Perumal Santhanam · Ajima Begum
Perumal Pachiappan *Editors*

Basic and Applied Phytoplankton Biology

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Editors
Perumal Santhanam
Marine Planktonology & Aquaculture Laboratory, Department of Marine Science
School of Marine Sciences, Bharathidasan University
Tiruchirappalli, Tamil Nadu, India

Ajima Begum
Department of Botany
Tihu College
Tihu, Assam, India

Perumal Pachiappan
Department of Biotechnology, School of Biosciences
Periyar University
Salem, Tamil Nadu, India

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Preface

Phytoplankton (in Greek: phytos-plants; plankton-wanderer/drifter) are the microscopic drifting organisms found in the surface and subsurface layers of aquatic environs like seas, oceans, lakes, rivers, and ponds. They are the pigmented autotrophs initiating the food chain of any aquatic ecosystems and are the primary producers upon which the primary consumers, zooplankton depend. The major types of phytoplankton are diatoms, dinoflagellates, cyanobacteria, green algae, and coccolithophores, and they are popularly known as "the grasses of the sea." And these chlorophyll-containing autotrophs perform photosynthesis using CO_2 and water to generate food for themselves, while giving out O_2 as a by-product. This reduces CO_2 in the water and allows water to take in more CO_2 in the atmosphere and thus help in CO_2 sequestration. While part of the synthesized carbon is transferred to higher levels of aquatic environs, the rest goes down the bottom after the phytoplankton death. In this way, phytoplankton help to transfer tens of gigatons of carbon to the deep oceans annually and keep the climate system in check.

A number of organisms like copepods, sea jelly, and even larger creatures like whales feed on the phytoplankton, which underlines their importance for the survival of major aquatic food chains. The other side of these plankton is the creation of anoxic condition and release of toxic compounds, after the formation of harmful algal blooms (HABs) and their death. These HABs reportedly cause serious setback to other inhabitants of the marine environment and also cause planktonic shellfish poisoning, diarrhetic shellfish poisoning, etc. to human beings who consume the contaminated fishes/seafood. The seafood and tourism industries incur the annual loss of US$ 82 million due to HABs.

On the other hand, phytoplankton provide immense scope for commercial exploitation through their mass cultivation and conversion of the harvested biomass into value-added products. Through phytoplankton biotechnology, a wide variety of applications are possible in the fields of nutraceuticals, cosmeceuticals, aquaculture feeds, agricultural fertilizers, fine chemicals, secondary metabolites/molecules, biodiesel, wastewater, and in food processing sectors. One such important development is the addition of phytoplankton-based PUFA oils to the infant feed formula as nutrient supplements. So far, only modest achievement has been made through

phytoplankton biotechnology, and in future, technology has to be developed towards large-scale production of biomass through genetic modification (of plankton strains) and to market desirable metabolites.

As such this book provides comprehensive information pertaining to the ecological, experimental, and applied aspects of marine phytoplankton to the wide array of readers and researchers. Methods on the collection, identification (through morphological and molecular methods), and preservation of phytoplankton besides culture and their use in aquafeed formulation and also in wastewater treatment have been highlighted here. The book is specifically intended for both graduate and postgraduate students and researchers in the fields of Marine Biology/Aquatic Biotechnology/Zoology. The editors are thankful to the authors and publishers.

Tiruchirappalli, India Perumal Santhanam
Tihu, India Ajima Begum
Salem, India Perumal Pachiappan

Contents

An Introduction to Plankton 1
P. Pachiappan, P. Santhanam, A. Begum, and B. Balaji Prasath

Methods of Collection, Preservation and Taxonomic Identification of Marine Phytoplankton 25
P. Santhanam, P. Pachiappan, and A. Begum

Study on Molecular Taxonomy and Phylogenetic Analysis of Phytoplankton 63
S. Jeyapandi, C. Rajthilak, P. Santhanam, A. Begum, S. Ravikumar, P. Pachiappan, and S. U. Ahmed

Replacement of Fishmeal with *Arthrospira* (*Spirulina*) *platensis* and Its Use in Freshwater Prawn *Macrobrachium rosenbergii* Production 77
T. Muralisankar, P. Santhanam, S. Radhakrishnan, N. Manickam, and P. Saravana Bhavan

A Method of Bio-efficacy Potential of Microalgae (Phytoplankton) for the Control of Vector Mosquitoes 109
S. Balakrishnan, P. Santhanam, N. Manickam, and M. Srinivasan

Isolation, Culture, and Application of Marine Microalga *Dunaliella salina* (Volvocales, Chlorophyceae) as an Aqua Feed Additive 123
A. Shenbaga Devi, P. Santhanam, S. Jeyanthi, and N. Krishnaveni

Strain Selection and Lipid Characterization of Marine Diatoms with Potential for Biodiesel Production 163
S. Jeyanthi and P. Santhanam

Bioremediation of Wastewater Using a Novel Method of Microalgae Immobilized on Twin-Layer Recirculation System (TLRS) 177
M. Divya and P. Santhanam

The Techniques in Microalgae Bioremediation and Algal Co-product Development 191
S. Dinesh Kumar, P. Santhanam, and F. Leena Grace Nancy

Detection of Cyanotoxins of Cyanobacterial (*Microcystis aeruginosa*) Strain Using Microtox® Bioluminescence Bioassay 211
B. Balaji Prasath, P. Santhanam, R. Nandakumar, and T. Jayalakshmi

A Method of Analysis of Pigments in Phytoplankton 221
N. Krishnaveni, A. Shenbaga Devi, and P. Santhanam

Surface Bioengineering of Diatom by Amine and Phosphate Groups for Efficient Drug Delivery 229
R. Sasirekha and P. Santhanam

A Study on the Impact of Acidification on the Morphometry, Photosynthesis, and Biochemical Composition of Phytoplankton 239
P. Bharatha Rathna and P. Santhanam

Distribution of Phytoplankton in Selected Salt Pans of Tamil Nadu, Southeast Coast of India 251
A. Shenbaga Devi, P. Santhanam, S. Ananth, and S. Dinesh Kumar

A Study of Carbon Sequestration by Phytoplankton 277
M. Divya, S. Dinesh Kumar, N. Krishnaveni, and P. Santhanam

Mass Scale Culture and Preparation of Microalgal Paste 285
A. Begum, S. U. Ahmed, S. Dinesh Kumar, M. Divya, P. Santhanam, and P. Pachiappan

An Estimation of Antimicrobial and Antioxidant Activity of Microalgae 303
C. Premkumar, M. Divya, N. Krishnaveni, P. Santhanam, and P. Pachiappan

A Method of Extraction, Purification, Characterization, and Application of Bioactive Compounds from Phytoplankton 315
C. Premkumar, S. Dinesh Kumar, S. Ananth, and P. Santhanam

Potential Harmful Microalgae in Muttukadu Backwater, Southeast Coast of India 323
B. Balaji Prasath, P. Santhanam, R. Nandakumar, and T. Jayalakshmi

About the Editors

Dr. P. Santhanam is an assistant professor at the Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India. He obtained his M.Sc, M. Phil and Ph.D Degrees in Marine Biology, from the C.A.S. in Marine Biology, Annamalai University. He has over 19 years of research and 11 years of teaching experience with over 130 publications in national and internationally respected journals, books and proceedings. He specializes in the areas of marine planktonology & aquaculture with reference to biodiversity, taxonomy, biology and culture of phyto and zooplankton for fish larviculture. He has successfully completed 5 major research projects funded by UGC, DST, DBT & SERB (Govt. of India) and is currently involved in 2 major research projects funded by the UGC & MoEF& CC (Govt. of India). He is a member in several scientific bodies and has served as an editorial board member for over 5 journals and as a reviewer for over 25 leading national and international journals. He received the DST Young Scientist Award in 2007, KTSTM Trust Best Researcher Award, and the Prof. Dr. M. Aruchami Award from Kongunadu Arts & Science College & Dr. M. Aruchami Research Foundation for his extraordinary contribution to Indian live-feed aquaculture, especially copepod. He has been expert-member of various committees.

Mrs. Ajima Begum is currently an assistant professor at the Department of Botany, Tihu College, Tihu, Nalbari, Assam, India. Before joining in Tihu College, she worked as an assistant professor at B. N. College, Dhubri, Assam. She also served as a research investigator at NIRD-NERC, in the project entitled "Training-cum-Documentation for Establishing Rural Industries of Medicinal and Aromatic plants in North Eastern Region". She has published over 10 research articles in leading national and international journals and participated in more than 6 national and international level trainings, conferences and seminars.

Dr. P. Pachiappan is a professor & head of the Department of Biotechnology, Periyar University, Salem, Tamil Nadu, India. Prior to his appointment at Periyar University, he was a faculty research associate, lecturer, reader and professor at the

C.A.S. in Marine Biology, Annamalai University, where he received his Ph. D., Degree in 1989. He is also passed the UGC-National Educational Test in 1985. He has over 30 years of research experience with about 150 publications to his credit. His research interests include molecular taxonomy and live-feed aspects of plankton, fish larviculture and antibiotic principles/drugs from marine bioresources. He has successfully completed many major research projects funded by ICAR, MoES, DRDO, UGC (Govt. of India) and is currently co-ordinating the DST-FIST programme. He is a member of the Academic Council, Senate, Board of Research Studies, chairman of PG-Board of Studies in Biotechnology & Bioinformatics (of Periyar University) and a member of the Board of Studies at various colleges and universities. He was the chairman of the Periyar University Institutional Animals Ethical Committee & co-ordinator of the Periyar University Patent Facilitation Centre & Industrial MOU, an expert-member of the European Commission (EC) Projects Review committee, scientist member of Govt. Mohan Kumaramangalam Medical College Institutional Animal Ethics committee and Scientific Advisory committees. He is also a reviewer/editor for several national & international journals.

An Introduction to Plankton

P. Pachiappan, P. Santhanam, A. Begum, and B. Balaji Prasath

Introduction

The word "plankton" is derived from the Greek word for drifting. Plankton are frequently described as organisms that drift on or near the surface of the water and are unable to swim sufficiently strongly to move toward tides, winds, or currents. This description is not strictly true, in that many planktonic organisms, even very small individuals, can propel themselves for very long distances in water columns in very short periods of time. Many planktonic organisms are single-celled plants, called phytoplankton, while others are single-celled animals, known as zooplankton. However, a few organisms referred to as plankton are the embryonic or juvenile forms of larger organisms, including fish and invertebrates. Planktonic organisms are inhabited in nearly all aquatic ecosystems and play a crucial role in aquatic food webs. The aquatic ecosystem including freshwater and marine environs forms a significant feature of our planet and provides livelihood to most of life on the earth. Millions of species have been discovered from the aquatic ecosystem and many

P. Pachiappan
Department of Biotechnology, School of Biosciences, Periyar University,
Salem, Tamil Nadu, India

P. Santhanam (✉)
Marine Planktonology & Aquaculture Laboratory, Department of Marine Science,
School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

A. Begum
Department of Botany, Tihu College, Tihu, Assam, India

B. Balaji Prasath
Marine Planktonology & Aquaculture Laboratory, Department of Marine Science,
School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

Beijing Normal University-Hong Kong Baptist University, United International College,
Zhuhai, Guangdong, China

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new species are still being explored daily. Of all plankton is considered to be an important biological component, which can act as primary factor of the aquatic food web. Plankton are microscopic organisms that drift on the water. They include organisms such as diatoms, dinoflagellates, krill, and copepods as well as the microscopic larvae of fin fish and shell fish. Plankton also include tiny photosynthetic organisms that are innumerable and capable of generating more oxygen than all other plants on Earth combined. Plankton serve as primary (phytoplankton) and secondary (zooplankton) producers and predominates the entire aquatic biodiversity. Plankton have paramount ecological significance as they form pelagic food links and serve as indicators of water masses. They constitute the largest and reliable source of protein in the rivers, lakes, and ocean for most of the fishes. Being a primary producer, its absence or reduction often affects fishery. It has been observed in many countries that the downfall of fishery has been attributed to reduced plankton population. Furthermore, trillions of little copepods produce countless fecal pellets contributing greatly to the aquatic snow and therefore accelerating the flow of nutrients and minerals from the surface waters to the bottom of the rivers and seas. They are phylogenetically highly successful groups as regards to phylogenetic age, number of living species, and success of adaptive radiation. The study on the taxonomy, ecology, biology, and biochemistry of plankton would give an index of the fishery potential in aquatic environment. This becomes important not only to know the facts about the aquatic ecology, but also to ensure society's long-term welfare directly or indirectly by providing food and livelihood for the millions of people living in inland and coastal areas. For this reason, we need to understand all the facets of plankton as well as their interactions with atmosphere and other life forms, including human. This chapter reveals the ecological and economical values of phytoplankton and zooplankton especially their biotechnological and industrial applications.

Classification of Plankton

Based on its protein content, plankton can be classified as phytoplankton (plant plankton) and zooplankton (animal plankton). Phytoplankton is categorized into five groups according to their cell structure and cell-wall arrangements; these groups are diatoms, dinoflagellates, blue-green algae, green algae, and coccolithophores. Zooplankton is categorized according to habitat, depth-wise distribution, size, and lifecycle (Omori and Ikeda 1984). In regard to habitat, zooplankton is classified as marine plankton, or haliplankton, and as freshwater plankton, or limnoplankton.

The classification of plankton according to habitat is shown in Table 1.

Zooplankton is classified according to depth-wise distribution (Table 2), size (Table 3), and life cycle (see below).

Zooplankton are classified into two types based on life cycle; namely, holoplankton and meroplankton.

Table 1 Classification of plankton according to habitat

Oceanic plankton	Plankters inhabiting waters beyond continental shelves
Neritic plankton	Plankters inhabiting waters overlying continental shelves
Brackish water plankton	Plankters inhabiting brackish water areas, such as estuaries, mangrove swamps, and lagoons
Limnoplankton	Plankters inhabiting freshwaters such as rivers, lakes, ponds, streams, canals, and valleys

Table 2 Classification of zooplankton according to depth-wise distribution

Pleuston	Living on the surface of the aquatic ecosystem
Neuston	Living within tens of mm of the surface
Epipelagic plankton	Living between 0 and 300 m
-Upper epiplankton	Living between 0 and 150 m
-Lower epiplankton	Living between 150 and 300 m
Mesopelagic plankton	Living between 300 and 1000 m
-Upper mesoplankton	Living between 300 and 700 m
-Lower mesoplankton	Living between 700 and 1000 m
Bathypelagic plankton	Living between 1000 and 3000 m
Abyssopelagic plankton	Living between 3000 and 4000 m
Epibenthic plankton	Living at the bottom of the aquatic ecosystem

Table 3 Classification of zooplankton according to size

Femtoplankton	0.02–0.2 μm (e.g., Marine viruses)
Picoplankton	0.2–2.0 μm (e.g., Chrysophyta)
Nannoplankton	2–20 μm (e.g., Diatoms, Dinoflagellates)
Microzooplankton	20 μm –200 μm (e.g., Rotifers, Copepod nauplii)
Mesozooplankton	200 μm-2 mm (e.g., Copepods, Cladocera, Chaetognatha)
Macrozooplankton	2-20 mm (e.g., Ctenophores, Salps, Doliolids)
Megalozooplankton	>20 mm (e.g., Sea jellies)

Holoplankton are organisms that are planktonic throughout their life cycle; this category includes tintinnids, copepods, chaetognaths, and cladocerans. Meroplankton are organisms that act as plankton for only part of their life (e.g., fish larvae and veliger larvae).

Ecological and Economic Importance of Plankton

Plankton has ecological and economic importance in various spheres. It is well known that phytoplankon is a primary producer in many aquatic systems. Even higher trophic organisms such as dolphins, whales, and sharks, as well as sessile

forms like benthic oysters, are dependent on phytoplankton for their main food. Almost all commercially important finfish and crustacean larvae depend mainly on phytoplankton for their initial feeding. In Indian waters, the presence of the diatoms *Fragilaria oceanica* and *Hemidiscus hardmanianus* indicates an abundance of clupeid fish and oil sardines. In British waters, an abundance of coccolithophores serves as a good indicator of the presence of herring. The diatom *Fragilaria antarctica* is known to indicate abundance of the Antarctic krill, *Euphausia superba*. Some phytoplankters, such as *Dunaliella tertiolecta* and *Phaeodactylum tricornutum*, serve as indicators of metal, especially copper, pollution. Some diatoms also serve as indicators of rich petroleum deposits on the sea bed. Diatoms are good filters for the filtration of fruit juice, syrup, and varnish, and they can also act as insulators in boilers, electric ovens, and refrigerators. Dinoflagellates emit light and this helps fishermen to identify certain fish shoals at night time. Researchers have reported that some kinds of bioactive compounds can be derived from phytoplankton. Phytoplankton is considered to be a rich source of biofuel, animal feed, human food, and biofertilizers. It is evident that phytoplankton removes carbon more efficiently than terrestrial plants and thereby helps in the control of global warming. The diatoms in phytoplankton produce an amorphous nanostructured silica skeleton known as a frustule. The insertion of different metallic oxides, including titanium and germanium dioxide, into the nanostructure of the diatom frustule could doubtlessly be applied to the manufacture of new dye-sensitized solar cells, nanostructured battery electrodes, and electroluminescent devices. The exploitation of diatom nanobiotechnology for the improvement of novel devices and for nanostructures is now an emerging area of research.

Similarly to phytoplankton, zooplankton serves as primary consumers in aquatic ecosystems, as well as secondary producers. Zooplankton can be used as live-feed for carnivorous finfish and shellfish. Zooplanktonic organisms such as Mysids and *Euchaeta norvegica* are directly consumed as food in the West Indies. Zooplankters such as *Lucifer* spp. and *Sagitta* spp. serve as bioindicators of water masses, especially mixed and unmixed waters. The presence of the calanoid copepod, *Calanus* spp. indicates rich herring shoals. Abundance of the krill *E. superba* helps fishermen in locating baleen whales, seals, and squid. Zooplankters such as those present in foraminiferal ooze are used as thermal insulators and chromatographic column filters. Marine copepods produce fecal pellets that contribute to the formation of marine snow, thereby accelerating the flow of nutrients and minerals from the surface to the bottom of the sea. It is interesting to note that some zooplankters are a potential source of bioactive compounds that emit light in the aquatic environment. With more scientific research being carried out on plankton, there could be discoveries about what plankton can do to enhance our lives and our health. It is incredible to consider how these small microorganisms can contribute to many distinctive fields, and how they could improve people's lives in terms of new clinical remedies. In time, plankton may be recognized as one of the most essential organisms that exist on this planet. Recently, a private Norwegian company developed a new bio-industry on the marine copepod *Calanus finmarchicus*, which is the largest renewable and harvestable resource in the North-Atlantic

Ocean, producing copepod oil with focus on human health and nutrition products including a novel marine oil with unique properties (third-generation omega-3). The main products, namely, Calanus® Oil and the hydrolyzed protein Calanus® Hydrolysate were launched in Norway and the USA (Arctic Ruby® Oil) in late 2012, with good response. The company is also aiming other products/markets such as protein ingredients for food, starter feed for aquaculture, and pet-food. The main product is a marine oil with favorable biomedical properties, which prevent metabolic changes due to food styles and counteract the development of obesity-related disorders. It is demonstrated that copepod (Calanus) oil is safe, digested, and bioavailable for human. Now the copepod oil has become a novel food in Europe and USA (Kurt Tande, Co-Founder/CTO, Calanus, Norway). This article reviews the biotechnological and industrial applications of phytoplankton and zooplankton.

Biotechnological Applications of Phytoplankton

Phytoplankton biotechnology includes the industrial exploitation of these organisms via mass cultivation, and conversion of the harvested biomass into value-added products; this is an unexpected growth enterprise worldwide. The industries involved in the biotechnological applications of phytoplankton include nutraceutical producers; cosmeceutical manufacturers; feed manufacturers (for aquaculture); fertilizer manufacturers (for agriculture); fine chemicals manufacturers; natural products manufacturers; biodiesel producers; municipal wastewater remedy corporations; and processors of meals using nutrient-enriched wastewaters. Phytoplankton biotechnology has advanced from the mid-twentieth century, and nowadays there are numerous commercial applications of phytoplankton are identified, as outlined below. Phytoplankton products may be regarded as a supply of fairly valuable molecules. From a business point of view, the purpose of phytoplankton biotechnology is to develop marketable products, and thus the first steps that need to be taken are to identify an acceptable metabolite and a phytoplankton that produces and accumulates that desired metabolite. For example, polyunsaturated fatty acids (PUFAs) can be used as dietary supplements and phytoplankton pigments can be used in herbal dyes. In future we will need to focus on the improvement of manufacturing structures and the genetic modification of phytoplankton strains. To date, there are few large-scale production processes for the preferred metabolites and the marketing of these metabolites, and the commercial achievements of phytoplankton biotechnology have been modest. Phytoplankters that produce many marketable products are now being recognized. High-throughput screening has identified many potential products, but successes in large-scale production and product advertising have been few. Phytoplankton biotechnologists are satisfied with the capacity of phytoplankton biotechnology, but we have little fulfillment to justify our optimism. Looking ahead, however, it seems that phytoplankton biotechnology will have various applications. Phytoplankton species and their present applications are shown in Table 4.

Table 4 Various biotechnological applications of phytoplankton

Species	Application areas	Product	References
Spirulina	Health food, cosmetics	Phycocyanin, biomass	Lee (2001) and Costa et al. (2003)
Chlorella vulgaris	Health food, food supplement, feed	Biomass	Lee (2001)
Dunaliella salina	Health food, food supplement, feed	Carotenoids, β-carotene	Jin and Melis (2003) and Del Campo et al. (2007)
Muriellopsis sp.	Health food, food supplement, feed	Carotenoids, lutein	Blanco et al. (2007) and Del Campo et al. (2007)
Haematococcus pluvialis	Pharmaceuticals, cosmetics, baby food	Carotenoids, astaxanthin	Del Campo et al. (2007)
Odontella aurita	Pharmaceuticals, cosmetics, baby food	Fatty acids	Pulz and Gross (2004)
Porphyridium cruentum	Pharmaceuticals, cosmetics, nutrition	Polysaccharides	Fuentes et al. (1999)
Phaeodactylum tricornutum	Nutrition, fuel production	Lipids, fatty acids	Yongmanitchai and Ward (1991) and Aciẽn Fernández et al. (2003)
Lyngbya majuscula	Pharmaceuticals, nutrition	Immune modulators	Singh et al. (2005)
Odontella sp.	Pharmaceuticals	EPA (eicosapentaenoic acid)	–
Euglenida	Pharmaceuticals	Paramylon starch (β-1,3-polyglucan)	Ragan and Chapman (1978)

Phytoplankters are extraordinary green solar power converters and they are able to produce high-quality metabolites. This potential and their ubiquitous distribution has led to their exploitation for a variety of functions (Borowitzka and Borowitzka 1988; Boussiba et al. 1992; Wilde and Benemann 1993; Johnson and Schroeder 1995; Borowitzka 1996; Dixon et al. 1997; Vilchez et al. 1997). These authors have appraised strategies for the cultivation of phytoplankton and potential industrial programs for exploiting phytoplankton biotechnology. The main biotechnological applications of phytoplankton are outlined in the following section.

Phytoplankton for Human Nutrition

Regardless of the reality that the biotechnological or genetic-technological exploitation of phytoplankton is, and could be, hampered by protective guidelines for human intake, there are already numerous suitable fields of application. The phytoplankton market is dominated by Chlorella and Spirulina (Becker 2004; Pulz and Gross 2004), particularly because of their high protein, carbohydrate, and lipid contents and their nutritive value, and their market may be easy to develop. The composition of different phytoplankton sources used in human and animal nutrition (as a percentage of dry matter), according to Becker (2004), is shown in Table 5.

Table 5 Nutritional characterization of various phytoplankton species

Microalgae	Protein (%)	Carbohydrate (%)	Lipid (%)
Anabaena cylindrica	43–56	25–30	4–7
Chlorella vulgaris	51–58	12–17	14–22
Dunaliella salina	57	32	6
Spirulina maxima	60–71	13–16	6–7
Synechococcus sp.	63	15	11

The documented bioactive properties of phytoplankton have led to an advanced marketplace for its dried biomass as a human dietary complement. In recent times, phytoplankton products have been marketed as drugs. They can also be integrated into cool beverages, snack meals, candy bars, gums, and drinks (Yamaguchi 1997). Owing to their various chemical properties, they act as dietary supplements and as natural food colorants (Borowitzka 1999; Soletto et al. 2005). The economic use of phytoplankton is dominated by four strains, i.e., *Arthrospira, Chlorella, D. salina*, and *Aphanizomenon flos-aquae*. In processed food products, chlorophyll is used as a natural pigment (Humphrey 2004). Owing to its strong green pigment and the consumer demand for herbal ingredients, chlorophyll is gaining prominence as a food additive. This, in turn, encourages food processors to change from using synthetic pigments to chlorophyll-based natural coloring, but a downstream process is required to purify chlorophyll 'a' and 'b' from phytoplankton. Spirulina, a blue green alga, is still used in food and dietary supplements because of its outstanding nutrient content and digestibility (Kumar et al. 2005). Spirulina contains a high amount of protein (60–70 wt%) and is a rich source of nutrients, in particular vitamin B12 and provitamin A (β-carotene) and minerals (Thajuddin et al. 2005). A few manufacturers of human dietary products originating from Spirulina are the Myanmar *Spirulina* Manufacturing Unit (Yangon, Myanmar), which sells capsules, chips, pasta, and liquid extract; and the Cyanotech Corporation (Kona, Hawaii, United States), which produces merchandise ranging from pure powder to packaged bottles of Spirulina extract, using the name Spirulina pacifica. Cyanotech Corporation has evolved a method for drying the biomass to avoid the oxidation of carotenes and fatty acids, which happens when the usual drying method is used. Proteins consist of various amino acids and therefore the nutritional quality of a protein essentially depends on the content, proportion, and availability of its amino acids. Amino acid profiles of various phytoplankton species and a reference pattern of a nicely balanced protein, endorsed by the World Health Organization (WHO)/Food and Agricultural Organization (FAO) (1973), are shown in Table 6).

Long-chain PUFAs with more than 18 carbon atoms cannot be synthesized by higher plant life or by animals, only by phytoplankton, which materials entire food chains with fatty acids (Pulz and Gross 2004). PUFAs, especially docosahexaenoic acid (DHA), in addition to the vital fatty acids in human breast milk, are critical for infant development, being crucial building blocks in brain and retinal development and in ongoing visual and cognitive development (Table 7).

Table 6 Amino acid profiles of different phytoplankton species (g per 100 g protein)

Source	Ile	Leu	Val	Lys	Phe	Tyr	Met	Cys	Try
Chlorella vulgaris	3.8	8.8	5.5	8.4	3.4	3.4	2.2	1.4	2.1
Dunaliella bardawil	4.2	11.0	5.8	7.0	5.8	3.7	2.3	1.2	0.7
Scenedesmus obliquus	3.6	7.3	6.0	5.6	4.8	3.2	1.5	0.6	0.3
Arthrospira maxima	6.0	8.0	6.5	4.6	4.9	3.9	1.4	0.4	1.4
Spirulina platensis	6.7	9.8	7.1	4.8	5.3	5.3	2.5	0.9	0.3
Aphanizomenon sp.	2.9	5.2	3.2	2.5	2.5	–	0.7	0.2	0.7

Note: Ile-Isoleucine; Leu-Leucine; Val-Valine; Lys-Lysine; Phe-Phenylalanine; Tyr- Tyrosine; Met- Methionine; Cys-Cystine; Try- Tryptophan

Table 7 Fatty acid profiles of different phytoplankton species

Source	Fatty acid	Reference
Arthrospira sp.	GLA (18:3)	Bandarra et al. (2003)
Porphyridium sp.	AA (20:4)	Donato et al. (2003)
Nitzschia sp., *Isochrysis* sp.	EPA (20:5)	Chini Zittelli et al. (1999)
Crypthecodinium sp., *Schizochytrim* sp.	DHA (22:6)	Molina Grimaa et al. (2003)
Dunaliella sp.	ALA (18:3)	Shenbaga Devi (2010)
Nannochloropsis sp.	Palmitic acid (16:0)	Shenbaga Devi (2010)
Chlorella marina	Palmitic acid (16:0)	Rekha et al. (2012)
Skeletonema costatum	EPA (20:5)	Rekha et al. (2012)

Note: GLA Gamma-linolenic acid, AA Arachidonic acid, EPA Eicosapentaenoic acid, DHA Docosahexaenoic acid, ALA α-Linolenic acid

Phytoplankton in Animal Nutrition

Thorough nutritional and toxicological investigations have validated the suitability of algal biomass for use as a high-grade feed supplement or as a replacement of traditional animal feed sources in aquaculture and farm animals (Dhargalkar et al. 2009). Phytoplankton produces valuable compounds that include crucial amino acids, pigments, and nutrients, in addition to other bioactive molecules. The most important pigments include chlorophyll a, b, and c; ß-carotene; phycocyanin; xanthophylls (astaxanthin, canthaxanthin, lutein); and phycoerythrin. These pigments are used in human food, animal feed, prescription drugs, and cosmetics, and there may be a growing demand for their use as natural colorants in textiles and as printing dyes. The cost of these pigments no longer depends only on their colorant properties, as they are also antioxidants with established health advantages. Phytoplankton is now used as feed in aquaculture for the production of bivalve molluscs, juvenile

abalone, crustaceans, and finfish species and as feed for the zooplankton that is employed in aquaculture food chains. The first aquaculture areas appeared in the 1970s (Pulz 1998), and phytoplankton is now ubiquitous as a feed supply in hatcheries for juvenile marine fish and shellfish. There are many marine hatcheries globally, generating billions of juvenile fish and shellfish annually. Of note, Tilapia fish showed a greater increase in weight and greater protein efficiency ratio when phytoplankton was used in the feed (Azaza et al. 2008). The marine cyanobacteria, *Phormidium valderianum*, was successfully used as feed for aquaculture, primarily based on their nutritional value and risk-free effects (Thajuddin et al. 2005). Dried phytoplankton biomass is also used extensively in formulating feeds for aquaculture species and terrestrial animals (farmed livestock, chickens, and personal pets), in which it has been validated to have healthy effects. The capacity of *Arthrospira* (Spirulina) as a factor in animal feed was studied by Belay et al. (1996). Another example of the use of phytoplankton in aquaculture is a conventional French approach called the greening of oysters. This includes using the diatom *Haslea ostrearia* to obtain a blue coloration on the gills and labial palp of oysters. This increased the product's market price by 40% (Muller-Feuga 2000). A further example of the use of phytoplankton is a herbal animal feed made with the algae *Chlorella* and *Arthrospira*, produced by the Institut für Getreideverarbeitung (Bergholz-Rehbrücke, Germany) and referred to as Algrow.

Phytoplankton in Molecular Farming

The concept of molecular farming, also known as gene pharming, in phytoplankton is to generate biomolecules that are essential for the manufacture of medicinal drugs that might be difficult or even not possible to generate in any other way, or that entail prohibitively high manufacturing costs in other systems. Phytoplankters have proven to be suitable for synthesizing vaccines. In this regard, strong expression of the hepatitis B surface antigen gene has been shown in *Dunaliella salina* (Sayre et al. 2001; Geng et al. 2003; Sun et al. 2003). As *Dunaliella* is also used for nutrients, there is no need for purification of the antigen, so the intact phytoplankton can be used to deliver a vaccine. Phytoplankters have also been shown to be beneficial for expressing insecticidal proteins; the green alga *Chlorella* is one feasible food for mosquito larvae, and the mosquito hormone trypsin-modulating ostatic factor (TMOF) was heterologously expressed in *Chlorella*. TMOF terminates trypsin biosynthesis in the mosquito intestine. After mosquito larvae were fed with recombinant Chlorella cells, the larvae died in 72 h (Borovsky 2003). In tropical regions, malaria, dengue fever, and West Nile fever are transmitted via mosquitoes, and mosquito abatement is expensive in these regions. The use of a transgenic phytoplankton is probably a far cheaper alternative.

Phytoplankton in Cosmetics Manufacturing

Phytoplankton is used in skin protection and hair care products, with phytoplankton extracts being used in skin care products (e.g., anti-aging creams, cleansing and regenerative care products, emollients, and anti-irritants in exfoliants). Phytoplankton additives are regularly used in cosmetics as thickening and hydrating agents, and as antioxidants. Cosmetics companies state that phytoplankton contents, such as carrageenan, other algal polysaccharides, algal proteins and lipids, and vitamin A, vitamin B1, as well as iron, phosphorus, sodium, copper, magnesium, calcium, and other elements, are beneficial for the skin. Some companies state that phytoplankton extracts inhibit the oxidative degeneration of collagen and hyaluronic acid and that these extracts have anti-aging properties. However, from a systematic point of view, these outcomes ought to be regarded as unsubstantiated and not scientifically validated. Some phytoplankton species have appeared in the cosmetics marketplace, with *Chlorella* and *Arthrospira* playing a major role in cosmetics. A few cosmetics organizations (e.g., the cosmetics arm of Louis Vuitton, Seoul, South Korea) have themselves invested in phytoplankton production. Phytoplankton has been investigated as a source of vitamins and diet precursors; namely, ascorbic acid; riboflavin; and α-, β-, and γ-tocopherol (Spolaore et al. 2006). Two examples of commercially available products and their properties are a protein-rich extract from *Arthrospira*, which repairs large pores and aging skin, exerting a tightening effect (Protulines; Exsymol S.A.M., Monaco) and an extract from *Chlorella vulgaris* that stimulates collagen synthesis in the skin, thereby assisting tissue regeneration and reducing wrinkles (Dermochlorella; Codif, St. Malo, France). Chlorophyll has also been investigated as a source of pigments in cosmetics. Brown and crimson algae are primarily used in the cosmetics industry (Miller 1996). Traditional species that might be used in cosmetics are *Spirulina platensis, Nannochloropsis oculata*, *Chlorella vulgaris*, and *Dunaliella salina.*

Phytoplankton as Biofertilizers

Phytoplankton biofertilizers, which are appropriate mainly for extensive agriculture, are a mixture of microalgae, water, and enzymes. This mixture is heated and, upon reaching a particular temperature, the phytoplankton breaks up and releases amino acids. This fertilizer, with the addition of the amino acids, accelerates the growth of plants. The principal characteristic of phytoplankton has very high concentrations of amino acids, polysaccharides, phytohormones, oligoelements, and antioxidants, which makes it the biological supplement par excellence. The useful outcomes originate now not the most effective from the production of polymers for particle adherence and water garage in soils or nitrogen-fixing, however additionally from the alga-derived bioactive compounds which impact higher vegetation (Borowitzka 1995; Metting 1996; Ördög et al. 1996). Benthic phytoplankton is now

being investigated by microalgal biotechnologists as a promising source of species with surprising properties. Nitrogen fixation with cyanobacteria, including *Spirulina*, *Scytonema*, *Nostoc*, and *Anabaena*, usually forms the basis of "inexperienced" fertilizers (Venkataraman 1986), and is vital for rice production in tropical and subtropical agriculture. surface solidification towards erosion tactics is likewise of interest in extra arid areas for the duration of the past decade, plant increase regulators from microalgae gained increasing attention substances or extracts have been located which promote germination, leaf or stem growth, or flowering. These kinds of substances, extracted the use of herbal and environmental-pleasant methods, act on the secondary root boom, cell boom multiplication of the plant's mass and multiplication of new shoots, as well as enhancing flowering, which in turn offers more uniformity in the culmination, each in phrases of length and their outside look. The most important usage of cyanobacterial fertilizers is in India, where two million hectares had been fertilized in this way in 1979 (Roger and Kulasooriya 1980). Improvement of phytoplankton biofertilizers for use in temperate environments has been investigated (Metting 1996), and industrial products have evolved; these include an agricultural fertilizer produced by considering soil technology and a lawn fertilizer produced by Cyanotech, which offers a consistent end result of extra shine. In olive cultivation, after the use of this biofertiliser, the petioles were longer, facilitating harvesting, and the flowering in the crop showed greater uniformity. Following treatment with Algafer (Datingbayan Agro-Industrial Corporation;) hydroponically grown plants in Almeria, Spain, were more resistant to water pressure than plants grown without this treatment. Biological phytoplankton products can also protect plants against diseases caused by viruses or bacteria. It is probably that phytoplankton can be a supply of a brand new magnificence of organic plant shielding substances. Eventually, all of this has the gain that this biofertilizers can be utilized in ecological farming and with biologically dealt with vegetation.

Phytoplankton in Pharmaceuticals

Many phytoplankton species produce bioactive compounds; these include antibiotics, algicides, other pharmaceutically active compounds, plant growth regulators, and pollutants (Metting 1996; Borowitzka 1988). Normally, these metabolites are produced in large quantities under conditions of stress. For instance, under stress situations associated with nitrogen challenge, excessive salt concentrations, and high or low temperatures, excessive levels of carotenoids are produced, but it is not known how and to what extent stress may be applied to phytoplankton. Antibiotics have been isolated from a wide variety of phytoplankton species and they display great chemical variance (fatty acids, bromophenols, tannins, terpenoids, polysaccharides, alcohols). Similarly, neurotoxic and hepatotoxic compounds produced by phytoplankton species display great chemical variance. Thus far only some species of phytoplankton have been investigated for their capacity to produce pharmaceuticals and nutraceuticals (Olaizola 2000). The great biodiversity of phytoplankton

Table 8 Pharmacological activities of phytoplankton

Microalgal source	Product	Activity
Spirulina	Vitamin B_{12}	Helps immune system
Odontella	EPA	Anti-inflammatory
Chlorella	Canthaxanthin, astaxanthin	Helps immune system, anti-flu
	Polysaccharides	
Haematococcus	Astaxanthin, canthaxanthin, lutein	Anti-inflammatory
Crypthecodinium	DHA	Aids brain development
Scenedesmus	Lutein, β-carotene	Antiviral

makes the discovery of new metabolites very likely. Therefore it is also possible that we will see the invention and production of high-price compounds. In recent years, various phytoplankton activities have been identified (Table 8).

The term "bioactive molecule" is commonly used for substances that can have an effect on the body, whether useful or harmful, even at low concentrations. Normally the term refers to secondary metabolites that appeal to the eyes of both scientists and industrialists. We assume that future drugs produced from phytoplankton products might be organized and packaged as different pharmacological compounds are today. Such drugs will certainly have applications in diagnostics and other pharmaceuticals, as well as in photodynamic therapy, and their applications will extend to their use in cosmetics and nutritional products (Hirata et al. 2000). EPA has been used for the treatment of coronary heart disease and inflammatory conditions; allergies; arthritis; migraine headache; and psoriasis. Chlorophyll presents a chelating agent hobby which may be used in ointments, remedy for pharmaceutical advantages specifically liver restoration and ulcer treatment. Chlorophyll also aids in cell maintenances and increases hemoglobin concentration in the blood and faster the cell growth. Some of the chemical compounds in phytoplankton, or compounds derived from them, have potential as prescription drugs (Metting 1996). The therapeutic cost of pollutants has not yet been investigated (Luckas 1995). Numerous screening investigations of phytoplankton and cyanobacteria to isolate their biologically active substances have been completed in the United States, Australia, Germany, and France. Consequently, cytotoxic activities were identified for use in anticancer treatments (Sirenko et al. 1999); antiviral activities were discovered in cyanobacteria, in apochlorotic diatoms, and in the conjugaphyte Spirogyra, where certain sulfolipids are active against the herpes simplex virus; and antimicrobial activity is under investigation to find new antibiotics. Although the success rate is about 1% (Muller-Feuga et al. 2003), there seem to be some promising substances from phytoplankton, e.g., the Cyanobacterium scytonema. Antifungal activity has been found in some extracts of cyanobacteria, and antihelminthic effects have been recognized for Spirogyra and Oedogonium. It has also been found that β-carotene from *Dunaliella* acts as a diet precursor in healthy meals. Besides for phytoplankton traces that may be determined in sufficient quantities and purity in nature (e.g., cyanobacterial mats), not less than laboratory scale up is necessary for this phase of the invention efforts.

Table 9 Biofuel production efficiency of phytoplankton

Microalgal species	Biofuel content (% dry weight)	References
Chlorella sp.	29	Sheehan et al. (1998)
Dunaliella tertiolecta	36–42	Tsukahara and Sawayama (2005)
Tetraselmis suecica	15–32	Chisti (2007)
Thalassiosira pseudonana	21–31	Brown et al. (1996)
Nannochloropsis sp.	46	Hu et al. (2006)
Botryococcus braunii	29–75	Metzger and Largeau (2005)
Nannochloris sp.	31	Sheehan et al. (1998)
Skeletonema costatum	87	Rekha et al. (2012)
Chlorella marina	70.6	Rekha et al. (2012)
Dunaliella sp.	66.6	Shenbaga Devi (2010)
Nannochloropsis sp.	68.5	Shenbaga Devi (2010)

Phytoplankton in Biofuel Production

Biofuels are appealing for various reasons; to apprehend the key reasons, one must understand the history and prospects of fossil hydrocarbon-based fuels. Phytoplankton has been studied for many years for the production of hydrogen (H_2), methane, oils (triglycerides and hydrocarbons, for biodiesel and jet fuels, and so on), and bioethanol; the idea of manufacturing biodiesel from phytoplankton that has a high oil content was recognized by Sheehan et al. (1998). It was proposed to use phytoplankton harvested from lakes to produce bio-oil by fast pyrolysis; this was an environmentally sound method to lessen algal blooms, and up to 24% of the dry biomass was recovered as bio-oil. The oils produced by pyrolysis have higher houses than the oil produced from lignocelluloses; however, they have a far better oxygen content than fossil-derived oil, and their heating cost is low, at 29 MJ kg^{-1}, compared with 42 MJ kg^{-1} for fossil-derived oil (Miao et al. 2004). With increasing expenses for petroleum and for international warming caused by the use of fossil fuels, the concept of biofuel production has attracted much attention (Chisti 2007). Many species of phytoplankton have a high content of oils consisting of triacylglycerols with free fatty acids bound to glycerol. The fatty acids are saturated or unsaturated, with carbon chains of various lengths. Non- or mono-unsaturated fatty acids of 16 or 18 carbon atoms in length are best for the manufacture of biodiesel. The biofuel production efficiencies of selected phytoplankton species are listed in Table 9.

Phytoplankton in Carbon Sequestration

There is worldwide awareness about global warming that is a result of increasing levels of various greenhouse gases, including CO_2, that are released from the burning of fossil fuels. The use of phytoplankton for biological CO_2 sequestration has

Table 10 Carbon-capturing efficacy of phytoplankton species

Microalgal species	Known maximum CO_2 concentration	References
Cyanidium caldarium	100%	Seckbach et al. (1971)
Scenedesmus sp.	80%	Hanagata et al. (1992)
Chlorococcum littorale	60%	Kodama et al. (1993)
Euglena gracilis	45%	Nakano et al. (1996)
Chlorella sp.	40%	Hanagata et al. (1992)
Dunaliella tertiolecta	15%	Nagase et al. (1998)
Nannochloris sp.	15%	Yoshihara et al. (1996)
Tetraselmis sp.	14%	Matsumoto et al. (1995)

been considered, as seawater can be used directly as a growth medium so that maintenance costs of phytoplankton culture can be reduced. Many CO_2-producing industries, including electricity plants, are in coastal areas. Some marine phytoplankton species that have been tested for CO_2 sequestration programs are *Tetraselmis* sp. (Laws and Berning 1991; Matsumoto et al. 1995), *Synechococcus* sp. (Takano et al. 1992), *Chlorococcum littorale* (Pesheva et al. 1994), *Chlamydomonas* sp. (Miura et al. 1993), *Nannochloropsis salina* (Matsumoto et al. 1995; Matsumoto et al. 1996), and *Phaeodactylum tricornutum* (Matsumoto et al. 1995). Some species showing high CO_2 tolerance were identified. The carbon-capturing efficacies of some species of phytoplankton are shown in Table 10.

Various methods for the sequestering or immobilization of CO_2 to prevent its release into the ecosystem include filtration and other mechanical and chemical strategies. The idea of the biological sequestration of CO_2 by phytoplankton, via their photosynthetic action, is seen as an alternative technique for decreasing the amount of CO_2 released into the environment (Sheehan et al. 1998; Chisti 2008). However, although the use of phytoplankton to mitigate the quantity of carbon released into the ecosystem is an attractive idea, several primary challenges must be overcome before this idea can be realized in practice. It is generally considered that the growth of phytoplankton is negatively influenced by means of increasing CO_2 (Lee and Lee 2003). Strains that develop well at CO_2concentrations of 5–10% show drastic decreases in their growth rate above 20% (Watanabe et al. 1992). A critical undertaking, therefore, has been to select strains that can cope with very high CO_2 concentrations and also have high boom costs. Screening has yielded strains that grow well at CO_2 concentrations between 30% and 70% saturation (Hanagata et al. 1992; Iwasaki et al. 1998; Sung et al. 1999). Also, as reported by Olaizola (2003), controlling pH modifications in the algae and freeing CO_2 to the algae led to increases in growth that could be sustained even at 100% CO_2. Another characteristic that would need to be optimized is the achievement of high thermal stability in the phytoplankton strains. It has been reported that flue gases delivered within phytoplankton cell cultures can affect the temperature (Ono and Cuello 2007). For CO_2 sequestration, depending on the species, phytoplankton can assimilate CO_2 from an ambient level (0.04%) to 100% v/v CO_2. The process also works under a wide

variety of thermal conditions, ranging from 25 to 100 °C. Adapting phytoplankton for CO_2 sequestration also leads to the production of useful byproducts, and the technology is environmentally friendly.

Phytoplankton in Effluent Remediation

Phytoplankton can be used to sequester pollutants, including excess vitamins, xenobiotics, and heavy metals, from wastewater. This process is known as phycoremediation. The treatment methods yield an output in the form of phytoplankton biomass, which can be used to supply chemicals, feed, fodders, biofuels, or biogas (Muñoz and Guieysse 2006). Phytoplankton has the capacity to reduce nutrient and organic molecules in wastewater, with the elimination of 75%, 84%, and 89% of ammonia, nitrite, and phosphorus, respectively (Dinesh Kumar et al. 2017). Wastewater remediation and phytoplankton CO_2fixation eliminates various chemicals from water and improves the environment. Moreover, a pathway for the removal of nitrogen, phosphorus, and metal ions from wastewater is provided, and the resultant phytoplankton biomass may be exploited for biofuel manufacture or for diverse innovative products. Owing to the supply of nutrient-rich water in commercial and home wastewater, the wastewater can be adapted for phytoplankton cultivation as an alternative to or complementing activated sludge bio-processing. Digestion of the phytoplankton biomass, or the residual biomass following preliminary processing for oil and high-cost products, can be used to generate methane for electricity production. Phytoplankton is often incorporated in maturation ponds for the tertiary treatment of home wastewater, and it may also be incorporated in small- to medium-scale municipal wastewater treatment structures.

The technology known as Advanced Integrated Wastewater Pond Structures (AIWPS) is commercially available (Oswald Green Technologies, North Carolina, USA) (Oswald 1994). The general design includes facultative ponds, which can be quite deep and support the surface increase of phytoplankton, and high-rate algal ponds (HRAPs), which can be shallow and depend on mechanical mixing to maximize phytoplankton production and the removal of oxygen. HRAPs are the most price-effective reactors for liquid waste management and the capture of solar energy, and are used to treat waste from pig farms. Phytoplankton biomasses can be harvested from the HRAPs for animal feed, and can be a factor in integrated approaches to the recycling of cattle wastes, wherein algal wastewater remediation is a second step following the initial anaerobic treatment of organic nutrient-rich wastewater (Ogbonna et al. 2000; Olguin 2003). Normally, no attempt is made to govern the composition of the algal species in wastewater treatment ponds, but including unique species that sediment, float, or flocculate successfully can substantially facilitate their harvesting. *Chlorella, Ankistrodesmus*, and *Scenedesmus* species have been used to deal with wastewater containing natural pollutants from pulp and paper mills and olive oil manufacture (Muñoz and Guieysse 2006). Heterotrophic phytoplankters are regularly outcompeted by microorganisms whose growth rates are

greater; however, phytoplankton and microorganisms can be blended into consortia that clean up wastewaters. Although the production of photosynthetic oxygen by phytoplankton reduces the need for the outside aeration of wastewater (and this is especially useful), volatile pollutants must be biodegraded aerobically but must not be permitted to evaporate during the process of mechanical aeration (Olguin 2003; Muñoz and Guieysse 2006). The phytoplankton produces oxygen that is used by bacteria that can degrade hazardous natural pollutants. These remedies have been shown to be feasible (Muñoz and Guieysse 2006). The biomass produced during wastewater treatment is not used in human food or for animal feed. The biomass may be used for the production of high-price chemical compounds, but it is much more likely that the biomass remaining after the phytoplankton-bacterial treatment of wastewater would be used for biomethane production and CO_2 mitigation.

Phytoplankton as Feedstock for Novel Chemical/Enzymes

Phytoplankton biotechnology for novel enzyme manufacturing and chemical feedstock has, up to now, been little explored. The investigation of high-cost compounds and novel enzymes will become feasible when there are successful efforts to relaxed a large amount of biomass of regular satisfactory to yield enough quantities of trace quantities of bioactive compounds via producing large portions of phytoplankton biomass for carbon seize and/or biodiesel manufacturing. Phytoplankters have diverse habitats, from the warm temperature zones of the tropics to the intense cold of Antarctica. Thus, enzymes may be revealed that operate at low temperatures, enabling low energy chemical practices. Phytoplankton has formerly been grown for a number of high worthy products and applications. A paradox exists in regard to the large-scale culture of phytoplankton. The value of expensive pigments and PUFAs is based on their rarity and cost of manufacturing. Phytoplankton cultivated on the large scale is highly necessary for biofuel production will devalue this merchandise, because the price of production will have to be decreased to a degree whereby the value of the biofuel is competitive with that of fossil fuel oil reserves.

Applications of Zooplankton

Copepods, which outnumber every other multicellular animal group, are a very ancient class, probably having diverged from different arthropod taxa between 388 and 522 million years ago. They are important components of the world's freshwater and marine ecosystems and are sensitive indicators of worldwide climate change; they play key roles in different ecosystems, and they are also parasites and predators of economically important aquatic animals and potential vectors of current water-borne ailments. Copepods preserve fisheries that nourish and help

human populations. They are an extraordinarily various group with regard to their morphologies, physiologies, life-cycles, and habitats, with adult sizes ranging from < 0.1 mm to 23 cm. Copepods are more abundant than any other group of modern multicellular animals, including hyper-abundant insects and nematodes (Humes 1994). They pervade aquatic systems, including those that are man-made, and inhabit domains that extend from nutrient-rich black ooze to abyssal ocean depths, and to the nutrient-poor waters of mountain tarns. Copepods show swarming behavior and can attain densities of as much as 92,000 nos. L-1 (Buskey et al. 1996). Some species have escaped their traditional aquatic habitats, and live in rainy wooded-area canopies, leaf-mulch, hot springs, sand, hyper-saline waters (NaCl~200 ppt) and caves, as well as in symbiotic relationships with other animal and plant species. Deeply divergent morphologies are found with regard to an independent-living or parasitic existence, with a few classes being classically "arthropodan", and others being unrecognizable. However, as the dominant secondary producers of the sea copepods are the linchpin of aquatic food webs. They devour microorganisms and are preyed upon by animals of higher trophic stages, such as fish and whales. In particular, they serve as the major prey for the juvenile forms of many fish species of economic significance (Beaugrand et al. 2003), such as cod, herring, anchovy, flounder, and salmon. Copepods make a considerable contribution to the food sources of many marine and freshwater animals, which have an anticipated value of $US 22.6 trillion per annum (Costanza et al. 1997). Fish provide more than 2.9 billion human beings with more than 15% of their daily animal protein, and fisheries generate a net profit of $US 24.6 billion per annum for developing countries [FAO News room (2006) http://www.fao.org/Newsroom/en/information/2006/1000301/index.html]. Copepods are critical players in marine fish production, and therefore play a crucial position in regard to the supply of nutrients, and thus the health and well-being of people who have few other sources of animal protein. In their vertical migration between surface and deeper waters, copepods also play a major function in the transfer of carbon into the deep sea, and thus they play a major role in worldwide carbon finances (Frangoulis et al. 2005). Copepods are sensitive indicators of weather changes, with warmer ocean temperatures affecting copepod network structure, abundance, distribution, and seasonal timing of breeding (Richardson 2008). Changing copepod distribution has led to the decreased recruitment of fisheries in general and decreased productiveness in modern regional fisheries, such as North Sea cod stocks. Copepods harbor a huge range of new human and fish pathogens. Pathogenic microorganisms, including *Salmonella* spp., *Enterococcus faecalis, Aeromonas* spp., and *Arcobacte*r spp., as well as numerous pathogenic *Vibrio* species and *Vibrio cholerae*, have been reported to be associated with copepods (Venkateswaran et al. 1989; Asmat and Usup 2002; Heidelberg et al. 2002). However, the role of copepods as vectors of water-borne bacterial pathogens in humans is poorly understood. Copepods are intermediate hosts for the guinea worm, *Dracunculus mediensis* (which causes the debilitating disease dracunculiasis), as well as for fish tapeworms (e.g., *Diphyllobothrium latum*) and nematodes that can also infect people. Many parasitic and predatory copepods are themselves pathogenic and affect freshwater and

marine fisheries worldwide, with important monetary impacts on aquaculture (Johnson et al. 2004). Although not many products are derived from copepods, there are three products—freeze-dried copepod, green fluorescent protein (GFP), and astaxanthin—that make a critical economic contribution.

Zooplankton as Live Feed

Cultured copepods have been used effectively in state-of-the-art larviculture for many marine fish. The use of copepods in aquaculture has grown since the 1980s (Schipp 2006). The use of cultured copepods as feed depends on the subculture of three essential orders- calanoida, harpacticoida, and cyclopoida. The other seven orders of copepods, being parasitic, are not appropriate for use as live feeds. The larvae of many marine fish require prey that are about 50–100 μm wide as feed. This requirement can be fulfilled only with the use of copepods, owing to their varying sizes for the duration of its metamorphosis. Copepods offer a huge range of sizes, species, and characteristics and contain high levels of protein, unsaturated fatty acids, carotenoids, and other crucial compounds. In addition, the presence of high levels of DHA and EPA in copepods lends credence to their use in state-of-the-art live feed enterprises.

Green Fluorescent Protein (GFP)

First isolated from the bioluminescent sea jelly *Aequorea victoria*, phylum cnidaria, GFP and its derivatives have been used as genetically encoded in-vivo markers. It was thought that fluorescent proteins might be present only in cnidarians and that these proteins could always be coupled with the luminescent structures in these marine animals. However, this view changed with the discovery of new GFP-like proteins in non-luminescent organisms such as corals (phylum cnidaria, class anthozoa), as well as in representatives of different phyla; for example, copepods (phylum arthropoda, class crustacea) and amphioxus (phylum chordata, subphylum cephalochordata) (Prasher et al. 1992). Seven GFP-like proteins have been identified to date in the copepod families pontellidae and aetideidae (Masuda et al. 2006). The GFP-like proteins from these animals show fast florescence development following protein synthesis, high brightness, and elevated photostability, properties that are all extremely important for use in biotechnology tools. The isolation and characterization of more GFP-like proteins in copepods will probably lead to the production of proteins with higher fluorescence for use in biomedical research.

Astaxanthin

Krill oil contains a unique and powerful antioxidant—astaxanthin. Antioxidants are useful substances that protect cells from molecules known as free radicals. Damage caused by free radicals is involved in many diseases, such as most cancers, heart disease, atherosclerosis, diabetes, and Alzheimers disease, as well as physical aging. Astaxanthin, whose content in krill oil is extraordinarily high, combats the damaging consequences of free radicals. Astaxanthin is one of only a few antioxidants that can penetrate the blood-brain barrier, and it may protect the brain cells from neurodegenerative disorders such as Alzheimer's disease. This antioxidant is also able to prevent and alleviate oxidative stress in the eyes that leads to glaucoma and related eye diseases. Astaxanthin can also reduce irritation in skin. Elite athletes take synthetic astaxanthin as a supplement to increase stamina, as confirmed in laboratory experiments; however krill oil is a natural product that may be superior to the synthetic form. Astaxanthin also reduces the formation of wrinkles that result from sun exposure, as the antioxidant prevents oxidative damage of the collagen in the skin, making the skin more supple. Astaxanthin is considered to be very safe, and it is not converted into diet A in the body. Krill in their natural habitat have an extremely long lifespan for animals of such small size. Researchers assume that the reason for the long lifespan of krill is their astaxanthin content, which slows aging, keeping the animals healthy for longer. This suggests that people who take krill oil could have longer lifespans because of reduction in free radical activity and the related oxidative strain. However, much more research is needed to confirm that the fitness impact of astaxanthin is real. Of interest, other copepods also contain astaxanthin, so that fish and crustacean larvae fed with such copepods would show stress resistance, and this would provide some fitness advantages to people who consume such fish.

Conclusion

The sea performs a major function in regulating the biosphere. Microscopic photosynthetic organisms that live in the ocean provide 50% of the oxygen we breathe, and much of our food and many mineral resources are extracted from the ocean. In a time of natural crisis and major adjustments in our society, it is essential to turn our attention toward the ocean to find more answers for a sustainable destiny. Various marine resources, specifically fisheries, whose planktonic compartment consists of zooplankton and phytoplankton, represent 95% of the current marine biomass, but the volume and constituents of this biomass remain largely unknown and unexploited. Thus, the capacity of plankton to be a bioresource for humanity is essentially untapped. With their diverse evolutionary backgrounds, planktonic organisms provide substantial opportunities as new sources for medicines, cosmetics, and food; as sources of renewable power; and as long-term solutions to the

problem of global warming. Our review of the biotechnological and industrial applications of phytoplankton and zooplankton has shown that marine planktonic biodiversity in the oceans is being investigated, and various bioactive extracts and purified compounds have already been identified.

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Methods of Collection, Preservation and Taxonomic Identification of Marine Phytoplankton

P. Santhanam, P. Pachiappan, and A. Begum

Introduction

The term plankton was coined by Victor Hensen in 1887. And the word "plankton" was derived from the Greek word for drifting. They are often defined as organisms that float at or near the surface of the water and are unable to swim strong enough to go against tides, winds or currents. The phytoplankton is considered as basis of food chain in aquatic environment. Phytoplanktonic organisms are found in virtually all-aquatic ecosystems and play a very important role in aquatic food webs. The food chain originates with phytoplankton, and they serve a vital role as food for tiny zooplankton that exist in different aquatic systems. Phytoplankton are extremely important from the standpoint of monitoring water quality since they are the first group to respond to changes in nutrient conditions in an ecosystem (Mitra et al. 2004). Being the base of the food chain, all higher trophic groups ultimately depend on them. The phytoplankton represents one of the world's simplest and basic organisms. Furthermore, the phytoplankton is one of the forefront basal organisms in the food chain. This means that they are the source of food for most of the tiny organisms that start the web. The phytoplankton is considered to be a rich source of biofuel, feed, food and biofertilizers. It is evident that the phytoplankton removes the carbon very efficiently than terrestrial plants and thereby helps in controlling the global warming. It is just unbelievable how this small microorganism can contribute in so many different fields and improve the lives of people that need new medical treatments. In

P. Santhanam (✉)
Marine Planktonology & Aquaculture Laboratory, Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

P. Pachiappan
Department of Biotechnology, School of Biosciences, Periyar University, Salem, Tamil Nadu, India

A. Begum
Department of Botany, Tihu College, Nalbari, Assam, India

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the course of time, phytoplankton will be recognized as one of the most important and yet subtle organisms that exist in this planet. But unfortunately the systematics and taxonomic identification of phytoplankton are still in critical phase. The number of people working on the taxonomy of phytoplankton is so meagre (Subrahmanyan 1946; Sampathkumar and Perumal 2002; Verlencar and Somshekar Desai 2004; Santhanam and Perumal 2008). In this context, this paper considered being an important one provides information on various techniques involving collection, preservation and morphological identification of some common phytoplankton.

Classification of Phytoplankton

Based on protein content, the marine plankton can be classified as phytoplankton (plant plankton) and zooplankton (animal plankton). Based on cell structure and cell wall arrangements, the phytoplankton is categorised into five groups, viz. diatoms, dinoflagellates, blue greens, greens and coccolithophores.

Bacillariophyceae (Diatoms) These are microscopic in size and have characteristics shell or frustules. The frustule is composed of translucent silica. The cell wall has two parts resembling a pillbox bottom known as hypotheca and lid called the epitheca. The diatoms possess beautifully sculptured shells. Based on the nature of valves and pattern of ornamentation in the valve surface, the diatoms are further grouped into centric and pennate diatoms.

Centric Diatoms The cell is discoid, solenoid or cylindrical in nature. Ornamentation is radial, i.e. the arrangement of markings is radiating from the centre.

Pennate Diatoms They are elongated and fusiform, ovate, sigmoid or roughly circular. Ornamentation is bilateral in nature, i.e. the arrangement of the markings is on either side of the apical axis.

Pyrrophyceae (Dinoflagellates) These are unicellular; some are naked, while others are armoured with plates of cellulose. The dinoflagellates possess two flagella for locomotion. Several of them are luminescent.

Chlorophyceae (Green Algae) This is largely occurring in coastal waters. It has dark-green colour due to the presence of chloroplasts. They are widely distributed in the tropical and subtropical seas, and only few species are found in the Arctic and Antarctic oceans.

Cyanophyceae (Blue-Green Algae) These are unicellular or multicellular organisms. The blue colour in them is due to the presence of a pigment called phycocyanin. Various species are belonging to this group, and the most important ones are

Trichodesmium erythraeum. In certain seasons its biomass increases largely and forms as clumps.

Chrysophyceae (Coccolithophores) These are the smallest category of phytoplankton having a size range between 5 and 20 microns. Some have flagella, while others are devoid of them. The soft body is covered by tiny, calcified circular plates or shields of various designs. These are commonly seen in the open sea.

On the basis of size, the phytoplankton is classified into five different groups:

Ultraplankton – <2 μm
Nannoplankton – 2–20 μm
Microplankton – 20–200 μm
Macroplankton – 200–2000 μm
Megaplankton – >2000 μm

Based on habitat, the marine phytoplankton is further divided as follows:

Oceanic plankton: plankters inhabiting waters beyond continental shelves
Neritic plankton: plankters inhabiting waters overlying continental shelves
Brackish water plankton: plankters inhabiting brackish water areas such as estuaries, mangroves, lagoons, etc.

Methods of Collection

There are three different types of plankton hauling such as horizontal hauling, vertical hauling and oblique hauling being followed for the collection of epipelagic, mesopelagic and bathypelagic plankton, respectively. Three methods would be followed for phytoplankton sampling, viz. bottle samples, plankton pumps and plankton nets.

Bottle Samples: Sampling by water samplers is a recommended method for qualitative and quantitative studies of phytoplankton. Water can be collected from the desired depth. Water samplers are generally used from vessels, ships or fish trawlers. Bottle sample method is the simplest method as it is generally used for the collection of water samples from any desired depth in shallow systems like the near shore water, estuaries and mangroves.

Mayer's Water Sampler: It is made up of an ordinary glass or perhaps bottles of about 1–2 L capacity and is enclosed with a metal band. It is weighted below with a lead weight, and there are two strong nylon graduated ropes, one tied to the neck of the bottle and the other to the cork. During the time of application, the bottle is let down to the desired depth in closed condition where the stopper is jerked open by a strong pull of the lid rope. Water flows into the bottles, and then the lid rope is

released to keep the lid closed. Afterwards, using the neck rope, the bottle containing the water sample is taken out of the water columns.

Friedinger's Water Sampler: It is made up of plexiglass or Perspex with two hinged covers. When handling, the sampler is dispatched down in an open state to the desired depth, and the sampler can be closed by sending drop-weight messenger, which falls down inside on sliding rail and closes the covers and makes the bottle water tight. By this method, the plankters are sampled from the desired depth.

Niskin Water Sampler: This water sampler is made up of non-metallic, free-flushing PVC sampling bottles. These samplers can be activated by GO Devil messengers (1000 MG) when individually or serially attached to a hydrocable. The samplers can be closed by remote command Rosette@ multibottle array or model Acoustic Command Control option. The spring closure is made up of latex tubing with optional stainless steel spring closure, clamp bolts for attachments on a cable and mounting blocks for multisampling system attachment. Delivery is made with lanyards for loading on both cable and multisampling systems. All metal parts are made out of special V4A stainless steel. Specially made V-LIP seal rings can be pasted to avoid leaking of the samples. The sampler is lowered in water column in open condition by opening the clamp at the lower end and upper end, so that water can pass through the sampler. The sampler is held in this position by the wire rope. When the messenger is dropped down the rope, it strikes the release, shutting the valves closed by a locking device. The water sample of the desired depth will be trapped in the bottle can and then be pulled up onto the vessel in a closed condition. For collection of plankton samples from different depths, a more number of water bottles can be used simultaneously; a series of water samplers are suspended one above the other from a wire rope and are lowered into the depths in the open state. In this case, the messenger releases another messenger that is attached to the wire clamp before lowering. The second messenger closes the next lower sampler releasing a third messenger and so on.

Pump and Hose

Pump on Deck

An electrically operated rotary pump or centrifugal pump with attached flexible inlet and outlet rubber or a plastic hose pipe is suitable. The inlet hose is marked off in metres in order to read off the depth. A weight is attached near the open end of the inlet hose to make sure that the hose descends vertically when sent down. The

pump may be used to constantly suck seawater with plankton into the vessel. The pumped water will be filtered through plankton mesh on deck.

Submersible Pump

The submersible pump can be attached near the open end of the inlet hose. The sample taken into the vessel from the specified depth is either transferred to a sedimentation chamber or poured through one or several hand nets of various sizes suspended one above the other. Several advantages encountered by using the plankton pump are collection of plankton from a desired depth, encountering of organisms at different depths in a particular stretch of the water column and continuous sampling from the moving ship itself which is not possible with water samplers.

Net Method

Closing Net

This net is provided with opening and closing mechanisms, and hence collection of plankton between the surface and bottom or from any desired depth in the vertical profile is possible by this method. The net is closed by means of a drop-weight (messenger) which releases the throttle nose. The net must be drawn out immediately without stoppage. No more plankton can enter the closed net during take up.

The Clarke-Bumpus Horizontal Closing Net

The net consists of a solid front section, a brass tube 15 cm long and 12.7 cm in diameter attached to the filtering net portion by a bayonet lock. Propeller vanes are encased in the brass tube and a counter placed on the outside to register the volume of water that passes through the net. Brass tube is so mounted in a frame as to be movable up and down so that, regardless of the position of the frame, the net lies horizontally. A metal bar fixed between the brass tube at the front end and the net jar at the tail end act as a stabilizer, and the two vanes located on the outer side of the brass tube help to keep the net in a horizontal position while in operation. The frame itself is so attached to a tow rope (draw line) that it can swing freely and that the opening of the brass tube is directed forwards by any movement of it. The brass tube can be opened and closed by a closing device.

Hardy's Continuous Plankton Recorder

It is torpedo-shaped plankton sampler. It consists of water tunnel, two rolls of the net silk, gear mechanism, propeller, stabilizing fin, horizontal stabilizing fin, diving plane, vibration damper, storage spool, formalin tank, etc. When the instrument is towed from a vessel, the propeller is turned by the passage of the water. The gears and adjoining spools containing the net silk strips are also simultaneously activated. When water with plankton flows through the tunnel, one of the rolls of silk from the lower spool runs up through the water tunnel in the same way as a film in camera and filters the plankton which streams through the small square mouth of the instrument. Before this net strip containing the filtered plankton reaches the spool immersed in the formalin tank, another roll of silk from the upper spool spins out of cover the collecting strip and holds the plankton in place. Then the two strips are wound on the storage spool located in a container filled with formalin solution to preserve the plankton.

The Indian Ocean Expedition Standard Plankton Net

The plankton net consists of a cone-shaped gauze bag equipped with a metal ring at the wider mouth and closed at the narrow tail by a detachable plankton-collecting bucket. The non-filtering portion generally made of coarse cloth is attached with metal ring. The filtering portion is made up of different materials such as bolting silk, nylon or polyethylene. The net is towed from a boat for about 15 min using three suspension bridle lines at a speed of 2 nautical miles/hour.

