

WINTER SCHOOL
ON
Recent Advances in
Mariculture Genetics
and Biotechnology

4th to 24th November 2003

Course Manual



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ISOLATION AND APPLICATION OF MARINE NATURAL PRODUCTS

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Introduction

The marine bioactive compounds or Marine natural products (MNPs) offer avenues for developing cost-effective, safe and potent novel drugs and other useful products. MNPs are organic compounds produced by microbes, sponges, seaweeds, and other marine organisms. The host organism biosynthesizes these compounds as non-primary or secondary metabolites to protect themselves and to maintain homeostasis in their environment. In the decade from 1977 to 1987, around 2500 new metabolites (MNPs) were reported from marine organisms ranging from microbes to fish. According to Dr. S.Z. Qasim, less than 1.0% of the total marine organisms have only been examined for MNPs research. Perusal of literature indicated that even the seawater has bactericidal properties. This could be attributed to the production of antibiotics by planktonic algae and bacteria respectively. Considering such vast resource, holistic approach of pharmacological research has to be given to develop potent therapeutics.

Marine bacteria

The discovery of penicillin in 1929 heralded the era of antibiotics, which led to the understanding that microorganisms are a rich source of useful natural products. Since that time and consequent to the continuous effort of R&D projects with heavy investments, nearly 50,000 natural products have been discovered from microorganisms. Of these substances, more than 10,000 are biologically active and more than 8000 are antibiotics. More than 100 microbial products are in use today as antibiotics, anti-tumor agents and agrochemicals. It is demonstrated that marine bacteria produce anti-microbial substances. Further array of research results indicated that the marine microorganisms are capable of producing potent and unusual bioactive natural products that are uncommon in terrestrial microbes.

The first documented identification of a bioactive marine bacterial metabolite was the highly brominated pyrrole antibiotic, isolated by Burkholder and co-workers from a bacterium obtained from the surface of the Caribbean Sea grass. Subsequently, this unique metabolite was identified by x-ray crystallographic methods, which composed of more than 70% bromine by weight. The metabolite exhibited impressive *in vitro* antibiotic properties against Gram-positive bacteria, with minimum inhibitory concentration (MIC) ranging from 0.0063 to 0.2 µg/ml. However, it was inactive for Gram-negative bacteria and animal assays.

As more evidence is obtained, it is becoming abundantly clear that bacteria form highly specific, symbiotic relationships with marine plants and animals. Experience in this area arose from a study of the pathogen resistance of the estuarine shrimp *Palaemon macrodactylus*. The eggs of *P. dactylus* possess significant bacterial epibionts, which, when removed by treatment with antibiotics, leads to the rapid infestation of the eggs by pathogenic fungi, especially of *Lagenidium callinectes*. Although there are many plausible mechanisms to explain this protective phenomenon, with *Palaemon* it could be anti-fungal agents produced by bacteria.

Marine Fungi

Although terrestrial fungi have represented a major biomedical resource (penicillin from *Penicillium*, for example), studies to develop the biomedical potential of marine fungi have been few. The isolation of a small lactone, leptosphaerin from *Leptosphaeria oraemaris* by Schiehsler in 1980 demonstrated that marine fungi may form important resource for unique metabolites. Later, the useful chemical, Gliovictin was isolated from marine fungus, *Asteromyces cruciatus*. Since then more than twenty useful bioactive compounds have been derived from marine fungi.

Marine Micro algae

Micro algae are significant resource for bioactive metabolites, particularly cytotoxic agents with applications in cancer chemotherapy. From the marine micro algae such as from the blooms of *Phaeocystis* sp., antibiotic substances are reported. *Phaeocystis pouchetii* produces chemicals such as Acrylic acid, which constitutes about 7.0% of the dry weight. The antibiotic substances thus produced are transferred throughout the food chain and found in the digestive tract of Antarctic penguins. Production of β carotene and vitamins by the halo tolerant alga *Dunaliella* sp., is documented.

