

PHOTO BIOREACTOR BASED MICROALGAE CULTURE FOR MARICULTURE APPLICATIONS

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Microalgae are considered as a valuable organic resource with a potential application in finfish, shellfish and molluscan culture. Being, rich source of essential fatty acids (EPA and DHA), the microalgae have very important role even in human nutrition. Microalgae culture forms an inevitable component in aquaculture venture especially seed production of either finfishes or shell fishes. Cultivated microalgae have long been integral to the hatchery production of many farmed finfish, shellfish and other commercially important aquaculture species. Molluscs like oysters, mussels and clams filter them from the sea water in all stages of life. Rotifers and brine shrimps also ingest algae, and are then themselves used as food for larval fish and prawns. The shrimp hatcheries use micro algae as food for the early larvae and later for the water quality maintenance. In many hatchery systems algae are added to the water containing larvae to improve the 'quality' of water as green water systems. The production of live algae is very critical in the successful hatchery management. In the natural environment, the larvae feed on any minute plant components which are readily available to them. But in a hatchery, the feed which are acceptable to the larvae for their growth and further development have to be identified and isolated. In the early critical stages of the rearing larvae of fin fishes and shellfishes, the phyto-flagellates (species of *Isochrysis*, *Pavlova*, *Dicrateria*, *Chromulina* and *Tetraselmis*) and other nanoplankters (species of *Chlorella* and *Synechocystis*) form the basic food. But in the post larval stages of crustaceans and spat or juvenile stages of bivalves, the diatoms (species of *Chaetoceros*, *Skeletonema* and *Thalassiosira*) form the primary food. Hence the culture of micro algae is an essential prerequisite for the rearing operations of economically important cultivable organisms in a hatchery system.

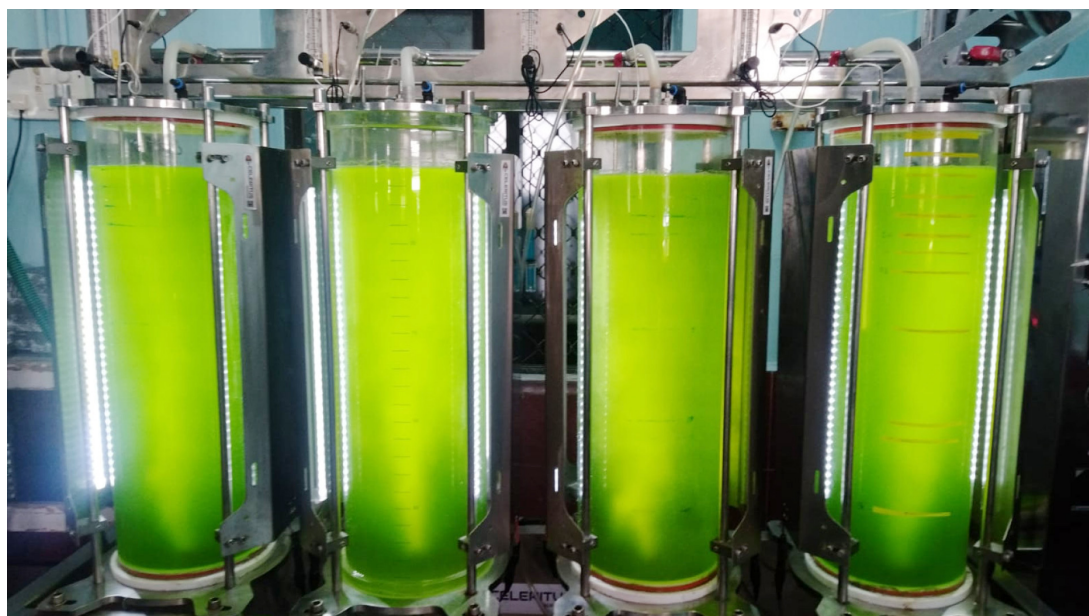
Large-scale production systems are necessary due to increasing demands for microalgal biomass and products originating from microalgae. Mass culture of microalgae on a commercial scale is essential to satisfy its huge requirement in the hatchery as well as for biomass production for application as functional food and nutraceuticals. Microalgae are enriched with a variety of high-value compounds natural pigments, anti-oxidants, vitamins, minerals, polysaccharide, and so

on. Microalgal biomass used an alternative fuel source in the form of biodiesel, and also for extraction of valuable products including bioactive compounds for human health and nutrition (omega -3 fatty acids), biopharmaceutical, cosmetic and feed industries. Value added products are also sourced from micro algae includes carotenoids, phycobiliprotein pigments, Vitamin C, E and biotin , fatty acids (linolenic, arachidonic, etc.); and recombinant proteins.