Flow-Through Harvesting

A propeller can be switched on from an anchored platform, bridge close to shore or on free-floating boat. The sucked water along with plankton can be filtered by using plankton net. The distance between the propeller and net should be maintained in a range from 0.3 to 1.5 m for proper filtration and to avoid the damage of nets.

Plankton Light Trapping

The standard light trap, equipped with a single high output LED light is powered by 2 C cells. They allow for deployment of up to 32 h. Upgrade to four batteries for improved system autonomy of up to 64 h. The phytoplankton especially some

species of dinoflagellates can be trapped by this method since the dinoflagellates have photoactive property.

Methods of Preservation

The process of fixation and preservation of phytoplankton are done by using various chemicals as detailed here:

Neutralized Formaldehyde

A 20% formaldehyde solution (HCHO) 1 Litre + hexamethylenetetramine 100gm is a widely used fixative and preservative applied for preservation of phytoplankton. 100 ml of water sample is added to 2 ml of the fixing/preserving agent. For net samples, fixing or preserving agent to make up about 1/3 of the volume is added if the sample is dense.

Acidified Formaldehyde

A 20% formaldehyde solution (HCHO) + 50% glacial acetic acid (CH_3COOH) (1:1) is a good preservative for all phytoplankton especially diatoms.

Lugol's Solution

This preservative is prepared by adding 100 gm of potassium iodide (KI) in 1 litre of distilled water, 50 gm of iodine (crystalline) and 100 ml of glacial acetic acid. This is used for all phytoplankton except coccolithophorids as the acid may dissolve the coccoliths. For this, preservation is done by adding 0.4–0.8 ml for 200 ml of phytoplankton sample.

Osmic Acid

200 mg of osmium tetroxide is added to 10 ml of distilled water. This preservative is added at the rate of 3–6 drops per 100 ml of phytoplankton sample.

Glutaraldehyde

An 8 g of glutaraldehyde is added to 100 ml of distilled water. The application of this preservative is done in the ratio of 1:1.

Bottling and Labelling of Plankton

Storage of Phytoplankton

Plankton especially diatoms can be stored in bottles made up of soft glass. Because surface water is usually undersaturated with silicate, storage in bottles of high-quality glass like Pyrex which does not release much silicate or plastic bottles may result in slow dissolution of delicate frustules or spines of diatoms. This may happen in plastic bottles in one to a few years. Further use of glass of very low quality for storage of phytoplankton may result in precipitates. The bottles are closed by a leak-proof cork. After the analysis of the plankton, contents of the bottles and for permanent storage of plankton, wax coating is given around the cork of the bottle after the latter's closure. This would help avoiding the loss of formalin by evaporation in the long run.

Labelling

Proper labelling of the collected phytoplankton samples is more essential. All types of information regarding phytoplankton collection should be written on the labels so that the plankton samples can be identified accurately. The label should contain enough information about the samples collected in order to assure proper identification of the sample. The label is written with a light-coloured waterproof marker or wax pencil. It is also essential that apart from an external label, an internal label written on water-resistant paper be replaced in every jar containing a phytoplankton sample. This internal label should contain the following information:

1. Station number
2. Date of sampling
3. Duration of sampling
4. Sampling depth
5. Type of net, mouth size and mesh size
6. Type of haul (horizontal, vertical, oblique, etc.)
7. Number of turns recorded by flowmeter
8. Collector's name

Details on External Label

1. Bottle number
2. Station number
3. Date of sampling
4. Day/night
5. Sky-nature
6. Time

7. Depth of sampling site
8. Type of net
9. Mesh aperture
10. Flowmeter reading
11. Collector's name

Phytoplankton Mounting

Cleaning

Phytoplankton in a test tube should be washed by rinsing and centrifuging in distilled water to remove the salt particles associated with the phytoplankton. Then test tube with the sample is allowed to dry by removing water.

Acid Cleaning

After cleaning and drying, some hydrochloric acid will be added to the test tube to dissolve the calcareous matter and also to loosen any diatoms that may be attached to the debris. After allowing the test tube with the sample for 1 or 2 days, the test tube is well shaken, and the solid matter including the diatoms is allowed to settle at the bottom. The acid is then decanted off, and the sediment is washed by adding water and pouring off again after allowing time for the solids to settle. Finally most of the water is poured off, and concentrated sulphuric acid is added slowly and carefully. Until red fumes are no longer evolved, small crystals of potassium dichromate is added at intervals. The sulphuric-chromic acid mixture is then poured off and water is added. Acid and dichromate treatment must be repeated until cleaning is complete if the diatoms are not yet properly cleaned with water.

Specimen Mounting

Diatoms are put in a drop of distilled water on a cover slip that has been smeared with a little Mayer's egg albumen which is prepared by mixing 50 ml of egg white with 50 ml of glycerin and 1 g of sodium salicylate. After allowing the water to evaporate, the diatoms on the cover slip are thoroughly dried by heating, and then using any mounting media like Canada balsam, Styrax, Hyrax or DPX, mounting can be done. After cooling the specimen-mounted slide, excess resin is trimmed off by a knife, and the preparation is finally sealed with nail polish or wax. Glycerin mounting and polyvinyl lactophenol mounting are other methods of mounting diatoms. These are more convenient to mount the diatoms in slides directly by

embedding them in polyvinyl lactophenol. Canada balsam is ideal for permanent mounts. For longer preservation, diatoms can also be cleaned and stained with methylene blue and Bengal pink. Subsequently they are embedded in Canada balsam in microscopic slides and covered with cover glasses.

Micrometry of Plankton

By micrometry, while viewing through a microscope, the length, breadth and other details of an organism are measured. The size determination of the phytoplankton forms an important aspect, especially, in preparing the report on the occurrence of new species or taxonomic studies for publication. In micrometry an ocular micrometre (graticule) plays an important role. The ocular micrometre is a circular glass piece which contains a scale of lines which are engraved or photographically reproduced. This scale is of 10 mm in length divided into ten equal divisions. Thus, on the scale of the ocular micrometre, there are 100 divisions of 100 μm each.

Calibration Ocular micrometre is mounted on the diaphragm inside the eyepiece of the chosen microscope at the focal of the eye lens. On the diaphragm inside the eyepiece, at one point, the image from the object is also focused, so that the two can be viewed simultaneously. Now, not only the object in focus, but superimposed on the object, the series of lines of the graticule is equally visible.

For the calibration of the graticule, a stage micrometre which is a microscopic slide of 7.5 × 2.5 cm, which has been engraved a scale of 1 mm long, is divided into 100 divisions of 10 μm (0.01 mm) each. While calibrating, the stage micrometre is first placed on the stage of the microscope. Then it is focused and aligned with the ocular micrometre scale. The stage micrometre is then moved carefully until its zero line is in exact coincidence with that of the ocular metre, in order to find out how many divisions on the ocular micrometre scale correspond with a certain number of divisions on the stage micrometre scale. From this, the value (in μm) of one division of the ocular micrometre under the chosen microscope with fixed objective and eyepiece powers is calculated. If 30 divisions of the ocular micrometre correspond with 10 divisions of the stage micrometre scale, then these 30 divisions are equivalent to 100 μm. In other words, these 30 divisions occupy 100 μm space of the stage micrometre (as 1 division occupies 10 μm of the space in the stage micrometre and the total length of the scale is 1000 μm −1 mm). Thus one ocular micrometre division is equal to 100/30 = 3.3 μm. This calibrated value of the ocular micrometre is of a particular objective and eyepiece of a microscope. If size determination of an object is to be done in different objective or eye lenses, the ocular micrometre scale is calibrated for all the combinations of the different objectives and eyepieces; all the values may be tabulated and can be used whenever it is required. The size of an individual phytoplankton cell of a species may be determined using the calibrated ocular micrometre and micrometre as follows. For size determination, on the stage of the microscope, the specimen for which the size is to be measured is now placed

instead of the stage micrometre. If the diameter of *Cyclotella* cell is to be determined, the zero of the ocular micrometre is focused against the edge of the cell, and the number of division of the ocular micrometre occupying the diameter of the cell is found out. The number of calibrated ocular micrometre divisions multiplied by the corresponding calibrated value would give a diameter of the said cell. For example, if the graticule divisions are 20, then the diameter of the cell is $20 \times 3.3\ \mu m = 66\ \mu m$.

Camera Lucida Drawings

The specimen must be accurately drawn in terms of proportions and magnifications. A camera lucida is invariably used to ensure accurate drawings of specimens.

Camera Lucida

A small right-angled and reflecting prism is mounted above the eyepiece of a microscope. The reflecting surface of the prism is silvered except for one small patch in the centre. The prism mount carries a horizontal arm with an inclinable mirror to the end. The mirror should be set an angle of 45 to the vertical over the drawing paper (though small variations are permissible in the angle of the mirror, too much deviation results in distortion of the drawing).

Light from the drawing paper is reflected by the mirror onto the reflecting face of the prism, which reflects it into the eye of the observer. Light from the microscope eyepiece passes through the small unsilvered patch on the reflecting surface of the prism and which enters the eye of the observer along with the light for the paper. As a result, the observer sees the object apparently superimposed on the paper. The observer concomitantly sees the pencil point if a pencil is used for the drawing.

Making a Camera Lucida Drawing

The camera lucida should be attached to the microscope and properly positioned before undertaking the drawing of an organism that is mounted on a slide and placed to keep flat on the right hand side of the microscope. Invariably, a fairly hard-leaded pencil should be used to obtain precision lines. In drawing with the right hand, the left eye should be at the eyepiece leaving the right eye free to look at what is being drawn without raising the head. By this method, only the main outline of the object is traced, which serves as the frame work in to which other details can be drawn later under the microscope but without the camera lucida.

Effective use of the camera lucida lies in the extract balancing of the intensity of light on the drawing paper and the object, respectively. For example, if the light from the object is too bright, the pencil point can't be seen; on the other hand, if the paper is too bright, the object disappears altogether from view, and if the light to the specimen is too intense, it can be reduced by closing the iris diaphragm of the sub-stage condenser.

Depicting Size of the Specimen in Drawing

To determine the size of a specimen, the stage micrometre is placed on the stage of the microscope immediately after removing the specimen slide but without altering the microscope set-up. Subsequently, a space equivalent to 100–200 μm of the millimetre scale is drawn alongside the object.

Taxonomic Identification of Phytoplankton

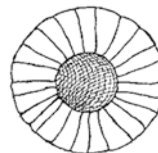
Cyclotella striata (Kiitzing) Grunow

Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Centrales/Biddulphiales
Suborder: Coscinodiscineae
Family: Thalassiosiraceae
Genus: *Cyclotella*
Species: *C. striata*

Cells are disc shaped with central area coarsely punctate. There are numerous regular striations with evenly striated border. Diameter of the cell is 15–30 μm.

Planktoniella sol (Wall.) Schiitt

Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Centrales/Biddulphiales
Suborder: Coscinodiscineae
Family: Thalassiosiraceae
Genus: *Planktoniella*
Species: *P. sol*



The cells are disc shaped, small with flat valves. It has a characteristic wing-like expansion with weakly silicified and radial rays hanging from the epitheca. Diameter of valve is 65–73 μm.

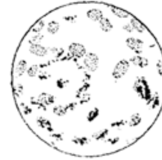
Skeletonema costatum (Grev.) Cleve

Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Centrales/Biddulphiales
Suborder: Coscinodiscineae
Family: Thalassiosiraceae
Genus: *Skeletonema*
Species: *S. costatum*

The cells form long and slender chains with the help of marginal spines. The valves are small and lens shaped, and the space between the cells is larger than the cells. Diameter of the cell is 12–15 μm.

Thalassiosira subtilis (Ostenfeld) Gran

Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Centrales/Biddulphiales
Suborder: Coscinodiscineae
Family: Thalassiosiraceae
Genus: *Thalassiosira*
Species: *T. subtilis*



Cells are disc shaped, forming a colony enclosed in mucilage, valves weakly silicified, and chromatophores numerous and disc shaped.

Stephanopyxis palmeriana (Grev.) Grunow

Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Centrales/Biddulphiales
Suborder: Coscinodiscineae
Family: Melosiraceae
Genus: *Stephanopyxis*
Species: S. *palmeriana*

Cells are cylindrical with slightly convex valves, and a number of cells are joined together by their spines to form chains. Cells are with hexagonal areolae; spines numerous, arranged in a ring and enlarged at the base; and chromatophores many and plate-like.

Leptocylindrus danicus Clave

Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Centrales/Biddulphiales
Suborder: Coscinodiscineae
Family: Leptocylindraceae
Genus: *Leptocylindrus*
Species: *L. danicus*

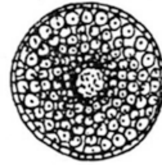
Cells are cylindrical, forming long chains, no structure visible on the valve and chromatophores numerous and disc shaped.

Coscinodiscus centralis Ehrenberg

Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Centrales/Biddulphiales
Suborder: Coscinodiscineae
Family: Coscinodiscaceae
Genus: *Coscinodiscus*
Species: *C. centralis*

Cells are disc shaped, valves are convex and areolated with a clear rosette at the centre, and the valve edge is narrow and striated. Marginal spines are clear, and numerous small plate-like chloroplasts are present.

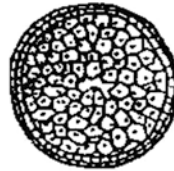
Coscinodiscus gigas Ehrenberg



Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Centrales/Biddulphiales
Suborder: Coscinodiscineae
Family: Coscinodiscaceae
Genus: *Coscinodiscus*
Species: *C. gigas*

The cells are large and flat very similar to a coin. Small areolae are found at the margins with large areolae at the centre and a central large area. Diameter of the cell is 472–536 μm.

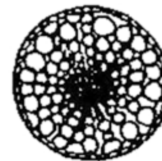
Coscinodiscus eccentricus Ehrenberg



Division: Thallophyta
Class: Bacillariophyceae
Order: Centrales
Suborder: Coscinodiscineae
Family: Coscinodiscaceae
Genus: *Coscinodiscus*
Species: *C. eccentricus*

The cells are disc shaped with two valves – epitheca and hypotheca. Cells are double walled with hexagonal markings and areolae of same size arranged in tangential series. Diameter of the cell is 34–104 μm.

Coscinodiscus radiatus Ehrenberg

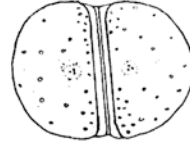


Division: Thallophyta
Class: Bacillariophyceae
Order: Centrales
Suborder: Coscinodiscineae
Family: Coscinodiscaceae
Genus: *Coscinodiscus*
Species: *C. radiatus*

Cells are disc shaped and arranged radially from the centre to periphery. This species is comparatively larger in size than *C. eccentricus* and *C. lineatus*. Diameter of the cell is 460–530 µm.

Hemidiscus hardmannianus (Greville) Mann.

Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Centrales/Biddulphiales
Suborder: Coscinodiscineae
Family: Hemidiscaceae
Genus: *Hemidiscus*
Species: *H. hardmannianus*



Valves are semicircular with central area large and hyaline. Ventral margins are more or less straight. Fine areolation is seen radiating from the centre.

Rhizosolenia styliformis Brightwell

Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Centrales/Biddulphiales
Suborder: Rhizosoleniineae
Family: Rhizosoleniaceae
Genus: *Rhizosolenia*
Species: *R. styliformis*

Cells are cylindrical with spines long and hollow and intercalary bands many and scale-like arranged into a dorsal and ventral row. Diameter is 21–101 µm and length is 210–385 µm.

Rhizosolenia alata Brightwell

Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Centrales/Biddulphiales
Suborder: Rhizosoleniineae
Family: Rhizosoleniaceae
Genus: *Rhizosolenia*
Species: *R. alata*

The cells are rod shaped, cylindrical with curved tube-like processes. The depression of each process fits the adjoining cell. Diameter is 6–31 µm and length is 412–668 µm.

Rhizosolenia hebetata (Bailey)Gran

Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Centrales/Biddulphiales
Suborder: Rhizosoleniineae
Family: Rhizosoleniaceae
Genus: *Rhizosolenia*
Species: *R. hebetata*

Cells are cylindrical and longitudinally drawn out, spines are hollow at base ending in a long straight or curved hairlike spine, intercalary bands are clear, and *Richelia intracellularis*, a blue-green endophyte, is common.

Rhizosolenia robusta Norman

Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Centrales/Biddulphiales
Suborder: Rhizosoleniineae
Family: Rhizosoleniaceae
Genus: *Rhizosolenia*
Species: *R. robusta*

Cells are crescent shaped or ‘S’ shaped, valve is curved with longitudinal line, intercalary bands are many, and collar-like, and apical process is hollow with a small spine.

Rhizosolenia crassispina Schroder

Division: Thallophyta
Class: Bacillariophyceae
Order: Centrales
Family: Rhizosoleniaceae
Genus: *Rhizosolenia*
Species: *R. crassispina*

Cells are cylindrical with truncated ends. Apical processes with hairlike after growths. Diameter and length of the cells are 41–54 μm and 145–278 μm, respectively.

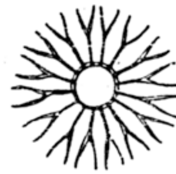
Rhizosolenia setigera Brightwell

Division: Thallophyta
Class: Bacillariophyceae
Order: Centrales
Family: Rhizosoleniaceae
Genus: *Rhizosolenia*
Species: *R. setigera*

Cells are rod shaped and cylindrical. Apical processes are long and end in a spine. Diameter of the cell is 7–8 μm and length is 512–528 μm.

Bacteriastrum hyalinum Lauder

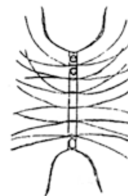
Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Centrales/Biddulphiales
Suborder: Biddulphiales
Family: Chaetocerotaceae
Genus: *Bacteriastrum*
Species: *B. hyalinum*



Cells form chains which are broader than long. Setae are many (12–25) and perpendicular to chain axis which fuse at the base and go apart as branches.

Chaetoceros affinis Lauder

Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Centrales/Biddulphiales
Suborder: Biddulpphiineae
Family: Chaetocerotaceae
Genus: *Chaetoceros*
Species: *C. affinis*

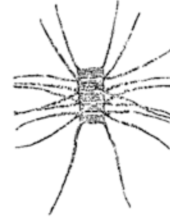


Cells are in straight chains, poles of adjacent cells touch, apertures are narrow, inner setae are thin and without basal part, terminal setae are large and strongly

divergent, both valves are with small spines, and chromatophore is one and plate-like.

Chaetoceros lorenzianus Grunow

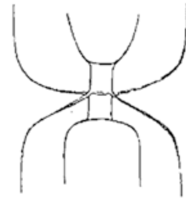
Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Centrales/Biddulphiales
Suborder: Biddulpphiineae
Family: Chaetocerotaceae
Genus: *Chaetoceros*
Species: *C. lorenzianus*



Cells of apical axis are long, forming straight chains. Setae are springing from the corners a very short basal part, terminal setae are thicker and somewhat shorter than the others, and setae are four sided, punctate areolate, punctate of neighbouring faces alternating with each other.

Chaetoceros diversus Cleve

Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Centrales/Biddulphiales
Suborder: Biddulpphiineae
Family: Chaetocerotaceae
Genus: *Chaetoceros*
Species: *C. diversus*



Cells apical, axis are forming straight chains, which are usually short; apertures are very small; some setae are hairlike and others thicker, tubular and spinous; and terminal setae are thin and hairlike.

Chaetoceros didymus Ehrenberg

Division: Thallophyta
Class: Bacillariophyceae
Order: Centrales
Family: Chaetocerotaceae
Genus: *Chaetoceros*
Species: *C. didymus*

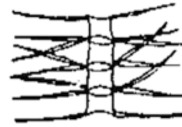
Cells form straight chains with semicircular knob in between each valve. Distinct interlocking of setae is seen. Length of the cell is 22–40 µm.

Chaetoceros curvisetus Cleve

Division: Thallophyta
Class: Bacillariophyceae
Order: Centrales
Family: Chaetocerotaceae
Genus: *Chaetoceros*
Species: *C. curvisetus*

Cells form curved long chains. All the setae are directed towards one side of the chain. Length of the cell is 8–20 µm.

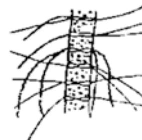
Chaetoceros diversus Cleve



Division: Thallophyta
Class: Bacillariophyceae
Order: Centrales
Family: Chaetocerotaceae
Genus: *Chaetoceros*
Species: *C. diversus*

The cells are compact with small apertures. Setae of cells are straight, thicker, tubular and spinous. Length of the cell is 5–9 µm.

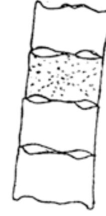
Chaetoceros compressus Lauder



Division: Thallophyta
Class: Bacillariophyceae
Order: Centrales
Family: Chaetocerotaceae
Genus: *Chaetoceros*
Species: *C. compressus*

Cells are longer than broad and form chains. Setae are thicker and twisted and run parallel to chain axis. Length of cell is 7–19 µm.

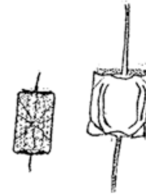
Bellerochea malleus (Brightwell) Van Heurck



Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Centrales/Biddulphiales
Suborder: Biddulpphiineae
Family: Lithodesmiaceae
Genus: *Bellerochea*
Species: *B. malleus*

Cells are flat, forming ribbon-like chains, and weakly silicified; valve with a rudimentary central knob punctuates in the margin; apertures are slit-like, closed in the middle by rounded valves; and chromatophores are numerous and disc shaped.

Ditylum brightwellii (West) Grunow



Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Centrales/Biddulphiales
Suborder: Biddulpphiineae
Family: Lithodesmiaceae
Genus: *Ditylum*
Species: *D. brightwellii*

Cells are prism-shaped, with strongly rounded ends and three-cornered valvar plane, valve margin is wavy, and a circle of short spines are on the valve surface and siliceous hollow spine at the centre.

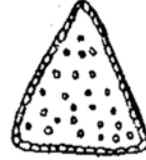
Triceratium favus Ehrenberg

Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyta
Order: Centrales/Biddulphiales
Suborder: Biddulpphiineae
Family: Lithodesmiaceae
Genus: *Triceratium*
Species: *T. favus*

Cells are triangular in shape with three-cornered valvar plane. Valve is strongly sculptured with regularly arranged hexagonal areolae of same size.

Triceratium reticulatum Greville

Division: Thallophyta
Class: Bacillariophyceae
Order: Centrales
Family: Lithodesmiaceae
Genus: *Triceratium*
Species: *T. reticulatum*



Cells possess three-cornered valvar plane with corners rounded. Areolae of different sizes are found rounded and scattered. A side of valve measures 24–128 µm.

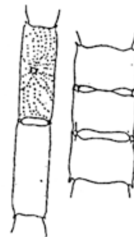
Odontella mobiliensis (Bailey) Grunow

Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Centrales/Biddulphiales
Suborder: Biddulpphiineae
Family: Eupodiscaceae
Genus: *Odontella*
Species: *O. mobiliensis*

Cells are flat, cell wall sculpturing pronounced with marked silicification; two horns (angular processes) at each end are embracing two bristles often in between; and bristles are thin, distant from the horns of the valve.

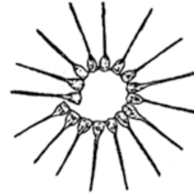
Odontella sinensis (Greville) Grunow

Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Centrales/Biddulphiales
Suborder: Biddulpphiineae
Family: Eupodiscaceae
Genus: *Odontella*
Species: *O. sinensis*



Cells form short chains with cylindrical to square shape in girdle view with presence of two thin blunt horns with two long and thin spines.

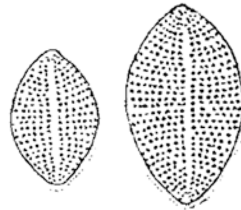
Asterionellopsis glacialis Castracane



Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Pennales
Suborder: Fragilariineae
Family: Fragilariaceae
Genus: *Asterionellopsis*
Species: *A. glacialis*

Cells form spiral colony, cells inflate at one end only and club-like, and cells are knob-like at base with slender long apex.

Rhaphoneis amphiceros Ehrenberg



Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Pennales
Suborder: Fragilariineae
Family: Rhaphoneidaceae
Genus: *Rhaphoneis*
Species: *R. amphiceros*

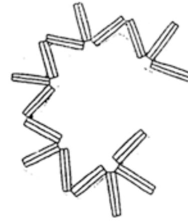
Cells solitary, valve outline broadly elliptical or lanceolate, inflated at the centre, broad, striae parallel or radiating, chloroplasts small and numerous.

Fragilaria oceanica Cleve

Division: Thallophyta
Class: Bacillariophyceae
Order: Pennales
Family: Fragilarioideae
Genus: *Fragilaria*
Species: *F. oceanica*

Cells are rectangular in girdle view with valves lanceolate and pseudoraphe narrow and linear. Transapical striae are 14/10 μm. Length is cell is 32 μm and breadth being 6 μm.

Thalassionema nitzschioides Grunow



Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Pennales
Suborder: Fragilariineae
Family: Thalassionemataceae
Genus: *Thalassionema*
Species: *T. nitzschioides*

Cells form zig-jag chains and are usually rectangular in shape; ends are similar cells linear forming colonies.

Thalassiothrix frauenfeldii (Grunow) Hallegraeff

Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Pennales
Suborder: Fragilariineae
Family: Thalassionemataceae
Genus: *Thalassiothrix*
Species: *T. frauenfeldii*

Cells are elongated, linear forming colonies, ends slightly dissimilar, and colonies stellate or zig-jag chains or both.

Thalassiothrix longissima Cleve & Grunow

Division: Thallophyta
Class: Bacillariophyceae
Order: Pennales
Family: Thalassionemataceae
Genus: *Thalassiothrix*
Species: *T. longissima*

These are solitary cells and thread-II in appearance. Valves are linear with rounded ends. Length of the cell is 495–1750 μm and breadth is 2.5 μm.

Asterionella japonica Cleve

Division: Thallophyta
Class: Bacillariophyceae
Order: Pennales
Family: Thalassionemataceae
Genus: *Asterionella*
Species: *A. japonica*

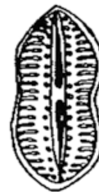
Cells form spiral colonies. Cells are knob-like at base with slender long apex. Length of the cell is 43–106 μm and breadth is 7–11 μm.

Phaeodactylum tricornutum Bohlin

Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Pennales
Suborder: Fragilariineae
Family: Phaeodactylaceae
Genus: *Phaeodactylum*
Species: *P. tricornutum*

It is a monospecific genus, solitary, with three types of cells, ovate, fusiform and, more rarely, triradiate. Ovate cells are motile with one siliceous valve for cell, and fusiform cells are nonmotile and lack a siliceous valve, one chloroplast.

Diploneis smithii (Brebisson) Cleve

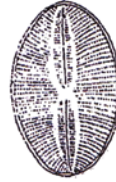


Division: Thallophyta
Class: Bacillariophyceae
Order: Pennales
Family: Diploneidaceae
Genus: *Diploneis*
Species: *D. smithii*

The valves are ovate with rounded poles and central small quadrate nodule. Raphe is lanceolate with transapical costae 9 in 10 μm and radial with alternating rows of alveoli in two oblique rows. Length is 55–60 μm and breadth is 35–38 μm.

Navicula forcipata Greville

Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Pennales
Suborder: Bacillariineae
Family: Naviculaceae
Genus: *Navicula*
Species: *N. forcipata*



Cells are boat shaped, elliptical with rounded ends; lateral areas are narrow and slightly constricted at the middle; and raphe is along the entire length of valves.

Navicula longa (W. Gregory) Ralfs

Division: Thallophyta
Class: Bacillariophyceae
Order: Pennales
Family: Naviculaceae
Genus: *Navicula*
Species: *N. longa*

Valves are rhombic with pointed ends. The central area is small and axial area narrow. Striae are 9–11 in 10 µm in length and breadth being 52–56 µm and 10 µm, respectively.

Cymbella marina Castracane

Division: Thallophyta
Class: Bacillariophyceae
Order: Pennales
Family: Naviculoideae
Genus: *Cymbella*
Species: *C. marina*

Cells are spindle shaped with raphe slightly broad. The axial area is narrow but central area rather large with striae radial and 15 in 10 µm. Length and breadth of the cell are 45–85 µm and 14–16 µm, respectively.

Pleurosigma angulatum (Quekett) W. Smith

Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Pennales
Suborder: Bacillariineae
Family: Naviculoideae
Genus: *Pleurosigma*
Species: *P. angulatum*

Cells single, valve lanceolate, ends obtuse, raphe sigmoid and eccentric at the ends; transverse and oblique striae equidistant

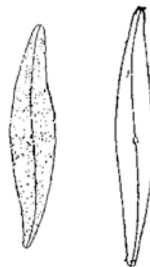
Pleurosigma elongatum W. Smith

Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Pennales
Suborder: Bacillariineae
Family: Naviculaceae
Genus: *Pleurosigma*
Species: *P. elongatum*

Valves are slightly sigmoid, elongated and gradually attenuated, ends are acute, and raphe is central and slightly sigmoid.

Pleurosigma normanii Raifs

Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Pennales
Suborder: Bacillariineae
Family: Naviculaceae
Genus: *Pleurosigma*
Species: *P. normanii*



Valves are broadly lanceolate and slightly sigmoid, with subacute ends, and raphe is nearly central, sigmoid with single curvature.

Gyrosigma balticum (Ehrenberg) Rabenhorst

Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Pennales
Suborder: Bacillariineae
Family: Naviculaceae
Genus: *Gyrosigma*
Species: *G. balticum*

Cells are single, valve is sigmoid, ends are obtuse, raphe is slightly eccentric and flexuose, and the central area is small and oblique and transverse and longitudinal striae are equidistant.

Bacillaria paradoxa Gmelin

Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Pennales
Suborder: Bacillariineae
Family: Bacillariaceae
Genus: *Bacillaria*
Species: *B. paradoxa*

Cells in girdle view are linear and rectangular, united by their valves to form a mat-like colony, the individual cells of which exhibit gliding movements in the living condition. Valves are linear spindle shaped in outline.

Nitzschia longissima (Breb.) Ralfs

Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Pennales
Suborder: Bacillariineae
Family: Bacillariaceae
Genus: *Nitzschia*
Species: *N. longissima*

Cells linear, spindle shaped and living singly, cells with central elongated portion, lanceolate, valve ends hairlike, elongated, generally straight.

Nitzschia sigma (Kiitzing) W. Smith

Phylum: Chrysophyta
Division: Chromophyta
Class: Bacillariophyceae
Order: Pennales
Suborder: Bacillariineae
Family: Bacillariaceae
Genus: *Nitzschia*
Species: *N. sigma*

Cells linear, slightly sigmoid in girdle view and in valve view almost straight and elongated.

Nitzschia closterium (Ehrenberg) W. Smith

Division: Thallophyta
Class: Bacillariophyceae
Order: Pennales
Family: Naviculoideae
Genus: *Nitzschia*
Species: *N. closterium*

Cells are spindle shaped with pointed ends and are usually found in chains. Ends of the cells are pressed against each other for a short distance. Length is 45–128 μm and breadth is 3–5 μm, respectively.

Nitzschia striata

Division: Thallophyta
Class: Bacillariophyceae
Order: Pennales
Family: Naviculoideae
Genus: *Nitzschia*
Species: *N. striata*

Cells are spindle shaped with pointed ends and are usually found in chains. Ends of the cells are pressed against each other for a short distance. Length is 45–128 μm and breadth is 3–5 μm, respectively.

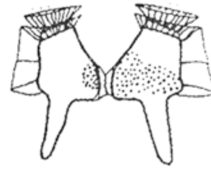
Prorocentrum micans Ehrenberg

Division: Pyrrhophyta
Class: Dinophyceae
Order: Prorocentrales
Family: Prorocentraceae
Genus: *Prorocentrum*
Species: *P. micans*

Cell is medium sized and heart shaped or teardrop shaped; in valve view, cell will have one convex side and one arched side, and in lateral view, the cell is flattened. Flagella insertion is anterior, cell wall is divisible into two longitudinal valves, and cells are with chloroplast.

Dinophysis caudata Saville-Kent

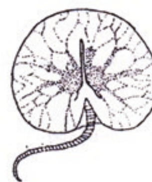
Division: Pyrrhophyta
Class: Dinophyceae
Order: Dinophysiales
Family: Dinophysiaceae
Genus: *Dinophysis*
Species: *D. caudata*



Cells are medium sized and have posterior finger-like process; cells often occur in pairs dorsally attached and body surface with areolations.

Noctiluca scintillans (Macartney) Kofoid and Swezy

Division: Pyrrhophyta
Class: Dinophyceae
Order: Noctilucales
Family: Noctilucaceae
Genus: *Noctiluca*
Species: *N. scintillans*



They are unarmoured, are spherical with two flagella and have a striated tentacle and radiating protoplasm; chloroplasts are absent.

Ceratium tripos (O.F. Muller) Nitzsch

Division: Pyrrhophyta
Class: Dinophyceae
Order: Gonyaulacales
Family: Ceratiaceae
Genus: *Ceratium*
Species: *C. tripos*

Cells are large, as well as broad; cells are anchor shaped, which are characterized by three horns, one apical and two antapical. The right antapical is less developed than the left, both are diverge, and the apical horn is longer.

Ceratium trichoceros (Ehrenberg) Kofoid

Division: Pyrrhophyta
Class: Dinophyceae
Order: Gonyaulacales
Family: Ceratiaceae
Genus: *Ceratium*
Species: *C. trichoceros*

They have large delicate cell with characteristic horn development, apical and hypothecal horns (open ends) in parallel plane.

Ceratium macroceros (Ehrenberg) Vanhoffen

Division: Pyrrhophyta
Class: Dinophyceae
Order: Gonyaulacales
Family: Ceratiaceae
Genus: *Ceratium*
Species: *C. macroceros*

They have large cells with angular box-like body that abruptly forms an offset apical horn directed to the right; left and right hypothecal horns are proximally of same width and formed abruptly from body.

Ceratium furca (Ehrenberg) Claparède and Lachmann

Division: Pyrrhophyta
Class: Dinophyceae
Order: Gonyaulacales
Family: Ceratiaceae
Genus: *Ceratium*
Species: *C. furca*

These are large species with two unequal, parallel or slightly divergent hypothecal horns; the right horn is shorter than the left. Epitheca gradually tapers into apical horn.

Gonyaulax polygramma Stein

Division: Pyrrhophyta
Class: Dinophyceae
Order: Gonyaulacales
Family: Gonyaulacaceae
Genus: *Gonyaulax*
Species: G. *polygramma*

They have elongated cell with tapered epitheca; epitheca is angular with short to moderate apical horn and hypotheca symmetrically rounded or truncate; cells typically have two antapical spines.

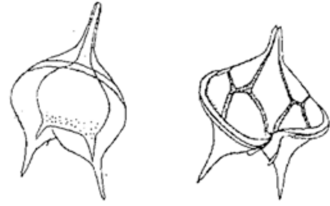
Protoperidinium depressum (Bailey) Balech

Division: Pyrrhophyta
Class: Dinophyceae
Order: Peridiniales
Family: Protoperidiniaceae
Genus: *Protoperidinium*
Species: *P. depressum*

Cells are broad, flattened obliquely dorsoventrally with prominent apical horn with hypotheca bearing two antapical horns.

Protoperdinium pentagonum (Gran) Balech

Division: Pyrrhophyta
Class: Dinophyceae
Order: Peridiniales
Family: Protoperidiniaceae
Genus: *Protoperidinium*
Species: *P. pentagonum*



Cell is medium sized to large and broadly pentagonal with truncate posterior margin with short antapical winged spines; sulcus is broad posteriorly not extending to antapex cell in cross section reniform.

Pyrocystis fusiformis C.W.Thomson

Division: Thallophyta
Class: Pyrrophyceae
Group: Dinophyceae
Order: Phytodiniales
Genus: *Pyrocystis*
Species: *P. fusiformis*

Cells are spindle shaped. Ends of the cell are pointed. Length is 600–1600 µm.

Trichodesmium erythraeum Ehrenberg

Division: Thallophyta
Class: Cyanophyceae
Genus: *Trichodesmium*
Species: *T. erythraeum*

Cells are filamentous. They have photosynthetic pigments called phycoerythrin. These cells are capable of fixing atmospheric nitrogen. In certain season of the year, its biomass increases greatly resulting in the formation of clumps.

Trichodesmium thiebautii Gomont ex Gomont

Division: Thallophyta
Class: Cyanophyceae
Genus: *Trichodesmium*
Species: *T. thiebautii*

Trichomes are blue green in colour. They are free-swarming found in bundles, thickened at apices. Cells measure 8–10 μm long and 7.5–10 μm broad.

Oscillatoria limosa C. Agardh ex Gomont

Division: Thallophyta
Class: Cyanophyceae
Genus: *Oscillatoria*
Species: *O. limosa*

Trichomes are more or less straight, not constricted at cross walls. End cells are flatly rounded with slightly thickened membrane. Cell measures 11–14 μm broad and 2–4 μm long.

Dictyocha sp.

Division: Thallophyta
Class: Chrysophyceae
Genus: *Dictyocha* sp.

The cell is qudriflagellate, with three pseudoflagella and one flagellum. Four chromatophores are present. These are motile in nature and possess internal skeleton of conspicuous siliceous spicules.

Tetraselmis gracilis (Kylin) Butcher

Division: Thallophyta
Class: Prasinophyceae
Genus: *Tetraselmis*
Species: *T. gracilis*

These are motile with four equal flagella. The size of this organism is around 2 μm. Cell consists of a single chloroplast, pyrenoid and protein coat.

Chlorella marina Butcher

Class: Chlorophyceae
Genus: *Chlorella*
Species: *C. marina*

It has a cell wall. The body of cell is ovate and green. This is non-flagellate and mucilaginous.

Chlorella salina Kufferath

Division: Thallophyta
Class: Chlorophyceae
Genus: *Chlorella*
Species: *C. salina*

Cell is very small with a cell wall. The cytoplasm is concentrated at the centre of the cell. The nucleus is present at one end of cytoplasm.

Dunaliella sp.

Domain: Eukaryota
Kingdom: Viridiplantae
Phylum: Chlorophyta
Class: Chlorophyceae
Order: Chlamydomonadales
Family: Dunaliellaceae
Genus: *Dunaliella* sp.

They are motile, unicellular, rod-to-ovoid-shaped (9–11 µm) green algae (Chlorophyceae), which are common in marine waters.

Isochrysis sp.

Kingdom: Chromalveolata
Phylum: Haptophyta
Class: Prymnesiophyceae
Order: Isochrysidales
Family: Isochrysidaceae
Genus: *Isochrysis* sp.

Cells of *Isochrysis* are showing the variable form of the cell, the anteriorly directed equal flagella and the two chromoplasts.

Nannochloropsis sp.

Domain: Eukaryota
Kingdom: Chromalveolata
Phylum: Heterokontophyta
Class: Eustigmatophyceae
Family: Eustigmataceae
Genus: *Nannochloropsis* sp.

The species are small, nonmotile spheres which do not express any distinct morphological features, and cannot be distinguished by either light or electron microscopy.

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Study on Molecular Taxonomy and Phylogenetic Analysis of Phytoplankton

S. Jeyapandi, C. Rajthilak, P. Santhanam, A. Begum, S. Ravikumar, P. Pachiappan, and S. U. Ahmed

Introduction

Algae are an enormous and diverse group of eukaryotic photosynthetic organisms found in wide variety of environments. The first algal groups arise 1–1.5 billion years ago (Douzery et al. 2004; Yoon et al. 2004) following the symbiogenesis of a heterotrophic eukaryotic organism through a photosynthesis. This event gave rise to the primary plastids found in the Glaucophyta, red algae and green lineage as well as land plants (Reyes-Prieto et al. 2007). These three lineages belong to a group Plantae or Archaeplastida (Cavalier-Smith 1981; Adl et al. 2005). Photosynthesis occurs in four of the six super groups: Archaeplastida (Glaucophyta, red algae, green plants), Chromalveolata (cryptophytes, Stramenopila or heterokonts counting diatoms and brown algae, haptophytes and dinoflagellates), Rhizaria (Chlorarachniophyta) and Excavata. Algae, which replenish themselves each single day, providing complete new plankton for water animals, supply almost 50% of the

S. Jeyapandi · P. Santhanam (✉)
Marine Planktonology & Aquaculture Laboratory, Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

C. Rajthilak · S. Ravikumar
Department of Oceanography and Coastal Area Studies, School of Marine Sciences, Alagappa University, Thondi, India

A. Begum
Department of Botany, Tihu College, Nalbari, Assam, India

P. Pachiappan
Department of Biotechnology, School of Biosciences, Periyar University, Salem, Tamil Nadu, India

S. U. Ahmed
Department of Biotechnology, Ministry of Science and Technology, Government of India, New Delhi, India

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total oxygen on earth (Felsenstein 1985). The organization of algae is presently going through an enormously attractive stage. The old, reproduction classification system is being replaced by a new, more usual, phylogenetic system. The introduction of molecular phylogenetic method into the systematics of green algae led to a novel revision of the concepts of higher taxonomic lineages such as division, classes and orders (Melkonian and Sure 1995; Friedl 1997; Leliaert et al. 2012). Aquaculture industries use marine microalgae as enhancement diet for the live feeds (Chakraborty et al. 2007). Microalgae build up the basis of food chain and provide the renewable reservoir of PUFAs in nature (Ferreira et al. 2008). Study on the fatty acid masterpiece of many marine microalgae exposed that PUFAs present in them are much healthier than many commercially accessible enrichment diets (Volkmann et al. 1989).

Marine photosynthetic microbial organisms are the major supporting components of ecosystem process and responsible for biogeochemical reaction that drives our climate changes. We can subsequently understand environmental, biological and evolutionary processes scheming and structuring marine ecosystem biodiversity.

The majority of developed world's energy supplies have been met by burning of fossil fuels such as coal, oil and natural gas. Fossil fuel combustion leads to emission of CO_2, SO_2, NOx, CO, particulate substance and unstable organic compounds which collectively is the main cause of emission of atmospheric greenhouse gasses. Hence, there is a need for environmentally and economically sustainable, renewable and carbon-neutral fuels. The current technologies are focusing on construction of renewable, carbon impartial fuel systems (Chisti 2007). Microalgae are various groups of photosynthetic microorganisms. They convert carbon dioxide into a mixture of valuable compounds including biofuels, foods, feed, pharmaceuticals and biologically energetic compounds (Mata et al. 2010). Algae are of exacting interest as a sustainable source of biodiesel since they are sunlight-driven oil factories which have the ability to produce and accumulate important quantities of lipids (Chisti 2007). Therefore, microalgae have great probable to be a major cause for renewable biofuel production (Li et al. 2011).

Morphological identification of algae significance is a challenging task to the researchers worldwide as it lacks specific morphological markers for identification. Microalgae can modify the cell size and shape during dissimilar stages of their life cycle; hence, it requires great effort to evaluate the microalgae by conventional microscopic techniques. Molecular identification by ribosomal DNA sequencing is a quick, accurate technique and has proved to be very useful in understanding the evolutionary relationship in the middle of diverse species of algae (Mutanda et al. 2010; Banerjee et al. 2012). This chapter deals the methods of molecular identification of phytoplankton.

DNA metabarcoding (high-throughput sequencing of DNA markers), which has unveil a vast and unsuspected multiplicity of microorganisms in current years, provides a leading new tool to assess the composition and ecological function of microalgae community. Environmental metabarcoding approach has also been used

for bioassessment and biomonitoring of lookout or indicator species, including microalgae (Pawlowski et al. 2014), and the study of diet regime in predators (Pi~nol et al. 2014).

More than a few models use a Bayesian modelling approach to generate the later probabilities of species assignments taking into account of reservations due to unknown gene trees and the inherited coalescent development (Yang and Rannala 2010), including the bPTP (Zhang et al. 2013), BAPS and STACEY. Some of these methods have been applied in modern studies on green microalgae (e.g. Darienko et al. 2015). We can then understand environmental preservation and evolutionary processes calculating and structuring marine ecosystem biodiversity.

Genomic DNA

DNA bases pair up with all others, A with T and C with G, to arrangement units called base pairs. Each base is also devoted to a sugar molecule and phosphate molecules (Alberts et al. 2014). Together, a base, sugar and phosphate are called a nucleotide. Nucleotides are arranged in two long strands that form a spiral called a double helix (Irobalieva et al. 2015). The structure of the double helix is rather like a ladder, with the base pairs establishing the ladder's rungs and the sugar and phosphate molecules forming the vertical sidepieces of the ladder.

An important stuff of DNA is that it can replicate. Both strands of DNA in the double helix can act as a pattern for replicating the sequence of bases.

Principle of Genomic DNA

The isolation and purification of DNA from cells is one of the greatest mutual procedures in contemporary molecular biology and symbolizes a transition from cell biology to the molecular biology (from in vivo to in vitro). The isolation of DNA from microalgae is a comparatively simple process. The organism to be used should be grown up in a capable medium at an optimal temperature and should be gathered in late log to initial immobile phase for maximum harvest. The genomic DNA isolation is essential to separate total DNA from RNA, protein, lipid, etc. Primarily the cell membranes must be interrupted in obligation to release the DNA in the extraction buffer. Once the cell is interrupted, the endogenous nucleases tend to source extensive hydrolysis (Ausubel et al. 1991). Nucleases speciously present on human fingertips are infamous for causing artificial degradation of nucleic acids during purification. DNA can be dwindling from endogenous nucleases by chelating Mg^{2++} ions using EDTA. Mg^{2++} ion is considered as a necessary cofactor for action of the

highest of the nucleases. Nucleoprotein relations are disordered with proteinase K. Proteinase enzyme is used to destroy the proteins in the disrupted cell chowder. Phenol and chloroform are used to denature and isolate proteins from DNA (Lis and Schleif 1975). Chloroform is also a protein denaturant, which alleviates the rather unstable boundary among an aqueous phase and pure phenol layer. The denatured proteins form a layer at the line between the aqueous and the carbon-based phases which are removed by centrifugation. DNA released from disrupted cells is advanced by cold absolute ethanol or isopropanol.

Agarose Gel Electrophoresis

Agarose gel electrophoresis is a method used to separate DNA and RNA molecules according to their molecular size (Maniatis 1982). This is achieved when negatively charged nucleic acids wander through an agarose gel matrix below the influence of an electric field (electrophoresis). Shorter molecules move faster and migrate beyond than the larger ones. This method is simple, rapid to perform and capable of resolving remains of DNA that cannot be separated by other processes such as density-gradient centrifugation. The position of DNA in the agarose gel is visualized by staining with low concentration of fluorescent intercalating dyes, such as ethidium bromide (Primrose et al. 2001).

Principle

Agarose is an unmodified polysaccharide of galactose with neutral charge, which is essential to obstruct interactions through charged DNA and protein molecules (Sambrook and Russell 2001). It forms large pores which are useful for parting of DNA and proteins by its molecular size. In aqueous solution, below 35 °C these polymer strands are held together in a permeable gel structure by non-covalent interactions like hydrogen bonds and electrostatic interactions (Kirkpatrick 1991). On boiling the solution, these non-covalent interactions are broken down, and the strands are separated (Devor 1983). As the solution cools, these non-covalent interactions are re-established, and the gel is formed (Helling et al. 1974). Purified agarose is insoluble in water or buffer at room temperature but dissolves on boiling water.

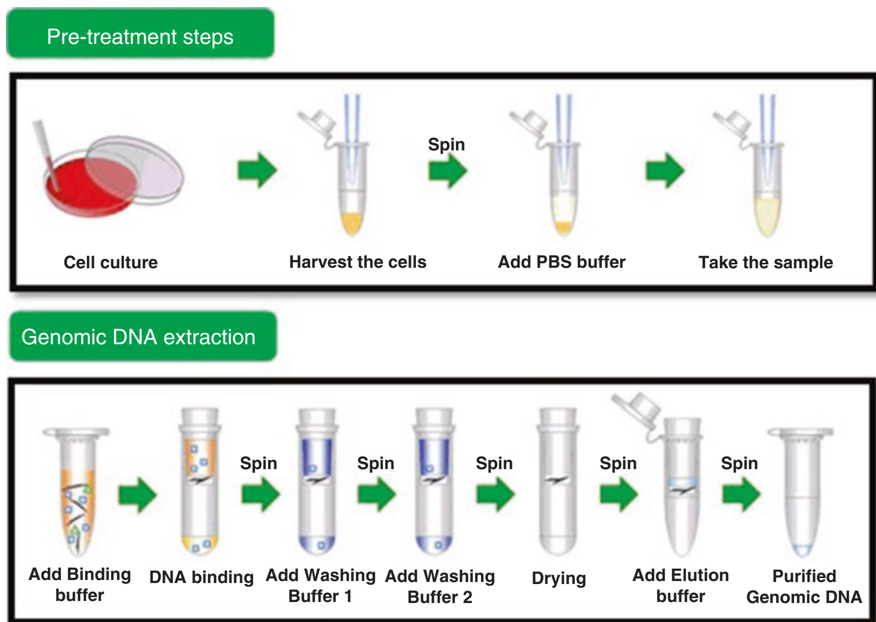
Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is a laboratory technique for DNA replication that allows a ‘target’ DNA sequence to be selectively enlarged (Bustin 2004). PCR can use the smallest sample of the DNA to be duplicated and amplify it to millions

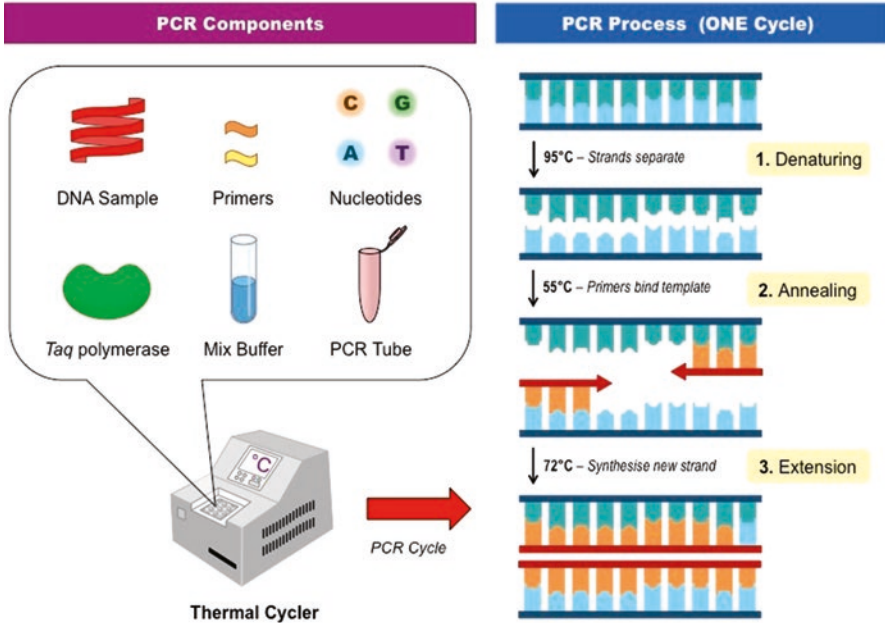
of reproductions in just a few hours. PCR was discovered in 1985 by Kerry Mullis, PCR has developed both crucial and routine tool in most biological laboratories.

Principle of PCR

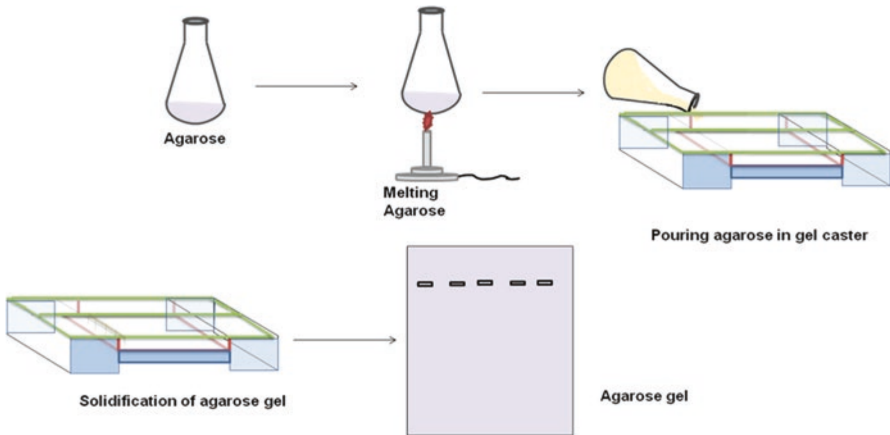
The PCR contains the primer-facilitated enzymatic amplification of DNA. PCR is based on using the capability of DNA polymerase to synthesize new strand of DNA corresponding to the offered template strand (Harris 1998). Primer is necessary because DNA polymerase can add a nucleotide solitary onto an earlier 3′-OH group to add the first nucleotide (Innis et al. 1990). DNA polymerase then elongates its 3′ end by accumulating more nucleotides to produce a prolonged region of double-stranded DNA (McPherson et al. 2000).



Workflow (1): Genomic DNA isolation (Source: *https://www.creative-biogene.com/blog/index.php/2016/01/19/isolation-and-purification-of-genomic-dna/***)**



Workflow (2): PCR (polymerase chain reaction) (Source: *http://ib.bioninja.com.au/standard-level/topic-3-genetics/35-genetic-modification-and/pcr.html*)



Workflow (3): Agarose gel electrophoresis (Source: *http://nptel.ac.in/courses/102103047/module3/lec17/2.html*)

Materials and Methods:

General Molecular Methods Following for Microalgae

1. Genomic DNA isolation
2. Polymerase chain reaction (PCR)
3. Agarose gel electrophoresis (AGE)
4. Phylogenetic tree analysis
5. DNA barcoding

Method (I): Liquid Nitrogen Method

Materials Required

Samples, distilled water, Eppendorf vial, liquid nitrogen, glass beads, centrifuge, PCR, 2-propanol, primers, QIAquick PCR purification kit (Qiagen), agarose gel electrophoresis and ethidium bromide staining

Isolation of Genomic DNA

A microalgae subsample is pulverized and rehydrated with 10–100 ml distilled water in a 0.5 ml Eppendorf vial. The slurry is homogenized and exposed to five freeze–thaw cycles, alternating immersion in liquid nitrogen and heating to 60 °C. Samples are additionally exposed to homogenization with glass beads at 5000 rpm for 5 min (0.10–0, 11 mm diameter glass beads, B. Brown Bideck; Mini-Bead-Beater, BioSpec Products). Rehydrated cells and the cell-free supernatant fractions are either used directly in PCR amplifications or the mixture is exposed to phenol/chloroform extraction and centrifuged, and the aqueous phase is mixed with the 0.6 volume of 2-propanol. DNA is recovered and stored in a minimal volume of sterile, redistilled water.

PCR Amplification

For the amplification of approximately 1200 bp of 16S rRNA gene fragments, primers described by Nadeau et al. (2001) will be used. In some cases, primers and PCR cycling conditions described are additionally applied. The reaction volume is 50 ml and contained 16 REDTaq PCR buffer, 200 mM each deoxynucleotide, 100 mg

BSA, 250 ng each oligonucleotide primer, 2.5 U of REDTaq DNA polymerase (Sigma-Aldrich) and 5 ml DNA extract. To minimize amplification after non-specific annealing of the primers to nontarget DNA, polymerase is added to the reaction mixture after the initial denaturation step (5 min at 94 uC) at 80 uC. Thirty-five incubation cycles are performed, each consisting of 1 min at 94 uC, 1 min at 55 uC and 1 min at 72 uC. The presence of PCR products is detected by standard agarose gel electrophoresis and ethidium bromide staining. Purified amplification products (QIAquick PCR purification kit, Qiagen) are commercially and directly sequenced in both directions.

Phylogenetic Analysis

Gene sequences obtained from the herbarium samples are aligned with selected GenBank entries using Clustal W 2.0 server (http://www.ebi.ac.uk/clustalw/index.html) at the default configuration, and a fragment of approximately 1154 bps (corresponding to *Escherichia coli* residues 269–1438) was used for the calculation of a neighbour-joining tree (Jukes and Cantor distance estimation, 1000 bootstraps) with TREECON for Windows 1.3b software (Van de Peer and De Wachter 1994). *E. coli* was used as the out-group.

Method (II): Phenol–Chloroform Method

Materials Required

Samples, distilled water, phenol–chloroform, Eppendorf vial, liquid nitrogen, glass beads, centrifuge, PCR, 2-propanol, oligonucleotide primers, QIAquick PCR purification kit (Qiagen), TAE agarose gels, agarose gel electrophoresis and ethidium bromide staining

Genomic DNA Isolation

DNA Extraction, PCR Amplification of 18S and 23S rRNA and Sequencing of phytoplankton (microalgae)

The total cellular DNA is extracted from algal isolates by using phenol–chloroform method. The genomic DNA is stored at 20 °C until used for PCR amplification. Two oligonucleotide primers (18SF: 50-CCAACCTGGTTGATCCT GCCAGTA-30; 18SR: 50-CCTTG TTACGACTTCACCTTCCTCT-30) and (23SF: 50GGACAGAA-AGACCC TATGAA-30; 23SR: 50-TCAGCCTGTTAT

CCCTAGAG-30) are used for PCR amplification of partial 18S and 23S rRNA genes, respectively (Yu et al. 2007; Sherwood and Presting 2007). Each PCR reaction mixture consisted of a 25 μl solution containing 1 μl of DNA template,1 μl of forward primer, 1 μl of reverse primer, 12.5 μl of 2X PCR TaqMixture (Himedia, India) and 9.5 μl of sterile water. The PCR amplification is performed in an Eppendorf Mastercycler ep gradient S thermocycler using the following thermal programmes:

18SrRNA amplification: preheating at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 63 °C for 1 min and extension at 72 °C for 1 min followed by another 10-min extension at 72 °C

23S rRNA amplification: preheating at 94 °C for 5 min followed by 35 cycles of denaturation for 1 min at 94 °C, annealing at 57 °C for 1 min and extension at 72 °C for 1 min followed by another 10-min extension at 72 °C

The PCR products are visualized by electrophoretic separation on 1.2% TAE agarose gels.

Method (III): Phenol: Chloroform: Isoamyl Alcohol

Materials Required

Samples, distilled water, Tris-HC1, Na_2EDTA, phenol/chloroform/isoamyl alcohol, sodium dodecyl sulphate (SDS), Eppendorf vial, liquid nitrogen, glass beads, centrifuge, PCR, 2-propanol, oligonucleotide primers, 'Gene clean' kit, QIAquick PCR purification kit (Qiagen), TAE agarose gels, agarose gel electrophoresis and ethidium bromide staining

DNA Extraction and Purification

About 300 m1 of phytoplankton culture is sedimented by centrifugation at 2600 X g for 15 min. Cell pellets are mechanically disrupted in the presence of 2 volumes of a mixture containing 50 mM Tris-HC1 pH 8.3, 50 mM Na2EDTA, 6% (v/v) redistilled phenol and 2% (w/v) sodium dodecyl sulphate (SDS). An equal volume of redistilled phenol which has been previously saturated with 50 mM Tris-HC1 pH 8.3 is added to the lysates and the whole suspension vortexed for 1 min and centrifuged for 3 min at maximum speed in a top-bench mini-centrifuge. The supernatant is transferred into a new tube, mixed with an equal volume of phenol/chloroform/isoamyl alcohol (24:24:1), vortexed for 30 S and centrifuged as described above. Eventually the supernatant is washed three times with

water-saturated ether, brought to 0.2 M (final) with sodium acetate, mixed with 2.5 vol of cold ethanol and stored overnight at −20 °C. DNA is precipitated by 10 min centrifugation in a top-bench mini-centrifuge, desiccated, resuspended in 30 μl sterile distilled water, desalted using a 'Gene clean' kit (Bio 101, La Jolla, CA, USA) and stored at −20 °C at a concentration of about 100 ng. The same protocol is used to extract DNA from natural phytoplanktonic populations. In this case, however, the cells are lyophilized for few hours after their recovery.

Amplification Conditions

The PCR is done in a 50 1.11 volume and contained about 100 ng DNA, 50 mM KC1, 10 mM Tris-HC1 (pH 8.4 at room temperature), 1.5 mM MgC12, 100 pg ml^{-1} gelatin, 0.25 pM of each oligonucleotide primer, 200 pM of each deoxynucleotide triphosphate and 2.5 units of *Taq* polymerase. The reaction is performed in a DNA 'Thermal Cycler' (Perkin-Elmer, Norwalk, CT, USA). Before adding the polymerase to the reaction mixture, template DNA is denatured at 95 °C for 7 min. Reaction conditions are as follows: 94 °C for 20 S (denaturation), 55 °C for 20 s (annealing) and 72 °C for 30 S (elongation) for a total of 40 cycles.

Method (IV): Molecular Data Analysis and DNA Barcoding

Sequences are aligned automatically using Clustal W (Thompson et al. 1994) under default parameters using MEGA5 software (Tamura et al. 2011). For similarity searches, the rbcL sequences are submitted to the Barcode of Life Data Systems (BOLD Systems) using the plant identification tool, while sequences are submitted to the Basic Local Alignment Search Tool (BLAST) for comparisons against nucleotide sequences deposited at the GenBank. The sequence secondary structures are predicted by either direct fold (energy minimization) or homology modelling (Koetschan et al. 2012). The barcode gap is inferred based on uncorrected pairwise (p) distance matrices. MEGA5 software is used for calculation. The dendrograms are constructed through the maximum likelihood (ML) method using MEGA6.0 software. The GTR model with invariable sites (I) and gamma distribution shape parameter (G) is chosen. The neighbour-joining (NJ) algorithm is used to generate the initial tree for ML computation. A phylogenetic test using the bootstrap method (1000 replicates) is used.

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Replacement of Fishmeal with *Arthrospira* (*Spirulina*) *platensis* and Its Use in Freshwater Prawn *Macrobrachium rosenbergii* Production

T. Muralisankar, P. Santhanam, S. Radhakrishnan, N. Manickam, and P. Saravana Bhavan

Introduction

Next to agriculture, aquaculture plays a major role in food production around the world. Finfish and shellfish have a vital role in aquaculture operations. Among these, crustaceans, such as prawns, shrimps, crayfish, lobsters and crabs have vital role due to their nutritious delicacy for mankind. *Macrobrachium rosenbergii* is one of the important cultivable freshwater prawn species due to its wide variety of environment tolerances like temperature and salinity. *M. rosenbergii* is a candidate species in rural aquaculture for economic development (Tayamen 2007). In prawn culturing industries, about 40–60% of the operational expenditure is incurred towards the feeds used. Among the feed ingredients, fishmeal plays a major role due to the presence of high digestible protein, however, it is an expensive one due to low or unpredictable availability (Maliwat et al. 2016). Hence, there is a need to search

T. Muralisankar (✉)
Aquatic Ecology Laboratory, Department of Zoology, Bharathiar University, Coimbatore, Tamil Nadu, India

Marine Planktonology and Aquaculture Laboratory, Department of Marine Science, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

P. Santhanam · N. Manickam
Marine Planktonology and Aquaculture Laboratory, Department of Marine Science, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

S. Radhakrishnan
Aquaculture Research Station, Department of Aridland Agriculture, College of Food and Agriculture, United Arab Emirates University, Al Ain, UAE

P. Saravana Bhavan
Crustacean Biology Laboratory, Department of Zoology, Bharathiar University, Coimbatore, Tamil Nadu, India

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the alternative protein sources to replace the fishmeal in prawn aquaculture. In regard to this, a number of studies have been conducted to find alternative source for replacing the fishmeal in the diets of prawns and shrimps (Sudaryono et al. 1999; Yang et al. 2004; Samocha et al. 2004; Muralisankar and Bhavan 2013).

Phytoplankton (microalgae) consists of unicellular organisms found in marine and freshwater ecosystem. About half of global primary production has been made by photosynthesis of phytoplankton which leads to provide the primary food source for the zooplankton and together form the base of food chain in the aquatic environment. In recent years, mass culturing of microalgae (diatoms, *Chlorella*, *Arthrospira*, *Tetraselmis*, etc.) has renewed interest to produce single-cell protein for pharmaceuticals, diet supplements, pigments and biofuels and also has been used as feeds in aquaculture. It has been used as potential feed for bivalve molluscs, fishes, crustaceans in the early larval stages, and the zooplankton (rotifers, copepods, etc.). Nowadays, researchers identified that microalgae can be used as an alternative protein source for fishmeal in aquafeed formulations (Goytortua-Bores et al. 2006; Radhakrishnan et al. 2014, 2015, 2016; Abdulrahman and Ameen 2014) due to its simple culturing method and elevated level of nutrients with easily digestible cell wall which makes the nutrients available to the organism (Raja et al. 2004; Patil et al. 2007).

Arthrospira (*Spirulina*) spp. are filamentous multicellular blue-green algae (*Cyanobacterium*) that have gained notable attention in the food industries due to presence of essential nutrients like protein (55–70% in dry weight), essential amino acids (methionine, cystine, and lysine), fatty acids (rich in γ-linolenic acid, linoleic acid, stearidonic acid, eicosapentaenoic acid, docosahexaenoic acid and arachidonic acid), carbohydrates, vitamins [B complex (B1, B2, B3, B6, B9, B12), vitamins C, D and E], minerals (Ca, Cu, Fe, Mg, Mn, P, Se, Na and Zn) and pigments (chlorophyll a, β-carotene, xanthophylls, echinenone, zeaxanthin, myxoxanthophyll, diatoxanthin, canthaxanthin, etc.). Also, *Arthrospira* stimulates the immune system on both cellular and humoral arms which improves their ability to regulate stresses from environmental toxins and infectious agents (Hayashi et al. 1994; Qureshi et al. 1995). It has been used as an alternative feed ingredient for fishmeal in the diet of fish, shrimp and poultry industries. The use of these microalgae in aqua feeds as alternatives to fishmeal can promote the growth performance, proximate composition of the muscle, immune response and antioxidant status in fish and crustaceans (Desai and Sivakami 2007; Teimouri et al. 2013; Yeganeh et al. 2015; Radhakrishnan et al. 2014, 2016). The present chapter has been focused to organize some analytical procedures for replacement of cost-effective fishmeal by *A. platensis* meal for better production of freshwater prawns.

Culture of *A. platensis*

Collection and Isolation

A. platensis can be collected from the aquatic environments by using planktonic nets (150–200 μm) or, if in sufficient quantity (1 L of green coloured water), by scooping a jar through the water. The collected water samples can allow overnight to settle the algae on the bottom of the container, followed by isolation of *A. platensis* can be made by serial dilution method with aseptic technique. For instance, dispense 9 ml of culture media (Zarrouk's medium) into each of the ten test tubes using sterilized automatic dispenser or 10 ml pipettes. Test tubes have to be labelled as 10^{-1}–10^{-10} to indicate dilution factor. One millilitre of enrichment sample is to be added to the test tube (10^{-1}) and mixed gently. One millilitre of this dilution is taken and added to the next test tube (10^{-2}) and then mixed gently. This procedure is repeated for the remaining test tubes (10^{-3}–10^{-10}). These tubes are incubated under controlled environment (temperature, 25–30 °C; light conditions, 2000 lux). After 2–4 weeks, cultures are examined microscopically by collecting a small sample aseptically from each dilution tube. The unialgal culture of *A. platensis* can be obtained in one of the higher-dilution (10^{-6}–10^{-10}) tubes.

Culture Media

Preparation of culture medium is one of the major constraints in the large-scale production of *A. platensis* at rural level. *A. platensis* grows in highly alkaline, saline and static waters of tropical and subtropical areas. The artificial culture medium has been developed by Zarrouk for culturing *A. platensis*, and it has been a widely accepted medium that satisfies the required nutrients like mineral salts for maintaining optimal environments to the algae. This medium is well suited for lab-scale cultures of *A. platensis*, whereas, it is expensive for outdoor mass cultivation. The detail composition of Zarrouk's medium is presented in Table 1 (Belay 2008). The culture environment should be maintained at 25–30 °C temperature and illuminating with florescence lamps at 2000 lux with 12:12 h light dark cycles and manual shaking of culture will made everyday. Further, freshwater culture medium can be maintained at 8–9 pH and 4–5 ppt of salinity (for algae collected from freshwater environment), whereas the salinity can be maintained at 21–24 ppt for seawater medium (for algae collected from marine environment).

