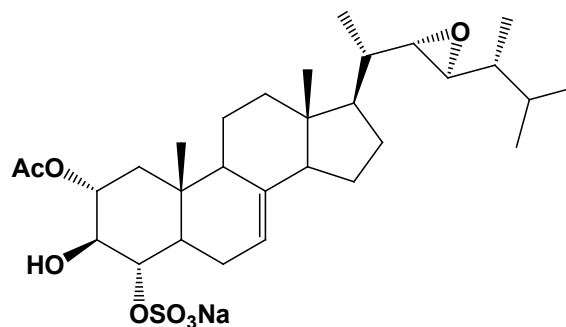


Pyrene

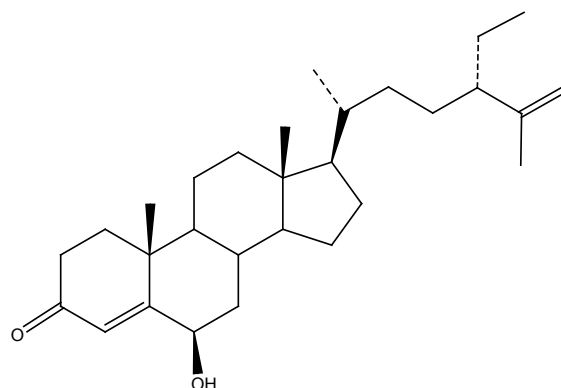
isomers like naphthaquinones, anthraquinones are reported in MNPs.

Steroids

They are crystalline compounds occurring in nature in free or ester of higher fatty acids.



Acanthosterol sulfate J

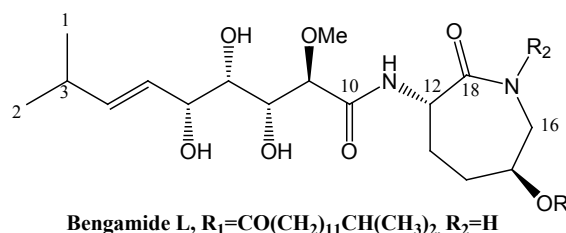


Oxygenated steroid

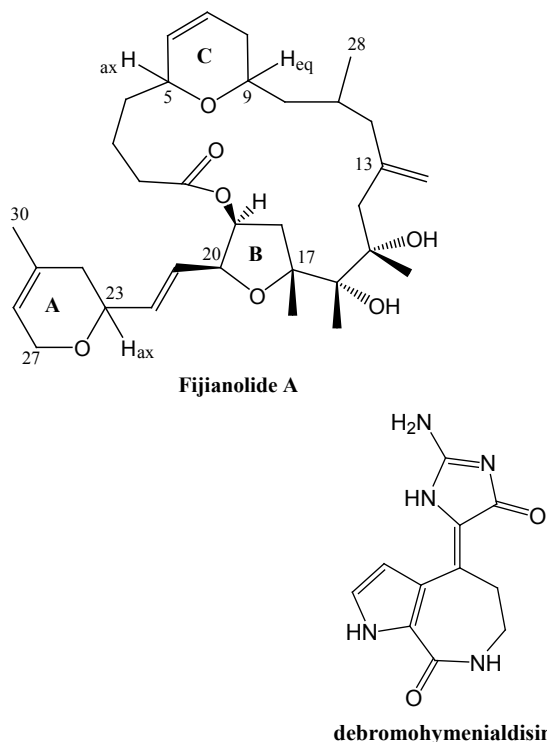
They occur in plants, animals, of both land and marine origins. Some are of the typical sterols of marine origin. Eg. A series of oxygenated steroids have been isolated from green algae *Codium arabicum*.

Heterocyclic compounds

They contain five or six membered heterocyclic rings



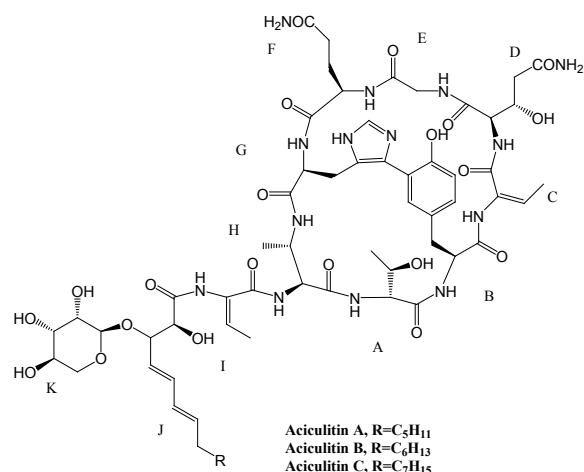
Bengamide L, $R_1=CO(CH_2)_{11}CH(CH_3)_2$, $R_2=H$



with aromaticity. The heteroatoms are oxygen, nitrogen and sulphur with prefixes oxa, thia and aza respectively while forming the names. Some heterocycles of marine origin are: Bengamides with anticancer activity.

Peptides and proteins

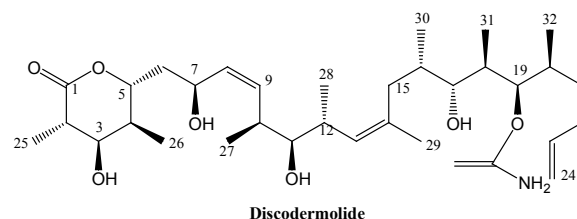
Proteins furnish a mixture of amino acids on hydrolysis with acids, alkalis or enzymes with the intermediate components of peptides of different and number of amino acids having different molecular weights. Amino acids are charged molecules of both positive and negative depending on the pH of the medium. Twenty amino acids are common and found in all proteins. However amino acids of different structural features



are extensively characterized from marine organisms and also land plants and microbes. Aciculitins A-C are bicyclic peptide that contain an unusual histidinotyrosine bridge with attachment to the bicyclic peptide.

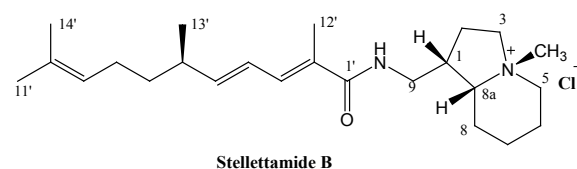
Polyhydroxylated lactone

Alkaloids

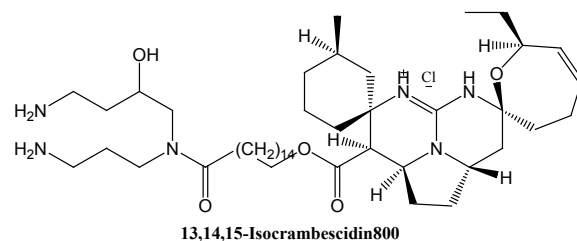


They are the bases of plant, animal and marine origin having a nitrogen atom predominantly. They are used as medicine in small quantities. Some of the compounds of substituted alkaloids of marine origin are:

Guanidine alkaloids



Pigments



They include number of compounds with chromophores responsible for light energy absorption of visible wavelength region. They are found in marine algae and based on their presence, algae are classified into red, green and brown algae. Eg. fucoxanthin – useful antioxidant and in treatment of obesity.

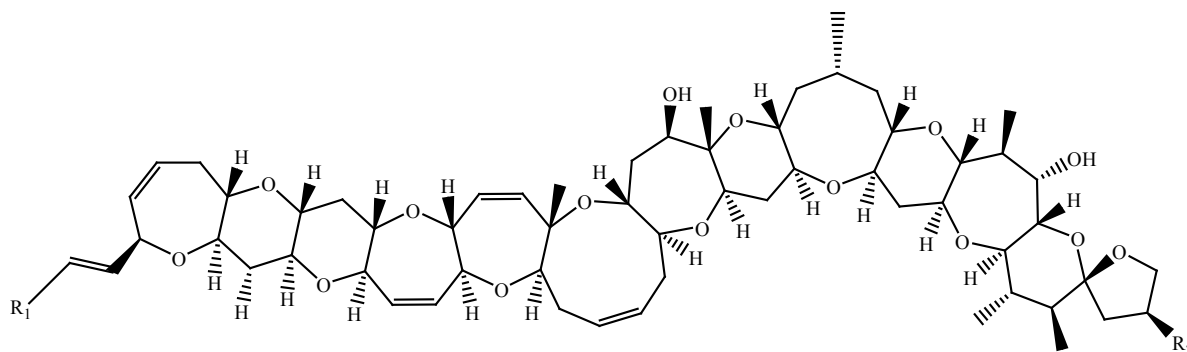
Toxins

They are defensive chemicals of both marine and terrestrial origin; that of marine origin are called

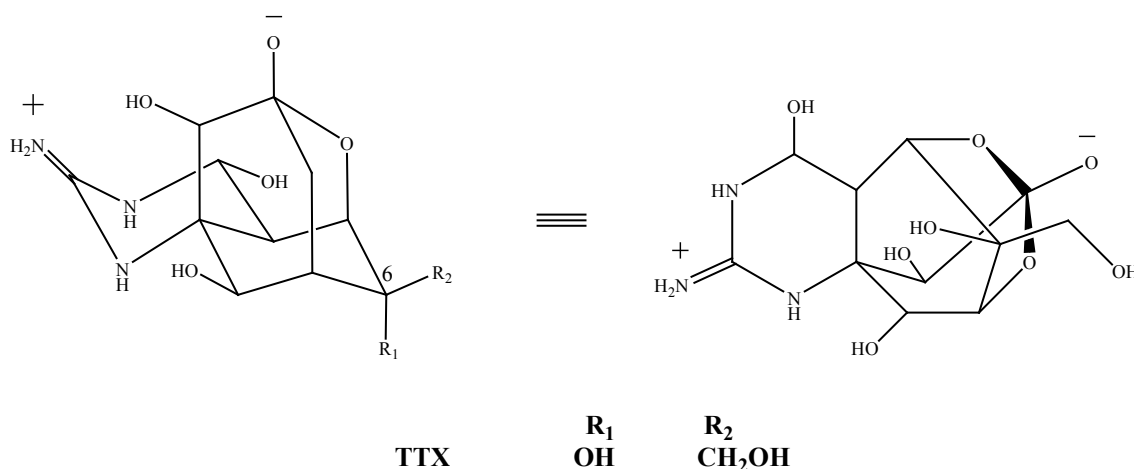
ichthyotoxins. Phenero toxin and crypto toxin are two types; the former having the venomous apparatus and the latter having the toxin incorporated in body tissues gained by food chain, symbiotic parasites and biosynthetic transformations of the acquired chemicals

origin are of important in pharmacological studies as molecular probes in retrieving biochemical reactions. Further unraveling the marine resources may throw light to the treasure of many more products for the welfare of the mankind.

Ciguatoxin (CTX)



Tetrodotoxin (TTX)



Conclusion

Classification of organic compounds in the context of bioactivity helps to document the compound in a convenient way to refer to them for any assay or isomer synthesis or a lead compound for the clinical trials. In this way compounds of marine

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Physical and Chemical Methods for Structural Elucidation and Identification of Organic Compounds

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Introduction

Organic compounds are derived from carbon and are of plant or animal origin. Any interpretation and study are done with respect to the pure organic compound only to know the molecular characteristics and its property. The pure compound is therefore isolated from its original mixture or extract by various methods. Unlike inorganic salts which are ionic and simple molecules, organic molecules are from simple to complex. Since it is available from living systems, it is formed by regular biosynthetic pathway either by the host or the microbes present in the host organism. Macro molecules *albeit* simple with respect to some structural features, are complex with secondary and tertiary structures which are responsible for their biological functions. With the latest developments in the structural interpretation with the help of MALDI-TOF-MS/MS tandem mass spectrometry, it is now possible to deal with molecules like peptides and acetogenins.

Separation of organic compounds

Separation of organic compound is a foremost step to proceed further for the determination of the structure. Previously solvent extraction, distillation, steam distillation were used. Later counter current separation, electrophoresis, dialysis, molecular distillation, ultracentrifugation, etc. Chromatography is most extensive method used for the isolation and purification of compounds from the reaction mixture or extract. Various forms of

chromatographic techniques are involved depending on the nature of the mixture that is handled. State-of-art instrumentation is now available to use this technique for a successful separation of a compound. The quantity of the final pure compound may not be sufficient in many cases and marine bioprospecting in particular. Various separation methods have been dealt with in other chapter extensively. The suitable method can be followed depending on the type and quantity of compound isolated.

Identification of organic compounds – Chemical methods

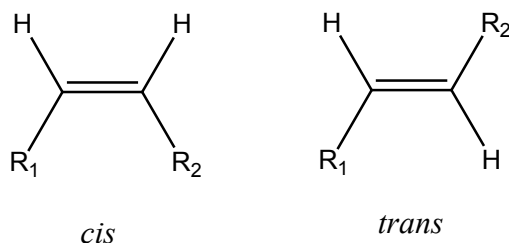
Qualitative analysis

The elements of an organic compound are: carbon, hydrogen, oxygen, nitrogen, sulphur, phosphorous, and metals. Preliminary qualitative chemical analysis of the elements is done using cupric oxide for C and H. Elements of N, halogens, and S are analyzed by *Lassaigne* test. Common functional groups present in organic compound are analyzed/estimated by standard methods. In this way, estimation of phenols, ketones, sugars, ascorbic acid, amino groups (aromatic amines), nitro groups (aromatic nitro compounds), amino acids (glycine), etc. are done. The unknown compound is subjected to various chemical reactions to study the plausible structure. The compound is also degraded with reagents to break them into small molecules of known structures. Integration of small molecules in a possible manner was done to

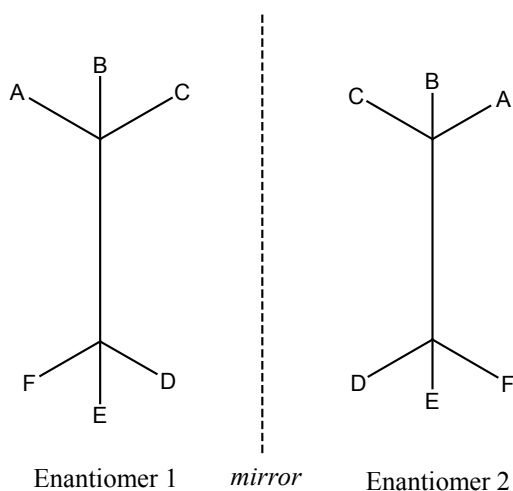
arrive at a probable structure. However execution of the structural determination methods depends on the quantity of the compound isolated from natural sources. If the quantity is low, analysis by chemical methods, degradative studies, etc. is limited and the problem is explored with instrumental analysis.

Stereochemistry

Stereochemistry is the chemistry of groups and atoms of a molecule with respect to their spatial arrangements. Stereoisomerism is exhibited by molecules having the same molecular formula but different spatial arrangement. It helps to understand the reaction course because the orientation of groups determine the conditions of the reaction. There are two types of stereoisomerism, optical isomerism and geometrical isomerism (*cis-trans*).



Optical isomerism: It is formed by the asymmetric carbons present in the molecules as these compounds rotate the plane of polarized light [dextro (+) or laevo (-) rotatory]. Optical isomers may rotate the plane of polarization by equal and opposite amounts. These particular isomers are called *enantiomers* and also can be visualized as the mirror image to each other with respect to their spatial arrangement.



Some compounds with same physical and chemical properties are not of mirror image arrangement of their groups and they are *diastereoisomers*. Some compounds do not possess rotation and are called *meso-* compound. The optical isomers can be represented by

Newman projection or saw-horse form. Racemic mixture is a mechanical mixture of enantiomers.

Geometrical isomerism

It is also called *cis-trans* isomerism. Eg. Maleic acid and fumaric acids. The *cis*-compound has identical or similar atoms or groups on the same side.

Identification of organic compounds – Physical methods

The pure organic compound obtained by appropriate physical method of separation is subjected to elemental as well as the functional group analysis to know the preliminary composition and nature of the composition. The elements are quantitatively estimated by elemental analyzer and atomic absorption spectrophotometer to arrive at the percentage composition. Physical methods involve sophisticated instruments for the structural and functional group analysis. These methods are very useful in the recovery of costly compounds after the analysis which is not possible in the case of chemical methods. Additional methods and improvements are being added up with the advancements in the instrumental analyses.

Molecular weight determination

It is done based on the physical parameters like vapour density, boiling point elevation, freezing point depression for simple molecules. Physical methods like rate of diffusion, rate of sedimentation, viscosity of the solution, osmotic pressure are used for the determination of molecular weight of larger molecules. X-ray (XRD) and mass spectrometry are also used.

UV-visible spectroscopy

It includes the wavelength range of 200-400 nm for UV and 400-750 nm for visible spectroscopy. The presence and nature of unsaturation can be effectively detected by this method. The concentrations of compounds are also estimated from the absorption parameters.

Infra-red (IR) spectroscopy

Apart from the conventional chemical methods, functional groups, H-bonding (inter- and intra-molecular), geometrical isomerism, conformational orientation of groups in both aliphatic and aromatic compounds can be detected by IR spectroscopy effectively for the absorption in the IR region of 4000-650 cm^{-1} . With the improved high resolution by fourier transform mode the spectra are nowadays recorded. The unsymmetrical charge distribution due to various vibrations in the molecule makes the molecule to be detected in IR spectra. The various vibrational motions are stretching and bending modes. Stretching regions have higher frequencies than the deformation regions. The spectrum of a compound can be recorded in gas, liquid (thin film), solid (thin film or mull) or solution in CCl_4 , CHCl_3 , CS_2 . Identification of compound is carried out by comparison with published spectral data. The region 1400-650 cm^{-1} is the "finger-print region" as it is having the vibrational energy changes of *molecular skeleton* which will be characteristic for every molecule. The absorption values of functional groups throw light on the type of compound. Characteristic bands of the functional group are also recorded together inevitably. Eg. C=O str. and C-O str. bands at 1750-1735 cm^{-1} and 1250-1170 cm^{-1} respectively. It is also the case that absence of a particular band is not a warrant that particular functional group is not present and this situation requires chemical information about the compound along with other spectral data by UV and NMR.

Nuclear magnetic resonance (NMR) spectroscopy

Nuclei with odd atomic and odd mass numbers, odd atomic and even mass number, and even atomic number and odd mass number are having magnetic

properties. Eg. $^1\text{H}_1$, $^{15}\text{N}_7$, $^2\text{H}_1$, $^{13}\text{C}_6$. These nuclei are having resultant spin behave as spinning magnet and they will orient themselves in an applied magnetic field with possible energy levels of $2I+1$, where I is the nuclear spin quantum number. Eg. The two possible orientations of the simple atom like proton are align or against the direction of the applied field. The energy levels of these orientations are different and are quantized. It is possible to change the alignment of proton to orient against the applied electromagnetic radiation with definite frequency which is absorbed by the proton to go from lower to higher energy level and the proton is said to be in resonance. In practice with a fixed frequency, the magnetic field is varied to get signals depending on the magnetic moment of the nucleus (proton) resulting in NMR spectrum. The protons in a molecule are in different chemical environment i.e. Shielding or deshielding depending on the electronic influence. Shielding causes a shift of the resonance frequency to higher values of the applied field, (*upfield*). Deshielding causes a shift of the resonance frequency to lower values of the applied field (*downfield*). The magnitude of the shift is known as chemical shift (Δ). Since these values can not be determined accurately, chemical shifts are measured relative to some standards like, tetramethylsilane which has protons which occur at relatively highest upfield.

It is usually in the range 1-10 and is quoted in parts per million (ppm). Eg. Ethanol. The position and the intensity of the peaks give information of the type of protons present in the molecule. In the high resolution NMR spectrum, the multiplicity of each type of proton gives the information about its environment and neighbouring protons leading to the partial structure of the molecule. Chemical equivalence of the protons is deduced from the spectral study. Chemical structure, configurations, conformations, tautomerism, H-bonding, molecular weight can be determined using NMR techniques.

Mass spectrometry

When a compound is bombarded with electron under vacuum, it is converted into positively charged ion with the loss of electron and this ion is called molecular ion. The excess energy present in the molecular ion

enables to break down further into neutral and positively charged fragments. Further break down is the case of excess energy fragments also.

These positive charged ions are accelerated in an electric field and separated by their passage through an electric field and then magnetic field. The ions of like charges of mass/charge (m/e) ratio are sorted out and so the masses of ions are determined. The instrument is capable of resolving adjacent beams of m/e and $(m+1)/e$ and also ions of masses differing in third decimal place.

The largest peak is the most abundant and is called base peak with the given value of 100. All other peaks are reported as percentages of the base peak. Fragmentation pattern is characteristic of a compound and by this pure compound is characterized. This interpretation is difficult when the ion undergoes rearrangement and gives the fragment pattern not expected from the structure of the compound. However mass spectrometry is valuable for the determination of molecular weights, molecular formula, structure elucidation, quantitative analysis of mixtures, ionization potential, etc. From the pattern of peaks of compounds having various functional groups, the structure of an unknown compound can be proposed. List of common peaks table is also useful for this work. It is also not necessary to identify every peak.

With the advancement in the instrumentation in mass spectrometry, it is now possible to interpret the structure of complex molecules like, metal complexes, supramolecules, nanostructures, biopolymers (peptides, proteins and nucleic acids) and other macro molecules with the help of fast atom bombardment mass spectrometry (FABMS) and inductively coupled mass spectrometry (ICPMS). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has revolutionized *in situ* identification of microorganisms by analysing them in a short time from colonies grown on culture plates.

Hyphenated techniques such as LC-NMR, LC-MS, LC-PDA35, UHPLC-PDA-TOF-MS and combinations thereof compounds could be unambiguously characterized. Hyphenation of HPLC separation

with different spectroscopic detection methods like PDA, MS or NMR offers two ways of identification; Coupling TLC/HPTLC with mass spectrometry either by compound extraction with specific interfaces or by ambient mass spectrometry significantly increased the spectral information on selected compounds. Recently, using a TLC-MS extraction interface and coupling to NMR; analysing biological material containing volatile constituents like essential oils, GC-MS analysis still represents the method of choice, taking advantage of the unsurpassed peak capacity of capillary GC columns.

X-ray diffraction (XRD)

Complete structure of any crystalline compound is possible by X-ray diffraction by the process of the data with the help of computer. It has become a powerful tool to determine the structure and molecular weight as well without involving any chemical methods in some cases. Crystalline form of the compound is the prerequisite for the analysis.

Chemical synthesis

The confirmation of the chemical structure deduced by various means be it a chemical or physical method, is done by chemical synthesis. It is the protocol of the chemical analysis of any unknown pure compound to illustrate the structure of the compound by synthesis. So whenever a novel compound is discovered in one place, the synthesis of the compound is followed by subsequent report elsewhere. It may not be an economic one involving multiple steps but it is the chemical confirmation of the structural entity they arrived at for a particular compound.

Conclusion

As the knowledge is expanding, means of identification of an organic compound is also expanding exponentially. Modern instrumental methods involving micro of quantity of the analyte, is indeed amazing compared to the conventional, laborious and lengthy methods used previously like degradative studies and various chemical analyses involving large quantity of samples. This situation is overcome with the help of modern

sophisticated instruments. Good candidate compounds have emerged paving way to medicinal screening and pharmacological studies especially the stereochemistry which is vital to the structure activity relationship.

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General Methods of Isolation Procedures and Separation Methods for Organic Compounds

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Introduction

Exploration of marine natural products (MNPs) from bioprospecting of marine environment is involving various steps like collection of source organisms, preservation, processing for crude component, screening of the crude extract for the targeted activity, isolation of the compound of activity by physical methods of separation and final identification of the compound by instrumentation. Inline multi analytical methods like LC-NMR or LC-MS gave the first hand information about the structure of the compounds prior to actual isolation of the compound. Once this information is obtained from the given extract, actual quantitative isolation of the product becomes easy with appropriate separation technique. The quantity of active ingredient of the mixture may be low after a series of steps involved in the isolation process, but with the modern powerful instrumentation, it is now comfortably possible to get the actual structural information.

Types of isolation techniques

The relative solubility of the compounds present in an extract based on their polarity determines their isolation/extraction in the lipophilic or lyophilic nature of the extractant. The complexity of the extract is thus fractionated with like group of compounds with respect to their polarity and solubility. This sub fractionated compounds can easily be analyzed and the components can be isolated with less difficulty. The isolation of active compound from the crude mixture is first of all tested analytically

to know the complexity. Before this the mixture is simplified by filtration through sieves to evade impurities. Preliminary analysis is done by thin layer chromatography (TLC), gas chromatography. Using a TLC-MS extraction interface and coupling to NMR, compounds can be identified and quantified as well.

Isolation protocol of MNPs starts with identification and collection of the biological material with the help of biologist. Extraction with different solvents from low to higher polarity of both organic and aqueous moieties follows. Prior to isolation of pure compounds, often by semi preparative high pressure liquid chromatography (HPLC) or liquid-liquid chromatographic techniques, several purification steps are necessary to remove most of the non-targeted matrix.

Solvent extraction

The majority of isolation procedures employ simple extraction procedures with organic solvents of different polarity, water and their mixtures. The methods include maceration, percolation, Soxhlet extraction, ultrasound-assisted extraction and turbo-extraction. Maceration is carried out at room temperature by soaking the material with the solvent with eventual stirring. It has the advantage of moderate extraction conditions but suffers from high solvent consumption, long extraction times and low extraction yields. Extraction yield is improved by percolation, *i.e.* packing the pre-soaked plant material in a container which allows the constantly controlled removal of the extract via a valve at the bottom

and adding fresh solvent from the top. Soxhlet extraction is a popular method for extraction due to its reduced solvent consumption; however, heat sensitive compounds might be degraded during the extraction process. For liquid samples extraction by organic solvents or heterogeneous solvent mixtures can be done, either simply in a separating funnel or similar to a Soxhlet apparatus in a perforator. On a smaller scale, extraction of the liquid sample absorbed on a porous matrix (like diatomaceous earth) packed in a column with non-miscible solvents is an option.

Ultrasound-assisted extraction (UAE)

In UAE the source material, usually in a glass container, is covered by the extraction solvent and put into an ultrasonic bath. It decreases extraction time and improves extraction yields due to mechanical stress which induces cavitations and cellular breakdown, and has gained increasing popularity. temperature-controlled water bath connected to an ultrasound probe, showed superior extraction efficiency compared to steam distillation or superheated water extraction.

Microwave-assisted extraction (MAE)

Nowadays extraction employing either diffused microwaves in closed systems or focused microwaves in open systems are established methods. MAE has been modified in different ways leading to vacuum microwave assisted extraction (VMAE), nitrogen-protected microwave assisted extraction (NPMMAE), ultrasonic microwave-assisted extraction (UMMAE) or dynamic microwave-assisted extraction (DMAE). Some recent examples of application of MAE to NP isolation employing ionic liquids are mentioned below.

Extraction with ionic liquids

These ILs, also designated as “designer solvents”, are organic salts in the liquid state consisting of an organic cation and an organic or inorganic anion. ILs are able to dissolve a wide range of polar to non-polar compounds, have a low vapour pressure, show a high thermal stability and low combustibility, and some of them are biodegradable. Ionic liquids with different

extraction technologies like liquid-liquid extraction (LLE), UAE, MAE or liquid-phase micro-extraction (LPME), N,N-dimethylethanolammonium octanoate (DMEA oct) and bis(2-methoxyethyl)ammonium bis(tri-fluoromethylsulfonyl)imide (BMOEA bst) showing the best performance

Accelerated (pressurized) solvent extraction (ASE)

In ASE, sequential extraction with solvents of different polarity and mixing of solvents is possible, ASE or similar instrumentation can also be used for subcritical water extraction (SWE) employing temperatures of 100–280°C. Subcritical water (superheated water, pressurized hot water) is heated to a temperature between the boiling point at atmospheric pressure (100°C) and the critical temperature (374°C) under pressure, thereby increasing its solution properties for organic lipophilic compounds. For phenolic type of compounds, SWE seems to be an attractive alternative to organic solvent extraction, however, artifact formation and degradation has to be scrutinized.

Supercritical fluid extraction (SFE)

Replacing extraction with organic solvents by extraction technologies which are less detrimental to environment and meet the increasing regulatory requirements certainly can be considered as a driving force for the increasing application of supercritical fluid extraction, above all using supercritical CO₂. The utilization of organic solvents as modifiers for supercritical CO₂ to increase its solvating capabilities to medium polar and polar compounds has broadened the spectrum of NP compound classes accessible to SFE, accepting the ecological problems related to organic solvent extractions which increase to a small extent.

Chromatographic techniques

Extraction processes which take advantage of adsorption of the analytes or unwanted impurities on a solid phase have gained a dominant role in purification of NP extracts, not least due to its integration into automated sample preparation and isolation systems. Most applications utilize solid-phase extraction (SPE) which employs a wide range

of stationary phases with diverse chemistry like silica gel, reversed-phase material, ion-exchange resins or mixed-mode material and hydrophilic interaction chromatography (HILIC) stationary phases in pre-packed glass or plastic columns. Elution of the compounds of interest might be done stepwise by applying a gradient with increasing eluting power, i.e. the procedure is then related to vacuum liquid chromatography (VLC). An exciting development of recent years was the design of molecularly imprinted polymers (MIP) to be used in SPE applications for selective enrichment of various compounds. Either ionic liquid-imprinted silica particles or copolymers of acrylamide and ethylene glycol dimethacrylate with the respective template compounds are used to create material which will have a high affinity to the template structures.

In a first elution step the unwanted material is removed from the SPE column whereas target compounds bound to the solid phase are obtained in a concentrated solution usually upon elution with organic solvents like methanol, though additional purification steps might be necessary. A sophisticated combination of SPE columns representing strong anion and cation exchangers, a mixed-mode polymeric RP-anion exchanger with a poly (divinylbenzen-co-vinylpyrrolidone) backbone and a size exclusion column of a hydroxypropylated dextran gel (Sephadex LH-20) were used for explorative fractionation of extracts

Chromatography is the process of separation of components of the mixture, by distribution between two phases, one stationary phase and the other mobile phase. The solubilities and polarity of the components present in the mixture determine the extent of elution/distribution from the matrix. To get systematic elution, the eluent should be either from non polar to polar or polar to non polar depending on the normal phase or reversed phase chromatography respectively. Eg. The order of increasing eluting power for silica gel: (Eluotropic series)

$PE < Cyclohexane < CCl_4 < CH_2Cl_2 < CHCl_3 < Et_2O < EtOAc < Acetone < PrOH < EtOH < MeOH < H_2O < AcOH$

This sequence is roughly reverse in the case of

activated carbon adsorbant. Some of the common adsorbents used are:

$Cellulose < Starch < Sucrose < Calcium\ carbonate < Magnesia < Silica\ gel < Alumina < Activated\ charcoal$

Alumina is widely used and is available in three forms – acidic, basic and neutral.

Preparative planar chromatography (PPC)

An attractive feature of PPC is the wide range of chemical detection methods characteristic for compound classes which can be carried out on a narrow section of the plate leaving most of the compound unchanged and available for isolation. In bioassay-guided isolation strategies, planar chromatography has the advantage of direct application of bioassays on thin layer chromatography (TLC) plates, making the rapid localization of bioactive compound zones possible. To overcome the disadvantage of classical TLC of uncontrolled flow rates of the mobile phase, forced-flow techniques such as centrifugal planar chromatography or over-pressured layer chromatography have been developed enabling elution and online detection of compounds. R_f value is the ratio of the distance travelled by a component to the distance travelled by the solvent front and it is characteristic of each component. This is the similar condition for RT value observed in HPLC. The spots can be visualized by I_2 vapour for most of the cases or by spraying the reagent solution on the plate for specialized structured compounds eg. Ninhydrin for amino acid detection. The visualization can also be done by viewing the plate under UV irradiation.

Vacuum liquid chromatography (VLC).

In contrast to other forced-flow column chromatographic techniques, not pressure but vacuum is applied in VLC to increase flow rate and hence speed up the fractionation procedure. Column beds in VLC usually consist of silica of 40–60 mm particle size or reversed-phase silica.

Flash chromatography (FC).

Similarly to VLC, FC is mainly used for rapid fractionation of crude extracts or coarsely purified fractions. By applying nitrogen or compressed air, the mobile phase is flushed through the stationary phase in a tightly closed glass column or prepacked cartridges

Low-pressure liquid chromatography (LPLC)

Column chromatographic methods which allow flow of the mobile phase at atmospheric pressure without additional forces either by vacuum or pressure are still a major tool in the fractionation protocols for NP isolation.

Medium-pressure liquid chromatography (MPLC).

MPLC is commonly used to enrich biologically active secondary metabolites before further purification by HPLC due to its lower cost, higher sample loading and higher throughput using RP-18 and polyamide CC 6 stationary materials afforded highly pure compounds.

High-performance (high-pressure) liquid chromatography (HPLC).

Octadecyl silica (RP-18) columns are still widely used for NP isolation and purification. However various laboratories have benefited from the availability of high-quality modern-generation HPLC columns with modified matrices such as cyano, phenyl, trimethylsilane, triazole, secondary and tertiary amines, β -cyclodextrine and dihydroxypropane for successful isolation and purification of different groups of MNPs.

Chiral chromatographic methods

This separation technique allows separating enantiomers either indirectly with chiral derivatization reagents or directly with chiral stationary phases or chiral mobile-phase additives.

Preparative gas chromatography (PGC)

For isolation of volatiles, PGC is an attractive option.

Distillation

Volatiles such as essential oils are still obtained mainly by distillation techniques, although working at elevated temperatures can lead to chemical changes. Liquid compounds/mixtures are separated by distillation under atmospheric pressure or vacuum at different boiling points which are specific to pure compounds. Distillation under vacuum also prevents the chemical changes or destruction of heat sensitive compounds.

Electrophoresis

Under the influence of applied potential difference, anions in the dispersion medium move to the cathode and cations to the anode resulting in the separation of the mixture. Eg. Amino acids and peptides with resultant charge on the molecule which decides the direction and extent of movement at a particular pH of the medium.

Conclusions

With the development and improvement of the existing extraction and analytical techniques, it is now comfortable to explore the complex sample to retrieve the components present in it. Hyphenation of chromatographic and spectroscopic or spectrometric techniques thus increases the possibility of the elucidation of the structures of known as well as novel compounds without the need for isolation.

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Instrumental Methods in Bioprospecting: Chromatography and Spectroscopy

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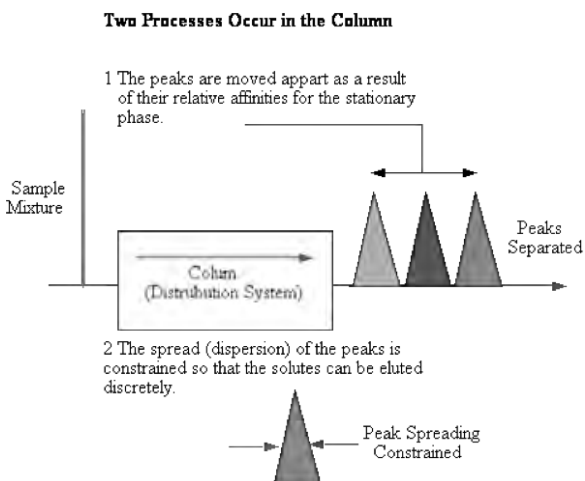
Chromatography: An overview

Chromatography, although primarily a separation technique, is mostly employed in chemical analysis. Nevertheless, to a limited extent, it is also used for preparative purposes, particularly for the isolation of relatively small amounts of materials that have comparatively high intrinsic value. In a single step process it can separate a mixture into its individual components and simultaneously provide an quantitative estimate of each constituent. Samples may be gaseous, liquid or solid in nature and can range in complexity from a simple blend of two enantiomers to a multi component mixture containing widely differing chemical species. The first scientist to recognize chromatography as an efficient method of separation was the Russian botanist Tswett, who used a simple form of liquid-solid chromatography to separate a number of plant pigments. The colored bands he produced on the adsorbent bed evoked the term chromatography for this type of separation (color writing). Although color has little to do with modern chromatography, the name has persisted and, despite its irrelevance, is still used for all separation techniques that employ the essential requisites for a chromatographic separation, viz. a mobile phase and a stationary phase. Today, chromatography is an extremely versatile technique; it can separate gases, and volatile substances by gas chromatography (GC), in-volatile chemicals and materials of extremely high molecular weight (including biopolymers) by liquid chromatography (LC). Chromatography is a separation process that is achieved by distributing the components of a mixture between two phases,

a stationary phase and a mobile phase. Those components held preferentially in the stationary phase are retained longer in the system than those that are distributed selectively in the mobile phase. As a consequence, solutes are eluted from the system as local concentrations in the mobile phase in the order of their increasing distribution coefficients with respect to the stationary phase; *ipso facto* a separation is achieved.

In practice, the distribution system, (that part of the chromatographic apparatus where the solutes are distributed between the phases) can take the form of a column such as a tube packed with particulate matter on which the stationary phase is bonded or coated. The mobile phase (which may be a gas or a liquid) passes under pressure through the column to elute the sample. The column form may also be a long, small-diameter open tube that has the stationary phase coated or bonded to the internal surface. Alternatively, the chromatographic system may take the form of a plate (usually glass) the surface of which is loaded with particulate matter to which the stationary phase is coated or bonded. The mobile phase (a liquid) is arranged to percolate up the plate (usually by surface tension forces) to elute the sample. The sample is injected into the mobile phase stream just before the front of the columns. The column is designed to allow two processes to take place that will produce the separation. Firstly, as a result of different forces between each molecular type and the stationary phase, each solute is retained to a different extent and, thus, the more weakly held will elute first and the more strongly held elute last. The process is diagrammatically depicted below.

Gas Liquid Chromatography



Classification of Chromatography

As all chromatographic separations are carried out using a *mobile* and a *stationary* phase, the primary classification of chromatography is based on the physical nature of the *mobile* phase. The mobile phase can be a gas or a liquid which gives rise to the two basic forms of chromatography, namely, gas chromatography (GC) and liquid chromatography (LC).

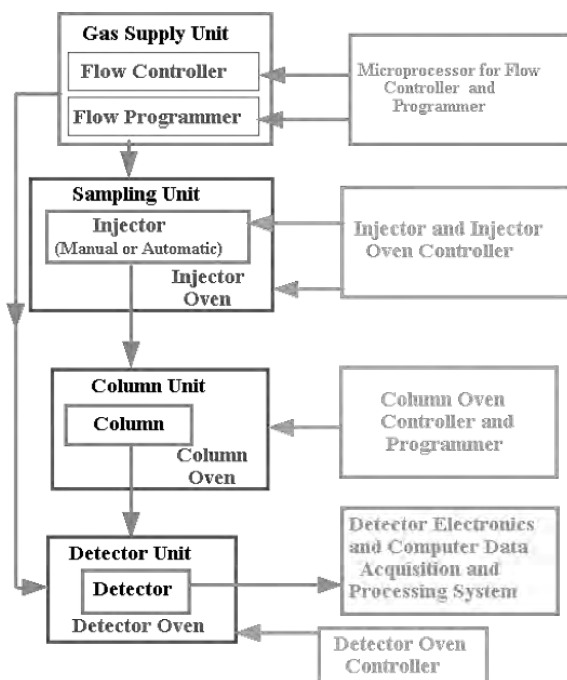
Table 1 The Classification of Chromatography

Mobile phase	Stationary phase
Gas	Liquid
Gas Chromatography (GC)	Liquid Gas-liquid chromatography (GLC)
	Solid Gas Solid Chromatography (GSC)
Liquid	Liquid
Liquid chromatography (LC)	Liquid-liquid chromatography (LLC)
	Solid Liquid solid chromatography (LSC)

The stationary phase can also take two forms, solid and liquid, which provides two subgroups of GC and LC, namely; gas-solid chromatography (GSC) and gas-liquid chromatography (GLC), together with liquid solid chromatography (LSC) and liquid chromatography (LLC). The different forms of chromatography are summarized in Table 1. Most thin layer chromatography techniques are considered liquid-solid systems although the solute normally interacts with a liquid-like surface coating on the adsorbent or support or, in some cases an actual liquid coating.

Gas-liquid chromatography (GLC) was invented by James and Martin and is a chromatography separation technique in which the mobile phase is a gas (usually helium or nitrogen) and the stationary phase is a liquid. In the original columns used by James and Martin, the liquid stationary phase was adsorbed on the surface of an inert support such as Celite (a diatomaceous earth) or calcined Celite (a form of brick dust). The support was usually deactivated before use by acid treatment and subsequent reaction with hexamethyldisilazane. The technique was extensively used for the separation of a wide range of volatile substances including fatty acids.

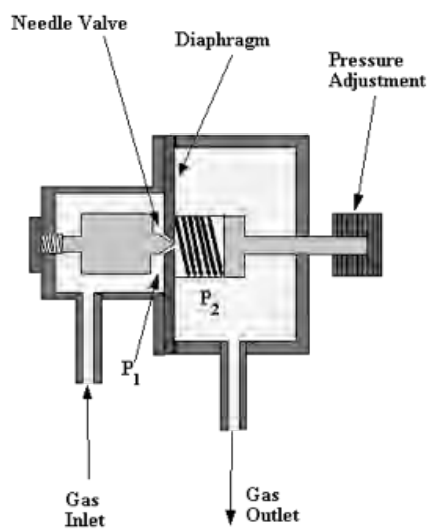
The modern gas chromatograph is a fairly complex instrument mostly computer controlled. The samples are mechanically injected, the analytical results are automatically calculated and the results printed out, together with the pertinent operating conditions in a standard format. However, the instrument has evolved over many years although the majority of the added devices and techniques were suggested or describe in the first three international symposia on gas chromatography held in 1956, 1958 and 1960. The layout of the modern gas chromatograph is shown as a block diagram:



Different components of GLC

Gas supplies

Gases (carrier gas-N₂ or He; and fuel gas-air and H₂) for use with the gas liquid chromatography were originally all obtained from gas cylinders fitted with reducing valves that are set to supply the gas to the instrument at the recommended pressure defined by the manufacturers. The reducing valves on the gas tanks are examples of simple pressure controllers and the flow controllers that are used for detector and column flow control often involve devices based on the same principles. The pressure controller consists essentially of two chambers separated by a diaphragm, in the center of which is a needle valve that is actuated by the diaphragm. The diaphragm is held down by a spring that is adjustable so that the pressure in the second chamber, and thus the outlet flow, can be set at any chosen value. When gas enters the lower chamber, the pressure on the lower part of the diaphragm acts against the spring setting, and opens the valve. Gas then passes into the upper chamber and pressure is built up in the upper chamber to the value that has been set at which time the diaphragm moves downward closing the valve. If the pressure falls in the upper cylinder, the diaphragm again moves upward due to the pressure in the lower chamber, which opens the valve and the pressure in the upper chamber is brought back to its set value.

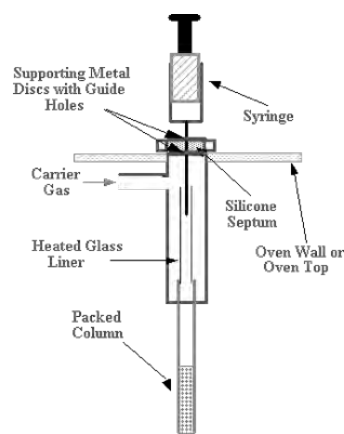


Pressure controller

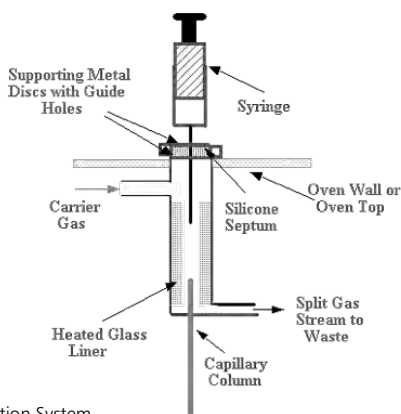
Injectors

The sample is injected by a hypodermic syringe, through a silicone rubber septum directly into the column packing or into a flash heater. An example of a septum injection system used for packed columns is shown in following figure. The silicone septum is compressed between metal surfaces in such a manner that a hypodermic needle can pierce it, but when it is withdrawn the hole is closed as a result of the septum compression and there is no gas leak. The glass liner prevents the sample coming in contact with the heated metal wall and thus, reduces the chance of thermal decomposition. The glass liner can be fitted with a separate heater and the volatilization temperature can, thus, be controlled. By using a syringe with a long needle, the tip can be made to penetrate past the liner and discharge its contents directly into the column packing. This procedure is called 'on-column injection' and, as it reduces peak dispersion on injection and thus, provides higher column efficiencies, is often the preferred procedure.

The basic difference between the two types of injection systems is that the capillary column now projects into the glass liner and a portion of the carrier gas sweeps past the column inlet to waste. As the sample passes the column opening, a small fraction is split off and flows directly into the capillary column, *ipso facto* this device is called a split injector. The split ratio is changed by regulating the portion of



Packed Column Injector



Split Injection System

the carrier gas that flows to waste which is achieved by an adjustable flow resistance in the waste flow line. This device is only used for small diameter capillary columns where the charge size is critical. Consequently, quantitative analyses carried out using the high efficiency small diameter capillary columns may have limited accuracy and precision, depending on the nature of the sample.

GLC Columns

There are two types of columns in common use in GC and they are the conventional packed column and the open tubular column. The former are usually 2 to 4 mm I.D. and 1-4 m long and, packed with a suitable adsorbent, are mostly used for gas analysis. As a result of the simpler injection procedure and the more precise sampling method, the packed column tends to give greater quantitative accuracy and precision. However, despite its problems with sample injection, the open tubular column is seen as the 'state of the art' column and is by far the most popular column system in general use. The length of open tubular or capillary columns range from about 10-100 m and can have internal diameters from 100-500 μm . The stationary phase is coated on the internal wall of the column as a film 0.2-1 μm thick.

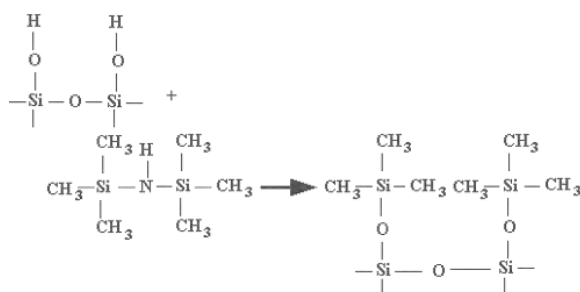
Packed GC Column

Packed columns are usually constructed from stainless steel or Pyrex glass. Pyrex glass is favored when thermally labile materials are being separated such as essential oils and flavor components. Longer columns

can be U-shaped but columns more than a meter long are usually coiled. Glass columns are sometimes treated with an appropriate silanizing reagent to eliminate the surface hydroxyl groups which can be catalytically active or produce asymmetric peaks.

Supports for GLC

There have been a number of materials used as supports for packed GC columns including, Celite (a proprietary form of a diatomaceous earth), fire-brick (calcined Celite), fire-brick coated with metallic silver or gold, glass beads, Teflon chips and polymer beads. Today however, the vast majority of contemporary packed GLC columns are filled with materials that are either based on of Celtic or polystyrene beads as a support. There are two processes used to modify Celite. One was to crush, blend and press the Celite into the form of a brick and then calcine it at a temperature of about 900°C. Under these conditions some of the silica is changed into cristobalite and traces of iron and other heavy metals interact with the silica causing the material to become pink in color. This material is sold under the trade name of Chromosorb P. The second process involves mixing the Celite with sodium carbonate and fluxing the material at 900°C. This causes the structure of the Celite to be disrupted and the fragments adhere to one another by means of glass formed from the silica and the sodium carbonate. As the original Celite structure is disrupted, the material exhibits a wide range of pore sizes which differs significantly from the material that was calcined in the absence of sodium carbonate. This material is sold under the name of Chromosorb W together with two similar materials called Chromosorb G and Chromosorb S. The residual deleterious adsorptive properties



of the support are due to silanol groups on the surface and these can be removed by silanization. The support is treated with hexamethyldisilazane which replaces the hydrogen of the silanol group with a trimethylsilyl radical. The reaction proceeds as follows,

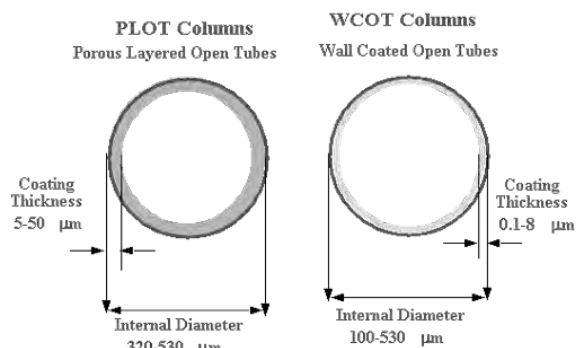
In this way the strongly polar silanol groups are methylated and assume dispersive characteristics that do not produce peak tailing. Although the major contributors to adsorption by the support are the silanol groups, a residual adsorption results from the presence of trace quantities of heavy metals such as iron, which can be largely removed by acid washing prior to silanization.

Capillary or Open Tubular Column

Capillary columns are fabricated from stainless steel. Metal columns provide the high efficiencies expected from open tubular columns and were used for the analysis of petroleum, fatty acids and fuel oils, etc. Metal columns, however, have some disadvantages as although easily coated with dispersive stationary phases (e.g., squalane, Apiezon grease etc.) they are not so easily coated with the more polar stationary phases such as CARBOWAX®. In addition, hot metal surfaces can cause decomposition or molecular rearrangement of many thermally labile materials such as the terpenes contained in essential oils. Metal can also react directly with some materials by chelation and adsorb polar material which results in asymmetric and tailing peaks. Nevertheless, metal columns are rugged, easy to handle and easy to remove and replace in the chromatograph consequently, their use has persisted in many application areas despite the introduction of fused silica columns.

Open Tubular Column Types

Open Tubular columns are broadly split into two classes, the *wall coated open tubular columns* or WCOT Columns (which have already been described and are by far the most popular,) and the *porous layer open tubes* or PLOT Columns. The two types of column are shown diagrammatically in the following figure. The external diameter of PLOT columns range

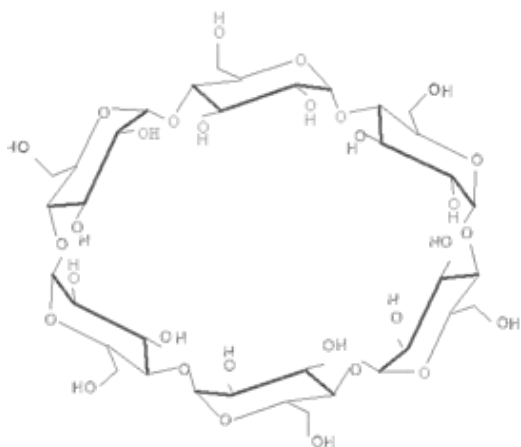


Open Tubular Column Types

from 320-530 μm with a porous layer that can be 5-50 μm thick.

Chiral stationary phases

Modern organic chemistry and pharmaceutical research are becoming increasingly interested in methods of asymmetric syntheses. This enthusiasm has been provoked by the differing physiological activity that has been shown to exist between the geometric isomers of pharmaceutically active compounds. A tragic example being the drug Thalidomide, which was made available as a racemic mixture of N-phthalylglutamic acid imide. The important physiological activity resides in the R-(+)-isomer and it was not found, until too late, that the S-enantiomer was probably teratogenic and caused serious fetal malformations. The separation and identification of isomers can, clearly, be very important and chromatography can be very effective in the resolution of such mixtures. The use of GC for the separation of asymmetric isomers is not as common as LC, but nevertheless there are some very effective optically active stationary phases that can be used in GC for the separation of enantiomers. Some of the more useful GC stationary phases are based on cyclodextrins already described. The columns are usually 30-60 m long 0.25 mm I.D. and have an operating temperature range of 30°C to 250°C. In order to employ the cyclodextrins as stationary phases for GC the permethylated cyclodextrins are often embedded in a siloxane matrix (e.g. 35% phenyl-65% methyl polysiloxane) which is deposited on the walls of fused quartz capillary tubes.



The Structure of Cyclodextrin

Derivatization of the base cyclodextrin structure can introduce groups to which only one enantiomer can interact, while the other(s) are partially or wholly entropically hindered from interaction. This increases the differential interaction between the enantiomers and the stationary phase, thus, increasing the separation ratio and hence the resolution.