Common Micro algae and applications in Aquaculture

SL No	Scientific Name	Size (µm)	Nutritional value	Practical application
1	<i>Nannochloropsis oculata</i> Eustigmatophyte (Yellow-green)	2-4	EPA	Rotifer culture, water conditioner in finfish hatcheries, reef tanks for feeding corals and other filter feeders
2	<i>Isochrysis galbana</i> Prymesiophyceae (golden brown flagellate)	3-5	DHA	Rotifers, copepods, brine shrimp, oysters, clams, mussels and scallops; enrichment of zooplankton ;
3	<i>Chaetoceros calcitrans</i> Bacillariophyceae(diatom)	3 - 6	EPA and Vitamins (B ₂ & C)	Shrimp hatcheries to increase the vitamin levels.
4	<i>Chlorella salina</i>	2-10	EPA	Used for feeding rotifers in finfish larval rearing.
5	<i>Pavlova lutheri</i>	3-10	DHA/E PA	Oysters, clams, mussels and scallops to increase the DHA/EPA levels in their broodstock.
6	<i>Tetraselmis suecica</i> (Prasinophyceae) green flagellates	4-5	amino acids	Feeding stimulant for oysters, clams, mussels and scallops. excellent feed for shrimp larvae and <i>Artemia</i>
7	<i>Thalassiosira weissflogii</i> Bacillariophyceae (diatom)	4 -32	EPA and Vitamins (B ₂ & C)	Single best algae for shrimp larvae and also good feed for copepods, brine shrimp, and broodstock conditioning of oysters (post set), clams and mussels.
8	<i>Skeletonema costatum</i> Bacillariophyceae (diatom)	2-21	EPA	extensive and intensive shrimp hatchery systems

Since the requirement for microalgae is huge for its application, mass culture of microalgae is inevitable. Outdoor culture of these microalgae in open systems is unsuccessful due to rapid contamination, especially with other algal species and also there is no control over illumination, temperature, nutrient level, contamination with predator and other competing algae which resulted in less density of the culture. This type of cultivation demands huge infrastructure occupying in large area, which will elevate the operational and maintenance cost of the aquaculture venture and polyvinyl chlorides). Uncertainty in continuous production with sufficient biomass become the major bottleneck in conventional culture systems, which requires large areas for commercial operation influenced by several environmental factors. In this juncture, microalgae cultivation in the closed system (photo bioreactors) can prove to be more effective, due to the possibility of better control over the production process in order to increase the productivity. Continuous microalgae production in PBR will be a good prospect for technology with high density culture, because it prevents culture contamination and thereby reduce the operational cost of the microalgae culture including the regular culture maintenance, manpower requirement etc. The issues such as temperature rising, inconsistent solar rays, weather fluctuation, photo inhibition due to excessive light intensities, night time biomass losses are among problems that remain to be



improved in the outdoor PBR. Indoor PBR using artificial lights may offer some solutions to overcome the problems in the outdoor PBR which depends to sunlight.

Interventions in PBR to increase the cell count / productivity

1. **Sea water Treatment:** Different sea water treatment used for culturing microalgae in photo bioreactors include sea water passed through slow sand filter, UV filtration, autoclaving and ozone treatment. For *Nannochloropsis* culture ozone treated sea water is found best with cell count of 36×10^6 /mL on 4th day.
2. **Different percentage of inoculum:** With the introduction of different percentages of inoculum for the culture it is resulted that 20% level is optimum for the progress of the microalgal culture in photobioreactor.
3. **Different culture medium:** Conway / F/2 Medium depending on the species of which microalgae need to be cultured. For *Nannochloropsis* and *Isochrysis* culture Conway medium found to be best in photobioreactor, whereas for *Thalassiosera* culture F/2 medium was best.
4. **Second dose of Culture medium:** It is proved to be advantageous that second dose of culture medium added to the exponential phase (second day) of the microalgal culture.
5. **Introduction of pure CO₂:** Different doses of CO₂ ranging from 10-30ppm in photobioreactor resulted that 10ppm CO₂ enhanced the better microalgal cell counts.
6. **Frequency of pure CO₂ :** Different frequency of pure CO₂ addition to the microalgal culture in photobioreactor resulted that maximum frequency of two times was found to be the better to enhance microalgal cell count.
7. **Photoperiod :** Difference in light intensity(24 light; 12:12 light & dark;6:18 light & dark; 18:6 light & dark etc.) during the particular growth phase of microalgal culture resulted in variation in microalgal cell density.