Table 1 Composition of Zarrouk's medium

Component	Concentration (g/L)
Sodium bicarbonate ($NaHCO_3$)	18.0
Sodium nitrate ($NaNO_3$)	2.5.0
Potassium sulphate (K_2SO_4)	1.0
Sodium chloride (NaCl)	1.0
Dipotassium phosphate (K_2HPO_4)	0.50
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	0.20
Disodium ethylenediaminetetraacetate (Na_2 EDTA)	0.08
Calcium chloride ($CaCl_2$)	0.04
Ferrous sulphate ($FeSO_4 \cdot 7\ H_2O$)	0.01
Trace elements solution[a]	1 ml
Final pH of solution	8.2

[a]H_3BO_3, 2.860; $MnCl_2 \bullet 4H_2O$, 1.800; $ZnSO_4 \bullet 7\ H_2O$, 0.220; Cu_2SO_4, 0.080; $(NH4)_6\ Mo_7\ O_{24} \bullet 4H_2O$, 0.020; vitamin B_{12} 5×10^{-6} (g L^{-1})

Stock Culture

A. platensis are kept on hand as stock culture during initial culture as a source of 'clean' algae when the mass cultures become contaminated or die or are completely harvested, the stock culture can be used as inoculum for mass culture. The culture system needs a set of stock cultures kept in heat-resistant and transparent test tubes or conical flasks (250 ml or 500 ml) closed with cotton wool bungs. The sufficient amount of stock culture should be kept for the next step in vessel size. Air is one of the potential sources of contamination of the culture, hence, it is necessary to avoid aeration. Partially filled containers can give a better surface area for gas exchange than full flasks. Therefore, each flask is partially filled with culture media, this practice provides the stable, long-lived and uncontaminated stock cultures.

Subculture

Algal stock cultures should be subcultured regularly on a weekly basis. This process involves inoculation of some cells from old into fresh culture medium and it allows that the cells can remain healthy and continue to grow. If this process is not carried out properly, the algal cells in the stock culture will die. It is important to prevent air from entering in the stock culture during subculturing of the algae. To start a subculture (new stock culture), 20 ml of algal cells are taken from the stock culture which

has been growing for 6–7 days and transferred into flask containing 250 ml of fresh culture medium, followed by the mouth of flask to be closed with cotton wool bungs, and the necks of flasks should be sterilized by gas flame. The new growing stock culture has to be placed near (about 20 cm) the fluorescent lamp constantly. After subculturing, the remaining old stock culture will be used to make a batch culture of up to 10 L.

Indoor Mass Culture

A large-scale culture of *A. platensis* is necessary for feeding larvae of aquatic animals including molluscs and crustaceans in hatcheries. The indoor mass culture of *A. platensis* can be performed with following containers: glass carboys (20 L capacity), polythene bags (10 L capacity), transparent Perspex tanks (100 L capacity), transparent cylindrical FRP tanks and FRP tanks inside coated with pure white (1 ton capacity). These containers can be placed on wooden racks and frames providing with optimum light and aeration facilities. For mass culture of algae in these containers, well-grown cells from the stock culture room are used as inoculums. About 100 ml, 250 ml and 100 L of the inoculums are used for the polythene bags, glass carboys and Perspex tanks, respectively, and they are adequately lighted and aerated. These containers will have the maximum concentration of algal cells in the growing phase on the fifth to sixth day which can be harvested. To continue the culture, 2 L of the same culture may be leaved and enriched with fresh medium in the same container.

Outdoor Mass Culture

To make outdoor mass cultures of algae, the density of algal cells should be kept at a high level during the process, because maximum level of light falling will be absorbed by surface culture. This process can reduce the cost of water handling during harvesting the algal cells from the culture medium. Also, outdoor mass culture must be designed to provide an adequate ratio of illuminated surfaces to the cells. FRP tanks and concrete tanks with the capacity of 1–5 ton and 5–10 ton will be used for this culture, respectively. Well-grown algal cells from the stock culture are used as inoculum for the mass culture along with these adequate quantities of chemical or fertilizer (10, 10, and 100 mg/L of urea 46, superphosphate and ammonium sulphate respectively) which may be added. Algal cells should be harvested when the containers having maximum concentration during growing phase of 3rd to 4th day or before going to the declining stage.

Growth Phases of Algae

The laboratory culture of microalgae has limited volume of medium which contains necessary nutrients along with a relatively small number of cells, and these are exposed to suitable environment conditions. Usually, the increase in cell volumes in this culture follows a characteristic pattern in which the following five growth phases may be recognized.

Lag or induction phase: Cells collected from the stock culture inoculated into a new container have to acclimatize to the new culture medium. It leads to preventing the cell division for a few hours. This stage is known as lag or induction phase. *Exponential phase*: The acclimatized cells start to multiply and grow rapidly within 12–18 h and this process continues until the cells reach maximum concentration. This growing stage is known as exponential phase. *Declining phase*: The growth and multiplication of cells will be arrested slowly when it reached maximum concentrations (it is a symptom of decline). This stunted growth stage of the cells is known as declining phase. *Stationary phase*: When the algal culture reached decline phase, it will be stationary for a few days without any further division. During this stage, cells may develop some features to thrive the unfavourable environments, which are used to start further growth and reproduction of cells when they moved to new environment. *Death phase*: Retaining the algal cells in the stationary phase for a long period, cells may lose their viability and start to die, and the culture will become ineffective for both re-culturing and feeding.

Determination of Algal Cell Densities

To make the production successful, it is essential to monitor the algal growth in mass cultures. By comparing actual cell numbers with the previous day's count, we can get accurate measure of growth. Sampling of mass culture can be made by using rubber tubing and a sterile pipette. Place the algal cells onto the Sedgewick-Rafter counting chamber, and allow standing on a flat surface for 20 min to settle the algal cells. Followed by cells are counted using upright light microscope with the 4x or more usually in 10x objectives of the compound microscope. In order to provide a sample grid, a Whipple disc may be inserted into the ocular lens. Determination of Whipple area is necessary when each set of ocular and objective lenses is used (this process is performed with a stage micrometre). Counting chamber of Sedgewick-Rafter comprises a total of 1000 fields which consisted of 50 and 20 fields in length and width, respectively (a horizontal strip corresponds to 50 fields). All cells are counted within the randomly selected fields.

The haemocytometer is also used for counting the algal cells. For this, algal cells are killed with a drop of eosin or formalin and then by stirring well. A drop of killed cells is taken with sterilized pipette, followed by placing the coverslip on the haemocytometer. Pipette should be brought to the edge of the cell counter, and touch it,

followed by pouring the sample. The poured sample will run inside the counting chamber, and cells should be equally distributed in the chamber which will be indicated by observing the thin film of the cell culture. Generally, the counter has 9 chambers (4 sides having 16 divisions and 5 chambers of multiple divisions), counting can restrict with minimum number of chambers (least 4) instead of all chambers. The average number of cells in 1 ml will be calculated, and the following equation can be used for calculating the total cells count:

$$\text{Total number of cells}\left(\text{cells / ml}\right) = \text{average count of chamber} \times 10^4$$

Harvesting and Processing of A. platensis

A. platensis is one of the easily harvestable algae species. It can be performed by simple filtration through 60 mesh fabrics. The harvested algal slurry must be rinsed with tap water in order to leave out remaining residues. This will be filtered and reused and will be used for further cultivation due to the presence of high level of minerals, especially bicarbonate, however, the exhausted chemicals have to be replenished. To prevent contamination in the culture, the replenished medium must be discarded after 4–6 cycles of cultivation. Finally, the harvested algae will be sundried, and this drying process can also be improved by solar driers.

Proximate Composition Analysis of A. platensis

Analysis of proximate composition of any algae is potentially important for it serves as a food or feed source for human and animals. The proximate composition of algae includes crude protein, amino acids, carbohydrates, lipids, ash, moisture and energy levels.

Crude Protein Analysis on Dry Weight Basis

The crude protein can be estimated according to the standard procedure of AOAC (1995) using Kjeldahl apparatus. For this determination, about 2 g powdered algae samples are digested along with 5:1 ratio of Na_2SO_4 and $CuSO_4$ (catalyst mixture) in a Kjeldahl digestion flask by boiling with 20 ml of concentrated H_2SO_4 at 420 °C for 45–60 min until the mixture is clear. When the digestion is completed, flasks are allowed to cool for 20 min, and then add 50 ml of distiller water to the digestion flasks. Place adequate volume of NaOH (40%) in alkali tank of steam distillation unit. Before starting the distillation, make sure that the sufficient amount (about 50 ml) of NaOH is released to neutralize the acid in the flask. Place a conical flask

(250 ml capacity) containing 25 ml of boric acid (4%) solution with an indicator (methyl red) on the receiving stage. Ensure that the condenser tube should touch below the surface of the boric acid in the conical flask. Further, connect the digestion tube (containing cooled and diluted digest) to steam distillation unit. Steam distills about 100–125 ml distillate to the receiver flask and then titrates against 0.1 N HCl solution until the solution changes into pink colour, and note the burette reading (amount of 0.1 N HCl dispensed). The total nitrogen and crude protein can be calculated as follows:

Total nitrogen (%) = atomic mass of nitrogen (14) × normality of HCl (0.1) × burette reading × 100/weight of the sample × 1000.

For determination of crude protein (%) = total N (%) × 6.25 (based on assumption that nitrogen constitutes 16% of protein).

Total Protein Analysis on Wet Weight Basis

The amount of total protein can be estimated following the standard procedure of Lowry et al. (1951) using alcoholic precipitated sample. Prepare 'solution A' by dissolving 2 g of Na_2CO_3 in 100 ml of NaOH (0.1 N), 'solution B' by dissolving 500 mg of $CuSO_4$ in 1% sodium potassium tartrate solution and 'solution C' by mixing 50 ml of solution A with 1 ml of solution B. For estimation of protein in the sample, homogenize a known amount of sample using 2 ml of 80% ethanol. Then centrifuge the sample at 5000 rpm at 4 °C for 15 min. Dissolve the precipitate in 1 N NaOH and make up to 5 ml. Take 0.5 ml of precipitated solution and then add 5 ml of the solution C, and incubate for 20 min in room temperature, followed by adding 0.5 ml of Folin-Ciocalteus reagent. The intensity of the colour developed can be read at 660 nm using spectrophotometer. Bovin serum albumin (1%) in 1% sodium dodecyl sulfate is used as standard solution and 0.5 ml of 1N NaOH is used as blank solution. The presence of total protein in the sample can be calculated as follows:

Protein present in the sample (mg/g) = OD of the sample/OD of the standard × conc. of the std. (mg)

Total Amino Acid Analysis

The concentration of total free amino acid will be assayed by the ninhydrin method (Moore and Stein 1948). Briefly, homogenate 1 g of sample with 2 ml distilled water, followed by adding 1 ml of 10% sodium tungstate and 1 ml H_2SO_4 (2/3 N) solutions. Centrifuge this mixture at 3000 rpm for 10 min and collect the supernatant. Three test tubes are taken and labelled as test, blank and standard, respectively. Further, 0.5 ml of supernatant (test) and 0.5 ml of 1% standard leucine (in 80% ethanol) solutions will be poured into the respective test tubes, and increase the volume up to 5 ml by adding distilled water. For blank solution, 5.0 ml distilled water will

be poured in the third test tube, followed by pipetting accurately 0.5 ml of ninhydrin into all test tubes closed with cotton bungs. The tubes are kept in boiling water bath until blue colour appeared. Then the tubes are cooled at room temperature, and the colour intensity will be measured with spectrophotometer at 540 nm. The presence of total amino acids can be calculated as follows:

Amino acid present in the sample (mg/g) = OD of the sample/OD of the standard × conc. of the std. (mg)

Total Carbohydrate Analysis

Total carbohydrate content will be estimated by the method of Roe (1955) using TCA-extracted sample. For this estimation, a known amount of sample will be homogenized well in 2 ml of ethanol (80%), and centrifuge the homogenate at 5000 rpm for 15 min. From this homogenate, take 0.5 ml of clear supernatant, and pour 5 ml of anthrone solution (50 mg of anthrone in 100 ml of H_2SO_4 (66%), to this 1 gm of thiourea will be added to stabilize the colour). Keep the tube in the boiling water bath for 15 min, and then keep it in the dark place to protect from the light. Read the optical density at 620 nm. Accurately 0.5 ml of 1% glucose (in saturated benzoic acid) is used as standard and 0.5 ml of 80% ethanol is used as blank solution. The presence of carbohydrate can be calculated as follows:

Carbohydrate present in the sample (mg/g) = OD of the sample/OD of the standard × conc. of the std. (mg).

Crude Lipid Analysis

Crude mixture of fat-soluble material present in a sample is known as crude lipid or crude fat. It is also known as ether extract or the free lipids in traditional measure of fat food products. Crude lipids can be estimated by standard methods of AOAC (1995) using Soxhlet method. For this analysis, known quality (minimum 2 g) of the dried algae sample is taken. Thimble is placed inside the Soxhlet apparatus, and dry solvent flasks (preweighed) are connected beneath the apparatus, followed by adding required quantity of solvent (petroleum or ethyl ether) and connecting the condenser to the extraction chamber. The heating rate is adjusted to give a condensation rate of 2–3 drops per second for 16 h as low setting. At the end of the extraction, thimble is removed, and the ether is distilled at 105 °C for 30 min and collected in another flask. Finally, the residual of crude fat is desiccated and weighed. Crude lipid can be calculated as follows:

$$\text{Crude lipid}\left(\%\text{of dry matter}\right) = \text{weight of fat} / \text{weight of sample} \times 100.$$

Total Lipid Determination

Total lipid extraction will be made using chloroform-methanol mixture (Folch et al. 1957) and estimated according to standard method of Barnes and Blackstock (1973). Weigh a known quantity of sample, and extract the total lipid using 2 ml of chloroform-methanol mixture (2:1 ratio). Add 0.2 ml of 0.9% NaCl solution to the extraction and shake well. Pour this mixture into a separating funnel, and allow standing for few hours until a clear biphasic layer is formed. Lower phase will be separated into a clean tube, and increase the volume to 2 ml by adding chloroform. To estimate the total lipid, take 0.5 ml of extract into a test tube, and allow evaporating the solvent at 50–60 °C for 5 hr. Further, dissolve the extract in 0.5 ml of concentrated H_2SO_4, and then plug the tubes with nonabsorbent cotton bungs and allow boiling in a water bath for 10 min, followed by cooling the tubes at room temperature. Take 0.2 ml of this acid digest in a separate clean tube with 5 ml of phosphovanillin reagent (0.2 g of vanillin in 80% of orthophosphoric acid), shake this mix and allow standing for 0.5 h. Finally, observe the optical intensity of the developed colour at 520 nm using spectrophotometer. Cholesterol (8 mg of cholesterol in 4 ml of 2:1chloroform-methanol mixture) will be used as standard. For blank, take 0.2 ml of chloroform, allow it to evaporate. Total lipid content of the sample can be calculated as follows:

Lipid present in the sample (mg/g) = OD of the sample/OD of the standard × conc. of the std. (mg).

Determination of Ash

The sample is burned at 600 °C, the nonvolatilized inorganic material at this temperature is known as ash. Exactly 1 g of sample is weighed and placed in the moisture-free preweighed crucible and placed in a muffle furnace at 600 °C for 6 h. Transfer the crucibles to the desiccator with the help of metal tongs and cool at room temperature. The crucibles are weighed as quickly as possible to avoid moisture absorption. The percentage of ash will be calculated using the following equation:

$$\text{Ash}\left(\%\right) = \text{WA} / \text{WS} \times 100$$

where WA is the weight of the ash and WS, weight of the sample.

Determination of Dry Matter and Moisture

The moisture of the sample is lost by volatilization caused by heat. The amount of moisture-free material is called as dry matter. Accurately 1 g of sample is placed into a preweighed dish and placed in oven at 105 °C for overnight. Then remove dishes from oven and place in a desiccator and cool, followed by weigh the sample

as an earlier time. The amount of dry matter and moisture can be calculated as follows:

$$\text{Dry matter}(\%) = \text{final weight of sample}(\text{g}) / \text{initial weight of sample}(\text{g}) \times 100.$$

$$\text{Moisture}(\%) = \text{initial weight of sample}(\text{g}) - \text{final weight of sample}(\text{g}) / \text{initial weight of sample}(\text{g}) \times 100.$$

where final weight is the weight of sample after drying and initial weight, weight of samples before drying.

Determination of Nitrogen-Free Extract or Crude Soluble Carbohydrates

The content of nitrogen-free extracts (NFE) or crude soluble carbohydrate is computed by the following formula:

NFE (%) = 100 − (percentage of moisture + percentage of crude protein + percentage of crude lipid + percentage of total ash + percentage of crude fibre).

Amino Acid Profile Analysis of **Arthrospira** *sp.*

The amino acids are analysed by high-performance liquid chromatographic (HPLC) system in samples (algal, tissues, feed ingredients and formulated feeds) according to Ali and Dayal (2006). Amino acids can be separated under gradient elution using a column packed with a Na^+-type strong acid cation exchange resin (styrene-divinylbenzene copolymer with sulfonic group). The amino acids are detected and quantified using a fluorescent detector after post column derivatization of the amine or amino (NH_2) functional group with ortho-phthalaldehyde (OPA) and β-mercaptoethanol (BME).

Reagents Required

- (i) Mobile phase A: 19.6 g of sodium citrate, 70 ml of ethanol and 14.2 ml of perchloric acid will be dissolved in 800 ml distilled water, then adjust the pH to 3.20 and make volume up to 1000 ml and filter the solution using 0.47 μm nylon membrane filter.
- (ii) Mobile phase B: 58.8 g of sodium citrate and 12.4 g of boric acid will be dissolved in 400 ml of distilled water, followed by adding 30 ml of 4 N NaOH, and adjust the pH to 10 and make volume up to 1000 ml, and filter this solution using 0.47 μm nylon membrane filter.
- (iii) Mobile phase C: 4 g NaOH will be dissolved in 500 ml distilled water, and filter through nylon membrane with pore size 0.47 μm.

(iv) Boric acid-carbonic acid buffer: 40.7 g of Na_2CO_3, 13.6 g of H_3BO_3 and 18.8 g of K_2SO_4 will be dissolved separately in distilled water, and make the volume up to 1000 ml.
(v) Brij-35 solution: 5 g of Brij-35 will be dissolved in 50 ml of distilled water and kept in a wide mouth bottle.
(vi) Sodium hypochlorite solution at less than 7%.
(vii) Reaction reagent-I: 500 ml of boric acid – carbonic acid buffer will be mixed with 0.2 ml of sodium hypochlorite solution and filtered using Whatman (No.1) filter paper.
(viii) Reaction reagent-II: 400 mg of ortho-phthalaldehyde will be dissolved in 7 ml ethanol, followed by adding l ml of β-mercaptoethanol and 2 ml Brij-35 solution, and make the volume up to 500 ml using boric acid-carbonic acid buffer. Filter the solution using Whatman (No.1) filter paper.
(ix) Hydrochloric acid (6 N)
(x) Hcl (1%).
(xi) NaOH pellets.
(xii) Diluent: 9.8 g of trisodium citrate will be dissolved in 400 ml distilled water, followed by adding 8 ml perchloric acid and 50 pl n-caprylic acid, and adjust the pH to 2.2 and make the volume up to 500 ml.
(xiii) High pure standard amino acids containing 10 μg of each amino acid per ml use (undiluted).

Acid hydrolysis of Protein Sample Take accurately 50 mg of powdered sample in a labelled clean glass ampule (10 ml capacity). Clean the ampule sides by using and adding 5 ml of HCl (6 N) solution. Evacuate and seal the ampule and hydrolyse at 110 °C for 22 h, and then cool and centrifuge few minutes at 2000 rpm. Then break the seal, filter the contents using filter paper (acid washed) and rinse the filter paper with HCl (1%) for several times. Further, the filtrate is pooled, and make up the volume up to 25 ml with HCl (1%). Then the 5 ml of filtrate will be evaporated using a flash evaporator, and traces of HCl will be removed by placing over NaOH pellets in a vacuum desiccator. Contents are dissolved with diluents and make the volume up to 5 ml. Before injection, sample should be filtered through 0.22 μ membrane.

Instrument Conditions for Amino Acid Analysis Column: Shimpack ISC-07/S1504 Na.

Mobile phase: Pump A (Mobile phase A – 0.4 ml per minute) and Pump B (Mobile phase B-Gradient); column temp should be at 60 °C.
Derivatization: Reaction reagent-I 0.2 ml per minute and reaction reagent-II 0.2 ml per minute.
Detection: Detection will be made by fluorescent detector (FLD-6A) and duration of running time will be 55 min.

Procedure The HPLC system should ready for analysis of amino acids in the samples. Feed the appropriate programme containing gradient parameters in the 'Master pump A' after obtaining the stable baseline, followed by setting of analysis file in

Table 2 The retention time of different amino acids under standard conditions

Amino acid	Retention times (min)
Asparagine	8.58
Threonine	10.50
Serine	11.30
Glutamic acid	12.48
Proline	13.78
Glycine	18.13
Alanine	19.53
Cystine	22.60
Valine	24.47
Methionine	25.89
Isoleucine	27.90
Leucine	28.62
Tyrosine	31.27
Phenylalanine	33.40
Histidine	39.62
Lysine	41.70
Tryptophan	43.94
Arginine	47.07

the Chromatopak. There will be four runs (Run 1, Run 2, Run 3 and Run 4). Run 1 – make the analysis without any injection to record the gradient profile, and Run 2: calibration parameters are specified by editing the analysis. The response factor of each amino acid will be obtained during this calibration run by the external standard calibration curve method using a single point calibration curve.

Accurately 20 μl of standard amino acid will be injected to initiate the analysis process, followed by recording the chromatogram after subtraction of the gradient profile obtained during Run 1. Further, different types of amino acids will be identified based on the specified retention time (Table 2). Corresponding response factors of each amino acid will be calculated automatically by the Chromatopak. Run 3: 20 μl of diluent will be injected, followed by recording the sample blank chromatogram. Run 4: 20 μl of pre-prepared sample will be injected and analysed. Further, the quantity of each amino acid concentration in the injected sample is estimated by the Chromatopak. Flush the column with 'Mobile phase C' for 20 min after analysis of each sample, and flush all flow lines and column with distilled water for 30 min before going to shut down the instrument.

Fatty Acid Profile Analysis of **Arthrospira** *sp.*

Fatty acid composition of algae and other samples can be analysed by gas chromatography (GC) method (Ho and Paul 2009).

Fat Extraction A known quantity (10 g) of sample is minced in a homogenizer using 4:4:1 ratio of chloroform, methanol and water, and the minced sample can be used for each batch. Vortex the slurry using vortex mixture for 10 min, and filter the homogenate using Whatman (No.1) filter paper, followed by centrifuging at 2400 rpm for 20 min at 5 °C. Then collect the chloroform from the lower layer, and remove the water drops using 10 g of Na_2SO_4 (anhydrous). Further, solvent will be dried under a nitrogen stream at 40 °C in a Dry Block Heater set. The extracted fat is stored at −18 °C until further analysis. Three similar extractions can be done for each sample as replicate.

Derivatisation of Fatty Acids Fatty acid methyl esters (FAME) can be prepared as per the standard procedures of AOCS described by Mehlenbacher (1998). Place 50 mg of fat extraction in a screw-cap tube which contains 0.5 mg of tricosanoic acid methyl ester as internal standard. Then add 1.5 ml alcoholic NaOH (0.5 N), and blanket the solution with nitrogen, followed by heating at100 °C for 5 min. Further, allow the solution to cool and add 2 ml of BF3/methanol and again heat at 100 °C for 30 min. After cooling, add 1 ml of isooctane and mix well using vortex for 30 s, followed by immediate pouring of 5 ml of saturated NaCl solution. Then the tubes are agitated vigorously, and allow standing to get clear isooctane layer (upper layer). Collect the clear upper layer and an additional extraction of the bottom layer using another 1 ml of isooctane. The isooctane extractions are combined and concentrated up to 1 ml under a nitrogen stream. The FAME samples are stored at −18 °C until used for further analyses.

Gas Chromatography FAME samples can be analysed using GC. For this, set the operation parameters of GC according to AOCS method Ce 1b-89 (Mehlenbacher 1998). Gradually increase the column temperature from 170 °C to 225 °C at 1 °C per minute. The temperatures in the injector port and the detector are to be 250 °C and 270 °C, respectively. Fix the column flow rate 2 ml per minute with a split ratio of 1:50. High purity helium gas (99.999%) should be used as carrier gas. Further, the 2 μl of FAME samples is manually injected into the GC. The results can be recorded and processed by CDS (chromatography data system). Finally, compute the percent of each fatty acid from the total peak area.

Determination of Mineral Contents

Mineral contents of algae will be determined by the atomic absorption spectrophotometry method (AOAC 1995). In this technique, elemental atoms are vaporized and atomized in the flame. The vaporized atoms absorb the light at a specific wavelength. The hollow cathode lamp can be used as light source which is made up of the same element (which has to be determined). Free atoms absorb the radiation produced by the cathode lamp in an appropriate wavelength, and the amount of energy absorbed by atoms is measured by a photodetector readout system.

Table 3 Operating parameters for determination of minerals

Element	Wave length, A	Flame	Range, μg/mL
Cu	3247	Air-C_2H_2	2–20
Fe	2483	Rich air-C_2H_2	2–20
Mg	2852	Rich air-C_2H_2	0.2–2
Zn	2138	Air-C_2H_2	0.5–5
Na	589	Air-C_2H_2	0.002–1.0
K	422.7	Air-C_2H_2	0.01–3

Concentration of the element in the sample is proportional to the amount of energy absorbed by the atoms.

Apparatus

Atomic Absorption Spectrophotometer

Several commercial models are available like PerkinElmer (model: 2380, 3030 and 403), Varian Spectra (model: A 400 and 220 AA), Bulk Scientific (model: 210 VGP), Elico (model: SL-194), etc. Since each design is different according to the different light sources, burner flow rate and detector sensitivity. Parameters of each instrument are usually given by the respective manufacturer in the form of manual. The general information of operating parameters is given in Table 3.

Reagents

EDTA solution (2.5%): 25 g Na_2H_2 EDTA will be dissolved in 1000 ml distilled water, and adjust the pH to 7.0 using 5 N NaOH (5 N).

Standard Stock Solutions

Copper stock solution (1 g Cu/ml) 1 g of pure Cu metals is dissolved in minimum amount of HNO_3, followed by addition of 5 ml HCl. Evaporate the solution almost to dryness and dilute to 1000 ml using HCl (0.1 N). **Iron stock solution (1 g Fe/ml):** Boil 1 g of pure Fe wires dissolved in 30 ml of HCl (6 N) and dilute to 1000 ml. **Magnesium stock solution (1 g Mg/ml):** 1 g of pure Mg is dissolved in 50 ml H_2O, followed by gradual addition of 10 ml HCl, and dilute to1000 ml. **Zinc stock solution (1 g Zn/ml):** 1 g pure Zn metal is dissolved in 10 ml 6 N HCl and diluted to1000 ml. **Sodium stock solution:** Accurately weigh out about 1 g of NaCl, quantitatively transfer into a 200 ml volumetric flask and dissolve in 1000 ml of deionized water. **Potassium stock solution:** 1 1.9070 g of KCl is dissolved in 250 ml of deionized water and diluted to1000 ml in a volumetric flask using deionized water.

Preparation of Sample Solution

Wet Digestion

2.5 g sample is boiled with 20–30 ml of HNO_3 in a 500 ml Kjeldahl flask for 30–45 min to oxidize all easily oxidizable matter. Then cool the solution, followed by adding 10 ml 70–72% $HClO_4$, and boil the solution very gently until the solution becomes colourless. After cooling the solution, add 50 ml of distilled water, and boil to drive out any remaining NO_2 fumes. Solution is cooled, filtered into 250 ml volumetric flask, and diluted with 25 ml HNO_3 and 100 ml of distilled water.

Determination

Set up instrument as shown in Table 3. Less sensitive secondary lines may be used to reduce necessary dilution if desired (Gatehouse and Willis 1961). Standard solutions read ≥4 within analysis range before and after each group of 6–12 samples. Flush burner with distilled water between samples, and re-set '0' absorption point each time. Calibration curve can be prepared from average of each standard before and after sample group. The concentration of samples can be read from plot of absorption against μg/ml.

Calculation

$$\text{Element}\left(\mu g/g\right) = \left(\mu\text{g/ml}\right) \times \left(\text{dilution factor/sample weight}\right)$$

Dilution factor = volume of diluted sample solution (ml) / volume of aliquot taken for dilution (ml).

Analysis of Pigments in A. platensis

Determination of chlorophyll a content: To estimate this pigment, harvest the algal cells by centrifugation at 6000 rpm for 10 min and wash with distilled water. Extract the chlorophyll a from the cell suspension with 90% (v/v) methanol at 4 °C in dim light by repeated freezing and thawing. Centrifuge until total pigment recovery. The chlorophyll content in the biomass can be calculated from the absorbance at 665 nm of the methanolic extract ($OD_{665} \times 13.9$) and expressed as μg/ml (Tandeu and Houmard 1988).

Determination of total carotenoids: Known volume algal suspension is homogenized and centrifuged at 3000 rpm for 5 min. Collect the pellet and wash with distilled water to remove traces of adhering salts. Add 2–3 ml of acetone (85%) into the pellet and then subjected to repeated freezing and thawing. Centrifuge 3000 rpm for 5 min and collect the supernatant containing pigment. To completely recover the carotenoids, repeat the extraction process till the supernatant became colourless, fol-

lowed by fractions of supernatants which are pooled and diluted up to known level. The optical density (OD) will be taken at 450 nm using acetone (85%) as blank, and the total amount of carotenoids can be calculated as follows (Saleh et al. 2011):

$$\text{Carotenoids}\left(\mu g\,/\,\text{ml}\right) = \text{OD} \times \text{volume of the extract} \times \text{dilution factor} / 2500 \times 100$$

Feed Ingredients and Preparation of Fishmeal Replacement Feeds

Feed Ingredients

Feed ingredients play a crucial role in formulation of artificial feeds in aquaculture. The commonly used feed ingredients are dry fishmeal, prawn head waste, squid waste, squilla meal, soybean meal, wheat flour, wheat bran, maize flour, ground nut oil cake, rice bran, poultry wastes, tapioca flour, fish oil, vitamin and mineral mixture. These feed ingredients may be selected based on their frequent availability, nutritive profile and low cost. For example, the feed ingredients, such as dried fishmeal and soybean meal, will be used as protein sources, wheat bran and tapioca flour will be used as carbohydrate sources, cod liver oil can be used as a lipid source, tapioca flour and egg albumin will be served as binding agents and vitamin B complex with vitamin C will also be added as a micronutrients.

Preparation of Fishmeal Replacement Diets

As a first step, separately ground the basal ingredients (fishmeal, groundnut oil cake soybean meal, wheat bran and tapioca flour) using a micro pulverizer, and sieve through a 60 μm mesh. Weigh these sieved feed ingredients at desired concentrations (Table 4) to formulate 40% protein diets which is optimum requirement for the rearing of freshwater prawn, *M. rosenbergii.* Thoroughly mix these weighed feed ingredients at different ratios for preparing different diets (Table 4). One diet should be served as a control (without replacement of fishmeal). Remaining diets can be prepared by gradual replacement of fishmeal replacement with *A. platensis* meal, for example, diet 1 (25% replacement), diet 2 (50% replacement), diet 3 (75% replacement) and diet 4 (100% replacement). Prepare the diets by thoroughly mixing the ingredient powders in sterilized water, followed by steam cooking in a closed aluminium container at 105 °C for 20 min and cooling at room temperature. Add cod liver oil, vitamin mix and egg albumin, and mix thoroughly until stiff dough is obtained. Pelletize the dough using an indigenous hand pelletizer with mesh size of 0.1 mm diameter, and cut it into about 3.0-mm-sized pieces. Dry the pellets at room temperature until reaching a constant weight (the moisture content should be below 10%). The prepared feeds will be stored individually in airtight plastic containers at

Table 4 Formulation and proximate composition of experimental diets (g/kg)

Ingredients (g kg − 1)	Control (FM)	Diet 1 (25% FMR)	Diet 2 (50% FMR)	Diet 3 (75% FMR)	Diet 4 (100% FMR)
Fishmeal	250	187.5	125	62.5	0
Groundnut oil cake	250	250	250	250	250
Soybean meal	250	250	250	250	250
Wheat bran	100	100	100	100	100
Tapioca flour	50	50	50	50	50
Egg albumin	70	70	70	70	70
Cod liver oil	20	20	20	20	20
Vitamin mix[a]	10	10	10	10	10
A. platensis	0	62.5	125	187.5	250
Proximate composition (g/kg)					
Protein	420.20	412.40	413.80	415.10	414.50
Carbohydrate	204.80	211.40	213.00	224.50	231.10
Fibre	55.60	55.43	54.75	54.36	54.15
Lipid	137	136.30	134.60	132.00	131.10
Ash (%)	11.86	12.80	13.30	141.30	143.60
Moisture (%)	9.93	9.20	8.63	7.93	7.40
Gross energy (kJ/g)	11.35	11.64	11.93	12.36	12.50

FM fishmeal, *FMR* fishmeal replacement

[a]Becosules capsules (manufactured by Pfizer), each capsule contains thiamine mononitrate 1P 10 mg; riboflavin 1P10mg; pyridoxine hydrochloride 1P 3 mg; vitamin B_{12} (as tablets 1:100) 1P 15mcg; niacinamide 1P100mg; calcium pantothenate 1P 50 mg; folic acid 1P 1.5 mg; biotin USP 100mcg; ascorbic acid 1P 150 m

−20 °C until it is used in the feeding trials. The proximate composition of the prepared feeds has to be analysed for determining whether the diets are composed of optimum nutrient for rearing of prawns (Table 4).

Determination of Water Stability of Pellet Feeds

Prawns and shrimps are slow and continuous feeders, hence, the feeds should be relatively stable in water for long durations, and it leads to consumption of feeds without waste. Poor water stability of feeds disintegrates rapidly, followed by feed waste, water pollution and poor feeding rate of prawns. Feed stability is influenced by different factors like feed composition, nature of used ingredients and moisture content. Water stability of dry shrimp/prawn feed pellets is determined by weight loss of pellets kept in water for a specified time interval by different methods. The small loss in weight of pellets indicates the stability, whereas, the higher loss indicates the poor stability.

The feed leaching can be analysed at specific time intervals like 2, 4, 6 and 8 h. Briefly, place the 1 g of feed sample in a glass bowl containing 100 ml of water.

Filter the feed containing water in No. 30 blotting silk cloth, and dry the residues using hot air oven at 65–70 °C until reaching a constant weight at each time interval. The mean weight before immersion and after drying will be used to calculate the percentage of dry matter loss. Percentage dry matter leaching can be calculated by the following formula:

$$\text{Feed leaching}(\%) = \text{dry weight}\left(\text{g}\right)\text{ at } t_0 - \text{dryweight}\left(\text{g}\right)\text{ at } t_1 \times 100$$

where t_0 is the initial weight (before immersion) and t_1 is final weight (after immersion).

Proximate Composition Analysis of Feeds

Crude Protein Estimation (See Section "Crude protein analysis on dry weight basis")

Crude Lipid Estimation (See Section "Crude lipid analysis")

Ash, Dry Matter and Moisture (See Sections "Determination of ash" and "Determination of dry matter and moisture")

Crude Carbohydrate Estimation (See Section "Determination of nitrogen Free extract or crude soluble carbohydrates")

Crude Fibre Estimation

Crude fibre is also known as indigestible carbohydrate portion of feed which consists of cellulose, hemicellulose and lignin. It is defined as the loss on ignition of the dried residues remaining after the consecutive digestion of feed sample with dilute acid and alkali under specific conditions (Natarajan 2006). For the analysis of fibre, digest moisture-free ether extracted sample with a weak acid, followed by a weak base solution, and collect the organic residue in a filter crucible. The weight loss of sample by ignition is known as crude fibre. For this analysis, place 2 g of dried fat-free sample into a 600 ml beaker, and add 200 ml of hot H_2SO_4 (0.25 N). Then place the beaker under the round bottom flask condenser unit, and boil gently for 30 min using distilled water. Filter the solution through Whatman No.541 paper in a Buchner funnel, using suction, and wash well with boiling water. Transfer residue to beaker and then add 200 ml hot 0.313 N NaOH. Replace the solution under the condenser and again boil for 30 min, and then filter through porous crucible and wash using boiling water, 1% (v/v) HCl and again boiling water. Further, wash twice using alcohol, dry at 100 °C for overnight, allow cooling and weigh. Ash the

processed material at 500 °C for 3 h followed by measuring the weigh and calculating the fibre by difference:

$$\text{Crude fibre}\left(\%\text{fat}-\text{free dry matter}\right)=\left(\text{weight of crucible with dried residue}\right)$$
$$-\text{weight of crucible with ash residue})\,/\,\text{weight of sample}\times 100$$

Estimation of Gross Energy Levels of Feed

Gross energy of feed can be calculated according to Natarajan (2006). Briefly, multiply the energy values contributed by the protein, nitrogen-free extract (NFE) and crude fat with the generalized physiological energy values of crude protein (19 kJ), NFE (15 kJ) and lipids (36 kJ), respectively. The following calculation may be performed to calculate the gross energy of feeds:

Energy contributed by crude protein (kJ/g) = content of crude protein in feed (%) × 19 = a
Energy contributed by NFE (kJ/g) = content of NFE in feed (%) × 15 = b
Energy contributed by crude lipid (kJ/g) = content of crude lipid in feed (%) × 36 = c

Therefore, gross energy (GE kJ/g) = (a + b + c)/100.

Mineral Analysis of Feeds (See Section "Determination of mineral contents")

Amino Acid Composition Analysis of Feeds (See Section "Amino acid profile analysis of Arthrospira sp.")

Fatty Acid Analysis of Feeds (See Section "Fatty acid profile analysis of Arthrospira sp.")

Feeding Experiment on Freshwater Prawn *M. rosenbergii*

Collection and Acclimatization of Prawns

Post-larvae (age PL 5–10) of prawns can be procured from hatcheries and culture ponds. Safely transport these species to the laboratory in plastic bags half filled with well-oxygenated hatchery pond water. Acclimatize the post-larvae to ambient laboratory conditions for 3 weeks in a large cement tank (1000 L) with groundwater. Adequate aeration should be provided during the acclimatization. The stocking

Table 5 Optimum water quality parameters for rearing of prawns

Parameters	Optimum level
Temperature (°C)	28–31
pH	7–8.5
Total hardness (mg/L)	30–180
Dissolved oxygen (mg/L)	3–7
Biological oxygen demand (mg/L)	10
Chemical oxygen demand (mg/L)	65
Ammonia (ppm)	<0.3
Salinity (ppt)	<10

density of post-larvae is 1–4 PL/L. The optimum water quality parameters for rearing of prawns are given in Table 5. During acclimatization period, prawns have to feed with boiled egg albumin, *Artemia* nauplii and control feed prepared from basal ingredients alternatively three times per day, and 70–80% of aquarium water must be changed daily at early morning.

Feeding Experiment

Assign five groups of uniform-sized *M. rosenbergii* post-larvae in triplicate for 90-day feeding experiment. One group will be served as control (to be feed diet without fishmeal replacement). The remaining four groups will be fed to different levels (25, 50, 75 and 100%, respectively) of fishmeal replacement diets. Each group may consist of 60 prawns in an aquarium maintaining with 60 L of groundwater. The rearing aquarium water has to be renewed every 24 h without disturbance to the prawns, and aerate adequately to maintain adequate level of dissolved oxygen. Feed the prawns with the experimental feeds at 10% of body weight twice per day. Further, collect the unfed feed, faeces and moult on daily basis while renewing the aquarium water.

Analysis of Growth Parameters of Prawns

At the end of the feeding trial, the survival, growth (length gain, weight gain, specific growth rate) and food index parameters (feed intake, feed conversion ratio and protein efficiency ratio) will be calculated by the following equations:

Survival (%) = no. of live prawns/no. of prawns introduced × 100
Length gain (cm) = final length (cm) − initial length (cm)
Weight gain (g) = final weight (g) − initial weight (g)
Feed intake (g day^{-1}) = feed eaten (g)/total number of days

Specific growth rate (% day^{-1}) = $_{log}$ final weight (g) − $_{log}$ initial weight (g)/total number of days×100

Feed conversion ratio = feed intake (g)/weight gain (g)

Protein efficiency ratio (g) = weight gain (g)/protein intake (g)

Digestive Enzyme Analysis of Experimental Prawns

A knowledge of digestive enzymes in an organism helps to determine its digestive capabilities, which helps in the selection of feed ingredients. The digestive enzymes of crustaceans have been studied over the last century for applications in physiology, biochemistry and food science. Determination of digestive enzyme activities includes preparation of crude enzyme extract and analysis of specific activity of each enzyme.

Crude Enzyme Source Preparation

The digestive tract including the hepatopancreas of prawn should be carefully dissected and homogenized in ice-cold distilled water and then centrifuged at 10,000 rpm at 4 °C for 20 min. Transfer the supernatant into new tubes, and it will be used as a crude enzyme source. Total soluble protein will be determined by the method of Lowry et al. (1951) using bovine serum albumin as standard for calculating the specific activity of enzymes.

Determination of Protease

Total protease activity can be determined by the casein hydrolysis method (Furne et al. 2005), where one unit of enzyme activity represents the amount of enzyme required to liberate 1 μg of tyrosine per minute under assay conditions. For this analysis, take the 250 μl of the substrate (1% casein) solution, 250 μl of 0.1 M phosphate buffer (pH 7.6) and 100 μl of crude enzyme extract (homogenate) in test tube. Shake and incubate the tubes at 37 °C for 1 h. After 1 h, stop the reaction by adding 600 μl of 8% TCA solution, and then keep it in ice for 1 h. After 1 h, the centrifuge the sample at 4000 rpm for 10 min. Read the absorbance of supernatant at 280 nm. Tyrosine (0.03%) can be used as standard and treated in a similar manner along with blank containing only the reagent, and total protease activity can be calculated as follows:

$$\text{Protease enzyme activity}\left(\mu\text{g} / \text{g} / \text{h}\right) = \text{OD of assay} \times \text{total volume of assay} / \text{volume of enzyme} \times \text{time}\left(\text{min}\right) \times \text{volume used in colorimetric determination.}$$

Specific protease enzyme activity (tyrosine liberated/U/mg protein) = Enzyme activity (μg/g/h)/Protein (mg/g).

Determination of Amylase Activity

Amylase activity is estimated by the starch hydrolysis method of Bernfeld (1955). One unit of amylase activity is defined as the amount of enzyme that produced 1 μg of maltose per min. For this assay, take 1 ml of 1% starch solution (substrate), 1 ml of 0.1 M phosphate buffer (pH 7.0), 1 ml of 1% NaCl and 1 ml of enzyme extract solution in test tube. Incubate the test tube for 1 h at 37 °C. After 1 h, stop the reaction by adding 0.5 ml of 3,5-dinitrosalicylic acid. Note the absorbance at 540 nm. Maltose monohydrate (0.1%) can be used as standard and treated in a similar manner along with blank containing only the reagent, and total amylase activity can be calculated as follows:

$$\text{Amylase enzyme activity}\left(\mu\text{g/g/h}\right) = \text{OD of assay} \times \text{total volume of assay/volume of enzyme} \times \text{time (min)} \times \text{volume used in colorimetric determination.}$$

$$\text{Specific amylase enzyme activity (maltose liberated/U/mg protein)} = \text{Enzyme activity } \left(\mu\text{g/g/h}\right)\text{/protein (mg/g).}$$

Determination of Lipase Activity

Lipase activity can be analysed by titration method (Furne et al. 2005). One unit of lipase activity can be calculated as the amount of free fatty acid released from triacylglycerol per unit of time estimated by the amount of NaOH required to maintain the constant pH and represented as mille equivalents of alkali consumed. Mix 1% polyvinyl alcohol (PVA) and 5 ml of 0.1 N HCl in 1 L of distilled water. Heat the mixture at 75–85 °C, followed by cooling at room temperature, and adjust pH to pH 8.0 with 0.1 N NaOH. Add virgin olive oil to an aliquot of the previous solution obtaining a substrate concentration of 0.1 M, and emulsify this mixture for 5 min. In addition, prepare the Mcllvaine buffer from 0.1 M citric acid and 0.2 M disodium phosphate. For determination of enzyme activity, a reaction mixture contains 1 ml of PVA solution-emulsified substrate, 0.5 ml of Mcllvaine buffer at pH 8.0 (0.5 ml) and 0.5 ml of supernatant from the homogenates. Incubate this mixture for 4 h at 37 °C. Afterwards, add 3 ml of 1:1 ethanol-acetone solution to stop the reaction and break the emulsion. Add phenolphthalein in ethanol 1% (w/v) to the reaction mixture and titrate against 0.01 M NaOH. For the blanks, the same procedure can be followed but boiled enzyme will be used. Lipase (0.02%) can be used as standard and treated in a similar manner. Lipase activity can be calculated as follows:

Lipase activity (meq/minute/g of sample) = volume of NaOH consumed × 0.1 (normality of NaOH)/volume of sample × time (minutes).

Specific lipase activity (lipase liberated/U/mg protein) = enzyme activity (mg/g/hr) /protein (mg/g).

Analysis of Muscle Composition of Prawns

Firmness of the muscle is considered as an important flesh quality trait in the aquatic animals for marketing. The nutritive compositions of crustaceans depend upon their biochemical constituents. Body composition is a good indicator of the physiological condition of an aquaculture organism and easy to assess. The muscle composition of prawns includes crude protein, total protein, amino acids, carbohydrates, lipids, ash, moisture and mineral contents.

Crude Protein Analysis (See Section "Crude protein analysis on dry weight basis")

Total Protein Analysis (See Section "Total protein analysis on wet weight basis")

Total Amino Acid Analysis (See Section "Total amino acids analysis")

Total Carbohydrate Analysis (See Section "Total carbohydrates analysis")

Total Lipid Analysis (See Section "Total lipid determination")

Determination of Ash and Moisture (See Sections "Determination of ash" and "Determination of dry matter and moisture")

Whole-Body Mineral Content Analysis of Experimental Prawns (See Section "Determination of mineral contents")

Amino Acid Composition Analysis of Prawns (See Section "Amino acid profile analysis of Arthrospira sp.")

Fatty Acid Analysis of Prawns (See Section "Fatty acid profile analysis of **Arthrospira sp.**")

Antioxidants and Metabolic Enzyme Analysis in Experimental Prawns

The antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT) are responsible for scavenging superoxide radicals and are involved in protective mechanisms within tissue injury following oxidative process and phagocytosis. Lipid peroxidation (LPO) refers to the oxidative degradation of lipids. It is the process in which free radicals steal electrons from the lipids in cell membranes, it leads to cell damage. This process proceeds by a free radical chain reaction mechanism. Generally, the elevated metabolic enzymes, such as glutamic oxaloacetic transaminase (GOT) and glutamic pyruvate transaminase (GPT), indicate the weakening or damage of normal functions of the hepatopancreas in crustaceans. The antioxidants and metabolic enzymes of crustacean are to be estimated in experimental prawns to determine the normal functioning of organism while feeding plant-based diets due to the presence of anti-nutritional factors.

For this estimation, homogenize the muscles and hepatopancreas of test prawns individually in 10% (w/v) ice-cold 50 mM tris buffer (pH 7.4), centrifuge at 1000 rpm for 20 min at 4 °C and use the supernatant to assay the enzyme activities. Soluble protein level will be determined by the method of Lowry et al. (1951).

Determination of Superoxide Dismutase (SOD)

SOD activity can be measured using pyrogallol (10 mM) autoxidation in tris buffer (50 mM, pH 7.0) (Marklund and Marklund 1974). The assay mixture contains 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of phenazine methosulphate (PMS), 0.3 ml of nitro blue tetrazolium (NBT), 0.2 ml of the enzyme prepared and water accounting to a total volume of 2.8 ml. Initiate the reaction by the adding 0.2 ml of NADH. Incubate the mixture at 30 °C for 90 sec., and add 1.0 ml of glacial acetic acid to arrest the reaction. Shake the reaction mixture with 4.0 ml of n-butanol, followed by allowing to stand for 10 min, and centrifuge to separate the butanol layer. Measure the intensity of the chromogen in the butanol layer at 560 nm using spectrophotometer. The specific activity of the enzyme can be expressed in unit/mg protein.

Determination of Catalase (CAT)

CAT activity can be measured using H_2O_2 as the substrate in phosphate buffer (Sinha 1972). Initiate the reaction by adding 1.0 ml of phosphate buffer (0.01 M, pH 7.1), 0.5 ml of H_2O_2 (0.2 M) and 0.4 ml of distilled water successively to 0.5 ml of enzyme prepared. After 60 sec., stop the reaction by adding 2.0 ml of dichromate-acetic acid reagent. Further, keep the tubes in a boiling water bath for 10 min and cool at room temperature. Read the absorbance of the chromophore at 620 nm. A system devoid of enzyme served as the control. The activity of CAT to be expressed as µmoles of hydrogen peroxide consumed/min/mg protein.

Determination of Lipid Peroxidation (LPO)

LPO in the tissue homogenates will be measured by estimating the formation of thiobarbituric acid reactive substances (TBARS) (Ohkawa et al. 1979). The tubes containing 1 ml of tissue homogenate (10% w/v in 50 mM phosphate buffer, pH 7.4) were subsequently mixed with 1 ml of tris buffer (0.02 M, pH 7.5), 1 ml of 10% trichloroacetic acid and 1.5 ml of thiobarbituric acid (1.5%). Boil the reaction mixture for 15 min in the boiling water bath, followed by cooling at room temperature. Centrifuge the content at 100 g for 20 min and collect the supernatant. Measure the absorbance of the supernatant at 535 nm against the reagent blank. TBARS to be expressed as nmoles of malondialdehyde (MDA)/mg protein.

Determination of Activities of Metabolic Enzymes

Preparation of enzymes: 100 mg of muscle and hepatopancreas tissues will be homogenized in 0.25 M sucrose and centrifuged at 10,000 rpm for 20 min in a high-speed cooling centrifuge at 4 °C. The supernatant is used as the enzyme source for analysis of metabolic enzymes, such as GOT and GPT (Reitman and Frankel 1957). For GOT analysis, add 250 µl (pH 7.4) buffered aspartate−α−ketoglutarate substrate in 20 µl enzyme sample, mix well and incubate at 37 °C for 1 h. Incubate 60 min, followed by adding 250 µl of 2,4-dinitrophenyl hydrazine colour reagent to each of the incubated mixtures, mix well and allow standing at room temperature for 20 min. After 20 min, add 3 ml of 0.4 M sodium hydroxide solution (freshly prepared) to each of the incubated mixtures, and mix well. Read OD of the colour developed at 505 nm in a spectrophotometer within 15 min. The enzyme activities can be expressed in micromole of pyruvate formed U/L.

For GPT analysis, add 250 µl buffered alanine−α−ketoglutarate substrate (pH 7.4) in 20 µl enzyme sample, mix well and incubate at 37 °C for 30 min. After 30 min incubation, add 250 µl of 2,4-dinitrophenyl hydrazine colour reagent to each of the incubated mixtures, mix well and allow standing at room temperature for 20 min. After 20 min, add 3 ml of 0.4 M freshly prepared sodium hydroxide solution to each of the incubated mixtures and mix well. Read the OD of the colour intensity

in the incubated mixtures at 505 nm in a spectrophotometer within 15 min. The enzyme activities can be expressed in micromole of pyruvate formed U/L.

Total and Differential Haemocyte Counts in Experimental Prawns

The health status of an organism is established by its immunological responses. In aquatic organisms, the immune status can be determined by studying the haematological parameters like blood cell counts. In crustaceans, enumeration of total and differential haemocytes will give good information about its immune system. To determine the total and differential count of haemocytes, collect about 100 μl of haemolymph from the ventral sinus in the first abdominal segment of prawns using a 26 gauge hypodermic needle on a 1 ml syringe. Each syringe must be prefilled with 200 μl of anticoagulant (10 mM Tris-HCl, 250 mM sucrose, 100 mM sodium citrate, pH 7·6). More anticoagulant can be added to make the volume up to 1 ml in the anticoagulated haemolymph. Further, fix about 200 μl anticoagulated haemolymph with an equal volume of formalin (10%) for 30 min, and use the fixed haemolymph to determine total and differential haemocyte count.

For total haemocyte count (THC), dilute 100 μl of fixed haemolymph at 1:2 ratio (v/v) with ice-cold phosphate buffer saline (PBS, 20 mM, pH 7·2). Allow the diluted haemolymph in 20 μl of rose bengal strain (1.2% rose bengal in 50% ethanol), and incubate at room temperature for 20 min. Haemocytometer can be used to determine THC under the light microscope at RP 1000×.

THC ($\times 10^6$ cells/ml) = counted cells × depth of chamber × dilution factor/number of 1 mm square

For differential haemocyte count (DHC), stain the fixed haemolymph in 10% rose bengal solution for 10 min, and smear on a clean slide. The numbers of differential haemocytes, such as hyalinocytes, semigranulocytes and granulocytes, can be characterized according to Tsing et al. (1989), and count about 350–400 cells from each smear under a trinocular inverted microscope at RP 1000×, and calculate the haemocytes according to your dilution for 1 ml of undiluted haemolymph.

Pathogen Challenge Test of Prawns Against *Aeromonas hydrophila*

Infectious diseases remain one of the greatest challenges to global aquatic animal health. *Aeromonas* spp., *Enterococcus* spp. and *Vibrio* spp. are among the most important bacterial pathogens of prawns which cause a number of diseases. In laboratory experiment, the pathogen challenge test can be done as follows (Dash et al. 2015).

At the end of the 90-day experiment, allow the prawns for pathogen challenge test against *Aeromonas hydrophila*. Anaesthetize (clove oil, 100 mg/L) the prawns in the treatment groups, and infect with *A. hydrophila* by injecting 20 μl of bacterial suspension (5×10^7 cfu/ml) to the ventral sinus of prawn to achieve a final pathogen concentration of about 10^6 cfu/prawn. Inject the same amount of *A. hydrophila* to control prawns, and it will serve as 'control challenged', whereas prawns in other control groups can be injected with same quantity of PBS (pH 7.2) for serving as 'control unchallenged'. The postinfection experiment will be conducted for 7 days. Renew the 25% of water during the experimental period. During challenge study, the basal diet has to be provided to prawns (10% of body weight) twice/day. The mortality should be recorded on daily basis to calculate the survival and mortality percent of challenged control and experimental prawn group. At the end of the 7-day challenge experiment, the haemolymph bacterial clearance efficiency, total and differential haemocytes (THC and DHC) count, phenol oxidase activity, respiratory burst, level of antioxidants and metabolic enzymes of prawns will be analysed for assessing their disease resistance ability against the pathogen *A. hydrophila.*

Determination of Survival (See Section "Analysis of growth parameters of prawns")

Haemolymph Bacterial Clearance Efficiency Analysis

After the bacterial infection of prawn group, 50 μl of haemolymph is withdrawn from the ventral sinus of infected prawns at 0 h and 3 h after the infection using a sterilized syringe containing equal amount of anticoagulant (0.114 M trisodium citrate, 0.1 M sodium chloride, pH 7.45, osmolarity 490 mOsm/kg). Dilute the haemolymph in tryptone soya broth (TSB), spread on tryptone soya agar (TSA) plates and incubate at 30 °C for 24–48 h to obtain bacterial colonies. Calculate the bacterial count by taking dilution factor into consideration, and record it as the mean colony-forming units (cfu). Bacterial clearance efficiency (CE) of haemolymph can be calculated as follows:

Haemolymph bacterial clearance efficiency (%) = 100 − (cfu of bacteria in test group after 3 h of challenge)/(initial cfu of bacteria injected) × 100.

THC and DHC Count (See Section "Total and differential heamocyte counts in experimental prawns")

Phenol Oxidase Activity Analysis

Phenol oxidase (PO) activity can be measured following a method of Dash et al. (2015) by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA). For this, centrifuge 1 ml of diluted haemolymph (1:9) at 700 × g at 4 °C for 20 min and collect the pellet. Resuspend the pellet gently in 1 ml cacodylate citrate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.10 M trisodium citrate, pH 7.0), and centrifuge at 700 × g at 4 °C for 20 min and collect the pellet. Suspend the pellets in 0.1 ml cacodylate buffer (0.01 M sodium cacodylate, 0.45 M NaCl, 0.01 M $CaCl_2$, 0.26 M $MgCl_2$, pH 7.0). Centrifuge this cell suspension at 15900 × g for 20 sec to induce degranulation, and incubate with 0.1 ml of trypsin (1 mg/ ml) for 10 min at 25 °C (activator of prophenoloxidase enzyme and active phenol oxidase enzyme). Add 0.1 ml DOPA (3 mg/ml) into the reaction mixture, allow standing for 5 min and adding 0.8 ml of cacodylate buffer. Read the absorbance at 490 nm, and express it as phenol oxidase activity in 0.1 ml of haemolymph.

Respiratory Burst (RB) Activity

RB activity of haemocytes can be quantified according to the method of Song and Hsieh (1994) using the reduction of nitro blue tetrazolium (NBT) to form a measure of superoxide anion ($O2^-$) formation. For this, take 100 μl of diluted (1:9) haemocytes in microplates previously coated with 100 ml 2% poly-L-lysine solution (for improving cell adhesion). Then centrifuge at 300 × g for 15 min, followed by removing the supernatant, add 100 μl sodium alginate (0.2 mg/ml in PBS) and allow reacting for half an hour at 26–27 °C. Then, discard the sodium alginate, and stain the haemocytes using 100 μl of 0.3% NBT solution at room temperature for 30 min. Further, remove the NBT solution, followed by fixing with absolute methanol, wash thrice with 70% methanol and air-dry. Dissolve the formazan by adding 120 μl KOH (2 M) and 140 μl DMSO. Read the optical density at 630 nm, and express the RB as NBT reduction in 50 μl of haemolymph.

Determination of Antioxidants and Metabolic Enzyme (See Section "Antioxidants and metabolic enzyme analysis in experimental prawns")

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A Method of Bio-efficacy Potential of Microalgae (Phytoplankton) for the Control of Vector Mosquitoes

S. Balakrishnan, P. Santhanam, N. Manickam, and M. Srinivasan

Introduction

Mosquitoes are the majority grave group of insects in the circumstance of public health, because they transmit various diseases, causing millions of deaths per annum. The recurrent exploit of systemic insecticides to deal with insect pests leads to the destabilization of ecosystems and enhanced confrontation to insecticides by pests, symptomatic of an obvious need for alternatives (Kalimuthu et al. 2013). Algae is a considerable part of the diet for a lot of kinds of mosquito larvae that nourish opportunistically on microorganisms, diminutive aquatic animals such as rotifers, and other tiny particulate food in their aquatic environment (Merritt et al. 1992). The larvae may filter algae from the water column, scrape them from the surface of containers or aquatic plants, or scoop them from the bottom of aquatic habitats where mosquitoes breed. Some species of phytoplankton provide nutritious food for mosquito larvae, whereas others produce allelochemicals that are toxic to mosquitoes at different stages (Kiviranta and Abdel-Hameed 1994; Gross 2003; Legrand et al. 2003; Graneli and Hansen 2006; Rey et al. 2009). It is common in nature for mosquito larvae to die before completing their development because they are poisoned by phytoplankton toxins, or they starve to death while feeding on phytoplankton

S. Balakrishnan (✉)
Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

Marine Aquarium & Regional Centre, Zoological Survey of India, Ministry of Environment, Forest & Climate Change, Government of India, Digha, West Bengal, India

P. Santhanam · N. Manickam
Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

M. Srinivasan
Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai, Tamil Nadu, India

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that are indigestible (Ahmad et al. 2001; Marten 2007). Mosquito indigestible phytoplankton has an excellent pasture distinctiveness as a biological control representative against mosquitoes because they are naturally present in the habitats of mosquito larvae and are able to multiply and persist in these habitats. An additional most vital expansion of phytoplankton for mosquito control is the expectation that mosquitoes will not evolve conflict to their exploit (Ahmad et al. 2001).

History of Microalgae Culture

Microalgae ethnicity is one of the contemporary biotechnologies. The first unialgal cultures were achieved by Beijerinck in 1890 with *Chlorella vulgaris*, and the use of such cultures for studying plant physiology was developed by Warburg in the early 1919s. Mass culture of microalgae really began to be a focus of investigation after 1948 at Stanford (USA), Essen (Germany), and Tokyo (Japan), and the classic book edited by Burlew (1953) summarizes many of these early studies. Interest in applied algal culture continued, especially with studies on the use of algae as photosynthetic gas exchanges for space travel and as microbial protein sources.

Profitable large-scale culture of microalgae commenced in the early 1960s in Japan with the culture of *Chlorella* followed in the early 1970s with the establishment of a *Spirulina* harvesting and culturing facility in Lake Texcoco, Mexico, by Sosa Texcoco S.A. In 1977 Dai Nippon Ink and Chemicals Inc. established a commercial *Spirulina* plant in Thailand, and by 1980 there were 46 large-scale factories in Asia producing more than 1000 kg of microalgae (mainly *Chlorella*) per month (Kawaguchi 1980), and in 1996 about 2000 t of *Chlorella* were traded in Japan alone. Other *Spirulina* plants were established in the USA (e.g., Microbio in California, Cyanotech in Hawaii). Commercial fabrication of *Dunaliella salina*, as a basis of ß-carotene, became the third most important microalgae industry when production facilities were established by Western Biotechnology Ltd. and Betatene Ltd. (Cognis Nutrition and Health) in Australia (1986). As well as these algae, the large-scale invention of cyanobacteria (blue-green algae) commenced in India at about the same time. More recently quite a lot of plants producing *Haematococcus pluvialis* as a source of astaxanthin have been established in the USA and India. Thus, in a short period of about 30 years, the industry of microalgal biotechnology has grown and diversified significantly.

Importance of Plankton

Plankton forms the beginning of almost all existence in the ocean. They exchange the sun's vigor into element energy, which is approved on up diverse grocery chains to support the enormous variety of ocean animals, including the fish and shellfish

that are harvested by humans. Ninety percent of the world's photosynthesis is carried out in the oceans by microscopic phytoplankton, providing the energy for this process. Phytoplankton, like plants, breaks down carbon dioxide releasing the oxygen and investment onto the carbon. If plankton sinks to the bottom devoid of being eaten by other organisms, this carbon is taken out of atmospheric circulation. Since carbon dioxide plays a role in the greenhouse effect, trapping the sun's heat and warming the atmosphere, this has an effect on the earth's climate. It has been suggested by some that the growth of phytoplankton could be encouraged by fertilizing the ocean with iron or other minerals as a way to counteract any global warming.

Biotechnological Applications of Microalgae

Microalgae are imperative biological possessions that have a wide range of biotechnological applications. Due to their high nutritional value, microalgae such as *Spirulina* and *Chlorella* are being mass cultured for health food. Algae represent an important group of organisms for biotechnological exploitation, especially for valuable products, processes, and services, with important impact in food and pharmaceutical industries as well as in public health. Microalgae possess a multitude of physiological, biochemical, and molecular strategies to cope with stress; they are capable of synthesizing a variety of bioactive chemicals. The bioactive compounds are usually secondary metabolites, which include various types of substances ranging from organic acids, carbohydrates, amino acids and peptides, vitamins, growth substances, antibiotics, and enzymes to toxic compounds. The metabolites show a wide range of biological activities, including anticancer, antiviral, antioxidant, and immunomodulatory personal property (Skulberg 2004).

Toxic metabolites from microalgae (phycotoxins) are a capable group of bioactive molecules for biotechnological exploitation. Such compounds are fashioned essentially by dinoflagellates and cyanobacteria, predominantly those that cause harmful algae blooms in either marine or freshwater environments. Whereas concerns of 300 species of microalgae have been reported to form algae blooms, nearly one fourth of these species are known to produce toxins (Hallegraef 2003). The toxins can cause a health hazard to humans, domestic animals, and wildlife with toxicological belongings as well as neurotoxicity, hepatotoxicity, cytotoxicity, and dermatotoxicity (Carmichael 2001). Microalgae contain a lot of applications in green biotechnology, especially for bioremediation, bioassay, and biomonitoring of environmental toxicants. In adding together to suspend cultures, immobilized microalgae systems can further enhance the efficiency in the elimination of environmental toxicants (De-Bashan and Bashan 2010). Microalgae can be used as bioassay organisms to evaluate the toxicity of pollutants such as heavy metals, pesticides, and pharmaceuticals.

Sampling of Microalgae

The collection of microalgae samples is a decisive step for isolation of microalgae starting their innate environment. Microalgae are originate in assorted environmental circumstances and habitats such as ice, hot water springs, freshwater, brackish water, rivers, oceans, dams, wastewater, rocks, saline bodies, coastal areas, soil, etc. Appropriate sampling technique, sampling season, habitat evaluation, and preservation of samples are essential factors for collection of microalgae. Varieties of techniques consist of hypodermic sampling, scraping, brushing, inverted petri dish method, etc. It is obligatory to documentation abiotic factors such as light, water temperature, dissolved O_2 and CO_2, nutrient concentration, pH, and salinity; it is also imperative to record biotic factors such as pathogens and any competitors at the variety site in order to mimic these circumstances at the laboratory (Mutanda et al. 2011). Comprehensive positioning system coordinates must be recorded for reference and resampling. Microalgae are often present in consortium with complex population dynamics in natural habitats, thus it becomes inevitable to isolate the strain of interest commencing the collected samples.

Isolation of Microalgae

The isolation of the required species of microalgae can be done by one of the following methods:

Density Centrifugation

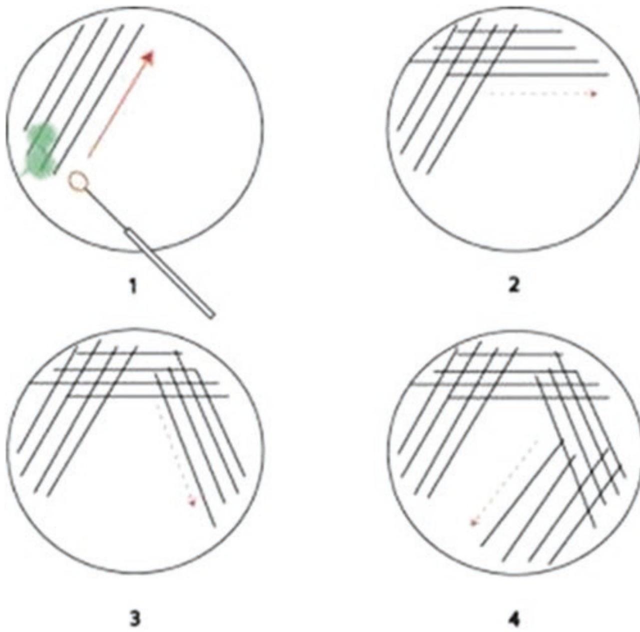
Centrifugation 1014 applies gravity settling to take apart organisms based on the cell size. This technique is usually applied to separate larger organisms from the microalgae cells. Density gradient (e.g., silica sol, Percoll) centrifugal performance separates the diverse species of microalgae into special bands. This technique is primarily used to concentrate the number of cells of a desired microalgae strain. When cells are concentrated, the isolation can be achieved by coupling this with other isolation techniques. Centrifugation speed and time vary depending upon the target microalgae species. Centrifugation can in addition damage the delicate cells via shear stress.

By Exploiting the Phototactic Movement

By this method, the phytoflagellates will move to one direction and with a micropipette can be isolated.

By Agar Plating Method

In favor of preparing the agar medium, 1.5% agar is added to 1 L of appropriate medium or constant natural seawater, and this agar solution is sterilized in an autoclave for 15 min under 150 lbs. pressure and 120 °C temperature. Then this intermediate is poured in sterilized petri dishes and left for 24 h. In case of culture tubes, the medium is poured in 1/3 part in tubes and properly plugged with cotton before autoclaving.