Column oven and accessories

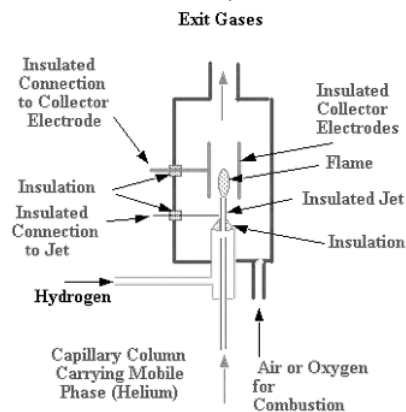
The column oven should operate over a fairly wide temperature range (e.g. from 5°C to 400°C). In practice, however, the maximum oven temperature needed is usually less than 250°C, particularly when synthetic stationary phases are being used, as many of them tend to be unstable and either decompose or volatilize at higher temperatures. Similarly, initial temperatures below 50°C are also rarely needed. The oven usually has air circulation driven by a powerful fan to ensure an even temperature throughout the oven. The temperature programmer (hardware and software) usually has a range of linear gradients from 0.5°C/min. to about 20°C/min. Some programmers include nonlinear programs such as logarithmic and exponential, but most GC analyses can be effectively accomplished using linear programs only. The program rate can be changed at any time in the chromatographic development or intermittent isothermal periods can be inserted where necessary in the program. The temperature programming limits are usually the same as those of the oven (viz. 5°C to 400°C).

GC detectors

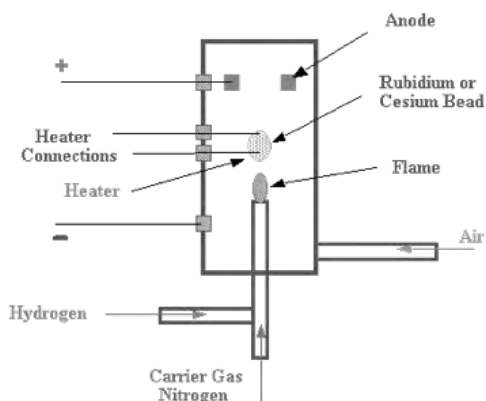
A large number of GC detectors have been developed and made commercially available. The detectors with the highest sensitivity tend to be specific and sense specific types of sample (e.g., halogenated substances by the electron capture detector). The detectors with a cathodic response are the most popular and the majority of GC separations are monitored by the flame ionization detector (FID). The most commonly used specific detectors are the nitrogen phosphorus detector (NPD) and the electron capture detector (ECD).

Flame Ionization Detector

The FID detector employs hydrogen as the combustion gas which is mixed with the column eluent (helium, nitrogen or other appropriate gas) and burnt at a small jet situated inside a cylindrical electrode. A potential of a few hundred volts is applied between the jet and the electrode and when a carbon containing solute is burnt in the jet, the electron/ion pairs that are formed are collected at the jet and cylindrical electrode. The current is amplified and fed to a recorder or to the A/D converter of a computer data acquisition system. During the process of oxidation, oxidized or partially oxidized fragments of the solute are formed in the flame which is thought to generate electrons by thermionic emission. The background current (ions and electrons from the hydrogen flame alone) is very small (1-2 x 10⁻¹² amperes) and consequently,



Flame Ionization Detector



Nitrogen Phosphorus Detector

the noise level is also commensurably small (about 10-14 amperes).

Nitrogen Phosphorus Detector

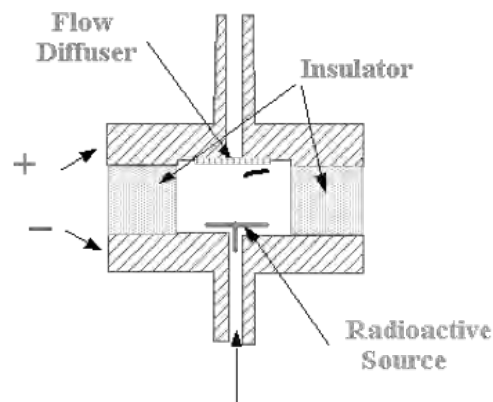
The nitrogen phosphorus detector (NPD) is a highly sensitive but specific detector and evolved directly from the FID. It gives a strong response to organic compounds containing nitrogen and/or phosphorus. Although it appears to function in a very similar manner to the FID, in fact, it operates on an entirely different principle. The actual NPD sensor is a rubidium or cesium bead contained inside a small heater coil. A potential is applied between the bead and the anode. The heated alkali bead emits electrons by thermionic emission which is collected at the anode and thus produces an ion current. When a solute containing nitrogen or phosphorus is eluted, the partially combusted nitrogen and phosphorus materials are adsorbed on the surface of the bead. This adsorbed material reduces the work function of the surface and, as consequence, the emission of electrons is increased which raises the anode current. The sensitivity of the NPD is about 10-12 g/ml for phosphorus and 10-11 g/ml for nitrogen).

Electron Capture Detector

The electron capture detector contains a low energy β -ray source which is used to produce electrons for capturing by appropriate atoms. Although tritium adsorbed into a silver foil has been used as the β particle source, it is relatively unstable at high temperatures, the Ni63 source was found to be preferable. The detector

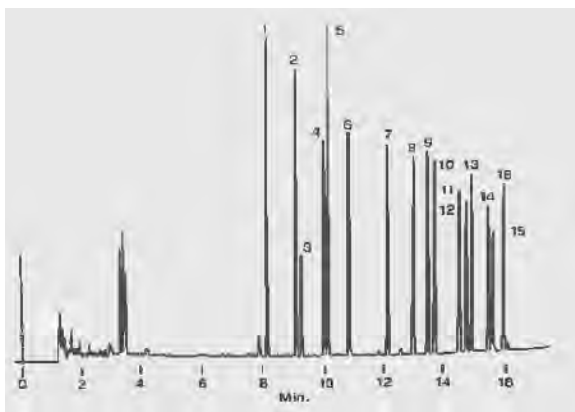
can be used in two modes, either with a constant potential applied across the cell (the DC mode) or with a pulsed potential across the cell (the pulsed mode). In the DC mode, hydrogen or nitrogen can be used as the carrier gas and a small potential (usually only a few volts) is applied across the cell that is just sufficient to collect all the electrons available and provide a small standing current. If an electron capturing molecule (for example a molecule containing a halogen atom which has only seven electrons in its outer shell) enters the cell, the electrons are captured by the molecule and the molecules become charged. The mobility of the captured electrons is much smaller than the free electrons and the electrode current falls dramatically. In the inactive period of the wave form, electrons having thermal energy only will attached themselves readily to any electron capturing molecules present in the cell with the consequent production of negatively charged ions. The negative ions quickly recombine with the positive ions (produced simultaneously with the electrons by the particles) and thus become unavailable for collection. Consequently the standing current measured during the potential pulse will be reduced.

The basic electron capture detector consists of a small chamber one or two ml in volume enclosing two metal



Electron Capture Detector

electrodes. The electrodes may be concentric cylinders or metal discs separated by an insulator. The cell contains the radioactive source, electrically connected to the entrance conduit and to the negative side of the power supply. A gauze "diffuser" is connected to the cell exit and to the positive side of the power supply. The output from the sensor is processed by suitable electronics and the output passed to



either a potentiometric recorder or a computer data acquisition system. The electron capture detector is very sensitive, probably the most sensitive GC detector available (ca. 10-13 g/ml) and is widely used in the analysis of halogenated compounds.

1	2	3	4 Heptachlor
5	6 Aldrin	7 Heptachlor Epox.	8 Endosulphan
9 p,p'-DDE	10 Dieldrin	11 Endrin	12 p,p'-DDD
13 Endosulphan	14 p,p'-DDt	15 Endin Aldehyde	16 Endosulp. Sulf.
11			

Analysis of chlorinated insecticides

Data acquisition and processing

Originally, analytical results were calculated from measurements made directly on the chromatogram provided by the chart recorder. The output from the detector (which is only rarely the direct output from the detector sensor) is usually in millivolts and is suitable for direct connection to a potentiometric recorder. The output from the detector usually passes directly to a scaling amplifier that modifies the signal to a range that is appropriate for the analog-to-digital (A/D) converter. The output can alternatively pass to a potentiometric recorder and produce the chromatogram in real time. The computer system can also produce a real time chromatogram but, to do so, the data must be processed and the chromatogram presented on the printer.

Quantitative analysis

There are three important stages in a GC analysis,

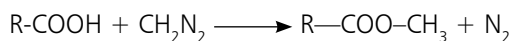
1. The preparation of the sample.
2. The development of the separation and the production of the chromatogram
3. The processing of the data and the presentation of the results.

Each stage is equally important and if not carried out correctly the results will be neither precise nor accurate. Sample preparation can be very simple involving no more than diluting a known weight of sample with mobile phase or be much more complex including an extraction procedure followed by derivatization and then dilution. Liquid extraction is a clumsy procedure, particularly when used on the micro scale which is often necessary in sample preparation. An alternative procedure is solid phase extraction. The procedure is relatively simple and involves the use of a short tube packed with an appropriate adsorbent such as silica, reversed phase silica or, for some applications, macro porous polymer beads. The adsorbent must be capable of removing the substances of interest from the liquid medium.

Derivatization

GC samples are usually derivatized to render highly polar materials sufficiently volatile so that they can be eluted at reasonable temperatures without thermal decomposition or molecular re-arrangement. Examples of such materials that need to be derivatized are the organic acids, amides, poly hydroxy compounds, amino acids etc. In order to render such materials more volatile, they are either esterified, silanated or acetylated using one of a number of different methods of derivatization. Acids can be esterified by treating them with an appropriate alcohol using an inorganic acid to catalyze the reaction. Hydrochloric acid was popular for this purpose because its strength was adequate and any excess could be easily removed. Other catalysts that have been found effective are trifluoroacetic acid, dichloroacetic acid, benzene sulphonic acid, *p*-toluene sulphonic acids and sulphuryl and thionyl chlorides. A volatile acid is recommended such as hydrochloric acid or thionyl chloride. However, the derivative must be sufficiently involatile not to allow loss when removing the excess alcohol and where appropriate the catalyst itself. The Lewis acid boron trifluoride or the equivalent reagent

boron trichloride is also very useful for forming ester derivatives. Boron trifluoride is supplied as a 14% solution in methanol. Boron trifluoride catalyzed reactions are very fast and can be complete in a few minutes. The esters can be extracted with *n*-hexane with vigorous shaking. Another popular esterifying reagent is diazomethane. Diazomethane is a yellow gas but is used in the form of an ethereal solution. Its reacts with an organic acid in the following manner,



When the reaction is complete, the yellow color persists and thus the reagent acts as its own indicator.

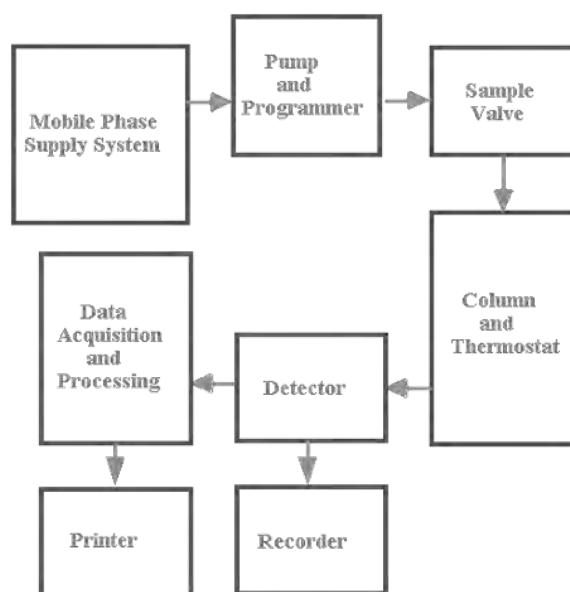
High Pressure Liquid Chromatography

Liquid chromatography (LC) was the first type of chromatography to be discovered and, in the form of liquid-solid chromatography (LSC) was originally used in the late 1890s by the Russian botanist, Tswett to separate and isolate various plant pigments. The colored bands he produced on the adsorbent bed evoked the term chromatography (color writing) for this type of separation. In the late 1930s and early 1940s Martin and Synge introduced a form of liquid-liquid chromatography by supporting the stationary phase, in this case water, on silica gel in the form of a packed bed and used it to separate some acetyl amino acids. Martin and Synge suggested the use of small particles and high pressures in LC to improve the separation which proved to be the critical factors that initiated the development of high performance liquid chromatography (HPLC). The statement made by Martin in 1941 contains all the necessary conditions to realize both the high efficiencies and the high resolution achieved by modern LC columns. Despite his recommendations, however, it has taken nearly fifty years to bring his concepts to fruition. The major impediment to the development of LC was the lack of a high sensitive detector and it was not until the refractive index detector was developed by Tiselius and Claesson in 1942 could the technique being effectively developed. The contemporary chromatograph, however, is a very complex instrument operating at pressures up to 10,000 PSI providing flow rates ranging from a few microliters per minute to 10-20

ml/minute depending on the type of LC that is carried out. Modern detectors can detect solutes at concentration levels of 1×10^{-9} g/ml and an analysis can be completed in a few minutes with just a few micrograms of sample.

Modern High Pressure Liquid Chromatography (HPLC)

HPLC is liquid chromatography which has been optimized to provide rapid high resolution separations. The basic liquid chromatograph consists of five basic units as follows. A block diagram of the basic liquid chromatograph is shown in the following figure.



The Basic Liquid Chromatograph

1. Mobile phase supply system and gradient mixers.
2. HPLC high pressure pumps and sample valves.
3. HPLC columns with inert packing materials.
4. High sensitivity low dispersion HPLC detectors.
5. High speed data acquisition systems.

Mobile Phase Supply System and HPLC Gradient Mixers

HPLC gradient mixers provide a very precise control of solvent composition to maintain a reproducible gradient profile. The mobile phase supply system consists of number of reservoirs (200-1,000 ml). At least two reservoirs would be necessary and are usually constructed of glass or stainless steel and contain an

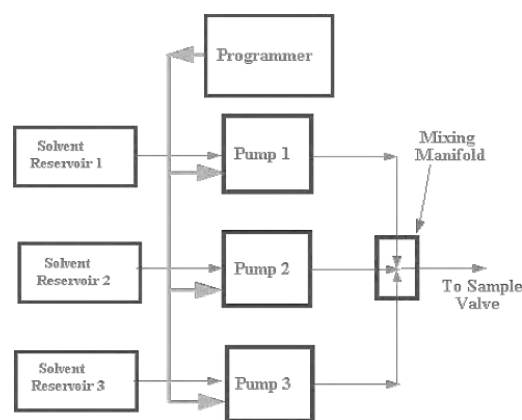
exit port open to air. Each reservoir is usually fitted with a gas diffuser through which helium can be bubbled. Many solvents and solvent mixtures (particularly aqueous mixtures) contain significant amounts of dissolved nitrogen and oxygen from the air. These gasses can form bubbles in the chromatographic system that cause both serious detector noise and loss of column efficiency. As helium is very insoluble in most solvents, it purges the oxygen and nitrogen from the solvent but does not produce bubbles in the system itself. Applying a vacuum to the reservoir is not a permanent solution to dissolved air as, on releasing the vacuum to allow the solvent to pass to the pump, air again dissolves in the solvent. The solvent is filtered through a stainless steel or sintered glass filter to remove any solid contaminants. Depending on the type of solvent programmer that is employed, the supply from each reservoir may pass either to a pump or to a valve blending device. Solvent reservoirs are not usually thermostatted but, when necessary, the solvent can be brought to the column temperature by the use of an appropriate heat exchanger.

Gradient Programmer

High Pressure Gradient Programmer

There are two basic types of solvent programmer. In the first, the solvent mixing occurs at high pressure and in the second the solvents are premixed at low pressure and then passed to the pump. Theoretically, there can be any number of solvents involved in a mobile phase program, however, most LC analyses require only two solvents, nevertheless, up to four solvents can be accommodated. The layout of a high pressure gradient system is shown in the following figure and includes, as an example, provision for three solvents to be mixed by appropriate programming.

Solvent passes from each reservoir directly to a pump and then to a mixing manifold from which it passes to the sample valve and column. The pumps control the actual program and are usually driven by stepping motors. The volume delivery of each solvent is controlled by the speed of the respective pump which is precisely determined by the frequency of its power supply. The controlling frequency can



High Pressure Gradient Programmer

be generated either by external oscillators or, if the chromatograph is computer controlled, directly from the computer itself.

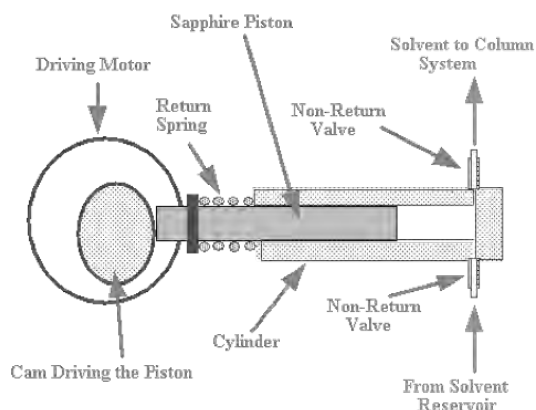
HPLC Pumps

Because of the small particles used in modern HPLC, LC pumps need to operate reliably and precisely at pressures of 10,000 PSI or at least 6,000 PSI. To operate at these pressures and remain sensibly inert to the wide variety of solvents used HPLC pumps usually have sapphire pistons, SS cylinders and return valves fitted with sapphire balls and stainless steel seats. For analytical purposes HPLC pumps should have flow rates that range from 0-10 ml/min., but for preparative HPLC, flow rates in excess of 100 ml/min may be required. There are a number of different types of pumps that can provide the necessary pressures and flow-rates required by the modern liquid chromatograph. In the early years of the LC renaissance, there were two types of pump in common use; they were the pneumatic pump, where the necessary high pressures were achieved by pneumatic amplification, and the syringe pump, which was simply a large, strongly constructed syringe with a plunger that was driven by a motor. Today the majority of modern HPLCs are fitted with reciprocating pumps fitted with either pistons or diaphragms.

Single Piston Reciprocating Pump

The single piston reciprocating pump was the first of its type to be used with high efficiency LC columns (columns packed with small particles) and is still very popular today. It is simple in design and relatively

inexpensive. A diagram of the single piston pump is shown in the following figure.



Single Piston Reciprocating Pump

Most pistons of modern LC pumps are made of synthetic sapphire to reduce wear and extend the working life of the pump. The cylinder is usually made of stainless steel and is attached to two non-return valves in line with the inlet and outlet connections to the pump. The piston is driven by a stainless steel cam which forces the piston into the cylinder expressing the solvent through the exit non-return valve. After reaching the maximum movement, the piston follows the cam and returns as a result of the pressure exerted by the return spring. During this movement the cylinder is loaded with more solvent through the inlet non-return valve. The shape of the cam is cut to provide a linear movement of the piston during expression of the solvent but a sudden return movement on the refill stroke. In this way the pulse effect that results from the refill action is reduced.

Rapid Refill Pump

In order to avoid the refill pulses resulting from a single piston pump, a number of rapid refill systems have been developed. The designs have ranged from cleverly designed actuating cams to drive the piston rapidly in the refill mode to electronically operated piston movements.

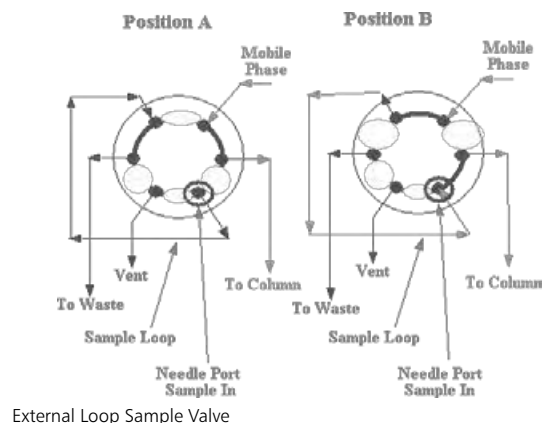
Diaphragm Pump

The unique property of the reciprocating diaphragm pump is that the actuating piston does not come into direct contact with the mobile phase and thus, the

demands on the piston-cylinder seal are not so great. The diaphragm has a relatively high surface area and thus, the movement of the diaphragm is relatively small and consequently the pump can be operated at a fairly high frequency.

HPLC Sample Valves

Since sample valves come between the pump and the column it follows that HPLC sample valves must also tolerate pressures up to 10,000 PSI. For analytical HPLC, the sample volume should be selectable from sub- micro liter to a few micro liters, whereas in preparative HPLC the sample volume may be even greater than 10 ml. The higher the operating pressure the tighter the valve seating surfaces must be forced together to eliminate any leak. It follows that any abrasive material, however fine, that passes into the valve can cause the valve seating to become scored each time it is rotated which will ultimately lead to leaks. This will cause the sample size to vary between samples and eventually affect the accuracy of the analysis. In LC, the sample valve contains an extra loading port and behaves like an internal loop valve. The basic difference between this type of valve and the normal external loop sample valve is the introduction of an extra port at the front of the valve. This port allows the injection of a sample by a syringe directly into the front of the sample loop. Position (A) shows the inject position. Injection in the front port causes the sample to flow into the sample loop. The tip of the needle passes through the rotor seal and, on injection, is in direct contact with the ceramic stator face. After injection, the valve is rotated to position (B) and the mobile phase flushes the sample directly onto the column. The sample is actually forced out



External Loop Sample Valve

of the beginning of the loop so it does not have to flow through the entire length of the loop. This type of injection system is ideally suited for quantitative LC, and is probably by far the most popular injection system in use.

HPLC Columns

HPLC columns are packed with very fine particles (usually a few microns in diameter). The very fine particles are required to attain the low dispersion that give the high plate counts expected of modern HPLC. Plate counts in excess of 25,000 plates per column are possible with modern columns, however, these very high efficiencies are very rarely found with real samples because of the dispersion associated with injection valves, detectors, data acquisition systems and the dispersion due to the higher molecular weight of real samples as opposed to the common test samples. LC columns, in general, achieve their separation by exploiting the different intermolecular forces between the solute and the stationary phase and those between the solute and the mobile phase. The column will retain those substances that interact more strongly with the stationary phase than those that interact more strongly with the mobile phase. In particular optically pure compounds can be used to make Chiral HPLC stationary phases.

Liquid Chromatography Stationary Phases

Traditionally the stationary phase used in LC has been silica gel which separates solutes largely on the basis of polarity, although, due to its unique structure, silica gel also exhibits strong exclusion characteristics. The bonded phases were introduced to provide a material that would separate solutes by dispersive interactions and also to provide some semipolar stationary phases. The bonded phases were also based on silica gel. More recently, polymeric stationary phases were introduced to provide materials that were insoluble in water and that were stable at extremes of pH.

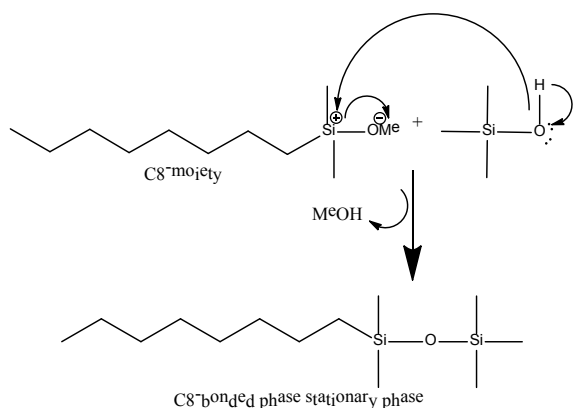
Structure of Silica Gel

The matrix of the primary silica gel particle consists of a core of silicon atoms joined together with oxygen

atoms by siloxane bonds (silicon-oxygen-silicon bonds). On the surface of each primary particle some residual, uncondensed hydroxyl groups from the original polymeric silicic acid remain. There are three types of hydroxyl group. The first is a single hydroxyl group attached to a silicon atom which has three siloxane bonds joining it to the gel matrix. The second is one of two hydroxyl groups attached to the same silicon atom which, in turn, is joined to the matrix by only two siloxane bonds. These twin hydroxyl groups are called Geminal hydroxyl groups. The third is one of three hydroxyl groups attached to a silicon atom which is now only joined to the silica matrix by only a single siloxane bond.

Bonded Phases

Bonded phases are formed by reacting the surface hydroxyl groups with an appropriate reagent to chemical link an organic moiety to the silica surface. The nature of the organic moiety will determine the type of interaction that will take place between the solute and the surface. The most efficient bonded phase has the maximum surface coverage. It is understood, that due to steric hindrance from the bonded moiety itself, only a proportion of the silanol groups can be bonded and there is little that can be done to avoid this problem. However, there are other reasons for incomplete silanization of the silica. Incomplete silanization can result from the reagent molecule being excluded from the smaller pores of the silica. Exclusion can be a particular problem when bonding relatively large molecular weight materials such as long chain hydrocarbons onto the silica surface. It is therefore, important to choose a silica gel that has a relatively large pore size (e.g., a mean pore diameter of 150Å) which may limit the surface area to between 150 and 250 sq.m per gram and thus, reduce the retentive capacity of the stationary phase. The solvents normally used in bonded phase synthesis are aromatic hydrocarbons e.g., toluene that boils at 110°C or mixed xylenes that boil 138-140°C. The procedure varies a little depending on the size of the batch and the type of silanizing reagent. A method of synthesis of bonded phase for the alkoxy silane reagents is illustrated below. The most reactive alkoxy reagents are the methoxy and ethoxysilanes and their reaction with a hydroxyl group is accompanied by the release of methanol or ethanol.



The final capping process is also the same as that employed in the method using the chlorosilanes reagents, utilizing hexamethyldisilazane as the capping reagent. The alkoxy-silanes are almost as readily available as the chlorosilanes and are easier and more pleasant to handle.

LC Mobile Phases

The choice of phase system can be very complex, particularly if multicomponent mixtures are to be separated. In the first instance the type of stationary phase needs to be chosen and this choice must be based on the interactive character of the solutes to be separated. If the solutes are predominantly dispersive then the stationary phase must also be dispersive (a reversed phase) to promote dispersive interaction with the solutes and provide adequate retention and selectivity. If the solutes are strongly polar then a polarizable stationary phase (one containing aromatic rings or cyano groups) would be appropriate to separate the solutes by polar and induced polar interactions. If the solutes are weakly polar then a strong polar stationary phase would be required (such as silica gel) to separate the solute by polar interactions.

Column Ovens

The effect of temperature on LC separations is often not nearly so profound as its effect in GC separations, but can be critical when closely similar substances are being separated. In LC a change in temperature will change the free energy of the solute in both phases, (generally in a commensurate manner) and so the net change in the free energy difference with

temperature, which controls the magnitude of the absolute retention, can be relatively small. Its effect on relative retention, however, can be very significant and, in fact, be the determining factor in achieving a satisfactory resolution. An increase in temperature will increase the diffusivity of the solute in both phases and thus increase the dispersion due to longitudinal diffusion and decrease dispersion due to resistance to mass transfer.

HPLC Detectors

A large number of LC detectors have been developed over the past thirty years based on a variety of different sensing principles. However, only about twelve of them can be used effectively for LC analyses and, of those twelve, only five are in common use. The dominant detectors used in LC analysis are the UV detector (fixed and variable wavelength), photo diode array detector, the electrical conductivity detector, the fluorescence detector and the refractive index detector. These detectors are employed in over 95% of all LC analytical applications.

UV Detector

The UV detector is by far the most popular and useful LC detector that is available to the analyst at this time. Although the UV detector has some definite limitations (particularly for the detection of non polar solutes that do not possess a UV chromophores) it has the best combination of sensitivity, linearity, versatility and reliability of all the LC detectors so far developed. Multi-Wavelength UV detectors utilize a single of wavelengths to detect the solute. Most multi wavelength UV detectors can also provide a UV spectrum of the eluted solute if appropriately arranged.

Electrical Conductivity Detectors

The electrical conductivity detector can only detect those substances that ionize and consequently, are frequently used in the analysis of inorganic acids, bases and salts. It has also found particular use in the detection of organic acids and bases that are frequently required in environmental studies and in biotechnology applications. The sensor is the simplest

of all the detectors consisting of only two electrodes situated in a suitable flow cell.

Fluorescence Detector

The fluorescence detector is one of the most sensitive LC detectors and for this reason is often used for trace analysis. Unfortunately, although the detector is very sensitive, its response is only linear over a relatively limited concentration range. In fact, the response of the detector can only be assumed to be linear over a concentration range of two orders of magnitude. Unfortunately, the majority of substances do not naturally fluoresce which is a serious disadvantage to this type of detector. It follows, that in many instances fluorescent derivatives must be synthesized to render the substances of interest detectable.

Refractive Index Detector

The refractive index detector is one of the least sensitive LC detectors. It is very sensitive to changes in ambient temperature, pressure changes, flow-rate changes and can not be used for gradient elution. Despite these many disadvantages, this detector is extremely useful for detecting those compounds that are nonionic, do not adsorb in the UV, and do not fluoresce.

HPLC Data Acquisition

The output from the detector, usually in millivolts, is passed to a scaling amplifier that converts the signal to a voltage that is acceptable to the analog to digital (A/D) converter. The A/D converter changes the voltage output to a binary number which is temporarily stored in a register. This process is continuously repeated at a defined rate, called the 'sampling rate'. The current binary number, stored in the register is regularly sampled by the computer and stored (usually on hard disk). On completion of the analysis the computer accesses all the data from store, calculates the retention report, compares peak heights or peak areas to provide the quantitative analysis according to the processing program that is used and finally prints out the results in tabulated form. Modern data processing software often includes routines that can process chromatograms where the components of the sample

are incompletely resolved. The routines deconvolute the individual peaks from the composite envelope and calculate the area of the individual de-convoluted peaks. Such algorithms can be used very effectively on peaks that are entrained in the tail of a major peak but are not so accurate for composite envelopes containing many unresolved peaks.

Spectroscopy

Spectroscopy is a technique that uses the interaction of energy with a sample to perform an analysis. The data that is obtained from spectroscopy is called a spectrum. A spectrum is a plot of the intensity of energy detected versus the wavelength (or mass or momentum or frequency, etc.) of the energy. A spectrum can be used to obtain information about atomic and molecular energy levels, molecular geometries, chemical bonds, interactions of molecules, and related processes. Often, spectra are used to identify the components of a sample (qualitative analysis). Spectra may also be used to measure the amount of material in a sample (quantitative analysis). Because the response of a compound to electromagnetic (EM) radiation depends on its structure, spectroscopy can be used to elucidate the structure of unknown chemical products. EM radiation behaves both as a particle of light (called a photon) and as a wave moving at the speed of light (c ; $c = 3 \times 10^8 \text{ m/s}$).

Properties of EM particles and waves

1. Wavelength (λ): Distance between two peaks or troughs in a light wave.
2. Frequency (ν): Number of wave cycles that pass a given point per line. Usually measured in Hertz (Hz; 1 Hz = 1 cycle/second).
3. Energy of a photon: $E = h\nu = hc/\lambda$, where h = Planck's constant = $6.6 \times 10^{-34} \text{ J/sec}$.

Types of Spectroscopy

There are several types of spectroscopy, and among all these three are important for bioprospecting.

- Nuclear magnetic resonance (NMR) spectroscopy:

Measures interaction of radio waves with atomic nuclei in a magnetic field.

- Infrared (IR) spectroscopy: Measures absorption of infrared light by chemical bonds.
- Ultraviolet/Visible (UV/Vis) spectroscopy: Measures absorption of ultraviolet or visible light by π bonds.

Nuclear magnetic resonance (NMR) spectroscopy

Atomic nuclei have a “spin” associated with them (*i.e.*, they act as if they were spinning about an axis) due to the spin associated with their protons and neutrons. Because nuclei are positively charged, their spin induces a magnetic field. When a magnetic field is applied to atomic nuclei, the magnetic fields of the nuclei align themselves either parallel or antiparallel to the applied magnetic field. The nuclei have a slight preference for the parallel alignment, as it has a slightly lower energy, but nuclei can flip between the two possible alignments. When EM radiation with energy equal to the energy difference between the two alignments is applied to the nuclei, it induces them to flip from parallel to antiparallel alignment. Rapid flipping between alignments occurs. The nuclei are said to be in resonance, and the energy they emit when flipping from the high to the low energy state can be measured. The energy at which a given nucleus achieves resonance depends on its chemical surroundings. NMR spectra are taken by applying a magnetic field to a sample, irradiating the sample with EM radiation whose energy is varied over a given range, and measuring the energy emitted by flipping nuclei at each energy.

1. The range of radiation energies is generally chosen such that emission from only one type of nucleus (*e.g.*, ^1H) in a molecule is seen.
2. NMR spectroscopy does not work for nuclei that have an even number of protons and neutrons—these nuclei have no net spin.
3. NMR spectroscopy is most commonly done on ^1H and ^{13}C .

Features of an NMR Spectrum

The distinguished features and terms related to NMR spectrum are as follows:

NMR spectra are displayed as plots of intensity of energy emission (due to resonance) versus the energy of the radiation applied to the sample. Peaks in the spectrum represent resonance energies for nuclei in a molecule.

Shielding: An electron cloud circulates around each nucleus and creates a small magnetic field opposing the applied magnetic field. The electron cloud around each atom depends on the surrounding atoms. As a result, each nucleus experiences a slightly different magnetic field (the sum of the applied field and the field from the electron cloud). For this reason, the energy at which a nucleus achieves resonance depends on its surroundings.

Chemical shift (δ): The resonance energy for a given nucleus is reported in an NMR spectrum as the difference (in parts/million) between the resonance frequency for a given proton and the resonance frequency for protons in a reference compound, which is usually tetramethylsilane, $(\text{CH}_3)_4\text{Si}$. Chemical shifts give information about the atomic surroundings of a given nucleus.

Peak intensity: The area under a peak in an NMR spectrum is proportional to the number of nuclei in a given chemical environment in a molecule (*e.g.*, if the area under a peak is two times the area under another peak, there are twice as many nuclei responsible for the larger peak than for the smaller one). The intensity of an NMR peak gives information about the relative number of a given type of nucleus in a molecule.

Spin-spin splitting: In ^1H NMR, a given hydrogen nucleus interacts with hydrogen nuclei on neighboring carbon atoms such that the peak from that nucleus is split into multiple peaks called a multiplet. Relative intensities of the peaks in a multiplet follow Pascal’s triangle. Spin-spin splitting gives information about the hydrogen atoms neighboring a given hydrogen nucleus.

Infrared (IR) spectroscopy

The principles and theory related to IR spectroscopy are detailed below:

- Covalent bonds are similar to springs—bonded atoms vibrate (*i.e.*, stretch and compress) and bend about their bonds. As a consequence of quantum mechanics, these bonded atoms can vibrate and bend only at frequencies that are integral multiples of a fundamental frequency that depends on the type of bond.
- Bonds about which vibration and bending occur can absorb light if the frequency of the light wave is the same as the frequency of the movement about the bond. The frequency of light absorbed by these bonds is generally in the infrared region of the EM spectrum.
- In IR spectroscopy, a chemical sample is irradiated with infrared light over a wide range of frequencies, and the light absorbed by the sample at each frequency is measured.
- Bonds about which a molecule is symmetric cannot absorb IR light and therefore cannot be detected by IR spectroscopy.

Features of an IR spectrum

The distinguished features and terms related to IR spectrum are as follows:

- IR spectra are displayed as plots of absorption versus wave number (cm^{-1} , $1/\lambda$), which is another measure of the energy of a light wave (similar to frequency). Peaks in an IR spectrum represent wavelengths at which light was absorbed by the molecules in the sample.
- Because each functional group that is IR-active has a characteristic set of frequencies at which it absorbs IR light, IR spectroscopy is useful in detecting the presence of specific functional groups in a molecule.
- Because nearly all organic molecules contain C-C and C-H bonds that absorb IR light in similar ways, IR spectroscopy is most useful in identifying functional groups that contain other bonds besides C-C and C-H bonds.
- Fingerprint region: Region between 1200 cm^{-1} and 1700 cm^{-1} in an IR spectrum; contains complicated absorption peaks that are characteristic of a specific molecule. An unknown compound can be identified with reasonable certainty if its fingerprint region matches that of a known compound.

Ultraviolet/Visible (UV/Vis) spectroscopy

If a molecule has π electrons, it can absorb UV or visible light to promote one of those electrons into a higher-energy orbital. UV/Vis spectroscopy generally involves the promotion of the π electron in the highest-energy occupied orbital to the lowest-energy unoccupied orbital. A UV/Vis spectrum is taken by irradiating a sample with UV/Vis light over a range of wavelengths and measuring the amount of light absorbed at each wavelength.

Features of UV/Vis spectra

The distinguished features and terms related to UV/Vis spectrum are as follows:

- UV/Vis spectra are displayed as plots of absorption versus wavelength. Peaks represent wavelengths at which light was absorbed by the molecules in the sample.
- The energy of the UV/Vis light absorbed by a π electron system in a molecule depends on the nature of the π system. As a result, the presence of a particular type of π system in a molecule can be identified by UV/Vis spectroscopy.
- The energy gap between the highest-energy occupied orbital and the lowest-energy unoccupied orbital decreases as the size of the π electron system increases. Consequently, the wavelength of UV/Vis light absorbed by a molecule increases as the size of its conjugated π electron system increases (because the energy of a light wave decreases with increasing wavelength).
- UV/Vis spectroscopy is generally used on conjugated hydrocarbon systems, but other molecules containing π electron systems, such as carbonyls, are also weak absorbers of UV/Vis light.

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Bioassays – Types and Evaluation

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Introduction

Bioassay is a tool for the determination of a biological activity, or the quantification of a target analyte based on this activity, using the recognition elements like bacteria, cells or tissues. It is the navigational pathway to find the active component present in the crude extract of marine organisms which have biological activity of interest for exploration. When a particular organism is found to have activity of definite interest, the extract was obtained by conventional methods from the marine organism. With the bioassay guided fractionation, the extract was sub fractionated to the limited fractions which have maximum activity of interest. When the complex mixture is simplified to few active fractions, it will be easy to isolate active pure component using the powerful isolation techniques depending on the quantity of the expected final product. Exploratory work would be complete only if the active component is retrieved from such extracts with the support of the bio assays. The final pure product of the extract usually happens to be in low yield when compared to the total weight of the source organism. If the compound/extract is to be screened against a wide spectrum of activity, it is normally inadequate for full screening experiments to find out the overall potential. The activity of drugs is dose dependent and toxic in higher doses. Eg. Pesticides at low doses act as PGR; at high dose as poison. Any prediction of the activity of a component can be done using dose response spectra library software.

Extraction techniques

Some of the latest isolation techniques include new state-of-the art extraction techniques, such as supercritical fluid extraction (SFE), pressurized liquid

extraction (PLE), accelerated solvent extraction (ASE), pressurized hot water extraction (PHWE), ultrasound-assisted extraction (UAE), and microwave assisted extraction (MAE) techniques.

Types of bioassays for screening

Methods and applications represent a unique attempt to describe assays to evaluate the bioactivities of therapeutic moieties resulting from chemical, biological, and natural processes and to explore the mechanism of action of therapeutic moieties in the cells, tissues, and organs of living beings, as well as in living beings themselves. Such assays are highly important in the pharmaceutical industry and are the prime engine for the advancement of chemistry, pharmacy, biology, and medicine.

As a routine record of bioactivities, in any exploratory work the samples are screened from simple activity to the much interested bio assay. The assays are of different kinds and can be performed in the following heads:

Anticancer, antiviral, antitubercular, antimicrobial (antibacterial or antifungal), antihelminthic, antimalarial, thrombus-related anticoagulation, analgesic, antiallergic, antiarrhythmic, hypolipidaemic, hypoglycemic, hypotensive, antihypertensive, diuretic, immunomodulatory, choloretic and anticholestatic, blood pressure, Parkinson's disease and Graves' Disease, Alzheimer's disease, antiosteoporosis, immunomodulation, anti-inflammation, antioxidant activity, epilepsy, diabetes, assays for toxins from microorganisms, hepatotoxicity and hepato protective assays, cytogenetic receptor and enzyme assays.

The screening models for the evaluation of these properties on the given analyte are available.

Types of screening experiments

The screening for the activities is done *in vitro* or *in vivo* modes depending on the nature and necessity of testing. Appropriate models for the screening by biological, toxicological and clinical evaluations are now available to find out the type of activity for the given sample. As a protocol, the extract of a marine organism is subjected to various kinds of activity tests depending on the type of activity chosen for screening. Based on this the active component is retrieved from the extract. *In vitro* assays usually consist of cell culture systems with neoplastic cell lines from human or other animal tumors as targets. The capacity of test compounds inhibiting the growth or reducing the survival of cancer cells in culture media and the potency of test compounds inducing structural change of cancer cells in culture media are generally correlated with the *in vivo* potency of a cancer therapeutic agent.

Anticancer tests

22 models used in anticancer research are described: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for six carcinoma cells, flow cytometric assay for cell apoptosis, DNA fragmentation assay, Bcl-XL/BH₃ interaction assay, dissociation enhanced lanthanide fluoro-immunoassay (DELFLIA), Ishikawa cell and rat assay for detecting antiestrogens, adenosine triphosphate (ATP) assay for eight cells, alkaline phosphatase (AP) activity assay, tumor endothelial cell tube formation assay, antiangiogenic assay, *in vivo* hollow fiber assay, VX2 rabbit lung assay, insulin-like growth factor-I (IGF-I)-induced kinase receptor activation assay, insulin-like gD. trkA-induced kinase receptor activation assay, UV spectra-based calf thymus DNA intercalation assay, fluorescence spectra-based calf thymus DNA intercalation assay, P-glycoprotein pump (P-gp) in MCF-7R cells assay, P-gp-related efflux carrier assay, substrate transport inhibition assay, lactate dehydrogenase release assay, functional assay of mitochondrial P-glycoprotein (P-gp), and resistance index value assay.

Neurosuppressives and muscle relaxants

Glutamate receptor antagonist, serotonergic receptor (SR) antagonist, IP₃-inhibitor, adrenergic receptor antagonist, Actomyosin ATPase inhibitor (antiasthmatic, uterine relaxation), Glutamate receptor antagonist (GRA)

Antiinflammatory

Antiinflammatory activity – Carrageenin-induced Oedema in Mice–Volume of both the paws is measured plethysmographically daily for ten days. Phospholipase A₂ (PLA₂) inhibitor

Antiviral

The potential antiviral agents should be evaluated in a living cell or animal host like cell culture, chicken eggs and animal models. The cells are infected with the virus and then exposed to the test substance. If the substance has antiviral activity the multiplication of the virus will be inhibited which will be evident from the morphology of the cell monolayer. Several viral targets are studied to estimate the antiviral effect of test substance in a cell culture system. Some of these are viral DNA polymerase activity; ribonucleotide diphosphate reductase; mRNA polyadenylation and RNA dependent RNA polymerase; terminal deoxynucleotidyl transferase; thymidine kinase; uracil DNA-glycolase; d-UTPase and reverse transcriptase. AV (HIV-1 integrase inhibitor), AV (herpes and polio), AV (feline leukemia, mouse influenza, mouse corona), UAG suppressor glutamine tRNA inhibitor, Interferon mediator, herpes simplex virus type 1 (HSV-1), HIV reverse transcriptase (HIV RT).

Antibiotic

Antibacterial, antifungal, antimalarial, antibiotic tests are carried out involving the pathogens: gram-(+) ve: *Bacillus subtilis*, *Staphylococcus aureus*, *Candida albicans*; Fungus- *Cladosporium cucumerinum*, Malarial parasite: *Plasmodium falciparum*. Antimicrobial testing involves various methods, viz. poison food technique; disc diffusion method, tube dilution method and microtitre technique. Antimalarial Activity–Against

Plasmodium berghei in primary screening in infected swiss mice. Subsequent screening is conducted in rhesus monkey infected with *P. cynomolgi*. Chloroquine (5 mg per kg for 7 days).

Pharmacological activities tests

Antitumor and Anticancer tests – They involve the following factors in identification of the activity of the given sample for screening. Protein kinase C (PKC) inhibitor, 3-Fucosyltransferase (FTase) inhibitor, Kinesin motor protein (KMP) inhibitor, Stabilization of microtubules (MTS), Tubulin polymerization (TP) inhibitor, Actin-depolymerisation (actin DP), Topoisomerase (TI) II inhibitor, Nitric oxide synthetase (NOS) inhibitor, NKT cell activator, Reverses drug resistance of cancer cells, v-ATPase inhibitor, Ca²⁺ channel blocker; Immunosuppressive (IS), cytotoxic (CTX), P-388 leukemia (P-388L) cells, Inhibitor of T-cell proliferation, IL-2 inhibitor, IL-8 inhibitor, Histamine release (HR) inhibitor, Inhibitors of proton pump activity (PPAI), Inhibition of DNA replication (DNAR), Lipoxigenase (LP) inhibitor, Cysteine Protease (CP) Inhibitor, phosphatase activity (PA) inhibitor, Ovarian human tumor (OHT) cell, Inhibitors of serine-threonine (STI)

Toxicity and CNS Activities

LD50 is determined in mice using the method of Horn *et al.* with a dose of 464 mg/kg (ip) and mortality in 24 hour recorded. The samples are evaluated against experimental animal to find out its toxicity. An experiment on albino rats is given. The following typical tests are undertaken while screening the samples. eg. The test substance is administered by the oral or intraperitoneal (ip) route in two or three adult albino mice of either sex and usually 15 to 20 g in weight. The test material is suspended in 0.1% agar or in 10% gum acacia in distilled water. Concentrations are so adjusted that a 20 g mouse receives a volume of 0.2 ml. The initial dose is at a level of 400 or 500 mg/kg going up or down by a factor of 2. Occasionally, an interval of 1.5 is used for closer approximation. Doses higher than 1000 mg/kg are not generally used. Control animals are administered only the vehicle (placebo). The animals are observed for

5 to 6 hours after dosage for toxic symptoms. If death occurs during this time, the cause of death is recorded. The approximate LD50 is estimated and the maximum tolerated dose is also recorded for use in subsequent investigations.

Clinical Trials

Any proven fraction/compound with promising activity from preliminary experiments on animals will be extended for human trials under different phases. Phase I clinical trials are performed on health human volunteers, Phase II on patients and Phase III on multicentric mode involving clinical trials protocol and duplicating trials.

Aquaculture

In aquaculture the extract is tested for the efficient fish culture practices. Antifouling – *repellent*; Inhibition–*fertilized seaurchin egg cell division*, Icthiotoxic–*artemia, fish, feed deterrent*, Nematocidal – Screening of harmful worms.

Blood-Related Diseases

Serine protease inhibitor (SPI), Thrombin receptor antagonist (TRA), VCAM-1 inhibitor, α-glucosidase inhibitor.

Anthelmintic Activity

Against parasites of hookworms, ascarids, oxyurids and filarids with test animal – hamsters.

Analgesic activity

It can be divided into two categories: (a) centrally acting analgesics, and (b) peripherally acting drugs.

Antiarrhythmic and antithrombotic activities

Ventricular arrhythmias

Hypolipidaemic activity

Testing lipid lowering activity

Hypoglycaemic activity

With test animals of Charles Foster albino rats in single and multiple doses. A fall of more than 30% in blood sugar is taken as active.

Hypotensive Activity

The test animal is generally anaesthetised with sodium pentobarbiton (35–40 mg/kg, iv or ip) or a chloralose (70–80 mg/kg, iv). The blood pressure is recorded. The test extract is given at 25 and 50 mg/kg (iv), and the effect noted. In rats with deoxycorticosterone acetate (DOCA)-salt model is used.

Diuretic Activity

In dogs at 5 mg/kg dose by intravenous administration.

Mechanism of drug action

The drug action on a living organism may be attributed to the counter biological functions stimulated by the host with the help of drug or by drug itself against the invading pathogens. In this way, immunomodulators are substances which have potential to modulate an immune response *in vivo* and/or *in vitro*. Immunostimulants enhance antigen specific (vaccines) and non-specific immune response against infection and malignancy. Immunotherapy of viral infection is done by potentiating the efficacy of drugs in immuno compromised host. Extracts from marine organisms show wide range of immunomodulation during the screening experiments.

Hepatoprotective Activity

It is known that the biochemical and physiological functions of liver and the activity of a wide range of hepatic enzymes are altered when the animals

are exposed to a variety of chemicals such as carbon tetrachloride, D-galactosamine, thioacetamide, heavy metals and drugs such as paracetamol, fungal toxins and parasitic infections. Some chemicals apart from these on exposure also result in carcinogenesis. Extracts from marine sources show hepatoprotective activity.

Conclusion

With the advancement of evaluation and screening techniques it is now possible to explore the range of activities for the given sample. The characterization of the active component of the active fraction is also simplified with low quantity of sample using powerful instrumental methods which require only micro level quantity. The required screening facilities are maintained both in private and public funded institutions to enable the activity testing. The work may be outsourced or collaborated to develop a drug.

Suggested readings

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Chitin, Chitosan and their Applications

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Introduction

Chitin is a nitrogenous polysaccharide (poly N-acetyl Amino D- glucose) found in the outer Skeleton of insects, crabs, shrimps and lobsters and in the internal structure of other invertebrates. It is the most abundant organic compound next to cellulose in the earth. It was first isolated by Braconnot (1811) from mushroom and was named 'fungine'. An identical material was isolated from insects in 1821 by Odier (1823) and named it as "chitine". He was the first person to observe remarkable similarity between cellulose and chitin. In 1859 chitosan, a partial deacetylated chitin was discovered by Rouget on boiling chitin in concentrated potassium hydroxide. It was finally named chitosan by Hoppe-Seiler (1894) but most information available now today has been obtained since 1950. The book "The Integument of Arthropods" by Richards (1951) gave thrust on chitin while Tracey (1957) reviewed the structure and detection and quantity analysis of chitin. Jeuniaux (1963) published a book on chitin and its enzymatic breakdown and in 1964 Brima Combe and Webber wrote a monogram on chitin. In 1967, Rudall first addressed the concept of chitin-protein complex which opened the door for additional work on the subject (Brine, 1984). A bibliography on chitin and its derivatives was published by Pariser and Boch (1972).

Resources

The major sources of chitin are shell fish, krill, clam, oysters, squid fungi and insects. Allan *et al.* (1978) quantified various sources of chitin (Table 1). Kong (1975) and Naczka *et al.*, (1981) estimated the chitin content of selected crustacean, insects, molluscan organs and fungi. Though the main source of chitin

is animals it is frequently present as cell wall material in plants replacing cellulose or sometimes occurring together cellulose. This polymer in the deacetylated form, *i.e.* chitosan is present in various fungi eg. Zygomycetes contain both chitin and chitosan (Austin *et al.*, 1981; Rudall, 1969)

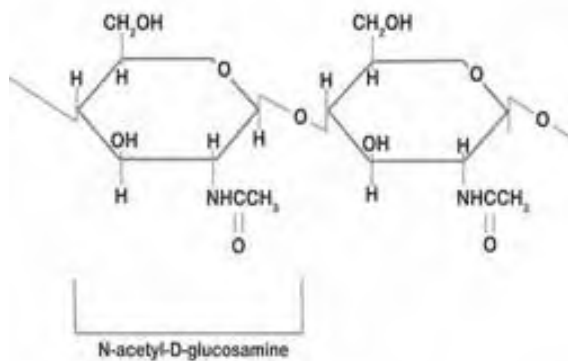
Table 1: Annual availability (global) of chitinaceous materials

Resources	Quantity harvested (103 tonnes)	Chitonous waste (103 tonnes)	Chitin potentials (103 tonnes)
Shell fish	1700	468	39
Krill (potential landing)	18200	3640	56
Clam/Oysters	1390	521	22
Squid	660	99	01
Fungi	790	790	32
Total	22740	5118	150

The utilizable resources of Antarctic krill 1×10^8 to 5×10^8 tonnes (Naczka, *et al.*, 1981) with chitin content of 0.33 to 1.74% (Sikrosky, *et al.*, 1980). Marine benthic organisms yield substantial amounts of chitin (Jeuniaux, 1978) marine surface zoo planktons are valuable source of chitin with a mean production of about 1 gm/m.sq/year which is higher than krill chitin production. Chitin production is the highest in eutropic fresh water ecosystems. Fresh water bryozans are able to produce colonies whose envelope is made of chitin and protein in a non-calcified form allowing easy extraction of chitin (Jeuniaux, *et al.*, 1989).