Application of microalgae in Aquaculture

The significant role of microalgae in aquaculture hatcheries include cultivation of microalgal strains for broodstock conditioning, larval rearing and feeding of newly settled spat, as all developmental stages of bivalve molluscs are directly dependant

on microalgae as a feed source. The planktonic larval stages of commercially important crustaceans are initially fed on microalgae. Farmed gastropod molluscs and sea urchins require a diet of benthic diatoms when they first settle out from the plankton, prior to transferring to their juvenile diet of macroalgae. The small larvae of most marine finfish species and some freshwater fish species also initially receive live prey in the presence of microalgae. These microalgae are allowed to bloom within the fish larval rearing tanks (green water) or are added from external cultures (pseudo-green water).

Commonly used microalgae in a specific area of application in aquaculture

Area of application in aquaculture	Commonly used microalgae
In formulated feed ingredient	<i>Arthrospira platensis</i> (Cyanophyceae); <i>Chlorella vulgaris</i> , <i>C.minutissima</i> , <i>C.virginica</i> , <i>Dunaliella tertiolecta</i> , <i>D. salina</i> , <i>Haematococcus pluvialis</i> (Chlorophyceae)
Feed for bivalve mollusks	<i>Thalassiosira pseudonana</i> (Bacillariophyta); <i>Pavlova lutheri</i> (Haptophyta); <i>Isochrysis galbana</i> <i>Chlorella minutissima</i> , <i>Gomphonema</i> sp, <i>Isochrysis galbana</i> , <i>Nitzschia</i> sp, <i>Phaeodactylum tricornutum</i> , <i>Tetraselmis subcordiformis</i> . <i>Tetraselmis suecica</i> , <i>T.chui</i> (Chlorophyceae); <i>Chaetoceros calcitrans</i> , <i>C. gracilis</i> ; <i>Skeletonema costatum</i> .
Rotifer and <i>Artemia</i> live prey	<i>Cryptocodinium cohnii</i> (dinoflagellates); <i>Schizochytrium</i> sp.; <i>Ulkenia</i> sp. <i>Chlorella</i> sp, <i>Chlamydomonas</i> sp, <i>Nannochloropsis oculata</i> , <i>Tetraselmis tetrathele</i> and <i>T. chuii</i> .
Feed for crustacean larvae (shrimps, lobsters)	<i>Tetraselmis suecica</i> , <i>T.chui</i> (Chlorophyceae); <i>Chaetoceros calcitrans</i> , <i>C.gracilis</i> ; <i>Skeletonema costatum</i> ;

	<i>Thalassiosira pseudonana</i> (Bacillariophyta).
Feed for gastropod molluscs and sea urchins	<i>Nitzschia</i> sp. ; <i>Navicula</i> sp.; <i>Amphora</i> sp.
Green water for finfish larvae	<i>Isochrysis galbana</i> ; <i>Nannochloropsis oculata</i>

Harvesting and production of microalgal concentrate

Finfish / Shellfish hatcheries typically use large quantities of single-cell phytoplankton (microalgae) to feed shellfish larvae. Microalgae are enriched with a variety of high-value compounds natural pigments, anti-oxidants, vitamins, minerals, polysaccharide, and so on. Algae production requires hatcheries to commit large square footage and equipment cost to culturing phytoplankton. Additionally, culturing algae requires committing significant labor and other operating costs to maintain algal cultures and equipment. The current microalgal production technologies are not cost-effective and are hindered by various bottlenecks, one of which is the harvesting of microalgal biomass. After cultivation of microalgae on a large scale, there is a need to reduce the volume for concentrating the microalgal cells. The development of microalgae concentrates for feeding larvae provides the opportunity to mitigate significant capital and operational costs.