Streak plating with wire loop

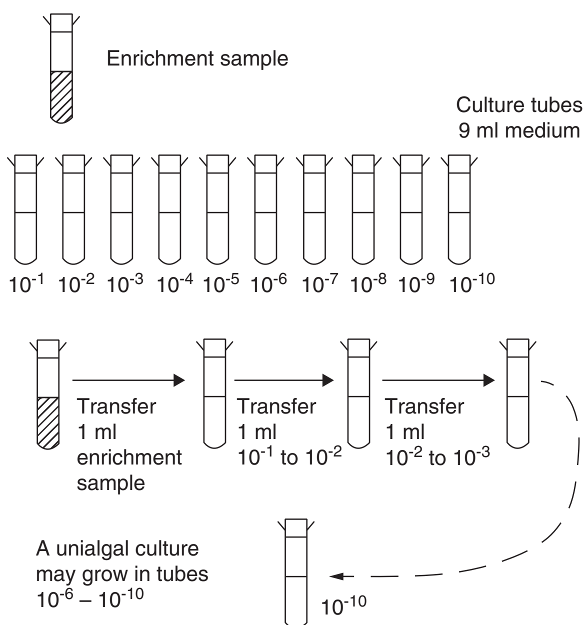
Micromanipulation

The algal cell is to be isolated in crash of enrichment illustration. Whereas observing, the cell is sucked up into micropipette. The cell is transferred to a drop of disinfected intermediate on agar plate. This succession is constant to "wash" the cell. The supplementary times cells are washed, the less likely is bacterial contamination. However, the risk of cell smash up increases with the quantity of times a cell is handled. The optimum quantity of washes will depend on the type of algae. Then

transfer the cell to dilute medium in a tissue culture plate, petri dish, or culture tube. Culture container is placed under low light at appropriate constant temperature. Growth is checked under the microscope, or we have to wait until macroscopic growth can be detected (3–4 weeks after transfer). Majestic unialgal culture consequences beginning this technique.

Serial Dilution

Tubes encompass to be labeled as 10^{-1}–10^{-10} to indicate the dilution factor. Aseptically 1 ml of enrichment, sample is to be added to the test tube (10^{-1}) and assorted tenderly. One ml of this dilution is taken and added to the next tube (10^{-2}) and then mixed gently. This procedure is repeated for the remaining tubes (10^{-3}–10^{-10}). Test tubes are incubated under controlled temperature and light conditions: the cultures are examined microscopically after 2–4 weeks by withdrawing a small sample aseptically from each dilution tube. An unialgal culture may grow in one of the higher-dilution tubes, e.g., 10^{-6}–10^{-10}. If the tubes contain two or three dissimilar species, then micromanipulation can be used to accomplish unialgal cultures.



Culture Media for Microalgae

The invention of microalgal biomass is very much influenced by the expansion medium, nutrient concentration, and nutrient type (Andersen 2005). Algal growth media can be grouped as freshwater and marine algal media. The suitability of growth media is monitored using photosynthetic health, chlorophyll content, and biomass yields of algae. The most important components of each media are nitrogen, phosphorus, inorganic carbon, and essential elements such as Fe, Mn, Zn, Cu, etc. Common examples of media used for cyanobacteria and freshwater algae are BG 11 (Grobbelaar 2004), COMBO medium, DY-V medium, DY-III medium, and MES Volvox medium, and for marine algae, Black Sea medium, ES medium, ASP medium, Aquil medium, Allen's Cyanidium medium, CCAP Artificial Seawater, Chry medium, ESAW medium, ESM medium, f/2 medium, K medium, L1 medium, MNK medium, Pro99 medium, SN medium, etc. The bold basal medium (BBM) is also used for freshwater algae and cyanobacteria. Dayananda et al. (2007) compared more than a few culture media (including BG-11 and BBM) for the culture of *Botryococcus braunii* and proficient that BG-11 was the most tremendous medium concerning biomass production.

Screening Criteria of Microalgae

Assortment criteria depend upon the desired use or product for which microalgae are being exploited. To sustain microalgae strain assortment and screening, a number of factors such as growth physiology, charity toward biotic and abiotic factors, metabolite production, nutrient requirements, etc. are taken into consideration. Screening for metabolite production includes determination of cellular components such as proteins, lipids, and carbohydrates of microalgae. Various chromatographic methods such as thin-layer chromatography, liquid and gas chromatography, and spectrophotometric methods such as near infrared, Fourier transform infrared, and nuclear magnetic resonance spectroscopy are applied to screen microalgae for metabolite production, which has significant applications in nutritional and pharmaceutical industries (Mutanda et al. 2011). Microalgae are a various group of organisms, thus rapid and efficient screening techniques necessitate to be developed to screen a number of phenotypic characters at the similar occasion.

Mass Culture of Microalgae

Large-scale cultures of microalgae, more than ever nannoplankton flagellates and elected species of diatoms, are obligatory for feeding the rearing larval forms in the hatchery. Since the molluscan larvae can feed organisms measuring less than

10 microns, these organisms contain to be isolated from the seawater, maintaining them as stock culture and utilizing the inoculums; mass culture can be done in the laboratory circumstances as well as in the outdoor 1 tonne tanks. The containers used for the mass culture of microalgae are 10 l capacity polythene bags, 20 l glass carboys, 100 l perspex tanks for the indoor culture, and 250 l or 1 tonne fiber glass tanks for the outdoor culture. For the indoor culture, the containers are kept in wooden racks with light and aeration facilities. Completely full-grown culture from the stock culture room is used as inoculums for the mass culture in these containers. About 100 ml of the inoculums is used for the polythene bags, 250 ml for the glass carboys, and 2 l for the 100 l perspex tanks which are properly lighted and aerated. These containers will have the maximum concentration of the cells in the growing phase of the 5–6th day and can be harvested. Behind estimating the cell concentration, using a hemocytometer, the culture is supplied to the hatchery for the rearing operations of the larval organisms. Leaving 2 l of the same culture, fresh, enriched can be added for further culture in the same container.

The technology to produce microalgae biomass depends on the selected application and profitable value of the compounds that can be extracted. In the pharmaceutical and cosmetic industries, closed systems are preferred for microalgae production to obtain the desired products. The costs of microalgae invention are supported by the high value of the extracted compounds. However, for environmental and energy applications, the production costs should be lower. Besides the selection of low-cost operating bioreactors, the integration of processes will enhance the economic viability of microalgae culture.

Harvesting Techniques

Microalgae harvesting is the elimination of biomass from the culture medium. This process can involve one or more steps (chemical, physical, or biological methods) and represents one of the most important challenges for biomass production on a commercial scale. Microalgae grow suspended in large amounts of water; they have a similar specific gravity of water; they keep a stable, dispersed state due to their negatively charged membrane; thus, dewatering is energy- and capital-intensive. The harvesting process can contribute 20–30% of the total costs of microalgae production (Goncalves et al. 2013; Pires et al. 2012). The selection of adequate harvesting methods depends on the characteristics of the target microorganism and also the value of the products that could be commercialized from the biomass. Conventional methods are centrifugation, flocculation, and filtration, which can be applied individually or in combination. They present some economic or technological disadvantages, such as an energy requirement (centrifugation), biomass contamination (chemical flocculation), or non-feasible scale-up (Oh et al. 2001; Rossignol et al. 1999).

Drying Techniques

Drying is the last harvesting step and is cured to take away moisture content (to 12% or less) to avoid interference with solvents used in downstream processes. It represents a significant fraction of the total production costs. It can be performed using dryers (spray drying, freeze drying/lyophilization, and fluidized bed drying) or by exposing the biomass to solar radiation (Brink and Marx 2013). Sun dries correspond to lower production costs as well as power consumption. Conversely, this process may not be efficient due to the high water content in biomass. Spray drying is the most recurrently functional methods.

Extraction and Characterization of Bioactive Compounds

The microalgae are air dried in the shade at room temperature and are smashed to construct a powder with a mortar and pestle. Each sample of 200 g is extracted with MeOH-H_2O (70:30) (5 × 200 ml) at room temperature. The combined extracts are evaporated under vacuum. The residues are subjected to silica gel (230) mesh and diluted successively with n-Hexane, $CHCl_3$, EtOAc, and methanol. Removal of the solvents resulted in the production of n-Hexane, $CHCl_3$, EtOAc, and MeOH-H_2O fractions.

Collection of Eggs and Maintenance of Larvae

The eggs of *Aedes*, *Anopheles*, and *Culex* are obtained from culture collections center using an "O" type brush. These eggs are brought to the laboratory and transferred to 18 × 13 × 4 cm, enamel trays containing 500 ml of water for hatching. The mosquito larvae are fed with pedigree dog biscuits and yeast at 3:1 ratio. The feeding is continued until the larvae transformed into the pupal stage.

Maintenance of Pupae and Adults

The pupae are scatterbrained from the culture trays and transferred to plastic containers (12 × 12 cm) containing 500 ml of water with the help of a dipper. The plastic jars are reserved in a 90 × 90 × 90 cm mosquito cage for adult emergence. Mosquito larvae are maintained at 27 ± 2 °C, 75–85% RH underneath a photoperiod of 14 l:10 D. A 10% sugar solution is provided for a period of 3 days before blood feeding.

Blood Feeding of Adult

The adult female mosquitoes are tolerable to feed on the blood of a rabbit (one rabbit per day, exposed on the dorsal side) for 2 days to ensure adequate blood feeding for 5 days. Subsequent to blood feeding, enamel trays with water from the culture trays are placed in the cage as oviposition substrates.

Larval and Pupal Toxicity Test

Laboratory colonies of mosquito larvae/pupae are used to test the larvicidal/pupicidal activity. Twenty five Ist to IVth instar larvae (I, II, III, and IV) and pupae are introduced into a 500 ml glass beaker containing 249 ml dechlorinated water, and 1 ml of the desired attentiveness of the compound marmesin is further added. Larval food is given to the test larvae throughout the investigational period. At each tested concentration, two to five trials are performed and each trial consisted of five replicates. The control is set up by mixing 1 ml of acetone with 249 ml dechlorinated water. The larvae and pupae exposed to dechlorinated water without ethanol served as the control. The control group's mortalities are corrected using Abbott's formula (Abbott 1925).

Corrected mortality = observed mortality in treatment – observed mortality in control × 100/100 -Control mortality.

Percentage mortality = No. of dead larvae/Pupae × 100/Number of larvae/pupae introduced

The LC_{50} and LC_{90} were calculated from toxicity data using probit analysis (Finney 1971).

Evaluation of Microalgae Isolates as Larvicides Against Mosquito Larvae

Larvicidal activity of the phytoplankton suspensions beside mosquito larvae is determined by transferring 200 ml suspension to a glass beaker containing 25 numbers of second-instar larvae of *Culex quinquefasciatus*. Three replicates are used each time and test is repeated two times. The control consisted of larvae in distilled water fed with 50:50 finely ground brewer's yeast and egg albumin (Rey et al. 2009). The groceries are added to the containers at a rate of approximately 0.5 mg/larva/day. The larval mortality is assessed after every 24 h, and lethal time (LT)

values are calculated using probit analysis (Finney 1971). The percentage of mortality for each test is calculated. Daily observations on larval and pupal mortality are continued through adult emergence or until termination of the test. Adult body size is determined by measuring the wing length (distance from the axial incision to the apical margin, excluding fringe of scales) of every creature (Rey et al. 2009).

The larval digestibility is tested by transferring 25 larvae into the 100 ml of phytoplankton suspensions. The larvae are permissible to nourish for 1 h and impassive from the postponement. The phytoplankton cells distant to larvae are washed with distilled water and sited in distilled water to tolerate for further digestion. The gut stuffing is then teased as of the membrane into avail containing sanitary distilled water and pragmatic beneath phase distinguish microscope. Cell counts are carried out to establish the percentage digestion of the phytoplankton cells.

Conclusions

The existing section demonstrates the green consequence of phytoplankton in the control of mosquito. The expose reveals that a number of species may serve as nutritious food for mosquito larvae, whereas others are harmful. The death of the larvae might be due to the poor digestibility of microalgae cells. Some species of blue-green algae kill mosquito larvae because of toxicity. The toxicity does not appear consistent enough to be of use for mosquito control. Blue-green algae toxins may have potential for use as insecticides. Numerous species of immature algae in the order Chlorococcales are resistant to digestion by mosquito larvae. Some are completely indigestible. Mosquito larvae are unable to complete their development if indigestible algae are numerous enough in the aquatic habitat to prevent the larvae ingesting enough other food to satisfy their nutritional requirements. This sometimes happens in scenery.

Consequently distant, no larvicidal algae have been put into operational use for mosquito control. Further research and development is necessary before their use is sufficiently consistent. The explanation improvement will be a method to ensure that larvicidal algae replace other algae in the aquatic habitat as completely as potential. One possibility is chemical treatment of the habitat to eliminate other algae facing introducing larvicidal algae. For indigestible algae, simultaneous preface of an herbivore that grazes on the digestible algae is a possibility that has already been demonstrated. In prospect, such phytoplankton can be used for the development of natural pesticides against larvae of disease-transmitting mosquitoes (Table 1).

Table 1 Microalgae species found to be promising biocontrol agents for mosquito control in different habitats

Species	Habitat	Mosquito species	Country	Reference
Microcystis aeruginosa, *Chlorella vulgaris*, and *Scenedesmus quadricauda*	Freshwater and marine environments	*Aedes aegypti*	Florida	Rey et al. (2009)
Nostoc commune, *Phormidium corium*, *P. tenue*, *Nannochloropsis oceanica*, *Chroococcus turgidus*, *Lyngbya confervoides*, *Oscillatoria fremyii*, *O. geminata*, *O. sancta*, *Phormidium corium*, *P. tenue*, *Spirulina*, *Chaetoceros calcitrans*, and *Skeletonema costatum*	Laboratory (200 ml glass beaker)	*Culex quinquefasciatus*	India	Rai and Rajashekhar (2015)
Anabaena sp.	Rice fields	*C. tarsalis*	California	Gerhardt (1956)
Anabaena unispora and *A. circinalis*	Laboratory	*Ae. aegypti*	Russia	Griffin (1956)
Anabaena cylindrica, *A. flos-aquae*, *A. sphaerica*, *Gloeotrichia echinulata*, *Plectonema boryanum,* and *A. flos-aquae*	Laboratory	*C. tarsalis*, *An. albimanus*, *An. freeborni*, and *An. quadrimaculatus*		Marten (1986)
Scenedesmus sp., *Ankistrodesmus convolutes*, *Chlorella* sp., *Chlorococcum* sp., and *Scenedesmus quadricauda*	Containers, coconut shells, and discarded tires	*Aedes aegypti*	Malaysia	Ahmad et al. (2004)
Chlamydomonas reinhardtii	Laboratory	*Anopheles gambiae*	Columbia (USA)	Anil Kumar et al. (2013)
Microcystis aeruginosa and *Akashiwo sanguinea*	Marine and freshwater	*Aedes aegypti*	Florida	Rey et al. (2009)
Scenedesmus sp., *Kirchneriella* sp., *Dactylococcus* sp., *Elakatothrix* sp., *Tetrallantos* sp., *Coelastrum* sp., *Selenastrum* sp., and *Tetradesmus* sp.	Laboratory	*Culex*, *Aedes*, and *Anopheles*	Hawaii	Marten (1986)
Microcystis aeruginosa, *Chlorella vulgaris*, *Oscillatoria*, and *Scenedesmus quadricauda*	Laboratory	*Ae. aegypti*	Florida (USA)	Rey et al. (2009)

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Isolation, Culture, and Application of Marine Microalga *Dunaliella salina* (Volvocales, Chlorophyceae) as an Aqua Feed Additive

A. Shenbaga Devi, P. Santhanam, S. Jeyanthi, and N. Krishnaveni

Introduction

Microalgae are microscopic unicellular organisms capable to convert solar energy to chemical energy via photosynthesis. They contain numerous bioactive compounds that can be harnessed for commercial use. The potential of microalgal photosynthesis for the production of valuable compounds or for energetic use is widely recognized due to their more efficient utilization of sunlight energy as compared with higher plants. Microalgae can be used to produce a wide range of metabolites such as proteins, lipids, carbohydrates, carotenoids, or vitamins for health, food and feed additives, cosmetics, and energy production (Adams et al. 2009). However, microalgal biotechnology only really began to develop in the middle of the last century. Nowadays, there are numerous commercial applications of microalgae have been identified for example microalgae can be used to enhance the nutritional value of food and animal feed owing to their chemical composition; they play a crucial role in aquaculture. Moreover, they are cultivated as a source of highly valuable molecules. For example, polyunsaturated fatty acid oils are added to infant formulas and nutritional supplements, and pigments are important as aqua feed additive.

Microalgae have three fundamental attributes that can be converted into technical and commercial advantages. They are genetically a diverse group of organisms with a wide range of physiological and biochemical characteristics; thus they naturally produce many different and unusual fats, sugars, bioactive compounds, etc. In recent years, microalgae apart from being used as single-cell proteins, they are projected as living-cell factories for the production of bio-fuels and various beneficial biochemicals used in food, aquaculture, poultry and pharmaceutical industries due to presence of different useful compounds. Nowadays, they are consumed throughout the world for their nutritional value. Some of the most biotechnologically relevant

A. Shenbaga Devi · P. Santhanam (✉) · S. Jeyanthi · N. Krishnaveni
Marine Planktonology & Aquaculture Laboratory, Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

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microalgae are green algae (Chlorophycea) *Chlorella vulgaris, Haematococcus pluvialis, Dunaliella salina*, and the cyanobacteria *Spirulina maxima* which are widely commercialized and used, mainly as nutritional supplements for humans and as animal feed additives (Priyadarshani and Biswajit 2012).

Microalgae and Aqua Feed

Microalgal pigment has commercial uses as a natural food coloring and cosmetic ingredient. Some microalgae contain substantial amounts of carotene (besides beta-carotene). Other types of coloring appear in microalgae as well. Beta-carotene is used as a food coloring (with a major application in providing the yellow color to margarine) and as a food additive to enhance the color of the flesh of fish and the yolk of eggs and is used to improve the health and fertility of grain-fed cattle (Borowitzka and Borowitzka 1987). *Dunaliella salina* is grown for a source of the photosynthetic pigment, beta-carotene. Beta-carotene is used as an orange dye and as a vitamin C supplement. Algae are a nutritionally good fish food. Besides the high levels of protein, lipids, and carbohydrates, it contains appreciable amounts of valuable vitamins.

In India, the culture of Pacific white shrimp *Litopenaeus vannamei* which is an exotic species has been practiced intensively. Fishmeal is the most important ingredient of balanced feed formulas used in aquaculture. Fishmeal is dry brown colored flour obtained after cooking, pressing, drying, and milling whole fish trimmings. Fishmeal provides an excellent source of highly digestible protein, beneficial fatty acids, and essential vitamins and minerals. Fishmeal production has not risen recently because catch of wild fish has flattened. This has constrained the growth of aquaculture as an industry worldwide.

Fishmeal is considered as an essential ingredient in marine shrimp diets because of its balanced amount of essential amino acids and fatty acids, vitamins, minerals, and palatability (Suarez et al. 2009). Tacon and Metian (2008) reported that the aquaculture industry consumed 68.2% of global fishmeal production in 2006; however, fishmeal production has remained relatively constant since 1985 at about 7 million tons per year (IFFO 2006). The steady growth of aquaculture and consequent increase in demand for fishmeal has caused a significant increase in fishmeal prices in the last decade (Duarte et al. 2009; FAO 2009). It has led to an increase in feed prices reducing the use of this ingredient in diets for animals (Naylor et al. 2009). Hence, all over the world, aquaculturists, aqua feed formulators, and researchers are trying to replace the fishmeal with cost-effective and nutritionally appropriate alternative feed ingredients. Microalgal meal is considered as one of the promising alternative feed ingredients in aqua feed formulation. This paper has been focused on the methods involving collection, isolation, biochemical analysis, and application of marine microalga *Dunaliella salina* as an aqua feed additive in rearing of shrimp *Litopenaeus vannamei* post larvae.

Materials and Methods

Collection, Isolation, and Stock Culture of Dunaliella salina

Salt pan water samples of green, orange, and red colors were collected in sterile plastic vials from salt pans and screened for *Dunaliella* under compound microscope. The samples contained *Dunaliella* was transferred to De Walne's medium and kept at 24 °C in thermostatically controlled room, illuminated with cool fluorescent lamps at irradiance, under 12 h/12 h light/dark photo period. After 10 days the samples were serially diluted up to 10^{-4} and 0.1 mL spread on 2% De Walne's medium for further investigation. The cyanobacterial contaminants were eliminated by treating them with 3000 ppm of the antibiotic, streptomycin sulfate. The cultures were made axenic by triple antibiotic treatment as described by Droop (1967).

Stock Culture Maintenance

The stock culture was maintained in special air conditioning room. The stock cultures were kept in 1 and 2 litres culture flasks and 5 and 15 liters plastic containers. The seawater was filtered using filter bag (1 μm), the filtered seawater was sterilized by using automated autoclave, and after cooling water was transferred to the culture flasks. The culture flasks were plugged with cotton or covered by aluminum foil. All the vessels used for algal culture were sterilized properly and dried in an oven before use. Conway's medium was used for stock culture (Table 1). For 1 litre of filtered seawater the following nutrients were added:

- Solution A: 1 ml
- Solution B: 0.5 ml
- Solution C: 0.1 ml

About 10 ml of inoculum in the growing phase was transferred to the culture flasks, and culture was provided with 12:12 h light and dark cycle with 5000 lux by using two tube lights. After 10–15 days, the maximum exponential phase was obtained. The temperature and salinity was maintained in the range of 23–25 °C and 25–35 ppt., respectively, for entire culture period. The continuous aeration was provided for culture.

Estimation of Biochemical Composition

The biochemical constituents such as moisture, protein, carbohydrate, lipid, and ash were analyzed in microalga *D. salina* according to the standard procedure as follows:

Table 1 Composition of "Conway's medium" as follows

Composition	Gm/L
Solution – A	
Potassium nitrate	100 gm
Sodium orthophosphate	20 gm
EDTA (Na)	45 gm
Boric acid	33.4 gm
Ferric chloride	1.3 gm
Manganese chloride	0.36 gm
Distilled water	1 l
Solution – B	
Zinc chloride	4.2 gm
Cobalt chloride	4.0 gm
Copper sulfate	4.0 gm
Ammonium molybdate	1.8 gm
Distilled water	1 l
Solution – C	
Vitamin B1 (thiamin)	20 mg in 100 ml dist. water
Vitamin B12 (cyanocobalamine)	10 mg in 100 ml dist. water

Moisture Estimation

For moisture estimation, a known quantity of sample was taken, and the excess moisture was removed using a filter paper (Rajendran 1973). Then the sample was dried in a hot air oven at a constant temperature of 60 °C until the wet sample dried completely. The moisture was estimated by subtracting the dry weight of the sample from the wet weight of the sample. Percentage of moisture content was calculated as follows:

$$\text{Moisture}\,\% = \frac{\text{Wet weight of the sample- Dry weight of the sample}}{\text{Wet weight of the sample}} \times 100$$

Protein Estimation

Dunaliella salina weighing around 1 gm was taken. The algal sample was homogenized with double-distilled water, and the extract was centrifuged at 4000 rpm for 10 min. The extract was dissolved in 4 ml of 1 N NaOH solution. About 5 ml of freshly prepared alkaline copper solution containing 1 ml of 0.5% copper sulfate in 1% potassium tartrate and 50 ml of 20% sodium carbonate in 0.1 N sodium

hydroxide were added to the redissolved extract and allowed to stand for 20 min. 1 N NaOH solution and 0.1% bovine serum albumin served as blank and standard solution, respectively. The color developed was read at 650 nm with UV visible spectrophotometer. Standard was also run simultaneously and based on the OD value; the total protein concentration of the sample was calculated using the following formula:

$$\frac{\text{OD of the Unknown}}{\text{OD of the Known}} \times \text{Concentration of standard} = \text{Concentration of protein present in the Unknown sample}$$

Lipid Estimation

Lipid was estimated by using chloroform-methanol method as described by Folch et al. (1957). 10 mg of dried *D. salina* powder sample was taken in a test tube; 5 ml of chloroform methanol (2:1) mixture was added. The mixture was incubated at room temperature for 24 h after closing the mouth of the test tube with aluminum foil. After the incubation, the mixture was filtered using filter paper. The filter was collected in a 10 ml pre-weighed beaker, which was kept on the hot plate. The chloroform-methanol mixture was evaporated leaving a residue at the bottom of the beaker. The weight of empty beaker was subtracted from the weight of beaker with lipid to estimate the total amount of lipid present in algal sample using the following formula:

$$\text{Lipid}\,\% = \frac{\text{Amount of lipid in the sample}}{\text{Weight of sample taken}} \times 100$$

Carbohydrate Estimation

Carbohydrate was estimated according to the procedure of Dubois et al. (1956). Dried *D. salina* sample weighing 5 mg was homogenized with double-distilled water and centrifuged for 10 min at 3000 rpm. To the supernatant, 1 ml of 5% phenol solution and 5 ml of Con.H_2SO_4 were added, and it is allowed to stand for 30 min, and OD was measured at 490 nm. D-glucose was used as standard. The amount of carbohydrate present in the sample was estimated using the following formula:

$$\text{Carbohydrate}\,\% = \frac{\text{Standard value} \times \text{OD of the sample}}{\text{Weight of sample taken}} \times 100$$

Ash Estimation

Ash content in *D. salina* was estimated according to the method of AOAC (1995). Two gram of dried algal sample was added to a pre-weighed crucible, kept in furnace at 400 °C for 4 h, cooled in desiccators, and reweighed.

HPTLC Analysis of Samples for Amino Acids

Sample Digestion

The 100 mg of *D. salina* sample was weighed in an electronic balance and transferred into labeled glass test tubes. 1 ml of 6 M hydrochloric acid solution was added with sample in specified test tubes. These test tubes were sealed at the top under vacuum by high temperature gas flame, conducted triplicates of samples. All the sealed tubes were kept in a hot air oven at 110 °C for 48 h continuously.

Test Solution Preparation

After completion of digestion process, the top of the tubes were broken, available gident in the tubes was tranfered to glass beaker and the tubes contains digest was rinsed five times with distilled water. The acid in the digest was evaporated to core dry under vacuum using Roto-vac evaporator. The residual content was dissolved with distilled water and make up to 2.4 ml in a centrifuge tubes. This solution contains 41.6 µg raw sample in 1 µl distilled water and used as test solution for amino acid profile analysis by HPTLC technique.

HPTLC Analysis

Standard Amino Acid Solution Preparation

All the standard 20 amino acids were classified into 4 groups according to their R_f values to avoid merging of individual amino acids while eluted with mobile phase (Table 2).

Table 2 Amino acid standards

Group I	Group II	Group III	Group IV
Lysine	Proline	Histidine	Glycine
Asparagine	Serine	Arginine	Alanine
Glutamine	Cystine	Aspartic acid	Valine
Glutamic acid	Tyrosine	Threonine	Isoleucine
Methionine	Tryptophan	Leucine	Phenylalanine

Standard Amino Acid Concentration

Group I, Group II, Group III, and Group IV contain 10 mg of each five amino acids dissolved in 10 ml distilled water.

Sample and Standard Amino Acid Loading

1 μl of each test solutions was loaded as 5 mm band in pre-coated silica gel $60F_{254}$ TLC plate (10 cm × 10 cm) using 100 μl Hamilton syringe and CAMAG-LINOMAT 5 instrument. 1 μl of each Group I, II, III, and IV standards was loaded in the plate for analysis as separate track.

Spot Development

The sample loaded plate was kept in TLC twin trough developing chamber with respective mobile phase (amino acids), 20 min for chamber saturation. After chamber saturation the plate was developed in respective mobile phase up to 90 mm.

Photo Documentation

The developed plate was dried by hot air to evaporate solvents from the plate. The plate was documented using photo-documentation chamber (CAMAG-REPROSTAR 3) at visible light, UV 254 nm and UV 366 nm mode.

Derivatization

The plate was sprayed with respective spray reagent (amino acids) and dried at 100 °C in hot air oven. After derivatization, the plate was documented at visible light and UV 366 nm using CAMAG-REPROSTAR 3.

Scanning

Finally, the plate was fixed in scanner stage and scanned at 500 nm using CAMAG-TLC SCANNER 3. The Rf value and peak area of each track were observed for quantification study. The win CATS 1.3.4 version software was used. The concentration of amino acids present in algae was estimated as follows:

Calculations:

Sample concentration	100 mg raw material in 2.4 ml of distilled water
Loaded volume of test solution	1 µl (41.6 *µg of raw material*)
Individual amino acid content in %	Conc. of amino acid in µg/41.6 µg × 100

GC-MS Analysis of Samples for Fatty Acids

Gas Chromatography

An Agilent 6890 gas chromatograph equipped with a straight deactivated 2 mm direct injector liner and a 15 m Alltech EC-5 column (250 µ I.D., 0.25 µ film thickness) was used to estimate the fatty acid concentration of microalga *D. salina*. A split injection was used for sample introduction and the split ratio was set to 10:1. The oven temperature program was programmed to start at 35 °C, hold for 2 min, and then ramp at 20 °C per minute to 300 °C and hold for 5 min. The helium carrier gas was set to 2 ml/minute flow rate (constant flow mode).

Mass Spectrometry

A JEOL GCmate II benchtop double-focusing magnetic sector mass spectrometer operating in electron ionization (EI) mode with TSS-2000[1] software was used for all analyses. Low-resolution mass spectra were acquired at a resolving power of 1000 (20% height definition) and scanning from m/z 25 to m/z 700 at 0.3 seconds per scan with a 0.2 second inter-scan delay. High-resolution mass spectra were acquired at a resolving power of 5000 (20% height definition) and scanning the magnet from m/z 65 to m/z 750 at 1 second per scan.

Mass Spectrometry Library Search

Identification of the components of the purified compound was matching their recorded spectra with the data bank mass spectra of NIST library V 11 provided by the instruments software.

HPLC Analysis of Samples for Carotenoid

The HPLC instrument used consisted of a quaternary low-pressure mixing dual piston pump (Waters 600), a photodiode-array detector (Waters 996), and a fluorimeter (Waters 474) set at a detection wavelength of 430 nm for excitation and 670 nm for emission. The HPLC system was coupled with a computer equipped with the Millennium 2010 chromatograph manager software package (Waters-Millipore). The separating column was a 300 × 3.9 mm Nova-pack C18 with 4 μm particles. The carbon load was 7%; the pore size, 60 Å; and the pore volume was 0.30 ml g^{-1}. The injected sample volume was 100 μl, and the flow rate 0.6 ml min^{-1} for 35 min. The solvents and gradients were adapted from Kraay et al. (1992), but with a longer column, as recommended by Waters in case of high number of peaks. Identification and calibration of pigments was performed with, namely, chlorophylls *a* and *b* and β-carotene from Sigma; lutein, astaxanthin, phaeophytin, and neoxanthin from "The International Agency for 14C Determination," VKI, Denmark; and also lutein and zeaxanthin, which were kindly provided by Hoffman-La Roche laboratories. Standards of breakdown products of chlorophylls *a* and *b* were prepared following the procedures given by Brotas and Plante-Cuny (1998) and Plante-Cuny et al. (1993). The absorbance spectra of other algal pigments and their elution order were compared with literature data (Brotas and Plante-Cuny 1998; Jeffrey et al. 1997; Le Bris et al. 1998; Wright et al. 1991). From the total pigments identified, six are calibrated and their concentrations in the samples were estimated. Results were given in μg/g^{-1} for dry algal cultures.

Formulation of Shrimp Feed with D. salina

Purchasing of Feed Ingredients

The branched feed ingredients such as soya bean, coconut oil cake, groundnut oil cake, tapioca flour, dry fishmeal, green gram, and egg were purchased from merchants; vitamin, mineral mix, and cod liver oil are obtained from medical shops. These ingredients were air dried and stored at desiccator in laboratory.

Pellet Feed Preparation (Bhavan et al. 2011)

The preparation of pellet feed was made according to the procedure of Bhavan et al. (2011) involving the following procedure:

Grinding

Dry fish, soya bean, coconut oil cake, groundnut oil cake, and green gram were ground well separately using micro pulverizer and sieved using commercial sieve.

Mixing

After grinding, the feed ingredients were weighed out as per the standard formula (proportion of feed shown in Table 1) and put into a mixer and homogenized the feed mix for 15 min, under room temperature.

Steam Cooking

The feed mixers were loaded in the thermostable trays, and trays are kept in streaming cooker. The feed mixers were cooked at 95–100 °C using stream cooker and kept for 5 min and allow the feed mixer to cool at room temperature.

Incorporation of Vitamin Mix, Cod Liver Oil, and Egg

The vitamin mix and cod liver oil was added to the stream cooked feed mix and thoroughly homogenized in a dough mixer. In this feed mix, 10% of distilled water and egg albumin were added and homogenized for another 10 min to bring the final mixture into a paste form.

Pelletization

The feed mix paste was loaded in the manual pelletizer fixed with 3 mm diameter disc, and pellets are collected in an aluminum tray.

Drying

The trays with moist feed was loaded into electrical trays at the temperature between 75 and 80 °C, and the feed was allowed to dry until the moisture content is less than 10%.

Checking the Quality of Feed

The dry feed pellets were physically examined for visual appearance such as uniformity, color, smooth surface, and fragment smell. The pellet feed was subjected to the analyses of proximate composition. The water stability of the pellets was also tested after 24 h of preparation.

Preparation of Experimental Feed

Combination of dry fish, groundnut oil cake, coconut oil cake, and soya bean was powdered in a grinder and was taken in different compositions as shown in Table 3. Along with this, the dried microalga *Dunaliella salina* powder was added in feed mix in known ratio as shown in Table 2. The feed without algae powder was considered as control feed. Tapioca flour and egg albumin were used as a binding agent. These feed ingredients are mixed well and brought into colloidal form. This feed mix paste was made into a pellet using pelletizer. Finally the pellets were dried in hot air oven at 27 °C for 48 h (Plates 1, 2, and 3).

Collection and Acclimatization of Experimental Animal

The PL 10 of *Litopenaeus vannamei* was obtained from the shrimp hatchery with proper aeration and then transported to the laboratory for further experiment.

Maintenance of Shrimp Larvae

During acclimatization, shrimp larvae were fed with control feed without *Dunaliella salina* powder. Culture water was routinely changed 7 days once to maintain a healthy environment for the shrimp apart from providing artificial aeration. This

Table 3 Composition of formulated experimental and control diets

S. No	Composition	G/100 g	
		Experimental feed with *Dunaliella salina*	Control diet without *Dunaliella salina*
1	Dry fishmeal	5	15
2	Groundnut oil cake	25	25
3	Coconut oil cake	10	10
4	Soya bean meal	25	25
5	Green gram	10	10
6	Wheat flour	5	5
7	Tapioca	3	3
8	Egg albumin	2	2
9	Cod liver oil	3	3
10	*Dunaliella salina*	10	–
11	Vitamin mix[a]	1	1
12	Table salt	1	1
	Total	100	100

[a]Becosules capsules
B-Complex forte with vitamin C for therapeutic use

Plate 1 Control feed ingredients

Plate 2 Experimental feed ingredients

Plate 3 Prepared formulated feed

Plate 4 Acclimatization of shrimp *Litopenaeus vannamei* larvae

ensures sufficient oxygen supply for the shrimp and an environment devoid of accumulated metabolic wastes (Plate 4).

Experimental Shrimp Rearing Condition and Feeding Regime

Salinity was maintained at 26‰. PL was stocked in 100 L tanks at 50 larvae/50 liter of filtered seawater and each diet is stocked in each tank. The shrimp larvae were fed twice a day for 21 days. The larvae were collected, measured, and weighed at weekly intervals for 3 weeks. PL was fed two times a day with formulated feed (control and experimental feed) at the rate of 10% of PL body weight. Survival, length, and weight measurements were made on weekly intervals. The mortality rate was assessed daily. The unconsumed feed, if any, was siphoned out 6 h after feeding. Likewise, fecal matter was also siphoned out prior to the next feeding.

Evaluation of Shrimp Larvae Growth Performance and Feed Efficiency Ratio

Growth performance of shrimp larvae was evaluated by individual weights which were taken at every 7 days interval throughout the experiment. The following response variables were determined from the experimental FRP tank:

$$\mathbf{Weight\ gain}\left(\%\right) = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100$$

Biochemical Analyses

The biochemical compositions, viz., moisture, protein, carbohydrate, lipid, amino acids, fatty acids, ash, and carotenoids, were estimated for both shrimp larvae and formulated feeds according to standard methods as explained previously.

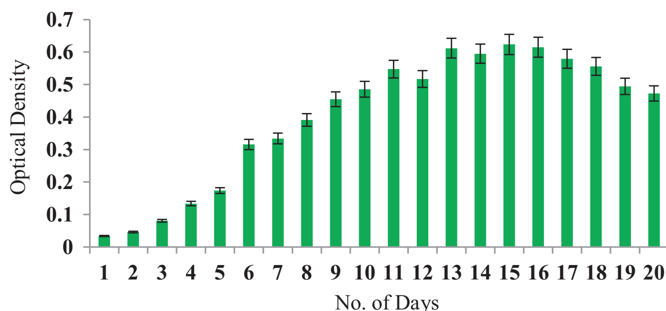
Results

Growth Rate of Dunaliella salina

A maximum growth rate of 0.623 ± 0.035 OD in *D. salina* was obtained on 15th day (Fig. 1).

Pigment Extraction

Dunaliella salina showed maximum Chl "a" (0.273 μg/ml) on 16th day, and minimum value (0.019 μg/ml) was noticed on 1st day. Maximum value of Chl "b" (0.089 μg/ml) was noticed on 16th day, and minimum value (0.015 μg/ml) was noticed on 1st day. Total carotene was found maximum (0.196 μg/ml) on 10th day, and minimum value (0.049 μg/ml) was noticed on 4th day (Fig. 2).



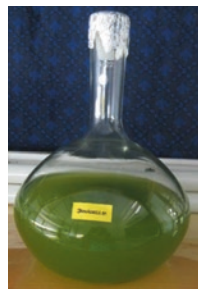


Fig. 1 Daily growth rate of *Dunaliella salina*

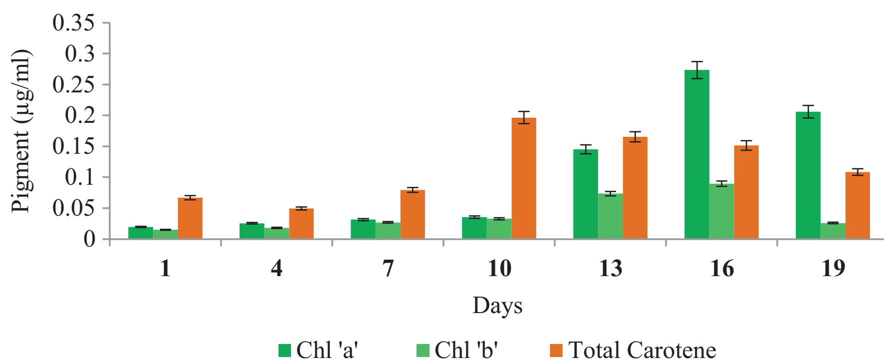


Fig. 2 Pigment extraction from marine microalga *Dunaliella salina*

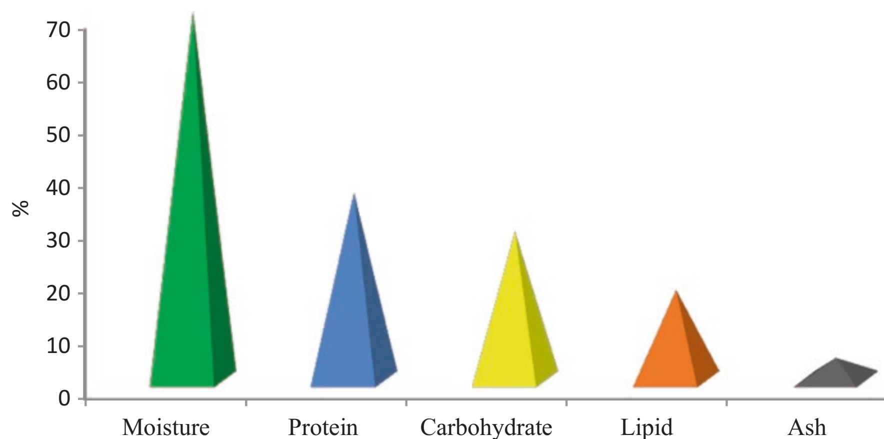


Fig. 3 Biochemical composition of *Dunaliella salina*

Biochemical Composition of D. salina

The biochemical composition showed high moisture content (69.44%). The recorded amount of total protein and carbohydrate in *Dunaliella salina* were 35.35% and 28.15%, respectively. The recorded concentration of lipid and ash were 17% and 4.07%, respectively (Fig. 3).

Amino Acid Profile

In the present study, the profiles of amino acids detected through HPTLC analyses from the marine microalga *Dunaliella salina* are presented in (Table 4). Totally 20 amino acids were detected; among these 9 are essential amino acids (histidine,

Table 4 Amino acid composition of marine microalga *Dunaliella salina*

S. No	Amino acids	*D. salina* %
	Essential amino acids	
1	Histidine	2.11
2	Isoleucine	1.27
3	Leucine	1.65
4	Lysine	2.30
5	Methionine	1.15
6	Phenyl alanine	1.51
7	Threonine	1.61
8	Tryptophan	3.72
9	Valine	1.20
	Nonessential amino acids	
10	Alanine	3.75
11	Arginine	3.75
12	Asparagine	–
13	Aspartic acid	2.11
14	Cystine	1.20
15	Glutamic acid	1.75
16	Glutamine	1.61
17	Glycine	1.75
18	Proline	6.63
19	Serine	–
20	Tyrosine	2.13

(−) denotes trace level

lysine, arginine, asparagine, threonine, isoleucine, tryptophan, leucine, and valine), and remaining 11 are nonessential amino acids (alanine, arginine, asparagines, aspartic acid, glycine, cystine, glutamic acid, glutamine, prolein, tyrosine, and serine). Among the essential amino acids noticed presently, tryptophan, lysine, and histidine were found dominant with 3.72%, 2.30%, and 2.11%, respectively. Similarly among the nonessential amino acids, prolein, alanine, and arginine were found predominant with 6.63%, 3.75%, and 3.75%, respectively.

Fatty Acid Profile

In the present study, the profiles of fatty acids detected through GC-MS analyses from the marine microalga *Dunaliella salina* are presented in Table 5 and Fig. 4. In this study, totally 12 fatty acids were detected, which include both unsaturated (essential) and saturated fatty acids (Table 5). There were ten saturated fatty acids

Table 5 Fatty acid composition of marine microalga *Dunaliella salina*

S.No	FAME formula	Common name	%
1	C11:0	Undecylic acid	0.85
2	C14:0	Myristic acid	1.87
3	C14:1	Myristoleic acid	7.75
4	C15:0	Pentadecylic acid	2.64
5	C15:1	Pentadecenoic acid	2.18
6	C16:0	Palmitic acid	4.29
7	C16:1	Palmitoleic acid	24.15
8	C16:2	Palmitic acid	32.92
9	C16:3	Phthalic acid	11.58
10	C17:0	Margaric acid	1.42
11	C20:0	Arachidic acid	5.97
12	C26:0	Cerotic acid	4.47

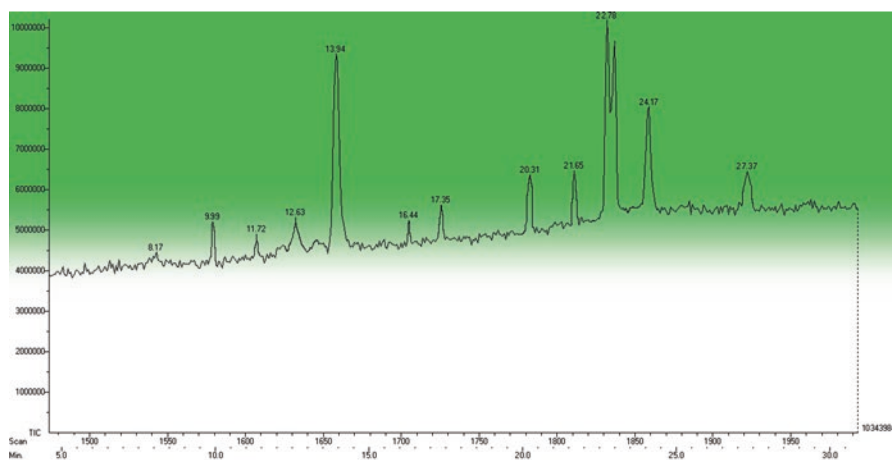


Fig. 4 Chromatogram of marine microalga *Dunaliella salina* showing fatty acid profile

(undecylic acid, myristic acid, pentadecylic acid, pentadecenoic acid, palmitic acid, phthalic acid and margaric acid, cerotic acid, arachidic acid); remaining two were unsaturated fatty acids (myristoleic acid and palmitoleic acid). In general, *Dunaliella salina* contains high level of saturated fatty acids such as C16:0 with 32.92% and C16:3 with 11.58% followed by unsaturated fatty acids C16:1 with 24.15% and C14:1 with 7.75%; other fatty acids are observed in the minimum percentage.

Table 6 Total carotenoids of marine microalga *Dunaliella salina*

Peak	Retention time	Pigments	References
1	3.159	Astaxanthin	Abd El-Baky et al. (2004)
2	5.681	Lutein	Abd El-Baky et al. (2004)
3	8.472	Chlorophyllide a	Wright et al. (1991)
4	12.143	Myxoxanthophyll	Garcia-Plazaola et al. (2012)
5	13.531	β-carotene	Abd El-Baky et al. (2004)
6	16.195	Chlorophyll “a”	Garcia-Plazaola et al. (2012)

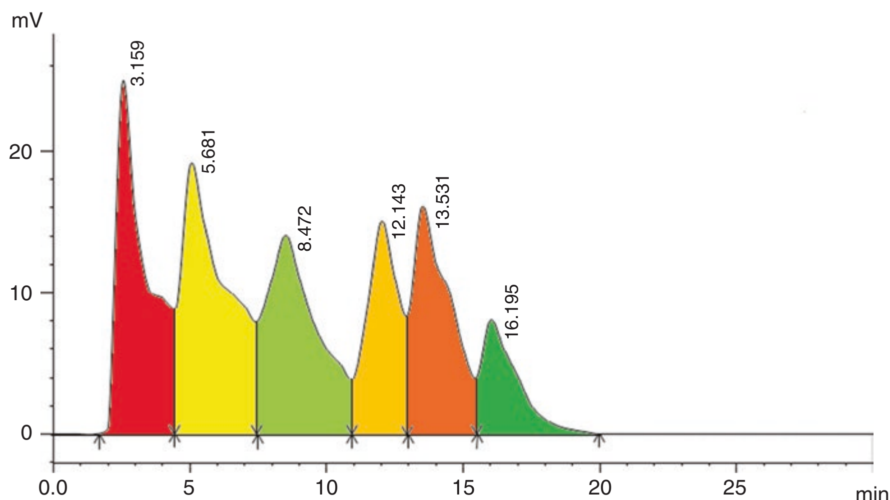


Fig. 5 HPLC profile of carotenoids of *Dunaliella salina*

Total Carotenoids Profile

A total of six pigments including astaxanthin, lutein, chlorophyllide “a,” myxoxanthophyll, chlorophyll “a,” and β-carotene were identified from the *Dunaliella salina* (Table 6; Fig. 5). The maximum pigment was determined to peak value of chlorophyll “a,” β-carotene, and myxoxanthophyll and recorded minimum pigments were astaxanthin, lutein, and chlorophyllide “a.”

Measurement of Length in Shrimp L. vannamei

The initial length of shrimp larvae was 1.34 cm. During the experiment trial, the length in control feed was 1.57 cm, 1.59 cm, and 1.6 cm, while in experimental feed, it was 1.58 cm, 1.73 cm, and 2.0 cm on 7th, 14th, and 21st day, respectively. Overall length was found to have increased gradually from 7th to 21st day in both the groups, whereas in experimental feed, higher growth rate was observed (Table 7).

Table 7 Measurement of length, weight, and survival in shrimp *L. vannamei* larvae

	Initial		7th day			14th day			21st day		
Feeding regimes	Length (cm)	Weight (gm)	Length (%)	Weight (gm)	Survival (%)	Length (cm)	Weight (gm)	Survival (%)	Length (cm)	Weight (gm)	Survival (%)
Control feed	1.34	0.001	1.57	0.003	97	1.59	0.013	90	1.6	0.027	88
Experimental feed	1.34	0.001	1.58	0.009	100	1.73	0.029	95	2	0.085	94

Measurement of Weight in Shrimp L. vannamei *Larvae*

The initial weight of shrimp larvae was 0.001 gm. During the experiment trial, the weight in control feed was 0.003 gm, 0.013 gm, and 0.027 gm, while in experimental feed, weight were 0.009 gm, 0.027 gm, and 0.085 gm on 7th, 14th, and 21st day, respectively. Overall weight was found to have increased gradually from 7th to 21st day in both the groups, whereas in experimental feed, maximum weight gain was observed compared to control feed fed animal (Table 7).

Biochemical Composition of Formulated Feeds

The maximum protein (40.01%) and lipid content (19.21%) were observed in experimental feed, while minimum moisture (80.51%), carbohydrate (22.54%), and ash (10.54%) were noticed in control feed (Fig. 6).

Total Carotenoids of Control and Experimental Feeds

The maximum total carotenoids (0.033 μg/gm) were noticed in experimental formulated feed, while the minimum total carotenoids of 0.023 μg/gm were noticed in control feed (Fig. 7).

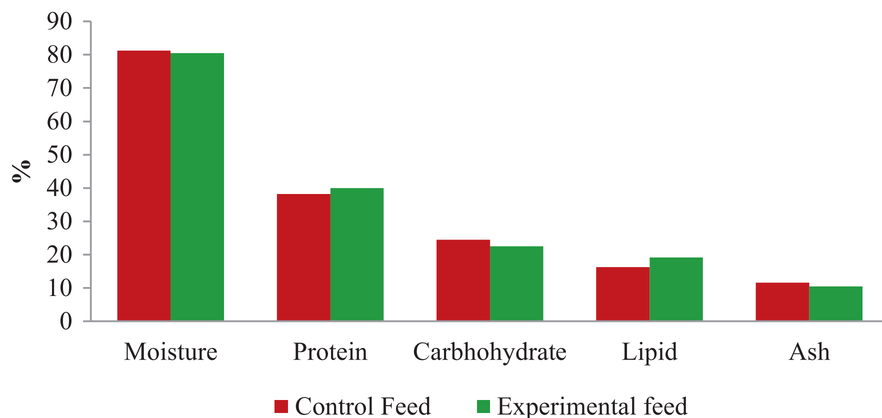


Fig. 6 Biochemical composition of formulated feeds

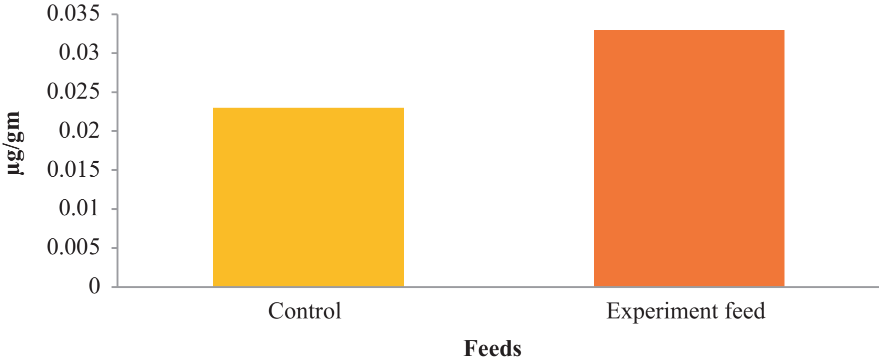


Fig. 7 Total carotenoids of control and experimental feeds

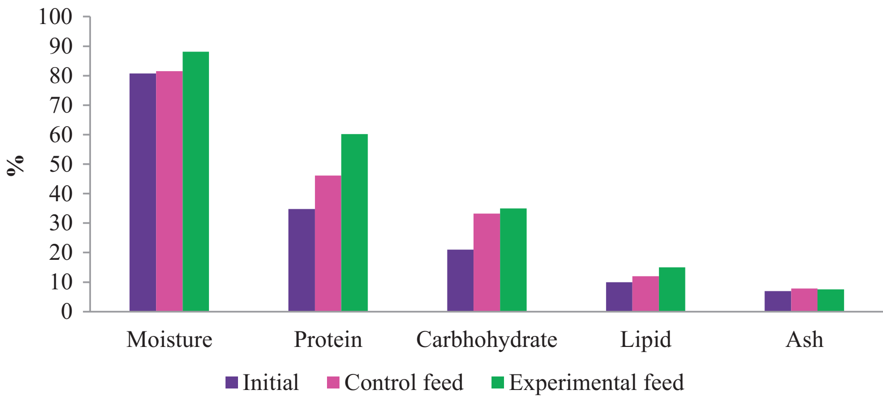


Fig. 8 Biochemical composition of shrimp larvae fed on different formulated feeds

Biochemical Composition of Shrimp Larvae Fed on Formulated Feeds

The recorded moisture, protein, carbohydrate, lipid, and ash contents in experimental feed fed shrimp larvae were 88.13%, 60.2%, 35%, 15%, and 7.5%, respectively, while the moisture, protein, carbohydrate, lipid, and ash contents in control feed fed shrimp larvae were 81.5%, 46.1%, 33.2%, 12%, and 7.8%, respectively (Fig. 8).

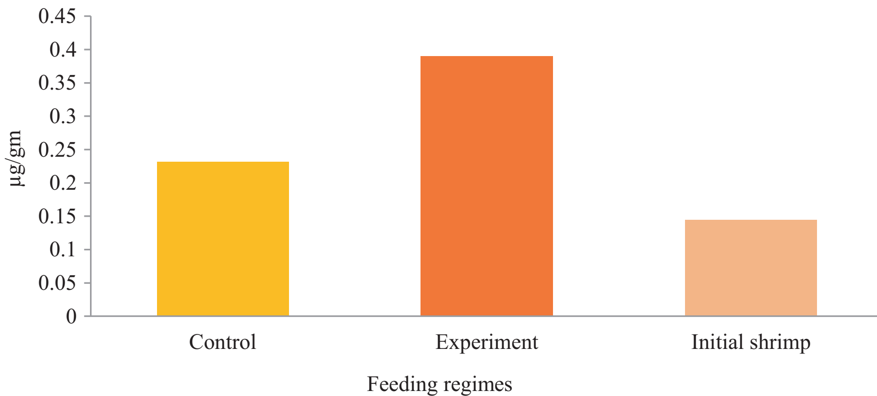


Fig. 9 Total carotenoids of shrimp larvae fed with control and experimental feeds

Total Carotenoid Contents of Shrimp Larvae Fed with Control and Experimental Feed

Total carotenoid contents of initial larvae, experimental feed fed shrimp larvae, and control feed fed shrimp larvae were 0.232, 0.39, and 0.145 µg/gm, respectively (Fig. 9).

HPTLC Amino Acid Analysis

In Feed

HPTLC analysis of various amino acids in formulated feeds is presented in (Table 8). The profile of amino acids detected through HPTLC in different formulated feeds is shown in Fig. 10. In the present study, however, the formulated feeds with alga showed almost equal proportion of various amino acids to that of control feed. The densitogram display of control and experimental feeds and control and experimental feed fed shrimp larva are shown in Fig. 11 HPTLC peak for standard amino acids and formulated feed amino acids are given in Fig. 12.

Table 8 Concentrations of amino acids detected through HPTLC in control and experimental feed and control and experimental feed fed shrimp larvae

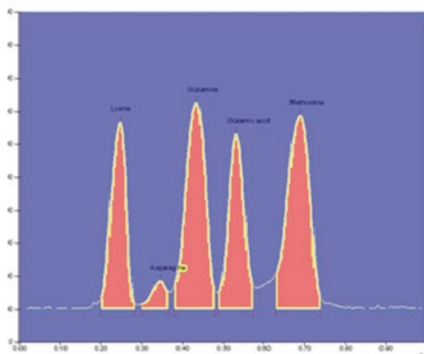
Amino acids	Control feed (%)	Experimental feed (%)	Control feed fed shrimp larvae (%)	Experimental feed fed shrimp larvae (%)
Essential amino acids				
Histidine	13.9	12.1	15.9	16.9
Isoleucine	2.59	2.16	2.64	2.69
Leucine	3.22	2.59	3.34	3.10
Lysine	2.62	2.28	2.98	3.17
Methionine	2.35	1.97	2.37	2.42
Phenyl alanine	2.90	2.35	3.02	2.81
Threonine	4.27	3.84	4.13	4.66
Tryptophan	7.13	5.79	7.45	6.92
Valine	3.17	2.86	3.07	4.36
Nonessential amino acids				
Alanine	3.50	3.50	4.37	3.72
Arginine	2.28	1.99	2.69	2.93
Asparagine	–	–	–	–
Aspartic acid	6.11	6.17	7.96	6.56
Cystine	2.11	1.97	2.47	2.11
Glutamic acid	4.71	4.23	4.56	5.12
Glutamine	2.30	2.16	2.69	2.28
Glycine	12.5	10.9	14.9	16.1
Proline	7.45	6.49	8.79	9.51
Serine	–	–	–	–
Tyrosine	4.39	3.65	4.44	4.54

In Experimental Animals

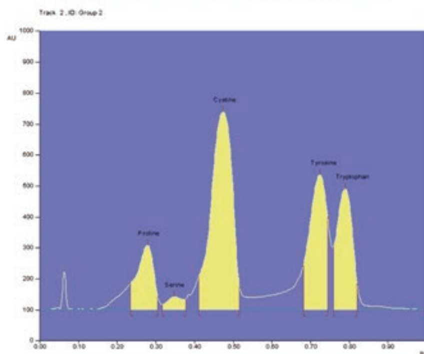
Concentrations of amino acids detected through HPTLC in experimental shrimp are shown in (Table 8). The small difference of certain amino acid levels in the whole body among the dietary treatments may be due to the differences in the tissue levels of free amino acids; this can be in experimental feed fed shrimp larvae showing higher amino acid levels when compared to control fed shrimp larvae.

In the present study, the profiles of amino acids detected through HPTLC analyses from the muscle of the prawns are presented in Table 8. Totally 20 amino acids were detected; among these 9 are essential amino acids (histidine, lysine, arginine, asparagine, threonine, isoleucine, tyrosine, leucine, and valine); remaining 11 are nonessential amino acids (alanine, arginine, asparagines, aspartic acid, glycine, cystine, glutamic acid, glutamine, prolein, tyrosine, and serine). In control formulated feed amino acids such as histidine (13.9%), glycine (12.5%), tryptophan (7.13%), and prolein (7.45%) were found dominant.

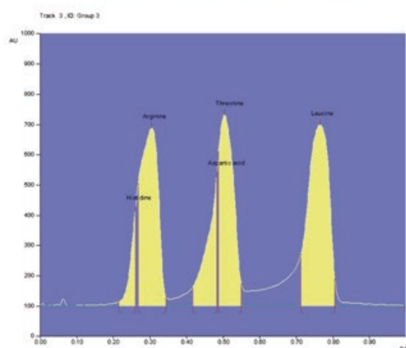
Track G1- Group 1 Standard amino acids - Peak densitogram display (500 nm)



Track G2- Group 2 Standard amino acids - Peak densitogram display (500 nm)



Track G3- Group3 Standard amino acids - Peak densitogram display (500 nm)



Track G4- Group 4 Standard amino acids - Peak densitogram display (500 nm)

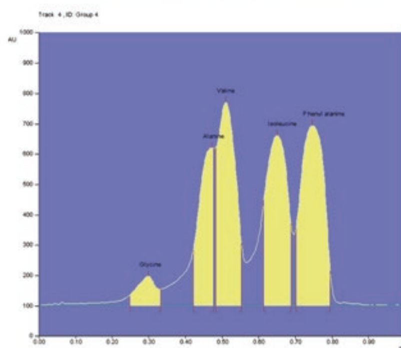
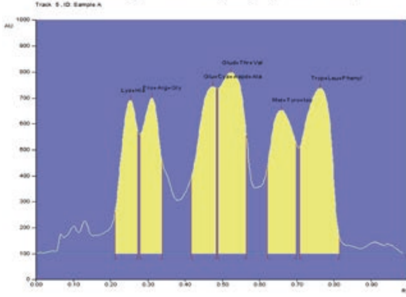


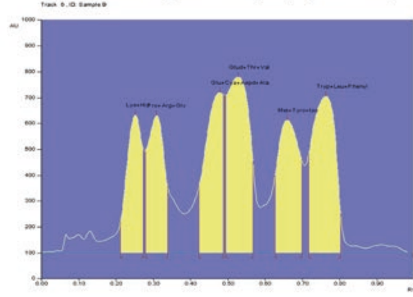
Fig. 10 HPTLC peak densitogram display for standard amino acids. Track G1 – Group 1 Standard amino acids – Peak densitogram display (500 nm). Track G2 – Group 2 Standard amino acids – Peak densitogram display (500 nm). Track G3 – Group3 Standard amino acids – Peak densitogram display (500 nm). Track G4 – Group 4 Standard amino acids – Peak densitogram display (500 nm)

The higher concentrations of amino acids were observed in experimental feed fed shrimp larvae which include histidine (16.9%), glycine (16.1%), and prolein (9.51%), while the lower concentration was noticed in control feed fed shrimp larvae including histidine (15.9%), glycine (14.9%), and prolein (8.79%). The experimental feed fed shrimp larvae showed maximum amino acids than control feed fed larvae.

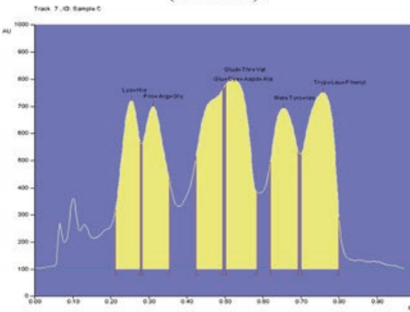
Track A- Control feed amino acids -Peak densitogram display (500 nm)



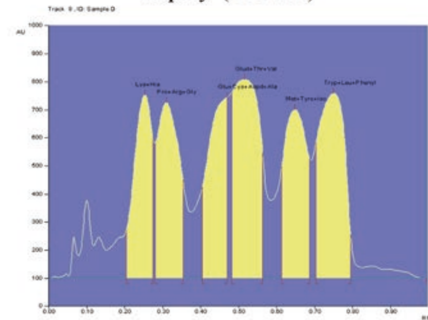
Track B- Experimental feed amino acids - Peak densitogram display (500 nm)



Track C- Control feed fed shrimp larvae amino acids -Peak densitogram display (500 nm)



Track D- Experimental feed fed shrimp larvae amino acids -Peak densitogram display (500 nm)



Track E- *D. salina* amino acids -Peak densitogram display (500 nm)

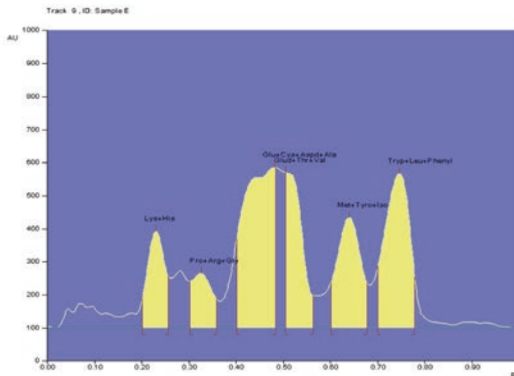


Fig. 11 HPTLC peak densitogram display for control and experimental feeds and control and experimental feed fed shrimp larvae. Track A – Control feed amino acids –Peak densitogram display (500 nm). Track B – Experimental feed amino acids – Peak densitogram display (500 nm). Track C – Control feed fed shrimp larvae amino acids – Peak densitogram display (500 nm). Track D – Experimental feed fed shrimp larvae amino acids – Peak densitogram display (500 nm). Track E – *D. salina* amino acids – Peak densitogram display (500 nm)

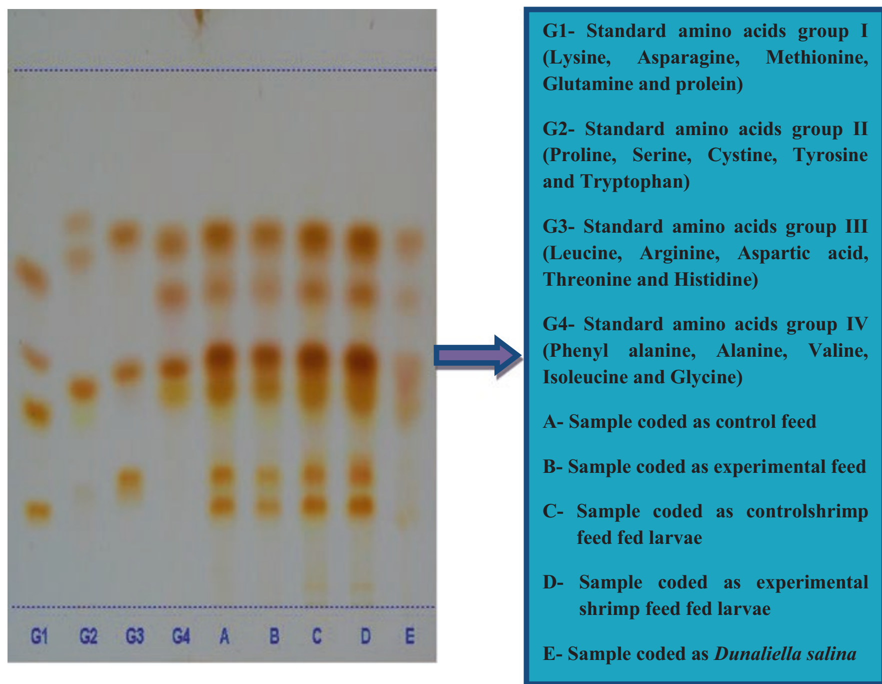


Fig. 12 HPTLC showing amino acids profiles of standard and experiment

Fatty Acid Profile

In the present study, the profiles of fatty acids detected through GC-MS analyses from the marine microalga *Dunaliella salina* are presented in Table 9 and Figs. 13 and 14. In this study, totally 17 fatty acids were detected, which include both unsaturated (essential) and saturated fatty acids (nonessential). There were 14 saturated fatty acids (myristic acid, pentadecylic acid, pentadecanoic acid, palmitic acid, margaric acid, and cerotic acid) which were recorded; remaining three were unsaturated fatty acids (palmitoleic acid, arachidic acid, and docosahexaenoic acid).

Control and experimental feed contains highest level of saturated fatty acids such as C18:0 with 31.69%, C16:1 with 3.19% and C26:0 with 2.41% and C18:0 with 24.75%, C26:0 with 17.89%, and C13:0 with 2.58%, respectively, followed by unsaturated fatty acids which was reported maximum in experimental feed that includes C16:2 with 42.37%. However, control feed showed the low level of unsaturated fatty acids, viz., C16:2 with 0.75% and C15: 0 with 2.12%. The other fatty acids are observed in the minimum percentage.