Marine Macro algae

It is reported that of the total marine algae so far evaluated, 25% showed one or the other biological activity. Metabolites of green algae were reported to contain 1,4 - diacetoxycyclohexadiene moiety, which exhibited ichthyotoxic property. Antimicrobial diterpene (dictyodiol) was isolated from *Dictyota spinulosa*. Both ichthyotoxic as well as cytotoxic diterpenoids from *Dictyota* sp. Among the red algae, halogenated lipids have been isolated, particularly from the *Laurencia* sp. The rare chemical prostaglandin was also reported to occur in *Gracilaria pichenoids*. *Ulva* meal supplementation was found to provide disease resistance to red sea bream in Japan. Similar results were also reported from Japan on the use of *Ulva* meal supplementation towards disease resistance and high growth rate in black sea bream. The polysaccharide fractions from marine algae, *Porphyra yezoensis* (PASF) was found to stimulate the *in vivo* and *in vitro* murine phagocytic function. The purified fractions of PASF gave stronger phagocytic activity.

An antiviral compound, UF-131 was isolated from the green alga *Ulva fasciata*. Extracts of *Caulerpa taxifolia* and *Ulva fasciata* with moderate antiviral activity was found to protect the animals. Among the 30 different species of algae collected and screened for activity biological activity was recorded in 26 species towards bacteria. Growth of gram-negative bacteria such as *Vibrio* and *Staphylococcus* and gram-positive bacteria such as *Bacillus sp* were found to be inhibited to varying degrees. Several species of *Halinida* produce terpenic di or tri aldehydes with ichthyotoxic activity. In addition, brominated aromatics, nitrogen heterocycles, nitrogen-sulfur heterocycle, sterols, terpenes, dibutenoloides, protein, peptides and sulfated polysaccharides were also reported. The sulphated polysaccharides grouped as carrageenens and agar isolated from red and brown algae were shown to possess antigenic properties and exhibited anticoagulant and antiviral activities. Other compounds from algae were shown to possess antifungal, antibacterial, ichthyotoxic, hypotensive and antihelminthic activities.

Under the ICAR Ad-hoc scheme sanctioned by the Council, basic data on the availability of different algae along the coasts of India with their secondary metabolite capabilities were carried out in CMFRI. Seventeen potential macro algal extracts were prepared and their bioactivity profiles were studied. Among the macro algal crude extracts, experiments conducted with pathogenic challenge among fish and shellfish indicated their potential therapeutic nature. The responses of the fish/shellfish towards treatment with a few of the algal metabolites have been also standardized in CMFRI laboratory.

Marine Sponges

In sponges, the secondary metabolites are synthesized to protect themselves and to maintain homeostasis. The wider biosynthetic capability of sponges could be attributed to their biological association with other symbionts. According to Bertrand and Vacelet, about 38% of the sponge body comprises of microorganisms. A wide variety of secondary metabolites were isolated from sponges and these have been associated with antibacterial, antimicrobial, antiviral, antifouling, HIV-protease inhibitory, HIV reverse transcriptase inhibitory, immuno- suppressent and cytotoxic activities. In addition to potential anticancer applications, the MNPs of sponges have a myriad of activities ranging from antibiotic activity including anticoagulant, antithrombin, anti-inflammatory, as well as immunomodulatory activities.

During the 1970s and 1980s, investigators also studied the type and specificity of sponge-microbe associations, often using microscopy to show the presence of specific symbiont morphologies within specific sponges. The molecular basis of symbiosis was also probed, albeit to a lesser extent. Bacteria, which live symbiotically with sponges, can be passed through their feeding chambers without being digested. This suggested some sort of encapsulation or recognition process. In the demosponge *Halichondria panicea*, an

association with the microbe *Pseudomonas insolita* may be lectin-based. An immunological basis for symbiosis in some sponges is claimed as evidence of a Precambrian origin for many symbioses.

A major problem with the early studies on sponge-microbe symbiosis was that most microorganisms were uncultured or uncultivable, so descriptions of symbioses usually relied either on morphology of symbionts or chemical measurements of nutrient transfer. Even in the cases where putative symbionts could be cultured, the ecological relevance of symbiosis could not be determined. The period following Wilkinson's review has been marked by the ascendance of molecular biological techniques in environmental microbiology, which have allowed investigators to focus on uncultured microorganisms.