Advantages and Disadvantages of Various Algae Harvesting Techniques (Barros et al., 2015).

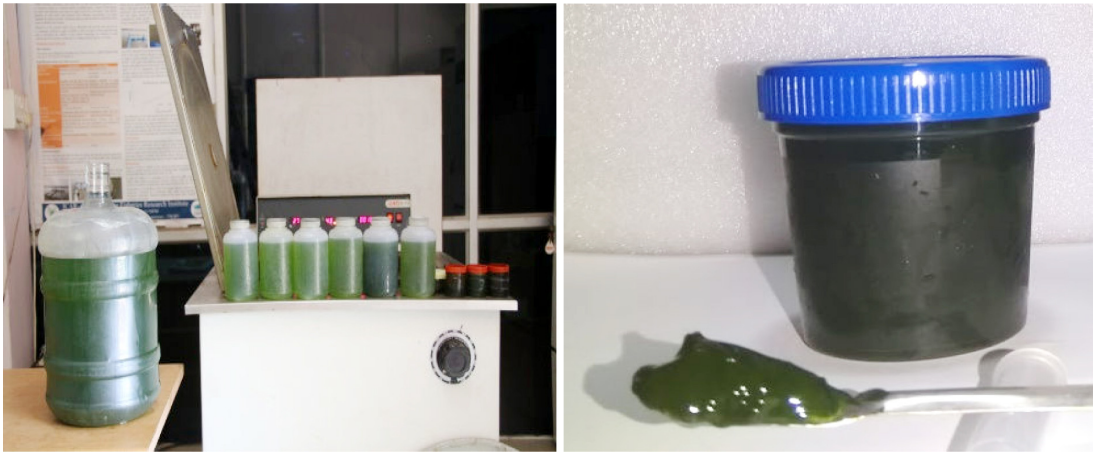
Harvesting Technique	Advantages	Disadvantages
Coagulation/flocculation	Fast and easy technique Used for large scale Less cell damage Applied to vast range of species Less energy requirements Auto and bioflocculation may be inexpensive methods	Chemicals may be expensive Highly pH dependent Difficult to separate the coagulant from harvested biomass Efficiency depends upon the coagulant used Culture medium recycling is limited Possibility of mineral or microbial contamination

Flotation	Suitable for large scale, Low cost and low space requirement Short operation time	Needs surfactants, Ozoflotation is expensive
Electrical based processes	Applicable to all microalgal species; No chemicals required	Metal electrodes required High energy and equipment costs Metal contamination
Filtration	High recovery efficiency Cost effective No chemical required Low energy consumption (natural and pressure filter) Low shear stress Water recycles	Slow, requires pressure or vacuum Not suitable for small algae Membrane fouling/clogging and replacement increases operational and maintenance costs High energy consumption (vacuum filter)
Centrifugation	Fast and effective technique High recovery efficiency (>90) Preferred for small scale and laboratory Applicable to all microalgae	Expensive technique with high energy requirement High operation and maintenance costs Appropriate for recovery of high-valued products Time consuming and too expensive for large scale Risk of cell destruction

Harvesting of *Nannochloropsis* by Centrifugation

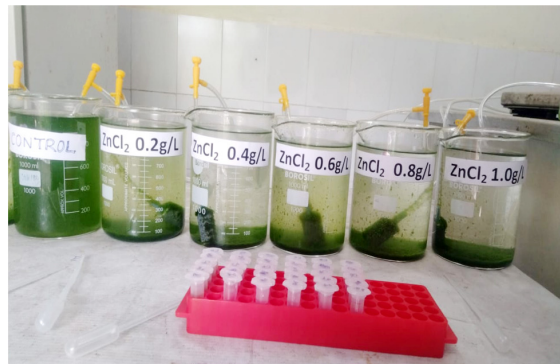
Harvesting of the *Nannochloropsis* culture in growing phase/log phase by centrifugation at 7000 rpm for 5 minutes at 4°C resulted in harvesting efficiency of 99.6%. Followed by preservation with different doses of glycerol (5 to 20%) in different storage method results that the preservation of the harvested *Nannochloropsis* by the mode of chilling can perform better than by freezing counterparts in terms of its efficiency as stock culture in the future. Microalgal concentrate is an alternative approach to ensure all-time availability of sufficient quantities of micro algae for larval rearing and zooplankton culture. Micro-algal