In the present investigation, experimental feed fed shrimp larvae contain highest level of saturated fatty acids such as C26:0 with 11.24%, C16:1 with 8.79%, and C15 with 8.59%. Similarly control feed fed shrimp larvae resulted the maximum of C26:0 with 7.26%, C14:0 with 6.54%, and C14:1 with 4.92%. The unsaturated fatty

Table 9 Fatty acids detected through HPTLC in control and experimental feed and control and experimental feed fed shrimp larvae

S.No	FAME	Common name	Control feed (%)	Experimental feed (%)	Control feed fed shrimp larvae (%)	Experimental feed fed shrimp larvae (%)
1	C12:0	Lauric acid	–	–	2.42	–
2	C13:0	Tridecylic acid	–	2.58	–	–
3	C14:0	Myristic acid	–	–	–	6.54
4	C14:1	Myristic acid	2.14	2.93	4.21	6.24
5	C14:2	Methyl myristate	2.31	–	–	–
6	C15:0	Pentadactyl acid	–	–	–	3.87
7	C15:1	Pentadecenoic acid	–	3.49	–	8.59
8	C16:0	Palmitic acid	2.12	–	–	–
9	C16:1	Palmitic acid	3.19	3.68	8.62	8.79
10	C16:2	Palmitoleic acid	0.75	42.37	7.59	–
11	C16:3	Methyl palmitoleate	–	–	3.64	–
12	C18:0	Stearic acid	31.69	24.75	–	–
13	C20:0	Arachidic acid	42.01	–	–	–
14	C22:0	Behenic acid	–	–	4.92	–
15	C22:6	Docosahexaenoic acid	–	2.14	61.28	48.62
16	C24:0	Lignoceric acid	–	–	–	5.92
17	C26:0	Cerotic acid	2.41	17.89	7.26	11.24

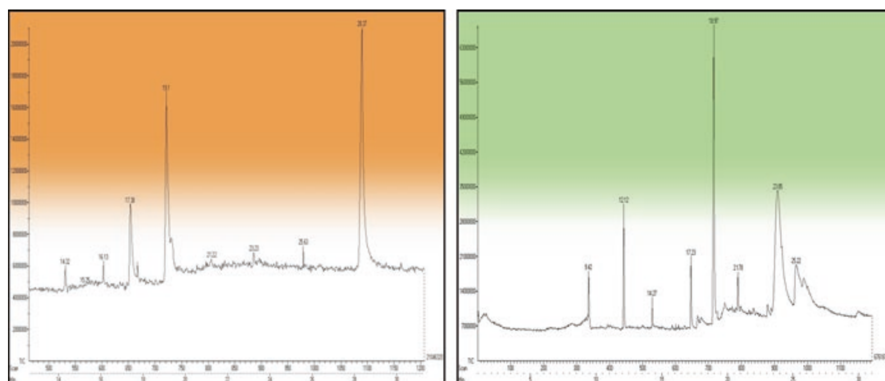


Fig. 13 Chromatogram of control and experimental feed showing fatty acid profile

acids were comparatively higher in control feed fed shrimp larvae (C22:6 with 61.28% DHA) than experimental feed fed shrimp larvae (C22:6 with 48.62% DHA). In general both the saturated and unsaturated fatty acids are comparatively higher in experimental feed and experimental feed fed shrimp larvae than control feed and control feed fed larvae.

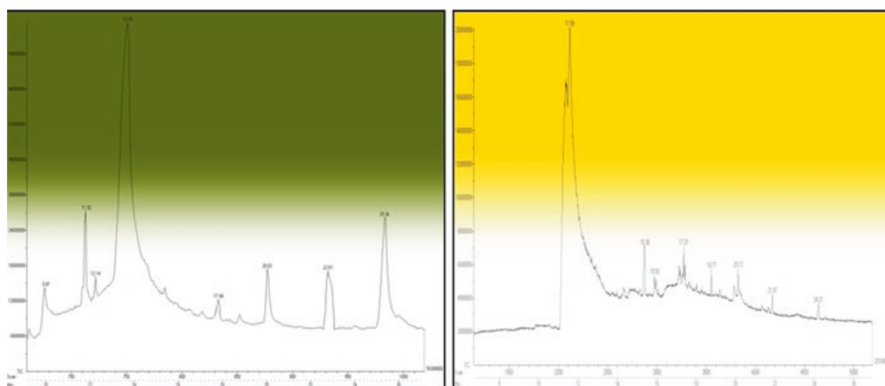


Fig. 14 Chromatogram of control and experimental feed fed shrimp larvae showing fatty acid profile

Table 10 HPLC profile of carotenoids of control and experimental feeds

Peak	Retention time		Pigments	References
	Control feed (%)	Experimental feed (%)		
1	8.428	8.596	Chlorophyllide "a"	Wright et al. (1991)
2	–	13.641	β-carotene	Abd El-Baky et al. (2004)
3	17.209	17.182	Pheophytin "a"	Brotas and Plante-Cuny (2003)

Total Carotenoid Profile

A total of three pigments were identified from the control and experimental feeds (Table 10) including chlorophyllide "a," β-carotene, and pheophytin "a." The maximum pigment was determined to peak value of experimental feed, and minimum pigments were observed in control feed in μg g^{-1} dry biomass (Fig. 15).

A total of five pigments were identified from the control and experimental feed fed shrimp larvae (Table 11) including chlorophyllide "a," lutein, neoxanthin, β-carotene, and pheophytin "a." The maximum pigment was determined to peak value of experimental feed fed shrimp larvae, and minimum pigment was observed in control feed fed shrimp larvae in μg g^{-1} dry biomass (Fig. 16).

Discussion

Algae with high nutritional value have remarkable potential as shrimp feed. *Dunaliella salina* has 90% of β-carotene and 10% of other carotenoids. Carotenoids are made up of α-carotene and xanthophylls like lutein, zeaxanthin, and cryptoxan. This is similar to those found in food and vegetables (Gouveia and Emphis 2003).

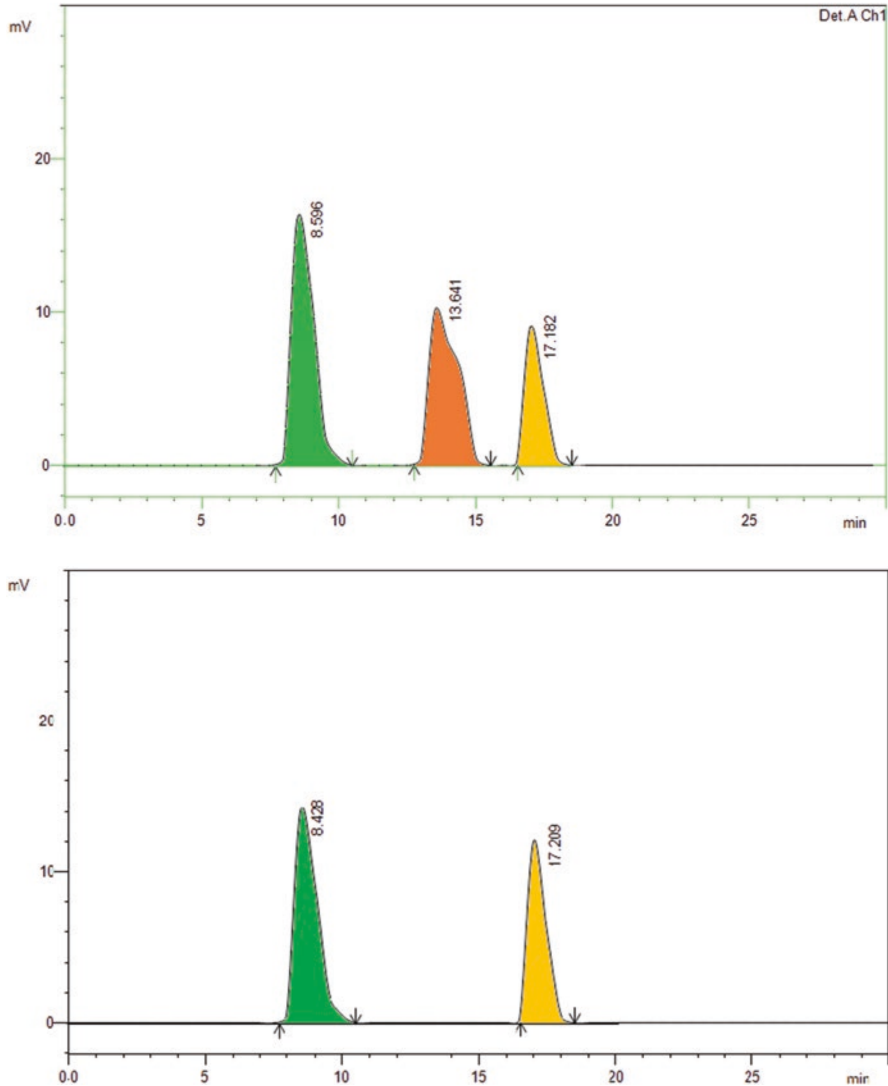


Fig. 15 HPLC profile of carotenoids of control and experimental feeds

Approximately 500 different carotenoids have been identified so far. *Dunaliella salina* which are widely commercialized and used, mainly as nutritional supplements for humans and as animal feed additives.

Fishmeal is an excellent source of protein and other essential nutrients to aquaculture species, but it is a limited natural resource. Fishmeal is considered as an essential ingredient in marine shrimp diets because of its balanced amount of essential amino acids, fatty acids, vitamins, minerals, and palatability (Suarez et al. 2009). Tacon and Metian (2008) reported that the aquaculture industry consumed

Table 11 HPLC profile of carotenoids of control and experimental feed fed shrimp larvae

Peak	Retention time			
	Control feed fed shrimp larvae (%)	Experimental feed fed shrimp larvae (%)	Pigments	References
1	5.154	5.225	Lutein	Abd El-Baky et al. (2004)
2	–	8.495	Chlorophyllide "a"	Wright et al. (1991)
3	11.529	11.657	Neoxanthin	Garcia-Plazaola et al. (2012)
4	–	13.562	β-carotene	Abd El-Baky et al. (2004)
5	17.089	17.148	Pheophytin "a"	Brotas and Plante-Cuny (2003)

68.2% of global fishmeal production in 2006. Fishmeal is a rich source of high quality protein, has relatively high-energy content, and is rich in important minerals such as phosphorus, B vitamins, and essential fatty acids. The steady growth of aquaculture and consequent increase in demand for fishmeal has caused a significant increase in fishmeal prices in the last decade (Duarte et al. 2009; FAO 2009). Hence in the present study, microalga *Dunaliella salina* was used as an alternative ingredient in formulated feed to replace the fishmeal partially.

Microalgae like *Spirulina*, *Chlorella*, *Scenedesmus*, *Dunaliella,* and *Nannochloropsis* are widely used as aquaculture feed for their high nutritional value (Venkataraman 1980; Avron and Ben-Amotz 1992; Lee 1997; Yamaguchi 1997). *Spirulina, Dunaliella,* and *Haematococcus* are also used as good sources of antioxidant pigments like carotenoids, lutein, astaxanthin, zeaxanthin, etc. in fish farming mainly for colored fishes (Chiu et al. 2001; Hanaa et al. 2003) for the intracellular protection of fish larvae against different diseases together with the bright coloration of fishes (Trinadha et al. 2003). *Dunaliella salina* food or food additive and a nutritional supplement mean that a high quality product is required. *Dunaliella salina* is also used as a source of natural pigments for the culture of prawns, salmonid fish, and ornamental fish. In the present study, the shrimp group fed with experimental diets showed a higher feed intake rate than that with the control feed during the experimental tenure. This might be due to the attractive color, flavor, and good nutrient composition of the experimental feeds.

The growth of the shrimp larvae depends on the quality of feed. The present study shows that the post larvae responded well to the experimental formulated diets which incorporated with microalga *Dunaliella salina*. The ingredients present in the formulated diets significantly influenced the performance of the juveniles, by resulting increase in final body length and weight. Survival rates obtained in this study with the experimental formulated diets seem to indicate that there were no overwhelming negative effects in utilization of nutrients by the microalga *Dunaliella salina.* Higher survival rate was reported in experimental shrimp larvae which could be due to the growth promoting substances occurred in the microalgae.

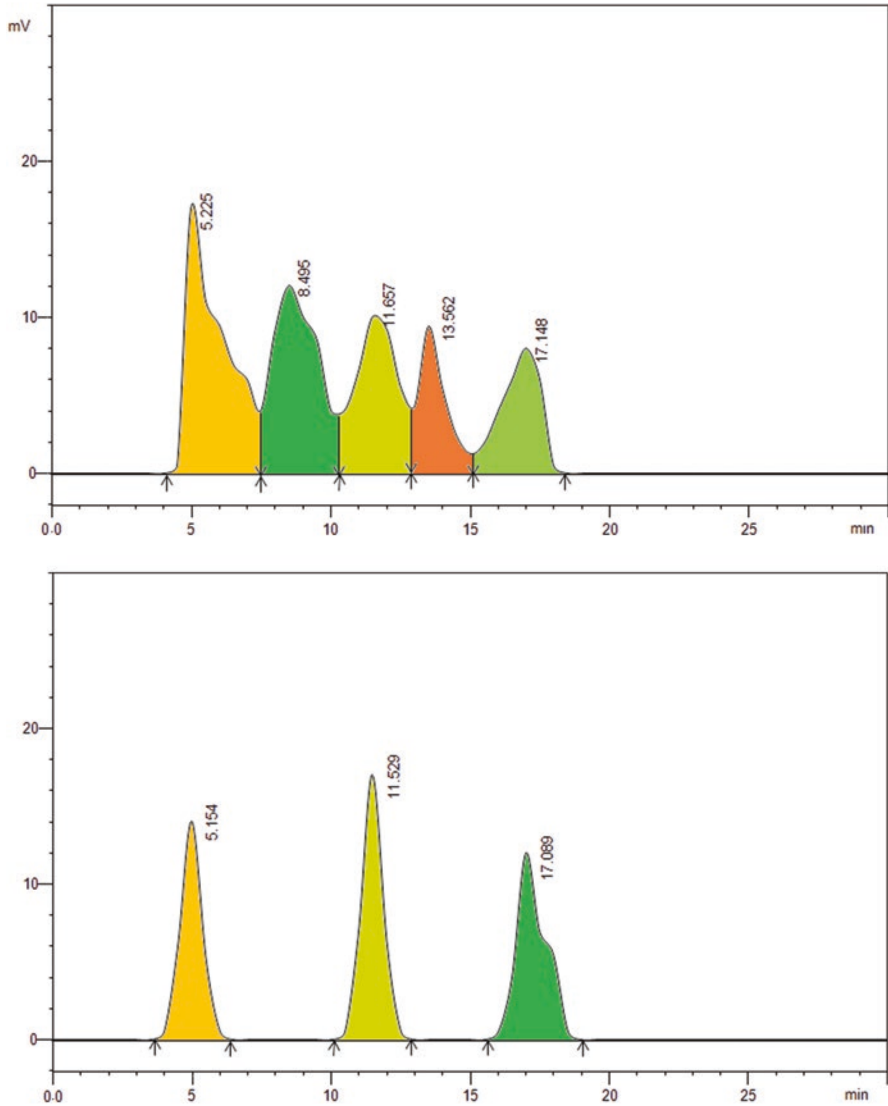


Fig. 16 HPLC profile of carotenoids of control and experimental feed fed shrimp larvae

The overall biochemical profile of the feed indicated the fact that the experimental feed had good nutritive value. As dietary protein is the most important factor affecting growth performance of shrimp (Kureshy and Davis 2002), most shrimp farmers prefer to use high protein feeds, especially in intensive culture systems. Amaya et al. (2007a) suggested that the successes of replacing animal protein in diets with alternative sources are due in part to the ability of shrimp to use natural productivity as a food supplement.

There is hardly any information on the use of microalgae as a dry feed component for shrimps, though there are ongoing efforts to replace fishmeal protein using terrestrial plant proteins. *L. vannamei* has been successfully grown on a predominantly plant protein diet containing solvent-extracted soybean meal, corn gluten meal, and corn fermented soluble, which together accounted for nearly 98% of the total dietary protein of 36% (Amaya et al. 2007a). The same research group has verified the concept of fishmeal-free shrimp feed in a pond trial (Amaya et al. 2007b). Furthermore, beneficial impact of algal inclusion on shrimp health has been reported recently; *L. vannamei* fed diets supplemented with marine algal meals rich in docosahexaenoic acid and arachidonic acid demonstrated significant improvement in immune responses (Nonwachai et al. 2010).

Various species of macroalgae and microalgae have been incorporated into fish feed formulations to assess their nutritional value, and many have been shown to be beneficial: *Chlorella* or *Scenedesmus* fed to Tilapia (Tartiel et al. 2008); *Chlorella* fed to Korean rockfish (Bai 2001); *Undaria* or *Ascophyllum* fed to sea bream (Yone et al. 1986); and *Ascophyllum, Porphyra, Spirulina*, or *Ulva* fed to sea bream (Mustafa and Nakagawa 1995). Often the algae chosen for fish feeding studies appear to have been selected largely for convenience, because they are low-cost and commercially available. For example, microalgae such as *Spirulina, Chlorella,* and *Dunaliella* can be produced by low-cost open pond technologies and are marketed as dry powders, and their nutritional profiles are well-documented.

Amino Acid Profiles

Proteins are made up of chains of amino acids which form the building blocks. They are utilized to form various cell structures and serve as a source of energy. An effective dietary protein source must satisfy an animal's requirement for both essential and nonessential amino acids (Guillaume 1997). Amino acids are required by all fish and shrimp species, and tryptophan is a precursor of the neurotransmitter serotonin (Savelieva, et al. 2008). Valine is involved in many metabolic pathways and is considered indispensable for protein synthesis and optimal growth (Wilson 2002). Histidine is also an indispensable amino acid involved in many metabolic functions including the production of histamines, which take part in allergic and inflammatory reactions. It plays a very important role in maintaining the osmoregulatory process and is related to energy production or is used in other metabolic pathways during certain emergencies/harsh conditions (Abe and Ohmama 1987).

From a diet formulation perspective, it should be noted that some responses to dietary protein source seem to be independent of their amino acid balance. Total protein content of a feed to a point where excessive amounts of many amino acids are included in an attempt to meet the requirement for one or more of the essential amino acids is shortest in supply. A diet should be formulated based on digestible amino acid values of feed ingredients and an ideal protein. *Spirulina* contains an unusually high amount of protein, up to 65% by dry weight, and is a complete

protein, containing all essential amino acids, along with good amounts of essential fatty acids, polysaccharide, phycobiliproteins, carotenoids, vitamins (especially B12), and minerals, making it a desired food source (Hu 2004).

The chemical composition of the experimental diets was equalled in protein and energy and at levels supposed to be optimal for Pacific white shrimp (Amaya et al. 2007a). All diets fed to shrimp in the present study had amino acid values similar to those reported by Suarez et al. (2009) for *L. vannamei*. In the present study, muscle composition of *L. vannamei* showed higher concentrations of histidine (16.9%), glycine (16.1%), and prolein (9.51%) in animal fed with diet supplemented with 10% of *Dunaliella salina*, whereas control feed fed group showed lower level of histidine (15.9%), glycine (14.9%), and prolein (8.79%). In the previous study, muscle tissue composition of *L. vannamei* resulted higher concentrations of arginine, lysine, and methionine in animals fed with diets supplemented with 10 and 40% of *Spirulina platensis*. However, it was found that the methionine content in animals fed with diet supplemented with 10% was lower when compared to the muscle demand values for this shrimp species fed with commercial diet. Whereas the imbalance, even if it is represented by a single essential amino acid, has an immediate effect on meeting the protein needs (Gadelha et al. 2013). As regards the energy balance (Fox et al. 2004), it was observed that the diet containing 40% *S. platensis* provided the best concentrations of amino acids for growth performance in animals.

The free amino acids have been shown to function in osmoregulation (Fang et al. 1992) and also have a major contribution to the flavor of seafoods (Thompson et al. 1980). Animals must consume dietary protein to obtain a continual supply of amino acids. After ingestion, it is digested or hydrolyzed to release free amino acids that are absorbed from the intestinal tract and then distributed to the various organs and tissues. The amino acid composition and concentration in the muscle of prawns may affect the quality of the prawn (Wang et al. 2004). Crustacean muscles contain high concentration of free amino acids, such as arginine, glycine, proline, glutamine, and alanine (Cobb et al. 1975). Amino acids are used by the tissues to synthesize new protein; thus animals do not necessarily require protein but do require the amino acids which comprise proteins. High protein diets are needed for good growth of most aquatic animals (NRC 1993). Hence, estimation of minimum requirement of essential amino acids (EAA) is indispensable to formulate cost-effective diets. The quantitative EAA requirements of fish and crustaceans are often determined by feeding experiments with diets containing graded levels of the particular amino acid to be examined (Wilson 1989). Deshimaru and Shigeno (1972) reported that the amino acid composition of the dietary protein should match of prawn tissue. These studies are all in agreement, indicating that arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine are essential in the crustacean diet. Although not strictly required, tyrosine and cysteine should be considered semiessential as their presence in a diet reduces the requirement for phenylalanine and methionine, respectively (Guillaume 1997).

Arginine was proven to be crucial in energy metabolism by maintaining glycolysis under hypoxic conditions (Gade and Grieshaber 1986). Arginine plays an

important role in cell division, the healing of wounds, removing ammonia from the body, immune function, and the release of hormones (Tapiero et al. 2002; Stechmiller et al. 2005; Witte and Barbul 2003). Glutamic acid turned into glutamine, which is deaminated to produce NH_3 (Shen and Wang 1990). Aspartic acid can be synthesized from other amino acid and carbohydrate. The present study revealed that the presence of essential amino acids (EAA) like lysine, arginine, asparagine, threonine, isoleucine, tyrosine, leucine and valine provide the best growth and survival rates during commercial farming. However, the nonessential amino acids such as (NEAA) alanine, glycine, cystine, and serine were also identified.

According to Holme et al. (2009), diets containing amino acids in proportions similar to those in the shrimp muscles provide the best growth and survival rates during commercial farming, and the diet quality was not necessarily related to the total amount of proteins, but a well-balanced supplementation of amino acids can be found in *Dunaliella salina* present in its composition, with a complete protein containing all the essential and nonessential amino acids (Di Lifetec Co LTD 2009).

Fatty Acid Profiles

In this present study, totally 17 fatty acids were detected, which include both unsaturated (essential) and saturated fatty acids (Table 9). Among these 14 were saturated fatty acids, remaining 3 were unsaturated fatty acids. In the present study, the following fatty acids such as myristic acid, palmitic acid, stearic acid, oleic acid, linolenic acid, EPA, and DHA were found to be significantly higher in microalgal supplemented feed fed groups when compared to control group. The linolenic acid, arachidic acid, behenic acid, and lignoceric acid were found to be lower in microalgal supplemented feed fed groups when compared with control group. The total quantity of the fatty acids in experimental groups was found to be higher in *Dunaliella salina* incorporated feed groups compared to control group.

Feed is the largest production cost for commercial aquaculture plants, so improving feed efficiency in industrial systems has high priority. Moreover, fishmeal prices have risen in real terms in the past three decades and are likely to increase further with continuously growing demand. Therefore, microalgae are some of the most important feed sources in aquaculture (live feed for larvae of bivalves, crustaceans, and marine fish; food for rotifers and shrimps), due to their nutritional value and their ability to synthesize and accumulate great amounts of ω3 PUFA.

The polyunsaturated fatty acids (PUFA) of the linoleic (n-6) and linolenic (n-3) families have been recognized as important nutrients for growth and reproduction in fish (Izquierdo et al. 2001), crustaceans (Jeffs et al. 2002), and mollusks (Caers et al. 2000; Navarro and Villanueva 2000; Nelson et al. 2002). Cavalli et al. (1999) suggested that the higher levels of linoleic acid (18:2n-6) and n-3 highly unsaturated fatty acids (HUFA) increased fecundity, egg hatching efficiency, and larval quality of *M. rosenbergii*. In the present study, the fatty acids such as myristic acid, palmitic acid, stearic acid, oleic acid, linolenic acid, EPA, DHA, arachidic acid, behenic acid, and lignoceric acid were found in both experimental and control groups. The

total quantity of the fatty acids in experimental groups was found to be higher in algal supplemented feed fed groups when compared with control group.

The control and experimental feed fed shrimp larvae in the present study had amino acid values similar to those reported by Suarez et al. (2009) for *L. vannamei*. In the present investigation, the recorded improved growth performance in the shrimp fed with algae diet might be due to higher HUFA content available in algae. Digestibility of fatty acids is known to be influenced by a number of factors, including their chain length, degree of unsaturation, level of incorporation in dietary fat and by other constituent fatty acids, and their melting points (Lin et al. 2006). Moreover, the levels of polyunsaturated fatty acids (PUFA), such as linoleic, linolenic, eicosapentaenoic (EPA), docosahexaenoic (DHA), octadecatrienoic, and arachidonic acids, were found to be higher in experimental feed fed shrimp larvae when compared with control feed fed shrimp larvae.

Total Carotenoid Profiles

Algae with high nutritional value have remarkable potential as fish feed. Previous finding by Khatoon et al. (2010b) suggested that algal feed could be a better supplement for animal protein like *Tubifex*. The fish group fed with experimental diets showed a higher feed intake rate than that with the control feed during the experimental tenure. This might be due to the attractive color, racy flavor, and good nutrient composition of the experimental feeds. The high dietary carotenoid content might have contributed to elevated muscle carotenoid deposition. The occurrence of carotenoid in the experimental fishes might protect them against different diseases as suggested by Trinadha et al. (2003). In the present investigation, the overall carotenoid pigments in *Dunaliella salina* and experimental feed fed shrimp larvae were found highest than control feed and control feed fed group. The potential market for microalgae-derived food coloring is vast. *Dunaliella salina* is grown for a source of the photosynthetic pigment, beta-carotene. Beta-carotene is used as an orange dye and as a vitamin C supplement. Microalgae such as *Dunaliella salina*, *Haematococcus pluvialis*, and *Spirulina* are also used as a source of natural pigments for the culture of prawns, salmonid fish, and ornamental fish. Over the last four decades, several hundreds of microalgae species have been tested as food, but probably less than 20 have gained widespread use in aquaculture.

Previous studies with prawns, mostly *Penaeus japonicus*, have shown that dietary astaxanthin, β-carotene, or canthaxanthin led to the deposition of mainly astaxanthin esters in the carapace, as in the present study (Yamada et al. 1990; Chien and Jeng 1992; NeÁgre-Sadargues et al. 1993). However in the work of Yamada et al. (1990), astaxanthin was reported to be more effective for pigmentation than β-carotene or canthaxanthin. The present study simply showed that feeding of algal β-carotene was effective and efficient with the *P. japonicas* (Chien and Jeng 1992), a higher survival rate for animals fed with astaxanthin-supplemented diets than for ones fed a supplement of β-carotene or algal meal was reported. This is contrast with the present results obtained with the different species, *Penaeus monodon*.

Conclusion

The present study revealed that the optimization of parameters can increase the carotene level in *Dunaliella salina*. It is understood that *Dunaliella salina* can be capable of producing more amount of pigment especially the carotene which has multi commercial applications and uses. The present study concluded that the marine microalga *Dunaliella salina* can be considered as good candidate species for mass scale culture and further application as aqua feed additive to replace the high-cost fishmeal as partially or fully. Furthermore, the *Dunaliella salina* will be cultured in large-scale level in the coastal arid lands wherever possible and used as aqua feed additive in shrimp feed manufacturing for sustainable aquaculture practices.

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Strain Selection and Lipid Characterization of Marine Diatoms with Potential for Biodiesel Production

S. Jeyanthi and P. Santhanam

Introduction

Phytoplankton is photosynthetic organisms that convert sunlight, water, and carbon dioxide into sugars, from which biological macromolecule, such as lipids, can be obtained. Due to their higher photosynthetic efficiency, higher biomass production, and faster growth rate, they have been suggested as very good candidates for biofuel production. High lipid content and fast growth rate are the major criteria for good candidate algal strains. Also, the algae should not be too difficult to harvest and must have a suitable and cost-effective cultivation system. Microalgae, like any other living organism, synthesize lipids which they use to maintain the integrity of their cells. Under specific circumstances, microalgae accumulate high concentrations of carbon in the form of TAG (Converti et al. 2009; Li et al. 2008; Liang et al. 2006; Melis and Mitra 2008).

Among the microalgae, diatoms are dominant variety in brackish water and marine environment and considered as promising raw materials for biofuel production. Diatoms are the major primary producer (40%) in marine ecosystems compared to other phytoplankton (Falkowski et al. 1998), and some species contains neutral lipids. The gravimetric method is an example of the first category which is time-consuming (approximately 3–4 days) (Doan and Obbard 2011; Govender et al. 2012). In contrast, the fluorescence method takes less than 30 min. Nile red stain is used to determine cell content of neutral lipids (Greenspan et al. 1985), which is advantageous over gravimetric method. It has been used to detect and quantify the lipid content of many microalgae strains for production of algal biodiesel (Isleten-Hosoglu et al. 2012). High carbon lipid is present in diatoms, which is the major source of sustainable oil production and thus is highly feasible for the development of third-generation biofuels.

S. Jeyanthi · P. Santhanam (✉)
Marine Planktonology & Aquaculture Laboratory, Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

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Research Strategy

The first section determines the isolation of diatom strains and the preparation of the culture medium. The second section covers the cultivation of the algae in laboratory. The third section explores the optimizing biomass production further by cultivating the strains and rapid screening method with diatoms for high lipid yield. The fourth section covers lipid extraction method. The fifth section of the research is transesterification of lipid to biodiesel production. The final section deals with the physical and chemical characterization of biodiesel.

Culture Media Conditions

Large and variable number of organic compounds and more than 50 known elements are available in natural seawater (NW) which has been used as a complex medium for algal culture. Without the addition of further macronutrients and trace metals enriched with vitamin solution, the yield of algae is usually too low for culture maintenance or laboratory experiments, and thus enrichment is normally required. The materials required for preparation of seawater culture media and the main recipes of stock solutions are macronutrients, trace elements, and vitamins.

Glassware Maintenance

Glassware and plasticware to be used for preparation of media should be kept separate. Washing protocols vary, but in general it is important to be aware that tap water often contains high amounts of nutrients, trace metals, and heavy metals. Therefore, if tap water is used for washing and rinsing, then make sure that distilled water is used for the final rinse. New glassware and plasticware should be degreased in dilute NaOH, soaked in dilute HCl, and then soaked in deionized water for several days before use. All the glassware should be autoclaved, and clean glassware and plasticware should be stored in closed cupboards.

Macronutrients (Nitrogen, Phosphorus, and Silicon)

Nitrogen, phosphorus, and silicate are considered as the major macronutrients. However, silicate is required only for diatoms, silicoflagellates, and some chrysophytes. Guillard f/2 (Guillard and Ryther 1962) is commonly used medium designed for growing coastal and marine microalgae, especially diatoms. Add the following components, viz., $NaNO_3$, $NaH_2PO_4.H_2O$, $Na_2SiO_3.9H_2O$, trace metals solution (see

mix below), and a vitamin solution (see mix below), into 1 l with filtered natural seawater. Prepared media should be autoclaved to reduce precipitation.

	Stocks	**Per liter**
(1)	**Trace elements (chelated)**	
	Na_2 EDTA	4.16 g
	$FeCl_3.6H_2O$	3.15 g
	$CuSO_4.5H_2O$	0.01 g
	$ZnSO_4.7H_2O$	0.022 g
	$CoCl_2.6H_2O$	0.01 g
	$MnCl_2.4H_2O$	0.18 g
	$Na_2Mo_4.2H_2O$	0.006 g
(2)	**Vitamin mix**	
	Cyanocobalamin (vitamin B12)	0.0005 g
	Thiamine HCl (vitamin B1)	0.1 g
	Biotin	0.0005 g
(3)	Sodium metasilicate $Na_2SiO_3.9H_2O$	30.0 g
	Macronutrients	**Per liter**
	$NaNO_3$	0.075 g
	$NaH_2PO_4.2H_2O$	0.00565 g
	Trace elements stock solution (1)	1.0 ml
	Vitamin mix stock solution (2)	1.0 ml
	Sodium metasilicate stock solution (3)	1.0 ml

Prepare 1 L medium from stock solution	
$NaNO_3$	1.0 ml
$NaH_2PO_4.2H_2O$	1.0 ml
Trace elements stock solution (1)	0.1 ml
Vitamin mix stock solution (2)	1.0 ml
Sodium metasilicate stock solution (3)	1.0 ml
For 1 litre with filtered and sterilized natural seawater	

Isolation and Cultivation of Microalgae

For isolation of microalgae, capillary pipette removal and serial (multiple) dilution technique are most widely used methods. Alternative isolation methods for diatom isolation (especially useful for smaller cells) include quadrant streaking cells in sterile petri dishes half full of media solidified with 1–1.5% agar. Pure diatom cultures are grown over a period of 20 days in sterile 250 mL Erlenmeyer flasks. 1000 lx illumination with white fluorescent bulb for 12:12 h light and dark

condition is set up for the cultures in a sterilized air conditioned room with a temperature of 25 ± 1 °C and salinity of 25–35 ppt. Diatom cells from the stationary phase of the stock culture are used as inoculum for the next cultures.

Optimization of Biomass Conditions

The optimal growth conditions for culture of microalgae are strain specific and the biomass productivity depends upon many factors. These include abiotic factors like temperature, minerals, carbon dioxide, pH, nutrients, photoperiod, and illumination. Based on the growth conditions such as medium composition, irradiance, and temperature, the composition of the lipid present in microalgae and productivity of microalgae will vary (Huerlimann et al. 2010). Other inorganic nutrients required for culture of algae include nitrogen, phosphorus, and silicate (Suh and Lee 2003).

Temperature

Temperature is the major factor which influences the rates of all chemical reactions related to algal growth rate and its metabolism (Sandnes et al. 2005). Adaptation to changes in temperature involves a variety of responses in both the physiology and biochemistry of microalgae. Temperature changes affect the biochemical composition of the cells specifically lipids and proteins. Temperature should be maintained at 25 ± 2 °C, particularly for marine microalgae which are less tolerant to temperature variations (Andersen 2005). Temperature variations were monitored regularly using thermometer. The algal growth rates were found by measuring their fresh weights.

Illumination and Photoperiod

Light is the important factor that affects biomass productivity of microalgae. The energy source for growing microalgae is provided by light via photosynthesis. Sufficient illumination must be effectively utilized to achieve higher biomass productivity. Algal cultures are placed outdoor in open sunlight; indoor cultures are maintained under fluorescent light. The effect of different light intensities on algae growth and lipid production is measured by carrying out the experiment at different light intensities, viz., 1500, 2000, 2500, and 3000 lux. The light intensity is measured by using lux meter and the values are expressed in lux or μmol m^{-2} s^{-1}. While optimal light-dark cycles have been found to vary from 12:12 to 16:08 for most cultures (Andersen 2005), some algae may be destroyed by continuous light (Price et al. 1998). Over illumination can cause photo-inhibition by photooxidative stress on the algae (Leon and Galvan 1999). The effect of illumination on the growth of algae is calculated by measuring the algal biomass.

pH

The pH determines the extent of ionization of chemical compounds and biochemical metabolites and consequently has a non-negligible influence on their uptake and reactivity. Cultivation of microalgae depends on pH levels and the carbon availability, metabolism, and biochemical composition of microalgae influenced by optimum pH (Richmond 2000). The culture media are adjusted with various pH ranges, i.e., 6.5, 7, 7.5, 8, 8.5, and 9. The algae samples are optimized within these pH ranges, the effect is observed, and the biomass of samples are analyzed after the specified time period has been elapsed.

Salinity

Salinity is the major factor for marine microalgal growth which relates to osmoregulation. Salinity is usually growth-limiting at the extremes of salt tolerance in some microalgae species, i.e., in hypotonic (low salt concentration) or hypertonic (high salt concentration) media. The effect of a different range of salinity levels (0 M, 0.2 M, 0.5 M, 0.8 M, 1.1 M) on growth rate of algal strains is tested to know the salinity tolerance. For increasing salinity, NaCl is added and to decrease salinity, distilled water is added to maintain the required salinity.

Dissolved CO_2

The availability of CO_2 and HCO_3^- in microalgal culture is largely pH-dependent (Emerson and Green 1938; Moss 1973; Owens and Esaias 1976) such that growth limitation related to carbon source in photoautotrophic microalgae can be conveniently managed by CO_2 supply or addition of carbonate salts. $NaHCO_3$ salt in the different concentration ranges of 20, 40, 60, 80, and 100 mg/L (1 mg/L = 1 ppm) is freshly weighed and added to each of the flasks for the bicarbonate growth study of microalgae, and the pH is measured according to the bicarbonate salt concentration.

Nutrients

Sustained fast growth and biomass production require that nitrogen, phosphorus, and carbon are available in non-limiting amounts as they are essential components of proteins, enzymes, lipids, genetic materials (RNA, DNA), energy-transfer molecules (ATP, ADP), and other cellular constituents. Nitrogen, phosphorus, and silicon

are the other inorganic nutrients required for algae production (Suh and Lee 2003). Nitrogen deprivation, phosphate limitation (Yeesang and Cheirsilp 2011; Rodolfi et al. 2009; Chiu et al. 2009; Phadwal and Singh 2003), iron supplementation, and silicon deficiency (Liu et al. 2008; Griffiths and Harrison 2009) are the factors which have been influencing the lipid content of algae.

Rapid Nile Red Fluorescence Screening Method of Lipid Determination

Nile red is a lipid-soluble dye and has been widely applied to determine the cellular lipid content of microalgae in qualitative and in situ quantitative analysis (Doan and Obbard 2011). Previous research by Elumalai et al. (2011) and Pick and Rachutin-Zalogin (2012) have mentioned that the Nile red staining technique is a useful tool for rapid determination of neutral lipids present in microalgae. Ahlgren and Hyenstrand (2003) and Hoffman et al. (2010) have reported that under nitrogen-deficient conditions, algal cells often accumulate a surplus of carbon metabolites as neutral lipids more than polar lipids.

Nile Red Stock Preparation

Nile red is a soluble phenoxazone lipid dye that partitions to cytoplasmic oil bodies in cells and becomes fluorescent (Greenspan et al. 1985). Nile red (9-diethylamino-5H-benzo[a]phenoxazine-5-one) is purchased from Sigma-Aldrich (USA). Analytical grade acetone is used for dissolving of Nile red. Stock solutions of Nile red 0.1 mg/ml in acetone are prepared and stored protected from light.

Fluorescence Microscopy

0.5 mL of microalgae culture (both nitrogen-rich and nitrogen depleted) is centrifuged at 1500 rpm for 10 min, and the pellets are washed with sterile distilled water (equal volume) for several times. 0.5 mL of Nile red is added into centrifuged algal sample and incubated for 10 min at room temperature (~25 °C) (Siaut et al. 2011). After washing once with the distilled water, stained microalgal cells are observed by a FLoid cell imaging system.

Observation of Oil Bodies

Nile red staining is applied in dark condition. This is used to visualize oil bodies and assess their morphology, localization, and numbers in the cultured cells. The mixtures are thoroughly mixed and a drop of culture is applied to a glass slide, covered with a cover slip after about 10 min, and observed under a fluorescence microscope.

Optimization of Diatom Cultures

Before each experiment, the stock cultures are grown approximately for two generations. The cultures are started by inoculating mother culture at a volume of 5–10%.

Growth Analysis

Optical densities of microalgae cultures are measured at 5-day interval of time by checking culture turbidity at 680 nm (Lichtenthaler 1987) with the help of spectrophotometer (Shimadzu UV/VIS). Vortexing of diatom cultures is done to get homogenous culture to prevent the settling and erroneous result while reading the OD.

Biomass Estimation

The biomass is harvested by centrifugation method. The centrifuged algal cells are washed with deionized water and dried (60 °C) using hot air oven to determine the dry weight (expressed as g/l). Cultures are harvested and dry biomass is estimated at 5 days of intervals.

Measurement of Photosynthesis Activity (Mackinney 1941)

Chlorophyll is extracted by taking 0.5 g of algae sample at every growth phase, to which 5 ml methanol or 80% acetone is added. The sample is kept undisturbed overnight at 4 °C and the optical density of the chlorophyll is measured next day with a UV/VIS spectrophotometer at 663 nm. The amount of chlorophyll extracted is calculated for following equations of Mackinney.

Calculation

$$\text{Chlorophyll } a \ \mu\text{g} / \text{ml} = \text{O.D} \times 12.63 \times \text{D.F.}$$

(where O.D is optical density and D.F dilution factor)

$$\text{Chlorophyll } b \ \mu\text{g} / \text{ml} = \text{O.D} \times 19.3 \times \text{D.F.}$$

(where O.D is optical density and D.F dilution factor)

Lipid Extraction

After finishing cultivation, microalgal cells are harvested at a stationary phase using the centrifugation method (7000 × g, 15 min, 4 °C). The cells are then collected and weighed. To get high concentrations from lipid extraction, cells are dried using lyophilization or freeze-drying method. The following are the various kinds of cell treatment investigated:

Mechanical/Physical Disruption

- Bead milling (Cerón-García et al. 2008)
- Mortar and pestle or sand (Chisti and Moo-Young 1986; Lee et al. 1998)
- Sonication, microwave, osmosis (Lee et al. 2010)
- Temperature shock (heat or freezing)

Solvent System

Lipids are typically distinguished from other water-soluble cellular components by their hydrophobicity and by their instantaneous solubility in solvents (Lundberg 1984). Methanol is the alcohol of choice for the transesterification process because of its low cost and chemical and physical properties (Fukuda et al. 2001), while alkalis are preferred as catalysts because the rate of reaction is 4000 times faster than with acid catalysts.

Single Solvent System

- Hexane
- Diethyl ether
- Isopropanol

Double Solvent System

- Chloroform and methanol (1:2)
- Dichloromethane and methanol (2:1)
- Propanol and cyclohexane (1:1.25)
- Acetone and dichloromethane (1:1)
- Hexane and isopropanol (3:2)

Extraction of Total Lipids and Fatty Acid Analyses

The total lipids are analyzed by Bligh and Dyer's method (Bligh and Dyer 1959). 100 mg of centrifuged (3000 rpm for 10 min) algal biomass (100 mg) is collected and transferred into a container. The algal biomass is suspended in 4 ml of chloroform and 2 ml of methanol mixture and shaken well. The cells are then subjected to sonication for the complete disruption of cells for 1 h. The chloroform-methanol forms a biphasic layer. The lower lipid layer is carefully separated by using micropipette and transferred into a centrifuge tube. And then 2 ml of distilled water is added and stirred well for further purification. The total lipid is transferred to clean dried and weighed glass centrifuge tube. The weight of total lipid is determined gravimetrically.

$$\text{Lipid content}\left(\%\right) = \text{lipid extracted}\left(\text{g}\right) / \text{original sample}\left(\text{g}\right) \times 100$$

The lower lipid layer is separated carefully using the micropipette and transferred into a centrifuge tube and processed for fatty acid analyses. GC-MS analysis is performed using SCION 436-GC Bruker TQ mass detector with BR-5MS column (30 × 0.25 mm × 0.25 μm film thickness). Helium is used as the carrier gas at a flow rate of 1 ml/min in split mode (50:1). Temperature of oven starts at 80 °C and is held for 2 min and then it is raised at rate of 20 °C per min to 160 °C without holding and raised at rate of 5 °C per min to 280 °C. Holding is allowed for 10 min at 300 °C at program rate of 20 °C per min. Total GC running time is 41 min. The mass spectrum of compounds present in samples is obtained by electron ionization at 70 eV. Total running time is 36 min. Mass scanning range is from 50 to 500 (m/z). Total MS running time is 46 min.

Biodiesel Production

The biodiesel from microalgae is produced by direct transesterification as described by Johnson and Wen (2009). 1 g of dried algal biomass is taken in a clean container. 3.4 ml of methanol, 0.6 ml of sulfuric acid, and 4.0 ml of chloroform is mixed with algal biomass and kept in a water bath at 90 °C for 40 min. When the reaction is completed, the tubes are allowed to cool at room temperature. Then 2 ml of distilled water is added to the tubes and mixed for 45 seconds. Then the samples are centrifuged at 3000 rpm for 10 min for phase separation. The organic layer that contained biodiesel (FAME) is carefully collected and transferred to a pre-weighed glass vial. The solvent is evaporated using nitrogen gas and the biodiesel is determined gravimetrically.

Washing and Drying

Biodiesel must be washed with 45 °C warm water to remove any remaining methanol, glycerin, catalyst, soaps, and other impurities. It is passed through the esters to allow soluble material, excess catalyst, and other impurities to stick to the water and be settled to the bottom of the vessel. The excess water is fully drained from the vessel periodically and the pH of the biodiesel becomes relatively neutral. If the excess water is remaining during washing of biodiesel, the biodiesel will look a bit cloudy. To remove the excess water from biodiesel, it should be heated slowly to 100 °C and held there until all moisture present is evaporated.

Physical and Chemical Characterization of the Biodiesel (Indhumathi et al. 2014)

Determination of Acid Value/Free Fatty Acid (FFA)

2 g of the oil is measured and poured in a beaker. 50 ml of neutral solvent (a mixture of petroleum ether and ethanol) is prepared and poured into the beaker containing the oil sample. The compound is stirred vigorously for 30 min. 0.56 g of potassium hydroxide (KOH) pellet is taken and kept in a separate beaker and 0.1 M KOH is measured; three drops of phenolphthalein indicator is added to the sample and is titrated against 0.1 M KOH till the color change observed turned pink and persisted for 15 min.

$$AV = 56.1 \times A \times N / Woil$$

where A represents the volume of standard a ali used, N is the normality of standard alkali used, and Woil represents the weight of oil used.

$$FFA = AV / 2$$

Determination of Saponification Value

The alcoholic KOH is freshly prepared by dissolving KOH pellet in ethanol. 2 g of oil is taken and transferred into a conical flask. 25 ml of the freshly prepared alcoholic KOH is added to it; a blank is used. The sample is well covered with foil and kept in a steam water bath for 30 min, shaking it periodically; 1 ml of phenolphthalein indicator is added to the mixture and titrated against 0.5 M HCl to get the end point.

$$SV = 56.1 \times B - A \times N / Woil$$

where B is the volume of standard ethanol potassium hydroxide used in blank titration, A represents volume of standard ethanol potassium hydroxide used in titration with the oil, N is the normality of standard acid, and Woil is the weight of oil used.

Determination of Moisture Content

In order to determine the moisture content in the oil (%), pre-weight the empty moisture pan and transfer the 48.15 g of oil into moisture pan and keep it inside an oven for 3 h at a temperature of 450 °C. The sample is cooled and weighed every 1 h until the weight before and after is approximately equal.

Determination of Specific Gravity/Density (ASTM D1298) by Hydrometer Method

This procedure is used to measure specific gravity of the biodiesel which is measured by hydrometer method. A clean dry empty pre-weighted 50 ml density bottle is filled up with distilled water and subsequently with the samples and the mass recorded as M, it. The mass of the bottle and water is taken and recorded as M1 and that of biodiesel as M2, respectively. Hence specific gravity is evaluated.

Flash Point of Biodiesel

The flash point of biodiesel is known as to minimize the level of unreacted alcohol remaining in the finished fuel. For fuel handling and storage, the flash point is important in connection with legal requirements and for the safety precautions. It is normally specified to meet the fire regulation (Prugh, 2007). The biodiesel sample is heated in a close vessel and ignited. The temperature is recorded at the time of sample burns, and the Pensky-Martens cup tester measures the lowest temperature at which application of the test flame causes the vapor above the sample to ignite. The biodiesel is transferred in a cup in such quantity as to just touch the prescribed mark on the interior of the cup. The cup with biodiesel sample is covered and the sample is heated at a rate of about 5 °C per min. During heating, the oil is constantly stirred. As the oil approaches its flashing, the injector burner is lighted and injected into the oil container after every 12 s intervals until a distinct flash is observed within the container. The temperature is recorded at the time of flash, it is repeated three times, and the average is taken.

Flue Gas Analyzer

The flue gas analyzer is composed of probe, hand set remote connection, and analyzer. While running the diesel engine, carbon monoxide (CO), hydrocarbon (HC), nitrogen oxide (NOx), carbon dioxide (CO_2), and oxygen (O_2) were released. Biodiesel blends and diesel fuel can be analyzed with the help of an analyzer kit.

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Bioremediation of Wastewater Using a Novel Method of Microalgae Immobilized on Twin-Layer Recirculation System (TLRS)

M. Divya and P. Santhanam

Introduction

Effluent is a general term used to represent the water with poor quality that contains more amounts of pollutants and microbes. The effluent is discharged into the nearby water bodies; it can cause serious environmental and health problems to human beings. Bioremediation is an ecofriendly technique to reduce the pollutant and other contaminants present in effluents. Effluent treatment involves several processes which can be classified as physical, chemical and biological based on the method adopted for treatment. Physical treatment includes sedimentation (clarification), screening, aeration, filtration, skimming, flotation and degasification. These treatment methods consume more energy and it involves higher costs. Chemical treatment includes chlorination, ozonation, neutralization, coagulation, adsorption and ion exchange. These methods could be expensive as well as harmful to the environment. Biological treatment is the best option for treating high-strength wastewater, because it is cost-effective, efficient and successful cleaning technique for treatment of effluents. The successful operation of biological waste treatment processes depends on the use of bacteria, algae, fungi, protozoa, etc. Microalgae are one of the best bioremediators for the treatment of effluent. Algal group are playing a very important role in bioremediation process. It has the capacity to produce oxygen during photosynthesis and it also provides the basis for maintenance of good water quality by means of self-purification, especially in those deeper surface waters that are still clean and healthy. During their growth, they trap sunlight and CO_2 from the environment for their photosynthesis. Bioremediation using microalgae has a number of positive applications over the conventional methods as it is cost-effective and useful in treatment of wastewater, in CO_2 sequestration, in sanitation and also in the production of renewable sources of energy such as biodiesel, biofuel, glycerol, methane gas, hydrogen gas, biofertilizers, etc.

M. Divya · P. Santhanam (✉)
Marine Planktonology & Aquaculture Laboratory, Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

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The use of algal biomass is a successful strategy to recycle nutrients from wastewater as these organisms have very efficient nutrient uptake mechanisms. Besides nutrient recycling, algal biomass and products derived from algal biomass have the advantage of offering additional value supporting the economic feasibility of this approach (Cai et al. 2013; Olguin 2003). Microalgae were introduced into wastewater treatment already more than 50 years ago with the high rate algal ponds (HRAPs) designed by Oswald et al. (1957) which are based on the cocultivation of microalgae and bacteria in open pond systems. Despite several successful applications of this technology, some major drawbacks of high rate ponds in wastewater treatment systems are the costs involved to separate the treated wastewater from the algal biomass, low biomass productivities (i.e. low nutrient uptake rates) and interferences with contaminating organisms (Cai et al. 2013). Early techniques of cell immobilization employed to remove nutrients and other types of pollutants from wastewater mostly relied on active immobilization by encapsulation of microalgae in synthetic or natural polymer bodies such as gel beads or screens De-Bashan and Bashan (2010). However, limited growth and nutrient uptake of encapsulated cells (De-Bashan and Bashan 2010; Mallick 2002) as well as the technically complex process of encapsulation make these methods unsuitable for large-scale applications. More recently, systems have been modelled on the basis of phototrophic biofilms and passively immobilized microalgae on various kinds of solid supports (Roeselers et al. 2008), thus avoiding cost-intensive active attachment procedures and matrices.

Through the use of immobilization techniques which effectively allow the separation of cells from the flowing liquid medium like the twin-layer (TL) technology, no loss of cells by leaching was reported, while nutrients (N, P) could be quantitatively removed from wastewater (Shi et al. 2007). The twin-layer system had previously been shown to support growth of a large variety of microalgae (Nowack et al. 2005) and has been scaled up to a technical photobioreactor (PBR) prototype for production of microalgal biomass (Naumann et al. 2013). This study can be used to identify possible strengths and weaknesses of the twin-layer photobioreactor (PBR) and to assess the technical feasibility of the system using real wastewater with different physico-chemical properties, to evaluate the potential of this technology to remove nutrients from wastewater and recycle the nutrients in the algal biomass.

Microalgal Isolation Techniques

Isolation of microalgal species can be done by one of the following methods.

Pipette Method

Large species of microalgae can be picked out using a micropipette under microscope and transferred to culture tubes having suitable culture media.

Centrifuge or Washing Method

By repeated centrifuging of the algal samples in different rpms and by inoculating the deposits, it is possible to get different groups of algae.

By Exploiting the Phototactic Movements

Most of the phytoflagellates can be isolated by this method. Make a dark chamber with a small hole on one side and keep the phytoplankton sample in a beaker nearer to the hole. Keep a candle near to the hole outside. The flagellates have a capability to move towards the light; it is visible after some time that these organisms accumulate near to the candle light. By pipetting, these organisms can be separated by using tube culture methods; it can be raised to a pure culture.

Agar Plating Method

By this method for the preparation of agar medium, 1.5 gm of agar is added to 100 ml of suitable culture medium, e.g. Schreiber's medium, Miquel's medium, TMRL medium and Conway medium or even natural seawater. This agar solution is sterilized in an autoclave for 15 min under 15 lbs pressure and 121 °C temperature. The sterilized agar is poured in sterilized 15 cm diameter Petri dishes and kept for solidification. For the isolation, the required species can be picked up by platinum needle or loop under microscope and streaked on the surface of agar plate. Quadrat streaking is the best method for isolation of microalgae. After inoculation, these Petri dishes are placed in an incubator for 7–8 days providing light (1000 lux) and optimum temperature (23–25 °C). Within this time, the required species, if it has grown into a colony, is picked by platinum loop under microscope and transferred to culture tubes. Further, from culture tubes to small conical flasks and larger flasks, the algae can be grown on a mass scale.

Serial Dilution Technique

For the serial dilution technique, nearly 25 culture tubes (15 ml) are required. The filtered seawater can be used for serial dilution technique. The filtrate has to be inoculated to five series of culture tubes in various concentrations. This has to be kept under sufficient light (1000 lux) with optimum temperature (23–25 °C) conditions. After 15–20 days, some discolouration can be seen in the culture tubes, due to the growth of microalgae. Further purification of this culture can be done by

subculturing it in 500 ml or 1 litre conical culture flasks. The purified culture will be transferred into a 3 or 4 litre Haufkin culture flask and maintained as stock culture.

Culture Media

Based on the organism involved, different culture media are used for culturing the microalgae. Though Erd-Schreiber's and Miquel's media (Miquel 1892) are suitable for culturing the diatoms and nanoplankton, several other media are also available with the addition of trace metals, vitamins and other organic and inorganic salts. The diatoms require silica for building up the cell walls; the culture media should have a compound of silicate besides the nitrates, phosphates, chlorides and trace metals. Usually for culturing the flagellates, 'Conway' or 'Walne's medium (Walne 1976) is used in the laboratory for the maintenance of the stock culture as well as mass culture. Since this culture medium contains various chemicals, trace metals and vitamins (B1 and B12), the phytoflagellates such as *Isochrysis*, *Pavlova*, *Dicrateria*, *Chromulina* and *Tetraselmis* are being cultured by using culture medium without silicate. However, TMRL (Gopinathan 1982) are suitable for the mass culture of nanoplankton flagellates. Still, the microalgal culture technique requires a clear understanding of their nutritional requirements, especially during the different phases of growth. The following are media used for the microalgal culture.

Schreiber's Medium

Potassium nitrate (KNO_3)	0.1 g
Sodium orthophosphate (NaH_2PO_4)	0.02 g
Soil extract	50 ml
Filtered and sterilized seawater	1000 ml

Soil extract is prepared by boiling 1 kg of garden soil with 1 litre of freshwater for 1 h. After 24 h, clear water is gradually transferred into a bottle. For 1 litre of sterilized seawater, 50 ml of this soil extract can be added. This can be used as a medium for the isolation of nanoplankton.

Miquel's Medium

Potassium nitrate (KNO_3)	20.2 g
Sodium orthophosphate (NaH_2PO_4)	4 g
Calcium chloride ($CaCl_2$)	2 g
Ferric chloride ($FeCl_3$)	2 g
Hydrochloric acid (HCl)	2 g
Distilled water	100 ml

TMRL Medium

Potassium nitrate (KNO_3)	10 gm
Sodium orthophosphate (NaH_2PO_4)	1 g
Ferric chloride ($FeCl_3$)	0.3 g
Sodium silicate (Na_2SiO_3)	0.1 g
Distilled water	100 ml

The chemicals are mixed with 100 ml of distilled water and kept in a reagent bottle. 1 ml of medium is added to each litre of sterilized seawater. This medium can be used for the mass culture of diatoms.

Conway or Walne's Medium (1974)

Solution A		Solution B		Solution C	
Potassium nitrate (KNO_3)	100 g	Zinc chloride ($ZnCl_2$)	4.2 g	Vitamin B1 (thiamin)	20 mg
Sodium orthophosphate (NaH_2PO_4)	20 g	Cobalt chloride ($CoCl_2$)	4.0 g	Vitamin B12 (cyanocobalamin)	10 mg
Sodium EDTA EDTA (Na)	45 g	Copper sulphate ($CuSO_{4.}5H_2O$)	4.0 g	Distilled water	100 ml
Boric acid (H_3BO_3)	33.4 g	Ammonium molybdate ($(NH_4)_6Mo_7O_{24}.4H_2O$)	1.8 g		
Ferric chloride ($FeCl_2.6H_2O$)	1.3 g	Distilled water	1000 ml		
Manganese chloride ($MnCl_2.4H_2O$)	0.36 g				
Distilled water	1000 ml				

Solutions A, B and C are prepared separately in reagent bottle. 1 ml of solution A, 0.5 ml of solution B and 0.1 ml of solution C are added to 1 litre of filtered and sterilized seawater.

Growth Phases of the Algal Culture

The usual way of the laboratory culture of microalgae is one in which a limited volume of medium containing the necessary inorganic and organic nutrients is inoculated with a relatively small number of cells and these are exposed to favourable conditions of light, temperature, photoperiod and aeration. Increase in cell density

in such a culture follows a characteristics pattern in which the following phases of growth may usually be recognized.

Lag or Induction Phase

The cells taken from the mother culture and inoculated to a new flask have to adapt the surroundings or in the new medium. Hence, there will be no cell division for a few hours and this stage is known as lag or introduction phase.

Exponential Phase

Once the microalgal cells are adapted to the surroundings, the cells start dividing and grow rapidly. The cell will divide into two within the period of 8–16 h and further these cells carry on the growth till the culture reached its maximum concentration. This growth phase is known as exponential phase.

Declining Phase

Once the cells reach its maximum concentration, the growth and multiplication of the cells will be stopped and slowly the cells starts decline. This arrested growth of the microalgal cells in the cultures is known as declining phase.

Stationary Phase

When the cell growth is stopped, the culture will be stationary without any further cell division for a few days. In the case of flagellates, the stationary phase is continuing for a long time. For this they may develop some cover or cyst or matrix around its body for thriving in the unfavourable conditions. If the cells get a new environment, they start further growth and reproduction in this phase.

Death Phase

The cells lose its viability and start to die and thus the culture becomes useless, either for reculturing or for feeding after the long period in the stationary phase.

Determination of Algal Cell Densities

The algal cell count must be made in order to schedule inoculation of the various culture flasks as well as mass culture containers, to monitor growth of the algal cultures. A small length of rubber tubing is helpful to work with and to connect it to the mouth piece end of a sterile serological pipette while collection of algal sample. To get the volume of algal sample, move the pipette around the tank while withdrawing algae up to the mark on the pipette and then place it in a flask. This is how the sample is to be counted. Since most of the nanoplankters measure less than 10 μ, a haemocytometer is used for counting most of the nanoplankters. The algal sample is treated with a drop of eosin or formalin to destroy the cells and stirred well; a drop of treated cell is taken with sterile pipette. After placing a coverslip on the haemocytometer, the pipette is brought to the edge of the haemocytometer to touch it. The sample runs inside the coverslip and thus a thin film of the culture is maintained, and the cells are equally distributed inside the chamber. The haemocytometer contains 9 chambers and 4 sides having 16 divisions and 5 chambers of multiple divisions; the counting is restricted for at least five chambers. The average number of cells in 1 ml is calculated as shown below:

$$\text{Total cell count} = \frac{\text{No of cells counted}}{\text{No of square counted in particular type}} \times \text{Total no of squares} \times 10{,}000$$

Stock Culture Maintenance

The microalgal stock culture is maintained in a special air-conditioned room adjacent to the mass culture room. Stock cultures are kept in 3 or 4 litre Haufkin culture flasks. The autoclaved or boiled seawater after cooling is transferred to the Haufkin flasks and required nutrient medium is added. Walne's or Conway medium is the quite ideal one for the maintenance of phytoflagellates. About 10 ml of the inoculum in the exponential phase is transferred to the culture flasks and kept in front of two tube lights (1000 lux). After 8–10 days, when the maximum exponential phase is reached, light is reduced to one tube light for further growth. The time required for the maximum cell densities varies based on the species. In the stationary phase, the flagellate can be maintained for 2 months in the stock culture room, under controlled conditions of light and temperature, with or without aeration.

Mass Culture

Microalgal mass culture can be maintained in the laboratory conditions as well as in the outdoor tanks. The containers used for the mass culture of microalgae are of 10 L capacity polythene bags, 20 L glass carboys, 100 L Perspex tanks and 250 L cylindrical transparent FRP tanks for the indoor culture. These containers can be

kept in wooden racks with sufficient light and aeration. Fully grown culture from the stock culture room is used as inoculum for the mass culture in these containers. The maximum concentration of the cells has been reached at the 5th–7th day and harvested. The haemocytometer is used for estimating the cell concentration. Once the culture reached the maximum density, the culture is supplied to the hatchery for the larval rearing. Leaving 2 L of the microalgal culture, fresh enriched medium is added for further culture in the same container.

Bioremediation of Wastewater Using Twin-Layer Recirculation System (TLRS)

Fabrication of Twin-Layer Recirculation System

Twin-layer recirculation system consists of two self-adhesion layers. (1) The substrate layer should be microporous in nature and ultrathin substrate (Table 1) and the source layer should be macroporous in nature (Table 2). The microalgae have a natural capability to attach to surfaces and grow on them. Using the twin-layer system, microalgae can grow effectively in an immobilized state. In brief, the TLRS could be horizontally (Nowack et al. 2005) or vertically (Shi et al. 2007) oriented and it consists of two different porous sheets, the substrate layer and the source layer. The substrate layer is a wet, microporous and ultrathin sheet, on which microalgae are immobilized, while the source layer subtending the substrate layer is a macroporous fibrous tissue, providing growth medium for microalgal growth. It is an ultrathin photobioreactor utilizing artificial illumination or natural sunlight. Nutrients, which is present in the medium, diffuse from the source layer through the substrate layer to the immobilized algae. The immobilized algae take up light, carbon dioxide and oxygen from the surface of the algal layer exposed to the ambient atmosphere.

Twin-layer recirculation system (TLRS) (Fig. 1) is mounted on a board made of polyvinylchloride (PVC) and is placed vertically inside a transparent PMMA tube. The board holds the twin layers and serves as the lid of the tube as well. Culture medium/wastewater is applied to the top of the source layer by a peristaltic pump at the with an optimal flow speed (1 m^{-2} h^{-1}). A continuous and gravity driven flow

Table 1 Material used for substrate layer

Material	Pore size (μm)
Protran reinforced nitrocellulose membrane	0.45
Printing paper	–
Nylon filter cloth	1.0
Non-woven polyester filter	–
PTFE filter	–

Table 2 Material used for source layer

Material	Pore size (μm)
Glass fibre lamina (80 g m^{-2})	–
Non-woven polyester lamina (AF 955 PE)	–
PTFE coated glass fabric mesh (7350)	4 × 4
Polyester mesh (PE4000)	4 × 4
Coated glass fibre mesh (8560)	4 × 4
PVC mesh (type 105)	4 × 4
Coated glass fibre mesh (110 g m^{-2}, blue)	10 × 10
Glass grid reinforced laminate (IGVP 271/540)	3 × 4
Polypropylene mesh (XN 0260)	4 × 6

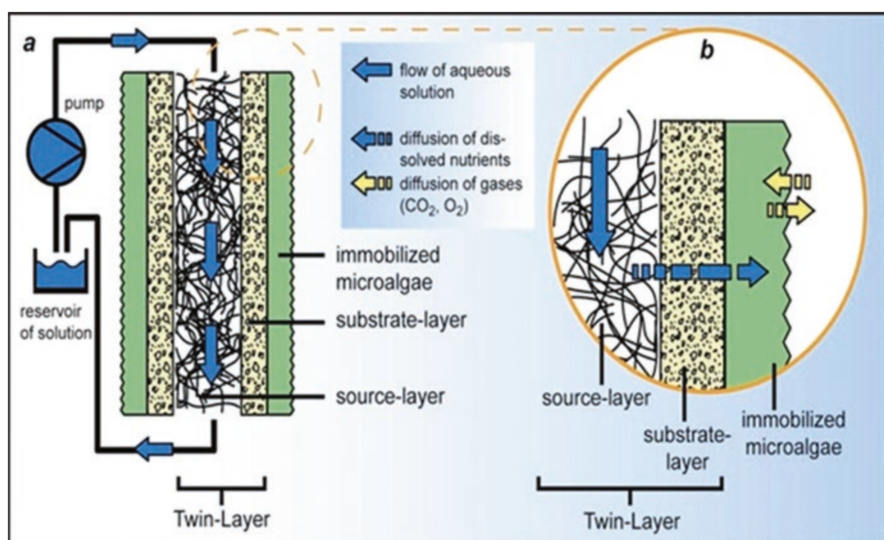

Fig. 1 Twin-layer recirculation system (TLRS) flow-through system (Shi et al. 2007)

through the source layer is established. After passing the twin layers, the fluid is collected in a darkened glass bottle. Nutrients diffuse from the source layer through the substrate layer to the immobilized algae. Light, carbon dioxide and oxygen are absorbed by immobilized algae from the surface of the algal layer exposed to the ambient atmosphere (Table 3).

Table 3 Optimized twin-layer recirculation system functional parameters

PMMA tube length (m)	Substrate layer (cm × cm)	Source layer (cm × cm)	Microalgae (cm × cm)	Liquid volume (L)
1	10 × 95	10 × 84	9 × 88	2
0.5	10 × 47.5	10 × 45.5	9 × 44.5	1

Large-Scale Twin-Layer Recirculation System (TLRS)

The large-scale twin-layer recirculation system includes three modules of twin layer mounted vertically on a metal rack. Culture medium is pumped from a reservoir to the filtration device (cartridge filter nominal, pore size 10 μm, Millipore, USA) and afterwards to the influent pipeline. The water medium dispersed to the source layers of three twin-layer modules. The immobilized algae absorbed nutrients from the source layer through the substrate layer. The fluid is collected at the bottom of the substrate layer and flows back to the container. The system operates with an optimal flow rate. The top of the twin layer is designed with irrigation drippings embedded in the influent pipeline to supply a homogenous water flow on the water channel located beneath. Once the culture medium is filled in the channel, it overflows through the weirs and supplies to the source layer. Nylon filter is used as the substrate layer on which the microalgae are immobilized. Microalgae are supplied with water medium by the source layer, which consists of a reinforced glass fibre mesh (Fig. 2 and Table 4).

Nitrogen and Phosphorous Removal

Nitrogen and phosphorous are the major key components for the algae for nutrient assimilation. Sewage is the major source of nitrogen and more than 50% phosphorous comes from the detergents. The principal forms in which they occur in wastewater are NH_3-N (ammonia), NO_2-N (nitrite), NO_3-N (nitrate) and PO_4 (orthophosphate). Together these two elements are known as inorganic nutrients and their removal is known as bioremediation. Microalgal culture offers a cost-effective approach for removing nutrients from wastewater (tertiary wastewater treatment) (Evonne 1997). Microalgae have a capability to utilize these inorganic nutrients and they can be grown in mass culture in outdoor solar bioreactor system. Biological treatment processes appear to perform well compared to the chemical and physical processes, which are, in general, too costly to be implemented in most places and which may lead to secondary pollution (de la Noue and Proulx 1992). The extensively used microalgae cultures for nutrient removal are species of *Chlorella*, *Scenedesmus* and *Spirulina. Scenedesmus* sp. plays an important role as primary producers, which is commonly found in all freshwater bodies, and it contributes to the purification of eutrophic waters (Mohamed 1994).