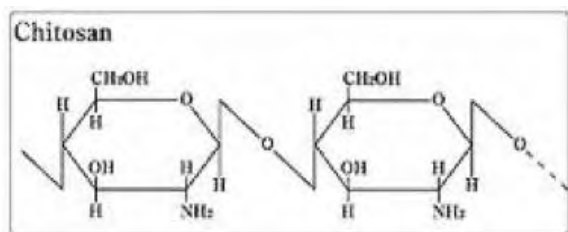
Structure

Chitin occurs in three polymorphic forms which differ in the arrangement of molecular chain within the crystal cell. α -chitin the tightly compacted most crystalline polymorphic form where the chains are



Chitin is a polymer of β (1-4)-N-acetyl- D- glucosamine (Fig.1).

arranged in an anti-parallel fashion, β - chitin is the form where the chains are parallel and γ - chitin is the form where the chains are “up” to everyone “down” (Muzzrelli, 1977a). Deacetylation of chitin with strong alkali yields chitosan, polymer of β (1-4)-D glucosamine (Fig.2).



Properties

Most commercial poly saccharides eg. Cellulose, dextran, pectin, alginc acid, agar, agarose, carrageenans and heparin are neutral or acidic. Chitin and chitosan are the only abundant basic polysaccharides. Their unique properties include solubility behavior in various media, solution viscosity, polyelectrolytic behavior, polyoxysalt formation, ability to form films, chelate metal ions and optical and structural characteristics (Austin *et al.* 1981). Although β -1-4 anhydro glucosidic bond of chitin is also present in cellulose, characteristic properties of chitin/chitosan are not shared by cellulose (Muzzrelli, 1978).

Chitin is highly hydrophobic and insoluble in water and most organic solvents. It is soluble in hexafluoro isopropanol, hexafluoro acetone (Capaozza 1975), Chlor-alcohols in conjunction with aqueous solution

of mineral acids (Austin, 1975) and dimethyl acctamide containing 5% lithium chloride (Rutherford and Austin, 1978). On hydrolysis of chitin with concentrated acids under drastic conditions gives relatively pure amino sugar D-glucosamine.

On deacetylation with the strong alkali yields the free base chitosan. Chitosan is insoluble in water but soluble in dilute acid. Pure chitosan is not hydrolysed by lysozyme while chitin or partially deacetylated chitin is hydrolysed. For hydrolysis to occur it is important that at least 6 contiguous actamido side groups should be present in the substrate. The probability of having 6 contiguous residues with the necessary acetamido side groups decreases as the deacetylation increases (Pangburn *et al.*, 1984).

Rutherford and Austin (1978) compared the molecular weight derived from intrinsic viscosities of chitin from crab and shrimp and found molecular weight from 0.4×10^6 to 1.8×10^6 for chitin. Anon (1991) obtained a molecular weight of 1.0 to 1.50×10^5 for chitin produced from Indian prawns.

The acetylated amino groups form hydrogen bonds which prevent swelling and dissolution in aqueous media. Depending on extent of deacetylation chitin contains 5 to 8 % nitrogen, which in chitosan in the form of primary aliphatic amino group. Chitosan undergoes the reactions typical of amines, of which N-acylation and schiff reactions are the most important. Chitosan derivatives are easily obtained under mild conditions and can be considered as substituted glucans.

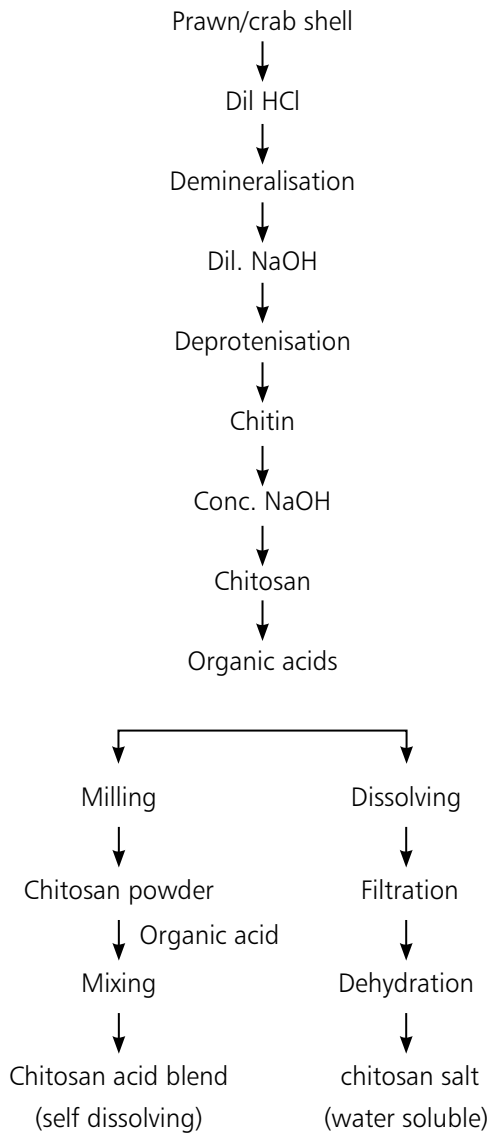
The properties of chitin and chitosan vary considerably depending on the source and production process. The quality requirements of chitosan and its derivatives vary with the end use.

Production

Chitin represents 14-27% and 13-15% of the dry weight of shrimp and crab processing waste respectively (Ashford *et al.*, 1977). Madhavan and Nair (1975) found that dry prawn waste contained 23% and dry squilla contained 15% chitin. Chitin is present as chitin protein complex along with minerals

mainly calcium carbonate. So the process of chitin production consists of deproteinisation with dilute alkali and demineralization with dil. acids (Fig. 3). The chitin thus obtained is deacetylated to chitosan using conc. alkali. Chitin and chitosan are now produced commercially in Japan, USA, India, China, Poland, Indonesia, Norway and Australia

Fig. 3: Production of chitosan from prawn/crab shells



Applications of chitin and chitosan

Chitosan is a versatile polymer and interest in chitosan is due to the large variety of useful forms that are

commercially available or can be made available (Ravikumar, *et al.*, 2004). The five international conferences on chitin and chitosan (1977, 1982, 1985, 1988 and 1991) have thrown light on various application in various fields. These applications can be classified under the following heads. Clarification and purification

1. Chromatography
2. Paper and textiles for photography
3. Food and nutrition
4. Medical and pharmaceuticals
5. Agriculture

Agriculture

Chitin and its derivatives have potential applications in agriculture for various uses such as germination and culturing to enhance self-protection against pathogenic organisms in plants and suppress them in soil to induce chitinase activity and proteinase inhibitor synthesis for antivirus activity, in encapsulation of fertilizers, in liquid fertilizers and controlled release of herbicides.

A thin coating of chitosan or its derivatives in seeds of radish or soyabean, rice and black pine induce and/or enhance chitinase activity which help the seeds to germinate with little infection (Hirano *et al.* 1984). The water holding capacity of soil was increased by mixing N-methyl chitosan gels with it and found that radish seedling could survive for 4 days after water was stopped (Hirano *et al.*, 1984). Chitinous materials are efficient to control parasitic nematodes in ornamental plants, in cucumber and tomato (Brown *et al.*, 1989). Chitosan has found application in pesticides and herbicides because of its sustained release property (Mc Cormick and Anderson, 1984). The chitosan is used in liquid fertilizers to reduce drying rate, controlled nutrient release and to reduce phyto-toxic affect (Struszyk, *et al.*, 1989). It can also be incorporated in foliar sprays for fixing of nutrients, slow release and to minimize moisture loss.

Activation of plants cells with chitosan and its derivatives may relate to (a) the induction and chitinase and chitosanase (b) an increase in the callus growth of cabbage leaves and (c) a yield increase in the field

culture of Japanese radish plants. The growth and chitinase activity of the callus increased in the presence of low molecular weight chitosan and chitosan oligosaccharides and the specific activity of chitinase also increased. This strongly suggests an increase in the protein synthesis in the presence of chitosan and its derivatives. Several investigators reported the induction of chitinase and chitosanase activities in entomorphogenic fungi with the degradation products of chitin and chitosan (St. Lager *et al.*, 1986). Silverstre and Tosti (2010) reported the efficiency of chitosan in protection of plants against diseases. It has also been reported by Boonlenthirum *et al.*, (2008) that rice production could be increased by foliar spray of chitosan which tended to show ability in disease control. (Uthairantnakij *et al.*, 2009) found that chitosan has improved orchid production and quality.

Conclusion

Many products are marketed based on chitosan for plant protection, growth promotion, seed coating etc., but these applications have not put in to large scale application so far. The shelf life of vegetables and fruits also can be extended with the application of chitosan coating. With the growing awareness of the adverse effects of hazardous chemicals in agriculture and popularization of organic farming, chitosan products will find use. Antivirus, antibacterial, nematocidal, insecticidal and pesticidal properties of chitin and chitosan have to be taken to the field by researchers to ensure safety of agriculture products.

Suggested readings

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Nutrition

Nutrient Requirement of Cultivable Brackishwater Fish and Aqua Feed Processing Techniques

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Introduction

Aquaculture farming have shown phenomenal growth in the last decade in India producing protein rich health food and earning valuable foreign exchange. Feed is a major input in fish farming. The development of nutritionally balanced feed involves understanding the dietary requirements of candidate species, selection of feed ingredients, formulation of feeds and appropriate processing technology for producing water stable pellet feeds. Depending upon the type of farming, a wide range of feeds are used for feeding stocked shrimp and fish. While no feed is used in traditional farming systems, supplementary and balanced feeds are used in extensive and semiintensive aquaculture.

All animals including fish requires food to supply the energy that they need for movement and all the other activities that they engage in for growth. However, they are 'cold-blooded' and as their body temperature is the same as the water they live in, they do not therefore need to consume energy to maintain a steady body temperature and they tend to be more efficient users of food than other farm animals. The nutrient requirement of different species of finfish vary in quantity and quality according to the nature of the animal, its feeding habits, size, its environment and reproductive state.

Fish diet should have adequate energy, not only to meet the needs of body maintenance called basal metabolism, but also for growth. In nature shrimp

and fish feeds on a variety of food items and derive their balanced nutrition for healthy growth. When they are cultured in confined pond they should be provided with a balanced diet as close to natural food as possible. This is the reason for understanding the nutritional requirement of candidate species which assumes paramount importance in developing the feeds for the candidate species.

Nutritional requirement of different brackishwater species

Protein

Protein is the most important nutrient in the diet of shrimp and fish. Protein requirement of aquatic organism is higher than terrestrial animals. Fish require food protein in the form of essential amino acids for maintenance of life, growth and reproduction and the requirement of protein depends on animal characteristics *i.e.*, species, physiological stages, size as well as dietary characteristics, *i.e.*, protein quality (digestibility and biological value), energy level etc. Scarcity of carbohydrate and abundance of protein and lipid in the natural aquatic food web is also probably responsible for the common trend of aquatic organisms to use protein as an energy source.

Protein is required in the diet to provide indispensable amino acids and nitrogen for synthesis of non-indispensable amino acids. A deficiency of

indispensable amino acid creates poor utilization of dietary protein and hence growth retardation, poor live weight gain and feed efficiency. In severe cases, deficiency reduces the ability to resist diseases and lowers the effectiveness of the immune response mechanism. Experiments have shown that tryptophan deficient fish become scoliotic, showing curvature of the spine, and methionine deficiency produces lens cataracts.

Protein requirement vary with the age of the fish. Younger animal generally require higher levels of protein (5-10% more protein) than older animals. Carnivores require high dietary protein (40-50%) than omnivores (25-35%). Among the brackishwater finfishes, requirement of protein for Asian seabass (*Lates calcarifer*), milkfish (*Chanos chanos*) and mullet (*Mugil cephalus*) is 40-45%, 40% and 27-35%, respectively.

Amino acids

The growth of fish is directly related to the quality of protein in terms of amino acids. After digestion of protein, amino acids are metabolized at tissue level to form new proteins for growth, maintenance and energy. Among 25 amino acids present in protein 10 amino acids must be supplied in the diet since fish cannot synthesize them and termed as essential amino acids (EAA). These are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. A large proportion of the amino acid consumed by a fish are catabolized for energy and fish are well adapted to using an excess energy in this way. It is found that if the amino acid composition of the protein in the feed matches with the amino acid composition of shrimp body tissue, such feed promotes good growth.

Table 1. Nutrient requirement of different brackishwater fishes

Nutrient	<i>L. calcarifer</i>	<i>Mugil cephalus/ Liza tade</i>	<i>Etroplus suratensis</i>
Energy (Kcal/kg)	4000-4500	4000-4500	4000-4500
Protein %	45-55	27-35	30-32
Lipid %	6-18	6-9	6-8
Carbohydrate %	10-20	30-40	30-40

Lipid

Lipid is a complex mixture of simple fat, phospholipids, steroids, fatty acids and other fat soluble substances such as pigments, vitamins A, D, E and K. Apart from its major role to supply energy lipid also act as precursors to many reactive substances. Phospholipids are responsible for the structure of cell membranes (lipid bi-layer). Fatty acids are the main active components of dietary lipids. Deficiency of essential fatty acid result in general reduction of growth and a number of deficiency signs including depigmentation, fin erosion, cardiac myopathy, fatty infiltration of liver and 'shock syndrome' (loss of consciousness for a few seconds following an acute stress). Fat levels of 6-8% are adequate in most of the fish diets. However, the quality of fat in terms of fatty acids is more important. Carnivorous fish such as seabass can utilize lipids more effectively and lipid level as high as 20% can be used in their diet. However, lipid level should be adjusted in diet considering the technological problems in feed manufacture and storage. Fish oil and soya oil are generally used as lipid source during feed formulation.

Fatty acids

Fish and shrimps are unable to synthesize fatty acids of the n-3 and n-6 series and must be provided in their diets. Aquatic animals require higher n-3 fatty acids than terrestrial animals. Among aquatic animals, marine habitat requires more HUFA than freshwater counterparts. Among the long chain fatty acids polyunsaturated fatty acids (PUFA) such as linoleic acid (18:2n6), linolenic acid (18:3n3), eicosapentaenoic acid (20:5n3) (EPA) and docosahexaenoic acid (22:6n3) (DHA) are essential for growth, survival and good feed conversion ratio. The n3 fatty acids are more essential than the n6 acids. The fatty acids, EPA and DHA, which are known as highly unsaturated fatty acids (HUFA) of n3 series, are particularly important. Quantitatively EPA and DHA are needed at 0.5% and 1.0% in the diet of larvae and fry of brackishwater fish. Fresh water fish show requirement for n6 and n3 essential fatty acids (EFA), whereas marine fish show requirement of n3 and also HUFA.

Phospholipids

Fish require phospholipids for growth, metamorphosis and maturation. Lipids of squid, clam, shrimp, fish and polychaetes are excellent natural source of phospholipids. The phospholipid phosphatidylcholine (lecithin) is essentially required in the diet of larval and fry stages of fish for fast growth and good survival. Soya lecithin is a good source of phospholipid. It is required at 1-2% level in the diet. The development and survival of larvae is significantly improved when the diet contains lecithin.

Carbohydrate

Carbohydrate is an inexpensive source of energy in fish diet. Among the different types of carbohydrates available, fish are found to utilize disaccharide and polysaccharide better than monosaccharide. Omnivorous fishes have enzymes to digest carbohydrates while carnivorous fishes have poor ability to digest carbohydrate. Polysaccharides are better utilized than monosaccharide. Generally carbohydrate utilization by fish is found to be lower than that of terrestrial animals. Fish can utilize dietary carbohydrate up to 40%. For carnivorous fish carbohydrate level in the diet may be in the range of 10-20 %. Depending upon the total energy content required in the diet, carbohydrate can be used from 10-40% level. Using starch as source of carbohydrate in diet has dual advantage. Besides being energy source, it can act as binder if gelatinized by cooking with moisture and hence improve water stability of diet. Corn flour, wheat flour, tapioca flour and other grain flours are good source of starch in shrimp and fish diet. Another polysaccharide, cellulose is required in the diet as roughage for improving the feed efficiency in fish.

Mineral requirement

Micronutrient such as vitamins and minerals significantly influence the growth and survival of fish and these cannot be synthesized by these organisms.

Fish can absorb minerals directly from aquatic environment through gills and body surfaces or by drinking. Hence, dietary requirement of minerals is

largely dependent on the mineral concentration of the aquatic environment. About 20 inorganic elements (macro and micro) are required to meet the metabolic and structural functions in the body of animals. The aquatic organisms regulate the mineral needs through dietary source and also through internal regulatory mechanisms in the kidneys and gills. In saline water, calcium (Ca) is abundant, which is absorbed by most aquatic animals. Since the availability of phosphorus (P) through water medium is poor, P should be made available through diet. Usually the preferred Ca:P ratio is 1:1 in feeds of aquatic species. Mono and dicalcium phosphate contain more available P than tricalcium phosphate. Incorporation of P should be very discrete in fish feeds, as most of it gets excreted leading to eutrophication. The dietary requirement of P ranges from 0.5-0.9% in fishes. The requirement of magnesium (Mg) in fish ranges between 0.04-0.3%. The requirement of zinc (Zn) ranges from 15-30 mg/kg diet for fishes. The requirement of iron (Fe) ranges from 150-200 mg/kg diet for fishes. Major deficiency symptoms of manganese (Mn) in fishes are cataracts and abnormal curvature of the backbone and malformation of tail. A dietary supplementation of 11-13 mg/kg restores normal growth in fishes.

Trace minerals like copper (Cu), cobalt (Co), selenium (Se), iodine (I) and chromium (Cr) have some role in general upkeep of the organism. Their dietary incorporation enhances growth and survival.

Vitamin requirement

Micronutrient such as vitamins and mineral significantly influence the growth and survival of fish and this cannot be synthesized by these organisms. Even though, some vitamin such as niacin can be synthesized by number of animals but are typically insufficient to meet physiological demand. Hence, supplementation of vitamins in feed becomes necessary for most of the aquatic organisms. Unlike domestic higher animals, the recommended doses of vitamin for aquatic animals are higher, as many vitamins are lost during the process of feed manufacture and also due to leaching. Destruction of vitamin - C due to oxidation is one of the biggest problem during feed manufacture. Many fishes cannot synthesize Vitamin - C from glucose due to

absence of enzyme L-gulonolactone oxidase. Major role of vit C is in the formation and maintenance of intracellular material having collagen or related basal

Table 3. Vitamin requirement of fish

Vitamin (mg/kg)	Fish
Thiamin	10
Riboflavin	20
Pyridoxine	10
Pantothenic acid	40
Niacin	150
Folic acid	5
Vit B12	0.1
Choline	3000
Inositol	400
Vit C	100
Vit E	30
Vit A (IU)	2500
Vit D (IU)	2400
Vit K	10

constituents in bones and in soft tissues. Among the 11 water soluble vitamins, three (vit C, inositol and choline) are required in large quantities. Sources of choline include cottonseed meal, fish meal, shrimp meal, soyabean meal and yeast. Stable form of vit C is available commercially.

Feed Processing

One of the important factors that determine the final quality of feed is the adoption of appropriate processing technology. With the best of machinery at the disposal, working out right combination of various factors in processing and standardizing them would only lead to production of feed of desired water stability. The following are the steps involved in processing of aquaculture feeds.

Processing of feed ingredients

The quality of feed ingredients has an important bearing on the quality of final feed. Feed ingredients should be fresh and confirm to the nutrient quality. Contamination with foreign matter, especially, sand, stones and earthen materials will affect the quality of the materials. Old stock of oil cakes may contain aflatoxin, while PUFA rich fish oils are oxidized leading to rancidity. Quality control of the raw materials should be done at the time of their procurement itself, to ensure the quality

of final feed. All the solid ingredients are procured in dry form with moisture levels preferably below 10%; otherwise the materials may be subjected to drying before they are processed.

Grinding

Pregrinding of solid ingredients to uniform particle size is essential for making homogenous mixture of a compounded feed. Fine powdering of materials increases the surface area and improves the digestibility besides helping in making compact pellets. Materials such as dry fish, prawn head waste, squilla and squid are subjected to two stage grinding process. First, size reduction, by passing through a hammer mill. In this the materials are roughly powdered so that they can be further powdered to finer particles. Subsequently these coarse materials are further powdered to fine particle size in a micropulverizer. Different kinds of grinding machines such as hammer mill, pulverizer, flour mill and impact pulverizers are employed for grinding feed ingredients.

Sieving

The powdered ingredients are passed through a standard mesh sieve for obtaining the desired particle size. In case the grinding equipment does not have an inbuilt sieving mechanism, the materials should be subjected to sieving. Feed materials that are commercially available in fine powder form may also be sieved to screen the presence of extraneous materials and metal pieces, which might otherwise inadvertently enter the pelleting equipment and cause damage. Sieving the ingredients helps in preparing feed pellets with uniform and attractive physical appearance.

Vibrating or gyratory type of sieve assemblies are available which are generally employed for sieving feed materials.

Mixing

The powdered ingredients after weighing according to the formulation are mixed together and homogenized into a feed mixture. The liquid materials such as fish oil may be added at the

end and further homogenized. Materials, which are heat sensitive and get destroyed, may not be added in the feed mix at this stage. Water required for increasing the moisture may also be added. Binders, which need mixing with water, should also be incorporated at this stage. Horizontal or vertical types of batch mixtures are employed for mixing feeds. For proper mixing of different feed ingredients into a homogeneous mass, the mixing time will vary based on the homogeneity of the material and formulations.

Feed Production

The final form of the feed is produced in the form of pellets. For shrimp compact sinking pellets are produced. For finfish floating pellet feeds are preferred even though sinking pellets are equally good for fish. However, for fish species such as Asian sea bass that feed the moving or live prey floating pellets are more desirable. The following technologies are for commercial production of pellet feeds.

Pelletization

Pelletization is a process in which the feed mixture is compacted into predesigned cylindrical pellets. Pelletization is done mainly using two types of machines namely, extruder and pelletizer.

Extruder technology

The basic components in an extruder are a barrel fitted with a die plate and a screw shaft conveyer, which is connected to a high-speed motor. The feed mixture is fed into an extruder by proper arrangement of water/steam injection facility. The extruder operates at high pressure (1498kg/cm²) and steam (Pressure 5-7kg/cm²) injection. Depending upon the characteristics of the feed mixture and moisture content, the pressure develops before the material passes through the die. Because of this the temperature rises and the material is forced through the die and the pressure suddenly drops. The temperature of the material rises to 110-130°C for a short spell of time and cooks the food, gelatinizing the starch present in the feed mixture. This imparts good binding and

water stability to the resultant pellets. However, the pellets expand as they come out of the die due to sudden drop of pressure and air gaps develop inside the pellet, which makes them float or sink very slowly. This is an excellent process for producing floating pellets for finfish culture. By adjusting the pressure in the barrel and moisture in the feed, it is possible to prepare sinking pellets by extruder. The new generation extruders are made with twin screwbarrel arrangement, which are more suited for sinking pellet feed manufacture. The resultant pellets are dried as the moisture will be high.

Pelletizer technology

Pelletizer is primarily used for making sinking pellets. The basic principle of pelletizer is that the finely ground feed mixture is pelleted by compression process. The main components are a pair of rollers and a die, which are driven by a high-speed motor. The pelletizer works with a combination of high pressure (42-1800kg/cm²) between rollers and the die, steam (0.5-3.5kg/cm²) and moderate temperature (75-95°C). Moisture is the limiting factor in the pelletizer. It works satisfactorily at 15% moisture and higher moisture levels choke the die. Because of this reason starch present in feed cannot be fully gelatinized for binding. Hence, additional binder, which works on the principle of thermo plasticity, has to be used. Conditions for proper reaction between binder and feed ingredients during pelleting should be standardized. Several steam conditioners in series are used to prolong contact between steam and ingredients for producing pellets with good water stability.

Wet pelletizer

For small and laboratory scale production of feed pellets, wet pelletizer which can work with high moisture levels (30%) is used. This is similar to noodle or spagatti making machine. Moist pellet can be successfully produced in this machine. Feed mixture is wetted with water (30%) and steam cooked in batches and passed through the wet pelletizer. Starch is well gelatinized and acts as an effective binder in this process. Because of higher moisture content, the pellets should be dried for longer period.

Drying

After pelleting, the feed should be dried to reduce the moisture content below 10%. This is essential for good shelflife of the feed. Different types of dryers are used for drying feed pellets. There are horizontal conveyer type, vertical hopper type and fluid bed dryers. Dry steam or hot air (heated either electrically or otherwise) is used for drying feed at temperatures 70-80°C. Higher temperature is not desirable. Feed pellets with low moisture obtained through a pelletiser should be dried in cooler dryer.

Conclusion

Nutrient requirements are the basic points on which the feed formulations are drawn and hence to develop a feed for any species understanding the nutrient requirement is of prime significance. The final feed quality is based on all the processing steps and hence all the machineries has to be suitably selected and the processing has to be carried out as per the requirement of finished feed. CIBA has developed feed processing technology for shrimp and fish and commercialized these technologies. The knowhow of the technology for commercial production can be obtained from CIBA on agreed terms and conditions.

Shrimp Nutrition

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Introduction

Aquaculture have shown phenomenal growth in the last three decades in India producing protein rich healthy food and earning valuable foreign exchange. Shrimp farming is a major component of aquaculture in coastal states of India. In exports of seafood's from India, farmed shrimp continued to be the major value item. Feed is a major input in shrimp farming which accounts about 50 to 60% of the production cost. The development of nutritionally balanced feed involves understanding the dietary requirements of candidate species, selection of feed ingredients, formulation of feeds and appropriate processing technology for producing water stable pellet feeds. Depending upon the type of farming, a wide range of feeds are being used for feeding shrimp and fish. While no feed is used in traditional farming systems, supplementary and balanced feeds are used in extensive and semiintensive aquaculture.

Whiteleg shrimp (*Litopenaeus vannamei*, formerly *Penaeus vannamei*), also known as Pacific white shrimp, is a variety of shrimp of the eastern Pacific Ocean native to the eastern Pacific, from Sonora in Mexico to northern Peru. The main sources of whiteleg shrimp are Thailand, Indonesia, Vietnam, Ecuador, Mexico, Brazil and India. Shrimp farming is now synonyms with *L. vannamei* culture. Since, it is endowed with many positive traits for current farming, it can readily accept low protein and low fishmeal diet, trouble-free seed production, suitable for intensive and super intensive farming, resistant to wide range soil and water quality fluctuation, increased disease resistance through SPF and good customer acceptance.

Nutrition and feeding of white shrimp have received a great deal of attention over the last 20 years.

Scientists, farmers, feed manufacturers, as well as suppliers of feed ingredients, all contributed to the better understanding of feeding shrimps raised under semi-intensive or intensive conditions.

Feeding behavior of shrimp

L. vannamei is an omnivorous scavenger, feeds voraciously on organic detritus and benthos (copepods, polychaeta, ostracods, nematodes and insects). Since, it can graze/nibble on microbial flocs, plankton sediments and faeces very effectively than *Penaeus monodon*, gives better feed conversion efficiency, improved water quality parameters and minimized organic load. Nutrients inputs and stocking densities are directly proportional. During high stocking density maintaining good water quality is real challenge; it requires feeds with high cohesive property, high digestible protein, optimal essential amino acids and fatty acids.

Significance of formulated feed for white shrimp

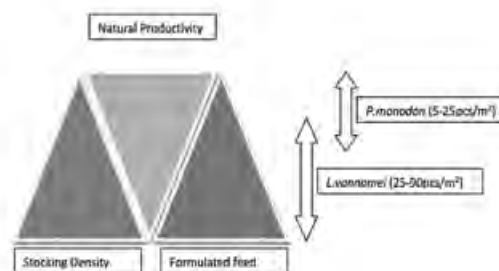


Figure: 1. Schematic demonstration of significance of formulated feeds with stocking density and natural productivity

In Indian conditions, different levels of stocking densities are being practiced 20 to 60 pcs/m², intended at 10-40t/ha/yr biomass production. Feed represents 40 to 60% of total production cost; hence, a range of feed formulations must be aimed to produce most economical and nutritionally rational as well. Currently essential nutrients and their digestibility and utilization are best known and with the recent advancement in analytical and experimental facilities in nutrition, feeds can be more precisely formulated to expand the industry further.

Protein nutrition

This is the most expensive and major nutrient in shrimp feed. Proteins are primary nutrient for the structure and function of shrimp growth and survival as in all living organisms. Since proteins are continually being used by the animal for growth and repair of tissues, a continuous supply of proteins or its constituent amino acids is considered obligatory. Protein is a major and the expensive component of formulated aqua feeds. Hence, nutritional studies of shrimp often start with investigating the optimal dietary protein level. As a consequence, the most researched nutrient in terms of the number of penaeid shrimp species being studied is proteins. Protein requirement varies from 18-50% under different stocking densities and primary productivity. Ideally, 32% crude protein in diets found to very effect for white shrimps at varied stocking densities. 32% protein diet was found to induce superior growth in juvenile and sub adult *L. vannamei* as compared to 16% and 48% protein diets. However, the 48% protein diet had higher Feed Efficiency (FE) on an isonitrogenous basis feeding. The lower weight gain, which resulted from feeding the diet containing 48% protein, is possibly due to the low energy to protein ratio of the diet, which would cause shrimp to utilize protein as a source of

Table: 1. Protein requirement for white shrimp

Dietary crude protein	Reference
15	Aranyakananda, 1993
25	Velasco <i>et al.</i> , 2000
30	Cousin <i>et al.</i> 1993; Colvin and Brand, 1977
32	Kureshy and Davis 2000
36	Smith <i>et al.</i> 1985
40-55	Sturmer <i>et al.</i> 1992; Treece and Fox, 1993

energy. Using protein as an energy source is relatively inefficient as compared with lipids and would reduce the amount of protein available for tissue deposition.

Among all penaeid shrimps, white shrimps utilize plant protein very effectively and nearly 2/3rd of dietary protein comes from plant by products. Plant proteins includes soya bean meal, rapeseed meal, ground nut/peanut meal, wheat gluten and corn gluten are widely used as source of protein in India. Protein that is utilized for energy and not deposited for growth contributes to the release of nitrogen metabolites into the culture medium. According to Wu (1995), Nitrogenous wastes are dietary in origin with estimates of up to 52-95% of feed nitrogen being excreted as waste, depending on the species and the diet; thereby high protein and imbalanced amino acid diet leads to environmental deterioration. Adequate dietary intake of high-quality protein is required to support rapid growth of fish and crustaceans, but shrimp do not have a dietary requirement for protein per se, but rather for amino acids.

Table: 2. Commonly used ingredients in white shrimps feed

Protein sources	Inclusion levels (%)
Fish meal	10-25
Squid meal	5-10
Crustacean meal (<i>Acetes spp.</i>)	5-10
Soya bean meal	25-50
Peanut meal	5-10
Rapeseed meal	5-10
Corn gluten meal	5-8
Wheat gluten	3-5
Yeast biomass	3-5
Energy sources	
Wheat	15-35
Broken Rice	10-20
Wheat bran	3-5
Rice bran	5-10
Lipid sources	
Fish oil	1-3
Squid oil	1-3
Soya lecithin	1-3
Cholesterol	0.15
Plant oil	1-3

Amino acid requirements

Optimal dietary amino acid profile will depend

Table 3: List of potential feed ingredients for use in commercial shrimp feeds in near future

	Moisture	Crude Protein	Crude Fat	Crude Fibre	Crude Ash
Protein sources of plant origin					
Mustard meal	10.15	32.2	8.9	8.1	9.2
Coconut meal	8.7	21.5	3.5	14.8	7.1
Cottonseed meal	10.0	32.9	1.7	21.8	6.0
Soy protein concentrate	7	59	5.4	1.5	7.9
Corn protein concentrate	10	76.2	4.5	1	1.3
Distillers dried grains with solubles (DDGS)	8.2	28.4	8.5	9.4	4.9
Corn gluten meal	8.6	56.1	4	2.9	2.1
Animal meal by-products of marine origin					
Fish soluble	7.4	55.9	6.5	3.2	12.6
Shrimp head meal	8.8	46.6	6.4	11.1	26.5
Crab meal	7.1	33.9	2.8	10.7	41.9
Squid viscera meal	10.3	50.3	18.6	1.5	9.8
Animal meals by-products of terrestrial origin					
Blood meal	9.0	85.5	1.4	0.9	5.3
Feather meal	8.4	84.0	4.2	1.0	3.6
Meat and bone meal	7.5	50.1	10.6	2.4	28.8
Poultry by product meal	7.4	59.0	12.4	2.6	15.3
Terrestrial invertebrate meals					
Silkworm pupae meal	11.1	55.1	23.2	5.5	3.8
Maggot meal	3.0	43.2	23.0	1.0	16.2
Earthworm meal	7.4	56.4	7.8	1.6	8.8
Polychaete worm meal	8.0	55.0	15.0	1.0	12.0
Protein sources of microbial origin					
Brewer's yeast	7.6	46.1	1.3	2.9	34.0
Fungal biomass	8.5	44.4	9.4	16.9	16.1
Spirulina	6.4	62.1	4.8	0.5	17.3
Chlorella meal (micro algae)	5.7	47.2	7.4	8.3	20.8
Methanol Substrate	6.4	73.1	5.7	0.4	2.7

on the amino acid requirement of an animal for protein synthesis and the use of individual amino acids as energy substrates or for other purposes. The amino acid composition of the food should be very similar to that of the animal's proteins. *Mente et al.*, (2002) found notable differences between the FAA concentrations in whole animal and in tail-muscle tissue. Tryptophan is a candidate amino acid that limits the rate of protein synthesis, *Forster et al.* (2002) reported that 50% of the fish meal could be replaced in the diet of *L. vannamei* with soy protein concentrate supplementation

with lysine alone, could be further substituted up to 75% level supplementation with arginine, methionine, and phenylalanine.

Lipid nutrition

Lipids constitute a broad group of naturally occurring molecules which include fats, waxes, sterols, fat-soluble vitamins (such as vitamins A, D, E and K), monoglycerides, diglycerides, phospholipids, and others. Dietary lipids are a highly digestible and concentrated source of energy which supply 9 kcal/g,

about double of that supplied by either carbohydrate or protein. The main biological functions of lipids include energy storage, as structural components of cell membranes, and as important signaling molecules. Although penaeid shrimp, like other animals use various biosynthetic pathways to both break down and synthesize lipids, some essential lipids cannot be made this way and must be obtained from the diet.

It has been demonstrated that shrimp have a limited ability to synthesize *de novo* the n-6 and n-3 families of fatty acids (FA), including the polyunsaturated linoleic (18:2n-6, LOA) and linolenic (18:3n-3, LNA) acids. They also have a limited ability to elongate and desaturate these polyunsaturated fatty acids (PUFA) to highly unsaturated fatty acids (HUFA) such as arachidonic (20:4n-6, AA), eicosapentaenoic (20:5n-3, EPA) and docosahexaenoic (22:6n-3, DHA) acids. Consequently, these FA are considered EFA. For *F. chinensis*, Xu *et al.*, (1993) observed that n-3 or n-6 HUFA had greater value than PUFA of the same family following this trend: DHA>AA>LNA>LOA.

Phospholipid (PL)

The importance of phospholipids (PL) in penaeid shrimp nutrition, including *L. vannamei* (Boone), has been demonstrated by researchers González-Félix *et al.*, (2002a). Phospholipids are a class of lipids and are a major component of all cell membranes as they can form lipid bilayers. Most phospholipids contain a diglyceride, a phosphate group, and a simple organic molecule such as choline; one exception to this rule is sphingomyelin, which is derived from sphingosine instead of glycerol. The first phospholipid identified as such in biological tissues was lecithin, or phosphatidylcholine, in the egg yolk, by Theodore Nicolas Gobley, a French chemist and pharmacist, in 1847. They maintain cell structure and function, and have regulatory activities within the membrane and outside the cell. For instance, they serve as second messengers in cell signaling, an essential process in regulating cell growth, proliferation, differentiation, metabolism, nutrient uptake, ion transport, and even programmed cell death. In addition, there is evidence that PL containing choline, sphingomyelin, and their metabolites are important mediators

and modulators of transmembrane signaling. PL act as emulsifiers and facilitate the digestion and absorption of FA, bile salts and other lipid-soluble matters. They also have a role in the transport of lipids, not only in the transport of absorbed lipids from the gut epithelium into the hemolymph, but also in the transport of lipids between tissues and organs since they are constituents of lipoproteins. Shrimp growth increased with PL levels up to 3-5% of diet.

Carbohydrate nutrition

Carbohydrates are classified among simple (glucose, trehalose) and complex (starch, glycogen, chitin, cellulose) and the bulk of organic matter in the environment is provided by carbohydrates. With disaccharides, the α 1-4 bond is broken down by amylase, and glycosidic bond is much more resistant. Most animals do not have enzymes for α 1-4 bonds. Within polysaccharides, starch presents a combination of α 1-4 (amylose) and branched chains (α 1-6 amylopectin), glycogen, cellulose and chitin, all are formed from monosaccharide chain units. Carbohydrate is the cheapest source of energy. Digestibility will be moderate if it is processed well. Critical component of feed processing because, only under optimal moisture and temperature it gets gelatinized completely which gives natural binding to the pellets that makes pellets more durable and water stable.

Carbohydrates utilization by shrimp varies with carbohydrate complexity and processing procedures. These differences are represented by the range of digestible energy values reported for Pacific white shrimp, *L. vannamei*, for typical ingredients processed under different conditions: whole wheat 3,571-3,857 kcal/kg, corn flour 3,037-3,917 kcal/kg, rice flour 3,093-4173 kcal/kg, and milo/sorghum 2,821-3,785 kcal/kg. Although these values are considerably lower than those of lipids, which contain about twice the energy of carbohydrates and proteins, the cost per energy unit is much lower for carbohydrate sources. Among botanical origin we can cite potato, corn (several forms), wheat, cassava, sago palm, rice basically. Digestibility of starch from various botanical origins is given in Table 7. Starch is rich in amylose

and is poorly digestible compared to starch rich in amylopectin. Native potato starch is less digestible than pre-cooked one. Wheat native starch is well digestible (92%). Corn starch with 76-99% amylopectin is equally digestible.

Chitin

It is the major structural component of the exoskeleton of shrimps and one of the carbohydrates that shrimp meet in natural environment might be chitin. Minimum of 0.5 % chitin is recommended in shrimp feeds because dietary chitin has growth promoting effect (Akiyama *et al.* 1992). Glucosamine, a monomer of chitin, has inhibitory effect on growth by diminishing the growth-promoting effect of cholesterol.

Vitamin and Mineral nutrition

Vitamins are organic compounds required by shrimp in very small quantities to maintain essential functions. Quite often they function as coenzymes in chemical reactions and hence are described as the “spark plugs” of cells. The qualitative and quantitative vitamin requirements of shrimp have not received major attention. The difficulties in determining the vitamin requirements are exemplified by the wide variations in vitamin supplementation utilized by researchers and the commercial industry. As there is considerable debate regarding the absolute requirements for shrimp, vitamin values can be tempered with those established with other species. Shrimp feeds are generally supplemented with a vitamin premix in a sufficient quantity to exceed the estimated vitamin requirements, including losses due to feed processing. Levels above those required for maximum growth may facilitate the ability of shrimp to respond to stress. Feed processing and storage can degrade many of the vitamins, and due to the slow feeding habits of shrimp, considerable leaching can occur prior to consumption. As exemplified in studies, the use of vitamin premixes may not be required under some culture conditions if the animals have access to natural foods and there are no environmental stresses. This stands more appropriate for *L. vannamei*, which prefers to feed on bioflocs, an assemblage of microorganisms and

decaying organic matter suspended in the water column of the rearing system. But with fishes and other species shrimp, a wide variety of factors affect the quantity, quality, and accessibility of natural foods. The decision to reduce feed costs through the utilization of nutritionally incomplete feeds is a viable strategy. However, it is difficult to predict and manage the availability and nutritional quality of highly dynamic natural foods. Consequently, if nutritionally incomplete feed is used, farmers increase the risk of suboptimal production due to nutritional inadequacies.

Vitamin-C

The vitamin C is essentially required in the diet to preventing the development of the black death syndrome in penaeid shrimp. Recommended dietary ascorbic acid (AA) levels for shrimp using ascorbic acid-polyphosphate (ApP) are 20 and 120–130 mg/kg for the postlarvae of tiger shrimp, *Penaeus monodon* and white shrimp, *L. vannamei* (Boone), respectively. A significant effect was shown to be produced on stress sensitivity for both the species by high dietary AA levels, and moreover, *P. vannamei* displayed a higher resistance to *Vibrio harveyi* infection.

Minerals

A variety of studies with shrimp illustrate the nutritional importance of the inorganic components of the diet. The quantitative mineral requirements of penaeid shrimp have not been established, but are reasonably well studied. Practical diets generally contain a substantial amount of endogenous minerals. Consequently, complete mineral premixes are not necessary. With the exception of phosphorus, macro minerals are generally not supplemented to commercial feeds, as there is no evidence these minerals are required in feed under normal production conditions. Since many of the trace minerals can have low biological availability or are found at relatively low levels, a number of trace minerals are commonly supplemented. Phosphorus is the most costly and problematic mineral. Its biological availability varies with the source, with the water-soluble forms having higher availability to shrimp.

Feed additives

Free amino acids: used individually or in combinations to reduce dietary protein levels and nitrogen excretion, to overcome dietary amino acid deficiencies resulting from fish meal replacements, or as feeding stimulants

Feed enzymes: used individually or in combinations to increase carbohydrate and mineral digestibility and reduce nutrient (e.g. phosphorus) loss to the aquatic environment.

Chemoattractants and/or feeding stimulants: used to increase feed palatability and stimulate feed intake (especially in plant-based feeds with low levels of marine protein sources), increase growth (by minimizing the time the feed remains uneaten in water and thereby minimizing nutrient loss through leaching), and reduce feed wastage

Probiotics: live micro-organisms and/or their processed products are used as dietary supplements or added directly to the water to stabilize or enhance a healthful and appropriate microbial community in the gastrointestinal tract of the cultured shrimp and/or within the culture system, so as to improve growth, survival, and/or disease resistance. However, it is important to mention here that although good scientific evidence exists concerning the beneficial effects of probiotics on shrimp performance and health under clearwater hatchery conditions, this has not always been the case under practical pond grow-out conditions, where resident aquatic/sediment microbial flora already exists.

Immunostimulants: used to stimulate the nonspecific immune system mechanisms of shrimp and thus increase disease resistance

Miscellaneous: Other additives that can be used in shrimp feeds include antioxidants, mold inhibiting compounds, pigments, and to a lesser extent chemotherapeutants and hormones

Feeding Management

Feed management starts immediately after the

introduction of post larval shrimps. During first 30 days of culture (DOC), feeding based on assumption and majority of the feed goes as nutrient for planktons rather for shrimps; which in turn feed and shelter for post larval shrimps. Initially, culturing adequate planktons is critical for better survival, good health and also reducing size variations of the post larvae. Second month onwards daily feeding must be controlled by introducing feed trays/check trays since 85% of total feeding left. Minimum of 4 feed trays for one acre pond is recommended. The dimension of the feed trays is 80cmX80cmX10cm, and made up of

Table: 4. The recommended physical dimension for white shrimps

Name	Dimension	Shrimp size (g)	% feed required
Starter I	0.3-0.6 mm	0.02-0.5	1
Starter II	0.5-1.0 mm	0.5-2.0	3
Starter III	0.8-1.2 mm	2.0-5.0	8
Pregrower 4s	1.6 x 3-4 mm	5.0-15.0	30
Grower 4	1.8/2.0 x 4-5 mm	15.0-25.0	30
Finisher 4L	2.0/2.2 x 4-5 mm	25.0-harvest	28

steel frame with nylon mesh. Physical size of feed also very important in controlling feed conversion efficiency and reducing pollution.

The role of feed boy is very important for white shrimp farming. He should have adequate knowledge of demand feeding, indication of over/under feeding, moulting on feed consumption, effect of cold weather

Table: 5. The recommended protein and lipid levels for whiteleg shrimp

Shrimp size	Protein	Lipid	Energy	E:P
0.02-3.0	38-36	7-8	3550-3650	9.5
3.0-15.0	36-34	6-7	3450-3550	10.0
15.0-40	34-32	5-6	3350-3450	10.5

and other climatic conditions. He should not follow feeding guides all the time since, it is based on a rough theoretical calculations. Actual feeding may vary greatly with temperature, dissolved oxygen, plankton bloom (natural productivity), pond bottom and pollution.

Table: 6. Feeding guide

Day	ABW (g)	Feeding Rate (%)	Daily Feed (Kg)	ADG (g)	Feed Ration	% of feed per tray	Checking hours	Meals per day	Remarks		
1	0.01	100.00	1.0	0.02	1	Blind feeding - No trays	Check prawn health only	2 to 3	Feeding based on calculation		
5	0.09	11.11	1.0	0.05							
10	0.34	10.29	3.5	0.10							
15	0.84	7.14	6.0	0.12				2		0.4	3 to 4
20	1.4	6.60	9.5	0.15							
25	2.2	5.94	13.0	0.18							
30	3.1	5.25	16.2	0.20				3		0.5	
35	4.1	4.80	19.6	0.21							
40	5.1	4.40	22.6	0.22							
45	6.2	4.00	25.0	0.24				4 S		0.6	4 to 5
50	7.4	3.80	28.3	0.25							
55	8.7	3.60	31.3	0.24							
60	9.9	3.40	33.6	0.25				4		0.7	
65	11.1	3.20	35.6	0.26							
70	12.4	3.00	37.3	0.26							
75	13.7	2.80	38.5	0.26	4 L	0.8	5 to 6				
80	15.0	2.60	39.1	0.26							
85	16.3	2.40	39.2	0.26							
90	17.6	2.30	40.6	0.26	1.0	1:00					
95	18.9	2.20	41.7	0.26							
100	20.2	2.10	42.5	0.26							
105	21.5	2.00	43.1	0.26	1.0	1:00					
110	22.8	2.00	45.7	0.25							
115	24.1	1.90	45.8	0.24							
120	25.3	1.80	45.5	0.22	1.0	1:00					
125	26.4	1.80	47.5	0.20							
130	27.4	1.70	46.6	0.19							
135	28.3	1.70	48.2	0.18	1.0	1:00					
140	29.2	1.70	49.7	0.17							
145	30.1	1.60	48.1	0.16							
150	30.9	1.60	49.4	0.15	1.0	1:00	5 to 6	Feeding based on meticulous feeding tray checking			

* Feed table is for 100000pcs, assuming stocking at 10pcs/m2

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Broodstock Feeds and Nutrition

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Introduction

In many cultured fish species the knowledge on nutritional requirement of broodstock is very scanty. This is mainly because the broodstock nutrition study requires huge facilities to keep considerably extensive group of large brood fish and also requires high cost for construction and maintenance of these facilities. And also, it is important to carry out long term feeding experiments on brood fish. This is the underlying reality in the broodstock nutrition studies. Improvement in the nutritional condition of broodstock diet have significant effects on the time of first maturity, number of eggs produced, egg size and egg quality as measured by chemical composition, hatchability and larval survival. Hence, the dietary supplementation of broodstock with essential amino acids, fatty acids, vitamins, minerals and carotenoids will produce desirable effects of egg and larval quality.

Preferred egg and larval quality

The broodstock performance is assessed in terms of quality and quantity of eggs and larvae produced by the brood fish. In a hatchery operation, the brood fish that produce more number of eggs per kilogram of body weight during the spawning cycle is most preferred. Good quality eggs are those exhibit low levels of mortality at fertilization, hatch and first feeding and produce fastest growing healthiest fry and adult fish. Egg quality in nutritional studies are indicated by fertilization rate, survival during egg stage, cleavage symmetry during 8 and 32 cell stage, blastomere morphology and hatching rate and later rearing success. The ovulated egg contains genetic code for new individual and also maternal

RNA, maternal deposited nutrients, hormones which all may affect the egg quality. The larvae with high quantity of yolk sac are considered as good yolk sac larvae.

Feeding period on broodstock performance

The feeding period plays an important role in reproductive function of brood fish. The length of the vitellogenic periods, the feeding history during reproduction, the time period needed to change the nutrient composition of gonads are the important factors that affects the reproductive success. For example, gilthead sea bream, *Sparus aurata* is batch spawners with group synchronous ovaries and have short vitellogenesis period where is possible to change or improve the spawning quality by modifying the nutritional quality of the diet even for few weeks of feeding. In sea bass, *Dicentrarchus labrax* vitellogenesis is slightly larger than sea bream and hence relatively extended period is needed for obtaining appropriate levels of n-3 HUFA in the eggs. In case of *Lutjanus campechanus* to obtain sufficient quantity of n-3 HUFA, will take at least 2 months before the spawning period commences. Salmonids are synchronous spawners and the vitellogenesis period extends up to 6 months. Hence, the brood fish should be fed with good quality diet for several months before spawning period. Only then, the fatty acid profile of muscle and developing eggs will started reflecting the dietary fatty acid profile.

Many fish species tend to decrease their feed intake during sexual maturation. The swelling of ovaries due to egg hydration may restrict

the space in the body cavity and hence the feed volume ingested is reduced. Endocrine changes in connection with sexual maturation and spawning may also interfere with appetite regulation. As a consequence, the energy and nutrient required for gonadal development may be obtained from body reserves. Rainbow trout mobilizes its lipid reserves from carcass and viscera where as African catfish utilizes their abdominal fat as major energy source for sexual maturation. Atlantic salmon uses its muscle protein and lipid where as cod uses the lipid reserve of the liver for sexual maturation. Gilthead sea bream brood fish continues to feed during sexual maturation and spawning period, and therefore producing an egg biomass equivalent to its body mass. In these circumstances, nutrients deposited in the ovaries may come from broodstock diets and the nutritional composition of eggs can be modified during spawning season.

Feed intake on spawning performance

Feed availability is the important factor determines the fecundity in the wild fish. The food deprivation in case of long lived species may delay in reproduction by favoring somatic growth over ovarian growth where as in case of short living species the ovarian growth is preferred than the somatic growth at times of inadequate feed supply. In cultured fish fecundity and other spawning quality parameters are affected by feed ration size and feeding rate. Inadequate feeding rate may inhibit gonadal development in many fishes.

The sea bass fed with half the actual feed ration showed decreased growth rate, delayed spawning period, smaller eggs and newly hatched larvae than the fish fed with full feed ration. The restricted feed ration to the brood fishes showed decreased plasma estradiol level and reduced fecundity. In tilapia feed intake influence the fecundity but did not affect the hatching percentage and egg diameter. High feed intake not only affects the fecundity increment but also egg size and female body size. The length of the time between successive spawns was lower in fish with high feed intake.

Specific nutrient requirement

Protein and amino acid requirement

The quality protein level in the broodstock diet affects the reproductive performance. The protein is the most abundant nutrient in fish eggs and is main energy source during embryonic development. The growth of embryos occurs through the deposition of protein. Hence, protein have important role in fertilization and embryonic development. A positive correlation exists between size at first spawning and protein content of the broodstock diet. Therefore, less protein (lower than optimum level) fed fishes spawn at smaller size, spawn less frequently and had higher relative fecundity (number of eggs per kg body weight), induce female to mature earlier and spawn over longer period of time. It is advisable to feed the broodstock with low protein-high energy diets than the high protein low energy diets. The need for correct protein requirement in the diets of brood fish has not been studied in detail in most of the fish species. But in general when the dietary protein level is optimal most of the fish species will spawns successfully.

Protein covering the yolk is significant for fertilization and their amino acid composition generally rich in proline and glutamic acid and low in cystine. The yolk protein precursors and vitellogenins are lipoproteins and their amino acid composition contains high level of alanine, glutamic acid and leucine but low in serine level. Free amino acids are available in high levels in eggs of pelagic fishes. The abundant free amino acids are leucine, lysine, valine, isoleucine, alanine and serine. Hence while formulating the diet for the broodstock these points should be kept in mind for better broodstock performance.

The sufficient amount of protein with balanced composition of amino acids is essential for the emerging embryo which should be obtained from yolk. Hence, the broodstock diet should be balanced with required protein and amino acid composition which improve the synthesis of vitellogenin. In most of the carnivorous marine fishes the broodstock diet

should contain at least 45-50% of protein level for better reproductive performance. This will increase fecundity and reduced the rate of deformed larvae. During spawning period if the diet is provided with insufficient level of protein that may altered the secretion of gonadotrophin releasing hormone (GnRH) and leutinizing hormone (LH) which play an essential role in the regulation of oocyte maturation and ovulation. The squid meal and cuttlefish meal incorporation in place of fish meal in the diet of red seabream showed significantly better reproductive performance.