concentrates are prepared and preserved with added preservatives and this could be used at the time of requirements. Major advantage of *Nannochloropsis oculata* concentrate (**NANNCON-** ICAR –CMFRI) with a cell count of 30 billions/ml compared to other commercial products include, cell viability more than 80% even after five months of storage in glycerol under chilled conditions. The concentrated cells perform as inoculums, after dilution, for mass scale culture as well as direct feed to rotifers.



Harvesting of *Nannochloropsis oculata* by chemical flocculation

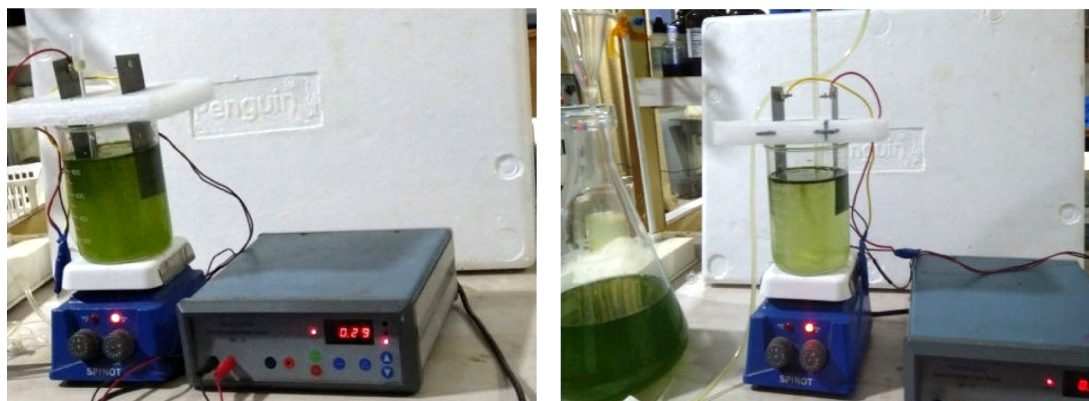
Chemical flocculation of *Nannochloropsis* was studied with $ZnCl_2$ and $ZnSO_4$ (0.2, 0.4, 0.6, 0.8 & 1.0 g/lit) as flocculants. On termination of the study, $ZnSO_4$ with 0.8gm/lit of *Nannochloropsis* culture performed better with 92% of harvesting efficiency. With various concentration of $ZnCl_2$, the harvesting efficiency varied from 70.55 % - 77.54 %. The highest efficiency was registered with 0.4 g/lit. Therefore,



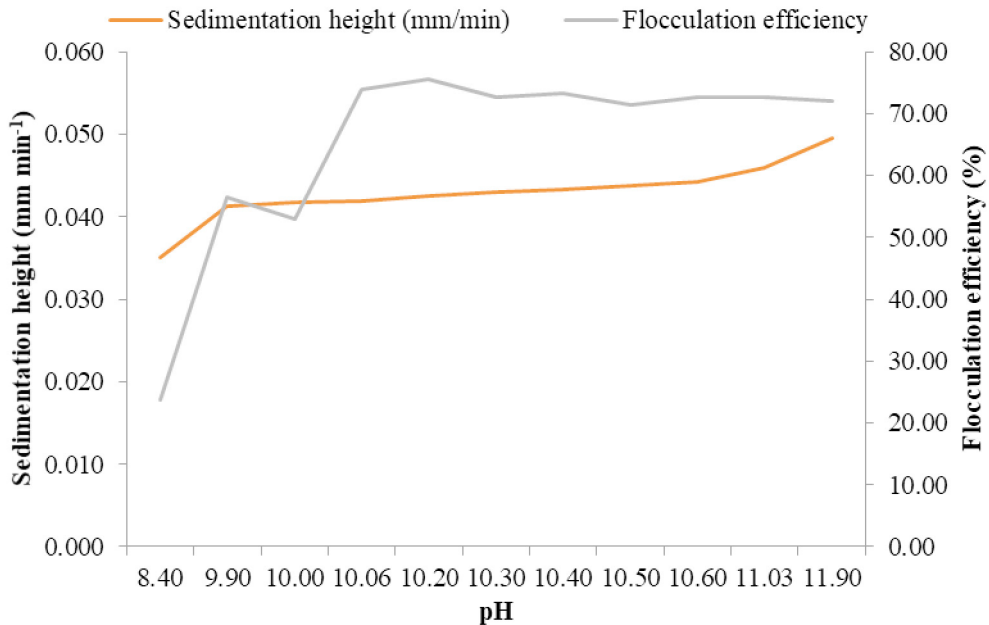
chemical flocculation of *Nannochloropsis oculata* with 0.8 g/lit $ZnSO_4$ will be an effective method for harvesting of large volume of microalgae culture with good harvesting efficiency.