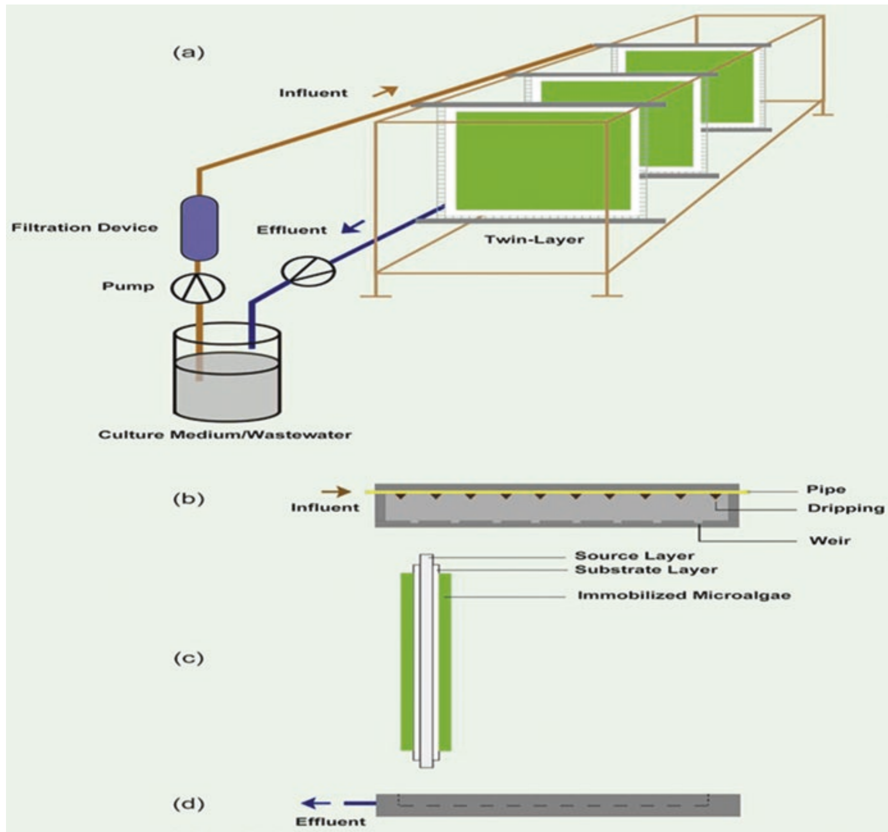

Fig. 2 Schematic diagram of the large-scale TLRS with its accessories. (**a**) The whole system, (**b**) influent supply, (**c**) twin layers and microalgae, and (**d**) effluent collection. (Source: Shi et al. 2007)

Table 4 Optimized large-scale twin-layer recirculation system functional parameters

Substrate layer (cm × cm)	Source layer (cm × cm)	Microalgae (cm × cm)	Liquid volume (l)
100 × 100	100 × 100	90 × 95	55

Estimation of ammonia-N, nitrite-N, nitrate-N and phosphate is used to study the nitrogen and phosphorus removal technology. Ammonia-N in the water samples is measured by indophenol method using a spectrophotometer at 640 nm. Similarly nitrite-nitrogen is determined using N-(1-naphthyl)-ethylene-diamine dihydrochloride (NEDD) at 543 nm. Nitrate-nitrogen is estimated using brucine sulphate method at 410 nm and phosphate is measured at 882 nm (APHA 2005).

BOD and COD Removal

Biological oxygen demand (BOD) is the amount of oxygen present in any water utilized by microorganisms for breaking down to simpler substances to the decomposable organic matter. It is also taken as a measure of the concentration of organic matter present in any water. BOD removal rate is measured using a 5-day incubation (20 °C) method. The reagents used for biochemical oxygen demand are phosphate buffer, magnesium sulphate, sodium sulphate solution (0.025 N), ferric chloride and calcium chloride. The preparation of dilution water and dilution of sample for BOD are done as per standard protocols as follows:

D_0 = DO in the sample bottle on the 0th day.
D_1 = DO in the sample bottle on the 5th day.
C_0 = DO in the blank bottle on the 0th day.
C_1 = DO in the blank bottle on the 5th day.
$C_0 - C_1$ = DO depletion in the dilution water alone.
$D_0 - D_1$ = DO depletion in the sample + Dilution water.
$(D_0 - D_1) - (C_0 - C^1)$ = DO depletion due to microbes.

BOD of the water sample is as follows:

$$\mathrm{BOD}(\mathrm{mg}/\mathrm{l}) = \frac{(\mathrm{D0} - \mathrm{D1}) - (\mathrm{C0} - \mathrm{C1})\,\mathrm{mg} * 1000}{\text{Volume of the sample used}}$$

Chemical oxygen demand (COD) which means the amount of oxygen required by organic matter for its oxidation by strong COD substance present in water. It can be used to measure pollution of domestic and industrial waste. The waste is assessed in terms of equality of oxygen required for oxidation of organic matter to produce CO_2 and water. Chemical oxygen demand is measured using the alkaline potassium permanganate method. The reagents used for chemical oxygen demand are sodium hydroxide, 0.01 N potassium permanganate, sulfuric acid, potassium iodide and sodium thiosulphate. COD of the sample is:

$$\mathrm{COD}(\mathrm{mg}/\mathrm{l}) = 0.8 \times \mathrm{N} \times 100(\mathrm{Blank} - \mathrm{Sample})$$

Biomass Estimation

10 ml from monoculture inoculum is used to analyse the total biomass (g/L) and biomass productivity (g/L/day). Microalgae samples are centrifuged at 10,000 rpm for 15 min to separate pellet from supernatant. All pellets are washed, resuspended with distilled water and centrifuged three times at same rpm to remove minerals and salts. Dewatering is accomplished by freeze-drying for 24 h followed by DCM

(deep chlorophyll maximum) determination from the difference between cell masses at the start and end of the experiment and divided by the time in days to determine biomass productivity.

Harvest

When the microalgal growth reached exponential phase, culture should be harvested and cell concentration determined. If the culture has reached the declining or stationary phase, the metabolites will be very high and the cells may not be in healthy condition. The rearing larval organisms may not show the expected growth if fed with this microalgal feed.

Preservation

During adverse weather conditions, the maintenance and constant supply of the algal culture whenever required is a major problem in the hatchery. To overcome this problem, sun-drying or freezing method can be used for preservation of microalgal cells. For the freezing technique, the culture is flocculated either by adding lime or by adjustment of pH using sodium hydroxide. The adjustment of pH with alum or lime will not give satisfactory results but by adding sodium hydroxide which is the advanced technique to raise the pH. After knowing the quantity of the culture to be flocculated, the volume of sodium hydroxide solution needed to flocculate to get one degree raise in pH is measured. If the pH of the culture is 8.4, by adding sufficient quantity of sodium hydroxide solution, it will be raised to 9.4. After vigorous mixing, the culture is left for 1 h to settle the algal mass at the bottom. The clear water is slowly discarded and the mass of algae can be collected in a plastic container. Then the original pH level of the culture is reduced slowly by adding of dilute hydrochloric acid. The collected algae are preserved by using freezing or sun-drying. The algae are dried by pouring the biomass in white enamel trays and keeping the same in the bright sunlight. If the algae are dried completely, the powder from the enamel tray is scraped and kept in a container. Some protective reagents like dimethyl sulphoxide or glycerol (a few drops) can be used before freezing the algal biomass. Finally, the concentrated algae are then poured into polythene bags after measuring. The polythene bags are labelled and kept in deep freezers. The frozen algae do not have the same protein content as in the live condition.

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The Techniques in Microalgae Bioremediation and Algal Co-product Development

S. Dinesh Kumar, P. Santhanam, and F. Leena Grace Nancy

Introduction

Absorption and adsorptions of pollutants involve the irons binding by either nonliving (biosorption) or living (bioaccumulation) biomass in addition to environmental factors influencing such remediation as mentioned in Fig. 1. Barron (1995) defined bioaccumulation process as gathering of pollutants, such as nutrients (nitrates, phosphates, sulfates, organic and inorganic carbon compounds), metals, pesticides, or other organic chemicals in an organism, and transferring of organic or inorganic pollutants inside of microbial organisms. Microbial organism uptakes the substances or pollutants (nutrients, metals, etc.), while the substance rate is higher than what they lost.

Treatment cost, pollutants types, strength and degree of pollution, and geographical and geological location of the polluted site are playing a major role in ex situ bioremediation techniques (Philp and Atlas 2005). Treating the pollutants on the spot is called in situ bioremediation. This technique is not involving any excavation or disturbance on the natural conditions; this treatment is comparably lower than the ex situ bioremediation (Fig. 2).

Microalgae are referred to as the aquatic microscopic plants (contains chlorophyll a and a thallus) and the oxygenic photosynthetic bacteria, that is, the cyanobacteria, formerly known as Cyanophyceae (Tomaselli 2004). Microalgae are tiny photosynthetic organisms that are available in marine as well as freshwater

S. Dinesh Kumar · P. Santhanam (✉)
Marine Planktonology & Aquaculture Laboratory, Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

F. Leena Grace Nancy
Marine Planktonology & Aquaculture Laboratory, Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

Bon Secours College for Women, Thanjavur, Tamil Nadu, India

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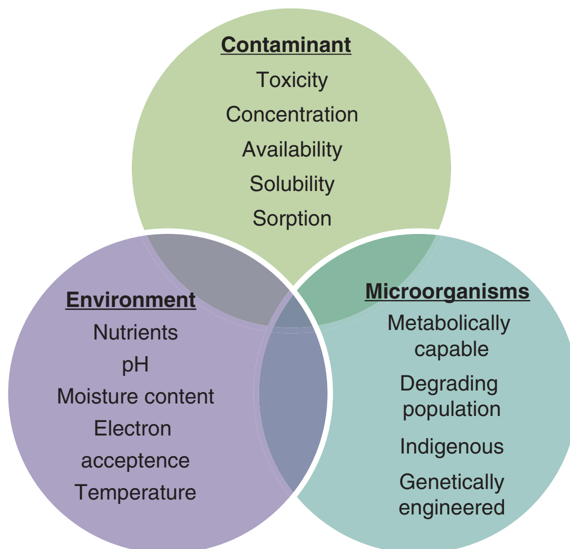


Fig. 1 Main factors influencing the bioremediation (Source: Bitton 2005)

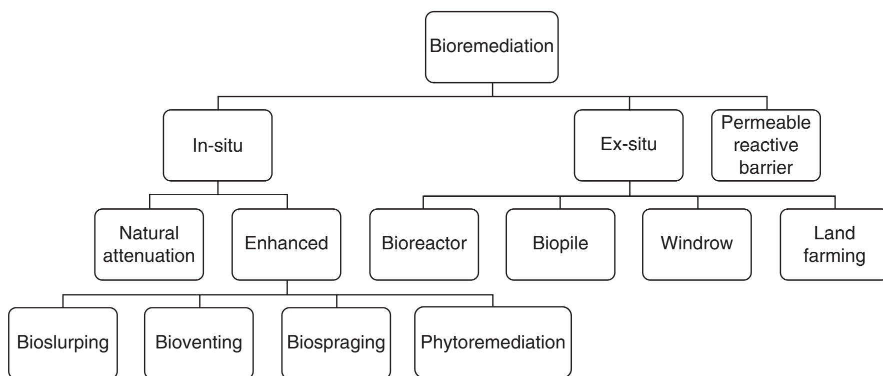


Fig. 2 The divergence of bioremediation techniques

ecosystem. Their photosynthetic methods are comparable to terrestrial plants. But compared to terrestrial plants, microalgae had higher efficiency to adsorb or absorb the CO_2 and other nutrients from the atmosphere and water; it might be due to simple cellular structure. Microalgae are the potential candidate species to convert the solar energy and wastewater nutrients to the valuable biomass while they are used as a bioremedient (Fig. 3). The aim of the present paper is to provide various bioremediation techniques (Fig. 4) by microalgae to treat a wastewater for removal of nutrients, metals, and other pollutants.

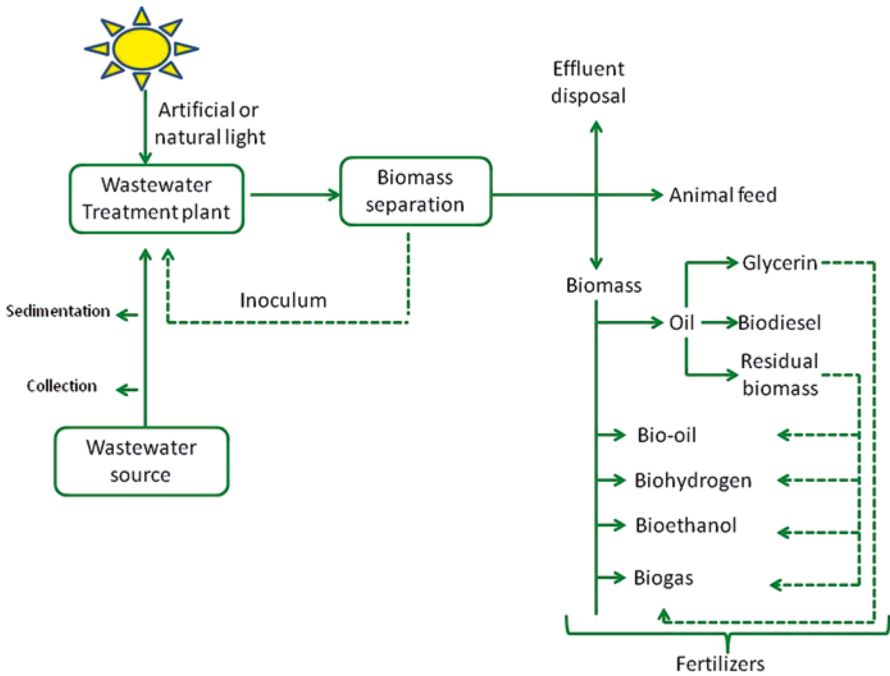


Fig. 3 Microalgal bioremediation process and co-product development

Analyses of Water and Wastewater

Hydrogen Ion Concentration (pH)

To analyze the pH of wastewater, use the portable bench top electrode pH meter with an accuracy of ± 0.1 pH units.

Turbidity

To analyze turbidity in wastewater, take a known volume (3.5 ml) of effluent sample, and the wastewater should be filled in the 4.5 × 1.2 cm cuvette, and the absorbance is measured using a UV-visible spectrophotometer at 425 nm and 580 nm (Palin 1955).

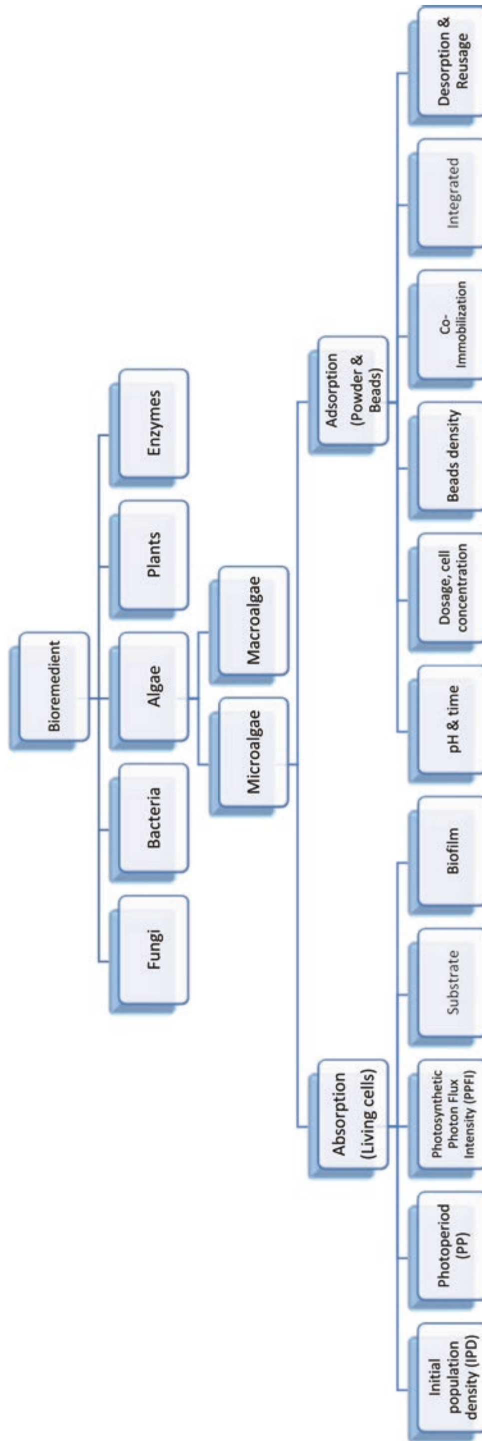


Fig. 4 Treatment tree of microalgae bioremediation

Total Suspended Solids (TSS)

To analyze a total suspended solid in wastewater, filter a 10 ml of wastewater using Millipore filtering apparatus fitted with 0.45 μm GF/C filter paper (APHA 1998). Concentration of TSS (%) will be calculated subtracting the initial weight from the final weight of the filter paper.

Total Hardness and Calcium Hardness

To analyze the water hardness (calcium and magnesium), a wastewater sample is to be buffered to pH 10.1, and an indicator is then added to the buffered sample. The indicator, when added to a solution containing Ca and Mg ions, turns red. EDTA, the titrant, complexes with Mg and Ca cations, removing them from association with the indicator. When all the Mg and Ca are complexed with EDTA, the indicator turns blue (AOAC 1995).

Nutrients

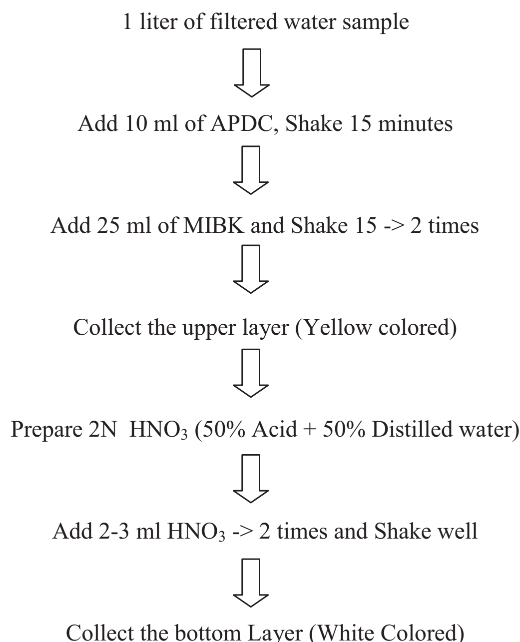
The inorganic nutrients (PO_4^{3+}, NO_3^-, NO_2^-, NH_4^+, SiO_3, TP, and TN) in the effluent water can be analyzed as per Strickland and Parsons (1972) and Jenkins and Medsker (1964) (Table 1). Phosphate is measured by using the acidified molybdate reagent to give phosphomolybdate complex which is then reduced to a highly colored blue compound (phosphorus molybdenum blue) using ascorbic acid as a reducing reagent in the wastewater. For the analyses of nitrate, the reaction between the alkaloid, brucine, and nitrate in acid medium that produces a yellow color is measured by standard spectrophotometric procedures. The nitrite is determined after diazotising it with sulfanilamide and coupling with N(1-Naphthyl)-ethylenediamine dihydrochloride. Ammonia nitrogen can be estimated by using the indophenol blue method based on the principle that in a moderately alkaline medium, ammonia is allowed to react with hypochlorite in catalytic amounts of nitroprusside to form indophenol blue. The determination of silicate in wastewater sample is based on the formation of a yellow silicomolybdic acid, when a nearly acidic sample is treating with a molybdate reagent. The yellow silicomolybdic acid is reduced to an intensely blue-colored complex using ascorbic acid as the reductant, and the color is measured spectrophotometrically at 810 nm. The estimation of total phosphorous and total nitrogen of wastewater sample is oxidized with the help of strong oxidizing agent (alkaline persulfate) by autoclaving in closed condition. The organic forms of phosphate and nitrate and also their inorganic forms in lower oxidation states are finally oxidized to inorganic phosphate and nitrate, respectively.

Table 1 Short notes of nutrient estimation in water and wastewater

Nutrients	Required amount of water sample	Required reagents	Preparation of reagents	Incubation time (min)	UV –NM
Phosphate	25 ml	Ascorbic acid – 0.5 ml	*Ascorbic acid* 10 g to 50 ml DW + 50 H_2SO_4	10–30	880
		Mixed reagent – 0.5 ml	*Mixed reagent* 125 ml of molybdate solution + 350 ml of H_2SO_4 + 20 ml of tartrate solution		
Nitrate	2 ml	Brucine sulfate – 1 ml H_2SO_4 – 10 ml	–	30	410
Silicate	25 ml	Molybdate reagent – 1 ml	*Molybdate reagent* 12.67 g $(NH_4)2MoO_4$to 100 mlDW	30	810
		Oxalic acid – 1 ml	*Oxalic acid* 10 g $C_2H_2O_4$ to 100 ml DW		
		Ascorbic acid – 1 ml	*Ascorbic acid* 2.8 g of $C_6H_8O_6$to 100 ml DW		
Ammonia	25 ml	Phenol reagent – 1 ml Buffer solution – 0.5 ml	*Buffer solution* 240 g $Na_3C_6H_5O_7$ to 500 ml DW + 10 ml NaOH	30	630
		Sodium hypochlorite – 1 ml	*Sodium hypochlorite* 0.5 g trione + 10 ml NaOH		
Nitrite	25 ml	Sulfanilamide – 0.5 ml	*Sulfanilamide* – 2.5 g + 25 con HCl make up to 250 with DW	10	543
		NEDA – 0.5 ml	*NEDA* – 0.25 g amine to 250 ml DW		

Metals

To analyze the metals from the wastewater, APDC and MIBK extraction methods will be followed (Brooks et al. 1967) as follows:



The collected bottom layer should be analyzed for heavy metals using atomic absorption spectrophotometer or ICP.

Bioremediation Using Living Cells

Effect of Initial Population Density

To estimate the pollutant removal with reference to initial population densities (IPDs) (Dinesh Kumar et al. 2015) of microalgal cells, different concentrations of initial population densities should be prepared. For preparing different IPDs, harvest the microalgae from the stock culture during the exponential phase (probably 6–8 days from the inoculation), and cells are centrifuged at 5000 × g for 10 min. Then the pellet dissolves into the different volumes of sterilized seawater to prepare different levels of cell density (e.g., 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, and 12 gL^{-1}). The dissolved cells should be transferred to a plastic container filled with wastewater, and vigorous aeration should be provided using fish aquarium motor to avoid

settlement of microalgae cells. Two fluorescent bulbs with 5000 lux capacity are furnished to maintain 12:12 h light and dark cycle. The experimental wastewater sample (100 mL) should be collected from the experimental container for every 3 days (if total experimental period is 15 days) or 5 days (if total experimental period is 25 days) for the analysis of nutrients and metals. The biomass production has been estimated according to Richmond et al. (2003).

Effect of Photoperiod (PP)

To estimate the pollutant removal with reference to photoperiod (Dinesh Kumar 2015), microalgae cells should be cultivated at different photoperiods (24:0, 18:06, 14:10, 12:12, 10:14, 8:16, 6:18, 4:20, 0:24 h light/dark) and at 150 µmol m^{-2} s^{-1} light intensity (PPFI) for 15 days. The optimized IPD should be used as inoculums from the stock culture as per the section "Effect of Initial Population Density," and microalgal cells can be transferred to the 1 L plastic container containing 500 ml of wastewater with an airflow rate of 0.5 vvm at 23 °C. The inoculated culture should be placed inside a culture chamber, which is equipped with fluorescent lamps (tubes). Temperature should be monitored regularly, and aeration can be supplied by aquarium pump through the experimental period. Chlorophyll 'a' is estimated following the standard procedure described by Welburn (1994). Before the experimental lasting, IPD and initial physico-chemical, and nutrients' characteristics should be analyzed.

Effect of Photosynthetic Photon Flux Intensity (PPFI)

To know the effects of photosynthetic photon flux intensity (PPFI) on pollutant removal of wastewater, Dinesh Kumar (2015) method is followed. In brief, algal cells can be cultivated in the culture chamber under different light illuminations such as 25, 50, 75, 100, 125, 150, 175, and 200 µmol m^{-2} s^{-1} with optimized PP and IPDs for 15 days. The light intensity should be measured using a quantum sensor connected to an LS Dual solar quantum light meter. Other experimental conditions should be followed as per the standard procedure shown in section "Effect of Photoperiod (PP)."

Effect of Substrates

The locally available polystyrene foam, cardboard, polyethylene landscape fabric, loofah sponge, polyurethane foam, and nylon sponge can be used as supporting materials for substrate experiment.

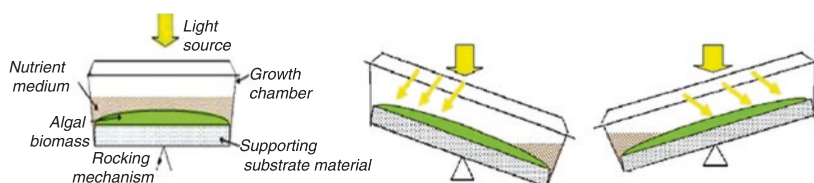


Fig. 5 Schematic representation of substrate algal culture system (Johnson and Wen 2010)

Figure 5 demonstrates the schematic representation of the microalgal bioremediation system with reference to substrates. In the bottom of the growth chamber, supporting material should be fixed as per the growth chamber size. Before the inoculation of the algal suspension, the chamber should be thoroughly washed and autoclaved. Then the chamber can be filled with 50–100 mL (containing 0.25–0.50gL^{-1} of IPD) of algal suspension and 150–250 mL of wastewater. The wastewater is collected from source site and preserved in temperature-controlled ice box and must be filtered before the treatment. Experimental growth chamber should be placed in the rocking shaker with continuous illumination or optimized PP with the PPFI of 100–150 μmol m^{-2} s^{-1}. The growth chamber is shaken (15–20 tips/min) using a rocking shaker at the rate of 15° from the horizontal plane. The supporting material should be submerged in the wastewater which provides a nutrient source for algal growth with illumination for algal photosynthesis. The experimental temperature should be maintained at 20 °C. After formation of thick algal mat on the surface of the substrate, the supporting material should be removed from the chamber and allowed to dry until the algal liquid stop dripping. Then the algal biomass is harvested from the supporting material by scraping using sharp-edged tool like knife. The substrate should be placed again into the culture chamber after harvesting the algal biomass for growing of next session of algae with fresh wastewater. For the next session of algal growth, residual algal cells are used as inoculums.

Effects of Biofilm Formation

This experiment will be performed in 250 mL Erlenmeyer flasks with 100 mL of the culture medium and a submerged supporting matrix for biofilm formation. The medium should be adjusted to an initial pH of 6.8 using 2 μmol/L HCl or 1 μmol/L NaOH and will be sterilized by heating (250 °F, 15 psi for 20 min) along with the matrix. The culture medium will be inoculated with the co-cultures of fungal spores and algae cells at a ratio of 1:300 (initial algae count, 2.50 × 10^{9}cells) unless otherwise specified and incubated in an orbital shaker at 150 rpm and 26 °C in the presence of light (light intensity of continuous illumination should be set to 100 μmol/s/m^{2}) for the entire cultivation period (8–10 days). Microalgal samples will be collected from the cultivation at regular time intervals to estimate the glucose and cell

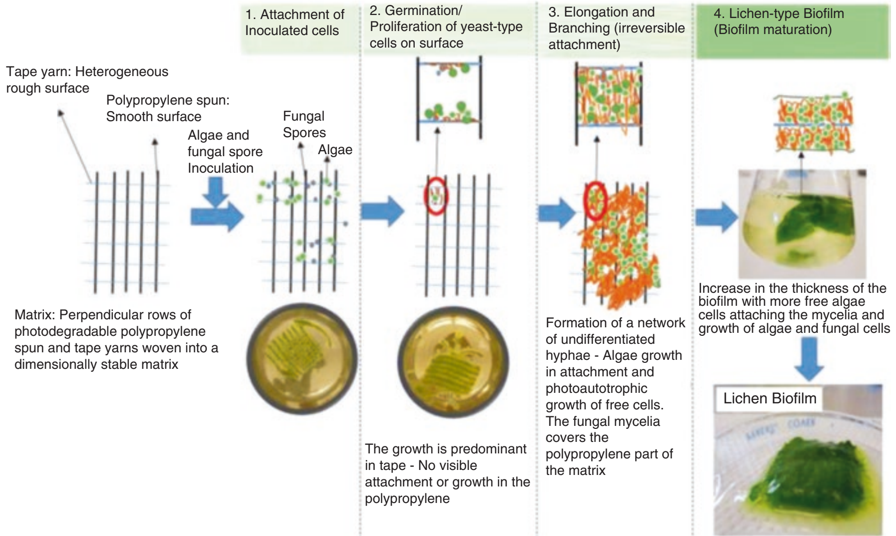


Fig. 6 The mechanism and stages of algae-fungal cell attachment and proliferation of the mycoalgae biofilm (Rajendran and Hu 2016)

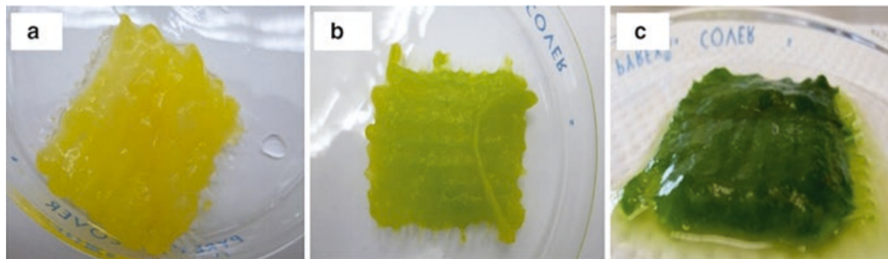


Fig. 7 (**a**) Biofilm of axenic *Mucorcircinelloides*, (**b**) *Mucorcircinelloides*, and *Chlorella vulgaris* mycoalgae biofilm at initial stages of the biofilm formation (48 h). (**c**) Mature mycoalgae biofilm at 168 h after complete attachment of the algae (Rajendran and Hu 2016)

counts of suspended algae without much change in the culture volume to maintain constant oxygen transfer. The algal cells are separated from the medium which will be suspended by centrifugation for 15 min at 4 °C and filtered through a 0.45 μm filter for residual nutrient analysis. Glucose, total phosphorous, and nitrogen will be estimated as per standard methods. Control experiments will be maintained using only wastewater without microalgae inoculums (Figs. 6 and 7).

Bioremediation Using Immobilized Cells

Effects of pH and Time

Beads with Dried Cells

The microalgal biosorbent powder is prepared according to Bishoni et al. (2004). Harvesting of algal biomass is performed by filtering the culture through Millipore filtering equipment using GF/C membrane filter paper (0.45 cm dia.). The obtained biomass will be scraped using sterile blades followed by washing twice with distilled water and dried. Algal biomass is also harvested by centrifugation at 3000 rpm for 10 min. The cell pellet will be washed twice with distilled water. Then the cell pellets are collected and dried in a hot air oven at 80 °C for 40 min. The algal biomass will be determined gravimetrically (g/l); then the cells are stored in desiccators for biosorption experiment. The microalgal beads with dried algal cells will be prepared according to Santos et al. (2002) with minor modifications; see section "Beads with Live Cells" for detailed methodology.

Beads with Live Cells

The algal beads with living cells will be prepared according to Santos et al. (2002). In brief, to prepare 100 mL of alginate solution with the required alginate concentration, the alginate will be first carefully dissolved by stirring in 70 mL of distilled water. In the remaining 30 mL of distilled water, 3.5 g of NaCl is dissolved to obtain 3.5 g/L of salinity final solution. When the alginate is completely dissolved, the two parts were mixed by a magnetic stirrer. The algal cells are harvested from the indoor mass culture during exponential phase and added into alginate solution. Cation solutions are prepared in nanopure water. Beads are prepared by adding dropwise in the alginate solution, by using 20 mL of syringe (0.8 mm × 40 mm needle) into cation solution, from a height of approximately one drop per second. Beads should be kept stirred in the cation solution for 45 min to allow complete hardening of the alginate and should be washed for several times with filtered (47 mm dia, 0.47 μm pore size) natural seawater to eliminate the remaining cations.

Effects of Dosage and Cell Concentration

The algal powder will be prepared as per section "Beads with Dried Cells," and dried algal powder will be weighed at different concentrations, viz., 0.1 g, 0.2 g, and 0.3 g, and dissolved into the alginate solution which contains the same volume. The mixed solution will be prepared as beads as explained in section "Beads with Live Cells." For preparing different cell densities, harvest the microalgae from stock culture during the exponential phase (probably 6–8 days from the inoculation), and

cells will be centrifuged at 5000 × g for 10 min. Then the pellets are dissolved into the different volumes of pellet to the same volume of sterilized seawater to prepare different levels of cell density (e.g., 260,000 cells/mL, 130,000 cells/mL, 95,000 cells/mL, 72,000 cells/mL, 48,000 cells/mL, and 25,000 cells/mL). The different algal cell concentrations will be prepared as individual beads (Dinesh Kumar et al. 2017a) as elaborated in section "Beads with Live Cells."

Effects of Bead Density

To evaluate the effect of algal bead density on nutrient removal in wastewater, Dinesh Kumar et al. (2017a, b) procedure is followed. The beads (4 mm) with optimum cell density (which is optimized in section "Effects of Dosage and Cell Concentration") are prepared as explained in section "Effects of Dosage and Cell Concentration." The beads are made out of each 1 ml of alginate algal suspension indicating that each algal bead occupied optimized concentration of volume. According to the bead volume, we will make different numbers of beads like 25, 50, 75, 100, 125, and 150.

Co-immobilization

The algal immobilization will be performed as per section "Beads with Live Cells." To co-immobilize both microorganisms (microalgae and bacteria), Shen et al. (2017) method is followed. In brief, first we should determine the initial ratio of microalgae and bacteria cells at which a microalgae-bacteria symbiotic relationship will exist best. Based on several preliminary evaluations, it is found that microalgae-bacteria ratio of 1:10 is better for maximum removal of nutrients. To immobilize bacteria and microalgae, the same procedure will be followed as shown in section "Beads with Live Cells" after careful preparation of microalgae-bacteria ratio. Blank beads should be prepared as a control which contains only alginate.

Integrated

To estimate the integrated experimentation by microalgae and copepod for nutrient removal, Dinesh Kumar et al. (2016) procedure is followed. In brief, 15 glass bowls of 3 l capacity are taken. The experiments consist of five treatments that are studied in triplicate. The first treatment (1) consists of copepod *Oithona rigida* (100 adults) alone, the second treatment (2) contains immobilized microalga *P. maculatum* beads (100 numbers) and 100 adult individuals of *O. rigida*, the third treatment (3)

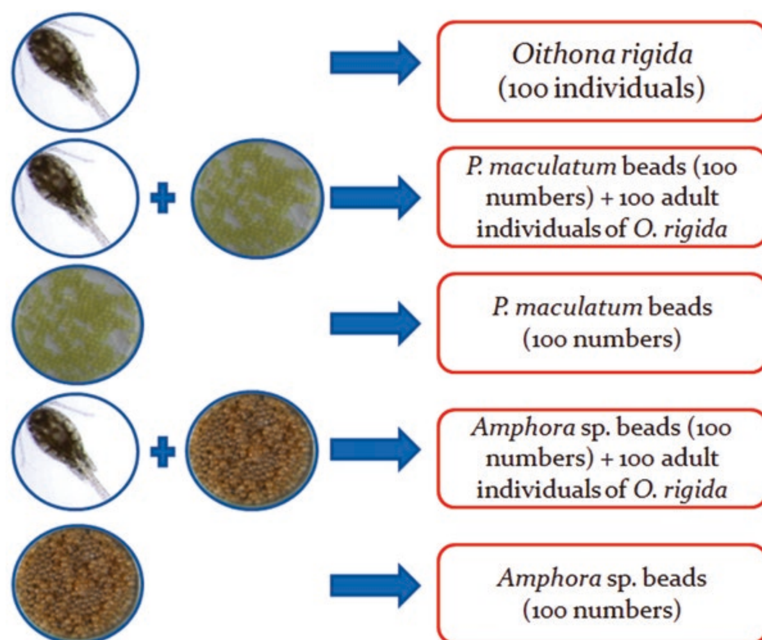


Fig. 8 Schematic representation of integrated wastewater treatment (Source: Dinesh Kumar et al. 2016)

consists of only immobilized *P. maculatum* beads (100 numbers), the fourth treatment (4) contains immobilized *Amphora* sp. (100 numbers) plus 100 adult individuals of *O. rigida*, and the final treatment (5) consists of only immobilized *Amphora* sp. (100 numbers). The experiment lasted for 6–9 days, with monitoring of physicochemical parameters (turbidity, pH, salinity, temperature, total suspended solids, and nutrients) for every 24 h. To know the stability or resistance of immobilized microalgae beads, the bead surface is monitored every 24 h using light microscope. The samples are taken from experimental containers, and algal cell density is counted for every 24 h once by using hemocytometer under the light microscope to determine if there are any algal cell leakages in wastewater. The copepod mortality in wastewater is assessed by collecting a known volume of sample (5 ml), and the number of dead copepods if any is counted under light microscope using Sedgewick counting chamber according to Santhanam et al. (2013). Total suspended solids in effluent are estimated by filtering 10 ml of effluent sample using Millipore filtering apparatus fitted with 0.45 μm GF/C filter paper (APHA 1998). The experiment should be conducted at optimized temperature, salinity, light exposure, and photoperiod. The turbidity of effluent water is measured according to Palin (1955). In brief, a known volume (3.5 ml) of the effluent sample is filled in the 4.5 × 1.2 cm cuvette, and the absorbance is measured using a UV-visible spectrophotometer at 425 nm and 580 nm (Fig. 8).

Desorption and Reuse

To examine the effect of desorption on the pollutant removal efficacy of microalgae beads (Revathi et al. 2017), three cycles of adsorption-desorption process can be carried out using HNO_3 as the desorbent. After treatment with microalgae, the wastewater should be separated by centrifugation (2500 rpm for 10 min). The alginate beads can be easily separated by pouring the mixture through a strainer. The algal mass and the beads are rinsed separately in 40 ml of deioniozed water. Removal of pollutants from algal beads is achieved by eluting with 0.1 M HNO_3 for 15 min, and the liquid is collected. The algal beads are rinsed again to remove any residual acidity.

Coproduct Development

Formulated Feed Production

Wastewater-cultured microalgae are used as feed for fin fishes and shellfishes. Many types of microalgae have been found to increase growth (protein accretion), feed utilization, physiological activity, stress response, starvation tolerance, disease resistance, and carcass quality (Mustafa and Nakagawa 1995) carotenoid and protein sources of shrimp. To replace the fish meal by microalgae meal, fish meal (trash fish) will be obtained from any local fish market or landing center. The collected fishes should be cleaned well with tap water, and the intestine will be removed from the fish. The flesh will be cleaned with hot distilled water. Then the flesh will be dried by natural sunlight or hot air oven to remove the moisture content. After drying of the flesh, the flesh is made as powder by an electric pulvelizer. The powder of fish meal will be sieved with an ordinary flour filter; then they are stored in desiccators for feed formulation. The feed ingredients such as soy meal, groundnut oil cake, wheat bran, and binding properties (egg and tapioca flour) can be obtained from local markets. The feed formulation by replacing fish meal with microalgae meal will be done according to Bischoff and Bold (1963), Vonshak (1986), Schuster et al. (1990), Schlosser (1994), and Sherief and James (1994).

The basal ingredients, such as processed fish meal, sundried soy meal, groundnut oil cake, and wheat bran, were grounded separately using a micro-pulvelizer and sieved through an ingredient sieve. The ingredients will be weighed and thoroughly mixed to different ratios for preparing different diets with one control using 100% fish meal and others replacing with fish meal with different microalga species at the concentrations of 25%, 50%, 75%, and 100%. Then the mixed feed ingredients will be steam-cooked for 15 min at 95–100 °C and allowed to cool at room temperature. Then the steam-cooked ingredients will be mixed with vitamin mix (1%), sunflower oil (2%), egg albumen, and tapioca flour (12%) for binding, and 10% of boiled

water will be added and then mixed well for 5 min until the mixture became paste-like form. Then the mixed paste will be made as pellet using manual pelletizer fixed with 3 mm diameter basal plate, and the pellets will be collected in aluminum trays. Then the pellets will be dried till the moisture content is less than 10%. After drying, pellets will be physically examined for visual appearance, such as uniformity, color, and fragrance.

Live Feed

Free-living algal cells are used for wastewater bioremediation, and the left out algal biomass can serve as live feed (Dinesh Kumar et al. 2017b). To evaluate the live feed suitability of wastewater-grown microalgae, we need six rectangular fiberglass-reinforced plastic (FRP) tanks (70 cm × 50 cm × 30 cm size and 6 mm thick; outside blue and inner white tanks, 100 L capacity) each containing 50 L clean, filtered (5 µm filter bag) seawater. Two experiments will be done fed with (i) artificial culture medium (ACM)-cultured microalgae and (ii) wastewater (WW)-cultured microalgae with both treatments in triplicates. Shrimp larvae (PL-10) are obtained from the commercial hatchery, and PL is stocked in 100 L FRP tanks filled with 50 L of filtered seawater at the density of 1PL L^{-1}. The constant aeration should be supplied to the rearing animal. Shrimp PLs should be monitored regularly, and every 8 h interval feeding will be given with ACM microalgae (treatment 1) and WW microalgae (treatment 2) at known densities (approximately 1,00,000 cells ml^{-1}). Twenty-one days of feeding experiment with shrimp or fish larvae is enough to assess the live feed suitability of wastewater-grown microalgae. Finally, growth, survival, and food conversion ratio (FCR) of larvae will be assessed with reference to feed according to Dinesh Kumar et al. (2017b), Derrien et al. (1998), Dubois et al. (1956), Lindroth and Mopper (1979), Lowry et al. (1951) and Radhakrishnan et al. (2014).

Biofuel Production

For biofuel production from microalgae, Moazami et al. (2012) method is followed. In brief, the microalgae are harvested from the wastewater treatment plant, then the algal biomass is dewatered by sieving the harvested biomass through 2 mm mesh nylon netting to get approximately 10% solid content, and then the biomass is air-dried for approximately 2 days using electric fans to get approximately 90% solid content. The dried biomass is grounded initially using a large-scale mill (based on their collected quantity), and then the algal powder is passed through 3 mm sieve and stored into the plastic bags with sealing at 20–25 °C. The algal cell density will be measured with the help of a spectrophotometer as per the Δ max values and cell

counting methods using a hemocytometer. Extraction of oil content from algae biomass is made according to modified Bligh and Dyer procedure (Bligh and Dyer 1959). Fatty acid has been estimated according to Na et al. (2011). Pre-weighed algal sample (100 g) is homogenized in a Waring Blendor for 2 min with a mixture of 100 ml chloroform and 200 ml methanol. The mixture will be blended for 30 seconds and then 100 ml distilled water is added, and the blend continues for another 30 s. The homogenate mixture is filtered through Whatman No. 1 filter paper on a Coors No. 3 Buchner funnel with slight suction. Filtration should be stopped when the residue becomes dry, and then the pressure will be applied with the bottom of a beaker to ensure maximum recovery of solvent. The filtrate will be transferred to a 500 ml graduated cylinder, and then the cylinder is kept undisturbed for a few minutes for complete separation and clarification. The alcoholic layer is removed by aspiration and keeps the chloroform layer. This total chloroform layer contains the purified lipid.

Bio-fertilizer Production

The bio-fertilizer is produced from the microalgae according to Garcia-Gonzalez and Sommerfeld (2016). In brief, 1 kg of wastewater-cultured microalgae biomass is suspended in double-distilled (DDI) water at a concentration of 150 g L^{-1}. The suspension is mixed on a stirring plate for 10 min to allow the biomass to dissociate. The suspension is processed through a Microfluidizer – a mobile high-shear fluid processor – at a flow rate of 450 mL min^{-1} at 172 mPa to disrupt the cell wall and obtain the intracellular extracts. The final extract is centrifuged at 8983 × g for 10 min at 22 °C to separate the cell extracts from the biomass residue. To minimize potential degradation, the final extract supernatant is collected into the flask and covered with aluminum foil and then stored into a cold room at 4 °C. The residual biomass is the bio-fertilizer. This should be stored in the cold room for further use. After preparation of bio-fertilizer, the application of fertilizer experiment should be conducted under greenhouse conditions at approximately 28 ± 2 °C, in 85% relative humidity. Plant seeds will be grown in sterilized potting soil with a mixture of vermiculture and peat moss. The seeds will be transplanted after 22 days using 28–30 cm pots (one seedling per pot). Two treatments, viz., 50 and 100 g with dry algal bio-fertilizer, should be applied for 22 days prior to seedling transplant, into pots containing potting soil (peat moss, vermiculture, perlite), and mixed thoroughly. Then the pots are watered once a week for 3 weeks prior to seedling transplant. Each treatment has three replicates and will be set up in a completely randomized block design. Plants will be grown for a period of 8 weeks. Plant height (cm), root length, shoot length, number of flowers, number of branches, chlorophyll content, and early fruit development should be recorded for all treatments to evaluate the efficacy of algal fertilizer on plant growth.

Conclusion

Rapid industrialization, energy reservoirs, and unsafe agricultural practices by human activities are the main culprits to the ecological pollution in the past few decades. Among the pollutants, heavy metals, nuclear wastes, pesticides, greenhouse gases, hydrocarbons, and excessive nutrients are highly dangerous. Remediation of pollutants using biological process (bioremediation) had proved the efficient and eco-friendly method, and it can be carried out by ex situ or in situ methods, depending on numerous factors like cost, type, and concentration of pollutants. Generally, ex situ techniques are more expensive than in situ techniques; however, selecting suitable remediation technique plays a key role on the processing cost as well as results. The present paper deals with the various bioremediation methodologies to treat the polluted wastewater by using microalgae and possible coproduct development using left out biomass after bioremediation process is over for zero waste management.

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Detection of Cyanotoxins of Cyanobacterial (*Microcystis aeruginosa*) Strain Using Microtox® Bioluminescence Bioassay

B. Balaji Prasath, P. Santhanam, R. Nandakumar, and T. Jayalakshmi

Introduction

The health hazards and economic losses associated with toxic cyanobacterial blooms are already well documented all over the world (O'Neil et al. 2012). Forty different types of cyanobacteria that can be accompanied by toxicity bloom were reported (Ahmed et al. 2008). The occurrence of the massive bloom of cyanobacteria in an aquatic ecosystem is a global problem of these blooms, has been found to be toxic and has been studied well in many countries. However, only a few studies are conducted in India on cyanobacteria toxic blooms (Prakash et al. 2009). This may be due to the lack of knowledge and awareness of the production of cyanotoxins with the injurious algal bloom (Sangolkar et al. 2009). According to the previous study in India, cyanobacterial harmful blooms that produce cyanotoxins especially MC-LR, MC-RR and its demethylated variant have been reported (Agrawal et al. 2006; Prakash et al. 2009; Sangolkar et al. 2009). The cyanobacterial bloom produces and release of cyanotoxins oftenly. These cyanotoxins represent, with the aid

B. Balaji Prasath
Beijing Normal University-Hong Kong Baptist University, United International College, Zhuhai, Guangdong, China

Marine Planktonology & Aquaculture Laboratory, Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

P. Santhanam (✉) · R. Nandakumar
Marine Planktonology & Aquaculture Laboratory, Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

T. Jayalakshmi
Marine Planktonology & Aquaculture Laboratory, Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

National Centre for Sustainable Coastal Management, Ministry of Environment, Forest and Climate Change (MoEF&CC), Chennai, Tamil Nadu, India

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of specific exposure routes, a vast risk to human fitness dangers and aquatic organisms (Oudra et al. 2001). Globally, several numbers of toxic blue-green alga species have been recognized to produce a range of hepatotoxic microcystins (Carmichael et al. 1988). Among the harmful blue-green algae, *M. aeruginosa* is the most common and cosmopolitan distribution genus from which 35 variants of microcystins have been reported (Sivonen and Jones 1999). *M. aeruginosa* information was well studied in ecology, toxicology and environmental implications by Watanabe et al. (1996). In the last few decades, for a diffusion of reasons, the damaging effect of cyanobacteria on human fitness has been reported (Oh et al. 2001 and Wang and Li 2002). In vivo bioluminescence assays, involving *Vibrio fischeri*, have been inserted as a rapid, reproducible and relatively cheap way of toxicity assessment. Since its presentation, the Microtox® assay, based upon *V. fischeri* bioluminescence, has been applied to evaluate the toxicity of a broad range of aquatic pollutants (Ribo and Kaiser 1987; Kaiser and Ribo 1988). In the present attempt, the suitability of bioluminescence assays for the preliminary screening of cyanobacterial blooms for toxicity was studied. Here we reported on the reaction of the Microtox® assay to purified microcystin and to extracts of field samples of bloom-forming hepatotoxic (microcystin-containing) cyanobacteria, *M. aeruginosa*.

Materials and Methods

Description of the Study Area

Muttukadu backwater (Lat. 12° 49′ N; Long. 80° 15′ E) extends for a distance of 20 km from the sea mouth. This backwater is normally cut off from the sea during summer and pre-monsoon season and leads to sandbar formation. During monsoon season, due to inundation by the freshwater from the upper reaches, the sandbar gets eroded and the connection with the sea is restored. The width of the estuary ranges from 800 m to 1050 m. The Muttukadu backwater is dominated by boating activities and recreational purposes for tourist people boating activities and as with many other tropical estuaries is threatened by the diverse uses to which humans put this water body and the land areas that drain into it. Therefore, the estuary has a large number of environmental problems (Fig. 1).

Sample Collection

Algal Bloom samples were collected with a phytoplankton net (mesh size, 48 μm) in Muttukadu backwaters, Chennai, Tamil Nadu, India. The bloom sample was observed under the microscope. The sample was identified as containing primarily of *M. aeruginosa*. Cyanobacterial bloom samples were lyophilized after collection

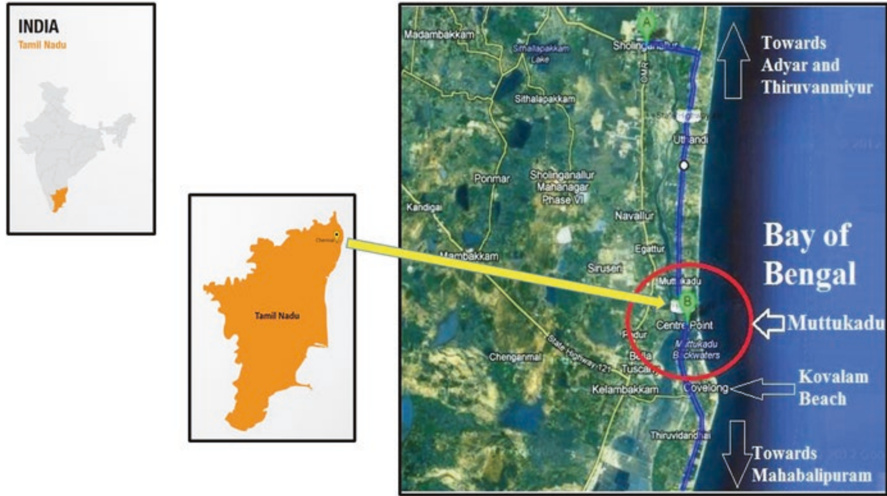


Fig. 1 Description of sampling site (Muttukadu backwater)

and stored at – 20 °C for further extraction and isolation of microcystin analysis. Extraction was carried out using the established method (Frias et al. 2006) as follows: *M. aeruginosa* bloom samples were centrifuged at 5000 × g for 15 min at 4 °C using centrifuge (Remi, India). Microcystin was extracted with MeOH/H_2O (3:1 v/v) from frozen samples (1 g) submitted to sonic disruption for 25 min. The extract was centrifuged at 10,000 rpm for 15 min, and the supernatants were taken in and the pellets obtained were re-extracted according to the same process. The supernatants have been blended and evaporated to dryness in a rotary evaporator (40 °C). The dried material was resuspended in MeOH and partitioned with $CHCl_3$/MeOH/H_2O (7:6:3 v/v/v) to remove hydrophobic compounds and pigments. The hydroalcoholic phase was evaporated and dissolved in 1 ml of MeOH/H_2O (7:3 v/v), and supernatants had been used for Microtox® bioassays (Lawton et al. 1990).

Microtox® Bioluminescence Assay

The standard Biotox tailor-made micro-toxicity kit (BO 1243-500 BioTox™ kit) was imported from AboatoxOy, Finland. It is used to measure the bioluminescence toxicity studies. The pH was adjusted to 7.0 ± 0.2 with NaOH or HCL if pH of the sample is not between 6 and 8.0. The freeze-dried *Vibrio fischeri* bacterial reagent (approx. 10^6 cells) was reconstituted, by adding the reagent diluents and equilibrates at 4 °C at 30 min. Then the reagent was stabilized at 30 min before pipetting into cuvettes. Then 300 μl of bacterial solution was added into cuvettes and allowed to

stabilize at 15 °C at 15 min. Then the kit-provided sample was diluted with diluents to 1:10 ratio with distilled water. Before every measurement, the pH was checked and adjusted to 7.0, if necessary. The natural bloom sample was diluted with the use of 2% NaCl sample as diluents. All the diluted samples were maintained at 15 °C for at least 15 min. Then all the samples and dilutions were kept at 15 °C during the whole measurements. The dilution series with 2% NaCl by means of a graduated dilution was prepared in accordance with ISO standards, i.e. 100, 10, 1, 0.1, 0.01 and 0.001 μg L^{-1}, and maximum dilutions were used. 2 ml of natural bloom sample was added with 1 ml of sample diluents and a stock solution was prepared by way of dilution series. As well, all the other dilution series was also prepared by adding 0.3 ml of sample diluents and 0.3 ml of stock solution in the first cuvette as dilution factor one. Then 0.3 ml of solution was transformed from the first dilution to second dilution factor and continues up to 5–9 dilution length aside. Control was prepared with the same volume of double distilled water. To measure the luminescence intensity, 'IO', the sample was immediately transferred into the first cuvette containing the bacterial suspension. It was prone to measurement with the luminometer. The '0' min luminescence intensity of all the diluted samples is measured using luminometer (Sirius single-tube luminometer), and the determination of luminescence intensity ('It') for 5 min ('IT5'), 15 min ('IT15') and 30 min (IT30) is measured continuously. The following formula was used to calculate % inhibition:

Calculation:

$$\text{INH\%} = 100 - 100 \times \left(\text{IT}_5 \text{ or } \text{IT}_{15} \text{ or } \text{IT}_{30} \,/\, \text{KF}\right) \times \text{IT}_0)$$

$$\text{KF} = \text{IC}_5 \text{ or } \text{IT}_{15} \text{ or } \text{IC}_{30} \,/\, \text{IC}_0$$

where

KF = Correction factor
$IC_{5, 15, 30}$ = Luminescence intensity of control after contact time in RLU
IT_0 = Initial luminescence intensity of control sample in RLU
$IT_{5, 15, 30}$ = Luminescence intensity of test sample after contact time in RLU
IC_0 = Initial luminescence intensity of test sample in RLU

Data Analysis

The percentage inhibition was calculated, and the EC_{50} value was determined by using standard linear regression analysis, by either graphically using a double logarithmic co-ordinate system or by using linear regression analysis. The INH% was plotted on the y-axis and the concentration on x-axis according to Lappalainen et al. (1999) and (2001). Measurements are based on BioTox™ kit (AboatoxOy, Finland) utilizing the bacterium *V. fischeri*. The outcomes are calculated as the inhibition % of light production and expressed using the corresponding EC_{50} values (Lappalainen et al. 2001).

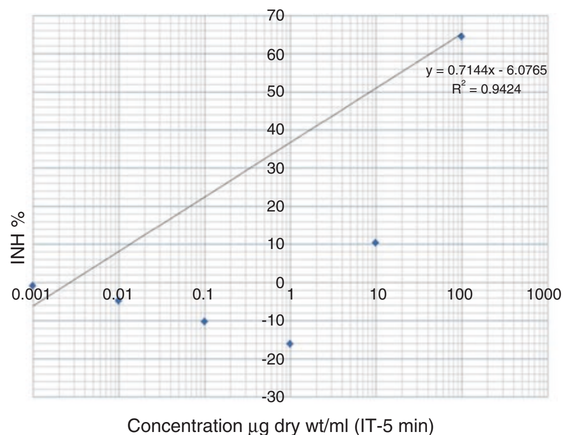
Results and Discussion

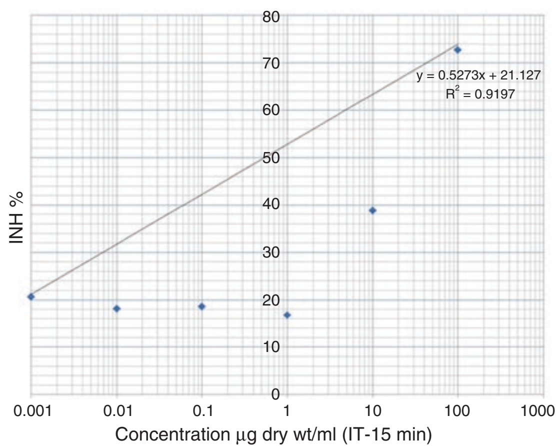
The original cyanobacterial bloom samples were collected during June 2012, and the cell density of Microcystis *aeruginosa* was reported as 6×10^8 cells L^{-1}. In the present study, *M. aeruginosa* was dominated by 95–98% of the total phytoplankton density during the bloom. Water in the region was green coloured and slimy in nature with a foul smell. Massive fishes were washed along the shore during the bloom (Plate 1). This was established due to the presence of the large concentration of M. *aeruginosa.* In this present study, the screening potential of the Microtox® assay was evaluated for testing cyanobacterial toxicity, based on the sensitivity and reaction time of the bioluminescence inhibition. This highest yielded an EC_{50} (concentration which causes a 50% reduction of light emission) of 100 μg dry wt/ml causing 84% inhibition in light emission in 30 min followed by 72% inhibition in light emission in 15 min and 64% inhibition in light emission in 5 min after toxin addition. The bioassay experiments clearly indicate that the luminescence inhibition rate was increased along with the concentration of each dilution increased along with the reaction times (Fig. 2) as agreed by Lappalainen et al. (2001). The present results inferred that the cyanobacterial toxicities to *V. fischeri* may cause damage to the membrane of the cell and induce the cell death after contracting for a certain time.

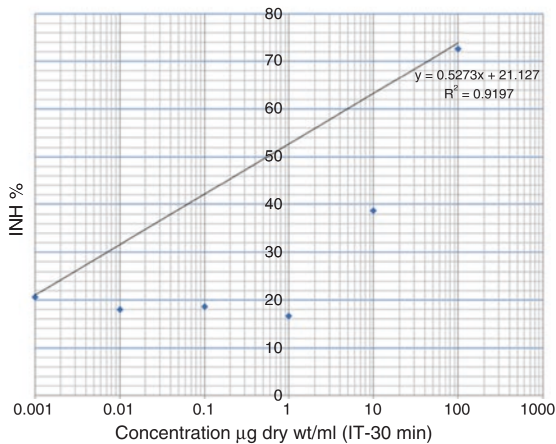
In the investigation of efficacy of toxicity test techniques, Microtox® assay has been reported as one of the most important methods of measuring the light intensity emitted by the test bacteria (*Vibrio fischeri*). The occurrence of *M. aeruginosa* blooms in Muttukadu backwater that produce cyanotoxin is a problem that is to be faced by human and aquatic organism, especially whenever the water is utilized for recreational purposes. Events of massive fish mortality are associated with algal blooms (Balaji Prasath et al. 2014). Local and tourist people use Muttukadu water for recreational purposes, even when bloom or scum is formed as they have no knowledge about the toxicity and, in some cases, they have no alternative. Our results revealed the positive effect of Microtox® should be used as a sensitive test for detection of microcystins in cyanobacterial samples, and the toxic effect was well noted. Similar results were also obtained with genetically engineered luminescent *P. putida* and *V. fischeri* (Blaha and Marsalek 2000). According to the present study, the toxic effect of cyanobacterial bloom as the fractionated extracts inhibited bioluminescence bacteria was clearly noted. Microtox® of the results presented here of EC_{50} (bioluminescence) for a system may be a useful tool for the preliminary toxicity screening of cyanotoxin associated with cyanobacterial blooms in Muttukadu backwater environments. Similarly, studies investigation of data evaluation became had to generate the statistics on cyanotoxin incidence, distribution and variety and close to climatic zones (Sangolkar et al. 2009). The present findings inferred that the toxigenic *M. aeruginosa* strains noticed in the Muttukadu backwater bodies of Southern Indian location has essential interest on the screening and mitigation techniques. Spatial and temporal dynamics measurements of cyanotoxin and toxic species composition in the backwaters are essential to assess the risks for the fitness of people and aquatic organisms.

Plate 1 Mass mortality of fishes along the Muttukadu backwater due to *M. aeruginosa* bloom

Fig. 2 The toxicity of microcystin calculated with the BioTox™ software







Conclusion

In summary, for rapid evaluation of the potential risk caused by blooms of specific *M. aeruginosa* strains, toxicity-based Microtox® provides a useful addition to the methods already available for detection of cyanobacterial toxins. *M. aeruginosa* toxicities detected by luminescence inhibition rate were increased along with the reaction time and were significantly higher than the cell death rate. In this present study, we have noted that it is possible to detect luminescence from cyanotoxin tests based on bioluminescent assay using inexpensive, fast and easy-to-use test toxicological screening systemic ideally suited for developing countries.

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A Method of Analysis of Pigments in Phytoplankton

N. Krishnaveni, A. Shenbaga Devi, and P. Santhanam

Introduction

Phytoplankton (phyto, plant; plankton, wandering) are the free-floating microscopic plant cells, which contain photosynthetic pigments found in both terrestrial and marine environments. They contribute nearly 25% of the total vegetation of the plant (Jefferey and Humphrey 1975). Pigments are chemical compounds which reflect only certain wavelengths of visible light. Because they interact with light to absorb only certain wavelengths, pigments are useful to plants and other autotropic organisms which make their own food using photosynthesis. Its value as a biomass indicator of oceanic microscopic marine plants has been recognized over the years. The inventory of pigments is a key characteristic of phototrophic organisms which is used as a criterion in the classification of autotrophic bacteria (Yacobi et al. 1996). Knowledge of phytoplankton dynamics in the World Ocean is central to the study of marine ecology and biogeochemical processes involved in climate change. Phytoplankton biomass can be estimated by the photosynthetic pigment. Phytoplankton pigment quantification is an integral part of inland water monitoring and general experimental research involving phytoplankton. Chlorophyll a (chl a) concentrations are widely used by plankton ecologists as an alternate for phytoplankton biomass and for estimating primary productivity. Photosynthetic and photoprotective pigments and their relative concentration can provide valuable taxonomical and physiological information of phytoplankton. Because pigment composition can be a reflection of taxonomic composition, presence or absence of certain marker pigments can be used to identify phytoplankton community composition. Pigment composition is an important physiological parameter. The environmental factors such as illumination and nutrient availability are influencing the relative pigment concentration (Thrane et al. 2015). Phytoplankton pigments were

N. Krishnaveni · A. Shenbaga Devi · P. Santhanam (✉)
Marine Planktonology & Aquaculture Laboratory, Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

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analysed by spectrophotometry and HPLC methods. Both techniques were significant advances because of their sensitivity and ease of measurement (Schluter et al. 2006).

Sampling and Processing

Surface water samples are collected with the help of the plastic bottle. Instantly after collection, the water samples are filtered through a mesh sieve to separate the zooplankton and the large debris. The sea water samples are obtained with alcohol-scrubbed 8.01 PVC Van Dorn bottles and the fresh water samples with 2–1 polythene bottles. Replicate aliquots of water are filtered at a vacuum of 25 cm Hg through either a 2.5 cm microfine glass fibre filter (Reeve Angel 984H), a 2.5 cm GF/C glass fibre filter (Whatman) or an HA membrane filter (Millipore) with a pore size of 0.45 micron.

Evaluation of Phytoplankton Pigments

Estimation of Chlorophyll

Phytoplankton biomass can be estimated by the photosynthetic pigment, which is found in all phytoplankton cells. The procedure consists of isolation, extraction and estimation.

Isolation

The concentrated phytoplankton were filtered by using Whatman GF/F, nominal pore size = 0.7 μm. The filters were soaked in a 90% acetone, which is the most efficient solvent for analysis of pigment without pigment alteration.

Three Methods of Extraction

Sonication

The cell suspension was taken in a plastic centrifuge tube then kept into the sonicator. A sonicator radiates 3000 watts of energy to agitate the cellular membranes; this idea is to break the chloroplasts so that the pigments can separate into the acetone without degradation (Laessee and Hansen 1961).

Grinding

Grinders manage mechanical force to cleave the cells.

Freezing

Another method is to simply freeze the cells using acetone for 24–48 h, to allow the pigments to separate out of the cell.

Acetone Extractions

Immediately after completion of filtration, the filter is folded in half and either (a) placed in a glass stopper centrifuge tube, to which is added 10 ml of 90% acetone, or (b) ground with 90% acetone in homogenizer. After which the slurry is transferred to glass stopper centrifuge tubes and taken to a final volume of 10 ml with 90% acetone. Samples are stored in complete darkness at room temperature during the extraction period, which varied from 10 min to many hours. At the end of the extraction period, the tubes are shaken by hand, centrifuged at 1000 × g for 5 min at 4 °C and then taken to room temperature by placing in a water bath for 10 min. The acetone used in these extractions is reagent grade, but it is neither distilled nor stored with $MgCO_3$ as suggested by Strickland and Parsons (1986).

Methanol Extractions

After filtration, the filtrate is located in 3 ml of concentrated methanol. After 1 h of extraction, the filter is wrung against the sides of the glass vessel using a glass roller and then rinsed twice in methanol; the final solution is filtered through a Whatman glass fibre filter to obtain a clear solution free from filter residues and cell debris (Holm-Hansen and Riemann 1978).

DMSO Extraction

Phytoplankton is filtered into glass fibre filters (Whatman GF/C or GF/F, 42.5 mm diameter). The filters are loosely rolled, placed in and stored at −20 °C at this point for later analysis. For analysis, the vials are brought to room temperature, 4 mL of DMSO is added, and then the vials are resealed. They are located in heating block at 65 °C for 10 min. After 10 min the vials are removed and mixed thoroughly, and the contents are filtered with the help of the filter (Nuclepore 0.2 μm membrane

filter or Whatman GF/C filter). The vials and filter are cleaned with 90% acetone and the filtrate volume brought to 10 mL with 90% acetone, and spectrophotometric readings are taken.

Measuring Chlorophyll

Basic Methods of Measuring Chlorophyll

The chlorophyll extracts have been taken by using 90% acetone. The clear extract has been collected by centrifugation method. This extract can then be measured in a fluorometer (to detect chlorophyll fluorescence) or a spectrophotometer (to detect light absorbance by chlorophyll). A spectrophotometer measures the absorption of light at a particular wavelength. Chlorophyll 'a' has two absorption maxima, one at 432 nm and another at 664 nm, or it can be used for chromatography to separate chlorophyll 'a' and all other phytoplankton pigments (Table 1).

Fluorometric Method

This fluorometric method is used to analyse the chlorophyll 'a' from phytoplankton cells which detects the fluorescence of the chlorophyll molecule (Mitchell and Kiefer 1984). There are two pigments, viz. chlorophyll 'a' and phaeopigment (Lorenzen 1967), which were extracted by using this method. After the extraction initial reading is taken by fluorescence, the initial reading only represent the chlorophyll 'a' concentration in the sample if any phaeopigment present, then 10% HCl is added to the sample to convert all of the chlorophyll 'a' to phaeopigment, and

Table 1 Various methods of analysis of different pigments in phytoplankton

Pigment	Solvent	Wavelength
Chlorophyll 'a'	90% acetone	664 nm
	100% acetone	662.7 nm
	Diethyl ether	660.7 nm
Pheophytin 'a'	90% acetone	667 nm
Chlorophyllide 'a'	90% acetone	664 nm
Pheophorbide 'a'	90% acetone	667 nm
Chlorophyll 'b'	90% acetone	647 nm
	100% acetone	629.1 nm
	Diethyl ether	643.3 nm
Chlorophyll C1&C2	90% acetone	631 nm
	100% acetone	629.6 nm

Source: Jefferey and Humphrey (1975)

another reading is taken (Fa), fluorometric measurement of chlorophyll 'a' and phaeopigments. 10 ml of water sample is filtered through 0.45 Millipore filters. The filters are transferred into 10-ml glass tubes and the sample is mixed with 5 ml of 90% acetone. The extracted phytoplankton samples were kept overnight at 4 °C in a dark refrigerator. Simultaneously run the blank also. Finally measure the OD at 647 nm.