The amino acids tryptophan and taurine are also very essential for fish reproduction. Tryptophan is a precursor of serotonin and can affect increase in the testosterone levels which favoring spermiation in males and induced female gonad maturation. Taurine being most abundant free amino acid in tissues of fish, the supplementation of taurine can improve fecundity, percentage of viable eggs and fertilization rate.

Lipids and Fatty acids

The broodstock required great amount of energy for production of primary sex products, secondary sexual characters development and for reproductive behaviors. The relative proportion of energy required between growth and reproduction varies according to type of breeding, sex and age. The energy required for reproduction is around 15-30% in most of the fishes. During the somatic growth phase of life around 40% of energy may be diverted for growth but after maturation hardly 10% of energy will be allotted for growth. When the energy supply from the feed is not sufficient most of the fishes produce the gonads at the expense of somatic growth and mobilize their body reserve for gonad development. Increased dietary energy in the form of fat is responsible for increase in gonadosomatic index. This will result in increased fecundity or increased egg size. The increased fat content in the diet will influence larval length, weight and survival rate. It is difficult to differentiate the beneficial effects obtained are solely through lipid or may be because of fatty acids not clear.

Fatty acid composition of fish egg and sperm is directly

affected by fatty acid composition of broodstock diets. However, fatty acid composition of lipids in fish eggs may vary with the species and even different batches of same species. In general ovarian lipids contain higher proportions of n3 HUFA, particularly 20:5 n3 and 22:6 n3 fatty acids. Fatty acids are essential sources of energy during early embryonic development, important in phospholipid synthesis and hence in formation of bio-membranes. The ratio between the saturated and unsaturated fatty acids regulates the fluidity of cell and organelle membranes and their functions.

Fatty acids, Arachidonic acid (ARA) and Eicosapentaenoic acid (EPA) are precursors of eicosanoids as well as production of prostaglandins and leukotrienes. Prostaglandins are involved in numerous reproductive processes such as production of steroid hormones, gonad development and ovulation. Optimal arachidonic acid may increase the fecundity, egg viability, hatching rate and larval survival. ARA will stimulate the release of testosterone through its conversion into prostaglandin E2 which in turn increase the fertilization rate. The ARA, EPA and DHA will compete with each other for eicosanoid production. Hence, the ratio of ARA/EPA and EPA/DHA will have the effect on the reproductive performance. The diet with higher lower EPA will have higher DHA/EPA that may affect the reproductive performance. In gilthead seabream broodstock dietary EPA and ARA levels show a correlation with fertilization rates. There should be a balance between n3 and n6 fatty acid ratios in broodstock diet and high n-3 to n-6 ratio will provide better maturation effect. The optimal n-3 to n-6 fatty acid ratio should be 2:1 to 3:1 for efficient performance of broodstock.

Diets deficient in essential fatty acids cause an increase in the number of droplets in fish eggs. Fusion of fat droplets into a single lipid droplet leads to increased percentage of hatching and normal larvae. Excess or deficiency of n-3 HUFA also causes negative effect on egg and larval quality. Very high levels of n-3 fatty acid caused a decrease in total number of eggs produced and egg quality. Low n-3 HUFA lowered the fertilization rate due to decreased sperm motility. The positive effects of n3 fatty acids on spawning performance may be by increasing

the fluidity of biological membranes. High levels of linoleic acid (18:2; n6) in the broodstock diet create problem on the larval quality such as body deformation and inability to inflate air bladder. The broodstock diet should possess at least 1.5% of n-3 HUFA with minimum of 50-60% DHA will be required for better larval survival and swim bladder inflation rate. The requirement of n-3 fatty acid in sea bream broodstock is between 1.5-2.5% being higher than the juveniles' requirement (0.5-0.8%) of the same species.

Among the phospholipids lecithin and cephalin are predominant in ovaries. The phospholipid requirement in broodstock diet is around 1.5-2%. Cholesterol is known to fulfill several endocrinological functions and its mobilization during maturation. Generally the cholesterol is supplied in the form of fresh feed such as squid, clam and mussel. The low membrane cholesterol-phospholipid ratios are correlated with a better sperm freezing resistance in cryopreservation.

Carbohydrates

It is an inexpensive source of energy with protein-sparing and lipid-sparing effects. It acts as basic source of energy in some of the tissues. Broodstock diets with low level of carbohydrates cause decrease in fecundity where as the increased level of carbohydrates reduce the spawning quality.

Vitamins

Vitamin C and E are antioxidant enzymes and are degraded as they fulfill their function. Broodstocks fed with trash fishes which are often low in C and E and also low in thiamin because of activity of thiaminase. And it is advisable to avoid trash fish in the broodstock diet. Vitamin E content is generally high in fish eggs and low in broodstock tissues after the spawning period. Vitamin E requirement is depend on the dietary content of HUFA, an increase in n-3 HUFA in the diet requires the vitamin E requirement increase also. Vitamin E deficiency inhibits the gonad maturation, loss of sexual coloration and decreased hatching and survival rate of larvae. Also cause the reduction in the percentage of fertilized eggs which is due to

decrease in number and motility of spermatozooids as well as sperm plasma tocopherol reduction and sperm viability. Elevated level of vitamin E up to 2000 mg/kg improved the percentage of buoyant eggs, ratio of larvae with normal development and larval survival. Optimum requirement of vitamin E in most of the broodstock diet is around 200-250 mg/kg.

Vitamin C plays a vital role in sexual maturation through the biosynthesis of steroid hormones. It generally affects the steroidogenesis and vitellogenesis. Also affects the level of vitamin C in seminal fluid which is directly related to sperm motility. In most of the fishes dietary vitamin C level reflects in the level of vitamin C in eggs. The level at least 1200 mg/kg in the diet improves the egg hatching percentage and rate of normal larvae. Fecundity and egg quality is not only by dietary vitamin C or E but also by the interaction of them. In rainbow trout vitamin C requirement is 8 times higher than the requirement of juveniles.

Vitamin A is required for reproduction and embryonic development. Vitamin A increase fecundity, viable egg percentage and normal larval percentage. The optimum level varies with species and is in the range of 10000-20000 IU/kg.

There is no much information is available on the effect of B complex vitamins on fish reproduction. In the broodstock diet thiamine is importance for normal embryo and larval development. Thiamine is supplied through injection into female Atlantic salmon shows reduction in mortality of progeny. Thiamine concentration in the egg and yolksac larvae is related to reduction of early mortality syndrome. Pyridoxine is an essential compound in the biosynthesis of steroid hormones and other vitamin, folic acid hence the deficiency may cause reduced cell division due to impaired DNA and RNA synthesis that plays a vital role in hatchability of eggs.

Minerals

There is scarce information available on the mineral requirement of broodstocks. Phosphorous deficiency in sea breams is related to decrease in fecundity, percentage of viable eggs and hatching rate, increase in number of abnormal larvae and lower fecundity. The

content of manganese, zinc and iron in the eggs found lower when the broodstock is not supplemented with sufficient level of these trace minerals.

Carotenoids

Carotenoids are being antioxidants and their inclusions improve the percentage of floating eggs, egg hatchability and percentage of normal larvae. Combined increase in the level of n3 HUFA and carotenoids significantly improve spawning quality such as fecundity, percentage of viable eggs, hatching rate and larval survival. Canthaxanthin and astaxanthin in the broodstock diets of red sea bream were incorporated into the egg without converted into beta carotene. During early maturation carotenoids accumulate in the liver and during secondary vitellogenesis it is mobilized from liver through blood to ovaries. Astaxanthin improve the larval survival this is because of the antioxidant properties which scavenges singlet oxygen and other free radicals and prevent the egg membrane deterioration. Carotenoid reserves in the embryo and prefeeding larvae will help in the development of chromatopores and eyes.

Nucleotides

Broodstock diets enriched with nucleotides showed an improvement in larval survival of haddock after 10 days of hatching. This may caused by better development of intestine of nucleotide supplemented larvae and as a consequence better utilization of first exogenous feed. In addition, broodstock fish fed with nucleotide enriched diet have also recorded higher fecundity.

Broodstock feeding practice

Generally, in most of the countries there are no specific feeds commercially available for marine broodstocks. And hence in most of the marine fish hatcheries the broodstock is mainly feed on fresh marine by-products or in combination with commercial diets. The fresh diet includes squid, cuttlefish, mussels, clam, shrimp and other small crustaceans. The use of these fresh and unprocessed fish products do not provide required levels of nutrients to broodstock fish and it also increases the risk of disease transmission and parasitic, bacterial and viral pathogens to the parents and offspring.

Nutritional Pathology

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Introduction

Due to rapid expansion of aquaculture industry, more research interest is being focussed on fish nutrition. Due to the lack of sufficient information on the nutrient requirements of cultured marine fishes, fish nutrition has not made much progress when compared to animal nutrition. Stress induced outbreak of diseases can be attributed to nutritional factors in addition to various chemical, physical and biological stressors. Hence, understanding the nutrient requirement of cultivable fishes is rapidly being recognized so as to develop proper feed for a particular fish species being cultured. Nutritional diseases of cultured marine fish may develop consequent to deficiency, excess, or imbalance of nutrients like protein, lipid and fat present in the feed. Nutritional diseases do not generally develop all of a sudden instead they develop gradually since the fish have body reserves of nutrients that make up for nutritional deficiency up to a certain extent. Clinical signs of nutritional deficiency develop only when supply of any diet component falls below critical level. Further, when fish consumes excess food, whatever in excess would be converted to fat which will be deposited in body tissues and organs. The deposited fat affects the physiological functions of the fish.

Pathological features of protein malnutrition

Dietary proteins are the source of essential amino acids for the fish. These proteins also supply nitrogen for the synthesis of non-essential amino acids. A total of 23 amino acids make up the protein present in the tissues of which 10 are essential amino acids

which must be supplied in the fish diet. Functions of proteins include maintenance, growth and reproduction. In addition to this some amino acids are readily converted to glucose to provide energy source for some tissues such as brain and red blood cells. Wild fishes especially carnivores depend largely on amino acids as precursors to glucose since their normal diet lack carbohydrates. Hence, in case of fish, some fraction of the dietary protein is used as an energy source.

Deficiency of essential amino acids may lead to improper utilization of dietary Protein resulting in growth retardation, lowered weight gain, and increased feed conversion ratio. In severe cases, deficiency of amino acid leads to poor resistance to diseases due to alteration in the immune response of the fish. Deficiency of individual amino acid like tryptophan may lead to scoliosis resulting in spinal curvatures and also renal calcinosis, cataract, caudal fin erosion, decreased carcass lipid content. Methionine deficiency affects the vision of the fish. Lysine deficiency leads to dorsal/caudal fin erosion. Lysozyme activity and C-reactive protein values are reduced in case of protein deficiency in fish.

Pathological features of fatty acids and lipids malnutrition

Fatty acids form the nutritionally active components of dietary lipids. Fish are unable to synthesize fatty acids that are unsaturated in the ω -3 or ω -6 positions unless a suitable precursor is supplemented in their diet. Unlike mammals, which

have a major requirement for ω -6 fatty acids, most of the marine fishes require ω -3 fatty acids. Hence, adequate quantity of essential fatty acids must be included in the dietary lipids.

When fish are affected with lipid liver disease, they exhibit severe anemia, bronzed, rounded heart and swollen liver with rounded edges. Microscopically, the conspicuous feature is extensive lipid infiltration of hepatocytes which appear as clear, round or oval vacuoles in H and E stained sections since the lipid is dissolved during routine tissue processing. Excess carbohydrates are known to induce damage to the liver and destructively affect the natural microbial flora in the gut of the fish.

Pathological features of vitamin deficiency

Vitamins are micro-nutrients that are required for growth, reproduction, and disease resistance. The

requirement of vitamins in case of fish depends on the size of the fish, uptake of other nutrients and environmental stresses. Deficiency of vitamin in fish may arise by their low content in feeds, environmental or physiological stressors and by diseases especially those, which occur in the early stages of development. Clinical signs of most of the vitamin deficiency disorders are non-specific in nature. It is rather complicated to identify them. Deficiency disorders caused by vitamin deficiency adversely affects the utilization of other nutrients and reduces the resistance to diseases.

Some of the deficiency disorders caused by vitamin deficiency in fish include:

Pathological features of trace elements deficiency

In higher vertebrates, trace elements like zinc, iron, copper and selenium are required for the immune

Vitamin	Clinical signs of deficiency
Ascorbic acid (Vitamin C)	Reduced growth, anaemia impaired collagen formation, scoliosis, lordosis, internal and fin haemorrhage, distorted gill filaments, slow wound repair, mortality, reduced hatchability, exophthalmia, caudal fin erosion, renal granuloma, club-shaped gill lamellae, fatty degeneration of liver, muscle degeneration, skin haemorrhage, swollen abdomen, changes in head bones, blindness, surface swimming, lower lip ulceration, scale loss, emaciation.
Pyridoxine (Vitamin B6)	Nervous disorders, hyperirritability, anorexia, ataxia, oedema of peritoneal cavity, erratic and rapid swimming, greenish-blue colouration of skin, anaemia, rapid breathing, poor growth, muscular spasms, abnormal pigmentation, convulsions, reduced food conversion ratio, lesions of lower lip, avoidance of schooling
Cyanocobalamin (Vitamin B12)	Anorexia, reduced growth, microcytic hypochromic anaemia, fragmented erythrocytes, low FCR, dark pigmentation, distended abdomen, skin and fin haemorrhage, loss of skin mucosa, grey-white intestine.
Choline	Reduced growth, fatty liver, low FCR, haemorrhagic kidney and intestine, exophthalmia, clouding and thickening of corneal epithelium, degeneration of the retina, elevated liver/muscle lipid content.
Vitamin E (Tocopherol)	Reduced growth, exophthalmia, ascites, anaemia, clubbed gills, mortality, erythrocyte fragility, muscle degeneration, reduced hatching rate/spawning efficiency, reduced antibody response, ceroid deposition in liver, spleen and blood vessels, splenic haemosiderosis.
Vitamin K3	Increased blood clotting time, anaemia, haemorrhage in gills, eyes, skin and vascular tissue.
Thiamine (Vitamin B1)	Anorexia, poor growth, nervous disorders, increased sensitivity to shock, fin haemorrhage,
Folic acid	Macrocytic normochromic anaemia, poor growth, anorexia, lethargy, dark colouration, pale gills, exophthalmia, distended abdomen with ascites fluid
Pantothenic acid	Anorexia, reduced growth, high mortality, clubbed gills, haemorrhage under the skin, fragile fins, oedema, rapid breathing, swelling at base of pectoral fins, pale gills, sluggish activity.
Riboflavin (Vitamin B2)	Anorexia, poor growth, corneal vascularisation, cloudy lens, snout erosion, spinal deformities, mortality, fin erosion, fin haemorrhage, muscular weakness, light or dark pigmentation, photophobia, in coordination, lethargy, anaemia.

system to function. However, in fish not much is known about the effects of these trace elements on the immune system. In case of fish, minerals execute important functions in osmoregulation, metabolism and in formation of the skeleton and scales. The minerals required in finfish diets include calcium, zinc, manganese, cobalt, selenium, iodine and fluorine. Iron is an essential requirement in fish diet. Low concentrations of free iron in mucus membranes and in other tissues are reported to be one of the non-specific host defences against bacterial infections. Iron deficiency causes microcytic

anemia in many fish species. Copper deficiency rarely occurs in fish since water is abundant in copper. In case of copper toxicity, susceptibility to bacterial infections like vibriosis increases. Manganese deficiency occurs in fishes when the diet contains high concentration of calcium or ash. Manganese deficiency in fish results in cataract. Deficiency of zinc is also associated with occurrence of cataract especially in young fish. Absorption of zinc will be hindered when feed contains excess amount of ash, calcium or phytate. Zinc deficiency also results in poor growth and darkening.

Feed Ingredients and Database

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Introduction

The rapid world-wide expansion of aquaculture and livestock production strongly indicates that a crisis will be predicted in the livestock and aquaculture feed industries in the near future. Nutrient content of individual feed ingredients varies widely from country to country or region to region depending on local climate, post harvest processing methods and storage. Some generalization can be made regarding the chemical composition of individual feed stuffs. Hence, the nutrient profiling of each ingredient should be done and database need to be developed for the better feed formulation. Ingredients used in commercial fish diets can be classified different means such as sources of protein (amino acids), energy, essential fatty acid content, vitamins and minerals. Special ingredients may be used as additives to enhance growth, pigmentation or sexual development and to prepare diets having the required physical quality, palatability and preservation properties.

Classification of Feed Ingredients Based on Protein Content

The ingredients used for fish feed based on protein content can be broadly classified into two major groups such as:

- Energy rich feed ingredients- Energy rich feed ingredients belongs to those groups which contain <20% protein and <18% crude fibres. These ingredients mostly plant origin.
- Protein rich feed ingredients- Those feed ingredients which contain more than 20% crude protein are

commercially referred as protein rich feed ingredients. These ingredients may be animal or plant origin

Classification Based on Commonness of Usage

The feed ingredients are broadly divided into two types based on its commonness of usage as:

- Conventional feed ingredients
- Unconventional feed ingredients

Conventional feed ingredients: Those feed ingredients which are commonly used in the commercial aqua feed preparation is known as Conventional feed ingredients. For example, Groundnut oil cake, Mustard oil cake, Soy bean meal, Sunflower oil cake, rice bran, Rice polish, Wheat bran, Fish meal, Meat meal, Blood meal etc.

Unconventional feed ingredients: Some of the ingredients which are not commonly used may be used in the aqua-feed after removal of some of the anti-nutritional factor present in the same ingredients. These ingredients are referred as unconventional feed. Some of the common examples are Guar meal, Squilla meal, Feather meal, Frog meal, Rubber seed cake, Neem seed cake, Karanja cake, Niger cake, Coconut meal, Tapioca flour, Dried whey, Crocodile meal, etc.

Commonly used ingredients in aquafeed

Cereals and cereal by-products

Cereals: Carbohydrates are the primary nutritional contribution of cereals/grains. Whole grains contain

62 to 72 % starch, which is 60 to 70 % digestible by warm-water fish but markedly less digestible by carnivores fishes. Starch in grains is an important binding agent in steam-pelleted and extruded fish feeds. It also contributes significantly to the protein and lipid content of the diet. Though deficient in some of AA (e.g. Lysine) they can be used to balance high protein animal and vegetable ingredients. Some of the common cereals used in aqua feed are sorghum grains, wheat, rice, sorghum/maize/wheat gluten meals.

Cereal by-products

Wheat flour: This ingredient is mainly used as a binder for shrimp and prawn feeds. Primarily Consists of wheat flour together with fine particles of wheat bran, wheat germ and the offal from the “tail of the mill”. This product usually obtained from commercial milling process and must contain less than 1.5 percent crude fibre. India is one of the world’s largest wheat producers and processors. Second-grade wheat flour, which is only marginally fit for human consumption, is used in animal feeds. Indian wheat has a low wet gluten index (<28 percent) relative to the ideal index for shrimp feed pelleting (32 percent).

Wheat Bran: Wheat bran is the coarse outer covering of the wheat berry as separated from cleaned and scoured wheat in the usual process of commercial milling. Wheat bran contributes bulk to ruminant feeds and is a source of carbohydrates, protein, minerals and vitamins. Too much wheat bran in a formulation results in pellets with poor water stability due to the water absorption characteristics of fibre.

Wheat Germ Meal: Wheat germ meal consists chiefly of the germs of the wheat berry, with some bran and middlings. A rather wide variety of wheat germ grades are produced, depending on the area and regional demand. The quality of wheat germ meal is influenced by the presence of screenings and the level, of other wheat products, which can lower the fat or protein level. Excessive storage time may result in rancidity, due to the oxidation of the rather high level of fat.

Corn germ meal: Corn germ meal is produced from corn germ by complete removal of oil through solvent

extraction. Corn germ meal contains high level of hemicelluloses fibres, which delivers good hydration and pelleting characteristics.

Rice Bran with Germ: Rice bran with germ is primarily the pericarp or bran layer and the germ portions of the rice kernel. The crude protein level is approximately 13.3%, crude fat 15%, and crude fibre 11%.

Tapioca flour: Tapioca- In the strictest sense, starch from cassava is to be referred as tapioca. Tapioca flour is rich in carbohydrates (starch), is mainly used as binder Tapioca flour have a considerably higher amount of amylopectin compared to other starch sourced, which helps in better expansion and functional properties of aquafeeds.

Corn Gluten Meal: Corn gluten is an excellent protein source, with minimum of 60 % protein. Corn gluten meal is commercial produced from dried residue of corn after the larger part of the starch and germ have been removed and the bran separated by the process employed in the wet milling manufacture of corn starch or syrup, or by enzymatic treatment. Corn gluten meal may contain fermented corn extractives and/or corn germ meal. White corn gluten meal will likely be priced at a premium to regular (yellow) corn gluten meal, but protein levels are at least 10% higher, which justifies the increased cost.

Wheat gluten meal: Wheat gluten meal is a good natural protein binder, containing 70-80% of protein. Wheat gluten meal having rich source of cystine and glutamine, but is very deficient in lysine. It is produced from wheat flour after starch extraction. Due to high level of yellow pigments, its use in aquaculture is restricted to only 10 %.

Oil Extractives/Oil Seed Cakes

Oil extractive or Oil seedcakes are by-products from vegetable oil extraction industry. The protein-rich residues left after removal of oil represent an immense resource upon which the world’s production of animal protein for human consumption largely depends. Oil extraction from seed or fruit is carried out by two methods: by pressing, or with chemical solvents. The

product obtained by pressing is termed oilcake and that by solvent extraction, oil meal.

Soybean Meal: Use of soybean products in the aquaculture industry has become the focus of protein substitution in fish food around the world. The high protein level makes it a key ingredient for aquaculture feeds since soybean meal is considerably less expensive than traditionally used marine animal meals. Soybean meal is universally available and has one of the best amino acid profiles of all protein-rich plant feedstuffs for meeting most of the essential amino acid requirements of fish. Some fish, such as young salmon, find soybean meal unpalatable while others, such as channel catfish, readily consume diets containing up to 50 % soybean meal. Soybeans contain several anti-nutritional factors but heating during commercial oil extraction destroys much of the activity. Soybean meal is produced in two major protein levels by different processes. Forty-four % soybean meal is usually mechanically extracted to produce a meal of 44 % crude protein; crude fat, 4.7 %; and crude fibre, 6.0 %. Forty-eight percent soybean meal is dehulled and solvent extracted to yield meal with a crude protein level of 48 %; crude fat, 0.9 %; and crude fibre, 2.8 %. Soybean meal must be heated (toasted) sufficiently to destroy the trypsin inhibitor. Soybean meal generally classified according to their protein level. The limiting amino acid (lysine and methionine) content of soybean meal is high, and is not on the level of Whole fish meal and especially egg.

Cottonseed Meal: Solvent extracted cottonseed cake is used for fish feed preparation. Cottonseed meal is a rich source of arginine (and to a lesser extent tryptophan and cystine), but is deficient in methionine and lysine. Most cottonseed meal contains free gossypol, which is moderately toxic to monogastric animals and limits its use in fish feeds.

Groundnut Meal/Oil cake (GNOC): Groundnut meal has been traditionally used as principal protein source for fish feed preparation. GNOC is obtained by grinding the cake, chips or flakes obtained by removal of most of the oil from peanut kernels by a mechanical or solvent extraction process. GNOC is known to be very poor in lysine and methionine, so the oilcake should be used with lysine and methionine supplements to achieve a proper balance of essential amino acids in the diet.

Sunflower seed meal: Sunflower meal is obtained by grinding the residue remaining after extraction of most of the oil from dehulled sunflower seed by a solvent extraction process. Sunflower meal contains crude protein level up to 46%. Sunflower seed meal is a rich source of tryptophan and arginine, but it is deficient in lysine and to a lesser extent tyrosine. In addition, sunflower meal may contain anti-nutritional factors, including protease inhibitors, tannins and arginase inhibitor.

Rapeseed meal/Canola meal: Meal from canola seed (low-glucosinolate rapeseed) has been used in experimental feeds for salmonids with success. It has an amino acid profile comparable to soybean meal, but it is lower in protein and higher in fat, sulphur containing amino acids particularly methionine, fiber and tannins.

Linseed cake: Linseed (flax) cake is the product obtained by grinding the flakes which remain after removal of most of the oil from flaxseed by a solvent extraction process. It must contain not more than 10 percent fibre. The oilcake has protein content comparable to cottonseed oilcake but is considerably richer in methionine.

Animal by-products

Fish Meal: Fish meal is the clean, dried, ground tissue of un-decomposed whole fish or fish cuttings, either or both, with or without the extraction of part of the oil. Fish meal provides a balanced amount of all essential amino acids, phospholipids, and fatty acids for optimum development, growth, and reproduction, especially of larvae and brood stock. The nutrients in "Whole Fish Meal" or "White Fish Meal" (such as Omega 3 fatty acids DHA or docosahexaenoic acid and EPA or eicosapentaenoic acid) also aid in disease resistance by boosting and helping to maintain a healthy functional immune system. Fish meals are manufactured by cooking fish, pressing to remove water and oil, and then drying. Fish meals are often made from a single species of fish, e.g., herring meal, Fishmeal made from fish parts, such as waste from fish processing and canning plants, has a lower percentage of high-quality protein than that of meal from whole fish. Whole Fish Meal

averages between 17% and 25% ash content. More ash indicates a higher mineral content, especially calcium, phosphorus, and magnesium. Calcium and phosphorus constitute the majority of the ash found in fishmeal. This makes Whole Fishmeal an important source of very essential minerals that fish need for osmoregulation. As it has high in ash content should be used prudently in fish diets because it can produce mineral imbalances. Over-all protein digestibility values for fishmeal are consistently above 95%. In comparison protein digestibility for many plant-based proteins varies greatly, for example, from 77% to 96%, depending on the species of plant. Whole Fish Meal is an excellent source of DL-methionine. Fishmeal also contains certain compounds that make the fish food more acceptable and agreeable to the taste. It is thought that the non-essential amino acid glutamic acid is one of the compounds that impart to fishmeal its palatability. This property allows for the feed to be consumed rapidly, and hence reduce the nutrient leaching.

Shrimp meal: Shrimp meal can be made from either cull shrimp that are being processed before freezing or from whole shrimp that is not of suitable quality for human consumption. The material to be made into shrimp meal is dried (sun or using a dryer) and then ground. Shrimp meal has been used in trout and salmon diets as a source of pigments to impart the desirable color in the tissues. Shrimp either whole or as a part of another prepared food is an excellent source for fats needed for the growth of hump heads in Flowerhorn Cichlids. Shrimp meal has been found to be an acceptable supplemental protein source for fish, but inferior to whole fish meal.

Meat and Bone Meal: Meat and bone meal is the dry rendered product derived from mammalian tissue, exclusive of hair, hoof, horn, manure and paunch contents. The relatively high fat content of meat and bone meal apparently helps protect the lysine content during rendering. Meat and bone meal generally contain a crude protein of 45-50 %; fat, 8-11 %; crude fibre, 3 % phosphorus, 4.4-5 %; calcium, 8.8-10 %; and ash, about 30 %. The quality of the protein in this product is less than that of whole fishmeal, and the ash content is usually high because a significant amount of

the material comes from bone and other non muscle tissue.

Squid Meal: Squid Meal is made from squid viscera portions from cannery plants including egg and testis. Squid Meal is high digestibility of protein source, which provides a full range of amino acids for fish. It provides various kinds of vitamins and minerals and also 1.0-1.5% of cholesterol that is suitable for fish fry and young fish.

Acetes meal: A small size shrimp called the Acetes shrimp is caught in large quantities seasonally in Karnataka, Maharashtra, and Gujarat. The sun dried and pulverized meal of Acetes shrimp is one of the good protein sources having 60% protein.

Blood Meal: Blood meal is produced from clean fresh animal blood, exclusive of all extraneous material such as hair, stomach contents, etc. Blood meal may be dried by several processes, but most often by spray drying. Spray dried blood meal has approximately a crude protein level of 85 %; crude fat, 0.5-3 %; crude fibre, 2.5 %; ash, 6 %; and lysine, 9-11 %, with an availability of 80-90 %. Flash or spray-dried blood meal is rich in protein (80 to 86 %) but low in methionine and unbalanced in branched-chain amino acids.

Hydrolyzed Poultry Feathers: Feathers from poultry are collected, ground and hydrolyzed under pressure. During processing the cystine linkage is broken, increasing the value of the meal. Not less than 75 % of the crude protein must be pepsin digestible. Generally, hydrolyzed poultry feathers will range in crude protein from 80 to 85 %; crude fat, 2.5 %; crude fibre, 1.5 %; phosphorus, 0.75 %; and ash about 3 %. Feather meal is high in crude protein (80 %) but, unless the feathers are thoroughly hydrolyzed during processing, digestibility is low.

Miscellaneous

Dried Distillers Grains with Solubles

This product is obtained by removal of ethyl alcohol by distillation from the yeast fermentation of corn or corn mixture by condensing and drying at least 75 % of

the solids of the resultant whole stillage. Corn distillers dried grain with solubles has a crude protein level of 26-27 %; crude fat, 7-8 %; and crude fibre, 8.5-9.5 %.

Brewers Yeast: Brewers dried yeast is the dried, sterilized, unextracted yeast (*Saccharomyces* sp.) resulting as a by-product from the brewing of beer and ale. Brewers dried yeast has a crude protein level of 45 %; crude fat, 1 %; and crude fibre, 2.7 %.

Brewers Dried Grains: Brewers dried grain is the extracted dried residue of barley malt alone or in mixture with other cereal grain or grain products resulting from the manufacture of beer and may contain pulverized spent hops in an amount not more than 3 %. The protein content may be around 25-30 %.

Dried Whey: Dried whey is the residue obtained by drying whey, a by-product of cheese manufacturing. It contains at least 65 % lactose; crude protein, 13 %; about 0.8 % crude fat; and no crude fibre.

Spirulina: Spirulina is blue-green algae rich in

raw protein and seven major vitamins: A1, B1, B2, B6, B12, C and E. Spirulina naturally contain beta-carotene color enhancing pigments (1500 mg/kg. Carotenoids; Orange/ Red pigment enhancers), and high amount of minerals. In addition, it contains all essential fatty acids and eight amino acids required for complete nutrition. Spirulina is similar to cyanobacteria in structure (spiral shape), which can be toxic. Spirulina is recognized by the body (fish in particular) as a bacterium, causing an increase in antibodies, which in turn increases disease resistance. Spirulina is also high in usable or digestible amino acids. Spirulina is probably one of the best fish food ingredients available, including for carnivores (usually fed via gut loading). Any staple fish food diet for community fish is improved by the addition of Spirulina Algae.

Silkworm pupae: It contains 72.5% crude protein. It is oil extracted, sun dried, powered and used as a source of animal protein in fish feed. It is widely utilized in carnivorous fish diets.

Table 1: Proximate composition of selected aquafeed ingredients

Ingredients	Moisture (%)	Crude Protein (%)	Ether Extract (%)	Crude Fibre (%)	Total Ash (%)	Nitrogen Free Extract (%)
Plant Origin						
Rice Polish	8.4-12.6	11.4-14.5	15.3-17.3	7.5-11	6-12.9	41- 46.8
Rice bran	7.8-10.1	2.9-12.6	4.2-11.3	5.3-19.3	3.1-20.5	36.5-37.5
Deoiled rice bran	7.2-8.1	12.1-14.3	1.3-1.8	15.2-16.7	23.8-29.1	40.4-43.3
Wheat bran	9-13	8.2-15.8	2.6-6.6	4-13.5	0.2-4.2	34.5-37.6
Wheat flour	12.6-12.9	14.5-15.6	3.7-3.9	2.7-2.9	2.3-2.8	64.2-64.6
Groundnut cake	7-10	42-48	7.3-13.8	13-13.2	2.5-13.4	25.2-29.9
Sunflower cake	8-10	31-32.6	2.1-2.9	18.4-24.7	1.5-6.2	39-40.1
Coconut cake	8.9-9.1	12.2-13.7	4.9-5.1	25.6-26.5	2.6-2.8	45.8-46.4
Soybean meal	-	26-32.8	2.1-2.9	18.4-24.7	1.5-6.5	39-40.1
Soybean meal, defatted, undehulled	-	42-44	-	6.5-7.0	.5-6.0	30-32
Soybean meal, defatted, dehulled	-	48-50	-	3.0	5.5- 6.0	30-31
Safflower cake	11-12.1	35.9-36.8	0.9-1.6	11.5-13.2	6.9-7.1	32.1-34.3
Cotton Seed cake	7-8.2	37-42.7	7-10	12.6-13	6.5-8.2	27.3-35.3
Sorghum	10-11.6	9-10.2	2.8-3.6	3-3.6	0.1-0.9	75.1-78.1
Lucaena meal	11.8-12.3	35.9-36.8	0.9-1.6	11.5-13.2	6.9-7.1	32.1-34.3
Coffee husk	12.3-14.1	14-15.2	1.2-1.7	20.8-23.1	8.2-9.2	43.5-45.4
Mulberry leaves	8.9-9.4	27.7-35.6	2.4-4.6	11.5-12.5	8.1-9.1	41.4-43.2
Salvinia leaves	2.6-2.8	16.2-17.1	1.1-1.8	18.5-18.6	22-23.5	39.6-40.4
Pistia meal	4.9-5.3	19.5-20.6	1.3-1.8	11.7-11.9	25.6-27.2	37.9-39.8
Colocasia meal	5.8-15.6	24.6-28.2	4.5-4.8	8.2-9.6	9.9-11.2	47-47.1

Sesame seed cake	8.3-10	29-42.7	6.9-12.9	5.7-18.3	10-14.8	19.8-21.8
Eichornia meal	3.3-4.1	19.5-20.2	2.3-2.6	18.3-18.4	9.3-9.6	47.3-48.5
Sal seed cake	8.6-8.9	8.2-9.1	2.9-3.5	1.7-1.8	10.2-11.6	68.4-69.7
Spirulina	8.7-10.1	50.5-51.3	1-1.8	2.1-2.6	11-11.7	26.7-27.5
Mustard oil cake	8.5-9.2	23.6-30.8	9.3-9.6	6.2-6.3	10.3-10.4	34.9-40.9
Gingely cake	7.9-9	34-40	2-7.8	7.9-9.6	2.9-3.1	38.2-38.4
Gingely extract	7-9	34-40	2-7.8	9.6-9.7	2.9-3.1	38.2-38.4
Corn / Maize	10.4-10.6	4.6-5	7.8-8	3.5-4	1-2	72.7-75
Corn gluten meal	-	60-64	-	8.0	45-50	-
Maize meal	10.4-13.5	4.6-9.5	4-7.8	3.5-4	1-1.5	67.5-72.7
Tapioca flour	8-11.5	1.8-3.1	1.3-2.3	1.8-2	0.2-2.3	78.8-86.9
Barley grains	10-12	8-10	2-3	4-6	2-3	70-80
Rice broken	10-10.5	12-12.6	4.2-4.8	5.3-5.9	3.1-3.6	65.4-69.1
Wheat broken	9-10	11.5-12	1.9-2	4-4.5	0.2-1	73.4-75.2
Palm kernel cake	8.9-10.2	12.2-12.5	4.9-5.1	25.6-26	2.6-2.9	45.8-48.2
Rapeseed cake	11-11.5	35.9-36.3	0.9-1.5	13.2-13.6	6.9-7.5	32.1-33.8
Niger extract	7-7.5	35-35.8	2-2.5	19-19.9	3.5-4	33.5-34.2
Copra cake	8.4-12	20.3-22	6-11.4	12-16.2	2.1-6.2	37.5-42.1
Tobacco seed ext	7.7-8.3	30.6-32.5	0.3-1	-	13.7-14	47.7-48.3
Coffee pulp	12.3-12.5	14-14.5	1.2-1.5	20.8-22.1	8.2-8.9	43.5-45.2
Animal origin:						
Fish meal	9-14.6	44.4-72	2.5-10.3	0.3-30	2.5-20.9	7-29.0
Shrimp meal	3.6-15.6	22.5-34.2	1.1-8	7.1-35.3	18.6-31.6	11-16.3
Squilla meal	14.1-14.9	46-47.3	2.6-3.3	13.5-15.2	18-20.1	5.8-6
Squid meal	8-8.5	75-76.9	6.5-7.1	4-5.3	-	-
Clam meal	7-8.1	50.7-52	8.9-11.6	3.9-5.5	6.4-6.9	22-23.1
Silk worm pupae	7.1-7.5	43.9-45.5	25.7-26.1	4.2-4.3	15.8-16.4	3.3-4
Defatted silk worm pupae	8.1-9	68-69.2	2.6-3.1	1.3-1.9	7.2-8.1	12.8-13.7
Blood meal	10-12.9	65.3-76.6	0.5-1.1	1-1.9	3.8-4.3	4.6-5.1
Meat meal	8-10	50-71.2	4.4-13.3	0.7-6.8	5-5.6	25.8-26
Liver meal	7-7.5	65-68.3	3.4-4.2	1.2-2	2.4-3.1	21-22.3
Earth worm pupae	5-6.5	51.7-55.1	3.4-4.1	12.8-13.6	12.5-13	14.6-15

Table 2: Nutritional composition of commercially important feed ingredients

	Fish meal	Poultry meal	Squid meal	Krill meal	Soybean meal	Sunflower meal	Rapeseed meal	Wheat bran	Corn meal
Moisture (%)	6-9	6-8	8-10	7-9	7-9	6-8	7-9	10-12	8-12
Crude protein(%)	57-64	66	78-80	58-62	45-48	26-29	34-36	15	8
Lipid(%)	7-9	10-14	2-3	16-20	2-4	2-3	2-4	3-4	3-4
Crude fibre(%)					5-7	24-26	10-13	9	2.2
Ash(%)	15-25	10-15	4-8	9-11	6-7	6-7	7	4.9	1.2
Gross energy	19-20	22-23	21-22	22-23	17-18	17-18	17-18	16.5	16
Essential Amino Acid									
Arginine(%)	3.7	3.4	7.9	3.8	3	2.1	1.8	0.9	1.55
Histidine (%)	1.4	0.9	2.0	1.4	1	0.6	0.8	0.4	0.4
Isoleucine (%)	2.5	2.0	4.1	2.8	1.8	1.1	1.2	0.4	0.8
Leucine(%)	4.3	3.6	6.9	4.7	3	1.6	2	0.8	1.3
Lysine(%)	4.5	2.3	8.2	4.9	2.4	0.9	1.7	0.5	1.3
Threonine(%)	2.5	2.0	4.0	2.7	1.6	0.9	1.3	0.4	0.7

Tryptophan(%)	0.6	0.4	0.9	0.5	0.5	0.3	0.4	0.2	0.2
Valine(%)	2.9	2.8	4.2	3.4	1.9	1.3	1.5	0.6	0.9
Methionine(%)	1.6	0.7	2.7	1.6	0.6	0.6	0.6	0.2	0.2
Cysteine(%)	0.5	1.3	0.4	0.7	0.6	0.4	0.7	0.3	0.3
Phenylalanine(%)	2.3	2.0	3.7	2.5	2	1.1	1.2	0.5	0.8
Tyrosine(%)	1.9	1.3	2.3	2.7	1.4	0.6	0.9	0.4	0.6
Non essential Amino Acid									
Alanine(%)	3.9	2.8	4.6	3.5	1.8	1.1	1.3	0.6	0.8
Aspartic acid(%)	5.6	3.5	8.1	5.7	4.5	2.3	2.1	0.9	2.1
Glutamic acid(%)	7.6	5.6	12.6	7.6	7.1	4.9	5	2.5	3.0
Glycine(%)	3.8	4.5	4.4	2.9	1.7	1.4	1.5	0.7	0.8
Proline(%)	2.5	4.1	3.6	2.4	2	1.1	1.8	0.8	0.8
Serine(%)	2.3	3.2	4.0	2.7	2	1.1	1.3	0.5	0.8

(Source : ARRAINA, Feed ingredients in aquaculture, Technical booklet)

Table 3: Vitamin content of selected commercially important feed ingredients

	Biotin	Choline	Folic acid	Niacin	Pantothenic acid	Pyridoxine	Riboflavin	Thiamin	Vitamin B12	Vit. E
	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
As fed basis										
Fish meal	0.2	4400	0.2	100	15.0	4.6	7.1	0.1	352	5
Squid meal		650		194	-	1.3	8	0.1	-	-
Krill meal		9000		-	1.6	-	0.1	0.1	-	2.6
poultry meal	0.3	1896	0.7	178	46	6.5	9.1	5.7		2.9
Corn gluten	0.2	352	0.3	60	3.5	7	2	0.3	-	23.4
Soybean meal	0.3	2731	1.4	22	15	6.4	3.1	3.2	-	2.3
Rapseed meal	1.0	6700	0.8	160	9.5	7.2	5.8	5.2	-	13.4
Sunflower meal	0.9	3700	2.3	242	40	13.7	3.5	3.1	-	11.1
Wheat bran	0.4	1232	1.8	2	28	8.5	3.6	8.4	-	14.3
Corn meal	0.1	504	0.3	23	5.1	4.7	1.1	3.7	-	21

(Source : ARRAINA, Feed ingredients in aquaculture, Technical booklet)

Table 4: Mineral content of selected commercially important feed ingredients

	Calcium	Phosphorus	Sodium	Potassium	Magnesium	Copper	Iron	Manganese	Selenium	Zinc
Unit	%	%	%	%	%	%	%	%	%	%
Fish meal	5.5	4.1	1.1	0.8	0.2	6	320	14	1.2	100
Squid meal	0.1	1.4	0.1	0.3	0.0	19	7	0	0.5	15
Krill meal	1.8	1.2	1.5	0.1	0.7	78	395	6	12	60
poultry meal	0.3	1.9	0.6	0.5	0.1	12	220	16		60
Corn gluten	0	0.4	0.1	0.1	0.1	12	100	8	0.8	34
Soybean meal	0.3	0.6	0	2.1	0.3	16	304	40	0.3	47
Rapseed meal	0.8	1.1	0	1.2	0.5	9	131	58	1.1	71
Sunflower meal	0.4	1	0	1.5	0.5	28	241	34	0.5	85
Wheat bran	0.1	0	0	1.2	0.4	12	137	98	-	77
Corn meal	0	0	0	0.3	0.1	2	32	4	-	18

(Source : ARRAINA, Feed ingredients in aquaculture, Technical booklet)

Table 5: Additives and other components used in the aquafeed industry in India

Additive/ Microingredients	Composition/details
Cholesterol	95 % purity, used in shrimp feeds
Mould inhibitor	Mixture of propionic acid and other organic acids and salts

Antioxidant	Mixture of BHT, BHA, ethoxyquin, etc
Pellet binder	Modified urea formaldehyde
Monocalcium phosphate	21-23% phosphorus
Dicalcium phosphate	18% phosphorus
Limestone powder	Mostly used as filler
Mineral premix	Typical micro-mineral premix for fish feeds
Salt	Feed grade
Potassium chloride	KCl 50%
Magnesium sulphate	MnSO ₄
Vitamin premix	Typical premix for fish feeds, without Vitamin C
Choline chloride	60%; Feed Attractant
Vitamin C, coated	35 % active ascorbic Acid
Inosital	Feed attractant

(Source: FAO Fisheries technical paper, 2007)

Suggested readings

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Feed Production Techniques

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Introduction

Fish feed manufacturing technology will differ based on the types of feeds produced. In aquaculture operation different types of feeds were used according to the life stage and needs of the fish. The aim of the feed production technology is to produce a high performance feed with lowest cost possible. The feed should have better water stability, less nutrient leaching, highly palatable, good feed acceptance and better efficiency.

Types of feeds

Based on life stage of fish

According to life stage of fish the feed can be classified as starter feed, fry feed, fingerling feed, grow-out feed and broodstock feed. These feeds are generally called as phase feeds. In most case, fingerling feed will not be commonly available.

Larval feed: The feed size is less than 400 μm and intended for the fish larvae which is not aggressive in their first feeding and with partially developed digestive system. The micro encapsulated diets, micro bound diets and micro coated diets falls in this category.

Starter feed: The starter feeds are larger than larval feeds and the size is above 400 μm . Starter feeds are produced for the fish species which are fully aggressive and have developed digestive system during first feeding. The feeds should be highly digestible and water stable and readily acceptable by the fish. The diet should contain, high protein, fat and balanced amino acid and essential fatty acid content.

Fry feed: Once the fish fry reaches the weight of 0.5 to 0.75 g they can be shifted to fry feeds. Generally the fry feeds are crumbles, flakes or small diameter pellet feeds. The nutritional composition of this feed is slightly lesser than the starter feed.

Fingerlings feed: The fingerlings are the fishes that metamorphosis into the size of about 10-20 g in weight. The nutritional composition of fingerling diet is lesser than the fry feed. For most of the species the fingerling diet is not available. They are directly fed to grow out diets of small diameter pellets.

Grow-out feeds: The major portion of commercial diet (around 80%) is produced for grow-out stages. The diet will be available in 2 or 3 different dimensions and nutritional compositions for different phase of grow-out stages. It is most necessary to ensure that the protein content of the grow-out diet should be used for the growth and not for the metabolic activity. Hence, faster body growth will be achieved in less feed which will reduce the production cost.

Broodstock diet: The broodstock diet should be enriched with essential fatty acids (EPA, DHA and ARA), essential amino acids, higher fat content to compensate the higher energy requirement, essential vitamins (A, E and C), trace minerals (Zinc, Magnesium, Iron and Selenium) for getting maximum fecundity, viable eggs, fertilization rate, hatchability rate and larval survival ship.

Based on moisture content

Wet feed: The wet feed comprises fresh ingredients such as fish flesh, salmon viscera, chicken viscera, beef liver, yeast and 2% salt added to congeal the mixture.

The moisture content of the feed will be 45-60%. Generally the wet feeds are used in hatcheries mainly for broodstock feeding served as wet balls or served as the texture similar to saw dust. FCR is ranged from 3 to 8. It requires frozen storage. Disease causing organisms also transmitted through unpasteurized wet feed. The thiaminase present in the wet fish meat may cause thiamin deficiency in fishes.

Semi moist feed and moist feeds: The semi moist feed are made by mixing the fresh ingredients and dry ingredients. The wet meat is pasteurized before it is mixed into the feed mixture. This feeds contains binding materials such as CMC, gelatin or other hydrocolloidal binding agents. The semi moist diet is made as pellets through cold extrusion. The moisture content of the diet is 20-40%. It can be store in refrigerator for certain period.

Dry feed: These feeds are made from dry ingredients through the addition of water. The feed contains less than 10% moisture. The feed is produced by either extrusion cooking or compressed pelleting or steam pelleting. The extrusion cooking is employed to produce floating feeds where as steam pelleting can produce compressed sinking pellets. The dry feeds based on shape may be divisible into pellet feed and crumbles.

Based on contents of ingredients and nutrients

High energy feeds: Contains high level of crude fat 15-36% which increases the total energy content of the diet and spare dietary protein for growth. It is mainly used in salmonid culture.

Low pollution feeds: The use of high quality raw materials with high bioavailability and high digestibility will reduce the faecal output and aquatic pollution. The feeds produced by extruder will increase the carbohydrate digestibility and inclusion of phosphorus with high digestibility and low solubility will greatly helpful in pollution reduction.

Medicated feeds: The feeds produced by the incorporation of antibiotics are generally known as medicated feeds for the control of bacterial diseases.

Generally the antibiotic is added by top dressing with oil or 5% of gelatin. Immunostimulant incorporated diets also come under medicated feeds.

Pigmented feeds: The inclusion of carotenoid pigments in the diets of salmonids for the production of pinkish red colour flesh is commonly practiced. Feed manufacturers are allowed to only 100 mg/kg either astaxanthin or canthaxanthin in salmon diets which is allowable to feed the salmon and trout fishes after 6 months of the age. The pigmented feeds also used in ornamental fish feeds.

Based on nature of the ingredients used

Purified feeds: The purified diets are generally made by the selection of different individual nutrients such as all amino acids, fatty acids, vitamins, minerals and binders. This diet is applicable for experimental purpose and the palatability is very poor.

Semipurified feeds: This diet is used for the experimental purposes. The diets are made by the combination of purified ingredients such as casein, gelatin, sucrose, glucose, cellulose, dextrin, refined fish oil, cholesterol, carboxy methyl cellulose, vitamins and minerals, Casein to gelatin in should be in the ratio of 4:1. These diets are not much palatable.

Practical feeds: The diet made with all the commercially available ingredients comes in this category.

Feed manufacture techniques

Aquafeed manufacturing is often a challenging task that involves in the production of feed with good water stability, manufacturing difficulties due to high fat content, good keeping quality due to high unsaturated fatty acids and protein content and protecting the water soluble vitamins and minerals from leaching. Though several kinds of feeds are available in the market basically two methods are employed for the production of most of the commercial feeds. They are steam pelleting and extrusion pelleting techniques. For both of the

techniques most of the preparatory procedures and equipments are common except the pelletizer and extruder respectively in steam pelleting and extrusion pelleting. The basic steps in feed manufacture:

Grinding/particle size reduction

The particle size of the different feed ingredients varies while arrives the feed mill. The grinding process reduces the particle size of the feed ingredients and increases the surface area to facilitate uniform mixing, pelleting and digestibility of finished feed as well as prevent blockages of die. Grinding is an expensive process in feed manufacture and hence over-grinding significantly increase the feed production cost. The ingredients with high fat contents mainly meat meal and dry fish meal are most difficult to grind than the low fat ingredients. There are different grinding mills available and the most popular size reduction system used in aquafeed manufacture is hammer mills. The other grinding mills are plate mills, attrition mills and air-swept pulverizers. The plate mills are not suitable for aquafeed production because they are incapable of producing fine particles. Air-swept pulverizers are common in aquafeed production when the required particle size is $100\ \mu$ or less. Also air-swept pulverizers are desirable for grinding high fat ingredients where air pressure is used for control the grinding rather screen. When the hammer mill is alone used for grinding high fat ingredients it is advisable to use along with low fat ingredients such as de-oiled rice bran and soy bean meal to avoid screen clogging with fatty materials. While using air-swept pulverizers, hammer mills are required for initial particle size reduction. In hammer mills the grinding chamber composed of moving or fixed hammers attached to the rotors which breakup the incoming material and forced through the different size of steel screen depending on the required particle size. There are lot of ferrous materials may present in the ingredients and hence magnetic protection should be given to remove the iron particles time to time before feeding the raw material into hammer mill.

Batching

Batching is the process in which the drawing of

different feed ingredients from its respective storage bins as per the feed formula and weighing in the weighing scale will be done mechanically by providing comments in the computer. The batching size may be one or two tones as per the capacity of the feed mill. Each batch will take 3 to 4 minutes to complete the operation.

Mixing

Finely ground ingredients mixed together properly in a desired proportion to form a homogenous blend. The uniform size particles of blend ingredients reduce the segregation of particles and produce pellets of uniform formulation. The mixing time should be optimum and insufficient or over mixing may cause particle segregation and non-homogenous mixing. The micronutrients should be mixed separately with fillers (rice bran or wheat bran) to make considerable volume before mix with other formula ingredients to reduce the non-homogeneity in the feed. At the time of batching, the dry ingredients should be mixed thoroughly first followed by the liquid ingredients addition should be done as the mixing continues. The mixer efficiency should be checked periodically by collecting the samples from the mixer for analyzing the known markers such as salt in the feed formula. Mixing can be done by mixers which are either batch or continuous mixture. The batch mixers (paddles, augers and ribbons) only useful in small scale feed production units where as in feed industries continuous mixers should be in place. Generally, in the feed industry horizontal and vertical ribbon mixers are used for uniform mixing. Nanta mixers and turbine mixers are also used less frequently. Twin-shell blenders are used in mixing the micronutrients with the carrier materials.

Pre-processing or Pre-conditioning

In a feed mill different kinds of diets are produced for same species or for different species. The diet production specifications may vary among the diets. Therefore, proper attention must be taken to ensure high quality feeds are produced consistently. Conditioning is the term used to prepare a feed mixture for pelleting which includes thermal and

mechanical processing. The preprocessing condition will vary for steam pelleting and extrusion pelleting. The conditioning is done at conditioning chamber that may be a same diameter cylinder or differential Diameter Cylinder (DDC), which is common in extrusion operation. This chamber contains agitators that mix the incoming feed mixture and conditions through the thermal processing which are achieved by addition of steam. For compressed pellets, the feed mixtures remain in the condition chamber for about 30 seconds where as for extrusion pellets it may be prolonged to about 2 minutes. Besides thermal processing, the heat generated by the shear force (pressure) applied along the barrel and frictional energy generated as the feed mixture forcedly pass through the narrow gap created by the presence of cone in the tapered outlet in the chamber that helps the starchy materials to get gelatinized for the production of extrusion pellets. A phase transition analyzer is used to determine the phase transition temperature of individual ingredients or complete feed. Generally for extrusion cooking the temperature is maintained around 120-150°C in the DDC.