The application of molecular biology to sponge-microbe symbiosis is yielding results that could not have been obtained by classical microbiological methods. The discovery of a member of the Archaea living specifically within a sponge similar to *Axinella mexicana* was a particularly exciting. The archaeal microorganism, *Cenarchaeum symbiosum* (P: Crenarchaeota), lives at a relatively cold 10 °C and is therefore considered psychrophilic. Subsequent *in situ* hybridization experiments showed which microorganism in the sponge was archaeal and allowed localization of the symbiont.

Unfortunately, despite the scientific and technological benefits of determining the source organisms of interesting and bioactive metabolites, a microbial origin of sponge compounds has rarely been demonstrated. Most of the literature is purely speculative, based on similarities, however slight, between compounds from sponges and those from cultivated microorganisms, especially cyanobacteria. Several researchers have attempted to culture microorganisms from invertebrates in the hopes of obtaining some of these bioactive compounds. Although they have been successful in the discovery of novel natural products, this research has rarely demonstrated the presence of sponge metabolites in the microbial isolates. In one case the same compound was found in a *Hyattella* sp. sponge as in a *Vibrio* sp. cultured from that sponge. These results demonstrate that traditional culturing approaches are not generally applicable to the environmental problems of sponge-microbe symbiosis. Two cases of symbiont production of sponge compounds have been clearly established. Both studies relied on cell fixation and physical separation techniques, bypassing the problem of culturing symbiotic microorganisms. Unson separated cyanobacterial symbionts from the sponge *Dysidea herbacea* by flow cytometry and showed that chlorinated amino acid derivatives could only be found in the cyanobacterial fraction, while terpenes were localized in the sponge cell fraction.

Sponges contain a standing population of bacteria inside; in some instances, the bacterial load may come up to 38% of the total volume of sponge. The bacterial population is seen even in the motile larval stages. How and when this bacterial population gets into the larvae of sponges is not known. It is believed that the sponge phagocytoses these bacteria when food becomes scarce. It is not sure where the various bioactive substances in

sponges are produced by the sponge itself or by the associate or through the combined action of both.

Avenues for further research and development:

Perusal of literature indicates that during the last three decades number of diverse biologically active compounds has been isolated from marine organisms, but the number of compounds taken-up for the field trial/clinical use are scanty. This may be due to the failure of successful collection of concerned source organism in bulk, which have same sort of secondary metabolites. Some of the future requirements are listed below:

Microbial Isolation/screening and culture techniques:

As the symbiotic microbes are difficult to culture under laboratory conditions, basic Research in Marine Microbiology is essential. Without considerable attention to developing the basic biology of marine microorganisms, explorations for new bioactive metabolites would be limited to those few classes of microorganisms, which are readily isolated and grown under "standard" conditions. Unfortunately, little is known about the specific nutrients and growth factors required by most of the marine microbes. For example, the common media components such as peptone, sugars etc., are unrealistic marine nutrients as complex carbon sources such as chitin, sulphated polysaccharides, marine protein etc., are found in the marine habitat. In addition, information is lacking on some of the uncommon inorganic elements such as lithium, silicon etc., abundant in the marine sediments. As a result of these difficulties, it is seen that less than 5% of the available microbial population is only cultivable under the standard laboratory conditions. Presently, this condition, certainly limits the scope and ability to isolate and culture majority of the interesting and new microbes.

Preparation of crude extracts for bioactivity:

As the goal is to obtain the widest possible screening for each crude extract so that no useful compound is over looked. Solvents such as methanol, chloroform or ether as independent solvents or as combinations can be used depending upon the nature of the MNPs. As soon as the crude extracts are obtained, there is need for immediate and simple *in vitro* assays such as: i. Antimicrobial and ii. Enzyme inhibition assay (very low quantity of sample only is required). This in turn helps in the 'bioassay – guided fractionation and purification' process.