Harvesting of *Nannochloropsis oculata* by Electro flocculation

It is based on the principle that the surface of microalgae is negatively charged and behaves as colloidal particles which can move in an electric field. Once they are attracted towards the anode, they are neutralised and form algal aggregates (flocs), which can be easily be collected. During electrolysis of water, H_2 and O_2 gas in the form of bubbles are produced in the electrodes and this will rise to the surface taking with them the algal aggregates (flocs) and forms a layer of microalgal cells.

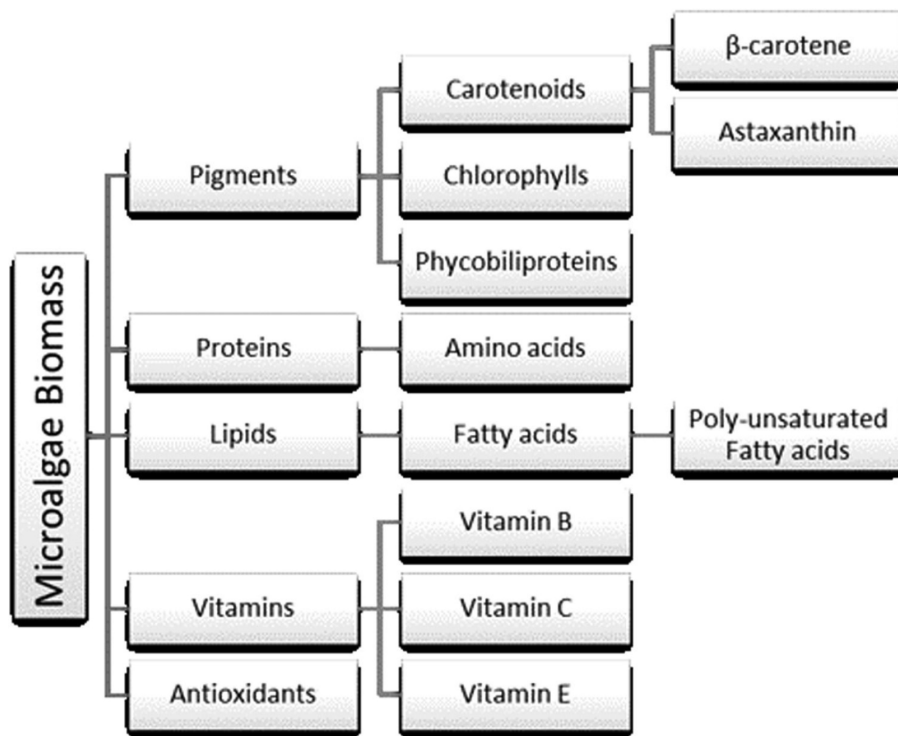


pH induced flocculation of *Chaetoceros calcitrans*: Flocculation of *Chaetoceros* culture with adjustment of pH using 5N NaOH concluded, that induced pH of 10.2 is optimum with better flocculation efficiency for the harvesting of *Chaetoceros calcitrans*. The induced pH for the flocculation study varied from 8.4 – 11.9.



Application of Microalgal concentrate in mariculture

From an aquaculture hatchery perspective, the key desired attributes for microalgal concentrates are high cell concentration without damage to cells, suitable nutritional composition; acceptable shelf life using standard cold storage methods; avoiding the use of preservatives that would be harmful to live prey; free from pathogens; avoidance of clumping and easy to suspend uniformly in water; regularly available and affordable.