Pelleting

Pelleting process converts the feed mixture into durable forms of physical characteristics of feed that is suitable for feeding. The feed mash is fed into the die and comes out the die whole where cutter assembly cut the pellets into defined length according to the speed of the cutter. The pellets may be a compressed one or expanded one according to the conditioning in the conditioning chamber. The different types of pellets are as follows:

Compressed Pelleting by pelletizer

Compressed pellet process forces the feed mixture into the conditioning chamber where dry steam is applied for about 30 seconds to increase the temperature of feed mixture to about maximum of 90°C together with the moisture content around 15-16% through the die holes in the metal die by the action of rollers in the ring die. Here the combination of heat, moisture and pressure helps to gelatinize the feed mixture into compressed pellet with the bulk density beyond 0.5 g/cm³ while it is coming out from the ring die; they are

cut into desired length. The pellet quality is influenced mainly by fat and moisture content of the feed. The fat content below 2 % makes the pellet too hard and increase dustiness whereas the fat content beyond 8-10% makes pelleting more difficult due to increased lubrication and insufficient compression. Hence optimum fat content is more advisable. The moisture content of the pellet affects the hardness of the pellet. The pellets with high moisture content results in softy pellets due to insufficient compression where are insufficient moisture content produce crumbly and dry pellets. The lubricating power of moisture and fat will reduce electric power consumption and also increase the life span of the die. The feed produce by the compressed pelleting will be a sinking feed with great water stability.

Extrusion dry pelleting by extruders

The extruded pellets also produced in the same way as compressed pellets produced but involves the use of different physical conditions and different type of dies. The process allows the feed mill operator to have a control over addition of large quantity of lipid as top dressing and buoyancy of pellet by managing the bulk density. There are two kinds of extruders, single screw and twin screw extruders are used for extruded feed production. As the name spells the single screw extruder contains single shaft in the DDC whereas in twin screw extruder, co-rotating twin shafts are present. The capital equipment cost of twin screw extruder is one and half to two times higher than single screw extruder with comparable hourly production capacity. The advantages of twin screw extruders are,

1. It can be used for the production of feed with very high internal fat content above 17% fat
2. Products with high levels of fresh meat (above 35%)
3. Ultra small sized products (0.5-2.0 mm dia products)
4. Co-extruded products.

In a single screw extruder it is very difficult to produce a product with more than 7% of fat content because the higher fat actually provides lubricity and reduce friction with in the extruder barrel. The extruder

works by increasing the temperature to 120-150°C in a pressurized DDC through shear force and increasing the moisture content to about 22-26% which makes the starch ingredients to get gelatinized. The preconditioned mixture becomes dough like consistency in the extrusion barrel and the pressure increases as the dough move towards the die which is sufficient to convert the water vapour into liquid. As the pellet leaves the die hole, the sudden reduction in pressure take place that cause the instant expansion of water vapor to evaporate and allow the pellet to form an air pockets. Because of the high gelatinization the durability and water stability of pellet will be excellent and the lower bulk density of 0.3-0.4 g/cm³ makes the pellets to float.

In the extruder, post extrusion pressure chamber is also available which is known as External Density Management System (EDMS) where desired pressures are maintained in the knife enclosure by a special airlock through which the product discharges. The special airlock system is created by compressed air steam which can be used to generate the required pressure in the chamber, as the pressure increases in the chamber the water vapour point increases which reduce product flash off expansion and thus reduce product bulk density. This technique used for the production of shrimp feed in the twin screw extruder with sinking capacity. The advantages of extrusion cooking are that increase the digestibility of carbohydrates and there by the energy supplied by carbohydrates and eliminates the need for non nutrient binders. The extruded feed reduce the fine production during transportation and water stability is better than steam pellets that is at least 10 h which can be very much suitable for slow feeding fishes.

Pellets from Universal Pellet/ Cooker (UP/C)

The universal pellet/cooker is combination of compressed pelleting and extrusion cooking and hence having the combined benefits of both systems. In UP/C process the preprocessing is around 3 minutes that results in 40-50 % of starch gelatinization. The feed mixture moisture is achieved about 16-18% due to less steam and moisture addition. The higher feed production of

18-20 mt/h is achieved in UP/C by accelerating the speed of the rotor in the extrusion barrel 2-3 times faster than conventional extruder which can produce 12-14 mt/h. Due to faster rotor rotation along with lower moisture content increase the frictional energy over the feed mixture which enhances the gelatinization up to 80%. The bulk density of UP/C produced pellets in the range of 0.5-0.65 g/cm³. Due to lower moisture content the pellets can be dried in cooler rather in drier. The pellets appear without glaze unlike compressed pellets hence, the top dressing of oil can be go up to 30%. By the faster knife speed, crumbles can be produced without much fines in UP/C method.

Drying, cooling and crumbling of compressed pellets

The compressed pellets are passed through cooler-drier assembly where it spread thinly in a moving belt in horizontal cooler and cool air is blown through the pellets. In case of vertical cooler the hot pellets are drop down from a cooling tower due to its temperature its moisture content gets dried and cooled due to air flow. The whole operation takes around 10-15 min and the final product reaches the moisture content of 10%. This product goes to storage bins or passed through the corrugated rollers of crumbler to make them crumbles of required size. Then the crushed crumbles separated according to the size in a shaking sieve. The fine particles produced send back to feed mixture for further feed pellet production.

Drying, oil coating and cooling of extruded pellets

Extruder pellets contains more moisture than the compressed pellets and hence required to heated at 70°C by hot air/steam to reduce the moisture content to 10-12%. In the extruded pellet drier the product has to move into 4 layers of beds and hot air need to pass over the pellets in a crosscurrent fashion which will take at least 30-45 minutes based on the diameter of the pellet. After drying the dried extruded pellets are shifted and sieved in a shaking, vibrating sieve assembly to remove the over sized and under sized pellets and the correct sized pellets are transferred to vacuum oil coater for oil coating at 60°C. Then

the pellet comes to cooler, where it gets cooled by blowing cooling air in a vertical cooler. Once the pellet is completely cooled sieved in a shaking sieve assembly to remove fines and then goes to storage bins.

Bagging and storage

The pellet feed is weighed automatically in a predetermined quantity in a computer assisted

electronic balance and transferred to polyethylene bags with inner liner and get stitched. After that stitched bags are sent through conveyer to the warehouse for storage where the bags will be shifted manually by labours and stored in wooden raised platform for proper ventilation. The stacked height of feed bags should be optimum to avoid physical damages to pellets.

Bomb Calorimetry

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Introduction

Calorimetry is the science of measuring quantities of heat and the instrument that is used for heat measurement is known as calorimeter. Bomb calorimeter is one such instrument used to measure the calorific values of solid or liquid ingredients. Heats of formation for organic compounds are generally determined by measurements of heats of combustion or calorific values. Heats of combustion or calorific value is defined as number of heat units liberated by a unit mass of a sample when burned with oxygen in an enclosure of constant volume that is bomb or in other words, the heat liberated by the combustion of all carbon, hydrogen and even other elements such as sulfur, nitrogen with oxygen to form carbon dioxide and water. Heat energy measured in the bomb calorimeter is expressed either in calorie (cal), British thermal unit (Btu) or Joule (J). 1 cal = 4.18 J; 1 Btu = 251.99 cal.

Bomb calorimeter

The bomb calorimeter consists of 4 important parts. They are bomb, bucket, insulating jacket and thermometer.

Bomb: The bomb or vessel in which the feed materials are burned hence, it must be a strong, thick-walled corrosion free chromium-nickel alloy and can be opened for inserting the sample, for removing the products of combustion. The bomb should be provided with valves for filling the bomb with oxygen under pressure and for releasing residual gases after completion of a test. It also provide with electrodes to carry an ignition current to a fuse wire. At times of combustion internal pressure may reach up to 1500 psig, hence, oxygen bombs are constructed to

withstand pressures of minimum 3000 psig.

Bucket: The bucket or container is provided for holding the bomb in a measured quantity of water along with a probe to read temperature and a stirrer to promote rapid thermal equilibrium without allowing excessive heat in the form of mechanical energy. Buckets are made with a highly polished outer finish to minimize the absorption and emission of radiant heat.

Insulating Jacket: The calorimeter jacket is holds the bomb and bucket that serves as a thermal shield by controlling any heat transfer between the bucket and surroundings. The jacket will minimize the effects of radiant energy and changes in room temperature during a test. Anyone of the 3 major types of jacket systems are employed in bomb calorimeter namely uncontrolled or plain insulating jacket, adiabatic system and isoperibol mode.

Thermometer: Precise measurement of temperature is very essential in bomb calorimeter which is achieved by any one of the thermometers like mercury-in-glass thermometers, platinum resistance thermometers, quartz oscillators and thermistor systems.

Standardization of calorimeter

Before analyzing a material with an unknown heat of combustion in a bomb calorimeter, the **energy equivalent** or **heat capacity** or **water equivalent** of the calorimeter must first be determined. This is the sum of the heat capacities of the components, the metal bomb, the bucket and the water in the bucket. Since, the exact amount of each of the metals used

in the bomb and bucket is difficult to determine and it is changing continually with use. Hence, energy equivalents are determined empirically at regular intervals by burning a sample of a standard material, benzoic acid with a known heat of combustion (6319 cal/g) under controlled and reproducible operating conditions. Benzoic acid is used as a reference material because it burns completely in oxygen and it is not hygroscopic and readily available in very pure form. The amount of heat generated by the reference sample is determined by multiplying the heat of combustion of the reference material by the weight of the sample burned which is divided by the temperature rise generated in the test, resultant energy equivalent for this particular calorimeter.

Water equivalent (W) = $(M \times H + C_1 + C_2) / (\text{Temperature rise})$

Where, M—Weight of benzoic acid; H- Heat of combustion of benzoic acid; C1- Heat of combustion of fuse wire (1400 cal/g); C2-Heat of combustion of acids

Calorimetric corrections

The factors which cannot be held constant will require corrections to compensate for their effects. The burning fuse wire in the bomb contributes additional heat to the bomb combustion. As the amount of fuse wire consumed in each test may vary, the energy contributed by the fuse must be determined for each test and a correction applied to compensate for this variance.

In normal combustion all sulfur in a feed material is oxidized to sulfur dioxide and discharged with the stack gases. Whereas if the same material is burned in an oxygen bomb, the oxidation is carried further to trioxide which then reacts with moisture in the bomb to form sulfuric acid. Similarly, in normal combustion nitrogen in the air is not affected. But when a feed sample is burned in bomb, some of the molecular nitrogen trapped in the bomb is oxidized and combined with water vapor to form nitric acid. Hence corrections required for the heat energy contributed by these acids. American Society for Testing and Materials (ASTM) and ISO test methods contain procedures for calculating the correction which must be applied to account for the heat liberated in the formation of these acids.

Energy estimation from test samples

After the energy equivalent of the calorimeter has been estimated, the calorimeter is ready for testing feed samples. Samples of known weight are burned and the resultant temperature rise is measured and recorded. The amount of heat of combustion or calorific value of each sample is determined by multiplying the observed temperature rise by the energy equivalent of the calorimeter and divide by the weight of the sample. The calorimetric corrections need to be considered for acid and fuse wire.

$$\text{Gross calorific value} = \frac{(T \times W - (C_1 + C_2))}{(\text{Weight of sample})}$$

Where, T- Temperature rise; W- Water equivalent of the calorimeter

Estimation of gross energy value from feed stuff

Principle

The gross energy is the measure of amount of heat produced from unit volume of feed in a bomb calorimeter when it is completely burnt down to its ultimate oxidation products, CO₂ and H₂O.

Procedure

1. Bomb assembly and filling the bomb: Weigh the feed material in the range of 0.1 – 0.2 g and place it in the metal crucible. Connect a 7 cm platinum wire to the two electrodes of the bomb. Keep the crucible over the spring stand in such a way that it should have contact with ignition wire loop. Close the bomb tightly without shaking and fill oxygen into the bomb through its inlet valve up to 30-lbs pressure per square inch.
2. Fill exactly 400 ml of water in the bucket and temperature of water should be 2-3°C lower than the jacket temperature (jacket-constant temperature).

3. Place the bomb in the holder fitted in the bucket and connects with electrodes. Then, place the stir, thermometer and lid of the calorimeter in their proper position.
4. Keep the bomb calorimeter in determination mode for test samples and for benzoic acid standardization, it should be in standardization mode.
5. Press start menu and enter sample weight, sample id and specify the bomb number.
6. The stirrer will start functioning and after 15 min the result will be obtained in the monitor of the bomb calorimeter. The gross energy value is represented as cal/g.
7. Remove the connecting wires, thermometer, stirrer and covering lids. Take out the bomb and release slowly the gas pressure inside the bomb. Open the bomb clean the interior surfaces with a jet of distilled water and wipe with tissue paper before store it.

Atomic absorption spectroscopy: Analysis of minerals

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Introduction

The minerals present in fish include iron, calcium, zinc, iodine, phosphorus, and fluorine. These minerals in the fishes are highly 'bioavailable' meaning that they are easily absorbed by the body. Iron is important in the synthesis of hemoglobin in red blood cells, which is important for transporting oxygen to all parts of the body. Iron deficiency is associated with anemia, impaired brain function and in infants is associated with poor learning ability. Due to its role in the immune system, its deficiency may also be associated with increased risk of infection. Calcium is required for strong bones (formation and mineralization) and for the normal functioning of muscles and the nervous system. It is also important in the blood clotting process. Vitamin D is required for its proper absorption. The intake of calcium, phosphorus and fluorine is higher when small fish are eaten with their bones rather than when the fish bones are discarded. Deficiency of calcium may be associated with rickets in young children and osteomalacia (softening of bones) in adults and older people. Fluorine is also important for strong bones and teeth. Zinc is required for most body processes as it occurs together with proteins in essential enzymes required for metabolism. Zinc plays an important role in growth and development as well in the proper functioning of the immune system and for a healthy skin. Zinc deficiency is associated with poor growth, skin problems and loss of hair among other problems. A deficiency of iodine may lead to goiter (enlarged thyroid gland) and mental retardation in children. It is evident that fish contribute more to people's diets than just the

high quality protein they are so well known for. Fish should therefore be an integral component of the diet, preventing malnutrition by making these macro and micronutrients readily available to the body. The contents of K, Na, Cl, Mg, P and Ca are up to 1 mg/100 g, whereas those of Fe, Zn, Cu and I are less than 1 mg/100g. However, it is difficult to generalize and to establish the mean mineral values, because they depend on several factors such as species, sex, biological cycle, and on the portion of fish analyzed and also on ecological factors, such as season, place of development, nutrient availability, temperature and salinity of the water. Fish flesh is a good mineral source of Ca and P however, it is interesting to take into account that the bony fish skeleton are richer in these elements. Because bone tissue, in its stage of highest crystallization, is constituted by the hydroxyapatite salt, which has Ca and P in a 2.15/1 ratio (w/w), its addition to the flesh could contribute to high Ca and P contents. Fish cannot be eaten with bone in its natural state, as it cannot be chewed or digested. However, it is technically possible to process some fish with bone by careful prior homogenization, which could be incorporated in some manufactured foods, increasing the Ca and P contents and the Ca/P ratio of the meal. Ca and P are necessary to maintain an optimal bone development more of both minerals being required during childhood and growing stages to prevent rickets and osteomalacia. Although Ca, Mg and P are important in bone metabolism and development, other minerals such as Fe, Cu, Zn and Mn are considered to be essential for normal growth and for avoiding several pathologies.

Mineral Composition Analyses by atomic absorption spectroscopy

Estimation of minerals needs to be carried out by atomic absorption spectrophotometer following the di-acid ($\text{HNO}_3/\text{HClO}_4$) digestion method (Astorga *et al.*, 2005).

Apparatus/ Reagents

Atomic absorption spectrophotometer, sand bath, fume hood, concentrated HNO_3 , HClO_4 , Whatman No. 1 filter paper.

Procedure

- Place the samples (2 g) in digestion tubes, to which add concentrated HNO_3 (7 ml), and the content to be kept for overnight digestion in a fume hood until no brown fumes appeared.
 - Continue the digestion over the sand bath with HClO_4 (6 ml) until the color of the solution became pale yellow to colourless.
- Cool the solution and filter through Whatman No. 1 filter paper. Dilute the filtrate with distilled water (50 ml) to be injected in atomic absorption spectrophotometer for determination of minerals.
 - The analyses of Ca, Mg, Na, K, Mn, Cu, Fe, and Zn to be performed by flame atomic absorption spectrophotometry equipped with a hollow cathode lamp containing D2 lamp background correction system.
 - For Se, continuous flow hydride generator coupled with atomic absorption spectrometer should be used (AOAC, 2005).

Suggested readings

- AOAC., 2005. Metals. In: Official methods of analyses of AOAC International, 18th edition, 2005 Chapter 9, (Editor) Dr William Horwitz; Published by AOAC International, Suite 500, 481 North Frederick Avenue, USA, pp. 22-46 (chapter 9), Official methods of Analyses, 18th edition, Association of Official Analytical Chemists, Washington D. C.
- Astorga Espana, M.S., Rodriguez Rodriguez, E.M., Diaz Romero, C., 2005. Sodium, K, Ca, Mg, Fe, Cu and Zn concentrations in molluscs from the Strait of Magellan (Chile): their contribution to dietary intake. International Journal of Food Sciences and Nutrition 56, 337–347.

Amino acids from marine fish and their implications in health and diseases

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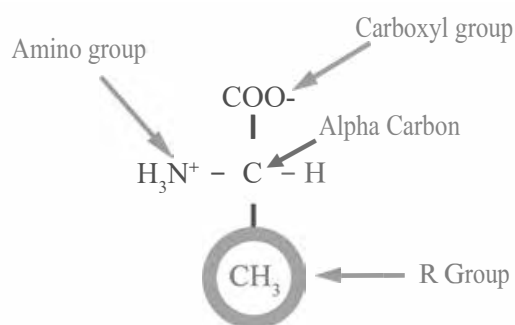
Introduction

An amino acid is any molecule that contains both amino and carboxylic acid functional groups. Amino acid is any one of a class of simple organic compounds containing carbon, hydrogen, oxygen, nitrogen, and in certain cases sulfur. These compounds are the building blocks of proteins. Amino acids are the building blocks (monomers) of protein, and are utilized by every cell in the body for a variety of crucial functions. The shape and other properties of each protein is dictated by the precise sequence of amino acids in it. Normally, we obtain them from our food sources, particularly those high in protein; the body breaks these proteins down into their constituent parts, and then our cells use these to build the specific types of protein each of them needs. Amino acids form short polymer chains called peptides or polypeptides which in turn form structures called proteins. Each amino acid has at least one carboxyl (COOH) group, which is acidic, and one amino (NH₂) group, which is basic.

Each amino acid consists of an alpha carbon atom to which is attached

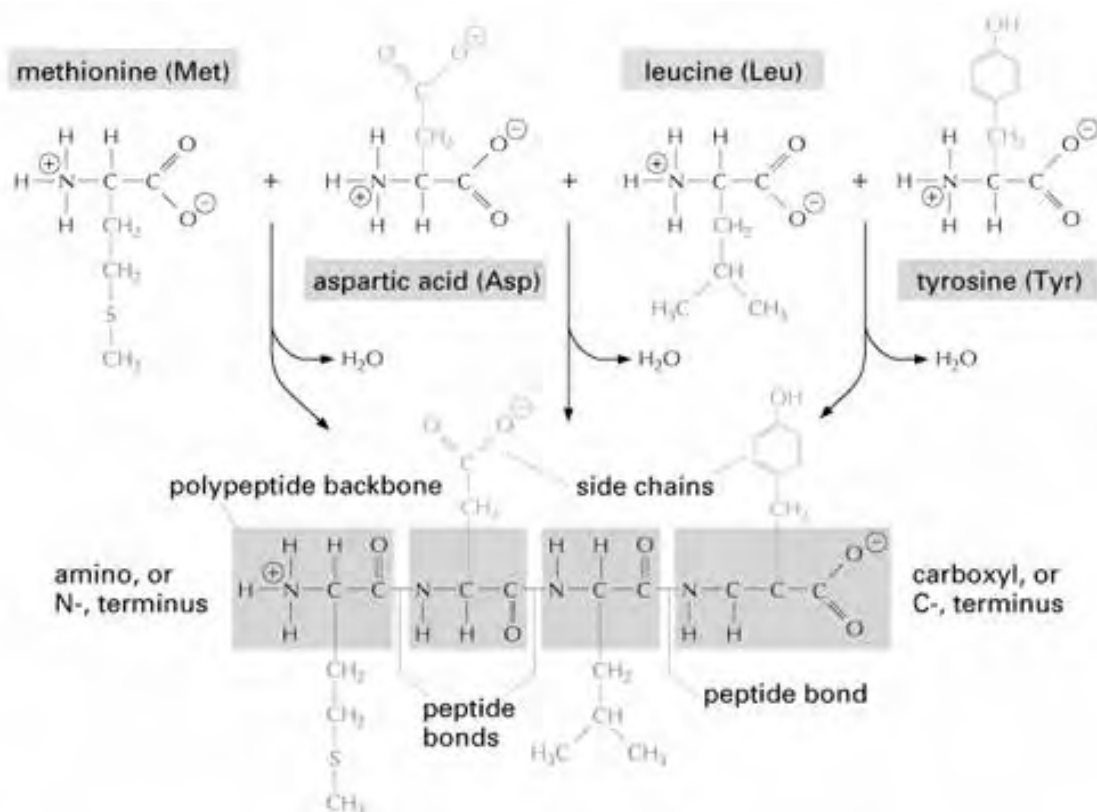
- A hydrogen atom
- An amino group (hence “amino” acid)
- A carboxyl group (-COOH). This gives up a proton and is thus an acid (hence amino “acid”)
- One of 20 different “R” groups. It is the structure of the R group that determines which of the 20 it is and its special properties. The amino acid shown here is alanine.

Amino acids join together in long chains, the amino group of one amino acid linking with the carboxyl group of another. The linkage is known as a peptide



bond, and a chain of amino acids is known as a polypeptide. Each type of protein differs in its amino acid sequence. Thus the sequential position of the chemically distinct side chains gives each protein its individual properties. The two ends of each polypeptide chain are chemically different: the end that carries the free amino group (NH₃⁺, also written NH₂) is called the amino, or N-, terminus; and the end carrying the free carboxyl group (COO⁻, also written COOH) is the carboxyl, or C-, terminus. The amino acid sequence of a protein is always presented in the N to C direction, reading from left to right.

There are two types of amino acids: essential and nonessential. Essential ones are defined as those which the body cannot manufacture on its own and must obtain from food sources (or supplements); nonessential ones, on the other hand, can be produced by our own bodies from an available source of nitrogen and a carbon skeleton, but can also be consumed as supplements. The essential amino acids are isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. The nonessential amino acids are arginine, alanine,



asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, proline, serine, and tyrosine. However, cysteine can partially meet the need for methionine (they both contain sulfur), and tyrosine can partially substitute for phenylalanine. Semi-essential amino acids are ones that can sometimes be made internally if conditions are right. Histidine is considered semi-essential because the body does not always require dietary sources of it. Other amino acids, such as carnitine, are used by the body in ways other than protein-building and are often used therapeutically.

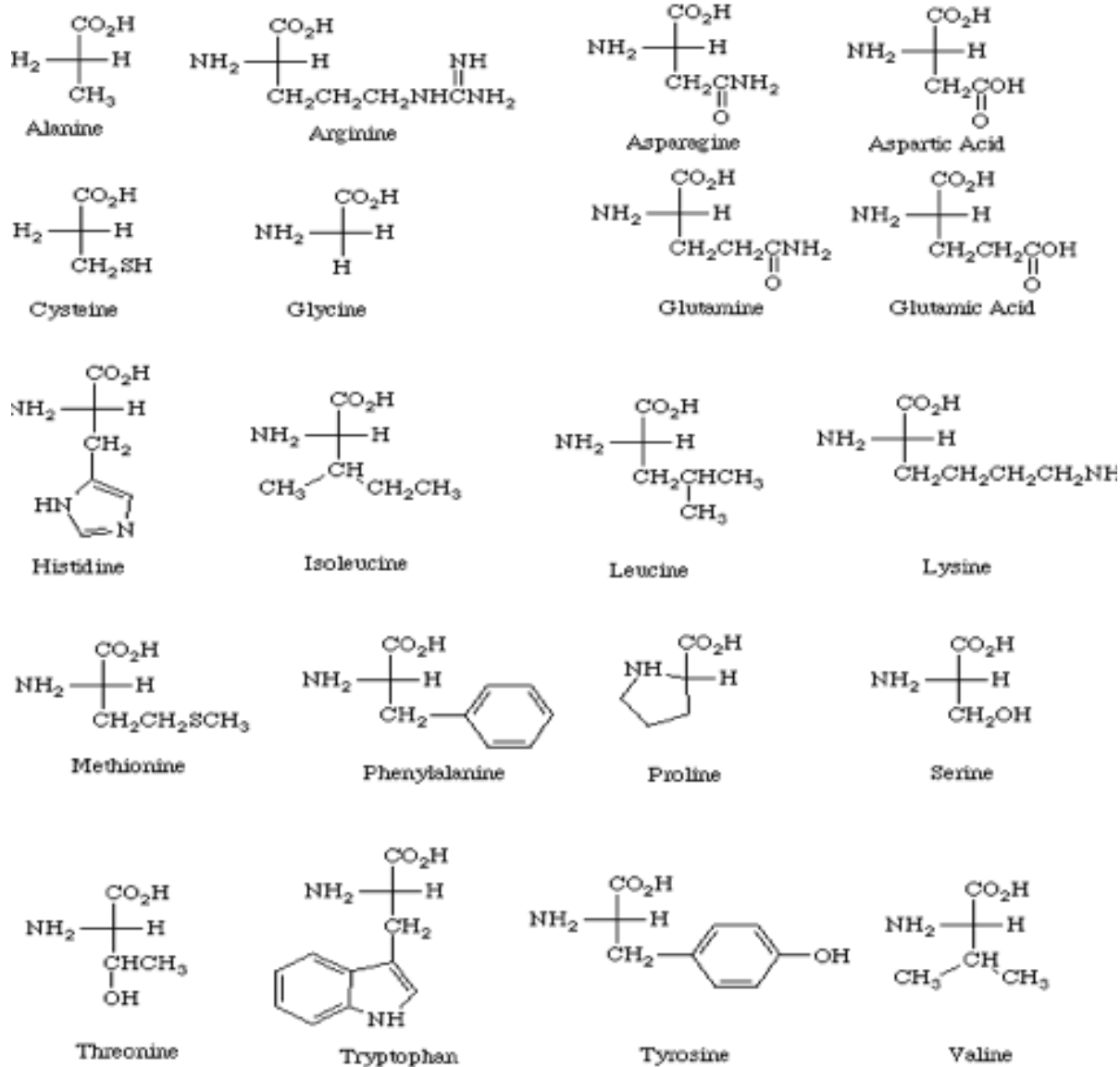
Leucine, isoleucine, and valine are called branched-chain amino acid (BCAAs) because human beings cannot survive unless these amino acids are present in the diet. The combination of these three amino acids makes up approximately one-third of skeletal muscle in the human body. In addition to their role in protein and enzyme synthesis, amino acids are extremely crucial for good health. Amino acids contribute significantly to the health of the nervous system, muscular structure, hormone production, vital organs and cellular structure. Some affects low levels of the essential amino acids result in hormonal imbalances, irritability, low concentration, and depression.

Essential amino acids and their importance

Isoleucine: Isoleucine belongs to a special group of amino acids called branched-chain amino acids (BCAAs), which are needed to help maintain and repair muscle tissue. Leucine and valine are other two branched-chain amino acids. Isoleucine is an essential amino acid that is not synthesized by mammalian tissues. Isoleucine is needed for hemoglobin formation and also helps to maintain regular energy levels. Isoleucine is important for stabilizing and regulating blood sugar and energy levels and is required through the diet as it cannot be produced by our bodies.

Leucine: Leucine is a member of the branched-chain amino acid family, along with valine and isoleucine. The branched-chain amino acids (BCAAs) are found in proteins of all life forms. Leucine ties glycine for the position of second most common amino acid found in proteins with a concentration of 7.5 percent on a molar basis compared to the other amino acids. Leucine is necessary for the optimal growth of infants and for the nitrogen balance in adults. It functions on balancing blood sugar level in the body. It also promotes in the development of the muscle

The molecular structures of the major amino acids are listed below:



tissue. It modulates the level of hormone production and energy regulation. It also provides support by preventing the breakdown of muscles. Deficiency in leucine may include dizziness, irritation, headache, fatigue, etc.

Lysine: Lysine is an essential amino acid that has a net positive charge at physiological pH values making it one of the three basic (with respect to charge) amino acids. Lysine is an essential amino acid because it cannot be synthesized in the body and its breakdown is irreversible. It is an essential building block for all protein, and is needed for proper growth and bone development in children. Lysine helps the body absorb and conserve calcium and it plays an important role

in the formation of collagen.

Methionine: Methionine is an important amino acid that helps to initiate translation of messenger RNA by being the first amino acid incorporated into the N-terminal position of all proteins. Methionine supplies sulfur and other compounds required by the body for normal metabolism and growth. Methionine reacts with adenosine triphosphate to form S-adenosyl methionine. S-adenosyl methionine is the principal methyl donor in the body and contributes to the synthesis of many important substances, including epinephrine and choline. It helps in breaking down of fatty acids, and hence it prevents in building up fatty elements on the artery walls. It also works

significantly in the normal detoxification of liver. It is essential in promoting energy production as well as in muscle building. Methionine, one of the essential amino acids, functions like an effective anti-oxidant by supplying sulfur for inactivating free radicals.

Phenylalanine: Phenylalanine is an essential amino acid that is also one of the aromatic amino acids that exhibit ultraviolet radiation absorption properties with a large extinction coefficient. Phenylalanine is part of the composition of aspartame, a common sweetener found in prepared foods (particularly soft drinks, and gum). Phenylalanine plays a key role in the biosynthesis of other amino acids and some neurotransmitters.

Threonine: This amino acid is perfect in assisting protein balance in the body. Additionally, it helps in the development of collagen and maintaining elasticity in the skin. It also functions of liver. It functions well in reducing liver fat. In addition to other essential amino acids, threonine promotes well balancing of immune system in terms of antibody production and thymus growth.

Tryptophan: Tryptophan is an essential amino acid formed from proteins during digestion by the action of proteolytic enzymes. Tryptophan is also a precursor for serotonin (a neurotransmitter) and melatonin (a neurohormone). This is an essential ingredient for the formation of vitamin B₃. It is responsible for the production of serotonin which is exclusively important for balancing nerve and brain functioning. It is beneficial for controlling hyperactivity among children. It aids in alleviating stress. It works effectively as an appetite suppressant. It also promotes in reducing weight.

Valine: Valine is a branched-chain amino acid (BCAA) that is closely related to leucine and isoleucine both in structure and function. Valine is a constituent of fibrous protein in the body. As a branched-chain amino acid (BCAA), valine has been found useful in treatments involving muscle, mental, and emotional upsets, and for insomnia and nervousness. Valine may help treat malnutrition associated with drug addiction.

Histidine: Histidine is one of the basic (with reference

to pH) amino acids due to its aromatic nitrogen-heterocyclic imidazole side chain. Histidine is the direct precursor of histamine; it is also an important source of carbon atoms in the synthesis of purines. Histidine is needed to help grow and repair body tissues, and to maintain the myelin sheaths that protect nerve cells. It also helps manufacture red and white blood cells, and helps to protect the body from heavy metal toxicity. Histamine stimulates the secretion of the digestive enzyme gastrin.

Non-essential amino acids

Alanine: Alanine is one of the simplest of the amino acids and is involved in the energy-producing breakdown of glucose. L-alanine is created in muscle cells from glutamate in a process called transamination. Alanine comes from the breakdown of DNA or the dipeptides, anserine and carnosine, and the conversion of pyruvate, a compound in carbohydrate metabolism. Alanine is used by the body to build proteins. Alanine is vital for the production of protein, essential for proper function of the central nervous system and helps form neurotransmitters. Alanine is necessary for the promotion of proper blood glucose levels from dietary protein.

Arginine: Arginine is a complex amino acid that is often found at the active (or catalytic) site in proteins and enzymes due to its amine-containing side chain. Arginine is involved in multiple areas of human physiology and metabolism. Arginine plays an important role in cell division, the healing of wounds, removing ammonia from the body, immune function, and the release of hormones. Arginine has a number of functions in the body such as assisting in wound healing, hormone production, immune function and removal of excess ammonia.

Asparagine: Asparagine is the β -amide of aspartic acid synthesized from aspartic acid and ATP (adenosine triphosphate). Asparagine is one of the principal and frequently the most abundant amino acids involved in the transport of nitrogen. Asparagine is very active in converting one amino acid into another (amination and transamination) when the need arises. Asparagine serves as an amino donor in liver transamination processes.

Aspartic acid: Aspartic acid is alanine with one of the β hydrogens replaced by a carboxylic acid group. Aspartic acid is a part of organic molecules containing an amino group, which can combine in linear arrays to form proteins in living organisms. Although aspartic acid is considered a non-essential amino acid, it plays a paramount role in metabolism during construction of other amino acids and biochemicals in the citric acid cycle. Among the biochemicals that are synthesized from aspartic acid are asparagine, arginine, lysine, methionine, threonine, isoleucine, and several nucleotides.

Cysteine: Cysteine is a naturally occurring hydrophobic amino acid which has a sulfhydryl group and is found in most proteins. Cysteine is one of the key components in all living things. N-acetyl cysteine (which contains cysteine) is the most frequently used form of cysteine. N-acetyl-L-cysteine (NAC) helps break down mucus and detoxify harmful substances in the body. Both cysteine and NAC have been shown to increase levels of the antioxidant glutathione.

Cystine: Cystine is the product of an oxidation between the thiol side chains of two cysteine amino acids. As such, cystine is not considered one of the 20 amino acids. This oxidation product is found in abundance in a variety of proteins such as hair keratin, insulin, the digestive enzymes chymotrypsinogen A, papain, and trypsinogen where it is heavily involved in stabilizing the tertiary structure of these macromolecules.

Glutamine: Glutamine is one of the twenty amino acids generally present in animal proteins. Glutamine is the most abundant amino acid in the body. Over 61% of skeletal muscle tissue is glutamine. It contains two ammonia groups, one from its precursor, glutamate, and the other from free ammonia in the bloodstream. Glutamine is involved in more metabolic processes than any other amino acid. Glutamine is converted to glucose when more glucose is required by the body as an energy source. Glutamine assists in maintaining the proper acid/alkaline balance in the body, and is the basis of the building blocks for the synthesis of RNA and DNA.

Glutamic acid: Glutamic acid is biosynthesized from a number of amino acids including ornithine and arginine. When aminated, glutamic acid forms the

important amino acid glutamine. Because it has a carboxylic acid moiety on the side chain, glutamic acid is one of only two amino acids (the other being aspartic acid) that has a net negative charge at physiological pH. This negative charge makes glutamic acid a very polar molecule and it is usually found on the outside of proteins and enzymes where it is free to interact with the aqueous intracellular surroundings. On a molar basis, glutamic acid is incorporated into proteins at a rate of 6.2 percent compared to the other amino acids.

Glycine: Glycine is the simplest amino acid and is the only amino acid that is not optically active (it has no stereoisomers). The body uses it to help the liver in detoxification of compounds and for helping the synthesis of bile acids. It has a sweet taste and is used for that purpose. Glycine is essential for the synthesis of nucleic acids, bile acids, proteins, peptides, purines, adenosine triphosphate (ATP), porphyrins, hemoglobin, glutathione, creatine, bile salts, one-carbon fragments, glucose, glycogen, and l-serine and other amino acids.

Proline: Proline is a non-essential amino acid that is involved in the production of collagen and in wound healing. Proline is the precursor for hydroxyproline, which the body incorporates into collagen, tendons, ligaments, and the heart muscle. Proline plays important roles in molecular recognition, particularly in intracellular signalling. Proline is an important component in certain medical wound dressings that use collagen fragments to stimulate wound healing.

Serine: The methyl side chain of serine contains a hydroxy group making this one of two amino acids that are also alcohols. Serine plays a major role in a variety of biosynthetic pathways including those involving pyrimidines, purines, creatine, and porphyrins. Serine has sugar-producing qualities, and is very reactive in the body. It is highly concentrated in all cell membranes, aiding in the production of immunoglobulins and antibodies.

Tyrosine: Tyrosine is metabolically synthesized from phenylalanine to become the para-hydroxy derivative of that important amino acid. Tyrosine is a precursor of the adrenal hormones epinephrine,

norepinephrine, and the thyroid hormones, including thyroxine. L-tyrosine, through its effect on neurotransmitters, is used to treat conditions including mood enhancement, appetite suppression, and growth hormone (HGH) stimulation.

Hydroxyproline: Hydroxyproline is derived from the amino acid proline and is used almost exclusively in structural proteins including collagen, connective tissue in mammals, and in plant cell walls. An unusual feature of this amino acid is that it is not incorporated into collagen during biosynthesis at the ribosome, but is formed from proline by a posttranslational modification by an enzymatic hydroxylation reaction. Non-hydroxylated collagen is commonly termed pro-collagen.

Non protein amino acids

In humans, non-protein amino acids also have important roles as metabolic intermediates, such as in the biosynthesis of the neurotransmitter gamma-aminobutyric acid. These class of amino acids are described in detail.

Carnitine: Carnitine is a non-essential amino acid produced in the liver, brain and the kidneys from the essential amino acids methionine and lysine. Carnitine is a nutrient responsible for the transport of long-chain fatty acids into the energy-producing centers of the cells (known as the mitochondria). Carnitine is recommended as a daily supplement to help maintain blood lipid profile and promote fatty acid utilization within heart muscle.

Carnosine: Carnosine is a dipeptide composed of the covalently bonded amino acids alanine and histidine and is found in the brain, heart, skin, muscles, kidneys and stomach. Carnosine is one of the most important and potent natural antioxidant agents which act as universal antioxidants both in the lipid phase of cellular and biological membranes and in the aqueous environment protecting lipids and water-soluble molecules like proteins (including enzymes), DNA and other essential macromolecules from oxidative damage mediated by reactive oxygen species and lipid peroxides.

Creatine: Creatine is a natural derivate of an amino acid and is synthesized in the liver, kidneys and pancreas out of arginine, methionine and glycine. Creatine functions to increase the availability of cellular ATP, adenosine triphosphate. Creatine works by acting on mechanisms of ATP by donating a phosphate ion to increase the availability of ATP. Creatine is stored in muscle cells as phosphocreatine and is used to help generate cellular energy for muscle contractions.

Citrulline: Citrulline is a precursor to arginine and is involved in the formation of urea in the liver. Arginine is a contributing member of the various amino acids found in the urea cycle, which is responsible for detoxifying ammonia. Citrulline supports the body in optimizing blood flow through its conversion to L-arginine and then nitric oxide (NO).

Gamma-aminobutyric acid: Gamma-aminobutyric acid (GABA) is a non-essential amino acid formed from glutamic acid with the help of vitamin B6. GABA (gamma-aminobutyric acid) is found in almost every region of brain, and is formed through the activity of the enzyme glutamic acid decarboxylase (GAD). GABA serves as a inhibitory neurotransmitter to block the transmission of an impulse from one cell to another in the central nervous system.

Glutathione: Glutathione (GSH) is a tripeptide composed of three different amino acids: glutamate, cysteine and glycine that have numerous important functions within cells. Glutathione plays a role in such diverse biological processes as protein synthesis, enzyme catalysis, transmembrane transport, receptor action, intermediary metabolism, and cell maturation. Glutathione acts as an antioxidant used to prevent oxidative stress in most cells and help to trap free radicals that can damage DNA and RNA.

Ornithine: Ornithine plays an important role in the urea cycle and is the precursor of the amino acids citrulline, glutamic acid, and proline. Another primary role of ornithine is being an intermediate in arginine biosynthesis, although this is due to its participation in the urea cycle (responsible for the production of urea). Ornithine is not directly incorporated into proteins and enzymes and does not have a codon in the genetic code.

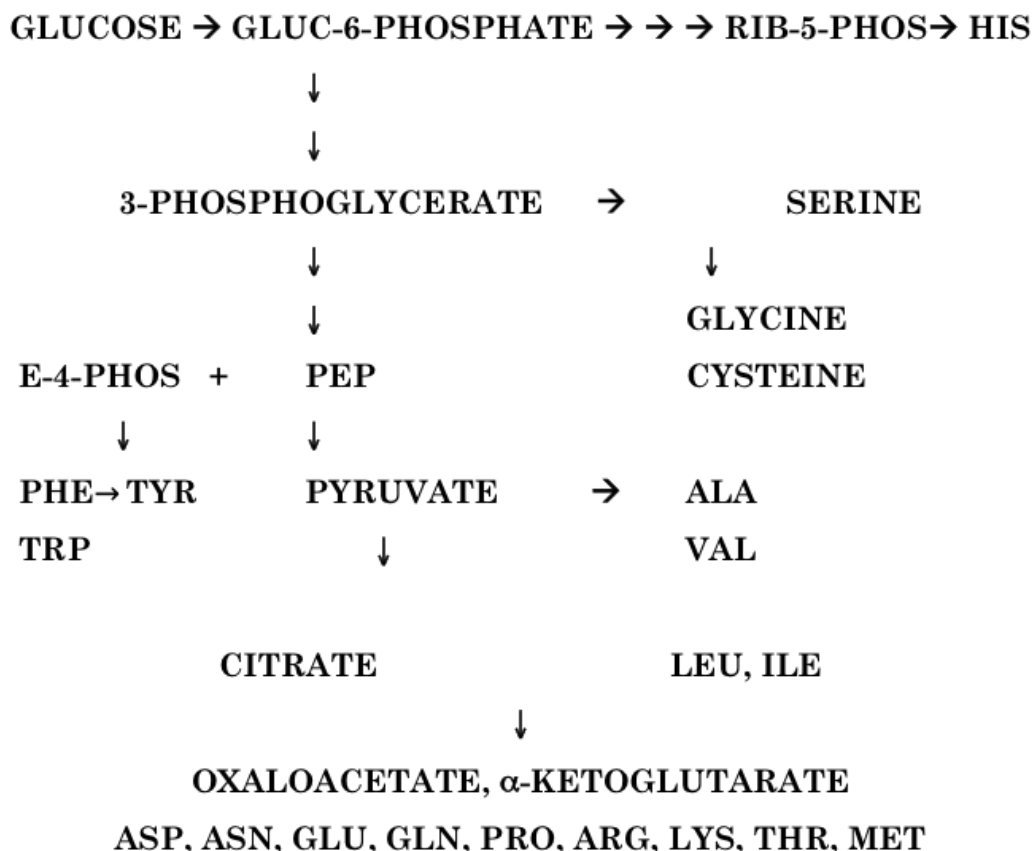
Taurine: Taurine is a non-essential sulfur-containing amino acid that functions with glycine and gamma-aminobutyric acid as a neuroinhibitory transmitter. Taurine is the body's water soluble anti-oxidant, and inhibitory neurotransmitter. The major antioxidant activity of taurine derives from its ability to scavenge the reactive oxygen species hypochlorite. Taurine plays an important role in numerous physiological functions.

Metabolism of amino acids

In plants, nitrogen is first assimilated into organic compounds in the form of glutamate, formed from alpha-ketoglutarate and ammonia in the mitochondrion. In order to form other amino acids, the plant uses transaminases to move the amino group to another alpha-keto carboxylic acid. For example, aspartate aminotransferase converts glutamate and oxaloacetate to alpha-ketoglutarate and aspartate. Other organisms use transaminases for amino acid synthesis too.

Transaminases are also involved in breaking down amino acids. Degrading an amino acid

often involves moving its amino group to alpha-ketoglutarate, forming glutamate. In many vertebrates, the amino group is then removed through the urea cycle and is excreted in the form of urea. However, amino acid degradation can produce uric acid or ammonia instead. For example, serine dehydratase converts serine to pyruvate and ammonia. Nonstandard amino acids are usually formed through modifications to standard amino acids. For example, homocysteine is formed through the transsulfuration pathway or by the demethylation of methionine via the intermediate metabolite S-adenosyl methionine, while hydroxyproline is made by a posttranslational modification of proline. Microorganisms and plants can synthesize many uncommon amino acids. For example, some microbes make 2-aminoisobutyric acid and lanthionine, which is a sulfide-bridged derivative of alanine. Both of these amino acids are found in peptidic antibiotics such as alamethicin. While in plants, 1-aminocyclopropane-1-carboxylic acid is a small disubstituted cyclic amino acid that is a key intermediate in the production of the plant hormone ethylene.



The health benefits of amino acids

Amino acids are needed to build the various proteins used in the growth, repair, and maintenance of body tissues. Amino acids play innumerable roles in human health and disease. Alanine is necessary for the promotion of proper blood glucose levels from dietary protein. Alanine stimulates lymphocyte production and may help people who have immune suppression. Alanine strengthens the immune system by producing antibodies. L-arginine is used by the immune system to help regulate the activity of the thymus gland, which is responsible for manufacturing T lymphocytes. The body uses arginine to produce nitric oxide. Nitric oxide is an endogenous messenger molecule involved in a variety of endothelium-dependent physiological effects in the cardiovascular system. In the central nervous system, asparagine is needed to maintain a balance, preventing over nervousness or being overly calm. Aspartic acid can help protect the liver from some drug toxicity and the body from radiation. Carnosine is the water-soluble counterpart to vitamin E in protecting cell membranes from oxidative damage. L-carnosine supports healthy aging and cellular rejuvenation by its effects on two mechanisms: glycosylation and free radical damage. Cysteine strengthens the protective lining of the stomach and intestines, which may help prevent damage caused by aspirin and similar drugs. The health benefits of glutamine include immune system regulation, nitrogen shuttling, oxidative stress, muscle preservation, intestinal health, injuries, and much more. Glycine is an inhibitory amino acid with important functions centrally and peripherally. Glycine may be indicated to help alleviate the symptoms of spasticity. Histidine is known to be vital in the maintenance of the myelin sheaths surrounding nerves, particularly the auditory nerve and is used to treat some forms of hearing disability. Isoleucine is necessary for the optimal growth of infants and for nitrogen balance in adults. Leucine is used as a source for the synthesis of blood sugar in the liver during starvation, stress, and infection to aid in healing. Lysine is used in managing and preventing painful and unsightly herpes sores caused by the herpes simplex virus (HSV). Methionine is both an antioxidant and lipotrope, meaning it helps remove fat from the liver. Phenylalanine is used to treated

depression, rheumatoid arthritis and osteoarthritis, menstrual cramps, Parkinson's disease, vitiligo, and cancer. Proline is an important component in certain medical wound dressings that use collagen fragments to stimulate wound healing. Serine is needed for the metabolism of fats and fatty acids, muscle growth, and a healthy immune system. Taurine helps regulate the contraction and pumping action of the heart muscle and it helps regulate blood pressure and platelet aggregation. Threonine may enhance immunity by assisting in the production of agents that fight viral infections. L-theanine reduces stress and anxiety without the tranquilizing effects found in many other calming supplements. Tryptophan is important for the production of serotonin. Increasing tryptophan may help to normalize sleep patterns. Tyrosine may act as an adaptogen, helping the body adapt to and cope with the effects of physical or psychological stress by minimizing the symptoms brought on by stress. As a branched-chain amino acid (BCAA), valine has been found useful in treatments involving muscle, mental, and emotional upsets, and for insomnia and nervousness. Creatine supplements fuels and enhances short bursts of high-energy exercise. Creatine prevents the body from relying solely on the process of glycolysis. Citrulline supports the body in optimizing blood flow through its conversion to L-arginine and then nitric oxide (NO). GABA has been used in the treatment of depression, manic-depressive (bipolar) disorder, seizures, premenstrual dysphoric (feeling depressed) disorder, and anxiety. Glutathione are necessary for supporting the immune system, glutathione is required for replication of the lymphocyte immune cells.

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Fatty acids from marine fish and their implications in health and diseases

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Introduction

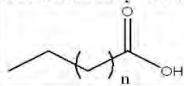
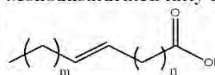

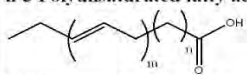
Fatty acids are carboxylic acids with long hydrocarbon chains (usually C_{12-22}). Dietary fats are used to build every cell in the body and cell membranes are made of a variety of individual fatty acids. The essential fatty acids from marine fish have protective mechanisms against coronary heart disease, which became apparent in the investigations of the health status of Greenland Eskimos who consumed diets very high in fat from seals, whales, and fish, and yet had a low rate of coronary heart disease events. This paradox was explained by the fact that Eskimos consumed contained large quantities of the very-long-chain and highly polyunsaturated fatty acids with C_{20-22} carbons and 5-6 olefinic bonds, which are abundant in marine fish, and are scarce or absent in land animals and plants. Classification of fatty acids is based on to denote hydrocarbon chain length and number and positions of olefinic bonds. However, the most accepted system of classification is based on the number of olefinic bonds. Saturated fatty acids (SFAs) do not possess olefinic bonds in hydrocarbon chain. Examples of SFAs are lauric acid, myristic acid, palmitic acid, stearic acid, arachidic acid, behenic acid, and lignoceric acid (Table 1). Monounsaturated fatty acids (MUFAs) possess one double bond, the typical examples being myristoleic acid, palmitoleic acid, elaidic acid, oleic acid, erucic acid, and nervonic acid. Fatty acids with ≥ 2 double bonds are termed as polyunsaturated fatty acids (PUFAs). The tetrahedral bond angles on carbon results in a molecular geometry for saturated fatty acids that is relatively linear. Olefinic bonds in hydrocarbon chain of unsaturated fatty acids results in kinks in their structure results in weak stacking. PUFAs are broadly divided into two major

families' viz., ω -3 and ω -6 PUFAs (otherwise termed as n-3 and n-6 PUFAs). However, ω -3 fatty acids are found to be abundantly available in marine sources particularly fish and phytoplanktons. These fatty acids affect many physiological processes including cognitive function, visual acuity, immunosuppressive, and anti-thrombic activities along with having major role on glucose and lipid metabolism. Table 1 illustrates the details regarding the differential changes of fatty acids and their structures including their abbreviated formulae, molecular formulae, and molecular weight.