Spectrophotometric Measurement of Carotenoids

The total carotenoids are isolated using n-hexane and isopropyl alcohol (1:1) and evaluated spectrophotometrically by the method of Devis (1976) by measuring absorption at 450 nm.

Determination of Beta-Carotene by Spectrophotometric Method

The beta-carotene standard solutions of different concentrations with pure acetone as solvent are detected under a wavelength of 453 nm where the characteristic spectrum of beta-carotene and contents of beta-carotene are determined spectrophotometrically. 2 ml of centrifuged (4000 rpm for 5 min) algal sample is placed in a container mixed with 2 ml of acetone solution. The pigment extract is transferred to a new tube. Repeat the above processes until the extract turned to white, then add 1/10 volume of 60% KOH at 49 °C and transfer the supernatant without chlorophyll and lipid to 10 mL volumetric flask and makeup with acetone solution. Finally measure the OD at 453 nm. The concentration of the beta-carotene solution can be found from the standard curve (Zhu and Jiang 2008) (Table 2).

HPLC Analysis

The HPLC analytical procedure proposed is by Van Heukelem and Thomas (2001). Briefly, microalgal sample is filtered through 25 mm Whatman GF/F filters (nominal pore size of 0.7 μm) and frozen immediately at −80 °C using liquid nitrogen until analysis. Samples are extracted by using 3 mL methanol (100%) for 2 h, and the extract was kept in a sonication bath for 10 min; again the sample should be centrifuged and filtered (Whatman GF/F) to remove excess cell debris (Coupel et al. 2015). A 1 ml of the filtered extract is pipetted and transferred to HPLC vials and placed in the cooling rack of the HPLC. 300 ml of water (HPLC grade) is injected into the filtered extract prior to HPLC injection, using a pretreatment programme of the HPLC involving careful mixing of the sample. The samples are then injected into a HPLC system with Class-VP software using the method of Wright et al.

Table 2 Various methods of measuring the carotenoids and other pigments

Carotenoid	Solvent	Wavelength (nm)
a-Carotene	Petroleum ether	444
	Hexane	445
b-Carotene	Petroleum ether	450
	Ethanol	450
	Chloroform	465
a-Cryptoxanthin/zeinoxanthin	Hexane	445
b-Cryptoxanthin	Petroleum ether	449
	Hexane	450
Lutein	Ethanol	445
	Diethyl ether	445
Zeaxanthin	Petroleum ether	449
Lycopene	Petroleum ether	470
	Ethanol	450
	Acetone	452

(1991). Carotenoids and chlorophyll ‘c’ and ‘b’ were detected at 450 nm, Chl a is measured at 676 nm and bacteriochlorophyll ‘a’ is detected at 770 nm with the help of HPLC.

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Surface Bioengineering of Diatom by Amine and Phosphate Groups for Efficient Drug Delivery

R. Sasirekha and P. Santhanam

Introduction

The self-assembled existent biomaterials have been synthesized naturally with enormous three-dimensional structured porous with multifunctional properties (Sarikaya 1999). Diatoms are benthic photosynthetic brown algae, which is the most terrific living thing that can produce their exoskeleton with amorphous silica particles organically (Morse 1999; Sumper and Brunner 2006). Those outer shells arrayed with porous biosilica with distinctive 3D architecture, called frustules, with extremely organized pore structures, patterns of species and hierarchical arrangements with peculiar mechanical, molecular transport, ocular and bioluminescence properties (Parkinson and Gordon 1999; Lopez et al. 2005; Losic et al. 2009). Their biocompatibility and mechanical potency with humans and other species are highly prominent (Kröger and Poulsen 2008). The pores are displaying more dominancy on diatoms; hence the diatoms become smaller at each generation, even though the pore size remains same on diatom's surface (Kröger and Poulsen 2008), so the identical pore size on diatom shell become a prospective biomaterial for the application of drug delivery. These features may well eradicate the low bioavailability of hydrophilic drugs and replace synthetic mesoporous silica materials as drug cargo loading (Lauritis 1968). Diatom frustules are available mainly as of two resources, including live diatom cultures with tiny amount of biomass and in the form of fossils diatomaceous earth (DE), in huge quantities. Owing to the silica chemistry of the diatom is having some limitations on those applications. Accordingly, to modify the biosilica material derived from diatoms, the significant efforts have recently been underwent technologically to convert it to be more suitable functional materials without disrupting the frustules morphologies and shapes. The conversion of biosilica into

R. Sasirekha · P. Santhanam (✉)
Marine Planktonology & Aquaculture Laboratory, Department of Marine Science,
School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

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inorganic (MgO, TiO2, zeolites), semiconducting (Si–Ge), metal (Ag, Au) or organic (polyaniline) scaffolds has been demonstrated through several approaches including gas/solid displacement, chemical deposition, sol–gel synthesis and polymerization (Losic et al. 2007). The possible surface modification will generate new properties like localized surface plasmon (LSPR) and surface-enhanced Raman scattering (SERS) on diatom explored via simple mechanisms, which will have been generated from diatoms, functionalization with Au nanoparticles (Bao et al. 2009). The potency of drug cargo delivery from diatom biosilica through their nanoporous is proven by controlled drug releases. Diatom biosilica can also be modified with antibodies and enzymes (Gordon et al. 2009; Singh and Singh 2009; Downs Jr. et al. 2005). The impact of drug loading and release on surface functionalised diatom's silica material, particularly investigating the properties of diatom surface and functionalization materials for both hydrophobic and hydrophilic drugs. The organosilanes and phosphonic acid such as 3-aminopropyltriethoxysilane [APTES] based self-assembled organic monolayers (SAM), N-(3-(trimethoxysilyl) propyl) ethylene diamine [AEAPTMS], 2-carboxyethyl-phosphonic acid [2-phos] were used for hydrophilic and 16-phosphono-hexadecanoic acid [16-phos] for hydrophobic drugs are used as chemical modifiers (Bariana et al. 2013a, b). Organosilanes APTES and AEAPTMS are amino functional groups containing amine modification agents and were mostly used for enhancing steady adsorption in numerous applications between organic compounds and silica substrates (Xu et al. 2003).

The challenges associated with drug delivery therapeutic agents have been overcome by the properties of diatoms biosilica such as structural, mechanical and chemical features (Yoysungnoen et al. 2008). A good drug loading capacity and sustained drug release of diatom biosilica have been reported as up to 2 weeks for drug cargo delivery (Bae et al. 2006). The characterization of cleaned diatom frustules before and after drug loading will have been carried through the series of characterization techniques, including light microscopy, scanning electron microscopy (SEM), energy-dispersive x-ray spectroscopy (EDXS), differential scanning calorimetry (DSC), thermogravimetric analysis (TGA), particle size, zeta potential and nitrogen adsorption Brunauer–Emmett–Teller (BET) analysis. The x-ray powder diffraction (XRPD) spectroscopy is used to analyse the crystalline state of drug-loaded diatom frustules. The solid state nuclear magnetic resonance (NMR) is used to characterize and demonstrate the feasibility of diatom biosilica for drug delivery; release behaviour of the model drugs will be presented and correlated to drug–diatom silica interactions.

Diatom Isolation and Identification

Diatoms can be isolated using various techniques, such as serial dilution, agar plating and micromanipulation. The culture was maintained in an axenic condition according to Perumal et al. (2012). The cultures are kept at 23 ± 1 °C and are supplied with continuous ambient cool-white fluorescent illumination with 1500 lux for 12:12 light-dark cycle's regime. The diatoms are cultured from a freshwater/marine water silicate-rich growth medium (F/2 medium) (Guillard and Ryther

1962) for 2 weeks. The cultures (typically ~1000 mL total) can be collected via centrifugation (3000 rpm; 10 min) and washed thrice with distilled water. Piranha solution (0.34 M $KmnO_4$, 1 mL; conc. H_2SO_4, 5 mL; 30% H_2O_2, 1.5 mL) was used to digested the organic portions of the diatoms to activate their cell walls for silane functionalization. Isolation of activated diatom cell walls are done by copious washing with distilled water and will be collected into a plastic centrifuge tube (15 mL) and suspended in a mixture of distilled water (5 mL) (Rosi et al. 2004). The structure, surface charges and property of diatom will be confirmed by FESEM images, DLS and EDS graph, respectively.

Surface Functionalization with Different Functional Groups

Surface functionalization of diatom frustules and surface modification using selected organosilanes can be prepared by a standard silanization process as previously described (Vrancken et al. 1995 and Jani et al. 2009). Briefly, 0.1 g of diatom frustules powder is mixed with 0.5 ml of N-(3-(trimethoxysilyl)propyl)ehtylenediamine (AEAPTMS) containing 10 ml toluene solution. The pure nitrogen gas will be purged to the suspension and sealed tightly. After an overnight incubation, the mixture will be sonicated to ensure an equal dispersion. The modified diatom microcapsules can be filtered after numerous washing with toluene, followed by ethanol before being dried under vacuum desiccator at room temperature.

2-Phos and 16-phos are used for surface treatment with 1 mM ethanol solution of either phosphonic acids added to the diatom microcapsules that are stored in hydrogen peroxide (5%). Then the sonication will be carried for diatom frustules in ethanol to reduce the formation of multilayer before the annealing process at 140 °C for 24 h in an oven, intended for a dense formation of monolayer on the biosilica surface. A base rinse in 5% triethylamine/ethanol via sonication is performed to remove any remaining physic absorbed molecules followed by copious rinsing with ethanol (Aw et al. 2012).

3-Aminopropyltrimethoxysilane (APTES) (500 μL) and glacial acetic acid (5 μL) are used to functionalize the diatom biosilica. The mixture will be allowed to react for 2 days on an inverting rotation device after which unreacted silane will be removed by centrifugation. The amino-functionalized diatoms are washed with distilled water 3times followed by acetone 3times to dry and remove excess water. FTIR spectrum and FESEM will be used to evaluate the functional group changes in order to surface modifications.

Fluorescence Labelling

The diatom frustules will be explored for their cargo loading ability. The quality of drug encapsulation will be analysed using fluorescein isothiocyanate (FITC) labelling of diatom biosilica. Before drug loading, cleaned diatom frustules (0.1 g) are

sonicated for 2 h with ethanol (2 mL) to acquire efficient pore wetting and enhance drug encapsulation within the pores as reported previously (Aw et al. 2012).After 2 h, leave the diatom frustules for drying. 1 mg/mL of FITC solution will be prepared for FITC labelling in distilled water, and it will be added to the powdered sample of diatom frustules drop by drop. Hence the FITC can be infused into the pores of diatom frustules into the hollow centre of diatom structure. Finally it will be centrifuged to remove excess dye at 5000 rpm continuously until the supernatant clear. The sample is dried under vacuum to obtain FITC-labelled diatom biosilica. Labelling will be confirmed with fluorescence microscopy and CLSM.

Drug Loading in Diatom Porous Biomaterial with Hydrophobic and Hydrophilic Drugs

To enhance drug encapsulation, the diatom frustules will be wetted with ethanol for drug delivery of both hydrophobic and hydrophilic drugs. 0.1 g of diatom biosilica taken for sonication with ethanol (2 ml) for 2 h, by the filtration ethanol, has been removed. Subsequently, 5 ml of ethanol as solvent used to dissolve the 0.03 g of hydrophobic drug with final concentration 6 mg/ml. Then the homogenous solution will be obtained from the mixture during sonication for 1 h, followed by repeated dropwise loading into the diatom frustule powder (Vallet-Regí et al. 2007). After the solvent evaporation, hydrophobic drug-loaded diatom frustules powder (hydrophobic drug/diatom frustules) will be obtained.

Hydrophilic drug-loaded diatom frustules (hydrophilic drug/diatom frustules) can be prepared as illustrated above, but Milli-Q water will be used instead of ethanol for wetting, then the water will be evaporated by the vacuum desiccator, and 50 mg/ml hydrophilic drug aqueous solution will be added for loading (Aw et al. 2011). Based on the solubility concentration, the drug will be selected for therapeutic loading (Zhang et al. 2013). Concentration of the model drug will have been chosen close to their saturation solubility point to increase the encapsulation efficiency. The drug-loaded nanocarriers will be collected by centrifugation at 8000 rpm for 10 min and washed thrice with distilled water (Wang et al. 2010). The supernatant will be used to determine the drug loading by high-performance liquid chromatography (HPLC) and also UV-VIS spectrophotometer with the ranges of target drugs wavelength. The pellets are used to study further. The drug-loading content and encapsulation efficiency can be calculated by the following equations:

$$\text{Encapsulation efficiency of drug}\left(\%\right) = \frac{\text{Initial amount of drug} - \text{Final amount of drug}}{\text{Initial amount of drug}} \times 100$$

In vitro Drug Release

The drug-loaded biosilica (15 mg) will be dispersed in 1.5 mL of acetate buffer and PBS buffer solution for in vitro drug release; the solution will be moved into a dialysis bag (molecular weight cutoff: 7000 Da). The particle stuffed dialysis bags are kept in 20 mL of 50 mM of two different pH buffer solutions (pH 4.0 and 5.2, acetate buffer; pH 6.8, 7.4 and 8.0, phosphate buffer) and smoothly spin at 100 rpm and 37 °C at prearranged time intervals; 10 mL of the solution will be withdrawn to determine the amount of released drug by UV-VIS spectrophotometry and HPLC; to replace withdrawn solution, an equal volume of fresh medium will be added to keep the volume of buffer remain constant.

Haemolysis Assay

The haemolytic properties of drug and diatom frustules will be evaluated to analyse the significant hemocompatibility. Hence, 4 mL of EDTA-stabilized human blood samples will be centrifuged with 20 mL of autoclaved PBS (pH -7) for 10 min, at 3000 rpm at 4 °C and further copiously washed with PBS. The serum-separated RBCs will be diluted to 20% with PBS buffer solution, and 200 μL of 20% diluted RBCs suspension was mixed with diatom frustules, surface functionalized diatom frustules and drug-loaded diatom frustules suspensions in 800 μL of PBS to achieve final 1000 μL concentrations, at different concentrations of samples from 2 to 50 μg/mL (2, 10, 25, 50). PBS and distilled water are used as negative and positive controls, respectively (Rajiu et al. 2015).The mixture will be swirled and incubated at 37 °C for 3 h and centrifuged at 3000 rpm for 3 min. 100 μL of supernatant was taken from the sample and is transferred to a microtitre plate. The absorbance values of the supernatants at 570 nm will be measured using microplate reader with 630 nm as the absorption reference wavelength (Malinauskas 1997). The percentage of haemolysis of RBCs from each sample will be calculated using the following equation:

$$\text{Haemolysis}(\%) = \frac{\text{Sample absorbance} - \text{Negative control absorbance}}{\text{Positive control absorbance} - \text{Negative control absorbance}} \times 100$$

Cytotoxicity Assay Against HeLa Cells

Cell viability will be measured by the MTT assay, HeLa cells cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. The cells will have been kept in a humid 5% CO_2 atmosphere at 37 °C. The old medium is replaced with fresh medium every 2 days. Cells are passaged thrice a

week. It will be subcultured at 80% confluence into the 96-well plates (Hansen et al. 1989; Aliabadi et al. 2010). Subsequent to the monolayer formation of cells that formed for 36 h, cells are treated with different concentration ranges of diatom frustules, drug-loaded diatom frustules and free drug. After 24 h treatment, the serum-free medium containing 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 0.5 $mg.ml^{-1}$) was renewed and incubated for another 4 h in a dark condition. The cell viability was measured by the MTT assay, by enzymatic reduction of yellow tetrazolium to a purple formazan, after incubation the aspirated medium will be replaced with DMSO. The absorbance will be measured at 570 nm using microplate reader (Moradi et al. 2012; Hong et al. 2011). The relative cell viability will be expressed as follows:

$$\text{Cell viability}\left(\%\right)=\frac{\left[\text{OD}\right]\text{Control}-\left[\text{OD}\right]\text{Test}}{\left[\text{OD}\right]\text{Control}}\times 100$$

From the cell viability percentages, the inhibition concentration (IC_{50}) will be calculated for the complexes used.

Intracellular Uptake and Intracellular Distribution

The cellular uptake and intracellular distribution of free drug, diatom frustules or drug-loaded diatom frustules will be evaluated through the confocal microscopic observations; HeLa cells (105 cells/dish) are seeded in 20 mm glass bottom culture dishes, and the cells are treated with IC_{50} concentration free drug, diatom frustules and drug-loaded diatom frustules (drug at 1 μg/mL) at 37 °C for 5 h. After washing with PBS in twice, cells will be fixed with 4% paraformaldehyde for 5 min and stained with FITC (1 mg/mL distilled water) already before drug loading. And the cellular uptake will be observed directly through 3D fluorescence and confocal laser scanning microscopy (CLSM) with respective wavelength of emission and excitation of model drug and cancer cells. For the diatom biosilica the correlation with the FITC emission ranges between 489 and 515 nm (Mosmann 1983).

Summary and Prospects

The micro- and nanoporous of diatom biosilica materials are the properties of interest for controlled drug delivery. Firstly, it is a promising drug delivery platforms due to their wide availability of diatom and bioactivity control on pore dimensions and drug release profiles of various therapeutics up to several weeks. Secondly, numbers of reports have been demonstrated that the oral drug delivery of porous biosilica materials is a useful contender for future in vivo investigation studies. The

toxicological investigation of diatom frustules and their major prerequisite and practical applications are attracting attention in the last few decades. The use of diatom frustules/biosilica as surface functionalized intracellular controlled release vector has been demonstrated in vitro. Further in vivo work is required in order to study the zero premature release anticancer and other therapies of loading and releasing potential of the drugs. In addition, in vivo biocompatibility, practical applications, biodegradability and bio fate of the diatom micro particles can also be envisioned in the future. From the first proof-of-concept studies, the microporous diatom material has experienced numerous growth and research development. Nowadays diatom biosilica materials that can release the entrapped cargo with response to functionalization agents have emerged as a special niche in drug delivery technologies. Furthermore, investigations of controlled release of bio molecules, such as genes, enzymes and proteins, are rarely reported. Finally it is required to study both in vitro and in vivo to understand the whole systems of drug carrier and surface chemistry made on diatom biosilica functions.

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A Study on the Impact of Acidification on the Morphometry, Photosynthesis, and Biochemical Composition of Phytoplankton

P. Bharatha Rathna and P. Santhanam

Introduction

Many marine organisms are affected by ocean acidification, particularly those with shells and skeletons built from calcium carbonate; e.g., corals, oysters, clams, mussels, snails, phytoplankton and zooplankton, the tiny plants and animals that form the base of the marine food web. These "marine calcifiers" face two potential problems associated with ocean acidification. The first one is that their shells and skeletons may dissolve as the ocean pH decreases; as CO_2 is dissolved in seawater it becomes more corrosive, and the water chemistry undergoes major changes, such that fewer carbonate ions, the primary building blocks of marine organisms, are accessible for uptake to shells and skeletons. To build their shells or skeletons, marine organisms generally undergo an internal chemical process that converts bicarbonate to carbonate in the form of calcium carbonate. Exactly how ocean acidification slows calcification or shell formation is not yet completely understood, but various mechanisms are being studied. Most investigations of this corrosive environment focus on the evolutionary defense mechanisms that organisms must have in order to build and maintain their calcium carbonate shells and skeletons in an increasingly acidified environment. In such environments, these organisms can be subject to greater energy expenditure and environmental stressors such as ocean temperature increases, low oxygen accessibility, disease, and habitat loss.

Sunlight-driven cells, including those present in phytoplankton, convert carbon dioxide to potential biofuels, foods, feed, and high-value bioactive agents (Banerjee et al. 2005; Spolaore et al. 2006). Phytoplankton is an essential component of aquatic ecosystems and is the base of food chains; it accounts for a large amount of food production in marine and freshwater ecosystems. Phytoplankton is a primary producer; it fixes most of the Earth's carbon and generates, through photosynthesis,

P. Bharatha Rathna · P. Santhanam (✉)
Marine Planktonology & Aquaculture Laboratory, Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

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much of the oxygen in our atmosphere. Phytoplankton is likely to affect organisms at higher trophic levels and may have important consequences for the health of the whole aquatic ecosystem. Phytoplankton consists of unicellular aquatic organisms with significant potential to produce valuable natural products (Davidson 1995; Plaza et al. 2009; Plaza 2010). These organisms are commonly present in fresh, marine, or brackish water, in which they may be suspended (planktonic) or live at the bottom (benthic).

Surface water pH is an important factor in the environment that can significantly affect the growth of the phytoplankton population (Agrawal and Singh 2000; Dnailov and Ekelund 2001). In tropical coastal waters, pH may fluctuate depending on biological activities in the surrounding environment. Phytoplankton abundance is affected by pH, and most organisms have an optimal range of conditions in which they thrive. The effect of pH in phytoplankton cultures has been discussed previously (Goldman 1981). It was reported that the pH tolerance limits of the tested phytoplankton were governed by chemical influence on the growth medium or by metabolic effects on the cells. It was concluded that the most tolerable pH was not influenced by the availability of inorganic carbon. Variations of seawater pH levels can also have a marked effect on the growth and survival of sea ice phytoplankton. A number of biological and physical processes influence pH. The study of sea ice has shown that in regions characterized by high primary production, the sea ice brine has a considerably low concentration of dissolved inorganic carbon (TCO_2) and elevated pH levels, as high as 10 (Gleitz et al. 1995; Thomas et al. 2001). Furthermore, changes in carbon chemistry alone can result in considerable changes in the pH of sea ice brine.

The pH of seawater is an important chemical factor for aquatic organisms because it affects the normal physiological functions of the aquatic ecosystem, including ion exchange in the water and respiration. The oceans absorb about 50% of the anthropogenic CO_2 emitted to the atmosphere. CO_2 dissolved in seawater will lower its pH and affect its carbonate cycle. It is commonly held that the average surface ocean pH has already declined by 0.1 from the pre-industrial level (Orr et al. 2005), and it is projected to decrease by 0.3–0.46 by the end of this century, depending on CO_2 emission scenarios (Caldeira and Wickett 2003). Hence, it is imperative to evaluate how marine organisms cope when these conditions arise. The lethal effects of low pH have been studied previously for several marine invertebrates, using strong acids such as hydrochloric acid (HCl) and sulfuric acid (H_2SO_4) (Knutzen 1981; Adams et al. 1997; Yamada and Ikeda 1999). Kikkawa et al. (2004) compared the acute toxicity of CO_2-acidified and HCl-acidified seawater for the eggs and larvae of marine fish and found that seawater acidified by CO_2 was more toxic.

Studies of the impact of acidification on marine organisms have focused on calcifying organisms, while researchers have paid less attention to non-calcifying organisms, such as phytoplankton and zooplankton, which play an indispensable role in the food web and can affect the growth and reproduction of higher trophic organisms (Shek and Liu 2010). However, to understand the effects of pH on the whole marine ecosystem, studies of organisms at lower trophic levels are also needed. Phytoplankters often constitute the majority of the ocean biomass and play a vital role in marine ecosystems, serving as crucial links in food chains and food

webs. Despite their ecological significance, the potential effects of ocean acidification on phytoplankton remain unclear, owing to the lack of appropriate experimental techniques. This chapter deals with the procedures involved in determining the impact of acidification on the growth, pigment production, and biochemical composition of phytoplankton.

Materials and Methods

Collection and Identification of Phytoplankton

Phytoplankton were collected from brackish water and marine environments, using 48-μm plankton mesh, and were immediately transported to the laboratory for identification and isolation. The phytoplankton was identified under a light microscope using standard taxonomic keys (Subramanyan 1946; Santhanam and Perumal 2008). Based on the taxonomic key, the strain was confirmed to the genus level and used for the acidification experimental study.

Phytoplankton Culture

Mixed phytoplankton samples were collected and transferred to Conway's Medium (Walne 1976), kept at 18–25 °C in a thermostatically controlled room, and illuminated with white florescence lamps with a light/dark ratio of 12:12 h. After 5 days, the samples were serially diluted to a concentration of 10^{-10}. Phytoplankton diatoms and green algae were isolated and identified based on their morphological characteristics (Subramanyan 1946) and an indoor stock culture was developed according to Perumal et al. (2012).

The sea water was filtered using a 1-μm filter bag. The filtered seawater was sterilized. After cooling, the water was transferred to culture flasks, and the flasks were plugged with cotton. Temperature and salinity were maintained in the range of 23–25 °C and 28–30‰, respectively, for the entire culture period. About 5 ml of inoculum in the growth phase was transferred to the culture flasks and the culture was provided with 1000 lux by using two tube lights. After 5–8 days, the maximum biomass of phytoplankton was obtained during the exponential phase.

pH – Potentiometric Method

The pH was measured using an ELICO grip pH meter (manufactured in Coimbatore, Tamil Nadu, India). The measurement of pH is a common water quality test. In pure water or dilute solutions, the activity of the hydrogen ions is measured by pH. In sea

water, potentiometric pH measurements are made using hydrogen-sensitive glass/reference electrodes.

Photosynthesis Measurements (Strickland and Parsons 1972)

Ten ml of the phytoplankton culture sample was filtered with a Millipore filtering system, fitted with a 4.5-cm-diameter GF/C filter paper. Before the sample was filtered, 2 ml of magnesium carbonate solution was poured onto the filter paper. After filtration, the filter paper was removed with clean forceps and the sample was mixed with 90% acetone, and ground with a mortar and pestle. The ground samples were transferred to screw-cap test tubes and kept in a refrigerator for 24-h incubation. After 24 h, the contents were reground with 90% acetone and centrifuged at 3000 rpm for 10 min. The supernatant was collected and the optical density of chlorophyll '*a*' and '*b*' at wavelengths of 630, 645, and 665 nm was measured.

Alkalinity

Carbonate and bicarbonate in culture water can be determined by titrating a known volume of sample against diluted H_2SO_4 solution, using phenolphthalein and methyl orange as indicators, as described by Strickland and Parsons (1972). When a drop of phenolphthalein is added to the water sample, a pink color develops when carbonates are present. If no pink color is shown, this indicates the absence of carbonate in the water. When a sample containing carbonate and bicarbonate is titrated against standard H_2SO_4, phenolphthalein loses its pink color; when half of the carbonate is converted to bicarbonate in this colorless solution, a few drops of methyl orange are added and titrated against H_2SO_4 until the straw-yellow color changes to a pinkish red color. The obtained values gave the amount of acidic required to neutralize the bicarbonate originally present.

Calibration of pH

The pH of the culture media was adjusted by two different methods. The first method consisted of bubbling the media with CO_2, compressed air, and a mixture of nitrogen and oxygen gases. The pH of the culture medium was raised by adding diluted NaOH solution. This solution was also bubbled with CO_2, compressed air, and a mixture of N_2 and O_2, for 2 h, producing pH levels of around 7, 8, and 9, respectively.

The second method of pH adjustment was done according to alkalinity and changes in pH levels. First, acid/base titration of the medium was conducted and

suitable acid and base additions were made to adjust the pH levels in one volume of culture medium.

Calculation of Carbon Speciation

The following equations were used to calculate the concentrations of CO_2 and HCO_3^-.

$$[CO_2] = \frac{CAa_H^{\ 2}}{K_1(a_H + 2K_2)}$$

$$[HCO_3^-] = \frac{CAa_H}{a_H + 2K_2}$$

in which CA represents carbonate alkalinity; a_H represents the 10 ^{PH} activity of hydrogen ions; and K_1 is the second apparent dissociation constant of carbonic acid.

Determination of Cell Density

The optical density (OD) of the phytoplankton cultures was measured at 5-day intervals by checking culture turbidity at 680 nm (Lichtenthaler 1987) with the help of a spectrophotometer. Vortexing of the phytoplankton cultures was done to obtain a homogenous culture, in order to prevent settling and the obtaining of erroneous OD results.

Cell Count

The Hemocytometer (Counting chamber) is used to determine the amount of cell growth in the phytoplankton culture. The sample was well stirred with a pipette; the pipette was then filled with the stirred sample and the sample was placed in the two-level counting chamber. We waited for a few minutes to allow the phytoplankton cells to settle. Then a hemocytometer was positioned in the lower chamber to start with, and the gridded area was found; the microscope was then set to ×10. The upper and lower chambers each contained nine large grid squares, and a total count of the algae was done in each of the 18 squares. The number of cells present in each grid was counted and the data was recorded. Finally, the number of cells present per ml of sample was calculated.

Chlorophyll Estimation (Mackinney 1941)

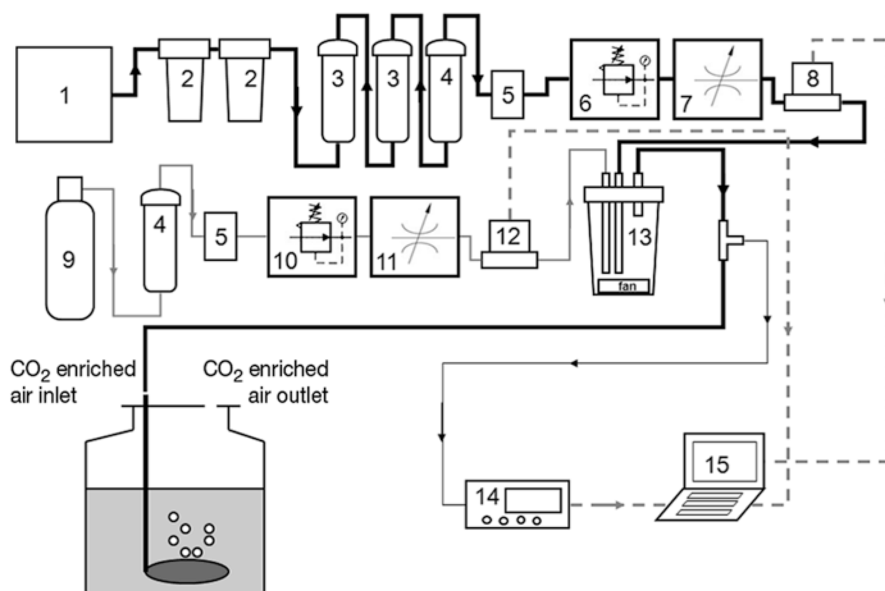
Dried algal samples (0.5 g each) were collected and chlorophyll was extracted with 5 ml methanol or 80% acetone. The reconstituted samples were kept in a refrigerator and incubated for 24 h; the OD values of the chlorophyll were measured after incubation with an ultraviolet and visible absorption (UV/VIS) spectrophotometer at 663 nm. The amount of chlorophyll present in the extract was calculated according to the equations below:

Calculation

Chlorophyll '*a*' μg/ml = optical density × 12.63 × dilution factor
Chlorophyll '*b*' μg/ml = optical density × 19.3 × dilution factor

Seawater CO_2 System

The experiment carried to mix with various ratio of mass flow rates using CO_2-free, dry air, and pure CO_2 (99.99% purity) to produce CO_2-enriched air at different pCO_2 (Fangue et al. 2010). With the facilitation of an oil-free, medical air compressor and 2 filters the atmospheric air was passed to remove water and sand particles, further the water will have been removed by 2 food-grade polypropylene (PP) columns (32 cm length, 6 cm diameter) filled with soda-lime to absorb the background CO_2, and then through a similar PP column filled with $CaCl_2$ anhydrate. Before entering into regulating utilities it will be passed through the 5 cm disk-type air filter. Then it will be delivered through pressure regulation valve and then a needle valve, so that the air flow could be maintained stably. To produce a stable CO_2 flow, the pure CO_2 will be delivered via pressure regulating valve and a needle valve. Two mass flow sensors will have been used to measure the mass flow of air and CO_2, upto the desired level by adjusting the needle valves. The equipment with a fan at the bottom was used to homogenize both air and CO_2 in a 2 L plastic jar to produce CO_2-enriched air with different CO_2 concentrations. A tiny proportion of gas mixture was directed through a bypass. It will be monitored with a CO_2 detector. The mass flow sensors and CO_2 detector were connected to a computer, and measurements were recorded with the associated software (version 2.1).



1. The pCO_2 manipulation system, showing 1: air compressor, 2: filter, 3: soda-lime column, 4: $CaCl_2$ anhydrate column, 5: disc-type air filter, 6: pressure regulation valve, 7: needle valve, 8: mass flow sensor, 9: CO_2 cylinder, 10: pressure regulation valve, 11: needle valve, 12: mass flow sensor, 13: plastic jar, 14: CO_2 detector, 15: computer. *Solid line*: air or CO_2 flow; *dashed line*: digital signal transferred to computer (Guo et al. 2015).

Nutrient Analysis

Phosphate

The sample was warmed to room temperature and 10 ml of mixed reagent was added to 100 ml of sample. After 5 min the OD values were determined using a UV/VIS spectrophotometer at a wavelength of 885 nm (Strickland and Parsons 1972).

Nitrite

A 50-ml sample of water was taken and 1 ml of sulfanilamide solution was added to the sample. After 2–8 min 1 ml of N1 - Naphthyl Ethylene DiamineDi – Hydrochloride (NNED) solution was added to the sample and the sample was immediately mixed. The procedure was carried out with standard nitrite solution

and then the nitrite concentration of the samples was estimated in a 1-cm cuvette at a wavelength of 543 nm, using a spectrophotometer (Strickland and Parsons 1972).

Silicate

A 50-ml sample was put into a beaker, to which was added, in rapid succession, 1.0 ml 1 + 1 hydrochloric acid and 2 ml of ammonium molybdate solution; this was mixed well and allowed to stand for 5–10 min, and then 1.5 ml oxalic acid was added. After 2 to 5 min, the color was read in a spectrophotometer at 410 nm.

Biochemical Analysis

Estimation of Protein (Lowry et al. 1951)

Samples weighing around 1 g were taken individually and homogenized in 80% ethyl alcohol to precipitate the protein. Then the homogenized samples were centrifuged at 4000 rpm for 10 min. The precipitate was re-dissolved in 4 ml of 1 N NaOH solution, after which 5 ml of freshly prepared alkaline copper solution, containing 1 ml of 0.5% copper sulfate in 1% potassium tartrate (W/V) and 50 ml of 20% sodium carbonate in 0.1 N sodium hydroxide, was added to the re-dissolved precipitate and this solution was allowed to stand for 20 min. We used 1 N NaOH solution and 0.1% bovine serum albumin (W/V) as blank and standard solutions, respectively. The color development was read at 650 nm with a UV/VIS spectrophotometer. Standards were also run simultaneously and, based on the OD value, the total protein concentration of the sample was calculated according to the equation below:

Calculation

$$\frac{\text{OD of the Unknown}}{\text{OD of the Known}} \times \text{Concentration of standard} = \text{Concentration of protein present in the Unknown sample}$$

Estimation of Lipid Folch et al. (1957)

The 10 mg of dried samples were placed in a test tube and 5 ml of chloroform/methanol mixture, at a 2:1 ratio, was added. This sample mixture was kept at room temperature for 24 h for incubation. After incubation, the mixture was filtered with

Whatman No. 1 filter paper. The filtered sample was collected in a pre-weighed beaker, and kept on a hot plate. After the solvent had evaporated the final weight of the beaker was measured. To determine the weight of the lipid present in the sample, the weight of the difference was calculated. The percentage (%) of lipid present in the sample was calculated by using the following formula:

$$\%\text{of lipid} = \frac{\text{Amount of lipid in the sample}}{\text{Weight of sample taken}} \times 100$$

Estimation of Carbohydrate (Dubois et al. 1956)

A sample weighing 5 mg was taken and homogenized with double-distilled water. This sample was then centrifuged for 10 min at 3000 rpm. The supernatant was collected and 1 ml of 5% phenol solution and 5 ml of concentrated H_2SO_4 was added. After 30 min the OD values were measured at 490 nm. D-glucose was used as a standard.

$$\text{Carbohydrate}\% = \frac{\text{Standard value} \times \text{OD of the sample}}{\text{Weight of sample taken}} \times 100$$

Estimation of Ash Content (Marsham et al. 2007)

A dried sample (2 g) was added to a pre-weighed crucible and kept in a furnace at 500 °C for 4 h, cooled in a desiccator, and re-weighed.

Estimation of Moisture (Association of Official Analytical Chemicals [AOAC] 1995)

Phytoplankton moisture is commonly determined by drying a sample at an elevated temperature and determining the loss of weight as moisture.

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Distribution of Phytoplankton in Selected Salt Pans of Tamil Nadu, Southeast Coast of India

A. Shenbaga Devi, P. Santhanam, S. Ananth, and S. Dinesh Kumar

Introduction

Salt is one of the world's best-known minerals and the chemical substances most related with the history of human civilization (Korovessis and Lekkas 2009). Solar evaporation is a process that has been profitably used for salt production for millennia. However still, the biology of a saline ecosystem in relation with the salt production process has not been well studied. Recently many countries have shown interest in maintaining and manipulating the hypersaline ecosystem for aquaculture and other related activities. Salt pan ecosystem is highly dynamic where the organisms are subjected to vulnerable physico-chemical disturbances. Salt pans are unique enclosed ecosystem that is characteristically exposed to a wide range of environmental stress and perturbations manifest mainly through salinity changes. In the extreme astatic physico-chemical conditions of these hypersaline habitats, only a few plant and animal species can live. Salt pan ecosystem offers a number of unique ecological niches having a strange combination of environmental factors. The nutrient-rich seawater in saltworks favours algal blooms in reservoirs and evaporators.

'Algae' is an historical and practical grouping of generally photosynthetic organisms of simple construction. It includes a diverse array of morphologies from single cell to large, multicellular organisms. They can be found in either freshwater or marine habitats. The distinction between marine and freshwater habitats is revealed in the variety of algae that occur in these environments. There are no exclusively

A. Shenbaga Devi · P. Santhanam (✉) · S. Dinesh Kumar
Marine Planktonology & Aquaculture Laboratory, Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

S. Ananth
Marine Planktonology & Aquaculture Laboratory, Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

Department of Animal Husbandry, Dairying and Fisheries, Ministry of Agriculture and Farmers Welfare, Krishi Bhavan, New Delhi, India

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freshwater divisions of algae, but certain groups exhibit greater abundance and diversity within freshwaters, especially the members of Cyanophyceae, Chlorophyceae and Charophyceae (Smith 1950).

The word plankton originates from the Greek meaning 'wandering'. Plankton as a term was first used by Victor Hensen (1887) for the aquatic communities of floating and drifting organisms that are carried primarily by movement of water current rather by their own swimming ability; many plankton animals are strong swimmers and are capable of moving through relatively long distances over a period of time, particularly vertical direction in salt pans. The organism living in every solar saltworks constitutes a biological system, which are able to aid or harm salt production. They also increase solar energy absorption (Coleman and White 1992) and prevent leakages (Jones et al. 1981). The microalgae produce a thick-layered mat on the bottom of the ponds which prevents leakage of the brine (Oren et al. 1992). The majority of microalgae belong to Chlorophyceae, Cyanophyceae and Bacillariophyceae present in salterns.

The biota of solar saltwork ecosystems has attracted the attention of both the scientific community and the general public (Britten and Johnson 1987; Sadoul et al. 1998; Walmsley 2000). Furthermore, the red halophilic bacteria (e.g. *Halobacterium*, *Halococcus*) and halotolerant microalgae (e.g. *Dunaliella*), as well as the 'brine shrimp' *Artemia*, all typically inhabiting the high-salinity ponds or the crystallizers, have been the subjects of considerable scientific research and applications in such fields as aquaculture and biotechnology (Oren 2002).

Physico-chemical disturbances can affect the quality and quantity of salt. Light and inorganic nutrient availability are the main environmental factors controlling microalgae growth in the saltworks (Coleman and White 1993). Light and nutrient are the major factors in primary productivity and aerial evaporation rate (Tett et al. 1996). In India, however, there are a few reports on algal flora of salt pans, although a number of publications are available on the occurrence of algae in brackish and marine waters (Thajuddin et al. 2002; Selvakumar and Sundararaman 2007; Velankar and Chaugule 2007; Reginald 2007). The present study dealt the physico-chemical characteristics and microalgae diversity of three different salt pans along the Tamil Nadu, Southeast coast of India.

Description of the Study Area

The water and phytoplankton samplings were made at three salt pans, namely, Marakkanam, Vedaranyam and Mimisal. Marakkanam salt pan is located at Villupuram district of Tamil Nadu nearby Pondicherry (latitude, 12°13′5.62″N; longitude, 79°58′18.79″E). Salt pans in the Marakkanam region, spreading over 2500 acres of the coastal area in Villupuram district, are beset with problems owing to natural and man-made causes. Vedaranyam salt pan is located in Nagapattinam district of Tamil Nadu (latitude, 10°22′31″N; longitude, 79°51′1″E). Vedaranyam salt swamp forms another great natural division. Mimisal salt pan is located in the Pudukkottai district of Tamil Nadu (latitude, 9°51′48″N; longitude, 79°7′15″E). The district has an area of 4663 km^2 with a coastline of 42 km. During January and

February, salt production process starts with the preparation of ground. Workers start preparing the platform to make the soil hard and flat to store the water to make the salt. This is an extremely hard process. Working under harsh sun and dry weather with bare feet is a very painful. In summer season (March to July) once the water is hard enough to prepare the salt, the actual production process starts. This process consists of storing water, separating salt from water, and carrying the salt to the storage area which is an extremely painful and dangerous process. The workers carry around 30 kg of salt on their head under very harsh conditions. October and November months are the worst season of the year for the workers. Their work is dependent on the presence of sun. With no sun in this season, the fields are full of water, and the workers have no job and thus no money. It is also very challenging to store the salt in this rainy season. The field stays full of water for the entire season. Once the season completes and the sun comes out, the salt process production starts again. For the present study, three different salt pans were selected for sampling (Fig. 1). From each salt pan, six stations were fixed (Plates 1, 2 and 3), and water and phytoplankton samples were collected.

Materials and Methods

The water and phytoplankton samplings were made at three salt pans, namely, Marakkanam, Vedaranyam and Mimisal.

Analysis of Physico-chemical Parameters

Atmospheric and surface water temperatures were measured using a standard mercury-filled centigrade thermometer. The pH was measured using an ELICO grip pH metre. Salinity was estimated with the help of a hand refractometer (ERMA, Japan), and salinity above 100‰ was estimated according to Knudsen's method (1901). The water samples were collected for the estimation of dissolved oxygen were fixed in the field and the same were analysed in the laboratory by Winkler's method as described by Strickland and Parsons (1972).

Pigment Extraction

Water samples were taken into a graduated test tube and centrifuged at 5000 rpm for 15 min. A known amount of pellet was homogenized with acetone using pestle and mortar. The supernatant was centrifuged at 3000 rpm for 10 min. The supernatant was collected and absorbance was measured spectrophotometrically (Shimadzu-160A, Japan) at 645 nm and 661.5 nm against acetone blank. Concentration of chlorophyll 'a' and 'b' were calculated by the equation of Lichtenthaler (1987) and modified

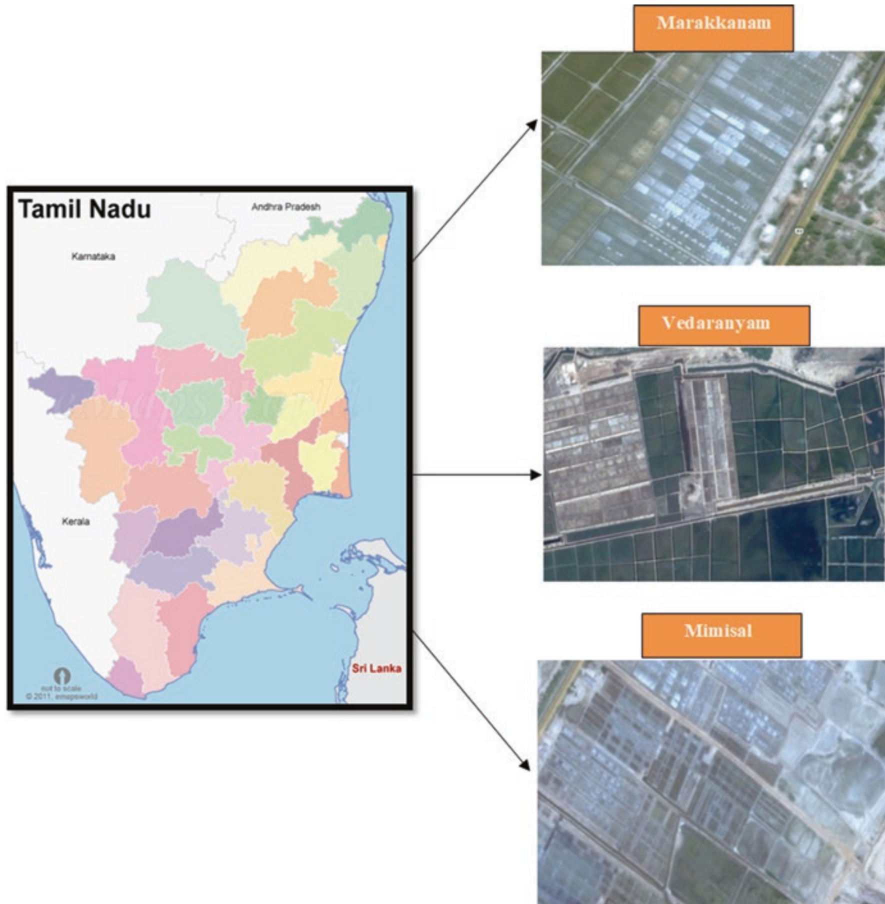

Fig. 1 Map showing the study areas

Parsons and Strickland (1963) and expressed as (µg/ml) of pigment. The chlorophyll 'a', chlorophyll 'b' and total carotene were calculated using the following formula:

$$\text{Chl a} = 11.24 \times \text{OD}661.5 - 2.04 \times \text{OD}654.0$$

$$\text{Chl b} = 20.13 \times \text{OD}645.0 - 4.19 \times \text{OD}661.5$$

$$\text{Total carotene} = 1000 \times \text{OD}470.0 - 1.82 \times \text{Chl a} / 198$$

Nutrients Analysis

For inorganic nutrients estimation, surface water samples were collected using clean polyethylene bottles and kept in an ice box and transported to a laboratory. At the laboratory, seawater samples were filtered through 0.45 cm dia GF/C filter

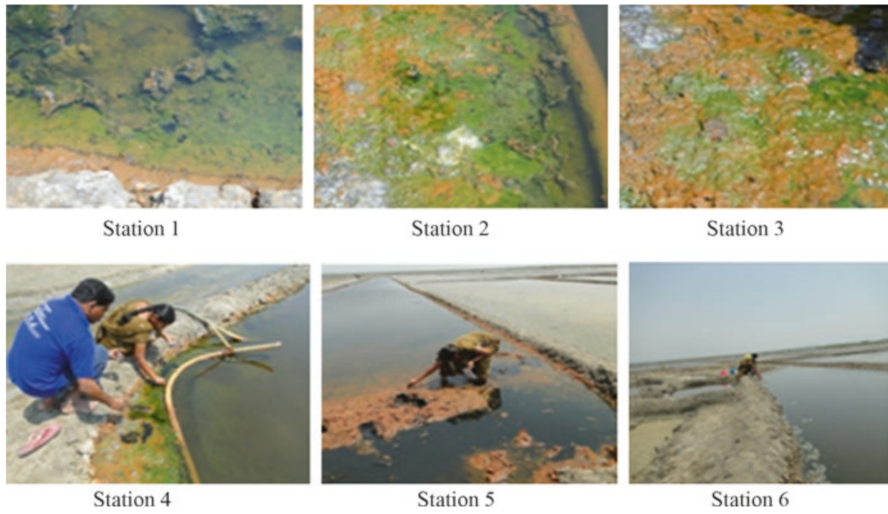

Plate 1 Marakkanam salt pan, Southeast coast of India

Plate 2 Vedaranyam salt pan, Southeast coast of India

paper using Millipore filtering apparatus. The filtered water sample was used for the analysis of dissolved inorganic nitrate, nitrite, phosphate and reactive silicate adopting the standard procedures described by Strickland and Parsons (1972) and Jenkins and Medsker (1964).

Biological Parameters

The phytoplankton (microalgae) samples were collected by filtering a known volume of water through 48 μm mesh, and species were identified based on the morphological characteristics, viz., cell shape, cell colour, cell length, width and flagella

Plate 3 Mimisal salt pan, Southeast coast of India

length and chloroplast arrangement using the standard books (Teodoresco 1906; Preisig 1992; Leonardi and Caceres 1997). For quantitate measurement, a known volume of water samples were filtered through plankton nets (48 μm), and retained microalgae were preserved in 5% formaldehyde for further analysis. The phytoplankton samples were analysed by using (1 ml capacity) Sedgwick-Rafter cell (Bernard 1971). After shaking the bottle, the 1 ml sample was drawn by pipette and poured in the rafter cell. All the 1000 squares on the chamber were screened and phytoplankton identified up to species level under binocular microscope. Species diversity index (H′) was calculated using Shannon and Weaner's (1949) formula, species richness (SR) was calculated using Gleason (1922) and evenness index (J′) was calculated using the formula of Pielou (1966).

Statistical Analysis

The data were expressed as the correlation coefficient of physical chemical parameters. Statistical correlation tests were employed to make statistically significant conclusions about the data using **SPSS Package ver. 16.0.**

Results

Analysis of Physico-Chemical Parameters

The hydrobiological and physico-chemical characters prevailing in a saltwork play an important role in the salt production strategy. On the other hand physico-chemical disturbances can affect the quality and quantity of salt. The physico-chemical

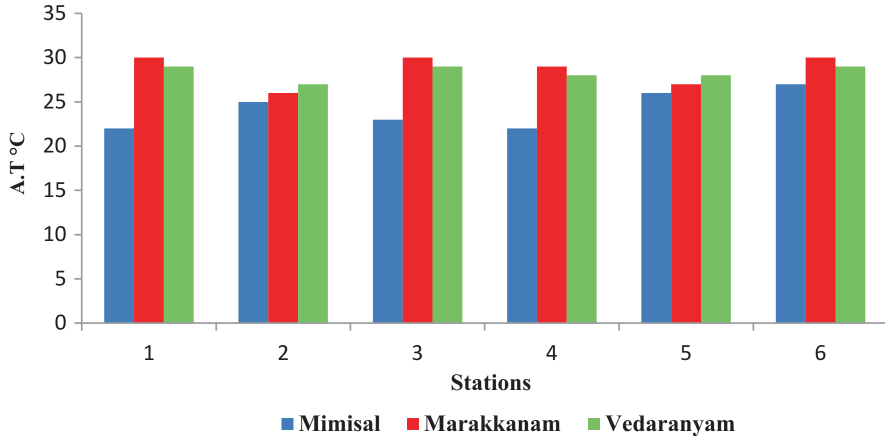

Fig. 2 Variations on atmospheric temperature in different salt pans

characters such as atmospheric temperature, water temperature, salinity, pH, dissolved oxygen, inorganic nutrients, phytoplankton and pigments were studied in the salt pans of Mimisal, Marakkanam and Vedaranyam for a one-time sampling.

Measurement of Atmospheric and Water Temperature

The recorded average atmospheric temperature (A.T) was ranged from 22 to 30 °C. Maximum A.T (30 °C) was recorded in Marakkanam and Vedaranyam at stations 1, 3 and 6, and minimum value (22 °C) was recorded in Mimisal at stations 1 and 4. The average water temperature (W.T) was ranged from 25 to 36 °C. Maximum W.T (36 °C) was recorded in Marakkanam at station 6, and minimum value (25 °C) was noticed in Mimisal and Vedaranyam at stations 1 and 4 (Figs. 2 and 3).

Measurement of Salinity and pH

Salinity was ranged from 70 to 255 ppt. Maximum salinity (255 ppt) was recorded in Vedaranyam at station 6, and minimum value (70 ppt) was found in Mimisal at station 5. The pH value was ranged from 7.16 to 8.43. Maximum pH (8.43) was recorded in Marakkanam at station 6, and minimum pH (7.16) was recorded in Mimisal at station 6 (Figs. 4 and 5).

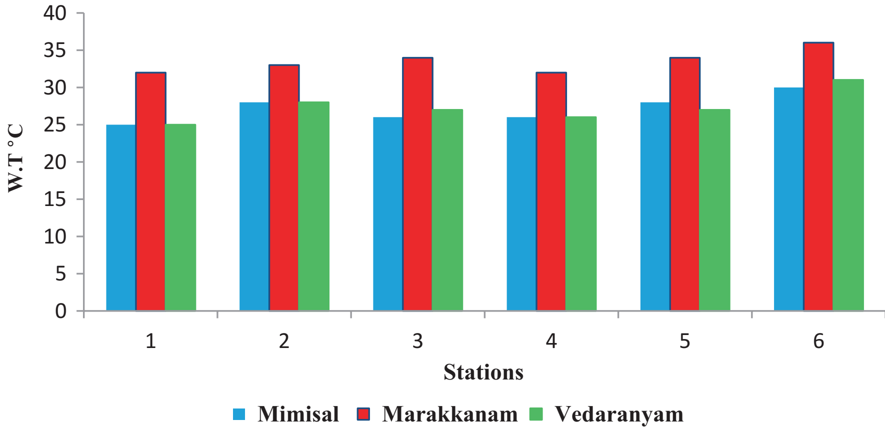

Fig. 3 Variations on water temperature in different salt pans

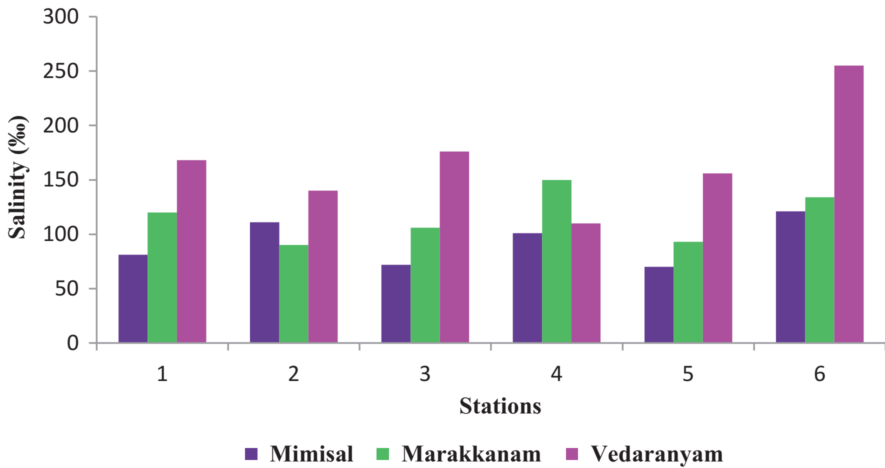

Fig. 4 Variations on salinity in different salt pans

Estimation of Dissolved Oxygen (DO)

The recorded DO was ranged between 0.62 and 7.25 mg/L. Maximum value (7.25 mg/L) was obtained in Vedaranyam at station 3 and minimum value (0.62 mg/L) noticed in Mimisal at station 5 (Fig. 6).

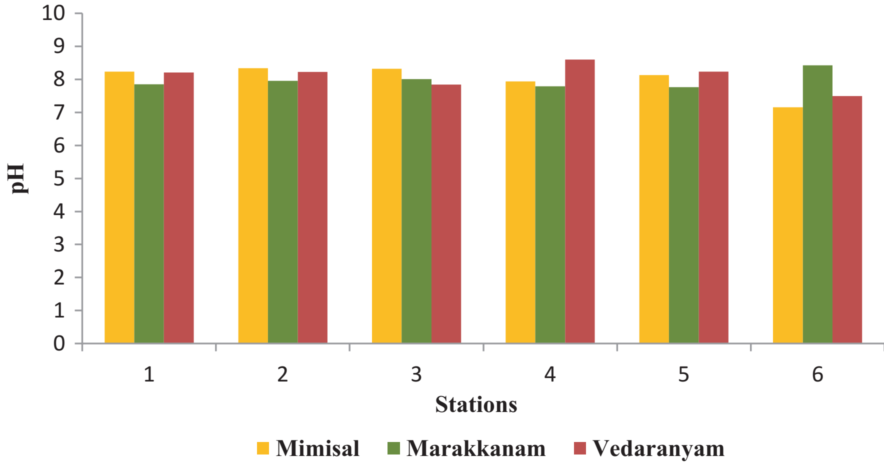

Fig. 5 Variations on pH in different salt pans

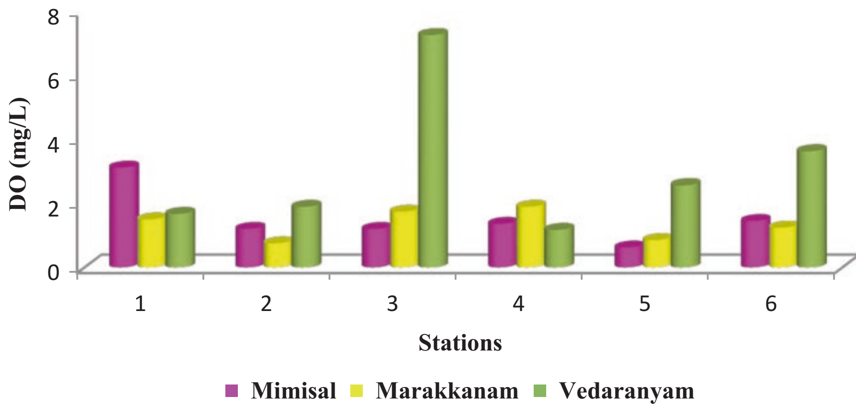

Fig. 6 Variations on dissolved oxygen in different salt pans

Pigments Concentration

The recorded Chl 'a' was ranged from 0.004 to 1.346 μg/ml. Maximum value (1.346 μg/ml) was obtained in Marakkanam at station 5, and minimum value (0.004 μg/ml) was observed in Mimisal at stations 4 and 5 (Fig. 7).

The recorded Chl 'b' was ranged from 0.027 to 1.125 μg/ml. Maximum value (1.125 μg/ml) was obtained in Marakkanam at station 5, and minimum value (0.027 μg/ml) was noticed in Marakkanam at stations 1 and 4 (Fig. 8).

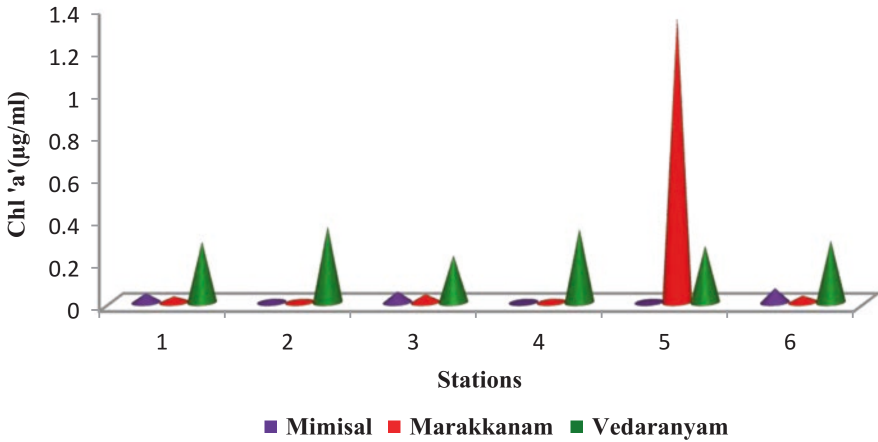

Fig. 7 Variations on chlorophyll ‘a’ in different salt pans

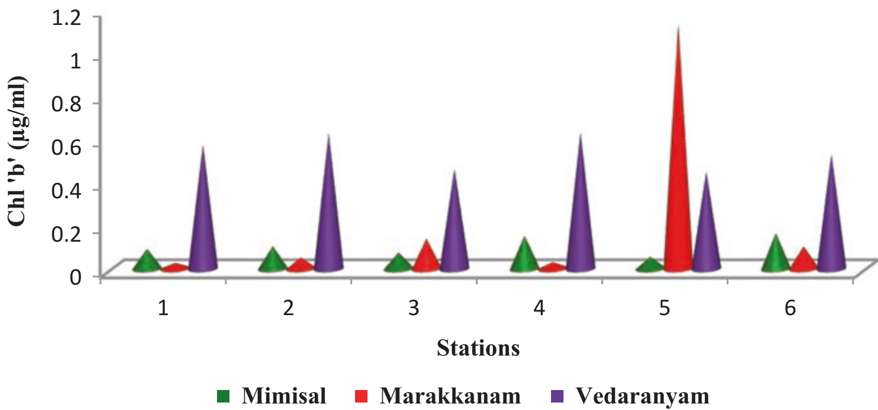

Fig. 8 Variations on chlorophyll ‘b’ in different salt pans

Total carotene values was ranged from 0.029 to 0.472 µg/ml. Maximum value (0.472 µg/ml) was obtained in Marakkanam at station 5 and minimum value (0.029 µg/ml) noticed in Vedaranyam at station 6 (Fig. 9).

Nutrients Analysis

Inorganic Nitrate

The recorded nitrate value was ranged from 0.1 to 2.11 µM/L. Maximum value (2.11 µM/L) for nitrate was observed in Marakkanam at station 5 and minimum value (0.1 µM/L) in Vedaranyam at station 4 (Fig. 10).

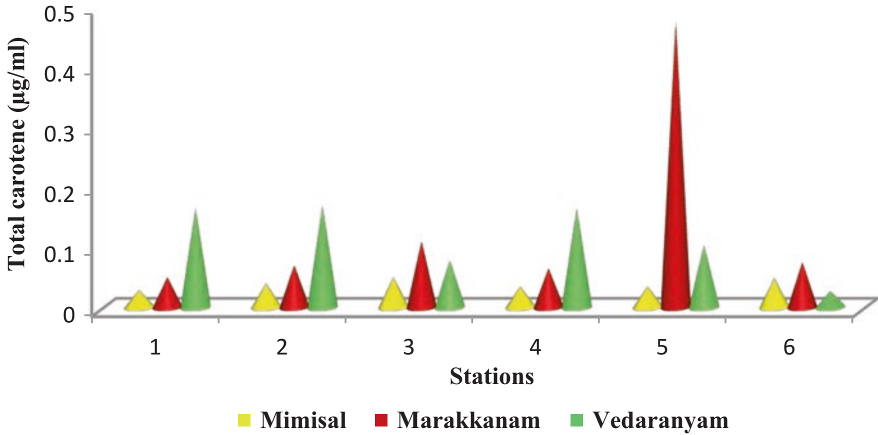

Fig. 9 Variations on total carotene in different salt pans

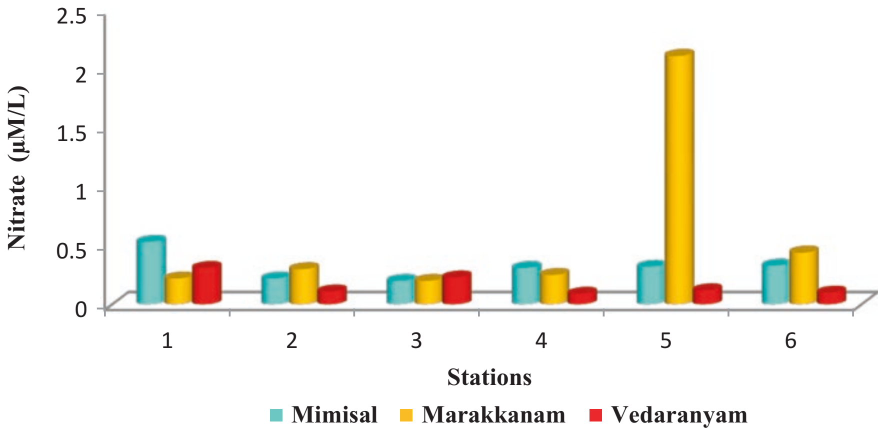

Fig. 10 Variations on nitrate in different salt pans

Inorganic Nitrite

The recorded nitrite value was ranged from 0.05 to 1 µM/L. Maximum value (1 µM/L) was recorded in Marakkanam at station 3 and minimum value (0.05 µM/L) observed in Vedaranyam at station 1 and 4 and in Mimisal at station 2 (Fig. 11).

Inorganic Phosphate

Phosphate value was ranged from 0.26 to 4.2 µM/L. Maximum value (4.2 µM/L) was obtained in Marakkanam at station 3 and minimum value (0.26 µM/L) in Vedaranyam at station 6 (Fig. 12).

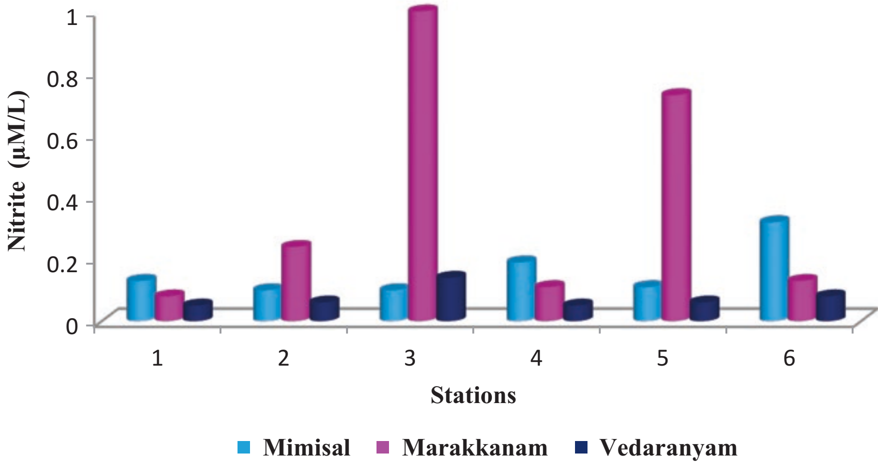

Fig. 11 Variations on nitrite in different salt pans

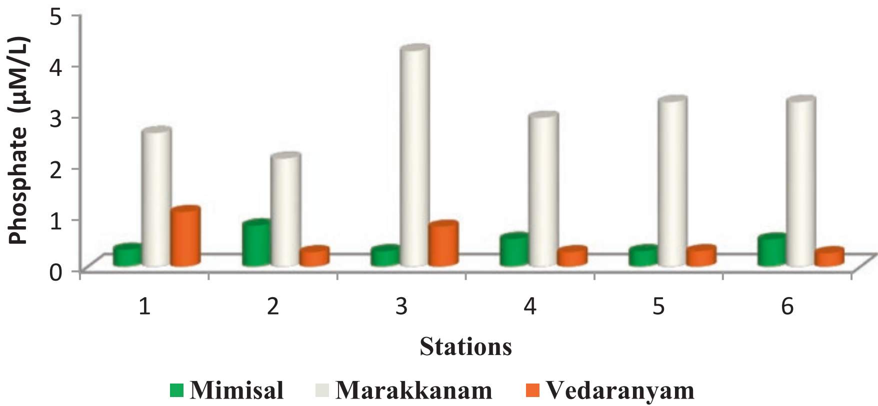

Fig. 12 Variations on phosphate in different salt pans

Reactive Silicate

The reactive silicate values were ranged from 0.5 to 10.2 µM/L. Maximum value (10.2 µM/L) was observed in Marakkanam at station 5 and minimum value (0.5 µM/L) in Marakkanam at station 3 (Fig. 13).