Bio-refinery of component extraction from micro-algal biomass (A.K. Koyande *et al* 2019)

1) Microalgae concentrate for Zooplanktonic Live Prey:

Microalgae have an important role in aquaculture as a means of enriching zooplankton for feeding to fish and larvae. The predatory finfish larvae and decapod crustacean larvae is to feed with zooplanktonic live prey rather than formulated inert diets. This reflects the technological challenge and high costs of providing nutritionally balanced feeds in the correct physical form for small planktonic larvae, whose digestive capacity is only partially developed. The nutritional quality of rotifer and brine shrimp were improved by manipulating their diet particularly to enhance n-3 highly unsaturated fatty acids (HUFA, e.g., docosahexaenoic acid and eicosapentaenoic acid) by microalgal strain selection or by incorporating dried microalgal biomass into formulated inert diets. Hatchery production of rotifers was initially based on feeding with live microalgae (*Nannochloropsis* sp., *Tetraselmis* sp., *Pavlova lutheri* and *Isochrysis galbana*) or baker's yeast.

2) Microalgae concentrate in Fish Larval Rearing Tanks

In “green water” technique, microalgae and zooplankton are bloomed within large tanks, into which the fish larvae are stocked. Cultured microalgal strains can be inoculated into rearing tanks for this purpose provided the system water has been pre-treated to exclude competing microorganisms. The “pseudo-green water” rearing technique relies instead on regular addition of cultured microalgae to the fish larval rearing tanks, to replace that removed by live prey grazing and dilution. This approach is required to sustain the higher larval stocking densities that are typical in most commercial marine fish hatcheries. Commonly used microalgal strains for this purpose are *Nannochloropsis* sp., *Isochrysis* sp. and *Tetraselmis* sp. Substitutes for live microalgae in mariculture includes bacteria, yeast, dried microalgae, microalgal concentrates etc.

3) Microalgae in human and animal nutrition

Microalgae has been utilized as food by humans for thousands of years and supplementation with help of microalgae will release the stress on intense resource demanding terrestrial food crop. Some of these species are cultivated at industrial scale to extract bio-active compounds for human & animal consumption, cosmetics and bio-fuel industry. For a healthy lifestyle, a balanced diet constituting of antioxidants, vitamins, PUFAs, etc is required. Numerous species of microalgae are reported to be rich in proteins, carbohydrates, lipids and other bio-active compounds. According to Becker et al., microalgae are excellent sources of vitamins such as vitamin A, B1, B2, B6, B12, C and E and minerals such as potassium, iron, magnesium, calcium and iodine. Currently the microalgae derived foods are marketed as healthy foods and are available in industry as capsules, tablets, powders and liquids. They are also mixed with candies, gums, snacks, pastes, noodles, breakfast cereals, wine and other beverages. The microalgae species widely used include *Spirulina plantesis*, *Chlorella* sp., *Dunaliella terticola*, *Dunaliella saline* and *Aphanizomenon flosaquae* due to their high protein content and nutritional value. Similar to human supplementation, microalgae is also a source of food for many aquatic species, ruminants, pigs, poultry and other animals. The microalgal species of *Spirulina*, *Chlorella*, *Tetraselmis*, *Nannochloropsis*, *Nitzschia*, *Navicula*, *Scenedesmus*, *Cryptothecodinium*, and *Chaetoceros* are reported to be used as feed for terrestrial as well as aquatic animals

Chlorella species are often marketed as 'healthy foods' and are being promoted as a functional foods to prevent, cure or help common diseases or acute diseases like Alzheimer's disease, cancer, etc. *Chlorella* sp. are excellent hepatoprotective and hypocholesterolaemic agents during malnutrition and ethionine intoxication, they lower blood sugar concentration and increase haemoglobin concentration. *Chlorella* also contains an active immunostimulator—1,3-glucan, which reduces blood lipids and acts as a free radical scavenger. *Spirulina* sp., also known as "superfood", a label given by World Health organization (WHO), belongs to the blue-green photoautotrophic genus of unicellular microalgae. Its cells are protein rich with protein content reaching up to 70% of dry weight. It is also an excellent natural source of vitamin A, B1, B2 and B12, essential fatty acids and useful pigments such as xanthophyll and carotenoids. These bio-active components cannot be synthesized by humans, therefore, importance is given on production of *Spirulina* or *Arthrospira*.