Biosynthetic route of fatty acids

Fatty acid synthesis is a metabolic process to combine eight C_2 – moieties ($-CH_2C(=O)$ group from $CH_3COSCoA$) to synthesize saturated fatty acid with C_{16} -moiety ($C_{16}H_{32}O_2$), which thereafter modified to form homologous fatty acid analogues. These modifications include: elongase-catalyzed chain elongation to synthesize fatty acids with longer hydrocarbon chain, e.g., stearic acid ($C_{18}H_{36}O_2$), arachidic acid ($C_{20}H_{40}O_2$), and so on. These SFAs, on desaturation yield unsaturated fatty acid analogues. In general, fatty acid synthesis takes place in cytoplasm of liver, adipose, central nervous system, and lactating mammary gland tissues of human. Glycolytic breakdown of glucose yields acetyl CoA through pyruvate ($CH_3COCOOH$) by aerobic glycolysis that is starting material for fatty acid synthesis. Acetyl CoA serves as substrate to synthesize citrate that transported out of mitochondria to cytosol and generates acetyl CoA. The overall reaction of anabolism of fatty acids to form unsaturated fatty acids is as follows:

Table 1. Nomenclature of fatty acids

Saturated fatty acids 	m	n	Abbreviated nomenclature		Molecular formulae	Molecular weight
			With respect to -COOH group	With respect to n (or ω)-group		
Butyric acid	0	1	4:0	4:0	C ₄ H ₈ O ₂	88.11
Caproic acid	0	3	6:0	6:0	C ₆ H ₁₂ O ₂	116.16
Caprylic acid	0	5	8:0	8:0	C ₈ H ₁₆ O ₂	144.21
Capric acid	0	7	10:0	10:0	C ₁₀ H ₂₀ O ₂	172.26
Undecanoic acid	0	8	11:0	11:0	C ₁₁ H ₂₂ O ₂	186.29
Lauric acid	0	9	12:0	12:0	C ₁₂ H ₂₄ O ₂	200.32
Tridecanoic acid	0	10	13:0	13:0	C ₁₃ H ₂₆ O ₂	214.34
Myristic acid	0	11	14:0	14:0	C ₁₄ H ₂₈ O ₂	228.37
Pentadecanoic acid	0	12	15:0	15:0	C ₁₅ H ₃₀ O ₂	242.4
Palmitic acid	0	13	16:0	16:0	C ₁₆ H ₃₂ O ₂	256.42
Heptadecanoic acid	0	14	17:0	17:0	C ₁₇ H ₃₄ O ₂	270.45
Stearic acid	0	15	18:0	18:0	C ₁₈ H ₃₆ O ₂	284.48
Arachidic acid	0	17	20:0	20:0	C ₂₀ H ₄₀ O ₂	312.53
Behenic acid	0	18	21:0	21:0	C ₂₁ H ₄₂ O ₂	326.53
Behenic acid	0	19	22:0	22:0	C ₂₂ H ₄₄ O ₂	340.58
Tricosanoic acid	0	20	23:0	23:0	C ₂₃ H ₄₆ O ₂	354.61
Lignoceric acid	0	21	24:0	24:0	C ₂₄ H ₄₈ O ₂	368.64
Monounsaturated fatty acids 						
Myristoleic acid	2	7	14:1Δ ⁹	14:1n9	C ₁₄ H ₂₆ O ₂	226.36
Cis-10-Pentadecenoic acid	2	8	15:1Δ ¹⁰	15:1n10	C ₁₅ H ₂₈ O ₂	240.36
Palmitoleic acid	4	7	16:1Δ ⁹	16:1n9	C ₁₆ H ₃₀ O ₂	254.41
Cis-10-Heptadecenoic acid	5	8	17:1Δ ¹⁰	17:1n10	C ₁₇ H ₃₂ O ₂	268.43
Elaidic acid	7	7	18:1Δ ⁹	18:1n9 trans	C ₁₈ H ₃₄ O ₂	282.46
Oleic acid	7	7	18:1Δ ⁹	18:1n9 cis	C ₁₈ H ₃₄ O ₂	282.46
Cis-11-eicosenoic acid	7	9	20:1Δ ¹¹	20:1n11	C ₂₀ H ₃₈ O ₂	310.51
Erucic acid	7	11	14:1Δ ¹³	22:1n13	C ₂₂ H ₄₂ O ₂	338.57
Nervonic acid	7	13	14:1Δ ¹⁵	24:1n15	C ₂₄ H ₄₆ O ₂	366.62
n-6 Polyunsaturated fatty acids 						
Linolelaidic acid	2	6	18:2Δ ^{9,12}	18:2n6 trans	C ₁₈ H ₃₂ O ₂	280.45
Linoleic acid	2	6	18:2Δ ^{9,12}	18:2n6 cis	C ₁₈ H ₃₂ O ₂	280.45
γ-Linolenic acid	3	3	18:2Δ ^{6,9,12}	18:3n6	C ₁₈ H ₃₀ O ₂	278.43
Cis-11,14-Eicosadienoic acid	2	8	20:2Δ ^{11,14}	20:2n6	C ₂₀ H ₃₆ O ₂	308.5
Cis-8,11,14-Eicosatrienoic acid	3	5	20:2Δ ^{8,11,14}	20:3n6	C ₂₀ H ₃₄ O ₂	306.48
Arachidonic acid	4	2	20:2Δ ^{5,8,11,14}	20:4n6	C ₂₀ H ₃₂ O ₂	304.47
Cis-13,16-Docosadienoic acid	2	10	22:2Δ ^{13,16}	22:2n6	C ₂₂ H ₄₀ O ₂	336.55
n-3 Polyunsaturated fatty acids 						
Linolenic acid	3	6	18:2Δ ^{9,12,15}	18:3n3	C ₁₈ H ₃₀ O ₂	278.43
Cis-11,14,17-Eicosatrienoic acid	3	8	20:2Δ ^{11,14,17}	20:3n3	C ₂₀ H ₃₄ O ₂	306.48
Cis-5,8,11,14,17-Eicosapentaenoic acid	5	2	20:2Δ ^{5,8,11,14,17}	20:5n3	C ₂₀ H ₃₀ O ₂	302.45
Cis-4,7,10,13,16,19-Docosahexaenoic acid	6	1	22:2Δ ^{4,7,10,13,16,19}	22:6n3	C ₂₂ H ₃₂ O ₂	328.49

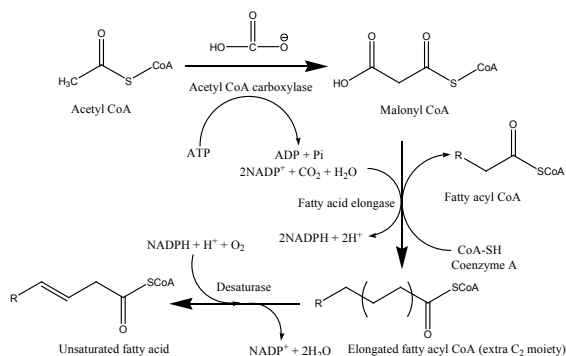


Figure 1. Synthesis of unsaturated fatty acids from acetyl CoA

Fatty acids are stored in adipocytes as triacylglycerol that must be hydrolyzed to release free fatty acids.

Polyunsaturated fatty acids and their importance in health and disease

To prevent cancer

Among dietary factors postulated to influence cancer development are long chain polyunsaturated ω-3 fatty acids, found in fish. Earlier studies revealed inverse

relation between marine fatty acid consumption and mortality rates of prostate (Hebert *et al.*, 1998) and breast cancer (Hebert *et al.*, 1996). The mechanisms proposed how the intake of marine fatty acids might lower the risk of cancer is the inhibition of eicosanoid biosynthesis from AA, a ω -6 fatty acid. Prostaglandins converted from AA by the cyclooxygenase-2 enzyme, notably PGE2, have been linked to carcinogenesis *viz.*, mammary tumor development, proliferation of breast and prostate cancer (Erickson, 1986). Tumor cells typically produce large amounts of AA-derived PGE2, which may impede immune system function, possibly through their role in the generation of suppressor T cells (Erickson, 1986). Marine fatty acids were reported to inhibit cyclooxygenase-2 and the oxidative metabolism of AA to PGE2. EPA and DHA also inhibit lipoxygenases which metabolize AA to HETEs and leukotrienes. 12-HETE has been linked to the suppression of apoptosis, stimulation of angiogenesis, stimulation of tumor cell adhesion, and expression of the invasive phenotype. It is apparent that both EPA and DHA can inhibit the biological activity of eicosanoids and androgens (Liang *et al.*, 1992), which are known to have a stimulating effect on cell growth and uncontrolled cell proliferation (Ghosh & Myers, 1997). It is well established that in animal models and in human cancer cell lines, EPA and DHA were found to suppress cell growth. However, because intakes of fish and marine fatty acids are highly correlated, it is difficult to disentangle the effect of fatty acids from the effect of fish *per se*.

To combat atherosclerosis and cardiovascular diseases

Eating ω -3 fatty acids abundantly available in marine fish were reported to protect human beings from heart failure (*European Heart Journal*. doi:10.1093/eurheartj/ehp111). Researchers in the USA and Sweden followed 39,367 Swedish men, aged between 45-79, from 1998 to 2004. They recorded details of the men's diet and tracked the men's outcome through Swedish inpatient and cause-of-death registers. PUFAs in the diet have long been considered essential to the growth and proper nutrition of humans and other vertebrates. It was reported that atherosclerosis and thrombosis represent essential fatty acid deficiencies, but rather

that the polyunsaturated fat may affect these pathological processes through other mechanisms. There is evidence from epidemiology that marine n-3 PUFA is associated with a reduced risk of coronary heart disease. This was originally found in Greenland Eskimos with an extremely high intake of n-3 PUFA (10–14 g/day) and later also reported in several other populations (Schmidt *et al.*, 2005; Kris-Etherton *et al.*, 2002) including Western populations with an average intake of marine n-3 PUFA below 0.2–0.4 g/day. Recently, a meta-analysis was published on fish consumption and CHD mortality from 13 cohort studies including a total of 222,364 individuals with an average of 11.8 years of follow-up (He *et al.*, 2004). Fish consumption was inversely related with fatal CHD and sudden cardiac death (He *et al.*, 2004).

Estimation of fatty acids in laboratory

Broadly fatty acid estimation is divided under the broad categories, *viz.*, (1) lipid extraction and acid-catalyzed transesterification of fatty acid to methyl esters (FAMES) and *N*-acyl pyrrolidides; and (3) gas-liquid chromatography and gas chromatography-mass spectrometry (GC/MS) analysis of FAMES. Below are illustrated the details under each head.

Lipid extraction

Lipid from the crude sardine oil was extracted by using CHCl_3 - CH_3OH - H_2O (Bligh, & Dyer, 1959). In brief, about 10 g tissue together with chloroform methanol mixture (2:1) ratio is homogenized, and CHCl_3 - CH_3OH mixture (15 times) was added and mixed (to 1/3rd of the total volume). The resulting solution was filtered, and the filtrate was collected. The process was repeated two more times with rest of the CHCl_3 - CH_3OH mixture. To the filtrate, add distilled water (20% of the total volume of the filtrate) and leave overnight. The water-soluble residue diffuses away from the solvent and occupies the top position in the separating funnel. Solvent containing lipid (bottom layer) is collected by filtering through anhydrous Na_2SO_4 . Evaporate to dryness and make up the volume using CHCl_3 . On extraction with CHCl_3 - CH_3OH , lipid (bottom layer) is separated from the sample and is collected by filtering

through anhydrous Na_2SO_4 . After saponification of the dried extract PUFA is determined using gas chromatograph as illustrated below.

Extraction and derivatization of fatty acids to fatty acid methyl esters (FAME) and N-acyl pyrrolidides

The lipid extract thus obtained was saponified with 0.5 N KOH in CH_3OH . After removal of the nonsaponifiable material with *n*-hexane and acidification with 1 N HCl, the saponifiable materials were extracted with petroleum etherdiethyl ether (1:1 v/v) and transesterified to furnish fatty acid methyl esters (FAME) by reaction (30 min under reflux) with a methylating mixture (14% $\text{BF}_3/\text{CH}_3\text{OH}$, 5 mL) in a boiling water bath under an inert atmosphere of N_2 (Metcalf, Schimtz, & Pleka, 1966). The FAME thus obtained was cooled to ambient temperature, and distilled water (20 mL) was added. The solution was extracted with *n*-hexane (10 mL X 6), and the upper *n*-hexane layer was removed and concentrated under an inert atmosphere of N_2 . The resulting FAME concentrate was reconstituted in petroleum ether, flushed with N_2 in glass vials, and stored in deep freeze (-20°C) until required for GC/GC-MS analyses. Analysis was performed in triplicate.

Gas-liquid chromatography and gas chromatography-mass spectrometry (GC/MS) analysis of fatty acid derivatives

Quantitative and qualitative analyses of FAME obtained by transesterification were performed on gas chromatograph using a flame ionization detector (FID). FAMES were identified by comparison of retention times with the known standards. In another process, FAMES were derivatized to *N*-acyl pyrrolidides by condensation of fatty acid methyl ester with a mixture of pyrrolidine (1 mL) and acetic acid (0.1 mL) at 100°C under reflux (2 h) for GC-MS analyses (Andersson, 1978). The GC-MS analyses need to be performed by GC interfaced with mass spectrometer for confirmation of fatty acid identification.

Mass Spectroscopic Analyses of FAME Derivatives

The following are the mass spectrometric data of FAME derivatives.

Methyl Palmitate. EI-MS *m/z* (relative intensity, %): 270 (M+, 61.11), 239 (15.74), 227 (31.48), 213 (7.41), 199 (14.81), 185 (12.96), 171 (12.96), 157 (7.41), 143 (31.48), 129 (11.11), 87 (74.07), 74 (100), 55 (18.52).

Methyl Oleate. EI-MS *m/z* (relative intensity, %): 296 (M+, 20.00), 111 (76.67), 264 (33.33), 222 (26.67), 180 (18.33), 166 (23.33), 152 (23.33), 123 (23.33), 110 (38.33), 97 (75.00), 83 (70.00), 74 (66.67), 69 (78.33), 55 (100).

Methyl Linoleate. EI-MS *m/z* (relative intensity, %): 294 (M+, 52.46), 263 (24.59), 220 (8.20), 178 (13.11), 164 (19.67), 150 (21.31), 136 (18.03), 123 (18.85), 109 (37.70), 95 (70.49), 81 (100), 67 (91.80), 55 (50.82).

Methyl Linolenate. EI-MS *m/z* (relative intensity, %): 292 (M+, 16.67), 261 (5.00), 236 (6.67), 173 (6.67), 163 (6.67), 149 (20.00), 135 (20.00), 121 (25.00), 108 (56.67), 95 (58.33), 79 (100), 67 (56.67), 55 (35.00).

Methyl Arachidonate. EI-MS *m/z* (relative intensity, %): 318 (M+, 1.82), 290 (1.82), 264 (1.82), 175 (5.45), 150 (7.27), 133 (7.27), 105 (30.91), 91 (70.91), 79 (100), 67 (80.00), 55 (49.09).

Methyl Eicosapentaenoate. EI-MS *m/z* (relative intensity, %): 315 (M+, 1.67), 175 (6.67), 161 (8.33), 145 (11.67), 131 (18.33), 119 (31.67), 108 (31.67), 91 (70.00), 79 (100), 67 (68.33), 55 (48.33).

Methyl Docosahexaenoate. EI-MS *m/z* (relative intensity, %): 342 (M+, 0.60), 145 (4.20), 131 (6.60), 119 (10.80), 108 (11.40), 91 (28.20), 79 (100), 67 (20.40). (Chakraborty et al., 2010).

Mass Spectroscopic Analyses of N-Acyl Pyrrolidide Derivatives

The following are the mass spectrometric data of N-acyl pyrrolidide derivatives.

1-(Pyrrolidin-1-yl)hexadecan-1-one/ Palmitoylpyrrolidine. EI-MS *m/z* (relative intensity, %): 309 (M+, 16.00), 294 (2.00), 168 (8.00), 140 (10.00), 126 (16.00), 113 (100), 98 (8.00), 70 (12.00), 55 (14.00).

1-(Pyrrolidin-1-yl)octadec-9-en-1-one. EI-MS *m/z* (relative intensity, %): 335 (M+, 27.56), 250 (8.62), 236 (10.34), 208 (6.90), 196 (5.17), 182 (12.07), 126 (53.45), 113 (100), 98 (18.97), 85 (8.62), 72 (20.69), 55 (27.59).

1-(Pyrrolidin-1-yl)octadeca-9,12-dien-1-one. EI-MS *m/z* (relative intensity, %): 333 (M+, 77.97), 290 (10.17), 236 (15.25), 222 (20.34), 182 (16.95), 168 (15.25), 140 (22.03), 126 (44.07), 113 (100), 98 (25.42), 70 (42.37), 55 (49.15).

1-(Pyrrolidin-1-yl)octadeca-9,12,15-trien-1-one. EI-MS *m/z* (relative intensity, %): 331 (M+, 44.00), 182 (22.00), 168 (24.00), 140 (26.00), 126 (60.00), 113 (100), 98 (30.00), 72 (64.00), 55 (42.00).

1-(Pyrrolidin-1-yl)icosa-5,8,11,14-tetraen-1-one. EI-MS *m/z* (relative intensity, %): 357 (M+, 18.97), 232 (10.34), 180 (10.34), 126 (13.79), 113 (100), 85 (17.24), 70 (22.41), 55 (27.59).

1-(Pyrrolidin-1-yl)icosa-5,8,11,14,17-pentaen-1-one. EI-MS *m/z* (relative intensity, %): 355 (M+, 3.85), 286 (7.69), 232 (7.69), 126 (13.46), 113 (100), 85 (17.31), 72 (26.92), 55 (21.15).

1-(Pyrrolidin-1-yl)octadeca-9,12-dien-1-one. EI-MS *m/z* (relative intensity, %): 381 (M+, 3.91), 312 (7.05), 272 (7.29), 232 (16.22), 218 (15.76), 192 (8.24), 166 (23.67), 153 (22.85), 113 (100), 98 (46.62), 72 (21.98) (Chakraborty *et al.*, 2010).

Conclusions

Research on exploring sources long-chain PUFAs, viz., DHA, EPA, and AA for use in nutrition have received considerable attention. These PUFAs, which are usually low in abundance in human, are regarded

as essential and must be supplied in diet. The importance of PUFAs in human nutrition has been extensively investigated during the past 20 years. DHA is one of the important PUFAs, which maintains structural and functional integrity in larval cell membranes in addition to the neural development and function, while AA and EPA are involved in, respectively, the production and modulation of eicosanoids. Docosahexaenoic acid (22:6 ω -3), which is a vital component of the phospholipids of cellular membranes, especially in the brain and retina, is necessary for their proper functioning. The ω -3 fatty acids favorably affect atherosclerosis, coronary heart disease, inflammatory disease, and perhaps even behavioral disorders. Membrane fluidity is essential for proper functioning of these tissues. In the retina, where ω -3 fatty acids are especially important, deficiency can result in decreased vision and abnormal electroretinogram results. The ω -3 fatty acids are essential fatty acids, necessary from conception through pregnancy and infancy and, undoubtedly, throughout life. AA has been an essential function of producing eicosanoids, making it an essential fatty acid because prostaglandins (PGF₂R) are produced from 20:4n₆, and has roles in reproduction. AA is the basis for cyclo-oxygenase (COX) action to produce PGF₂R. AA, being a major component of phosphoinositol, was reported to have a vital role in the transduction signal mechanism. An imbalance in ω -3/ ω -6 ratio can accentuate ω -3 fatty acid deficiency state, as shown by earlier studies. The ratio may have increased in industrialized societies because of increased consumption of vegetable oils rich in ω -6 fatty acids, ie, linoleic acid (18:2n₂₆), and reduced consumption of foods rich in ω -3 fatty acids. Another important feature of ω -3 fatty acids is their role in the prevention and modulation of certain diseases that are common (Importance of n₃ fatty acids in health and disease (W. E. Connor *Am J Clin Nutr* 2000;71(suppl):171S–5S). Below is appended a partial list of diseases that may be prevented or ameliorated with ω -3 fatty acids:

1. Coronary heart disease and stroke
2. Cancers of the breast, colon, and prostate
3. Retinal and brain development);
4. Immunostimulant
5. Hypertension

The first two functions are extremely important and are related directly or indirectly with other diseases as listed earlier.

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Nutrigenomics

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Introduction

Nutrition research has the long tradition for recommending the species specific diets to farmed fishes for more production. Over last two decades, nutritional research has also made a progress in the application of molecular biological tools to understand the basics of gene regulation that affected by dietary factors. Nutrigenomics (Nutritional genomics) refers to the research that investigates the interaction between nutrition and the genome. From the view point of nutrigenomics, nutrients are act as dietary signaling molecules and detected by cellular sensing mechanism, that influence the gene, protein and metabolite expression. Nutrients may act directly as ligands for transcription factor receptors; may be metabolized by primary or secondary metabolic pathways, thereby altering concentrations of substrates or intermediates involved in gene regulation or cell signaling; or alter signal transduction pathways and signaling. Nutrigenomics is the collective terms, includes genomics, transcriptomics, proteomics and metabolomics. Application of these modern research tools may yield new knowledge in molecular responses

of an organism reflected by dietary bio-molecules.

In nutrition research studying the Gene Expression Profiling (GEP) may be used for three distinct purposes such as to assist in the identification and characterization of basic molecular pathways that may be impacted by nutrients, to provide insights upon specific mechanisms that trigger either beneficial or negative effects and to identify specific genes altered by nutrients that might prove valuable as molecular biomarkers or nutrient sensors.

Genomics

Generally the genomic study covers the development of molecular markers, linkage mapping, quantitative trait loci (QTL) analysis and characterization of genomic resources for understanding all physiological functions. The development of genomic resources that is cDNA libraries is the first step in any genomic studies which represents the maximum of expressed RNA and the expressed sequence tag (EST) resources getting increased as well. The EST analysis is the most useful method for gene identification, gene expression profiling and cataloguing. The EST analysis is also used for the development of cDNA or oligonucleotide microarrays. The information on the fish genes responsible for metabolic pathways is helpful in understanding the nutritional metabolism in molecular level.

Transcriptome and Transcriptomics

The genome is only a source of information and it must be expressed to understand the function. The transcription of genes to produce RNA is the first stage

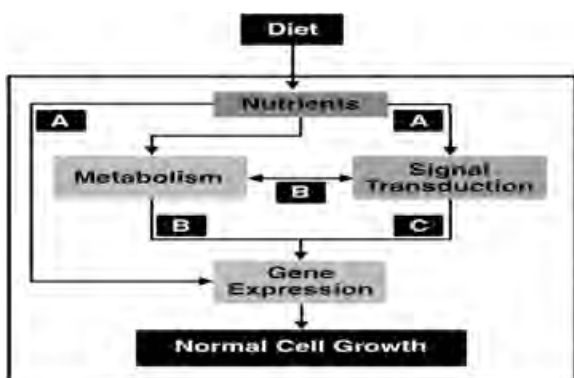
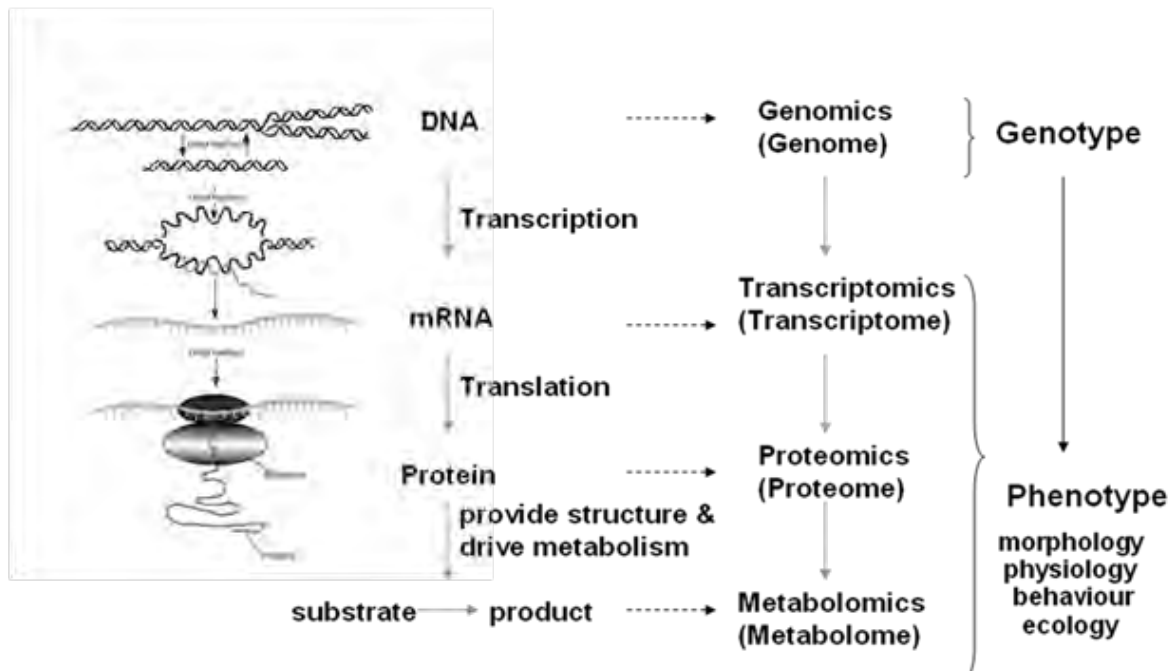


Figure: Fate and activities of nutrients in the cell
(Adopted from: Kaput and Rodriguez, 2004)

The -Omics Study



of gene expression. Transcriptome is the complete set of RNA transcripts produced by the genome at any one time and is extremely dynamic and varies with differing circumstances due to different patterns of gene expression. And hence, transcriptomics refers to the study of the complete set of RNAs encoded by the genome of a specific cell or organism at a specific time or under a specific set of conditions. Transcriptomics, also referred to as gene expression profiling, examines the expression level of mRNAs in a given cell population. Understanding the transcriptome is essential for interpreting the functional elements of the genome and understanding the functions such as growth, health, physiological well-being and flesh quality. Transcriptomics uses DNA microarray technology for studying the gene profiling expression. In fishes, the variations of transcriptome expression analysis is used to study the stocking stress, handling stress, chemical contaminants, salinity, temperature and hypoxia conditions and its mitigation through dietary intervention. The effects of dietary nutrients on the hepatic transcriptome expression will help to learn the nutrient requirement and its effects in fish species. The protein nutrition in fish affects the transcription of number of genes through the regulation of transcription regulators expression. The protein requirement study can be effectively studied by the

hepatic transcriptome of fish using cDNA microarray techniques. The rainbow trout fed with low protein levels showed that the overexpression of one gene that is responsible for the inhibitor of growth that is cell multiplication or division than the high protein fed fishes. Polyunsaturated fatty acid is being an essential component in the central nervous system, the dietary PUFA leads to the brain gene expression through the transcriptional modulators. Hence, the functional genome studies with high density microarrays are useful in the discovery of regulatory pathways linked to dietary components in fish.

Nutritional regulation of candidate genes in fish

Larval Nutrition

The production of marine fish larvae in hatcheries depends on the supply of live feed and hence substituting the live feed with inert compound diet reduces the cost of production of juveniles. Hence, it is necessary to determine the time of initiation of compounds in fish larvae. The digestive enzyme of seabass larvae is altered by dietary composition of diet, which was detected by its gene expression. During larval development of

seabass larvae specific activity and mRNA levels of amylase enzyme is regulated transcriptionally based on the dietary carbohydrate level.

In larval nutrition, physical abnormalities particularly skeletal abnormalities observed is due to malnutrition. In Japanese flounder larvae high levels of dietary retinoic acid results in the development of bone deformities which occurs due to retinoic acid dependent depression of a transcriptional factor, sonic hedgehog (shh).

Lipid metabolism

The substitution of fish oil with plant based oil is the prevailing scenario in aquafeed industry. The knowledge on molecular level of fatty acid biosynthesis particularly C_{20} and C_{22} from C_{18} will give the information on the manipulation of fatty acid bioconversion pathways through the series of fatty acid desaturation and elongation reactions for efficient utilization of plant based oils in aquafeeds. In general freshwater fishes can express almost all the enzymes responsible for long chain highly unsaturated fatty acid but marine fishes are inefficient in the production of sufficient level of DHA due to the deficiencies of one or more enzymes of the pathway. The expression of $\Delta 6$ desaturase by the dietary lipids and carbohydrate sources was noticed in marine fishes in recent studies. But, the $\Delta 5$ desaturase gene was not characterized in marine fish in contrast to freshwater fish.

Dietary lipid content influences the body particularly the muscle lipid content in fish. The key enzymes mainly the lipoprotein lipase is necessary for lipid transport and storage. In rainbow trout and red sea bream, this enzyme expression studies revealed that this enzymes works similar to the lipase enzymes of higher vertebrates. In some fishes, lipoprotein lipase gene expressed in a tissue specific manner according to the nutritional status of the fish that indicates the lipid storage based on the tissue.

Glucose metabolism

The carnivorous fishes generally do not utilize dietary carbohydrates and the level of utilization varies species

to species. In fish different types of glucose receptors such as Glut 1, Glut 2 and Glut 4 are available for transport and utilization of glucose. The nutritional control of these genes and its expression for their important function was started studied in few fishes. The carnivorous fish fed with high level of dietary carbohydrates expresses the lower level of its utilization due to the atypical hepatic regulatory mechanism. In rainbow trout and gilthead sea bream, dietary carbohydrate feeding induce the synthesis of first phosphorylation enzyme, glucokinase which is related to higher levels of glucokinase gene expression. In case of endogenous glucose production the enzymes such as glucose 6 phosphatase, fructose 1,6 bis-phosphatase and phosphoenolpyruvate carboxykinase are very much essential. These are expressed based on the dietary levels of carbohydrates in most of the fishes but in some of the fishes especially in rainbow trout, the exogenous glucose production is not affected by dietary glucose level. The reason for absence of the hepatic gluconeogenic enzymes regulation may be due to high levels dietary fatty acids and gluconeogenic amino acids. The need of the hour is to study the gene level expression on the utilization of dietary carbohydrates.

Phosphorous regulation

Dietary phosphorous utilization in most of the fish species is around 50% of the original level. This can be tackled by the understanding the mechanism of absorption of dietary phosphorous by fish. The genes associated with phosphorous metabolism such as type II sodium phosphate co-transporter, intestinal meprin 1A and cysteine sulphinic acid decarboxylase are essential bio-indicators for the dietary phosphorous optimization in addition to the traditional phosphorous estimation methods.

Proteomics

Proteomics is the study of all the proteins (proteome) in a particular cell, tissue or an organism. The proteome represents the protein equivalent of the genome, which is determined by the sequence, the type and number of its nucleotides. In contrast to static nature of the genome, the proteome represents a tremendously dynamic object, which is influenced

Reproductive endocrinology in Aquaculture with special reference to captive maturation of penaeid shrimps

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Introduction

Fish and fisheries rank top among all the global natural resources and fish has been considered to be the last wild food of humans (Walsh 2011). It contributes about US \$ 100 billion annually to global trade, which is greater than the GDP of 70 world nations (McClanahan *et al.* 2015). About 520 million people, *i.e.* 8% of world population, directly or indirectly depend on marine fisheries (Sumaila *et al.* 2011). Fish has been acknowledged as the major nutrient dense animal source protein for a significant proportion of nutritionally vulnerable people. It overshadows most terrestrial generated animal foods, and fish production was double that of poultry and three times of cattle. The unique long chain poly unsaturated fatty acids and highly bioavailable essential micronutrients, which are not readily available in most terrestrial food resources, make it as unique food which is highly essential for the adult health and cognitive development for the children. With all these widely acknowledged importance, there has been a growing concern about the sustainability of fisheries. Sustainable harvest of wild fisheries has been central theme of many international fora, media headlines, scientific publications and environmental campaigns as most wild fisheries are under crisis due to overfishing. Aquaculture has often been considered to be the best option to meet the deficit of the capture fisheries.

Although aquaculture has been vogue in Asia, the modern scientific aquaculture is youngest of all

food production system. During 1980s there was an upsurge for the export oriented agricultural crops, and shrimp farming in India is paradigmatic example for this. In aquaculture, there are certain areas where science can play crucial roles. For example: reproduction, early development, nutrition, health and genetics (Donaldson, 1996). The most essential characteristic of an aquaculture species is the ability to control reproduction and produce viable offspring from captive broodstock. Many aquaculture species, however, faces some form of reproductive dysfunction in captivity. This dysfunction is largely due to dramatic difference in the environmental conditions faced by animal in natural environmental conditions and captivity. The captive environmental conditions, which lack the natural stimuli, fail to induce appropriate endogenous response in fish. It is almost impossible to simulate the natural conditions for breeding under the captivity for many aquacultured species.

Complete control over the reproduction is the most elusive goals of almost all form of aquaculture. It is crucial for seed production, selective breeding, growth rate, feed efficiency, meat quality and biosecurity (Weber, 2009). Among all, seed production is the primary need of development of any form of aquaculture. Sustainability of any aquaculture, therefore, largely depends on how efficiently aquaculturists can manage reproduction under captivity. Some species reproduce more easily than others. For example, Indian white shrimp, *Penaeus indicus*, is relatively easy to breed species when compare to tiger shrimp, *Penaeus monodon*.

Table: Selected candidate genes in fish nutrigenomic study:

Genes	Species	Nutritional regulation	Selected reference (one for each gene)
<i>Digestion</i>			
Amylase (intestine/pancreas)	European seabass	+ by dietary carbohydrate levels	Geurden <i>et al.</i> (2007)
Trypsin (intestine/pancreas)	European seabass	- by dietary protein levels	Zambonino Infante and Cahu (2001)
<i>Nutrient transport</i>			
Sodium-phosphate co-transporter	Rainbow trout	- by dietary phosphorus levels	Sugiura <i>et al.</i> (2003)
Lipoprotein lipase (mesenteric fat tissue)	Gilthead sea bream	- by dietary plant protein inclusion	Saera-Vila <i>et al.</i> (2005)
<i>Protein metabolism</i>			
Glutamine synthase (liver)	Rainbow trout	- by dietary plant protein inclusion	Panserat <i>et al.</i> (2008b)
Atrogin (muscle)	Rainbow trout	- by refeeding	Selliez <i>et al.</i> (2008b)
<i>Lipid metabolism</i>			
Cholesterol biosynthetic genes (liver)	Atlantic salmon	- by dietary vegetable oil inclusion	Leaver <i>et al.</i> (2008)
Delta-6 desaturase (liver)	Rainbow trout	- by dietary vegetable oil inclusion	Selliez <i>et al.</i> (2001)
<i>Glucose metabolism</i>			
Glucokinase (liver)	Rainbow trout	- by dietary carbohydrate levels	Panserat <i>et al.</i> (2000a)
Glucose-6-phosphatase (liver)	Rainbow trout	No regulation by dietary carbohydrate levels	Panserat <i>et al.</i> (2000b)
<i>Hormonal actions</i>			
Growth hormone receptor (mesenteric fat tissue)	Rainbow trout	- by dietary protein levels	Gomez-Requeni <i>et al.</i> (2005)
Insulin-like growth factor I (liver)	Atlantic salmon	- by dietary lysine levels	Hevroy <i>et al.</i> (2007)

All the genes code for proteins involved in nutritional physiology (digestion, transport, metabolism).

+, induction of gene expression; -, inhibition of gene expression.

(Source: Panserat & Kaushik, 2010)

by a variety of parameters. However, arraying of proteins is more difficult than the arraying of DNA, because they have to maintain their correctly folded conformations. The major tools of proteomics study are two dimensional gel electrophoresis (2D) for separation of complex protein mixtures and mass spectrometry (MS) for identification and structure analysis of protein. In a typical proteomic study, protein extraction followed by high resolution 2D electrophoresis along with gel image analysis permit the identification and expression of n-numbers of protein molecules. The hepatic proteomes of fishes is used to study the effect of quality protein, plant based protein in fish meal replacement and fasting on the transport and primary energy generation, cellular protein degradation, bile acid biosynthesis etc.

Metabolomics

The study of metabolites present in a cell, tissue or organism is known as metabolomics. The metabolomic study includes the essential steps such as separation of analytes, detection, identification and quantification of the analytes/metabolites. The separation of analyte is done by chromatographic techniques such as Gas Chromatography (GC), Capillary Electrophoresis (CE), High Performance Liquid Chromatography (HPLC) and Ultra Performance Liquid Chromatography (UPLC). The detection and identification of metabolite is carry out by Nuclear Magnetic Resonance Spectroscopy (NMR) and Mass Spectrometry (MS). In fish nutritional research the metabolomic study is still in its infancy stage.

However, this relative easiness in breeding may not be a desirable quality in grow out production system, where reproductive growth adversely affect the somatic growth of the animal. Conversely, delayed maturation in *P. monodon* is a desirable characteristic in grow out production system. However, delayed maturation of a species hampers the seed stock production and selective breeding. These diverse needs illustrate that each species require different levels of control over reproduction, and even within the same segment of the industry. Whatever the goal of aquaculturists to control or manipulate reproduction, the progress in gaining control over reproduction process is dependent on our greater understanding of mechanism controlling these processes. This chapter summarizes the present understanding on control of reproduction in aquaculture with special reference to crustacean reproductive control. The first part of this chapter is a preamble to the general endocrinology providing the general concepts and principles in endocrinology. The second part deals with history of endocrinological applications of aquaculture, and third part provides present understanding of penaeid endocrinology, endocrine methodologies and future perspective for the application of endocrinology

Endocrinology: a preamble

When the life began as a unicellular organism, about three billion years ago, the time was simpler and communication between cells (organisms) were modest than that those required to maintain multicellular organisms. During the course of evolution the multicellular organisms developed a communication system through chemical signals that coordinate multiple organic functions. The substances that provide a chemical basis for communication between cells are called hormones. The investigation of these chemicals in early years of twentieth century gave birth to a new discipline, the endocrinology (Fingerman, 1997). In strict sense, endocrine glands are specialized organ that delivers their products, the hormones, directly into the interstitial spaces and enter circulatory system. In early years, the endocrinology is confined to the study of these specialized cells. Later, it was demonstrated that nervous system is also closely associated with the functioning of endocrine system in order to respond to varieties of stimulus and contingencies in their internal

and external environment. These two systems have been evolved in animals to initiate and coordinate appropriate responses. These stimuli may either be an active danger posed by the predator or may be a gradual threat posed by seasonal environmental change. Although these two systems are morphologically different, both the system will operate fundamentally in similar ways. The nervous system is organized as cellular network, where axons direct information via chemical messengers called neurotransmitters, and these neurotransmitters are secreted into synapses and act on target located on the other side. Neurotransmitters can also diffuse into circulatory system and act on distantly located target tissue as hormones do. These neurotransmitters that act outside the synaptic cleft are called neurohormones. In addition to endocrine and neurohormones, hormones have a broader usage in recent years (Table 1).

Method of hormone delivery: The specific receptors in the target cells determine the selectivity hormone action. Receptors are protein present in the target cells and bind a particular hormone and initiate a response. There are two types of receptors: cell surface receptors and intra cellular receptors (Fig 1). Protein/peptide hormones use cell surface receptors whereas steroid hormone uses intracellular receptors.

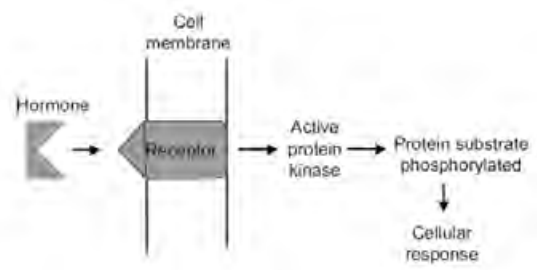


Figure 1 Action of peptide hormone on target tissue

How do hormones function: Hormones modulate the activity of target tissues, and effects of hormones are found even long after the levels of hormones returned to the basal levels. They are usually present in tracer levels ranging from 10⁻⁶ g/ml to 10⁻⁹ g/ml. Combined effect of more than one hormone on a biological response will be in number of different ways (Fig 2). The action of these hormones may be additive, if they cause same response. Thus the combined effect is simply sum of the response of


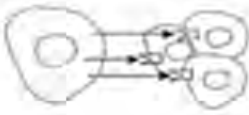



Hormone type	Action	
Endocrine	Signals are released into the blood and exert action at different site	
Paracrine	Signals diffuse from one cell type to target cell located in the proximity within the same organ.	
Neurocrine	Signals are exerted either as neurotransmitter or neurohormone	
Autocrine	Signals produced by one cell population and regulate the same population	
Pheromone and photocrine	Pheromones are signals are secreted into the environment targeting individuals of same species. In photocrine system day light affects structure within the eye and signals transmitted to the brain and influences reproduction	

Table 1 Terminology of different hormone producing organ

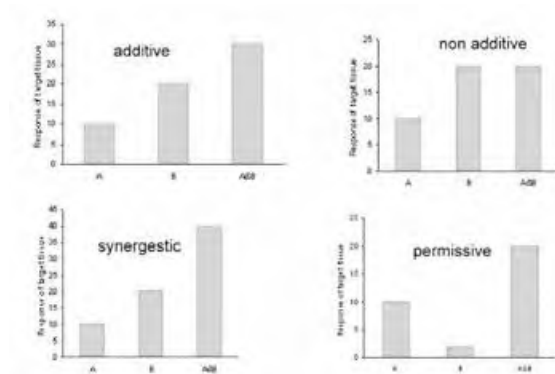


Figure 2 Various forms action of hormones on target tissue results different types of responses.

two individual hormones. Sometimes two hormones have same biological effect, but the combined effect may be non-additive. In still different way the combined effect will be more than the additive effect of individual hormone effect, and it is known as synergistic. In some cases some hormones will not have effect on their own but must be present

for another hormone to have an effect

Hormones and aquaculture

Husbandry of aquatic organisms is an ancient enterprise. However, the modern science and technology applied to it is only in the past quarter of the century. Fish endocrinology has been developed as a basic science with little applied interest. The past fish endocrinologists, largely confined the physiological and evolutionary aspects and not with fish production. Only recently, the endocrinologists have become concerned on aquaculture. Now aquaculture and ranching of salmon are considered to be the domain of fish endocrinologists.

Initial studies on endocrinological applications in biology largely confines to describe the effect of a hormone at organismal level. For example: how does hormonal therapy influences viable spawning? Although target and applicability of this approach

is obvious; this approach fails to investigate the fundamental biology of a particular hormone.

Hormonal application in induced breeding started in Brazil, when fish pituitary glands were injected to induce maturation. This is originally described by Argentinean physiologist, B. A. Houssay who recorded precocious spawning in viviparous fishes when they administered with crude extract of the pituitary gland. The role of pituitary gland (hypophysis) in the reproduction of vertebrates may considered to have commenced three years before, when S. Aschheim and B. Zondek found that pituitary implants accelerated the sexual development and cycle of female mice, and P. E Smith for the first time unequivocally demonstrated the effect of hypophysectomy and subsequent replacement therapy on the gonads of the laboratory rats (Allen, 1939). This new finding came to the attention of R. von Ihering, who was the Director of the *Comissao Tecnica de Piscicultura do Nordeste at Ceara*, Brazil, who had been facing the problems of failure in spawning in native fresh water food fish. Even when caught in nearly ripe condition these species rarely completed the maturation. In nature, spawning precipitously followed the first storms of rainy season, but these environmental cues could not be duplicated in the captivity. By 1934, von Ihering had developed successful techniques to induce ovulation using fish pituitary- this is generally known as hypophysation. During almost similar period, Russians were also discovered successful spawning and breeding of sturgeon using freshly removed pituitary gland extract. These findings were in fact a fortunate discovery as hydroelectric stations and dams were built in rivers and it prevents the upstream spawning migration of sturgeon.

Although hypophysation revolutionized fish culture, and contributed enormously towards the development of finfish aquaculture, it suffers from number of limitations. Crude pituitary extract vary in their content of gonadotropin depending upon state of maturity of donor fish, and in addition to the gonadotropin it contain a range of other hormones which may have a synergistic and antagonistic effect on reproductive process. It is extremely difficult to quantify the gonadotropin content of pituitary extract and standardize the dosage (Zohar, 1989). The chances to the transmission of pathogen via pituitary

extract are another threat of the use of pituitary extract. Although not totally reliable, hypophysation was not abandoned.

This first generation technology paved the way for the second generation technology, which involve the purified human chorionic gonadotropin (hCG). Although it has been proved to be success in many species, owing to its species specificity it did not work in many species (Zohar, 1989). This hormone has high molecular weight and when injected these hormones stimulate the immune system of fish and adversely affect the health of the animals. The discovery of the portal (closed) circulation between pituitary and brain initiated the discovery of the gonadotropin releasing hormone (GnRH) (Bowers *et al.* 1970). These discoveries radically changed our thoughts on the organization of the hormonal system, but some time passed before they affected aquaculture practices. Bretton and Well (1973) used the brain hormone for the first time to release gonadotropic hormones from the pituitary in fresh water carps. Later many workers used the GnRH for inducement of spawning in fishes. The advantages of use of brain hormones are obvious: pituitary gonadotropins differ in species used for aquaculture in their molecular structure and success rate is unpredictable in many cases. Brain hormones have undergone little change during evolution (McCeery *et al.* 1982), and thus same peptide molecule may well activate the gonadotrophs of pituitary of any vertebrates. Further, being low molecular weight compound, the immunogenitic potential of GnRH is far less than the pituitary hormones. GnRH molecules can be modified by replacing amino acids, and the resulted products in vivo degraded far slower than the native forms. The use of GnRH in the induced breeding of finfish is the third generation technology and it has attracted significant interest in recent years especially: the discovery of new natural forms of GnRH, the development and testing of potent GnRH analogue and the development of novel means of administration of hormones (Donaldson, 1996). In some teleosts gonadotrophs of pituitary are innervated by nerve fibers producing the dopamine, the neurotransmitter inhibits the production of gonadotropin 2. A combination of therapy with dopamine antagonists have been found successful in some cases, where GnRH alone does not provide

successful spawning. The most recent development in induced maturation of fin fishes is the incorporation of GnRH analogue into a polymeric sustained-release

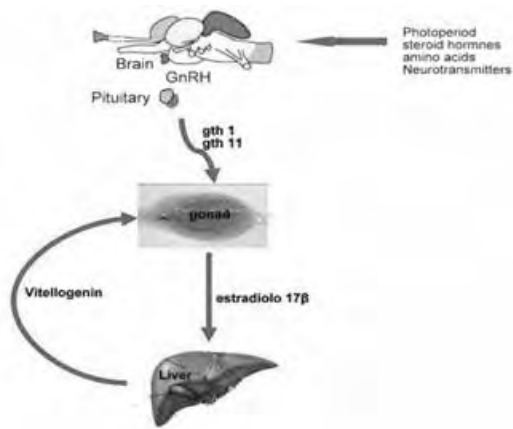


Figure 3 Schematic representation of hypothalamus-pituitary-gonad-liver axis in finfish

delivery system, which releases the hormone over a period of days. The current model for the control of reproduction of fin fishes is presented in the figure 3.

Reproductive endocrinology of penaeid shrimp and shrimp aquaculture

Endocrinological/neuro-endocrinological studies on crustaceans started in first half of the 20th century. Generally, in invertebrates, oogenesis can be stimulated by a release from inhibition or by a secretion of a stimulator (Schuetz, 1969). Ovarian maturation (oogenesis) in Crustacea is said to be stimulated by the gonad stimulating hormones secreted by the brain and thoracic ganglia and inhibited by Gonad Inhibiting hormones (GIH) of the eyestalk (Adiyodi and Adiyodi, 1970). The antagonism of eyestalk may be reduced by a decline in the titre of the GIH as the shrimp grows and moves into an environment suitable for spawning. Final spawning act may, in fact, be triggered by a stimulus, either visual or hormonal originating in the eyestalk (Muthu and Laxmyraran, 1982). Panouse (1943) demonstrated that the removal of the eyestalk of palaemonid shrimp would lead to ovarian development and spawning. However, the first use of eyestalk ablation procedure for inducement

of reproductive maturation is successfully conducted by Aquaculture team of Centre Océanologique du Pacifique (Aquacop). Alain Michael, the head of the Aquacop, serendipitously found that marine shrimp, *Penaeus aztecus*, who had lost the eye, matured and spawned in the tank. This observation, he connected with the work of Panouse, and developed the most revolutionized technology in the captive breeding of shrimp. It had far reaching impact on crustacean aquaculture in general and penaeid shrimp farming in particular. The first successful maturation and spawning of *P. monodon* was achieved by Santiago (1977), although Alikunhi *et al.* (1975) achieved maturation with unviable spawning. The great majority of the captive maturation has been from ablated females, although few workers have reported maturation in unablated females (Santiago, 1977, Primavera, 1978. Emmerson, 1983), only Emmerson (1983) was successful in obtaining viable spawning (16.7 to 82% hatch rate). Although eyestalk ablation started as a stop-gap procedure to induce maturation and spawning in penaeid shrimp in early 1970s, this procedure has been continuing in commercial seed production industry across the world.

The most acknowledged consensus of crustacean reproductive endocrinology is that reproduction is controlled by two antagonistic hormones, one inhibits (Vitellogenin inhibiting hormone or Gonad inhibiting hormone, V/GIH) and other stimulates (Vitellogenin stimulating hormone, Gonad stimulating hormone, V/GSH). This simple endocrine axis has been questioned by many recent researchers and postulated a multi hormonal system involving several neuroendocrine and endocrine pathways, involving neurotransmitters (serotonin or 5 hydroxytryptamine), steroids (progensterone, estradiol), terpenoids (methyl farnesoate) and vertebrate peptide hormones (GnRH). Additionally the role of other neurohormones in the CHH family (*i.e.* MIH and CHH) in reproduction in penaeid shrimps has also been reported. Thus crustacean reproduction is an end result of multiple vitellogenic related endocrine cascades (Figure 4). Nevertheless the bi hormonal axis is still central to the shrimp reproductive endocrinology.

The neural portion of the decapod eyestalk is an extension of brain (supra-esophageal ganglion). A

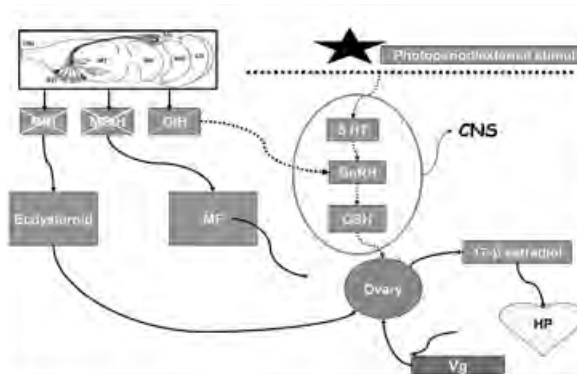


Figure 4 Schematic representation of shrimp reproduction and various factors controlling reproduction. CNS: central nervous system; GIH: Gonad inhibiting hormone; GnRH: Gonadotropic releasing hormone; GSH: Gonad stimulating hormone; 5HT: 5 hydroxy tryptamine; HP: hepatopancreas; LG: Lamia ganglioris; MF: methyl farnesoate; MT: Medulla terminalis; ME: Medulla externa; MI: Medulla interna; MIH: Molt inhibiting hormone, MOIH: Mandibular organ inhibiting hormone; Vg: vitellogenin

group of cell bodies usually found as faint blue white in live specimens is located in the middle portion of the medulla terminalis is termed as X organ. At least eight neuro-hormones appear to be synthesized in the X organ and it contains about 150 -200 neurosecretory cells. The neuro hormones produced in these cells are transported via axon and ends in the blood sinus called sinus gland in the medulla externa (Figure 5). These hormones regulate several physiological functions for example, gonad activity, molting, and blood-sugar level. CHH or crustacean hyperglycemic hormone family are structurally related neuro-hormones of X-organ. Two sub types of cHH

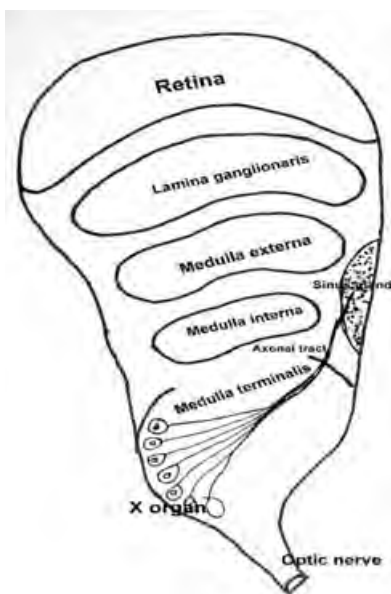


Figure 5 Schematic diagram of eyestalk showing X organ, sinus gland and optic nerve

peptides are recognized type 1 and type 11; type 1 peptides are cHH *sensu stricto* are with typically 72 amino acids. Their protein precursor contains cryptic peptide, cHH precursor related peptide (CPRP), between signal peptide and cHH progenitor sequence. This type 1 cHH is named because upon injection it elicit hyperglycemia in animals. Type 11 peptides are not with CPRP and it contains three neuro-hormones: Gonad/vitellogenesis inhibiting hormone (G/VIH), Molt inhibiting hormone (MIH) and Mandibular organ inhibiting hormone (MOIH).

Gonad/vitellogenesis inhibiting hormone:

G/VIH actively participate in ovarian development and is a key hormone for the reproduction in crustacea. It is believed that GIH is more intense than any other hormone in crustacea. Gene coding for GIH has been characterized and cloned from several crustaceans: For example, terrestrial isopod (*Armadillidium vulgare*), lobsters (*Homarus americanus* H. *gammarus* and *Nephropse norvegicus*) and shrimps (*P. monodon*, *Metapenaeus ensis*, *Litopenaeus vannamei*), prawn (*Macrobrachium nipponense*) and deep sea shrimp (*Rimicaris Kairei*). GIH is expressed in both male and female; it has been expressed in tissues such as eyestalk and brain. The presence of GIH has also found in larval crustacean as well. The expression of GIH mRNA is found to be lower in immature stage, however it was found to be higher in previtellogenic stage.