Purification:

Once bioactivity is detected in the crude extract, the next step is to purify the same. It is important to employ non-destructive method such as spectroscopic method, which conserves the materials for further bioassays. In addition, techniques such as: TLC, MS/IR/uv and H nMR – (for structural elucidations) are to be adopted for purification of the crude extract and for determining the structure.

Pharmacological screening:

The next step after purification and structural elucidation is pharmacological screening. Studies such as determining the LD₅₀ of the extracts in mice, in addition to brine shrimp assay, fertilized sea urchin assay and starfish assay are to be carried out in established laboratories. Further tests such as: antiviral (AIDS/anti-HIV), cytotoxic, anti-inflammatory, anti-tumor, tumor promoter (protein kinase), analgesic, anti-coagulant / anti-thrombic (ex: heparin), anti-ulcer, anti-cholesterol / anti-lipemic, wound dressing, anti-parasitic, anti-protozoa are to be conducted.

Commercial development of bioactive (MNP) products:

The 'co-operative drug development programme' as suggested by Faulkner (1993) is the best method, which will solve the problems arising on issues such as: patent rights, academic freedom and industrial secrecy.

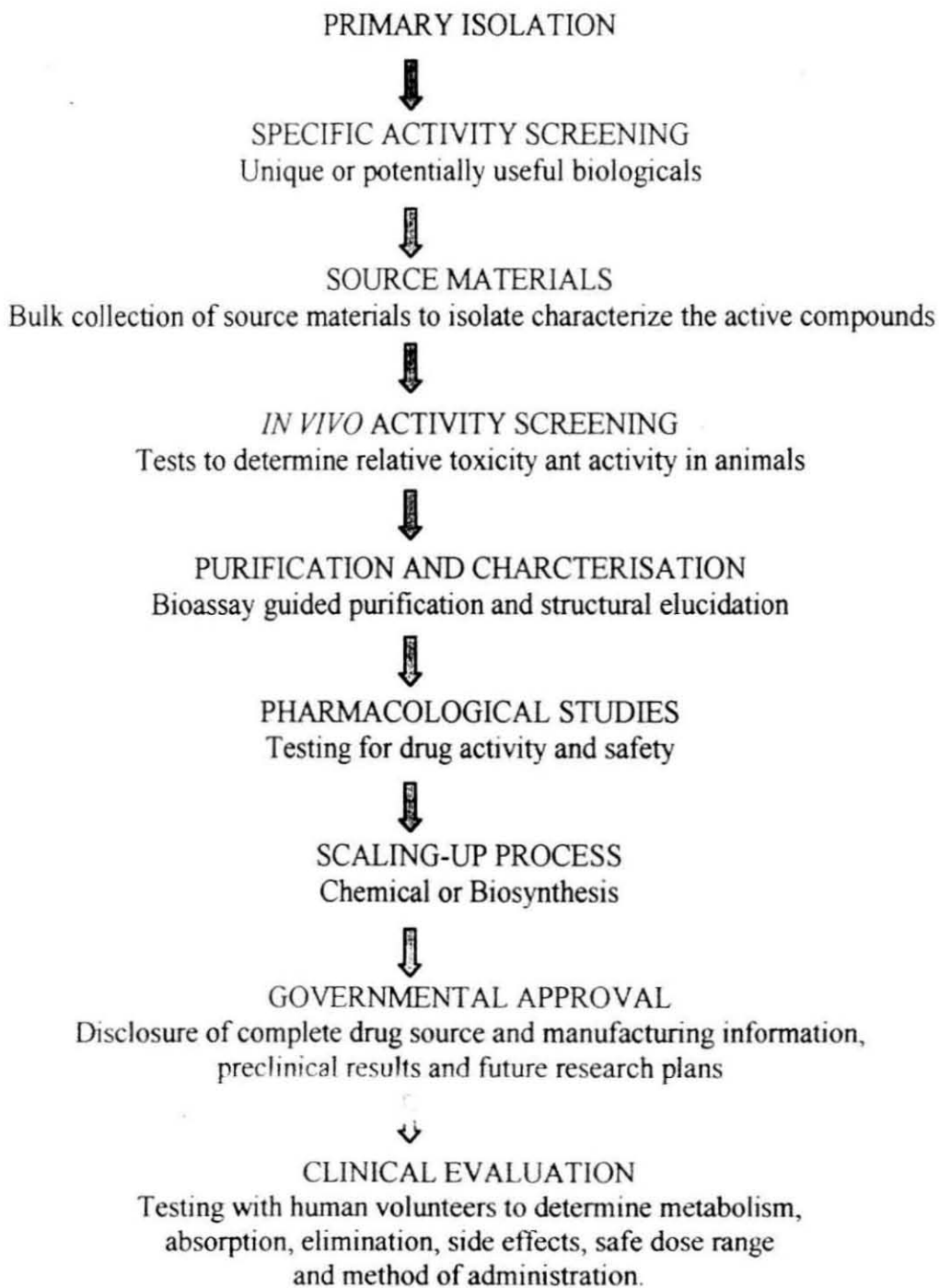
Conservational aspects of source organisms:

Eco-friendly collection of the source organism and required supply of them in bulk for scaling-up process.

The role of Industry and Academia:

Considering the less microbiological and intensive pharmacological training to the industrial personnel, relevant microbiological training has to be imparted to the industrial pharmacologists. New isolation methods, media development etc., are to be included in the curricula of academic/research institutes. Collaborative programmes which combine biomedical and microbiological expertise of the pharmaceutical industry with the marine resources available in the marine R&D Institutes will in the long run help in the better utilization of the marine organisms or resources for biotechnological aspects.

DEVELOPMENTAL PROCESS IN MARINE PHARMACEUTICALS



Mollusc foot adherence assay

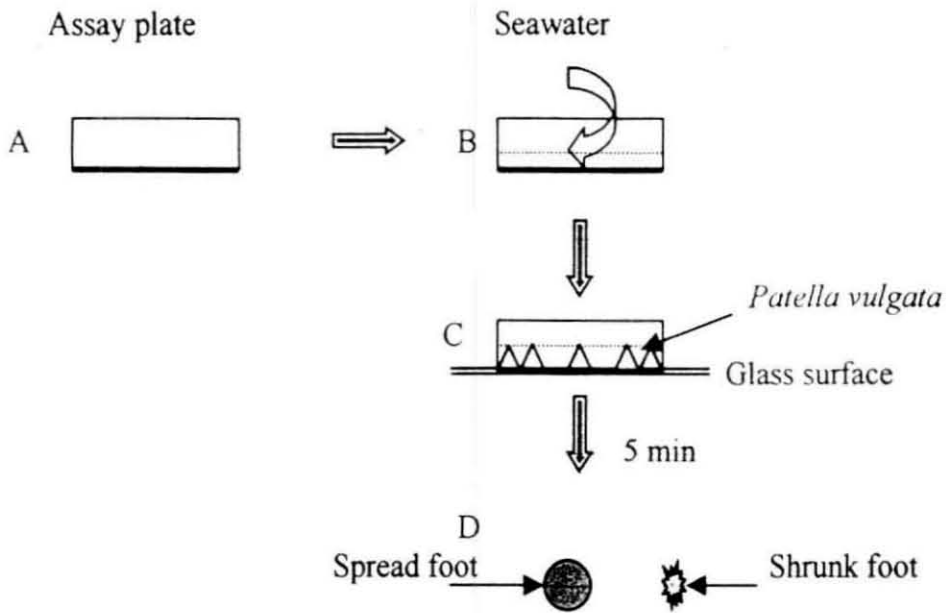


Figure 1. Procedure of the foot adherence assay:

- Preparation of the assay plate with 1 ml of selected concentration of extract/fraction.
- Filled with seawater without disturbance to the extract layer
- The limpets were removed carefully from the tank and introduced in to the triplicate experimental plate and kept on an illuminated glass surface to observe the foot reflex.
- Based on the foot adherence or shrinkage, the fouling rate can be estimated