4) Pigments (carotenoids, chlorophyll)

Pigments are used for variety of products including food additives/colorants, aquaculture, pharmaceutical and nutraceutical products. There is rising demand for naturally produced pigments due to the safety and environmental concerns associated with synthetically produced pigments. Microalgae are photosynthetic species that produce various pigments depending on the species and their corresponding colours. For example, green microalgae contains chlorophyll, red and blue microalgae possess phycobiliproteins and yellow, orange and red microalgae synthesize carotenoids. Among the various sources, microalgae are superior source of pigments due to their ability to synthesize natural pigments in higher concentration compared to others. Carotenoids are isoprenoid structured lipophilic pigments which are found in non-photosynthetic organisms, microalgae and higher plants. These pigments have a therapeutic effect on humans and animals due to their strong anti-oxidant properties therefore protecting the organisms from oxidative and free-radical stresses. Astaxanthin is the second most important carotenoid pigment and is mostly extracted from *Haematococcus pluvialis*, a freshwater green microalgae. Apart from *H. pluvialis*, *Chlorella zofingiensis* also produces Astaxanthin. Consumption of food rich in astaxanthin is promoted as a beneficial dietary supplement as its antioxidant strength is 100 times that of tocopherol. Chlorophyll contains chlorophyllin (CHL), a sodium-copper-salt water-

soluble derivative, which is readily absorbed by human body. Nagini et al., reported that addition of CHL in diet, has a potential to inhibit the progression of cancer as it targets multiple pathways of carcinogens and invades their cell cycle. Phycocyanin, a fluorescent blue-coloured phycobiliprotein, is extracted from *Spirulina* sp. as a natural dye. It is also used in popsicles, chewing gum, confectionery, wasabi, dairy products and soft drinks.

5) Proteins and amino acids

Microalgae is an excellent source of EAAs. *Chlorella* and *Spirulina* species are reported to constitute around 70% protein with respect to its mass. According to WHO/FAO/UNU recommendations, microalgae such as *Chlorella* sp. and *Spirulina* sp. contain well balanced EAA content required for human consumption. Microalgae is identified as an alternative high-protein source which can meet the requirements of malnourished population. In recent years, microalgae derived proteins have been incorporated in biscuits, sweets, bread, noodles, drinks and beer. The amino acid content in some microalgae are also found to be comparable to that of high protein content sources. Amino acids such as isoleucine, valine, lysine, tryptophan, methionine, threonine and histidine were found to be present in microalgae in quantities that are comparable or larger compared to protein-rich sources such as eggs and soybean.

6) Vitamins

Vitamins play a major part in energy metabolism of humans. Algal food products are rich in vitamins. *Dunaliella tertiolecta* was able to synthesize vitamin B12 (cobalamin), vitamin B2 (riboflavin), vitamin E (tocopherol) and provitamin a (carotene). Moreover, *Tetraselmis suecica* was an excellent source of vitamin B1 (thiamin), vitamin B3 (nicotinic acid), vitamin B5 (thnic acid), vitamin B6 (pyridoxine) and vitamin C (ascorbic acid). *Chlorella* spp. are reported to contain Vitamin B7 (biotin) in high concentration.

7) Antioxidants

Human body continuously produces free radicals or reactive oxygen species (ROS) as a result of external stresses like smoking, chewing tobacco, excessive exposure to sunlight. Microalgal biomass is considered to be superior source of nutritional anti-oxidants due to its higher production capacity compared to

conventional plant-derived sources. It is also reported that antioxidant such as astaxanthin extracted from microalgae have greater antioxidant activity compared to carotene, lycopene, lutein and Vitamin E.

Conclusion

Microalgal derived food and nutraceutical products possess a huge potential to decelerate the rate of malnourishment in developing nations. The abundance of proteins and other essential nutrients in microalgae can develop a massive algae based food industry, dedicated towards commercialization of healthy and functional foods. Apart from complete EAA profile, microalgae contain various bio-active components which act as anticarcinogenic, antioxidative, antihypertensive and hepatoprotective agents. Moreover, microalgal derived biomass is utilized for other purposes like production of biofuels and feeds for animals, poultry and fishes. The utilization of microalgae-derived products have been growing exponentially in recent decades.

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