Gonad stimulating hormones/ gonadotropins

It is generally accepted that identification of gonad stimulating hormones (GSH) in crustaceans would greatly expedite domestication and closing of life cycle of commercially important aquacultured crustaceans that do not reproduce readily in captivity. Research on GSH has been started in 1960s. A substance known to stimulate vitellogenesis has been found in brain and thoracic ganglion of several crustacean species (Otsu, 1963; Gomez, 1965). But the chemistry of this species is not yet elucidated. De Kleijn and Van Herp (1998) suggested that this hormone might be a crustacean hyper glyceimic hormone.

Methyl Farnesoate: Parallel to the search for GSH, studies on other gonad stimulating hormones (gonadotropins) have been carried out by several researchers. These investigations results in the discovery of a crustacean hormone, Methyl farnesoate (MF) (Laufer, 1987). MF is an intermediate compound produced during the juvenile hormone biosynthetic pathway (Fig 6) juvenile hormone is well known and extensively studied in insects where they play several regulatory roles both as gonadotropin in adults and morphogens during development. Juvenile hormone as such is not found in crustaceans, but MF is isolated from mandibular organs of crustaceans. Mandibular organ actively synthesize MF during vitellogenesis and become less active during non-reproductive periods. The secretion of MF is tissue specific and circulated through the blood, and circulating levels are positively correlated with the reproductive state of females. It has also been shown that mandibular organ is negatively regulated by an eyestalk neuro hormone, mandibular organ inhibiting hormones

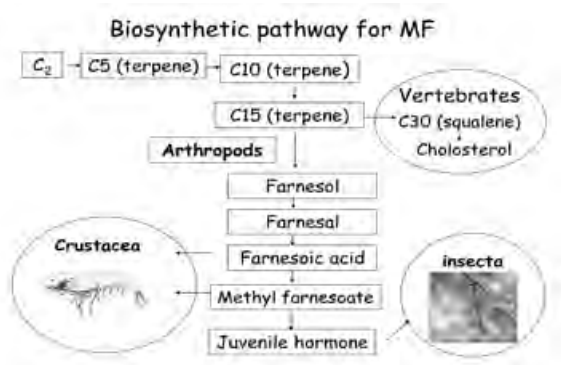


Figure 6 Schematic diagram of eyestalk showing X organ, sinus gland and optic nerve

(MOIH). The inhibition is artificially reversed *in vivo* by eyestalk ablation. However, MF can be applied directly, and in many cases successful ovarian maturation and spawning have been achieved as well. Table 2 shows that experimental results of MF treatment to study the effect of this hormone on reproduction of females.

Ecdysteroid: Ecdysteroid is a polyhydrated keto steroid, found in most arthropods and have a primary function as molting hormone. In crustacea ecdysteroids are synthesized in 'Y' organ (=ecdysal gland), and the alternative sources of Ecdysteroid is the epidermis, and ovary. It has been reported that Ecdysteroid has a possible role in reproduction and maturation as in the case of insects (Subramoniam 2000). It has been suggested that crustacean hormones are multifunctional in nature, a single hormone can mediate different functions. Ecdysteroid mediate as hormone that promotes protective membrane in embryos, then they function as molting hormone from larvae to adult life. In adult they function as gonadotropin. Chang (2001) called it as an 'amazing economy of nature'

Vertebrate-like steroids: Several decapod crustaceans have the ability to synthesize the vertebrate-type steroids (e. g. progesterone, 17- β - estradiol, and testosterone). Some of them fluctuate during the reproductive cycles, and therefore, indicates their role in the reproduction. Many crustaceans have been found to be responding to the injections of these steroids. In additions to the steroids, biogenic amines such as, 5 hydroxy tryptamine (serotonin) are also found to have ovarian stimulatory effect. Recently Wongprasert (2006) reported that serotonin injected *P. monodon* had ovarian maturation

Table 2 Effect of methyl farnesoate on crustacean reproduction

Species	Mode of study	Nature of response	Reference
<i>Triops longicudatus</i>	In vitro	No effect	Riley and Tsukimura 1998
<i>Triops longicudatus</i>	In vivo	Inhibition	Tsukimura <i>et al.</i> 2006
<i>Litopenaeus vannamei</i>	In vitro	Reproductive stimulation	Tsukimura and Kamemoto 1991
<i>Litopenaeus vannamei</i>	In vivo	Improvement of reproductive performance	Laufer 1992
<i>Sicyonia ingentis</i>	In vivo	Reproductive stimulation	Balasubramanian <i>et al.</i> 2010
<i>Penaeus monodon</i>	In vivo	Improvement of reproductive performance	Hall <i>et al.</i> 1999
<i>Penaeus monodon</i>	In vivo	Reproductive inhibition	Marsden <i>et al.</i> 2008
<i>Macrobrachium rosenbergii</i>	In vivo	No effect	Wilder <i>et al.</i> 1994
<i>Homarus americanus</i>		No effect	Tsukimura <i>et al.</i> 1993
<i>Procambarus clarkii</i>	In vivo	Reproductive stimulation	Laufer <i>et al.</i> 1998

and spawning similar to that of unilateral eyestalk ablated females. Although the mode of action of these hormones/neuro transmitters is not properly understood, there seems to be a tremendous potential in using these hormones to stimulate gonadal maturation in the aquacultured species.

In summary, the crustacean egg production is controlled by a cascade of hormonal activities which is triggered by environmental and nutritional factors. There fore, all these factors should be considered when any reproductive technology is developed.

Endocrine methodologies

Like most other discipline progress in endocrinology is largely depends on the analytical techniques. The most important step in the endocrinological study is the characterization of endocrine system, and understand how different organs function. This knowledge can be used to improve the reproductive performance of the animals. The information needed to characterize the hormone are: chemical structure of the hormone, biosynthesis and storage, and pathway of metabolisms. In most cases the fist assay when dealing with an unknown hormone is a bioassay. It is based on the measurement of physiological response caused by a hormone. In a bioassay, the hormone is injected directly into the animal to observe the phenotypic effect, or it is dissolved in a medium in which an appropriately responsive tissue can be cultured. An ideal bioassay is one that produces a graded response to the hormone, rather than an all or none response. The response of the assay is then transferred to numerical scale, by which the relative activity of the hormone can be quantified. Hormone concentrations are generally expressed in titers because they are usually determined indirectly by progressive dilution (titration) of the hormone sample being tested until its activity in the bioassay is no longer detectable. The goal of the bioassay is to detect the approximate concentration of hormone normally occurs in the animal. When excessive hormone is used in bioassay a non-specific response may occur. This abnormal physiological response is called pharmacological response

Chemical structure, biosynthesis and pathways of hormones can be studied by various chemical

analytical methods (Table 3). Today, assay of peptide hormones take advantages of powerful recombinant DNA and antibody technologies. If aminoacid sequence of a hormone is known, it is possible to synthesis nucleic acid probes that can recognize gene that codes for the hormone. The knowledge gene sequence also helps to understand the temporal and spatial pattern of expression of mRNA

Future Directions of reproductive physiological research

Basic research on reproductive physiology/endocrinology inevitably leads to the advances in applied research and eventually the practices of aquaculture. Achievements in the fin fish reproductive physiology is an excellent example for how basic endocrinological findings can be effectively integrated into broodstock management (Zohar and Mylonas, 2001). On the contrary, we are

Table 3 Conserved hierarchy of endocrine methodologies.

Sl no	Study level	Techniques
1	Gene	mRNA expression pathways (RT PCR, qRT PCR, microarrayn, Northern Blot)
2	Protein	PAGE, western blot, ELISA
3	Functional cell	Histology Histochemistry Immunohistochemistry, In-situte hybridization

still far from achieving adequate understanding on control of reproduction in penaeid shrimp. The growth of penaeid shrimp aquaculture has been spectacular and regarded as a remarkable success story of modern aquaculture. Twenty five years ago, Primavera (1985) identified the replacement of eyestalk ablation and development of low cost maturation diet as key goals to increase spawner yield and reduce the production cost of shrimp culture industry. Unfortunately, even after twenty five years later, endocrine replacement for eyestalk ablation yet to be achieved. When we look into the global scenario of farmed shrimp production, there is an obvious shift at production level. While *P. monodon* production remains at constant level, there is a drastic hike in the production of *Litopenaeus vannamei*. The poor performance of *P. monodon* is essentially due to the non-availability of specific pathogen free (SPF) broodstock. The most significant hindrance for the development of SPF broodstock is the poor reproductive performance of captive *P. monodon*. Although findings on reproductive physiology of penaeid shrimps have been increased during the recent years, findings are

still inadequate to resolve sufficient background for successful broodstock management. Information on the key hormones involved in reproduction is being just published.

Many of the research work in crustacean physiology are criticized as semi scientific (Adiyodi and Subramoniam, 1985) or lack sound experimental design (Benzie, 1997). Further, most endocrinological studies in aquaculture are based on non-model wild caught animals, and, therefore, the likelihood for the genetic and physiological heterogeneity is extremely high. The data generated by these studies does not have much value to develop a model for reproductive control.

The endocrine manipulation for induced maturation and spawning should be made at different physiological command levels. The choice at which level these interventions to be made are largely determined by at which level reproductive cycle disruption occurs due to the impact of captivity. These disruptions vary from simple inhibition of spawning to complete lack of gonad development. The success of endocrine manipulation largely depends upon the physiological stage of female. If female is not competent for oocyte maturation, it is less likely for having responded for hormonal therapy.

Modern technology has shifted endocrine research from traditional reductionist approach (part by part study) to an integrative approach. Although traditional approach progressed the science of endocrinology, it has limitations. The integrative approach investigates the complex system at a time. The modern molecular techniques such as microarray techniques would assist to unravel the many complexities of endocrinology. The selective breeding for the high reproductive trait is found to have tremendous scope in improving the captive maturation of penaeid shrimps.

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Protocols

Basic Tools in the Biotechnology Laboratory

A training in Molecular Biology can't be initiated without imparting a basic knowledge about the common lab equipment pieces and describe their function.

Measurement of Volume

Erlenmeyer flasks: are used primarily to prepare solutions prior to an accurate volume adjustment. Although there are volumetric markings on these flasks, they are not calibrated and should not be relied upon for exact volume measurements.

Beakers: are also used for preparing solutions, especially when a pH adjustment requires access to the solution by a pH probe. The volumetric markings on beakers are also not reliable.

Graduated cylinders: are calibrated with sufficient accuracy for most volume measurements when preparing solutions. For example, the calibration of most 100 mL graduated cylinder can be relied upon to accurately measure to within ± 0.6 mL.

Volumetric flasks: are used to measure a specific volume with the highest degree of accuracy, and are used to make standard solutions for analytical assays. For example, the calibration of a 100 mL volumetric flask can have an accuracy of ± 0.1 mL.

pipettes: are glass or plastic devices that are routinely used to measure and transfer liquids by drawing the liquid into the tube with a bulb or mechanical pump.

a. **Pasteur pipettes:** are small glass tubes used with a bulb to transfer volumes as small as a

single drop and as large as a few milliliters. They are not graduated and are not used to measure volumes.

- b. **Beral pipettes:** (transfer pipettes) are plastic pipettes with a bulb at one end used for transfer of liquids. Sometimes they have calibration marks, which have a low level of accuracy. They are often disposable, sterile and individually wrapped.
- c. **Serological:** or "blowout," pipettes are graduated glass tubes used to measure anywhere from 0.1 to 50 mL. When the liquid has drained from this pipette, the final drop in the tip is transferred with a puff of air.
- d. **Mohr:** or "to deliver," pipettes are similar to blowout pipettes, but do not require a puff of air to accurately deliver the desired volume. They can be identified by the label 'TD' on the top.
- e. **Volumetric pipet:** s are not graduated, but are carefully calibrated to deliver a single, highly accurate volume, and are used for the transfer of exact volumes.
- f. **Micropipettes:** are calibrated to deliver highly accurate volumes generally less than 1.0 mL, and as little as 0.1 microliter. They are often adjustable for measuring different volumes and they always use dispensable plastic tips to actually transfer the liquids.
- g. **Multichannel micropipettes:** can deliver the same volume from as many as 12 tips simultaneously. All micropipettes need regular maintenance, calibration, and validation.

Measurement of Weight

Instruments for weighing materials are called balances, and most laboratories have more than one

type of balance, depending on the amount of material being measured and the degree of accuracy required.

- a. **Mechanical balances:** weigh an object on a pan hanging from a beam that has a counterbalanced weight. We do not use mechanical balances in our lab.
- b. **Electronic balances:** have replaced most mechanical balances due to their greater accuracy and ease of operation. They are easier to use because they usually have a digital readout, and weighing dishes can be tared to read zero mass before using. Most balances used for preparation of solutions have a sensitivity of ± 0.01 g, but electronic analytical balances can be sensitive to ± 0.1 mg or less. Electronic balances require routine maintenance and recalibration

Measurement of pH

Most solutions prepared in the biological laboratory must have a carefully controlled pH. Buffers are prepared by adjustment to a specific pH with strong acid and base solutions, using a meter to monitor the pH. A pH meter is a volt meter that measures the electrical potential between two electrodes. One electrode is in contact with your solution, and the other is in contact with a reference solution. Usually both of these electrodes are combined in a single pH probe that you place in your solution. These meters can read to the nearest 0.1 pH unit, but require frequent calibration with reference buffers of known pH.

Measurement of light

Solutions are often analyzed in the biotechnology lab by measuring how the solutes interact with light. A spectrophotometer measures the amount of light that is absorbed by a solution at a specific wavelength or over a range of wavelengths. If you know a wavelength at which a specific substance absorbs light, you can calculate the amount of that substance in a solution from the measured absorbance of that solution at that wavelength.

- A visible (VIS) spectrophotometer measures absorbance of light in the visible region of the spectrum (wavelength of about 400-700 nm). A small vessel called a cuvette, which is generally

plastic or glass and which usually has an internal diameter of 1.0 cm, is filled with the solution and placed in the spectrophotometer for measurements.

- An ultraviolet/visible (UV/VIS) spectrophotometer can also measure absorbance of light in the ultraviolet region of the spectrum (about 100-400nm). These spectrophotometers require a halogen light bulb that emits ultraviolet light and require special cuvettes that don't absorb UV light.
- A scanning spectrophotometer can measure the absorbance of a solution over a range of wavelengths, creating an absorbance spectrum that can be used to identify substances in a solution.
- A NanoDrop spectrophotometer is a brand of scanning UV/VIS spectrophotometer that allows the user to measure the absorbance of a very small sample of liquid (1-2 uL). This instrument makes it easy to quickly evaluate the quality and quantity of nucleic acids or proteins in a small sample prep.
- A microplate reader is a spectrophotometer that can measure the absorbance in the individual wells of a plate. Usually the plates are 96 wells, but other formats are available, such as 48 wells and 384 wells. This allows the user to prepare and read many small samples at once, saving time and money. Microplate readers may also be capable of reading fluorescence and chemiluminescence, which are two types of light emission that are frequently used in biological research.

Solution Preparation

Solution preparation involves mixing liquids and dissolving solids in liquids. There are many specialized devices in addition to balances, volume measuring devices, and pH meters involved in these processes.

- Magnetic stirrers come in the form of a box with a magnet inside attached to a motor that spins the magnet. When a vessel containing a magnetic stir bar is on top of the magnetic stirrer, the stir bar spins and stirs the contents of the vessel.
- A vortex mixer rotates the bottom of a tube rapidly; setting up a vortex in the liquid that rapidly mixes the contents.

Microbiological techniques

Specialized equipment is required to isolate, transfer, and grow up cultures of microbes and tissues in the laboratory.

- Autoclaves are machines that achieve a high internal temperature and pressure and are used to sterilize solutions and glassware. The kitchen pressure cooker achieves the same results and can be used instead of an autoclave.
- A biological safety or cell culture hood filters small particles out of the air in order to avoid contamination of cultures or sterile media. The filters are similar to those used to decontaminate air for operating rooms in hospitals or clean rooms used in the semiconductor industry.
- Fermenters are used to grow up a large quantity of cells with automatically controlled pH and levels of oxygen and other nutrients.
- Since most cells are generally too small to be seen with the naked eye, microscopes are used to magnify their images. Light or Bright field microscopes and inverted microscopes are the most common types found in biotechnology laboratories.

Preparation of biological samples for analysis

There are many pieces of equipment that are used to prepare biological samples for analysis.

- A Sorvall type centrifuge, or preparative centrifuge, has a balanced rotor that holds vessels and spins them at high speed, up to 20,000 rpm. This will cause most insoluble particles such as cells and many subcellular components to rapidly form a pellet at the bottom of the vessel. Rotors are available that hold vessels as small as a few milliliters to as large as a liter. These centrifuges are often refrigerated so that heat sensitive compounds are not damaged during centrifugation.
- A tabletop, or clinical, centrifuge is generally not refrigerated and spins at a much slower speed than a preparative centrifuge. Rotors for clinical centrifuges generally hold tubes with a capacity of 15 mL or less.

- A microcentrifuge holds microcentrifuge tubes that can hold about 1.5 mL of liquid. These microcentrifuges can also spin at high speeds and are sometimes refrigerated.
- A sonicator emits ultrasonic waves that can be used to disrupt cells, allowing their contents to be released into the surrounding buffer in -grind and find strategies.

Separation of macromolecules

Since there are thousands of different macromolecules in each cell, purification of a specific one from all the others requires powerful separation techniques, such as chromatography and electrophoresis. Both of these approaches take advantage of physical and chemical properties that differ between the individual macromolecules.

In gel electrophoresis, the macromolecules are placed in a solid matrix, called a gel, which is under a liquid buffer. An electric field is applied to this system, and since biological macromolecules carry ionic charges, they will be attracted towards one pole of the electric field and repelled by the opposite. Thus, macromolecules characteristically migrate in either direction in the field. The migration speed is determined by the charge to mass ratio of the macromolecule.

- In a flat gel, also called a horizontal or submarine gel, electrophoresis system, an agarose gel lies horizontally below the electrophoresis buffer. This technique is mainly used to separate large nucleic acids (DNA and RNA).
- A vertical electrophoresis system holds a polyacrylamide gel in the vertical position, and is mainly used to separate proteins or small sized nucleic acids.

Chromatography is a family of methods used to separate macromolecules through their relative affinity to a stationary phase (generally, solid chromatography beads) and a mobile phase (generally, an aqueous buffer). The chromatography beads are loaded into a tube, called a chromatography column, and buffer is dripped, or pumped, through

the column to carry the macromolecules along. The macromolecules separate on their affinity for the mobile front. Some chromatography beads separate by charge (ion exchange chromatography), by hydrophobicity (hydrophobic interaction chromatography), or by a specific property of that protein (affinity chromatography). Macromolecules can also be separated by size otherwise known as size exclusion or gel filtration chromatography.

- To overcome this limitation, high performance (or high pressure) chromatography (HPLC) uses high pressure pumps and metal jacketed columns to operate at high pressures and speed up the process.
- A fraction collector collects the released mobile phase (eluent) of a chromatography column. It automatically measures a programmed volume (sometimes by the number of drops of liquid) into a line of test tubes or microcentrifuge tubes.

Manipulation of Nucleic Acids

Some of the specialized pieces of equipment used in the isolation, transfer, and analyze DNA in the

molecular biology procedures will include:

- A **thermal cycler** is a machine that is used for amplification of a specific section of DNA by PCR (polymerase chain reaction). The machine cycles through several temperatures, which allows an enzyme called DNA polymerase to use chemicals in solution to build DNA molecules identical to a template provided.
- An **electroporator** is used to discharge a high voltage, high amperage pulse of electricity of very short duration through a cuvette containing suspended cells to disrupt their plasma membranes, allowing DNA to be introduced.
- A **real time PCR** machine amplifies and measures the production of amplicons in one step. It is a thermal cycler and fluorescent analyzer in one instrument and is usually computer controlled. You do not have to load your product onto a gel to determine if it was made; the machine measures its production photometrically.

Nucleic acid Isolation

Research in biotechnology field largely depends on the genome analysis and recombinant DNA technology. Good quality nucleic acid is an essential prerequisite for consistent results in most of the downstream applications in the genome analysis and recombinant DNA technology.

The general principle underlying the isolation of nucleic acids is common with few modifications depending on the type of nucleic acid being isolated. Firstly the nucleic acid is to be made free from the other biological macromolecules and cell debris. This is achieved by properly lysing the cell wall/cell membrane and then by selectively denaturing the other macromolecules like proteins. Nucleic acids thus recovered in its native form is precipitated by alcohol and suspended in sterile buffer or distilled water. Finally the qualitative integrity of the isolated nucleic acid is checked by agarose gel electrophoresis and ethidium bromide staining. Quantitative estimation of nucleic acid is carried out by spectrophotometric methods.

The types of nucleic acids usually isolated on a routine basis are, total genomic DNA (gDNA), total RNA, extra chromosomal DNA (Plasmid, Mitochondrial DNA, Chloroplast DNA etc.). Earlier DNA was one of the most difficult molecules to analyze. But now, DNA has become the easiest macromolecule of the cell to study. DNA can be easily purified from the cells and once isolated it is much more stable than any other macromolecule. It can be manipulated precisely and reproducibly with various molecular tools like restriction enzymes, PCR, cloning etc. enabling genome analysis and recombinant DNA technology with much ease.

Isolation of total genomic DNA (gDNA)

Breaking of the bacterial and plant cell wall as well as solubilizing the cell membrane of animal cells are to be

carefully carried out under optimum conditions. Even rapid stirring can break high molecular weight DNA into shorter fragments. If physical disruption is necessary, as in the case with certain tissues, it should be kept to the minimum, and should involve cutting or squashing of cells, rather than the use of shear forces. Ultrasonic sound is used to disrupt cell wall of certain bacteria.

Care has to be taken to prevent degradation of DNA by deoxyribonucleases (DNase). These enzymes are found in most cells, and may also be present in dust, which could contaminate laboratory glassware. Hence all the glassware, plastic wares and the buffers are to be made sterile by autoclaving. These enzymes activity can also be inhibited by using EDTA in lysis buffer, which will chelate the Mg^{2+} ions needed for the DNase activity and also essential for preserving the overall structure of the cell membrane thus helps in cell lysis. Cell disruption and most of the subsequent steps should be performed at 4°C. The cell wall could be lysed enzymatically as well. The bacterial cell wall is usually lysed by the enzyme Lysozyme. The cell membrane on the other hand are solubilized by including suitable detergent like Sodium dodecyl Sulfate (SDS) in the lysis buffer. SDS aids in disrupting the cell membranes by removing the lipids of the cell membranes.

Upon lysis the entire content of the cell including nucleic acids (DNA, RNA), cytoplasm, organelles etc. will be released in to the lysis buffer and now the target molecule DNA is to be made free from RNA and other associated proteins. The RNA molecules can be selectively denatured by enzymatic treatment with RNase. The other major contaminant, protein, is removed by enzymatic treatment with proteinase K followed by treatment with Tris saturated Phenol (pH 8.0) or Phenol: Chloroform mix (1:1), either of which will denature proteins. Centrifugation of the emulsion formed by this mixing produces a lower, organic phase, separated from the upper aqueous

phase by an interface of denatured proteins. The upper aqueous phase was recovered and deproteinised repeatedly until no material is seen at the interface and washed with chloroform: isoamyl alcohol (24:1), to remove traces of phenol. Finally by the addition of two volumes of ice-cold absolute alcohol in the presence of salt (3M Sodium Acetate, pH 5.2) the high molecular weight DNA can then be efficiently precipitated out of the solution in a -20°C freezer.

After centrifugation, the DNA pellet is dissolved in a buffer containing EDTA for protection against DNases, and this solution can be stored. DNA solutions can be stored frozen, but repeated freezing and thawing tends to damage long molecules by shearing and hence the DNA preparations in frequent use are normally stored at 4°C.

Quantitative estimation of nucleic acid.

DNA can be spectrophotometrically estimated by taking optical density (OD) at 260nm, 1 OD corresponds to 50 microgram of DNA. Purity of the DNA can be checked spectrophotometrically by taking OD at 260 & 280 nanometers (nm). The ratio of 260 and 280 will result a value of 1.8 with pure DNA preparations.

Protocol for the isolation of DNA from animal tissue using Phenol Chloroform method.

Equipment and reagents required

- Lysis buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0, 400mM NaCl)
- Sodium dodecyl sulfate (SDS) solution (10%)
- Proteinase K (20mg/ml)
- RNase A (10mg/ml)
- Tris saturated Phenol (pH 8.0): Chloroform (1:1 v/v)
- 24:1 (v/v) chloroform-isoamyl alcohol
- 3 M sodium acetate, pH 5.2
- Ice cold Ethanol (100%)
- Ethanol (70%)
- TE buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0)
- Micro centrifuge

- Micro centrifuge tubes (1.5ml)
- Micropipettes

Procedure/Protocol

- 50 mg of tissue (muscle/fin clips) was homogenized with 400µl of lysis buffer in a sterile 1.5 ml micro centrifuge tube.
- 100ul of 10% SDS solution and 10 µl of Proteinase K was added to the homogenate
- The above mixture is mixed well and incubated at 55°C for an hour in an incubator / water bath.
- 500 µl of phenol-chloroform (1:1) mixture is added to the contents, mixed well by inverting several times and incubated at room temperature for 5 minutes.
- The contents are centrifuged at 10,000 rpm for 10 minutes at 4°C.
- The supernatant is collected using cut/wide bore tips and is transferred to a fresh tube.
- 10µl of RNase A was added to the supernatant and incubated at 37°C for an hour in an incubator
- The phenol: chloroform extraction is repeated and after centrifugation at 10,000 rpm for 10 minutes at 4°C the supernatant is collected in a fresh tube.
- Equal volume chloroform-isoamyl alcohol (24:1 v/v) is added to the supernatant, mixed well by gently inverting several times.
- The contents are centrifuged at 10,000 rpm for 10 minutes at 4°C.
- The supernatant is collected using cut/wide bore tips and is transferred to a fresh sterile micro centrifuge tube.
- 1/10 volume of 3M sodium acetate (pH 5.2) is added to the contents and is mixed gently.
- 1 ml of ice cold ethanol (100%) is added and mixed gently by inversion till white strands of DNA precipitates out. If no precipitate is observed the tube is incubated overnight at -20°C.
- After incubation the tube is centrifuged at 10000 rpm for 10 min to pellet the DNA.
- The pellet was washed in 70% ethanol by centrifugation at 1000 rpm for 10 min at 4°C
- Decant the ethanol and air dry the pellet
- Dissolve the pellet in 50 µl to 100µl of TE buffer or sterile dH₂O.
- The stock of the DNA samples is then stored at -20°C and diluted working solution can be stored at 4°C.

Isolation of DNA from animal tissue using salting out procedure

Reagents required:

Stock solutions:

0.5M Tris Cl (pH-8.0)

Tris base - 3.028 g
Distilled water - 40 ml
Adjust pH to 8.0 using HCl.
Make up the volume to 50 ml, autoclave and store at 4°C.

0.5M EDTA (pH-8.0)

EDTA - 9.31 g
Distilled water - 40 ml
Adjust pH to 8.0 using NaOH.
Make up the volume to 50 ml, autoclaved and stored at 4°C.

10mM Tris Cl (pH-7.5)

Tris base - 0.030 g
Distilled water - 20 ml
Adjust pH to 7.5 using HCl.
Make up the volume to 25 ml, autoclaved and stored at 4°C

RNAase buffer

10mM Tris Cl (pH 7.5)- 10 μ l
15mM NaCl - 30 μ l
Distilled water - 960 μ l
Autoclaved and stored at 4°C.

Working Solutions

Solution 1:

Tris-HCl (pH8.0) - 50 mM
EDTA (pH8.0) - 20 mM
SDS - 2 %
Prepared in double distilled water. Autoclave and store at 4°C

Solution 2:

NaCl solution (saturated)–(6 M)
Prepared in double distilled water.
Autoclave and store at 4°C

Proteinase K

Proteinase K – 20 mg/ml
Prepared in autoclave double distilled water and store at -20°C.

TE buffer

Tris Cl (pH-8.0) - 10 mM
EDTA (pH-8.0) - 1 mM
Prepared in double distilled water. Autoclave and store at 4°C

RNAase

RNAase–10 mg/ml of RNAase buffer (autoclaved)

Protocol

Tissue stored in alcohol was washed with TRIS buffer (pH 8.0) by spinning and then followed below listed steps.

- Placed tissue sample in 1.5 ml tube & added 500 μ l Solution 1.
- Homogenized tissue sample with sterile homogeniser or melted filter tip.
- Added 5 μ l of Proteinase K (20 mg/ml)
- Incubated at 55°C in water bath for 2 hours (with occasional mixing).
- Chilled on ice for 10 minutes.
- Added 250 μ l Solution 2 and inverted several times for through mixing.
- Chilled on ice for 5 minutes.
- Centrifuged at 8000 rpm for 15 minutes.
- Carefully collected clear supernatant (\sim 500 μ l) with wide-bore filter tip into a newly labeled 1.5 ml tube.
- Added 1.5 μ l RNase (final conc.20 μ g/ml) and incubated at 37°C on heating block for 15 minutes.
- Added twice the volume (\sim 1 ml) of ice cold 100 % molecular biology grade Ethanol to precipitate the DNA.
- Incubated overnight at -20°C.
- Next day, centrifuged at 11000 rpm for 15 minutes and removed supernatant.
- Rinsed DNA pellet in 250 μ l of ice-cold 70% ethanol.
- Centrifuged at 11000 rpm for 5 minutes.
- Carefully removed supernatant and partially dry with lid off at room temperature.
- Resuspended partially dried DNA in 50-200 μ l (depending on size of pellet) of TE buffer (pH-8) by gently pipetting the sample with wide-bore filter tip until dissolved.

Isolation of DNA from animal tissue using Kits

QIAGEN DNeasy Blood & Tissue Kit

1. Cut up to 25 mg tissue (up to 10 mg spleen) into small pieces, and place in a 1.5ml microcentrifuge tube. For rodent tails, place one (rat) or two (mouse) 0.4–0.6cm lengths of tail into a 1.5 ml microcentrifuge tube. Add 180 μ l Buffer ATL.
2. Add 20 μ l proteinase K. Mix thoroughly by vortexing, and incubate at 56°C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample, or place in a thermomixer, shaking water bath, or on a rocking platform.
3. Vortex for 15 s. Add 200 μ l Buffer AL to the sample, and mix thoroughly by vortexing. Then add 200 μ l ethanol (96–100%), and mix again thoroughly by vortexing.
4. Pipet the mixture from step 3 (including any precipitate) into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at $\geq 6000 \times g$ (8000 rpm) for 1 min.
5. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 μ l Buffer AW1, and centrifuge for 1 min at $\geq 6000 \times g$ (8000 rpm). Discard flow-through and collection tube.
6. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 μ l Buffer AW2, and centrifuge for 3 min at 20,000 $\times g$ (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.
7. Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube, and pipet 200 μ l Buffer AE directly onto the DNeasy membrane.
8. Incubate at room temperature for 1 min, and then centrifuge for 1 min at 6000 $\times g$ (8000 rpm) to elute.
9. Recommended: For maximum DNA yield, repeat elution once as described in step 7.

Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is a technique widely used in molecular biology. It derives its name from one of its key components. A thermostable DNA polymerase isolated from a thermophilic bacterium *Thermus aquaticus* (*Taq* polymerase) is most often used and since it is heat stable, it does not have to be replaced after each cycle. Developed in 1984 by Kary Mullis. PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications. PCR is a technique that employs in-vitro enzymatic amplification of a particular or desired DNA fragment of up to several kilobases (kb) in size from a complex genome. PCR allows the production of more than 10 million copies of a target DNA sequence from only a few molecules.

- Basic PCR set up requires several components and reagents. These components include:
- DNA template that contains the DNA region to be amplified.
- Two primers, which are complementary to the DNA regions at the 5' or 3' ends of the DNA region.
- *Taq* polymerase to amplify the DNA
- Deoxynucleoside triphosphates (dNTPs); the building blocks from which the DNA polymerases synthesizes a new DNA strand.
- Buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.

The PCR usually consists of a series of 20 to 40 repeated temperature changes called cycles; each cycle typically consists of 2-3 discrete temperature steps. Most commonly PCR is carried out with cycles that have three temperature steps

- Denaturation step: This step consists of heating

the reaction to 94-98°C for 30 seconds. It causes melting of DNA template and primers by disrupting the hydrogen bonds between complementary bases of the DNA strands, yielding single strands of DNA.

- Annealing step: The reaction temperature is lowered to 50-65°C for 30 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3-5 degrees Celsius below the T_m of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis.
- Extension/elongation step: At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction.
- Final elongation: This single step is occasionally performed at a temperature of 70-74°C for 5-15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.
- Final hold: This step at 4°C for an indefinite time may be employed for short-term storage of the reaction.

In PCR, the double stranded DNA is denatured by heat and then the temperature is lowered to allow annealing of two specific primers by complementary base pairing on the opposite strands of the DNA. *Taq* polymerase directs the synthesis of the new strand from the primed sites in both directions that results in double stranded DNA—and the procedure is repeated. In each cycle, the target DNA is replicated by a factor of 2 so that, after 30 cycles millions of copies of DNA

are available for subsequent manipulation.

PCR based techniques require only a small amount of template DNA of relatively inferior quality and therefore, the amount of material required for analysis is greatly reduced in comparison to all other methods. PCR can be used to identify a target organism even in small numbers in mixed infections. For methods involving PCR, the major potential drawback is the risk of contamination of the sample with material from another sources, especially previously amplified DNA. A control reaction, omitting template DNA, should always be performed, to confirm the absence of contamination

Materials required

Thermocycler, micro centrifuge, vortex mixer, thin-walled PCR tubes, Micropipettes and tips.

Reagents required

Template DNA, Primers, 10mM dNTP mix, 25mM MgCl₂, Taq DNA polymerase with 10x buffer

Components of the reaction mixture

Template DNA

Usually the amount of template DNA is in the range of 0.01-1 ng for plasmid or phage DNA and 0.1-1 µg for genomic DNA, for a total reaction mixture of 50 µl. Higher amounts of template DNA usually increase the yield of nonspecific PCR products.

Primers

PCR primers are usually 15 -30 nucleotides in length. The GC content should be 40-60 %. More than three G or C nucleotides at the 3'-end of the primer should be avoided, as nonspecific priming may occur. The primer should not be self-complementary or complementary to any other primer in the reaction mixture, in order to avoid primer-dimer and hairpin formation.

MgCl₂ concentration

Since Mg²⁺ ions form complexes with dNTPs, primers

and DNA templates, the optimal concentration of MgCl₂ has to be selected for each experiment. Too few Mg²⁺ ions result in a low yield of PCR product, and too many increase the yield of non-specific products and promote mis-incorporation. The recommended range of MgCl₂ concentration is 1-4 mM under the standard reaction conditions specified.

dNTPs

The final concentration of each dNTP in the reaction mixture is usually 200 µM. It is very important to have equal concentrations of each dNTP (dATP, dCTP, dGTP & dTTP), as inaccuracy in the concentration of even a single dNTP dramatically increases the misincorporation level.

Taq DNA polymerase

Usually 1-1.5-units of Taq DNA polymerase are used in 50µl of reaction mix. Higher Taq DNA polymerase concentrations may cause synthesis of nonspecific products.

Preparation of reaction mixture

Reagent	Final Conc.	Volume for 50µl reaction mix
10x buffer	1x	5.0
dNTP mix (10mM each)	200 µM	1.0
MgCl ₂ (25mM)	1mM	2.0
Forward primer (10pmol/µl)	10pmol	1.0
Reverse primer (10pmol/µl)	10pmol	1.0
Taq polymerase enzyme(2U/µl)	1U	0.5
Template DNA	50ng	1.0
Sterile H ₂ O		38.5

To perform several parallel reactions, prepare a master mix containing water, buffer, dNTPs, primers, Taq DNA polymerase and MgCl₂ in a single tube, which can then be aliquoted into individual tubes. This method of setting reactions minimizes the possibility of pipetting errors and saves time by reducing the number of reagent transfers. Template DNA solution is added after aliquoting into tubes.

Gently vortex and briefly centrifuge all solutions after thawing.

Add the following in a thin-walled PCR tube placed on ice:

A Negative control can be maintained by replacing the Template DNA with sterile water.

Gently vortex the sample and briefly centrifuge to collect all drops from walls of tube.

Place samples in a thermocycler and start PCR.

Thermocycling conditions

Initial Denaturation

The complete denaturation of the DNA template at the start of the PCR reaction is of key importance. The initial denaturation should be performed over an interval of 1-3 min at 95°C if the GC content is 50% or less. This interval should be extended up to 10 min for GC rich templates.

Denaturation

Usually denaturation for 30–120 sec at 94-95°C is sufficient, since the PCR product synthesized in the first amplification cycle is significantly shorter than the template DNA and is completely denatured under these conditions

Annealing

Annealing temperature of the primers is calculated using the following formula

$$T_m = 4(G + C) + 2(A + T)$$

$$\text{Annealing temperature (°C)} = T_m - 5^\circ\text{C}$$

Where,

T_m = Melting temperature; G, C, A, T = number of respective nucleotides in the primer.

Extension

Usually the extending step is performed at 72°C. The rate of DNA synthesis by *Taq* DNA Polymerase is highest at this temperature. Recommended extension time is 1 m for the synthesis of PCR fragments up to 2 kb.

Number of cycles

The number of PCR cycles depends on the amount of template DNA in the reaction mix and on the expected yield of the PCR product. 25-35 cycles are usually sufficient.

Final extension

After the last cycle, the samples are usually incubated at 72°C for 5-15 min to fill in the protruding ends of newly synthesized PCR products.

Visualization of PCR products

The PCR product was visualized by electrophoresis in agarose gel stained with 0.5 µg/ml ethidium bromide.

RNA Isolation Protocol – Using TRIZOL

Reagents required

- DEPC-treated water
- TRIZOL Reagent (Invitrogen)
- 75% ethanol
- Isopropyl alcohol

Equipment and supplies

- Refrigerated Microcentrifuge
- Micropipettors
- Aerosol-barrier tips
- Vortex mixer
- Powder-free gloves
- Centrifuge tubes

Homogenization

- Homogenize 50 to 100 mg of tissue samples in 1 ml of TRIZOL reagent using a glass-Teflon or power homogenizer. The sample volume should not exceed 10% of the volume of TRIZOL Reagent used for the homogenization.
- Incubate the homogenized sample for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Centrifuge to remove cell debris. Transfer the supernatant to new tube.

Phase Separation

- Add 0.2 ml of chloroform per 1 ml of TRIZOL Reagent. Cap sample tubes securely. Vortex samples vigorously for 15 seconds and incubate them at room temperature for 2 to 3 minutes. Centrifuge the samples at no more than 10,000 rpm for 15 minutes at 4°C.

- Following centrifugation, the mixture separates into lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. Transfer upper aqueous phase carefully without disturbing the interphase into fresh tube. Measure the volume of the aqueous phase (The volume of the aqueous phase will about 60% of the volume of TRIZOL Reagent used for homogenization).

RNA Precipitation

- Precipitate the RNA from the aqueous phase by adding equal volume ice-cold isopropyl alcohol (100%). Incubate samples at -20°C for 10 minutes and centrifuge at 10,000 rpm for 10 minutes at 4°C. The RNA precipitate, often invisible before centrifugation, forms a white gel-like pellet on the side and bottom of the tube.

RNA Wash

- Remove the supernatant completely. Wash the RNA pellet once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of TRIZOL Reagent used for the initial homogenization. Mix the samples by vortexing and centrifuge at 10000 rpm for 5 minutes at 4°C.

Redissolving RNA

- Air-dry or vacuum dry RNA pellet for 5-10 minutes to remove all leftover ethanol. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. Partially dissolved RNA samples have an A260/A280 ratio < 1.6. Dissolve RNA in DEPC-treated water by passing solution a few times through a pipette tip.

Spectrophotometric Analysis

- Dilute 1 μ l of RNA with 39 μ l of DEPC-treated water (1:40 dilution). Take OD at 260 nm and 280 nm to determine sample concentration and purity. The A260/A280 ratio should be above 1.9. Apply the convention that 1 OD at 260 equals 40 μ g/ml RNA to calculate the exact conc. of RNA.

and proteoglycan-rich sources. *Biotechniques* 19(6): 942-5.

TRIZOL Reagent technical insert (Invitrogen).

Suggested readings

Chomczynski P, Mackey K. (1995) Short technical report. Modification of the TRIZOL reagent procedure for isolation of RNA from Polysaccharide-

Isolation of total RNA by modified guanidine thiocyanate method

Preparation of glassware and reagents

Glasswares and plasticwares

All glass wares used for RNA isolation were treated with 0.1 % Diethyl pyrocarbonate (DEPC) solution prepared in Milli-Q water for 4 h at 37°C and then kept at 150°C in hot air oven for 3 h. Sterile disposable RNase free plastic wares were used for the preparation and storage of RNA. Utmost care was taken to prevent the action of RNase during the entire isolation process by ensuring the biochemical and microbiological sterility of the workbench by alcoholic sterilisation. Disposable gloves were used during the preparation of reagents, isolation and analysis of RNA.

Reagents

All reagents were prepared using molecular grade chemicals (SIGMA Inc, USA) in 0.1 % DEPC treated sterilized Milli-Q water. The autoclavable reagents were made sterile at 121°C for 15 min at 15 lbs pressure.

Phosphate Buffered saline (PBS)

137 mM NaCl

2.7 mM KCl

4.3 mM Na₂HPO₄

1.47 mM KH₂PO₄

Adjust to a final pH of 7.4.

Dissolved the chemicals in minimum volume of

DEPC treated water and made upto 1 liter and sterilized by autoclaving.

Solution D

4 M Guanidium thiocyanate

25 mM Sodium citrate

0.5 % w/v N-lauryl sarcosine

0.1 M β-mercapto ethanol*

Filter sterilized through 0.2 μm filter and store at room temperature

*Add β-mercaptoethanol just before use. (3.6 μl into 500 μl of sol D)

Phenol

Saturated with 0.1 M citrate buffer, pH 4.3

Chloroform

Isopropanol

75% Ethanol

Protocol

Tissue homogenization:

- A single pair of eyestalk neural tissue was homogenized in 500 μl PBS in a 1.5 ml micro centrifuge tube using a sterile pestle.
- Homogenized samples were incubated on ice for 5 min to ensure the dissociation of cell wall and pigments.
- The homogenate was then centrifuged at 10,000 rpm for 5 min at 4°C and the upper aqueous phase was saved and the pellet discarded.

Phase separation

- The aqueous phase (0.4 ml) was transferred to a fresh 1.5 ml micro centrifuge tube.
- Solution D (0.4 ml) was added to the preparation and the tube was vortexed vigorously for 15 sec followed by incubation on ice for 3 min.
- Saturated phenol (0.4 ml) and chloroform (0.1 ml) was added and vortexed vigorously for 30 sec followed by incubation on ice for 15 min.
- The preparation was centrifuged at 12,000 rpm for 15 min at 4°C

RNA precipitation

- The upper aqueous phase was transferred to a fresh 1.5 ml micro centrifuge tube and equal volume of isopropyl alcohol was added to precipitate the RNA and kept at -80 °C for 10 min .
- Precipitated RNA was recovered by centrifugation at 12,000 rpm for 10 min at 4°C.

RNA wash

- Precipitated RNA pellet was washed once with 1 ml of 75 % ethanol and centrifuged at 12,000 rpm for 5 min at 4°C.

- The resulting pellet was saved carefully and the ethanol was decanted .

Dissolving RNA

- The RNA pellet was air dried and dissolved in the 20 μ l sterile RNA storage solution (Ambion Biosciences)

Quantification of RNA

- Dilute 1 μ l of RNA with 39 μ l of DEPC-treated water (1:40 dilution). Take OD at 260 nm and 280 nm to determine sample concentration and purity. The A260/A280 ratio should be above 1.9. Apply the convention that 1 OD at 260 equals 40 μ g /ml RNA to calculate the exact conc. of RNA.

Suggested readings

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Reverse transcriptase PCR (RT-PCR) for First strand cDNA synthesis

The ability to synthesize DNA from an RNA template, via reverse transcription, enables researchers to study RNA with the same molecular approaches used for DNA investigations. cDNA generated by reverse transcription can be amplified using polymerase chain reaction (PCR). The combination of reverse transcription and PCR (RT-PCR) allows the detection of low abundance RNAs in a sample. In the first step of the PCR process, the cDNA is denatured by heating to 95°C, which disrupts the hydrogen bonds between complementary strands, yielding single-stranded molecules. The temperature is then lowered in order to allow primers complementary to the sequence(s) of interest to anneal. The DNA polymerase included in the reaction will then begin DNA synthesis. At this point, the temperature is raised to the optimal activity temperature of the DNA polymerase (usually 72°C) to synthesize a new strand complementary to the template. The process of denaturing, annealing, and extension can be repeated multiple times, with a two-fold increase in the amount of DNA molecules with each cycle. Because PCR can selectively amplify a template, it is an important method for detecting specific nucleic acid molecules in a particular cell or small populations of cells. PCR Products can be used in many downstream applications, such as cloning into plasmid vectors and sequencing using next generation sequencing platforms.

In the one-step protocol, the components of RT and PCR are mixed in a single tube at the same time. The one-step protocol generally works well for amplifying targets that are reasonably abundant.

One-step RT-PCR

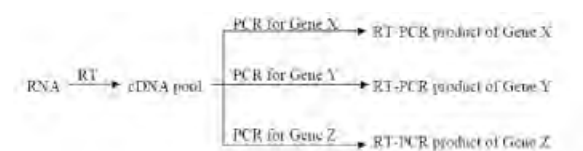
Convenient



Alternatively, RT-PCR can be done in two steps, first with the reverse transcription and then the PCR. The two-step protocol is usually more sensitive than the one-step method; yields of rare targets may be improved by using the two-step procedure.

Two-step RT-PCR

- Saves RT reagents. One RT reaction will provide templates for multiple PCR's
- Can be more sensitive than one-step RT-PCR



Two-step Protocol

RT-PCR involves use of reverse transcriptase enzyme to synthesize cDNA from mRNA. The two-step RT-PCR reaction mainly involves two major steps, 1) Synthesis of the first strand cDNA and 2) Synthesis of second strand by PCR amplification.

Step One: Reverse Transcription

After ensuring the qualitative integrity by gel electrophoresis and quantification by spectrophotometer, total RNA isolated was reverse transcribed with First Strand cDNA Kit using Oligo(dT)24 primer following the instructions given by the manufacturer.

Reagents

- First Strand cDNA synthesis Kit
 - Reverse Transcriptase MMLV-RT (100 units/ μ l)
 - 10X RT Buffer (500 mM Tris-HCl, pH 8.3, 750 mM KCl, 30mM MgCl₂, 50 mM DTT)
 - Oligo-dT primer [d(T)₂₄] (50 μ M)
 - dNTP (2.5mM each dNTP)
 - RNase Inhibitor (10 units/ μ l)
 - PCR Thermocycler

Place RNase Inhibitor and Reverse Transcriptase ON ICE directly from the box.

Component	Stock	Final amount	Experiment (+RT)	Control (-RT)
Total RNA			~1-2 μ g	~1-2 μ g
Oligo dT primer	50 μ M	5 μ M	2 μ l	2 μ l
10X RT Buffer	10X	1X	2 μ l	2 μ l
dNTP mix	2.5mM	0.5mM	4 μ l	4 μ l
RNase Inhibitor	10 U/ μ l	10U	1 μ l	1 μ l
Reverse Transcriptase	100 U/ μ l	100U	1 μ l	0 μ l
Nuclease-free water			x μ l (to total of 20 μ l)	x μ l (to total of 20 μ l)

Thaw 10x reaction buffer, random decamers, and dNTP mix quickly in your hands and place ON ICE; Use small 0.25ml PCR tubes.

- 1) Assemble your reaction as follows on ice. Add the enzyme last.
- 2) Mix gently, spin briefly.

Component	Working Stock	Final Conc.	Experiment	Control 1 (-RT in step 1)	Control 2 (no template)
RT reaction			1-2 μ l	1-2 μ l	0 μ l
10X PCR Buffer	10X	1X	2.5 μ l	2.5 μ l	2.5 μ l
Forward primer	5 μ M	0.25 μ M	1.25 μ l	1.25 μ l	1.25 μ l
Reverse primer	5 μ M	0.25 μ M	1.25 μ l	1.25 μ l	1.25 μ l
dNTP mix	2.5mM	0.125mM	1.25 μ l	1.25 μ l	1.25 μ l
Taq Polymerase	5U/ μ l	1U	0.2 μ l	0.2 μ l	0.2 μ l
dH ₂ O			Up to 25 μ l	Up to 25 μ l	Up to 25 μ l

- 3) Incubate in the thermocycler at:
 - a. 42°C for 1 hr.
 - b. 95°C for 10 min to inactivate the reverse transcriptase.

4) Store reaction at -20°C or proceed to the PCR.

Step Two: PCR

Setting up PCR reactions

Negative Controls

Use two negative controls among the PCRs.

- I. The minus-RT control from the previous step, or alternatively, untreated RNA can simply be subjected to PCR.
- II. A minus-template PCR, it should have all the PCR components, but use water as template instead of an aliquot of the cDNA (RT reaction). This control will verify that none of the PCR reagents is contaminated with DNA.

Positive Control

- I. Perform PCR to amplify a cDNA that corresponds to a basal/housekeeping gene transcript
- II. Use genomic DNA isolated from cells as template. If your primers span intron(s), note the size of the expected PCR product and if necessary, adjust annealing temperature of the PCR program.

Reagents:

Taq DNA polymerase with 10X Buffer (-MgCl₂) and 50mM MgCl₂

dNTP mix

The following table outlines the components needed for PCR.

Making Master Mixes:

Component	Experiment	Neg Control 1 (-RT in step 1)	Neg Control 2 (no template)
RT reaction	~1-2*(n+1) μ g	~1-2*(n+1) μ g	0
10X PCR Buffer	5 *(n+1) μ l	5*(n+1) μ l	5 *(n+1) μ l
dNTP mix	1.25*(n+1) μ l	1.25*(n+1) μ l	1.25*(n+1) μ l
MgCl ₂	*(n+1) μ l	*(n+1) μ l	*(n+1) μ l
Taq polymerase	0.2*(n+1) μ l	0.2*(n+1) μ l	0.2*(n+1) μ l
Nuclease-free water	Variable	Variable	variable
Total Volume	100 *(n+1) μ l	100*(n+1) μ l	100*(n+1) μ l

Consider making master mixes if you are testing multiple sets of primers at once. A master mix will contain everything except the PCR primers. If you are testing n sets of primers, make a master mix enough for n+ 1 tests.

Mix the components gently but thoroughly. Aliquot 22.5 μ l of your master mix to each tube. Add 1.25 μ l of each of the appropriate primer at 5 μ M working stock concentration.

Assemble reactions on ice. Incubate in Thermocycler:

- a. Initial denaturation: 94°C for 4 min
- b. 30 cycles: Denature at 94°C for 30 sec

Anneal at 55°C for 20-30 sec**

Extend at 72°C for 45 sec ***

- c. Final extension: 72°C for 5 min

**Start with the annealing temperature suggested by your primer design software. An annealing temperature of ~55°C used with the cycling times shown is often a reasonable starting point, but the optimal temperature and cycling times for your primer and template combination may need to be determined empirically.

***The rule of thumb is to use an extension time of 1 min per kilobase of target.

Run 6 μ l of the PCR reaction product on 1-1.5% Agarose gel to check for the presence of amplified products.

Agarose Gel Electrophoresis

Agarose gel electrophoresis is used to analyze and quantitate nucleic acid. The agarose, for agarose gel electrophoresis is purified from agar. Agarose is a linear polysaccharide made up of repeating units of agarobiose which comprises of alternating units of galactose and 3, 6 anhydrogalactose. Agarose has an average MW of 12,000 and contains about 35-40 agarobiose units. Agarose in solution exist as a left handed double helices. About 7 to 11 such helices form bundles which extend as long rods and appear to intertwine with one another, further strengthening the frame work of the gel. The cross links are held together by hydrogen and hydrophobic bonds. The pore size of the gel is controlled by the concentration of the agarose. Higher the concentration, smaller the pore size of the gel and vice versa. Because of large pore size even at low concentration, agarose gels are widely used for separation of DNA and RNA.

Effect of agarose concentration on separation ranges

The following table describes the relationship between agarose concentration and separation range of nucleic acid

Agarose Concentration (%)	Separation range (Kb)
0.3	5 to 60
0.6	1-20
0.8	0.8 to 10
1.0	0.4 to 8
1.2	0.3 to 6
1.5	0.2 to 4

Factors which affect the rate of migration of nucleic acids in agarose gels

Rate of migration of nucleic acids in agarose gels depends mainly on several factors.

a. Agarose concentrations

Higher concentration of gels are used for the separation of lower molecular weight DNA and RNA fragments and vice-versa

b. Molecular weight

Duplex DNA fragments migrates at rates inversely proportional to the log Molecular weight. A plot of log M.W vs Mobility gives a straight line

c. Conformation

Supercoiled DNA moves fastest followed by linear forms and relaxed open circular forms.

d. Applied Voltage

At low voltage (<5V/cm) the rate of migration is directly proportional to the applied voltage. However, if the voltage is increased, mobility of high molecular weight DNA fragments increased differentially

e. Presence of Ethidium Bromide (EtBr)

The presence if ethidium bromide in the gel causes DNA to run slower, as EtBr intercalates and uncoils the DNA.

Reagents

10X TBE/50X TBE, EtBr, distilled water, gel loading buffer, molecular weight marker.

Buffers

There are a number of buffers used for agarose gel electrophoresis. The most common being Tris acetate EDTA (TAE) and Tris borate EDTA (TBE) buffer. TAE has the lowest buffering capacity but provides the best resolution for larger DNA fragments. This means a lower voltage and more time, but a better product. On the other hand TBE will give a faster run time with higher voltage and a good resolving power.

TBE buffer

Tris borate (1X TBE)
89mm Tris base
89mm Boric acid
25mm Na ₂ -EDTA

TAE buffer

Tris acetate (1X TAE)
50mm Tris base
25mm Glacial acetic acid
1mm Na ₂ -EDTA

Sterilize the stock solutions by autoclaving

Ethidium bromide (stock solution)

Weigh 10 mg ethidium bromide into a sterile tube and dissolve in 10 ml sterile distilled water. The stock is stored at 4°C

Sample loading dye Glycerol & bromophenol blue (6x)

3ml glycerol (30%), 25mg bromophenol blue (0.25%) dH₂O to 10mL

Preparation of agarose solution for casting the gel

Weigh the required quantity of agarose on to 1x

buffer of choice (TBE/TAE) and dissolved completely by heating the flasks using a microwave oven. (Usually the percentage of agarose ranges between 0.7- 2.0 depending on the size of DNA to be electrophoresed). Allow the gel solution to cool down to 60°C at room temperature and 1.5 µl of EtBr stock solution was added to it and mixed by gentle swirling, without forming any air bubbles.

Assemble to gel tray and place the comb at about 1 cm from the top of the tray and pour the melted agarose without making any bubbles, keeping the thickness of the gel around 0.5 to 0.9 cm. Allow the gel to set for 20 mins and take off the combs to uncover the wells. Place the gel tray in the running unit and pour the 1x buffer over it. Make sure the gel is completely covered with the buffer.

The DNA sample (100 to 200 ng) is mixed with the loading dye (for 5 µl of DNA sample 1µl of 6x dye is used) and loaded in to the well carefully, using a micro pipette. The maximum volume that can be loaded on to a well formed from a 1.5 mm thickness tooth of the comb is 30 µl. Load one well with a DNA ladder.

Once the sample is loaded in to the well, the cathode (Black negative terminal) is connected towards the top end of the gel and the anode (Red positive terminal) is connected towards the bottom end of the gel.

The electrophoresis is started by switching on the DC power pack. The gel is run at 5v/cm till the bromophenol blue (the tracking dye) has moved 1 cm above the bottom end. Then the current is switched off, the power supply is disconnected and the gel is photographed under UV light on a gel documentation system.

Preparation of competent *E. coli* cells

Competence is the ability of a cell to take up extra cellular DNA from its environment. Competency can be artificially induced by treating the cells with CaCl_2 prior to adding DNA. The calcium destabilizes the cell membrane and adheres to the cell surface favoring the formation of the pores for the entry of DNA.

Solutions

Luria-Bertani (LB) media (1 L):

Mix 10 g of Bacto-tryptone, 5 of Yeast extract, and 10 g of NaCl.
pH to 7.5 w/ NaOH and dH_2O to 1 L (Autoclave)

1M CaCl_2 (1 L):

Mix 111 g of CaCl_2 (anhydrous) and 1 L of dH_2O .
Filter sterilize through a 0.22μ filter

0.1M CaCl_2 (1 L):

Mix 100 mL of 1M CaCl_2 with 900 mL of dH_2O .
Filter sterilize through a 0.22μ filter

50% Glycerol (500 mL):

Mix 50 mL of Glycerol with 50 mL of dH_2O (Autoclave)

0.1M CaCl_2 + 15% glycerol:

Mix 100 mL of 1M CaCl_2 , 300 mL of 50% Glycerol, and 600 mL of dH_2O

LB plates:

Mix 500 mL of LB media with 7 g of Agar (Autoclave). Cool to $\sim 55\text{-}65^\circ\text{C}$ prior to pouring. The addition of antibiotics should be made before pouring and at a temperature not higher than 55°C .

Procedure

1. Streak *E. coli* cells on an LB plate
2. Allow cells to grow at 37°C overnight
3. Place one colony in 10 mL LB media (+antibiotic selection if necessary), grow overnight at 37°C
4. Take 2 ml LB media and save for blank. Transfer 5 mL overnight culture into 500 mL LB media in 1 L conical flask
5. Allow cell to grow at 37°C (250 rpm), until $\text{OD}_{600} = 0.4$ ($\sim 2\text{-}3$ hours)
6. Transfer cells to 2 centrifuge bottles (250 mL), and place cells on ice for 20 mins
7. Centrifuge cells at 4°C for 10 mins at 3,000 g
8. Subsequent resuspensions may be done in the same bottle. Cells must remain cold for the rest of the procedure: Transport tubes on ice and resuspend on ice in the cold room
9. Pour off media and resuspend cells in 30 ml of cold 0.1 M CaCl_2 . Transfer the suspended cells into 50 ml polypropylene falcon tubes, and incubate on ice for 30 mins.
10. Centrifuge cells at 4°C for 10 mins at 3,000 g
11. Pour supernatant and resuspend cells (by pipetting) in 8 mL cold 0.1M CaCl_2 containing 15% glycerol. Transfer 140 μl into (1.5 mL) Ependorff tubes placed on ice. Freeze the cells in liquid nitrogen. Cells stored at -80°C can be used for transformation for up to ~ 6 months

Transformation of competent *E. coli* Cells

Changing the genotype of a cell or organism by transferring foreign DNA is called transformation. The transferred DNA may be maintained as extra-chromosomal elements or integrated into the genome.

eg: ampicillin susceptible genotype of *E. coli* strain TOP10 can be changed to ampicillin resistant genotype by transferring pUC18 plasmid that carries a gene for ampicillin resistance and the selection is done in a selection medium with ampicillin.

pUC18 vectors have a short segment of *E. coli* DNA, which contains the regulatory and coding sequences of Lac Z gene that codes for β -galactosidase enzyme. Isopropyl thiogalactoside (IPTG) is an inducer of Lac Z gene expression. β -galactosidase reacts with the chromogenic substrate 5-bromo-4-chloro- β -D-Galactoside (X-gal) and yields a blue colored product. A multiple cloning site (MCS) is engineered inside the coding region of the Lac Z gene. The MCS as such does not disrupt the reading frame and results only in insertion of a few amino acids in the amino terminal fragment of the β -galactosidase. Therefore, the colonies appear blue in color in the presence of IPTG and X-gal. However, when an insert is cloned in the MCS, that becomes a harmful insertion to the functional properties of β -galactosidase and it can no longer react with X-gal, and therefore, the colonies appear white in color. This is a simple visual color test that can be used to screen thousands of colonies to identify the presence of recombinant plasmids.

Materials

- Competent TOP10 cells
- Foreign DNA /plasmid
- LB medium
- LB plates with 100mg/L Amp
- 42°C water bath
- Isopropyl thiogalactoside (IPTG) 100mg/ml, Sterilize by filtration.
- 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) 20mg/ml dissolved in DMSO.

Procedure

1. Competent cells were taken out from -80°C freezer and thawed in the ice.
2. To transform the CaCl₂-treated cells transfer 50 μ l suspension of competent cells to a sterile chilled microfuge tube using a chilled micropipette tip. Add DNA (no more than 12.5 ng in a volume of 2.5 μ l or less) to each tube. Mix the contents of the tubes by swirling gently. Store the tubes on ice for 30 minutes.
3. Transfer the tubes to a rack placed in a preheated 42°C circulating water bath. Store the tubes in the rack for exactly 90 seconds. Do not shake the tubes.
4. Rapidly transfer the tubes to an ice bath. Allow the cells to chill for 1-2 minutes.
5. Add 200 μ l of SOC medium to each tube. Incubate the cultures for 45 minutes in a water bath set at 37°C to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid.
6. About 100 μ l of the inoculum of transformed competent cells were plated onto LB agar plate containing appropriate antibiotic, IPTG and X-gal
7. About 100 μ l of untransformed competent cells were plated on a LB agar plates containing appropriate antibiotic, IPTG and X-gal as a negative control.
8. Store the plates at room temperature until the liquid has been absorbed.
9. Invert the plates and incubate at 37°C for overnight. Transformed colonies should appear in 12-16 hour.

Cloning of the PCR amplified products

Bacterial Strain used for cloning

TOP10 (Invitrogen, USA) *E. coli* cloning host cells were made chemically competent and used for transformation with the recombinant plasmid.

CloneJET™ PCR Cloning Kit

pJET 1.2 Blunt-end vector (50 ng/μl)

In order to clone PCR amplified products, the pJET1.2/blunt vector (Fermentas, Germany), was used. pJET1.2/blunt is a linearized cloning vector, which accepts inserts from 6 bp to 10 kb.

Blunt-end PCR products generated by proofreading DNA polymerases (eg. *Pfu*) can be directly ligated with the pJET1.2/blunt cloning vector. All common laboratory *E. coli* strains can be directly transformed with the ligation product. Only recombinant clones containing the insert appear on culture plates as the re-circularized or self-ligated pJET1.2/blunt vector

expresses a lethal restriction enzyme in the host after transformation and hence blue/white screening is not required.

Ligation of cDNA

The ligation reaction of the PCR amplified cDNA with the cloning vector was carried out as described in the manufacturer's protocol (Fermentas, Germany). The ligation protocol in brief was as follows:

2X reaction buffer	10.0 μl
PCR product	x μl
pJET1.2/blunt end cloning vector (50ng/μl)	1.0 μl
Milli-Q water	x μl
T4 DNA ligase	1.0 μl
Total	20.0 μl

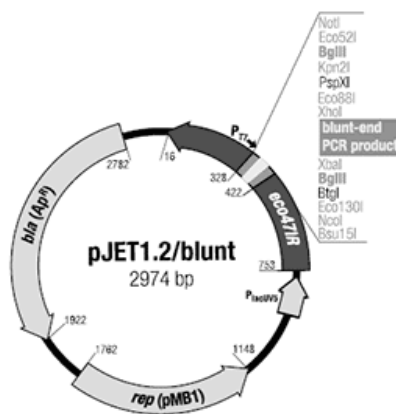
After giving a short spin, the ligation mixture was incubated at 22°C for 10 min and directly used for transformation.

Transformation protocol

An aliquot of 5 μl of ligation mixture was added to 50 μl of competent cells and mixed gently by flicking. The mixture was incubated on ice for 30 min.

The tubes were then transferred to water bath maintained at 42°C and held for exactly 90 sec. The tubes were immediately transferred back on ice and allowed to chill for 1 min.

To the above mix, 50 μl of SOC media was added and mixed. The tubes were incubated at 37°C for 1 h in a rotary shaker to allow the bacteria to recover and develop the antibiotic resistance. After incubation, 75 μl of transformed mixture was spread evenly on LB



Schematic diagram of PCR cloning vector pJET1.2

agar plates with ampicillin (100 $\mu\text{g/ml}$) and incubated at 37°C for 16-20 h.

Confirmation of the cloned genes by Colony PCR

The transformants obtained on LB agar plates with ampicillin (100 $\mu\text{g/ml}$) were screened using colony PCR with vector specific primers pJET1.2F (5'-CGA CTC ACT ATA GGG AGA GCG GC-3') and pJET1.2R (5'-AAG AAC ATC GAT TTT CCA TGG CAG-3') to confirm the presence of the insert DNA.

A small portion of selected colonies picked up from the transformed plate using sterile toothpicks were dispensed into the PCR reaction mix composed of 1x PCR buffer with, 2 mM MgSO_4 , 0.2 mM each dNTP, 0.5

μM of each primers (pJET1.2F and pJET1.2R) and 0.5 U Pfu DNA polymerase. The PCR reaction conditions were 95°C for 5 min then 35 cycles of 95°C for 30 sec, 58°C for 30 sec and 72°C for 60 sec followed by a final extension at 72°C for 10 min. Electrophoresis was performed on 1 % agarose gel prepared in 1x TBE buffer and stained with ethidium bromide.

Colonies with expected sized products were inoculated into LB broth containing ampicillin (100 $\mu\text{g/ml}$) and incubated in shaking incubator at 37°C for 12-16 h after which the cells were pelleted and used for plasmid isolation.

Minipreparation of Plasmid DNA by Alkaline Lysis with SDS

Plasmid is an extra chromosomal DNA present in most of the bacteria and in some yeast. Most of them are circular and they vary in size and in numbers. The plasmids are modified and used as vectors (carrier) in rDNA technology. In this experiment one such modified plasmid pUC18 is isolated from an *E.coli* culture by alkaline lysis method

This method is based on the principle that exposure of bacterial suspensions to the strongly anionic detergent at high pH opens the cell wall, denatures chromosomal DNA and proteins and release plasmid DNA into the supernatant. Although the alkaline solution completely disturbs chromosomal DNA, the circular plasmids DNA are unable to separate from each other because they are topologically intertwined. During lysis, bacterial proteins, broken cell wall and denatured chromosomal DNA become enmeshed in large complexes that are coated with dodecyl sulphate. These complexes are efficiently precipitated when solution with sodium ions are replaced by potassium ions. After the denatured materials have been removed by centrifugation native DNA plasmid can be recovered from the supernatant by precipitation.

Alkaline lysis is a flexible method that works well with all strains of *E. coli* and with bacterial culture ranging in size from 1ml to 500ml. The plasmid DNA obtained by this method is devoid of nuclear DNA.

Buffers and Solutions

- Alkaline lysis solution I
- Alkaline lysis solution II
- Alkaline lysis solution III

- Ethanol
- Phenol:chloroform (1:1, v/v)
- TE (pH 8.0) containing 20 µg/ml RNase A

Media

LB broth

Method

1. Inoculate 2 ml of rich medium (LB Broth) containing the appropriate antibiotic with a single colony of transformed bacteria. Incubate the culture overnight at 37°C with vigorous shaking at 200-300 rpm.
2. Pour 1.5 ml of the culture into a microfuge tube. Centrifuge at maximum speed for 30 seconds at 4°C in a microcentrifuge. Store the unused portion of the original culture at 4°C.
3. Remove the medium by aspiration, leaving the bacterial pellet as dry as possible.
4. Resuspend the bacterial pellet in 100 µl of ice-cold Alkaline lysis solution I by vigorous vortexing.
5. Add 200 µl of freshly prepared Alkaline lysis solution II to each bacterial suspension. Close the tube tightly, and mix the contents by inverting the tube rapidly five times. Do not vortex! Store the tube on ice.
6. Add 150 µl of ice-cold Alkaline lysis solution III. Close the tube and disperse Alkaline lysis solution III through the viscous bacterial lysate by inverting the tube several times. Store the tube on ice for 3-5 minutes.
7. Centrifuge the bacterial lysate at maximum speed for 5 minutes at 4°C in a microcentrifuge. Transfer the supernatant to a fresh tube.

8. (Optional) Add an equal volume of phenol:chloroform. Mix the organic and aqueous phases by vortexing and then centrifuge the emulsion at maximum speed for 2 minutes at 4°C in a microcentrifuge. Transfer the aqueous upper layer to a fresh tube.
9. Precipitate nucleic acids from the supernatant by adding 2 volumes of 100% ethanol at room temperature. Mix the solution by vortexing and then allow the mixture to stand for 2 minutes at room temperature.
10. Collect the precipitated nucleic acids by centrifugation at maximum speed for 5 minutes at 4°C in a microcentrifuge.
11. Remove the supernatant by gentle aspiration as described in Step 3 above. Stand the tube in an inverted position on a paper towel to allow all of the fluid to drain away. Use a tissue paper or disposable pipette tip to remove any drops of fluid adhering to the walls of the tube.
12. Add 1 ml of 70% ethanol to the pellet and invert the closed tube several times. Recover the pellet by centrifugation at maximum speed for 2 minutes at 4°C in a microcentrifuge.
13. Remove all of the supernatant by gentle aspiration as described in Step 3. Take care with this step, as the pellet sometimes does not adhere tightly to the tube.
14. Remove any beads of ethanol that form on the sides of the tube. Store the open tube at room temperature until the ethanol has evaporated and no fluid is visible in the tube (5-10 minutes).
15. Dissolve the pellet in 50 μ l of TE (pH 8.0) containing 20 μ g/ml DNase-free RNaseA. Vortex the solution gently for a few seconds. Store the plasmid solution at -20°C.

Reagents

Alkaline Lysis Solution I

50 mM glucose
 25 mM Tris-Cl (pH 8.0)
 10 mM EDTA (pH 8.0)

Prepare Solution I from standard stocks in batches of approx. 100 ml, autoclave for 15 minutes at 15 psi on liquid cycle, and store at 4°C.

Alkaline Lysis Solution II

0.2 N NaOH (freshly diluted from a 10 N stock)
 1% (w/v) SDS
 Prepare Solution II fresh and use at room temperature.

Alkaline Lysis Solution III

5 M potassium acetate, 60.0 ml
 Glacial acetic acid, 11.5 ml
 H₂O, 28.5 ml
 The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. Store the solution at 4°C and transfer it to an ice bucket just before use.

EDTA

To prepare EDTA at 0.5 M (pH 8.0): Add 186.1 g of disodium EDTA•2H₂O to 800 ml of H₂O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (approx. 20 g of NaOH pellets). Dispense into aliquots and sterilize by autoclaving. The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to approx. 8.0 by the addition of NaOH.

Glycerol

To prepare a 10% (v/v) solution: Dilute 1 volume of molecular-biology grade glycerol in 9 volumes of sterile pure H₂O. Sterilize the solution by passing it through a pre rinsed 0.22- μ m filter. Store in 200-ml aliquots at 4°C.

NaOH

The preparation of 10 N NaOH involves a highly exothermic reaction, which can cause breakage of glass containers. Prepare this solution with extreme care in plastic beakers. To 800 ml of H₂O, slowly add 400g of NaOH pellets, stirring continuously. As an added precaution, place the beaker on ice. When the pellets have dissolved completely, adjust the volume to 1 liter with H₂O. Store the solution in a plastic container at room temperature. Sterilization is not necessary.

Potassium Acetate

5 M potassium acetate, 60 ml
glacial acetic acid, 11.5 ml
H₂O, 28.5 ml

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. Store the buffer at room temperature

SDS

Also called sodium lauryl sulfate. To prepare a 20% (w/v) solution, dissolve 200 g of electrophoresis-grade SDS in 900 ml of H₂O. Heat to 68°C and stir with a magnetic stirrer to assist dissolution. If necessary, adjust the pH to 7.2 by adding a few drops of concentrated HCl. Adjust the volume to 1 liter with H₂O. Store at room temperature. Sterilization is not necessary. Do not autoclave.

TE

100 mM Tris-Cl (desired pH)
10 mM EDTA (pH 8.0)
(10x Tris EDTA) Sterilize solutions by autoclaving for

20 minutes at 15 psi on liquid cycle. Store the buffer at room temperature.

Tris-Cl

Dissolve 121.1 g of Tris base in 800 ml of H₂O. Adjust the pH to the desired value by adding concentrated HCl.

pH	HCl
7.4	70 ml
7.6	60 ml
8.0	42 ml

(1 M) Allow the solution to cool to room temperature before making final adjustments to the pH. Adjust the volume of the solution to 1 liter with H₂O. Dispense into aliquots and sterilize by autoclaving. If the 1 M solution has a yellow color, discard it and obtain Tris of better quality. The pH of Tris solutions is temperature-dependent and decreases approx. 0.03 pH units for each 1°C increase in temperature. For example, a 0.05 M solution has pH values of 9.5, 8.9, and 8.6 at 5°C, 25°C, and 37°C, respectively.

Restriction Enzyme digestion

Restriction enzymes form part of the restriction-modification system of bacterial cells that provides protection against invasion of the cell by foreign DNA – especially bacteriophage DNA. Restriction enzymes are Nucleases which can cleave the sugar-phosphate backbone of DNA, found in bacteria. As they cut within the molecule, they are commonly called restriction endonucleases. They specifically cleave the nucleic acids at specific nucleotide sequence called Restriction sites to generate a set of smaller fragments. Most of the restriction recognition sequences are palindromic and vary in lengths between 4 and 8 nucleotide. Restriction enzyme makes two incisions, once through each sugar-phosphate backbone (*i.e.* each strand) of the DNA double helix. Some restriction enzymes cut the double stranded DNA in two different positions and generate ends that are staggered, with 5' or 3' protruding terminal nucleotides; others cut at the same position and produce blunt ends. Discovery of restriction enzymes lead to the development of recombinant DNA technology and are routinely used for DNA modification and manipulation in laboratories.

Types of restriction enzymes

Four different classes of restriction endonucleases are recognized, Type I, Type II, Type III and Type IV, each distinguished by the difference in their mode of action. Type I and III are complex and have a limited role in genetic engineering since the sites of actual cleavage are at variable distance from these recognition sites, and can be hundreds of bases away. Type II restriction enzymes target only methylated DNA.

The unit definition of restriction enzyme activity is based on the amount of enzyme required to cut 1 μ g of bacteriophage lambda DAN to completion in one hour's time in a reaction volume of 50 μ l under optimal concentration of salt, pH and temperature.

Nomenclature

The first three letters of the restriction enzyme refer to the organism from which the restriction enzyme was originally isolated, the fourth letter (if present) refers to the strain, and the Roman numerals serve as indices if the same organism contains several different restriction enzymes.

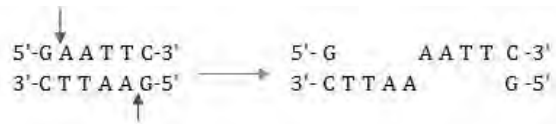
e.g. EcoR I and EcoR V are both from *Escherichia coli*, strain R; I and V are the order in which they were discovered.

Table 1. Restriction Enzyme Nomenclature

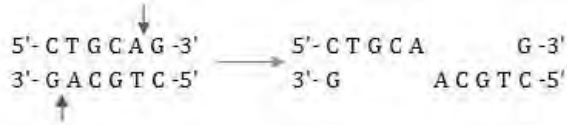
Enzyme	Enzyme Source
<i>EcoRI</i>	<i>Escherichia coli</i> , strain R, 1 st enzyme
<i>HindIII</i>	<i>Haemophilus influenzae</i> , strain d, 3 rd enzyme
<i>BamHI</i>	<i>Bacillus amyloliquefaciens</i> , strain H, 1 st enzyme
<i>SmaI</i>	<i>Serratia marcescens</i> , 1 st enzyme
<i>HaeIII</i>	<i>Haemophilus aegyptius</i> , 3 rd enzyme

Restriction Enzyme cleavage:

Class II restriction enzymes generate three types of DNA ends, all possessing 5'-phosphate and 3'-hydroxyl groups:



a) Cohesive 5' ends:- For example, ends generated by EcoR I:



b) Cohesive 3' ends:- For example, ends generated by Pst I:



c) Blunt ends:- For example, ends generated by Hae III

Sticky ends (Blunt ends) are produced by cutting the DNA in a staggered manner within the recognition site producing single stranded DNA ends. These ends have identical nucleotide sequence and are sticky because they can bind to complementary tails of other DNA fragments cut by the same Restriction enzyme.

Some enzymes require special conditions. Some requires BSA (bovine serum albumin) added in to the mixture. This is usually provided with the enzymes at 100x concentration. BSA stabilizes the enzymes, binds to some impurities and prevent enzyme adsorption to the surface of the tubes. Some require weak detergents (eg. Triton X-100) to reduce surface tension. Some require to be incubated at temperatures other than the standard temperature 37°C.

All the enzymes are usually supplied with a 10x buffer. Most companies have different kinds of buffer giving various levels of efficiency and occasionally a unique buffer for a particular enzyme optimized for 100% efficiency. The enzyme storage buffer contains antifreeze (glycerol) to allow it to be preserved at -20°C, but it will inhibit the digestion if present in more quantities. So the volume of enzyme in the reaction mix should not be more than 10% of the final reaction volume.

Reagents

Restriction enzymes, DNA, 10x buffer

Procedure

1. Combine the following in a PCR tube for a single reaction of 10 ml

Components	Volume
10X RE buffer	1.0 ml
Milli-Q water	6.5 ml
DNA	2.0 ml (depending on the concentration)
Enzyme	0.5 ml (1 to 5U/mg of DNA. Maximum volume to be added is 1/10 th of the totoal reaction volume)
Total volume	10 ml

2. It is always recommended to prepare a premix if you are doing many digestions at a time with the same enzyme.
3. Incubate the tubes in a water bath or thermal cycler at 37°C for 1-2 h.
4. Incubate the tubes at 65°C for 10 minutes to inactivate the enzyme.
5. Run a 1.5% agarose gel to see the restriction pattern.

Ligation

Ligation of a segment of insert DNA to a linearized plasmid vector involves the formation of phosphodiester bonds between DNA molecules. Ligase catalyze the formation of phosphodiester bonds between the directly adjacent 3' hydroxyl and 5' phosphoryl termini of nucleic acid molecule. The ligation process consumes ATP as energy source. When cohesive ends are present the ligation occurs efficiently, but when blunt-end fragments have to be ligated the efficiency is very low. Salt and phosphate concentration is very important for the efficiency of ligation. Incubation times and temperatures vary a great deal in the literature but the following seem to work well in most cases.

For "sticky ends": incubate 2-4 hrs. at 16°C; for blunt ends incubate overnight at 4°C.

Materials and Reagents

- Linearized vector
- Insert DNA

- T4 DNA Ligase
- 5X Ligase buffer
- 0.2ml PCR tube
- Incubator

Procedure

The following components were added in a 0.2ml PCR tube.

Linearized Vector (100ng/ μ l)	9 μ l
Insert DNA (100ng/ μ l)	3 μ l
5x ligase buffer	4 μ l
T4 DNA Ligase (0.1U/ μ l)	1 μ l
Sterile distilled water	4 μ l
Total	20 μ l

Mixed gently and centrifuged briefly

Incubate the tubes in thermal cycler at 16°C for 2 hrs and after incubation the samples were stored at -20°C for future use

Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS PAGE)

SDS-PAGE is widely used to analyze the proteins in complex extracts. The most commonly used methods are derived from the discontinuous SDS-PAGE system first described by Laemmli (1970). The system actually consists of two gels—a resolving (aka running) gel in which proteins are resolved on the basis of their molecular weights (MWs) and a stacking gel in which proteins are concentrated prior to entering the resolving gel. Differences in the compositions of the stacking gel, resolving gel and electrophoresis buffer produce a system that is capable of finely resolving proteins according to their MWs.

The Laemmli (1970) SDS-PAGE system can be considered a 3-component system. The stacking and running (resolving) gels have different pore sizes, ionic strengths and pHs. The third component is the electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH ~8.3), which contains large amounts of glycine. The ionization state of the glycine is critical to the separation. At neutral pH, glycine is a zwitterion, with a negatively charged carboxyl group and a positively charged amino group. The pKa of the amino group is 9.6, considerably higher than the pH of the chamber buffer. Consequently, very little glycine has a negative charge in the chamber buffer or stacking gel, and significant ionization does not occur until the glycine enters the more alkaline pH 8.8 environment of the running gel.

Let's follow the progress of protein samples during SDS-PAGE to see how differences in the composition of these three components generate the high resolving power of SDS-PAGE gels. The sample buffer used for SDS-PAGE contains a tracking dye, bromophenol blue (BPB), which will migrate with the

leading edge of the proteins being separated on the gel. The sample buffer also contains glycerol, which allows the protein samples to settle into the bottom of the gel wells. The gel is vertically positioned in the electrophoresis apparatus and covered with chamber buffer containing glycine.

Once a voltage is applied, the chloride ions in the sample buffer and stacking gel move rapidly toward the positive pole, forming the leading edge of a moving ion front. Glycine molecules have very little charge in the stacking gel, so they migrate at the rear of the moving ion front. This difference in chloride and glycine mobility sets up a steep voltage gradient in the stacking gel that sweeps along the negatively charged protein-SDS complexes. The large pores of the stacking gel present very little resistance to the movement of protein-SDS complexes, which then "stack up" into a very concentrated region at the interface between the running and stacking gels (right). Protein-SDS complexes remain concentrated at the interface until the slowly migrating glycine molecules reach the boundary between the two gels.

Dramatic changes occur as the glycine ions enter the running gel. The pH of the running gel is closer to the pKa of the glycine amino groups, so a significant fraction of the glycine molecules assume a negative charge. Negatively charged glycine molecules begin to move at the same rate as the chloride ions, thereby eliminating the voltage difference that controlled protein mobility through the stacking gel. The pores in the running gel are much smaller than those of the stacking gel, so the pores present frictional resistance to the migration of proteins. Proteins begin to migrate at different rates, because of the sieving properties of

the gel. Smaller protein-SDS complexes migrate more quickly than larger protein- SDS complexes (right). Within a certain range determined by the porosity of the gel, the migration rate of a protein in the running gel is inversely proportional to the logarithm of its MW.

Proteins are visualized with stains.

To visualize the positions of proteins after electrophoresis is complete, we stain the gels with various dyes that bind noncovalently and with very little specificity to proteins. During the staining process, proteins are also “fixed” in the gel, meaning that proteins become insoluble and unable to diffuse out of the gel. In our experiments, we will use a colloidal suspension of Coomassie Brilliant Blue G-250. Brilliant Blue G-250 binds proteins nonspecifically through a large number of ionic and Van der Waals interactions. In this procedure, gels are rinsed with water to remove the buffer salts used for electrophoresis and then treated with the colloidal G-250 suspension. Protein bands appear rapidly, and when necessary, the gels can be destained to lower the gel background. Brilliant Blue staining intensity is considered to be a quantitative procedure, because with some exceptions, the intensity of a stained band is directly proportional to the amount of protein in a band.

Reagents

Preparation of stock solution and buffers:

- 30% acrylamide
 - Acrylamide: 29.2g
 - N, N-methylene-bis-acrylamide: 0.8g
Added water, dissolved and made upto 100mL and filtered with Whatman no.1 filter paper.
- Separating gel buffer:
 - Tris-HCl: 1.5M, pH 8.8
18.171g of Tris was dissolved in 60mL of water and adjusted the pH to 8.8 with HCl and finally made upto 100mL with water.
- Stacking gel buffer:
 - Tris-HCl: 1M, pH 6.8
6.057g of Tris was dissolved in 60mL water

- and adjusted the pH to 6.8 with HCl and upto 100mL with water.
- 10% SDS solution: 1g of SDS in 10mL of distilled water.
 - N,N,N',N'-Tetra methylene diammine(TEMED)
 - 10% Ammonium per sulphate (APS): 1g of APS in 10mL of distilled water.
 - Electrophoresis Buffer:
 - Tris: 25mM, pH 8.3
 - glycine: 250mM, pH 8.3
 - SDS: 0.1%: Dissolved in minimum amount of water (500mL) and then added SDS. Allowed to settle and dissolved. This was finally made upto 2.5liters.
 - Sample buffer 4x: 5.0mL
 - Tris (1M, pH 6.8): 2.1mL
 - 2% SDS: 100mg
 - Glycerol (100%): 1.0mL
 - b-mercaptoethanol: 0.5mL
 - Bromophenol blue: 2.5mg
 - Distilled water: 0.4mL
 - Staining solution (100mL):
 - Alcohol: 40%
 - Acetic acid: 10%
 - Commassie Brilliant Blue (CBB): 259mg
 - Distilled water: 50%
 - Destaining solution (100mL)
 - Alcohol: 50%
 - Acetic acid: 10%
 - Distilled water: 40%

Casting SDS-PAGE gels

These instructions are designed for constructing two 12% SDS-PAGE gels with the BioRad Mini Protean system. The plates were washed in warm detergent solution, rinsed subsequently in tap water, deionised water and ethanol and dried.

Assemble the gel casting apparatus

- Assemble the components that you will need for casting the gel: a tall glass plate with attached 1 mm spacers, a small glass plate, a green casting frame and a casting stand.
- Place the green casting frame on the bench with

the green “feet” resting firmly against the bench and the clamps open (perpendicular to the frame) and facing you.

- Place the two gel plates in the frame. Insert the taller spacer plate with the “UP” arrows up and the spacers facing toward you into the casting frame (the BioRad logo should be facing you). Insert the short glass plate in the front of the casting frame. There should be a space between the plates.
- Secure the plates in the casting frame by pushing the two gates of the frame out to the sides. **IMPORTANT:** the bottom edges of the two plates should be flush with the lab bench before you clamp the frame closed to ensure a watertight seal. To do this, rest the frame vertically on the bench **BEFORE** closing the gates.
- Clamp the casting frame with glass plates into the casting stand, with the gates of the casting frame facing you.
- Repeat steps 1-5 to prepare a second gel in the casting frame.
- Check to see if the assembled plates in the casting stand are sealed properly by pipetting a small amount of deionized water into the gap between the plates. If the glass plates hold water and don't leak, you are ready to make the gels. Pour the water out by holding the entire casting platform over a liquid waste container or sink. Use paper towels or tissues to absorb any residual water. If the gel leaks, disassemble the frame, dry the plates and go back to step 3

Prepare two resolving gels.

Safety Note: Acrylamide and bisacrylamide monomers are weak neurotoxins. Gloves and goggles should be used when working with acrylamide.

Assemble the chemicals that you will need to pour the gels. The table below shows the quantities of each chemical that you will need to pour two gels with the Mini-Protean system.

Reagent	Resolving gel	Stacking gel
Deionized water	3.5 mL	2.1 mL
30% acrylamide:bis-acrylamide (29:1)	4.0 mL	0.63 mL
1.5 M Tris-HCl, 0.4% SDS, pH 8.8	2.5 mL	————
0.5 M Tris-HCl, 0.4% SDS, pH 6.8	————	1.0 mL
0% ammonium persulfate (catalyst)	100 μ L	30 μ L
TEMED (catalyst)	10 μ L	7.5 μ L

Polymerization occurs rapidly, so be sure to follow the step-by-step instructions below.

NOTE: catalysts should NOT be included into the mixture until you are ready to pour the gels!!

- Label two 15 mL beakers “Resolving gel” and “Stacking gel”.
- Prepare **ONLY** the resolving gels at this time. Mix the acrylamide solution, pH 8.8 Tris buffer and water, as shown in the chart above. Mix the ingredients gently, trying not to introduce air. Oxygen inhibits polymerization of acrylamide gels.
- To the resolving gel mixture, add 100 μ L of a 10% ammonium persulfate (APS) solution. Gently mix the solution, trying not to introduce air. Oxygen inhibits acrylamide polymerization.
- Add 10 μ L of TEMED catalyst. Once again, gently mix in the catalyst trying not to introduce air bubbles. **CAUTION:** TEMED has an unpleasant odor. Cover the tube immediately after you aliquot this reagent.
- Working quickly, use a plastic transfer pipette to fill the space between the two plates until the resolving gel solution reaches a height just above the green clamps on the gel casting frame. Draw up any remaining acrylamide into the transfer pipet. (You will know that the acrylamide has polymerized when you can no longer push the acrylamide out of the pipet.)
- Using a transfer pipet, add deionized water so that it gently flows across the surface of the polyacrylamide mixture. The water layer ensures that the polyacrylamide gel will have a level surface once it polymerizes.
- Allow the gel to polymerize, which takes ~15-20 minutes. You will note that the interface between the polyacrylamide and water overlay disappears temporarily while the gel polymerizes. A sharp new interface then forms between the two layers, indicating that polymerization is complete. (You can also check the remaining polyacrylamide in

- the transfer pipette to see if it has polymerized.)
- When polymerization is complete, remove the water from the top of the resolving gel by tilting the gel to the side and using a paper towel or Kimwipe to wick out the water.

Prepare the stacking gels

- Prepare the stacking gels. Mix the acrylamide solution, pH 6.8 Tris buffer and water, as shown in the chart above.
- Add 30 μL 10% APS and 7.5 μL TEMED to the stacking gel acrylamide mixture. Mix the contents by gently inverting the tube twice.
- Use a transfer pipette to pipette the stacking gel on top of the resolving gel between the two glass plates. Add enough stacking solution until it just reaches the top of the small plate.
- Carefully, but quickly, lower the comb into position, being careful not to introduce air bubbles. (The Bio Rad logo on the comb should be facing you.) Adding the comb will force some solution out of the gel, but this is fine. If air bubbles become trapped below the comb, remove the comb and reposition it.

Running SDS-PAGE gels

Set up the electrophoresis apparatus

- Carefully remove the gels from the casting stand and then from their green frames.
- Carefully remove the comb from the spacer gel.
- Remove the casting frame from the gel cassette sandwich and place the sandwich against the gasket on one side of the electrode assembly, with the short plate facing inward. Place a second gel cassette or a buffer dam against the gasket in the other side of the electrode assembly.
- Clamp the green clamps on the sides of the electrode assembly (below).

- Lower the chamber into the electrophoresis tank.
- Fill the space between the two gels with Tris-glycine running buffer. This forms the upper chamber for electrophoresis.
- Add Tris-glycine running buffer to the outer (lower) chamber until the level is high enough to cover the platinum wire in the electrode assembly.

Load and run samples on the SDS-PAGE gel

- Retrieve the cell extracts and mix with gel loading dye, vortex vigorously for ~ 10 seconds to thoroughly mix the contents and boil at 100°C immediately for 3-5 minutes.
- Using gel loading micropipette tips (tips have very long, thin points and fit P20s or P200s), load up to 15 μL of sample into each well. Load 5 μL of a molecular weight standard into one lane of the gel. Load samples slowly and allow the samples to settle evenly on the bottom of the well.

NOTE: Be sure to record the order of samples loaded onto the gel.

- Connect the tank to the power supply. Fit the tank cover onto the electrodes protruding up from the electrode assembly. Insert the electrical leads into the power supply outlets (connect black to black and red to red).
- Turn on the power supply. Run the gel at a constant voltage of 120-150 V. Run the gel until the blue dye front nearly reaches the bottom of the gel. This may take between 45-60 min.

Staining SDS-PAGE gels

- After the run is complete, turn off the power supply.
- Remove the gel apparatus from the tank. Open the clamping frame and remove the gel cassette sandwich. Carefully, pry the two plates apart with a spatula. With the spatula, remove the lower right or left corner of the gel to serve as an orientation marker. Be sure to indicate in your lab notebook

whether the notched corner corresponds to lane 1 or lane 10 of the gel. You may also remove the stacking gel with the spatula, if you desire.

3. Place the gel in a small plastic tray and gently free the gel from the glass plate, allowing it to slide into the water. The gel should move freely in the water. Place the gel and tray on a rocking platform. Rock the gel for ~2 minutes.
4. Drain the water from the gel and add enough staining solution to cover the gel, while allowing the gel to move freely when the tray is rocked. Cover the gel container with saran wrap and rock for 30 min. Make sure that the gel does not stick to the bottom of the tray.
5. After that, drain the staining solution into an

appropriately labeled waste container.

6. Destain the gel by filling the container about half full with destaining solution. Shake the gel in the destaining solution for 30 minutes. Pour off the destaining solution and add new destaining solution. Repeat, if necessary, until protein bands become visible.
7. When individual bands are detectable, record your data. You may photograph the gel with the gel documentation system against a white background. Alternatively, place the gel in a clear plastic page protector and scan the gel.
8. After recording the data, dispose of the gel in the Biohazard waste container.

Tricine–SDS Poly Acrylamide Gel Electrophoresis (Tricine–SDS-PAGE)

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Tricine–SDS-PAGE is used to separate proteins in the mass range 1–100 kDa and is the preferred electrophoresis system for the resolution of proteins smaller than 30 kDa.

Composition of stock solutions for use in Tricine SDS-PAGE:

Acrylamide / bis acrylamide (37.5% T, 1% C) 30%

$$\text{Total monomer concentration (\% T)} = \frac{[\text{Acrylamide (gm)} + \text{bis - acrylamide (gm)}]}{(\text{Total volume})} \times 100$$

$$\text{Cross linking monomer (\% C)} = \frac{\text{bis - acrylamide (gm)}}{[\text{Acrylamide (gm)} + \text{bis - acrylamide (gm)}]} \times 100$$

Dissolved 30 g Acrylamide and 0.8 g bis–acrylamide in 50 ml Milli-Q water and final volume was made to 100 ml with Milli-Q water, and stored at 4°C in an Amber coloured bottle.

3x Gel Buffer (3 M Tris-Cl, 0.3 % SDS, pH 8.45)

Dissolved 36.4 g Tris base in 50 ml Milli-Q water. The pH was adjusted to 8.45 with 6 N HCl and final volume was made to 100 ml with Milli-Q water and finally 0.3 g SDS was added and stored at 4°C.

10x Cathode Running Buffer (0.1 M Tris, 0.1 M Tricine, and 0.1 % SDS)

Dissolved 12.11 g Tris base, 17.92 g Tricine and 1g SDS in 50 ml Milli-Q water, and with out adjusting the pH the final volume was made to 100ml with Milli-Q water and stored at 4°C

10x Anode Running Buffer (0.2 M Tris-Cl, pH 8.9)

Dissolved 24.22 g Tris base in 50ml Milli-Q water. pH was adjust to 8.9 with 6 N HCl and final volume was made to 100 ml with Milli-Q water and store at 4°C.

1 M Tris-HCl, pH 6.8

Dissolve 12 g Tris base in 50ml Milli-Q water. The pH was adjusted with 6 N HCl and final volume was made to 100 ml with Milli-Q water and stored at 4°C.

2x Tricine sample buffer

Mixed 1 ml 1 M Tris-Cl ph 6.8, 2.4 ml glycerol, 0.8 g SDS, 2 mg Coomassie blue G-250, 1 ml β-mercapto ethanol and final volume was made to 10 ml with Milli-Q water and stored at 4°C.

Ammonium persulfate(APS) 10%

Tetramethylethylenediamine (TEMED)

15% Separating gel Preparation

Milli-Q water	0.516 ml
Glycerol	1.00ml
3X gel buffer	3.33ml
Acrylamide / Bis (30% stock)	5ml
10% APS	150µl
TEMED	4 µl
Total Volume	10ml

5% Stalking Gel Preparation

Milli-Q water	2.4ml
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3X gel buffer	1.67ml
Acrylamide / Bis (30% stock)	840 μ l
10% APS	75 μ l
TEMED	5 μ l
Total Volume	5ml

Tricine SDS-PAGE Electrophoresis

The gel was cast in a Mini-PROTEAN®Tetra Cell (10x8cm) (Bio-Rad Laboratories, Inc, USA). After the polymerization, the gel assembly was transferred in to a tank containing the 1x running buffers. The cathode and anode tanks were filled and electrophoresis was carried at constant current of 10 mA per gel till the dye reaches separating gel, then the current was

increased to 20 mA/gel. After the electrophoresis the gel was removed and proteins were fixed by staining in the Coomassie stain solution.

Coomassie Blue staining and Destaining

Fixing and staining was carried out simultaneously in a solution containing 0.1 % Coomassie blue R-250 in fixative (40 % Methanol and 10 % glacial Acetic acid). Afterwards the destaining of the gel was carried out by several changes of 40 % methanol + 10 % glacial acetic acid mixture to remove the background stain. The gels were documented under a gel documentation system.

Separation of DNA in Polyacrylamide Gels

Polyacrylamide gels can separate DNA that differs by 0.2% in length, well beyond the resolving capabilities of agarose (2% difference in DNA length). Another advantage to using polyacrylamide gels is that they can accommodate large amounts of DNA (up to 10 μg) without any loss in resolution. Depending upon the application, TBE gels can be prepared as denaturing or nondenaturing gels.

Applications

Denaturing gels: concentrations range from 8–20%

- Oligonucleotide purification
- Separation of single-stranded DNA
- Isolate radiolabeled DNA probes
- S1 nuclease assay
- DNA footprinting
- RNase protection assays

Nondenaturing gels: concentrations range from 3–20%

- Separation of di-nucleotide repeats
- Separation of DNA ranging from 20 bp–2000 bp in length
- Study DNA-Protein interactions (Gel Shift Assays)

Buffers for Electrophoresis

To ensure adequate buffering power during vertical electrophoresis, TBE Buffer is used for polyacrylamide gel electrophoresis at a working strength of 1X. Lower dilutions of the buffer or the use of TAE Buffer may cause gels to overheat and result in band smiling throughout the gel.

Reagents required

1.	Acrylamide- Bis acrylamide Solution Acrylamide (19:1) 30% stock - 4 ml Double distilled water - 6 ml 10 x TBE - 1.25 ml 10% Ammonium persulphate - 80 ml (freshly prepared) TEMED - 8 ml
2.	TBE buffer 10X (pH-8.0) Tris base - 10.8 g Boric acid - 5.5 g EDTA - 0.75 g Make up the solution to 100 ml with double distilled water. Autoclaved and stored at 4°C
3.	Gel loading buffer Bromophenol blue - 0.5% Glycerol (mol. grade) - 30% Prepared in 1X TBE Store at 4°C.
4.	1X TBE buffer 10X TBE - 10 ml Distilled Water - 90 ml

Protocol

1. Clean the glass plates and spacers thoroughly. Hold the plates by the edges or wear gloves, so that oils from the hands do not become deposited on the working surfaces of the plates. Rinse the plates with deionized water and ethanol and set them aside to dry. The glass plates must be free of grease spots to prevent air bubbles from forming in the gel.
2. Assemble the glass plates with spacers in gel caster.
3. Prepare the gel solution with the desired polyacrylamide percentage according to the table below, which gives the amount of each component required to make 12 ml (sufficient for 2 Hoefer minigels of 1 mm thickness):

Volume of Reagents Used to Cast Polyacrylamide Gels

Gel %	30% Acrylamide (29:1)	H ₂ O (ml)	5x TBE (ml)	10% APS (μ l)	TEMED (μ l)
8 %	3.2 ml	6.4	2.4	200	10
10%	4.0 ml	5.6	2.4	200	10
12%	4.8 ml	4.8	2.4	200	10

Stock solutions other than 29:1 (% w/v) acrylamide:bisacrylamide can be used to cast polyacrylamide gels. However, it is then necessary to recalculate the appropriate amount of stock solution to use. Gels can be cast with acrylamide solutions containing different acrylamide:bisacrylamide (cross-link) ratios, such as 19:1 and 37.5:1, in place of the 29:1 ratio recommended here. The mobility of DNA and dyes in such gels will be different from those given in this protocol.

- Wear gloves. Work quickly after addition of TEMED to complete the gel before the acrylamide polymerizes.
- Immediately insert the appropriate comb into the gel, being careful not to allow air bubbles to become trapped under the teeth. The tops of the teeth should be slightly higher than the top of the glass. Clamp the comb in place with bulldog paper clips. If necessary, use the remaining acrylamide gel solution to fill the gel mold completely. Make sure that no acrylamide solution is leaking from the gel mold.
- Allow the acrylamide to polymerize for 30-60 minutes at room temperature.
- After polymerization is complete, surround the comb and the top of the gel with paper towels that have been soaked in 1x TBE. Then seal the entire gel in Saran Wrap or plastic bag and store it at 4°C until needed.
- When ready to proceed with electrophoresis, remove gels from gel caster, carefully clean spilled gel from back of white plates and insert gels into Hoefer gelbox. Add running buffer and carefully pull the combs from the polymerized gel.
- It is important to use the same batch of electrophoresis buffer in both of the reservoirs and in the gel. Small differences in ionic strength or pH produce buffer fronts that can greatly distort the migration of DNA.
- Use a Pasteur pipette or a syringe to flush out the wells once more with 1x TBE. Mix the DNA samples with the appropriate amount of gel-loading buffer. Load the mixture into the wells using a micropipette equipped with a drawn-out plastic tip.
- Connect the electrodes to a power pack, turn on the power, and begin the electrophoresis run.
- Run the gel until the marker dyes have migrated the desired distance. Turn off the electric power, disconnect the leads, and discard the electrophoresis buffer from the reservoirs.
- Detach the glass plates. Lay the glass plates on the bench. Use a spacer or plastic wedge to lift a corner of the upper glass plate. Check that the gel remains attached to the lower (white) plate. Pull the upper plate smoothly away. Remove the spacers.
- Stain gels with silver nitrate to visualize the bands.

Visualization of DNA in Polyacrylamide gels using silver staining

Polyacrylamide gels were stained to visualize the DNA using the silver staining kit supplied by Amersham Pharmacia.

Reagents

	Solution	Composition
1	Fixing solution (5X)	3.0% Benzene sulphonic acid (w/v) in 24% ethanol (v/v)
2	Staining solution (5X)	1.0% Silver nitrate (w/v), 0.35% Benzene sulphonic acid (w/v).
3	Developing solution (5 X)	12.5% Sodium carbonate (w/v). 37% Formaldehyde (w/v) in water 2% Sodium thiosulphate (w/v) in water
4	Stopping and Preserving solution, 5X	5% Acetic acid (v/v) 25% Sodium acetate (w/v) 50% Glycerol (v/v)

Protocol

50 ml of fixing solution were used for 20 minutes to fix the gels (diluted five times with 30.4 ml double distilled water and 9.6 ml ethanol) and silver-impregnated (with 1X staining solution) for 20 minutes. This followed by washing the gels with double distilled water for another 1 minute. This followed by keeping gels in the 1X developing solution in darkness for 10 minutes. When the bands were dark enough, developing solution was poured out. Then stopping and preserving solution (1X) was immediately added and the gel was documented.

Production of Antibodies

Principle

A substance that can induce a detectable immune response in heterologous species is known as an antigen. When foreign proteins (antigen) are injected into rabbits, they induce immune response and produce antibodies to that particular antigen. These antibodies can be separated from serum of the rabbits injected with antigen.

Materials

Antigen: Soluble antigen: Proteins, virus, etc

- Particulate antigen: Bacterial cell
- Freund's adjuvant
- 2 ml syringe
- 20 gauge needle
- Millipore filter

Procedure

- Pass the antigen through 0.45 mm millipore filter in case of soluble antigen and pure bacterial cell preparation in case of particulate antigen.

- Mix 0.5 ml of soluble antigen to 0.5 ml of adjuvant. The mixture is forcibly passed through needle until water in oil emulsion is achieved.
- Clean skin of rabbit (preferably on thigh region) with 70% alcohol and hold a fold of skin between thumb and finger.
- Insert the needle into the skin lying behind the skin fold and inject the desired volume of toxin adjuvant mixture.
- Four to five injections of the same dose are given at weekly intervals.
- In case of particulate antigen, inject the antigen intravenously on 0, 3, 5, 9, 13 and 15 day without adjuvant.
- Bleed the rabbit from marginal ear vein, separate the serum and test for antibody formation by agar gel precipitation test for soluble antigen and by agglutination test for particulate antigen.

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This training manual is a compilation of the lecture notes delivered in the areas of marine biology and molecular biology related to fisheries research, fish genetics and genomics, fish health management, microscopy, fish nutrition and marine bioprospecting as part of the DBT sponsored 3 months National Training on Molecular Biology and Biotechnology for Fisheries Professionals at CMFRI, Kochi. Its uniqueness is in integrating marine biology with marine biotechnology where the stress was on introducing the marine biodiversity to the participants to begin their journey in marine biotechnology. This manual also documents standard operating procedures and laboratory protocols in marine biotechnology. The support from Department of Biotechnology, Govt. of India for putting this together was a need of time.



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