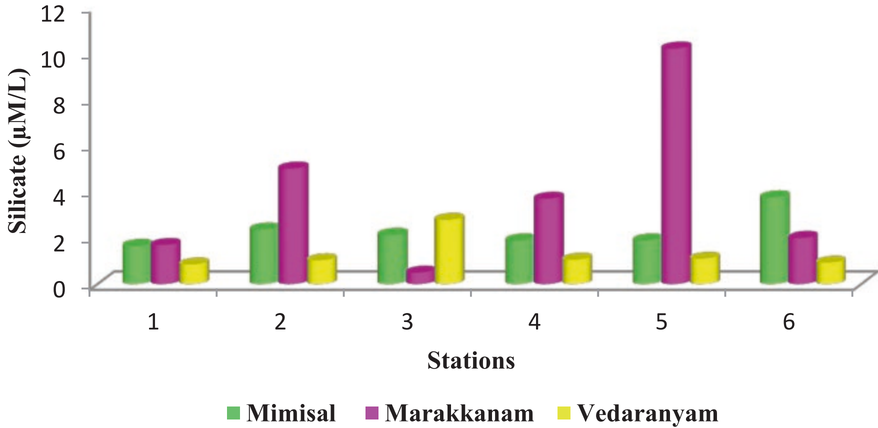

Fig. 13 Variations on silicate in different salt pans

Biological Parameters

Species Composition

In the present study, totally 11 species of phytoplankton were recorded in 3 salt pans comprising of 7 Bacillariophyceae, 3 Cyanophyceae and 1 Chlorophyceae. In Mimisal station, totally six species of phytoplankton were identified. Among these, four Bacillariophyceae, one Cyanophyceae and one Chlorophyceae were recorded. In Marakkanam station, totally ten species of phytoplankton were identified. Among these, six Bacillariophyceae, three Cyanophyceae and one Chlorophyceae were recorded. In Vedaranyam station, totally eight species of phytoplankton comprising four Bacillariophyceae, three Cyanophyceae and one Chlorophyceae were recorded. Species composition was dominated by diatoms in all salt pans.

Percentage Composition

Percentage composition of phytoplankton in different salt pans showed predominance by diatoms (50%) followed by green algae (25%) and Cyanobacteria (25%) in Mimisal (Fig. 14a). In Marakkanam salt pan, diatoms contributed 60% followed by Cyanobacteria with 30% and green algae with 10% (Fig. 14b). The percentage compositions of phytoplankton at Vedaranyam salt pan were dominated by diatoms with 50% followed by green algae (25%), and Cyanobacteria (25%) (Fig. 14c).

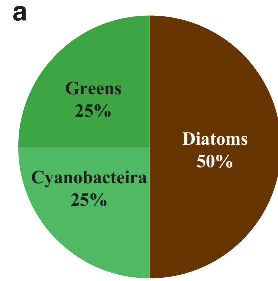

Fig. 14a Percentage composition of phytoplankton in Mimisal salt pan

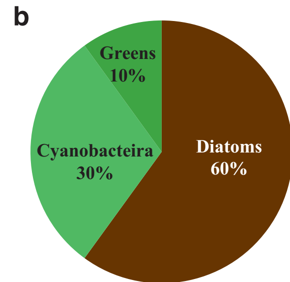

Fig. 14b Percentage composition of phytoplankton in Marakkanam salt pan

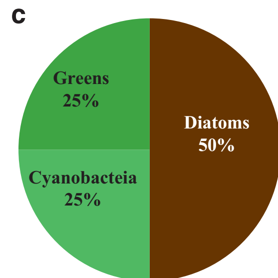

Fig. 14c Percentage composition of phytoplankton in Vedaranyam salt pan

Population Density and Species Diversity

The phytoplankton population density was ranged from 810 to 3591 cells/L. Maximum density (3591 cells/L) was recorded in Mimisal at station 6, and minimum of 810 cells/L was recorded in Marakkanam at station 3 (Fig. 15). The recorded species diversity was ranged from 0.38 to 2.57. Maximum (2.57) was recorded in Marakkanam at station 5, and minimum of 0.38 was recorded in Mimisal at station 6 (Fig. 16).

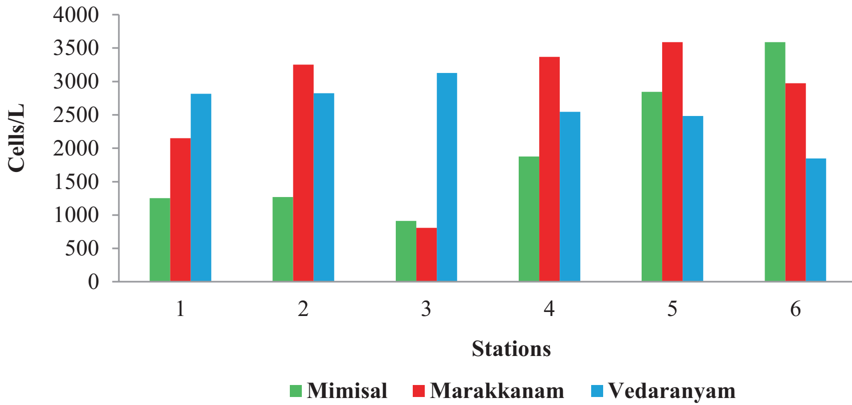

Fig. 15 Variations on phytoplankton population density in different salt pans

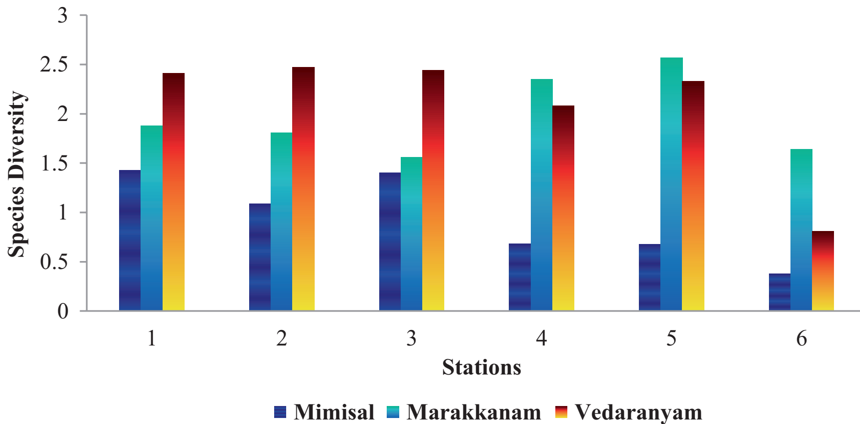

Fig. 16 Variations on species diversity in different salt pans

Species Richness

Species richness was ranged from 0.13 to 0.80. Maximum (0.80) was recorded in Vedaranyam at station 2, and minimum (0.13) was recorded in Mimisal at station 6 (Fig. 17).

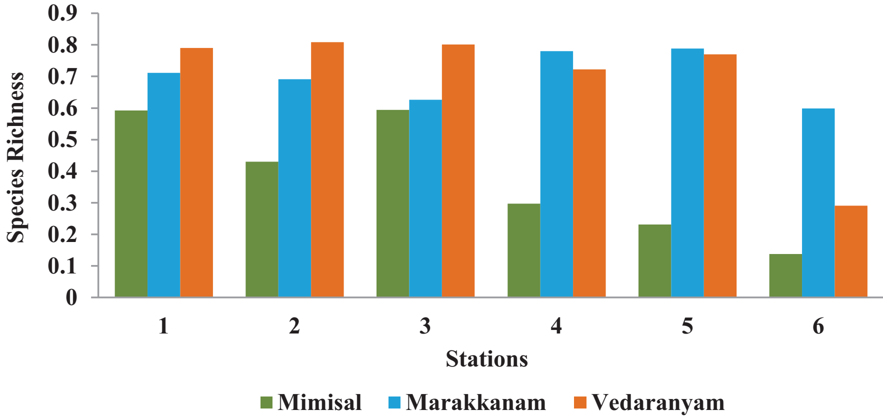

Fig. 17 Variations on species richness in different salt pans

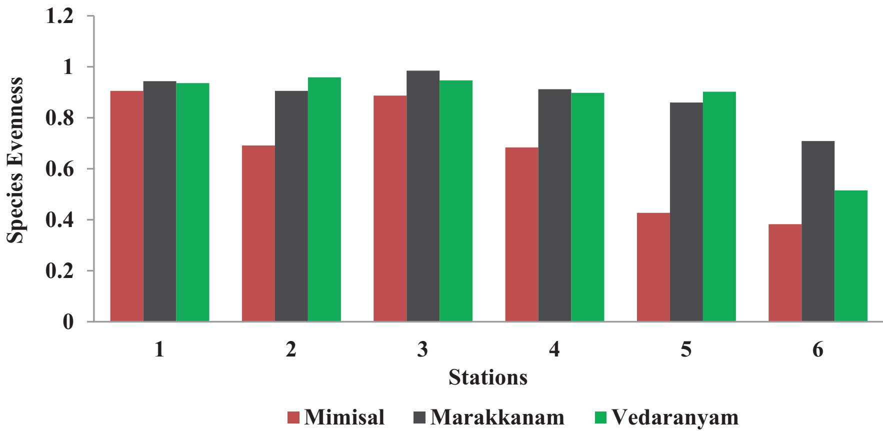

Fig. 18 Variations on species evenness in different salt pans

Species Evenness

Species evenness was ranged from 0.38 to 0.98. Maximum of 0.98 was recorded in Marakkanam at station 3, and minimum of 0.38 was recorded in Mimisal at station 6 (Fig. 18, Tables 1, 2, 3 and 4).

Discussion

The phytoplankton showed significant correlation to the temperature, pH, dissolved oxygen, nitrate, nitrite silicate and phosphate at respective stations. The temperature was shown as positive correlation, and it indicates the growth and population is

Table 1 List of phytoplankton recorded in different salt pans

S. No	Name of phytoplankton	Mimisal salt pan	Marakkanam salt pan	Vedaranyam salt pan
Bacillariophyceae				
1	*Coscinodiscus centralis*	+	+	+
2	*Fragilaria intermedia*	–	+	–
3	*Nitzschia microcephala*	+	+	+
4	*Amphora turgida*	–	+	+
5	*Pleurosigma elongatum*	–	+	–
6	*Navicula ramosissima*	–	+	+
7	*Pseudo-nitzschia seriata*	+	–	–
Cyanophyceae				
8	*Oscillatoria salina*	–	+	+
9	*Spirulina subsalsa*	–	+	+
10	*Microcystis littoralis*	+	+	+
Chlorophyceae				
11	*Dunaliella salina*	+	+	+

+ Denotes presence
– Denotes absence

favourable with increasing temperature (Adesalu and Kunrunmi 2012). The solubility of oxygen has been increased by low temperature. Atmospheric temperature in all salt pans was found maximum during the pre-monsoon period (January to March). This was in accordance with earlier work (Arif Shaikh et al. 1997). During the study period, atmospheric temperature was recorded in the range 22–30 °C and water temperature ranged from 25 to 36 °C. Chakraborti et al. (1985) have also reported that temperature varied from 25.6 °C to 29.8 °C in the brackishwater. The temperature in the Veppalodai salt pan (Tuticorin) varied from 24.8 °C to 30.7 °C (Bensam and Marichamy 1975).

Salinity is an ecological factor of considerable importance, influencing the type of organisms that live in a body of water. Any life form adapted to saline condition is classified as halophiles. The salinity of the water samples from the three salt pans showed maximum values (255 ppt) at Marakkanam. Salinity is one of the key factors that determine the distribution of phytoplankton. In the present study, salinity varied from 70 to 255 ppt. The similar salinity values were also noticed earlier in the Veppalodai salterns, Tuticorin (Marisamy and John Motha 1986). Reginald and Banu (2009) also reported the maximum salinity of 209.25 ppt in Thamaraikulam solar salt pan, Kanyakumari, Tamil Nadu, India.

The pH was shown as positive correlation and which indicates high pH and high phytoplankton production. The changes in pH levels in marine systems appear to correlate with changes in temperature, dissolved oxygen and phytoplankton production (Celia et al. 1994). pH is a measure of the intensity of acidity or alkalinity and is also a measure of the concentration of hydrogen ions in the water. In the present study, pH value was ranged from 7.16 to 8.43. The pH of brine at different stages of the salt pan was alkaline (Cumming and Kay 1968). In the present study, maximum pH was recorded in Marakkanam at station 6. The decrease in pH may be attributed

Table 2 Correlation coefficient between physico-chemical and biological parameters in Vedaranyam salt pan

pH	Salinity	DO	W.Tem	A.Tem	Phosphate	Silicate	Nitrite	Nitrate	Chl ‘a‘	Chl ‘b‘	T.C	P.Den	S.Div	S.Rich	S.Even	
1	−0.952**	−0.644	−0.761	−0.570	−0.052	−0.282	−0.597	−0.075	0.522	0.487	0.882*	0.390	0.645	0.688	0.711	pH
	1	0.422	0.751	0.622	0.032	0.020	0.357	0.059	−0.431	−0.415	−0.856*	−0.591	−0.774	−0.812*	−0.837*	Salinity
		1	0.252	0.504	0.270	0.912*	0.992**	0.262	−0.801	−0.686	−0.648	0.283	−0.034	−0.059	−0.077	Do
			1	0.040	−0.584	−0.078	0.234	−0.563	0.026	−0.148	−0.752	−0.703	−0.809	−0.829*	−0.835*	W.Tem
				1	0.596	0.316	0.450	0.575	−0.719	−0.468	−0.564	−0.141	−0.377	−0.390	−0.403	A.Tem
					1	0.320	0.250	0.995**	−0.508	−0.156	0.184	0.579	0.414	0.389	0.380	Phosphate
						1	0.937**	0.294	−0.681	−0.531	−0.318	0.583	0.296	0.287	0.283	Silicate
							1	0.234	−0.730	−0.596	−0.589	0.330	−0.006	−0.028	−0.037	Nitrite
								1	−0.523	−0.193	0.171	0.569	0.422	0.392	0.378	Nitrate
									1	0.905*	0.598	−0.207	−0.043	−0.013	0.031	Chl ‘a’
										1	0.673	−0.009	0.022	0.052	0.109	Chl ‘b’
											1	0.535	0.709	0.731	0.759	T.C
												1	0.907*	0.899*	0.904*	P.Den
													1	0.998**	0.992**	S.Div
														1	0.998**	S.Rich
															1	S.Even

*Correlation is significant at the 0.05 level (2-tailed)
**Correlation is significant at the 0.01 level (2-tailed)

Table 3 Correlation coefficient between physico-chemical and biological parameters in Marakkanam salt pan

pH	Salinity	DO	W.Tem	A.Tem	Phosphate	Silicate	Nitrite	Nitrate	Chl 'a'	Chl 'b'	T.Caro	P.Den	S.Div	S.Rich	S.Even	
1	0.212	−0.078	0.821*	0.376	0.203	−0.463	0.758	−0.304	−0.39	−0.337	−0.362	0.028	−0.696	−0.873*	−0.714	pH
	1	0.728	−0.086	0.652	0.066	−0.459	0.695	−0.442	−0.466	−0.486	−0.495	0.305	0.023	−0.014	−0.26	Salinity
		1	−0.301	0.781	0.472	−0.66	0.157	−0.549	−0.498	−0.494	−0.479	−0.333	−0.145	−0.063	0.367	DO
			1	0.151	0.448	−0.009	0.574	0.246	0.17	0.238	0.218	−0.114	−0.354	−0.645	−0.743	W.Tem
				1	0.544	−0.769	0.46	−0.469	−0.455	−0.433	−0.445	−0.454	−0.439	−0.464	−0.031	A.Tem
					1	−0.266	0.044	0.094	0.129	0.199	0.214	−0.763	−0.207	−0.348	0.103	Phosphate
						1	−0.28	0.896*	0.884*	0.858*	0.859*	0.371	0.815*	0.7	−0.165	Silicate
							1	−0.18	−0.273	−0.261	−0.292	0.414	−0.199	−0.409	−0.847*	Nitrite
								1	0.993**	0.990**	0.984**	0.05	0.707	0.511	−0.243	Nitrate
									1	0.996**	0.994**	−0.026	0.722	0.551	−0.133	Chl 'a'
										1	0.999**	−0.077	0.672	0.487	−0.146	Chl 'b'
											1	−0.093	0.677	0.499	−0.11	T.Caro
												1	0.399	0.362	−0.511	P.Den
													1	0.941**	0.019	S.Div
														1	0.294	S.Rich
															1	S.Even

*Correlation is significant at the 0.05 level (2-tailed)
**Correlation is significant at the 0.01 level (2-tailed)

Table 4 Correlation coefficient between physico-chemical and biological parameters in Mimisal salt pan

pH	Salinity	DO	W.Tem	A.Tem	Phosphate	Silicate	Nitrite	Nitrate	Chl 'a'	Chl 'b'	T.Caro	P.Den	S.Div	S.Ric	S.Even	
1	−0.637	0.067	−0.686	−0.549	−0.118	−0.847*	−0.983**	−0.113	−0.459	−0.752	−0.39	−0.847*	0.796	0.776	0.694	pH
	1	−0.008	0.593	0.374	0.822*	0.728	0.696	−0.128	0.178	0.860*	0.277	0.38	−0.529	−0.499	−0.396	Salinity
		1	−0.529	−0.536	−0.164	−0.206	0.053	0.818*	0.381	0.126	−0.454	−0.33	0.499	0.476	0.572	DO
			1	0.961**	0.4	0.846*	0.584	−0.333	0.205	0.337	0.511	0.792	−0.754	−0.781	−0.877*	W.Tem
				1	0.229	0.727	0.415	−0.278	0.187	0.078	0.439	0.766	−0.65	−0.704	−0.862*	A.Tem
					1	0.354	0.174	−0.364	−0.219	0.518	0.106	−0.009	−0.243	−0.224	−0.174	Phosphate
						1	0.816*	−0.228	0.608	0.617	0.704	0.662	−0.603	−0.589	−0.612	Silicate
							1	0.162	0.476	0.844*	0.351	0.75	−0.729	−0.696	−0.576	Nitrite
								1	0.147	0.044	−0.687	0.104	0.118	0.056	0.141	Nitrate
									1	0.225	0.592	0.133	0.129	0.137	0.086	Chl 'a'
										1	0.218	0.395	−0.563	−0.494	−0.287	Chl 'b'
											1	0.18	−0.176	−0.117	−0.203	T.Caro
												1	−0.910*	−0.939**	−0.941**	P.Den
													1	0.991**	0.926**	S.Div
														1	0.958**	S.Ric
															1	S.Even

*Correlation is significant at the 0.05 level (2-tailed)
**Correlation is significant at the 0.01 level (2-tailed)

to the reduction of photosynthesis, and maximum can be the result of the increased photosynthesis.

The dissolved oxygen was showed positive correlation which indicates the amount of productivity was high. The dissolved oxygen concentration depends on the photosynthetic rate and subsequently on nutrient concentrations. The dissolved oxygen concentration increases with increasing of photosynthetic rate (Wheeler et al. 2002). In the present study, DO was found ranged between 0.62 and 7.25 mg/L. The dissolved oxygen maximum value (7.25 mg/L) was obtained in Vedaranyam at station 3 and 6. The dissolved oxygen also showed negative correlation due to high temperature at station 3. Generally high temperature and salinity cause the oxygen to be relatively low (Badran 2001).

The chlorophyll ‘a’ concentration was found to be high in Marakkanam at station 5, owing to high phytoplankton population recorded in the station. The chlorophyll ‘b’ concentration was high in Marakkanam at station 5. The total carotene concentration was found high in Marakkanam at station 5. The low Chl ‘a’, Chl ‘b’ and total carotene that were obtained in Mimisal may be due to low phytoplankton production. These findings are in agreement with those reported earlier in most hypersaline environments (Por 1980; Javor 1983; Javor and Castenholz 1984).

Nutrients values were very low in all the stations. High nutrient concentration was recorded during low saline periods. The nitrate is an important source of nitrogen for phytoplankton. It is used by plants for their growth and utilized in the synthesis of organic nitrogenous compounds. Nitrate is the most oxidized forms of nitrogen and the end product of the aerobic decomposition of organic nitrogenous matter. In the present study, nitrate recorded ranged from 0.1 to 2.11 μM/L. The maximum value (2.11 μM/L) was observed in Marakkanam at station 5. The low value recorded may be due to its utilization by phytoplankton as evidenced by high photosynthetic activity and also due to the high salinity, which dissolute the negligible amount of nitrate (Rajashree and Panigrahy 1996; Das et al. 1997; Govindasamy et al. 2000). The higher concentration of nitrite may also be attributes to the variation in phytoplankton excretion and oxidation of ammonia (Kannan and Kannan 1996). Low values of nitrite observed because of higher salinity. The recorded nitrite values were ranged from 0.05 to 1 μM/L. The nitrite maximum values were recorded in Marakkanam at station 3.

Phytoplankton had a negative correlation with phosphate due to the high rate of phytoplankton phosphorus uptake at low concentrations in Marakkanam at station 3. The other nutrients are not shown significant correlation due to lower concentrations or rapid recycling (Steinhart et al. 2002). High concentration of phosphate observed may possibly be due to salt pan water which increased the level of phosphate. Similar observations were reported by Ramalingam et al. (2011) from Vedaranyam Swamp of the Point Calimere, Southeast coast of India.

Gopalakrishnan et al. (1994) recorded the phytoplankton population of 3859–12,804 cells/m^3 in Uppodai salt pan area, Tuticorin. In the present study, phytoplankton population density of 810–3591 cells/L was noticed. Among the phytoplankton, diatoms were recorded to be more dominant in all the stations followed by Chlorophyceae and Cyanophyceae members. Presently recorded Shannon

weaner's diversity index was 0.38–2.57. The low species diversity noticed might be due to the presence of minimum number of species recorded. The abiotic factors were responses to the phytoplankton diversity (Swati et al. 2012), and they were change both from the spatially and temporally according to the seasons (Puspalata Moharana et al. 2012). The increase in phytoplankton population during higher salinities was mainly due to the abundance of hypersaline algal species like *Dunaliella salina* (Ortega and Martinez 1987; Rahaman et al. 1990).

The range of phytoplankton species diversity was recorded to be more in Marakkanam compare to other stations, which could be due to stable environmental conditions prevailed in the study area and occurrence of salterns species in the salt pan. As the salinity increases, the species diversity decreases mostly due to environmental stress, while the various population densities increase. Similarly, decreasing trend in species richness of micro-autotrophic plankton community along the salinity gradient in salterns was reported by Estrada et al. (2004). In the present investigation, species richness was ranged from 0.13 to 0.80. The minimum phytoplankton species richness was noticed in Mimisal at station 6. However, maximum species richness was recorded in Vedaranyam at station 2. The microplankton assemblage was dominated by diatoms which accounted for about 50% of the total abundance. However, the diversity and species richness remained poor in the salt pans. Poor diversity and species richness of phytoplankton have also been reported by earlier worker from the salt pan (Rahman 2006). Phytoplankton species evenness was varied from 0.38 to 0.98. The least species evenness was recorded in Marakkanam station. Similarly, the maximum species evenness was recorded in Marakkanam at station 3, and minimum of 0.38 was recorded in Mimisal at station 6.

Microalgae species belonging to Cyanophyceae, Chlorophyceae and Bacillariophyceae were recorded in different salt pan during the study period. In the present investigation, 11 species of phytoplankton were recorded in 3 salt pans comprising of 7 Bacillariophyceae, 3 Cyanophyceae and 1 Chlorophyceae. In Mimisal station, totally six species of phytoplankton were identified. Among these, four Bacillariophyceae, one Cyanophyceae and one Chlorophyceae were recorded. In Marakkanam station, totally ten species of phytoplankton were identified. Among these, six Bacillariophyceae, three Cyanophyceae and one Chlorophyceae were recorded. In Vedaranyam station, totally eight species of phytoplankton were identified. Among these, four Bacillariophyceae, three Cyanophyceae and one Chlorophyceae were recorded. It was found that diatoms was the dominant group in all the salt pans surveyed.

Bacillariophyceae or diatoms that were represented by seven species belonging to one genus were recorded in all salt pans. Temperature and pH play a key role in the distribution and abundance of diatoms. The maximum abundance of diatoms was encountered in all salt pans studied currently. Present findings are agreeable with earlier reports (Sommer et al. 1986). Goel et al. (1992) have stated that Bacillariophyceae occurs in all types of waters. Among the Bacillariophytes *Coscinodiscus centralis*, *Fragilaria intermedia*, *Nitzschia microcephala*, *Amphora turgida*, *Pleurosigma elongatum*, *Navicula ramosissima* and *Pseudo-nitzschia seri-*

ata dominantly occurred in the present study. Cyanophyceae are well known to occur in diverse physico-chemical conditions with varying degree of abundance and can tolerate wide fluctuations in chemical factors. Anand and Venkatesan (1985) reported 17 species of Cyanophyceae from the salt pans near Madras, India. Thajuddin et al. (2002) recorded 36 species of Cyanophyceae out of which none was heterocystous. In the cyanophytes, *Oscillatoria* sp., *Spirulina* sp. and *Microcystis* sp. were found dominant in the present study. Chlorophyceae are free living and planktonic, mostly confined to high saline water and shallow water and are attached to the submerged plants or found in moist soil (Huisman et al. 2005). This group was represented by one species (*Dunaliella salina*) with the maximum density as agreed by Sommer et al. (1986).

Summary and Conclusion

The present investigation consists of the water and phytoplankton samplings made at three salt pans, namely, Marakkanam, Vedaranyam and Mimisal, Southeast coast of India. Thus the present study gives an idea about the phytoplankton biodiversity in salt pans located at Vedaranyam of Nagapattinam district, Marakkanam of Villupuram district and Mimisal of Pudukkottai district, Tamil Nadu, India. Although phytoplankton are ubiquitous, they vary in their morphological characteristics depending on the physico-chemical environment of the habitats.

The physical and chemical parameters and their fluctuations in the various stages of different salt pans were studied. In the absence of prior knowledge of environmental conditions, this short-time monitoring established a baseline for future comparisons. The following are the major conclusions derived by the investigation. The physico-chemical conditions such as atmospheric and water temperature at Marakkanam salt pan showed peak at station 6. Salinity showed a wide variation; the high brine water was observed in Vedaranyam at station 6. In the case of nutrients, major peak was observed mostly in Marakkanam salt pan at all the stations than other salt pan stations.

The diatoms formed the predominant group among the phytoplankton studied presently which might be due to their wide tolerance to changing environmental conditions and high reproduction capacity. In the present investigation, phytoplankton population that was found high at Mimisal salt pan could be attributed to favourable conditions prevailing in the salt pan. The recorded plankton density which was 810–3591 cells/L reached as maximum. Among the phytoplankton, diatoms were recorded predominant in all the salt pans followed by Chlorophyceae and Cyanophyceae members. In the present study, totally 11 species of phytoplankton were recorded in 3 salt pans comprising of 7 Bacillariophyceae, 3 Cyanophyceae and 1 Chlorophyceae. The Bacillariophytes such as *Coscinodiscus centralis*, *Fragilaria intermedia*, *Nitzschia microcephala*, *Amphora turgida*, *Pleurosigma elongatum*, *Navicula ramosissima* and *Pseudo-nitzschia seriata* were reported, while in cyanophytes *Oscillatoria salina*, *Spirulina subsalsa* and *Microcystis litto-*

ralis were noticed, and Chlorophyceae *Dunaliella salina* was observed in the study period. Species diversity and species richness were found to be high at Marakkanam salt pan at station 5 and at Vedaranyam salt pan at station 2; this could be due to the stable hydrographical conditions that prevailed and consequent high density of phytoplankton. High evenness values were noticed in Marakkanam salt pan at station 3.

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A Study of Carbon Sequestration by Phytoplankton

M. Divya, S. Dinesh Kumar, N. Krishnaveni, and P. Santhanam

Introduction

Climate change is one of the most serious threats for sustainable development of human society. The release of carbon dioxide (CO_2) from the atmosphere due to anthropogenic activities is one of the main causes of global warming and climate change. Reducing CO_2 emission and increasing carbon storage are the two major solutions to control greenhouse effects. China is now one of the world's largest greenhouse emitters, and actively dealing with climate change by reducing emission and increasing storage has become China's strategic consensus for economic and social development. Oceans cover approximately 71% of the Earth's surface, and the carbon content present in the ocean is 50 times that in the atmosphere and 20 times that in the soil (Holmén 2000). Therefore, oceans are the largest carbon pools on Earth, and it also serves as a "buffer" for climate change. Approximately 30% of the CO_2 produced by anthropogenic activities is absorbed by oceans (Le Quere et al. 2014) (otherwise global warming would have become more intense). In particular, the coastal oceans are mostly affected by anthropogenic activities, which account for only 8% of the global ocean area, but the amount of 20% CO_2 is taken by open oceans (Field et al. 1998).

Carbon sequestration can be defined as the capture and secure storage of carbon from the atmosphere. The idea is to keep carbon emissions produced by anthropogenic activities from atmosphere by capturing and diverting them to secure storage or to remove carbon from the atmosphere by various means and stores it. There are two methods to be analyzed for the sequestration of carbon in the ocean: (1) decarbonized fuel production system, power plant released a relatively pure CO_2 stream, that could be introduced directly into the ocean. (2) The injected CO_2 may become

M. Divya · S. Dinesh Kumar · N. Krishnaveni · P. Santhanam (✉)
Marine Planktonology & Aquaculture Laboratory, Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

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trapped in ocean sediments or icelike solids, called hydrates. The net oceanic uptake from the atmosphere could be enhanced through a method such as iron fertilization.

Phytoplankton has evolved over many millions of years, interacting with other organisms to produce a great diversity of life-forms. This group is the basis of marine food web, and it is a major driver in the cycling of elements. These primary producers involved oceans' ecological and biogeochemical services. Diatoms and coccolithophorids play a major role in the regulation of atmospheric carbon dioxide. "Biological pump" which means the carbon uptake by phytoplankton, and its export as organic matter to the ocean interior, reduces the partial pressure of CO_2 in the upper waters and also the diffusive drawdown of atmospheric CO_2. The aim of this paper is to understand the mechanisms that control phytoplankton distribution in the oceans and its impact in the regulation of atmospheric CO_2 levels.

Ocean Carbon Sequestration

Carbon sequestration in the ocean may leads to several biological processes through photosynthesis. Photosynthesis by phytoplankton is one such mechanism (Rivkin and Legendre 2001), which fixes approximately 45 Pg C $yr.^{-1}$(Falkowski et al. 2000). Some of the particulate organic material formed by phytoplankton is deposited at the ocean floor and is thus sequestered (Raven and Falkowski 1999). One of the limiting factors on phytoplankton growth is availability of Fe in oceanic ecosystems. Thus, several studies have determined the importance of Fe fertilization on biotic carbon sequestration in the ocean (Martin and Fitzwater 1988; Falkowski 1997; Martin et al. 2002; Boyd et al. 2004). It is also argued that incremental C could be sold as credits in the developing global C marketplace. Ecology of ocean may change to deep injection of C and ocean fertilization (Chisholm et al. 2001). However, with the current state of knowledge, the topic on ocean fertilization remains a debatable issue (Johnson et al. 2002).

Units used:

$$1\,\text{Pg} = \text{Petagram} = 1\,\text{billion metric ton} = 10^{15}\,\text{grams} = 1\,\text{Gt giga ton} = 10^{9}\,\text{Mt}\left(\text{M tons}\right)$$

Global Carbon Cycle

The carbon cycle is a complex series of processes through which all the carbon atoms on Earth recycle. The carbon cycle is of major role in basic and applied ecology (Chapin et al. 2002). Most biological and anthropogenic activities are powered by biochemical transformations of carbon; CO_2 uptake and energy fixation in organic compounds through photosynthesis, subsequent oxidation of organic

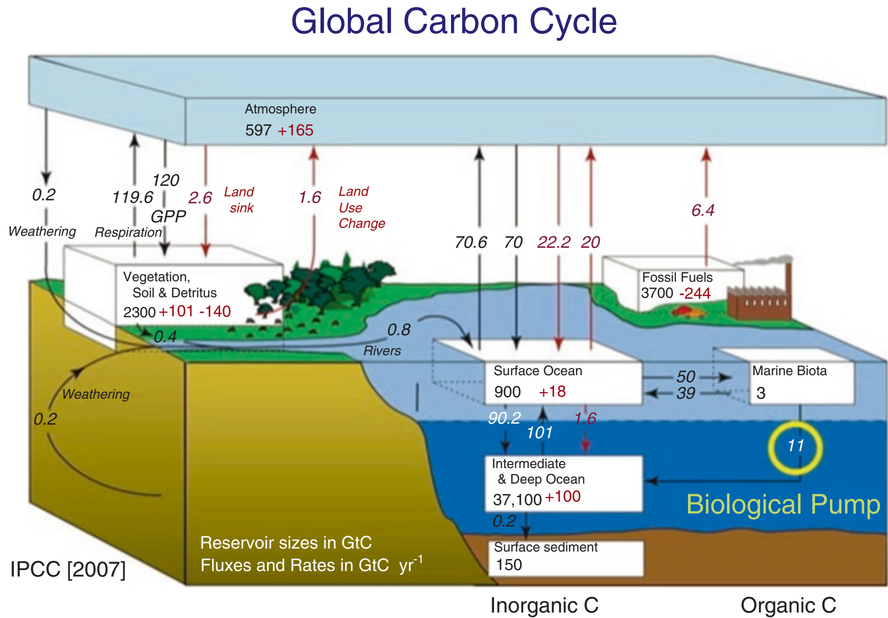

Fig. 1 Global carbon cycle. Black arrows and values indicate the natural carbon cycle, and red arrows indicate the anthropogenic perturbation. (Source: IPCC 2007)

compounds, and release of energy; and CO_2 through respiration and fossil fuel combustion. Carbon sequestration is intimately tied with two carbon cycles, the natural and anthropogenic cycles. Both cycles provide a context for developing carbon sequestration options. Although transfer of CO_2 natural environment is approximately 20 times greater than those due to anthropogenic activities, they are normally in a near balance position. The additional carbon resulting from anthropogenic activities due to the burning of fossil fuels, industrial processes, and land-use changes has led to emissions of various greenhouse gases and has altered the global carbon cycle (IPCC 2007). An average of 7.2 GtC/year is added to the atmosphere through fossil fuel combustion, while land and ocean vegetation (including microalgae) sequester about 2.8 GtC/year, thus leaving a major share of carbon in the atmosphere reported by Folger (2009) (Fig. 1).

Estimation of C Limitation from Phytoplankton

Consider a population of a certain species of phytoplankton that lives in a water column of 20 °C with a certain alkalinity, at a certain density, and under steady-state conditions (growth = mortality), and let C be the limiting factor and the air the main C source. When C is incorporated by the species, the concentration of CO_2 in the water will decrease and the pH will increase. This will reduce the C assimilation of

the phytoplankton and enhance the air–water flux of CO_2 until the C assimilation equals the air–water flux and the whole system is in steady state. This steady state is characterized by the pH.

This study should be performed in 300 mL bottles filled with 150 mL of a nutrient-rich medium (Lurling and Beekman 1999) without the TRIS buffer added, and the alkalinity of the medium will be maintained at 0.25 meq L^{-1}. A 5 cm water depth was maintained in the bottles. Two CO_2 treatments are determined by refreshing the headspace above the solution with 100 L air per day containing 350 or 700 ppm CO_2. Eighty rotations per minute with amplitude of 1.8 cm should be maintained for all the reagent bottles, but bottles with a high air–water exchange rate are also bubbled without changing the residence time of the air. Coefficient values of CO_2 exchange are 2.1 and 5.1 (m day^{-1}), respectively (Schippers et al. 2004). The experimental setup has to be maintained at a constant temperature of 21 °C. The illumination at the surface of the water is 135 μmol m^{-2} s^{-1}. An initial concentration of chlorophyll was 80μgL^{-1}. Electronic particle counter (Coulter Mulitisizer II, Coulter Electronics, Luton, London, UK; 150 lm capillary, orifice width 100 lm) in the range of 2.5–25 lm equivalent spherical diameter has been used to estimate the biovolume of the samples. The rate of growth is calculated from the last four measurements where species grow under C-limiting conditions.

The C assimilation or phytoplankton productivity (G) can be described as follows:

$$G = Fc\mu W \quad (1)$$

where

W = algal biomass (g C m^{-2})
μ = maximum growth rate (day^{-1})
Fc = C assimilation effect

Fc can be modeled using a Monod equation (Clark and Flynn 2000; Schippers et al. 2001). HCO_3^- and CO_2 are the major C sources absorbed by phytoplankton species (Maberly and Spence 1983; Burkhardt et al. 2001; Rost et al. 2003). C-limitation factor can be modeled as:

$$F_c = \left\{ \frac{[CO_2] + [HCO_3^-]}{Fr_{CO2}H_{CO2} + Fr_{HCO3^-} - H_{HCO3^-} + [CO_2] + [HCO_3^-]} \right\} \quad (2)$$

where

$[CO_2]$ = sum of free carbon dioxide and carbonic acid (M)
$[HCO_3]$ = bicarbonate concentration (M)
H_{CO2} = actual half-saturation concentration for CO_2, if CO_2 is the only C source
H_{HCO3^-} = actual half-saturation concentration for bicarbonate, if bicarbonate is the only C source
Fr_{CO2} and Fr_{HCO3^-} = fractions of the C species relative to the sum of [HCO3−] and [CO2]

At various pH levels, this equation is used to successfully predict C limitations as reported by Allen and Spence (1981). The C species are the ions determining alkalinity and pH in both fresh and marine water (Stumm and Morgan 1981). Alkalinity (eq L^{-1}) can be defined (Allen and Spence 1981; Maberly 1996) as following equation:

$$A = \left[HCO_3^-\right] + 2\left[CO_3^{2-}\right] + \left[OH^-\right] - \left[H^+\right] \quad (3)$$

Alkalinity of water body does not change because of the uptake of HCO_3^- and CO_2 by phytoplankton (Stumm and Morgan 1981) and can be regarded as a conservative characteristic of natural waters. HCO_3^- can be calculated from Eq. (3) for certain pH and alkalinity and the dissociation constants K_2 of HCO_3^- and K_w of water:

$$\left[HCO_3^-\right] = \frac{A\left[H^+\right] + \left[H^+\right]^2 - Kw}{2K_2 + \left[H^+\right]} \quad (4)$$

The concentration CO_2 can be derived from the pH and its dissociation constant K_1:

$$\left[CO_2\right] = \frac{\left[HCO_3^-\right]\left[H^+\right]}{K_1} \quad (5)$$

The flux E of CO_2 from water to air is known as the CO_2 concentrations. This can be calculated (Siegenthaler and Sarmiento 1993) as following equation:

$$E = \left(\left[CO_2\right]_{sat} - \left[CO_2\right]_{act}\right)K_c \quad (6)$$

where

$[CO_2]_{act}$ and $[CO_2]_{sat}$ = water CO_2 concentrations under actual and air-saturated conditions (M)

Kc = gas exchange coefficient of CO_2 between water and air (m day^{-1})

For estimation of the C limitation (Fc) of our algal population, substitute the results from Eqs. (4) and (5) in Eq. (2) and calculate the C limitation (Fc). If we know the pH and alkalinity of a water body, we can predict the half-saturation constants of an algal species and the C limitation (Fc). These results can be used to explore the effect of an increase in atmospheric CO_2 on the phytoplankton productivity, if the other conditions are stable. The relative productivity increase Ip can be calculated from Eq. (1):

$$IP = \frac{G_{ele}}{G_{ref}} = \frac{W\mu Fc,_{ele}}{W\mu Fc,_{ref}} = \frac{Fc,_{ele}}{Fc,_{ref}} \quad (7)$$

where

$Fc_{,ref}$ and $Fc_{,ele}$ = C-limitation factors at present and elevated air CO_2 concentrations

The productivity increase can be described as an increase in CO_2 flux. And the productivity increase can be calculated for the following formula:

$$Ip' = \frac{E_{ele}}{E_{ref}} = \frac{[CO_2]_{sat,ele} - [CO_2]_{act,ele}}{[CO_2]_{sat,ref} - [CO_2]_{act,ref}} \tag{8}$$

where

E_{ele} and E_{ref} = fluxes of CO_2 at elevated and reference air CO_2 concentrations.

As we assume steady state, both elevated and reference conditions G equals E and therefore Ip equals Ip′. We have already calculated how Fc and $CO_{2\ act}$ under the reference condition; however, we cannot follow the same method for elevated CO_2 because the new pH is unknown. To overcome this problem, calculate Ip and Ip′ at various pH_{ele} levels and search for the steady-state criterion Ip = Ip′. We can determine the productivity increase (Ip) at elevated atmospheric CO_2 concentrations for all species if we know the pH and alkalinity at reference atmospheric CO_2 concentrations. From this we can determine the half-saturation concentrations of CO_2 and HCO_3^-. This we can use to calculate the potential increase in productivity (Ip) because of an elevated atmospheric CO_2 concentration of 700 ppm given the pH at a reference atmospheric CO_2 concentration of 350 ppm (pH 350). This pH serves as an indicator of the effect of algal productivity on the steady-state concentrations of the C species. In an extreme condition, the concentration of pH will increase when carbon depletion happened by phytoplankton and the actual concentration of CO_2 concentration will approach zero. The algal productivity is determined by flux from the air to the water, which is simply in proportion with the atmospheric concentration (Eq. 8).

We can analyze the effects of an increase of atmospheric CO_2 from 350 ppm to 700 ppm on the C limitation, and productivity increase will be studied in marine water species at various pH and alkalinity (Stumm and Morgan 1981). By substituting known concentration of $HCO_3^-$and CO_2, we can calculate the half-saturation parameters and C-limitation effects (Fc) measured at two pH levels, producing two equations with two unknown half-saturation parameters. The following results were already derived from some of the algal species: for example, *Stichococcus bacillaris* (Chlorophyta) H_{CO2} = 7.5 μM, H_{HCO3^-} = 300 μM (Munoz and Merrett 1988); *Emiliania huxleyi* (Prymnesiophyceae) H_{CO2} = 115 μM, H_{HCO3^-} = 200 μM (Nimer and Merrett 1992); *Heterosigma carterae* (Raphidophyceae) H_{CO2} = 668 μM, H_{HCO3^-} = 668 μM (Clark and Flynn 2000); *Phaeodactylum tricornutum* (Bacillariophyceae) H_{CO2} = 20.1 μM, H_{HCO3^-} = 30 μM (Burns and Beardall 1987; Clark and Flynn 2000); and *Oscillatoria woronichinii* (Cyanobacteria) H_{CO2} = 69.3 μM, H_{HCO3^-} = 15.4 μM (Burns and Beardall 1987). The C species dissocation constant of marine and fresh water were derived at 20 °C (Stumm and Morgan 1981). The half-saturation values that are derived vary between 200 and 1000 μmol $m^{-2}\ s^{-1}$ illumination and temperature of 19 or 20 °C.

Conclusion

CO_2 capture by phytoplankton is a promising technology to solve the environmental problem and also reduce the concentration of GHGs in the atmosphere in a sustainable manner. The major task of research in this area of carbon sequestration is to identify the algal species and their specific growth conditions, viz., temperature, light intensity, nutrient concentration, photoperiod, aeration, and physiochemical characters, which increase the growth rate of microalgae. The integration of CO_2 fixation from flue gas, nutrient removal from effluents, and production of biomass from algae may provide a very promising alternative technology to current CO_2 capture strategies. This carbon capture and storage methodologies are economically feasible. To conclude, phytoplankton can safely be termed as the favorable system for carbon sequestration compared to forests and other vegetations. Due to their potential of faster growth, high photosynthetic efficiency, eco-friendly utilization of effluent nutrients and flue gas, and also providing a spectrum of value-added commercial products with no waste by-products, these could be used for sequestration of carbon.

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Mass Scale Culture and Preparation of Microalgal Paste

A. Begum, S. U. Ahmed, S. Dinesh Kumar, M. Divya, P. Santhanam, and P. Pachiappan

Introduction

For the past two decades, microalgae are cultured commercially for secondary wastewater treatment and production of human food, animal feed, fertilizer, biofuel, fine chemicals and secondary metabolites. But, success of mass culture of microalgae depends on bacteria and other contaminated cells. If it is efficient method to culture of microalgae in mass scale means, it should be contains higher biomass productivity, can grow in low light conditions and maximum utilization of carbon dioxide. In recent years, most of the world researchers focused on to develop sustainable outdoor mass scale culture techniques in low cost. Most outdoor culture techniques result in low algal density, high contamination, problem in harvesting and lipid separation from the algal cells. The low algal cell productivity of mass scale techniques has prompted the development of enriched outdoor mass culture methods like raceway, photobioreactors and attached algal culture system (Fig. 1). This chapter reveals the various techniques involving culture, havest and preparation of microalgae paste.

The photobioreactors (PBRs) and raceway pond design should be optimized based on the physiological and growth characteristics (Fig. 2) of the individual algal

A. Begum
Department of Botany, Tihu College, Tihu, Assam, India

S. U. Ahmed
Department of Biotechnology, Ministry of Science and Technology, Government of India, New Delhi, India

S. Dinesh Kumar · M. Divya · P. Santhanam (✉)
Marine Planktonology & Aquaculture Laboratory, Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

P. Pachiappan
Department of Biotechnology, School of Biosciences, Periyar University, Salem, Tamil Nadu, India

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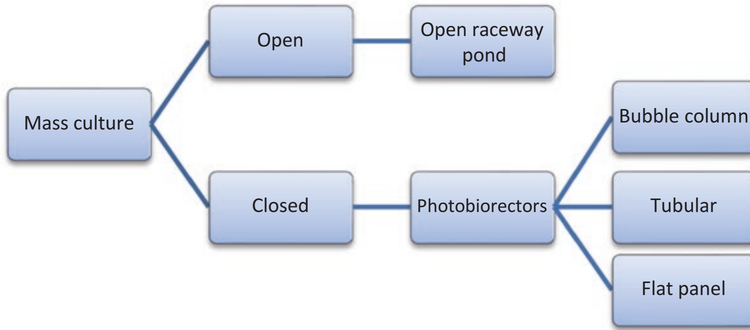


Fig. 1 Different methods of microalgae mass culture

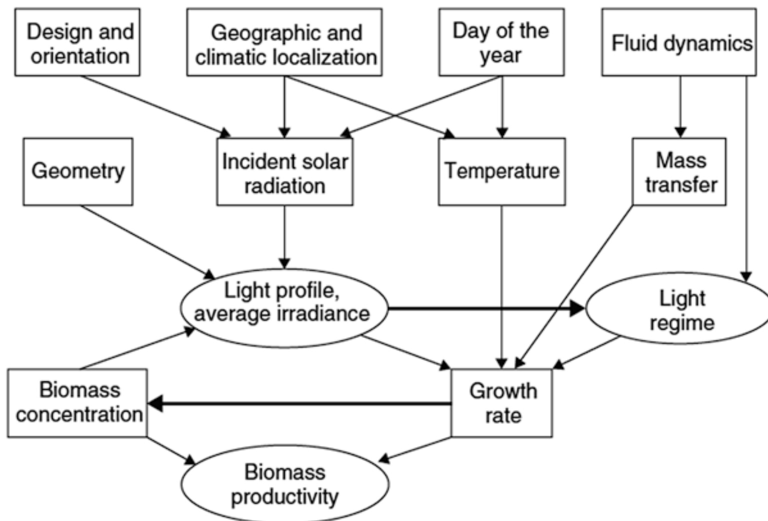


Fig. 2 Major factors influencing microalgae during mass scale culture (Grima et al. 2010)

species (Richmond and Cheng-Wu 2001). PBRs meet the proper balance of engineering and biological factors, resulting in the successful mass production of algal species with outstanding PBRs. The same time PBR and race way pond construction and operation should be in the low cost while microalgae culture going to the commercial purpose and also their physical (the fluid dynamics, temperature control, light delivery, mixing, the gas–liquid mass transfer) and biological (production of metabolites, photosynthesis and growth) rate processes should be in concern. Hence, the present chapter deals with major types of mass scale productions of microalgae with the help of raceway pond and closed bioreactors. The basic requirements and methodologies for successful outdoor culture and different harvesting methods are discussed.

Mass Culture Methods

Open Raceway Pond

Open raceway pond is introduced in mid of twentieth century for mass production of microalgae by Oswald (1988). Dodd (1986) has reviewed the main principles for constructing open raceway pond and shallow paddle-stirred raceway for producing mass microalgae. The simplified diagrams of open raceway pond are shown in Fig. 3 with major parts of the nomenclature.

Culture Flow

The flow in a raceway conduit needs to be turbulent to keep the cells in suspension, enhance vertical mixing, prevent thermal stratification and facilitate removal of the oxygen generated by photosynthesis. Generally, the culture flow should be maintained under 4000–8000 Reynolds number (R_e) values (Chisti 2012).

Flow and Mixing

As per Chisti (2012) and Becker (1994) methodologies, the requirements of power for maintaining flow and mixing in the pond fully depend on the flow velocity. Although a flow velocity of 0.05 m s^{-1} is sufficient to prevent thermal stratification, a higher velocity of around 0.1 m s^{-1} is needed to prevent sedimentation of algal biomass.

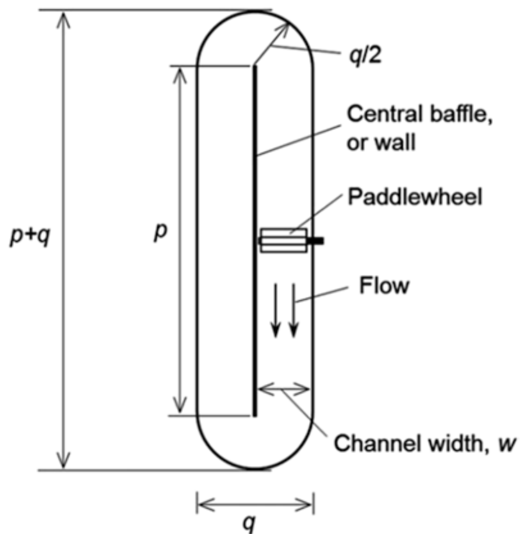


Fig. 3 An aerial view of raceway pond. (Source: Chisti 2016)

The Paddle Wheel

Paddle wheel is one of the most inexpensive methods to mix the algae while culturing in the mass scale level. Generally to avoid the interference between the multiple paddle wheels, single paddle wheel is typically used to mix the microalgae. Paddle wheel should be fixed in the mid-channel by 22.5° to encounter the power demand and load oscillation (Dodd 1986).

Climatic Conditions

The main factors influencing productivity are the prevailing temperature. Preferably, the temperature should be maintained in the pond around 25 °C with minimum seasonal changes (Chisti 2012). As per the previous researcher (Becker 1994), the prominence biomass productions in raceway pond while the average water evaporation rate of 10 L m^{-2} d^{-1} has been noted for some tropical regions (Becker 1994).

Temperature

The culture temperature strongly affects the algal biomass productivity and in some cases the biochemical composition of the biomass. According to Chisti (2012) survey, the optimal growth temperature for microalgae culture in the raceway pond is 24–40 °C.

pH

Algal biomass typically contains 50% carbon by weight. All carbon in photoautotrophically grown biomass comes from carbon dioxide or dissolved carbonate. If carbon dioxide is consumed rapidly and not replenished, the pH becomes alkaline. Growth under alkaline pH may not be possible for oceanic algae as marine salts precipitate at pH values of >8 (Chisti 2012).

Medium

The commercial fertilizers can be used (urea, 46 mg L^{-1}; superphosphate, 10 mg L^{-1}; ammonium sulphate, 100 mg L^{-1}) for mass culturing of diatoms and nanoplankters.

Cost of Pond Construction and Operation

Plastic-lined earthen raceways are apparently the least expensive to build. Unlined earthen ponds are used in wastewater treatment operations, but not generally considered satisfactory for producing algal biomass. Ponds enclosed in glass houses or plastic-covered greenhouses are relatively protected from contamination compared

to open ponds and allow a better control of the growth environment. Such ponds may be suitable for high-value low-volume products such as nutraceuticals and have been commercially used (Becker 1994; Lee 1997). Based on the recommendations by previous researchers, the raceway-based production of biomass is generally claimed to be the least expensive production option.

Operation

The raceway pond culture mostly starts as a batch operation. Once the algal biomass had a growth to a sufficient level, the operation is change to a continuous run mode. In continuous culture, the raceway is filled with the fresh culture medium at some precise flow rate. Just forward of the paddlewheel, the fresh medium should be filled. During filling with fresh medium, the algal broth is withdrawn or harvested from the raceway at a rate equal to the feed flow rate. Feeding and harvesting occur only during daylight and must stop at night, or the biomass may wash out of the raceway overnight.

Photobioreactor

Horizontal Photobioreactor

Design

The horizontal photobioreactor can be designed according to Dogaris et al. (2015), and it contains two plastic films forming the top and bottom surfaces of the horizontal raceway, sealed to each other and connected to two vertical airlift units (Fig. 4). The plastic film will fabricate from inexpensive transparent polyethylene (PE) sheet of 0.15 mm thickness. The horizontal photobioreactor prototype unit is designed 133.5 cm long × 68 cm wide, and the raceway depth should be 5 cm enabling increased light exposure of the microalgae culture medium as well as reducing water consumption for further culture. For fair mixing of culture medium, two airlifts will be made from acrylic pieces with 0.5 cm thickness, and each should be enriched with CO_2. A pump will be used for transferring liquids via silicon tubing into and out of the bioreactor. The bioreactor will be connected with pH probe with automatic temperature recorder. To conduct indoor experiments, two tube lamps can be used for exposing lights to the photobioreactor.

Culture

After sterilization of bioreactor and fresh growth medium, the inoculum will be inserted in the bioreactor. While culture reaches the exponential phase, the culture is harvested and implemented to the growth test. The pH of the culture water will be maintained at 7.50 ± 0.10 by using automatic pH detector with the help of adjusting

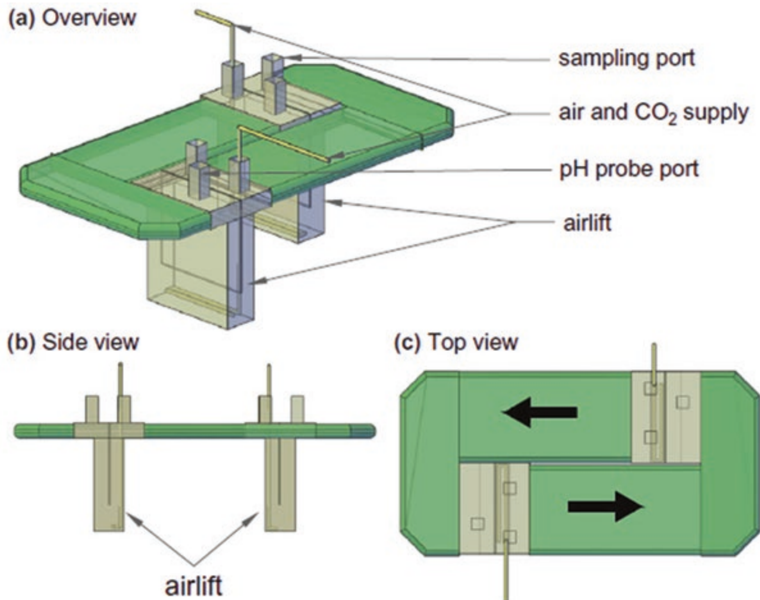


Fig. 4 Schematic representation of horizontal photobioreactor. (Source: Dogaris et al. 2015)

the CO_2/air mix between 2% and 3% (v/v) CO_2 in air. The experimental conducting room should be air-conditioned and the temperature will be maintained at 22 °C. While sampling is conducted for analysing the growth, biomass and biochemical composition, the deionized water should be added to compensate for the water loss in the culture chamber.

Bubble Column Photobioreactor

The schematic of the bubble column photobioreactor is shown in Fig. 5. The bioreactor is 2 m high and 0.15 m in diameter for lift force; a water pump will be used to circulate the culture medium. The accurate pump solution velocity inside the circulation pipeline (pipeline solution velocity) will be measured by weighing method (Yue et al. 2008). Pump solution volume ($V_{solution}$) from the pipeline will be collected within a certain time (t); therefore, pipeline solution velocity ($v_{velocity}$) is calculated as $v_{velocity} = V_{solution}/t/S$, where S is the cross-sectional area of the circulation pipeline. Bubble generation time and residence time will be measured according to the method described in Cheng et al. (2015). The rectangular areas filled with hatching lines represent the HSP measurement areas. The bubble column photobioreactor will be used to carry out the culture of microalgae at 24 °C under continuous illumination of 40,000 ± 2000 lux. The culture medium will be continuously aerated with 15% CO_2 at a rate of 1920 mL/min, and the gas flow rate is controlled by a mass flow meter.

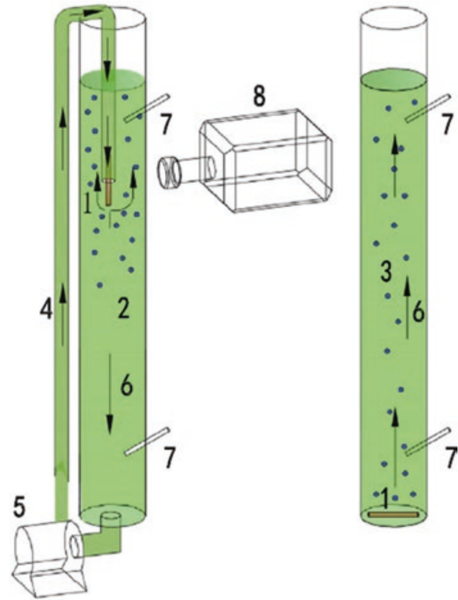


Fig. 5 Schematic representation of bubble column photobioreactor. (Source: Yang et al. 2016). *1*. Gas aerator, *2*. water-circulating column, *3*. airlifting column, *4*. circulation pipeline, *5*. pump, *6*. flow directions, *7*. DO and pH probes, *8*. camera

Bag Photobioreactor

The bag photobioreactor is housed in a greenhouse, and the core photobioreactor consists of 10 separate production modules, and each V-bags will be mutually interconnected (more than 100 bags), and their capacity can adopt 25 L of culture medium (Fig. 6). In each module, the culture will be circulated sequentially through all the V-bags. The V-bags are approximately 1.5 m in length with a diameter of 0.15 m. The suspension is sparged by 1.4–1.8% CO_2 with a gas flow rate of approx. 1.6 L per bag per minute, and pH should be measured daily without manipulation. The temperature is maintained in the greenhouse with the help of centralized air condition or automatic windows for cooling and a fan heater.

Flat Panel Photobioreactor

The pilot-scale flat panel photobioreactor (PBR) is made of clear toughened glass supported in unplasticized polyvinyl chloride (UPVC) frame (Fig. 7) according to the Mhatre et al. (2017). In this modular photobioreactor, we can culture multiple modes for studying individual as well as multiple panels. The single panel photobioreactor (0.6 m long, 0.08 m wide and 2.8 m in height) will occupy a land area of 0.048 m^2 and a volume of 86 L, while the total ground surface area occupied by the entire photobioreactor system of eight panels is 0.67 m^2. To circulate algae within bioreactor, two air blowers (work in succession) will be provided aeration and agitation at the rate of 1 VVM through a sparger (perforated pipe) installed longitudinally along the bottom centreline of the individual bioreactor. Each bioreactor is

Fig. 6 Bag photobioreactor. (Source: https://www.pinterest.co.kr/pin/530791506060698316)

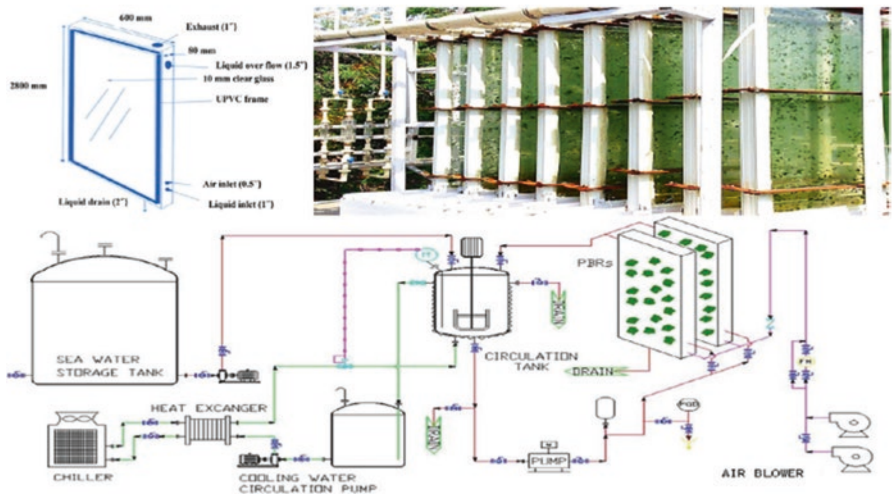


Fig. 7 Schematic representation and digital image of pilot-scale flat panel photobioreactor. (Source: Mhatre et al. 2017)

closed at the top by a sliding metal frame comprising single perforation at the centre (1″ diameter) and functions as the air exhaust. Additionally, the UPVC frame contains circular aperture (1″ diameter) that will connect to pipeline functioning as liquid overflow header. The aperture contains a 0.5 mm mesh screen to prevent loss of biomass. Further, UPVC frame contains two perforations near the base of the reactor which house inlets for airline and liquid media. Single aperture at the bottom of the reactor (2″ diameter) is connected to PVC pipe (drain pipe) with a drain valve to harvest microalgae from the bioreactor. Drain pipes of individual bioreactor will be connected to a single large pipe (5″ diameter) running longitudinally below the flat panel system. The open end of the large harvest pipe contains a drain valve which in its open mode allows biomass outflow from bioreactor. A makeshift mesh and bucket system allows to collect biomass through 500 μm nylon mesh funnel, and the utilized media is recirculated back to the media tank using 0.5 HP domestic water pump. The media tank is thermally insulated, and it contains 300 L of water always for efficient maintenance of temperature. For controlling temperature, the system comprises the cooling/heating water circuit. The water within the thermal jacket will flow by gravity though chiller/heater unit. The unit contains a heat exchanger that optimizes the water temperature which is then pumped to the thermal insulated media tank. The utilized media through the overflow header of the bioreactor will flow continuously by gravity towards media tank for re-optimization.

Biocoil Photobioreactor

A biocoil-type photobioreactor is a tubular photobioreactor composed of a transparent, plastic or negligible rubber tube with a small diameter (2.4–5.0 cm), which is screw-wrapped around a vertical tube with a large diameter. A few parallel systems of tubes are coupled through collectors with a pumping system that is realized through the airlift system or a variety of pumps. The reactor can be equipped in a gas exchange system. To maintain temperature we can use air coolers. This reactor's design assures uniform mixing and minimizes the adherence of algae cells to the internal walls of the tubes. The reactor can operate with full automation, which enables reducing costs of the production process (Fig. 8).

Until now there are many systems to culture algae at mass scale level; unfortunately none of them may be found cost-effective. Photobioreactors are too expensive and require high exploitation inputs (lighting, supply of carbon dioxide, operation); they additionally pose some difficulties in exploitation, e.g. due to overgrowing and restricted light penetration. In contrast, some technological systems are applied in the technical scale that merge certain elements of open and closed systems. In this case, Table 1 shows the advantages and disadvantages of commonly used systems for the mass cultivation of algae.

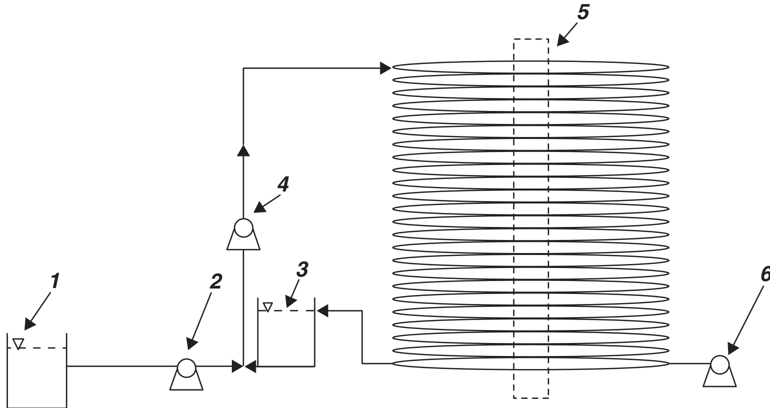
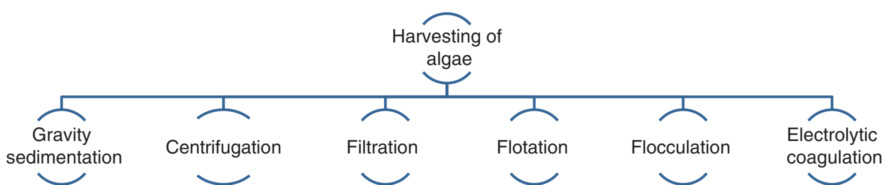


Fig. 8 Schematic representation of biocoil photobioreactor. (Source: Dębowski et al. 2012). *1*. Medium tank, *2*. nutrient dosing pump, *3*. discharge of biomass, *4*. circulation pump, *5*. light source, *6*. air pump

Harvesting Techniques

Microalgae have been identified as a potential alternative resource for biofuel and aqua feed production. Significant drawbacks to algal culture include dilute culture density and the small size of microalgae, which translates into the need to handle large volumes of culture during harvesting. This energy-intensive process is therefore considered a major challenge for the commercial-scale production of algal biofuels and feed. The harvesting of microalgae will be done following various methods as follows:



Gravity Sedimentation

In this technique, the microalgae are separated from one another by gravitational forces as shown in Fig. 9 (Salim et al. 2011). Microalgae suspensions will be taken and diluted in a beaker. After mixing, the suspension will be allowed to settle at 27 °C in the dark room. After certain period the upper layer will be taken and subjected to the optical density measurement.

Table 1 Advantages and disadvantages of the mass algal cultivation methods

Type of system		Advantages	Disadvantages
Open system	Round ponds, racetrack-type ponds	Relatively economical, easy to use and clean after completion of culture, good for the cultivation of algae on a large scale	Low ability to control culture conditions, difficulty with cultivation algae in the long term, low productivity, large size, limited number of culture species, cultivation susceptible to external factors (predators, disease, pollution)
Closed system	Photobioreactors column (vertical)	The high mass transfer, good mixing and low stress, low energy consumption, high potential for scalability, easy to ensure sterility, good for the immobilization of algae, reduce photoinhibition and photooxidation	A small area of exposure, their construction requires the use of sophisticated materials, the possibility of hydrodynamic stress, decrease in surface exposure with increasing diameter of the column
	Plate photobioreactors	The large surface area exposure, suitable for outdoor culture, good for the immobilization of algae, good availability of light, good productivity, biomass, relatively cheap, easy to clean, low concentration of oxygen	Increasing the size of the reactor requires the use of multiple chambers and supporting structures, the problems of controlling the culture temperature, the risk of fouling the walls, the possibility of hydrodynamic stress in some species of algae
	Horizontal tubular photobioreactors	The large surface area exposure, suitable for outdoor culture, good productivity, biomass, relatively cheap	Fluctuations in pH, dissolved oxygen and CO_2 in the pipe length, the risk of fouling the walls, requires a large surface

Source: Dębowski et al. (2012)

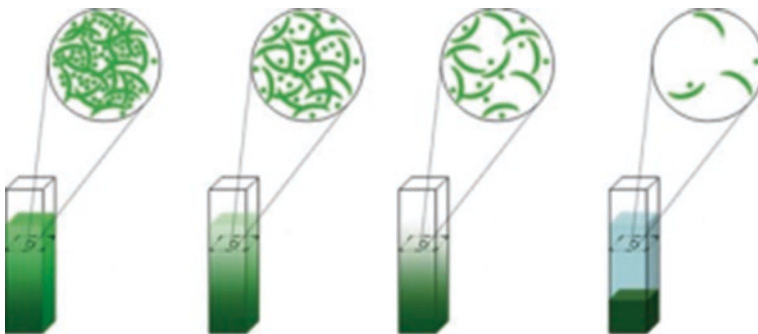


Fig. 9 Gravitational sedimentation techniques. (Source: Salim et al. 2011)

Centrifugation

This type of harvest mechanism is widely used in beverage, food and pharmaceutical industries. Centrifugation is a process in which a centrifugal force is used to enhance the separation of solids. Spinning the suspension creates the pressure differential necessary for particle separation from the liquid suspension. As per the previous researchers, if we use 13,000 g force in motor, we can get 95% of algal recovery from the culture medium (Heasman et al. 2000).

Filtration

Amid all filtration techniques, filtration is one of the most competitive methods that separate the microalgae from the culture medium. This technique needs a high pressure difference crosswise the filter which can be determined by vacuum, pressure or gravity (Brennan and Owende 2010). The membrane filters can be categorized based on the pore size into macro-filtration (greater than 10 μm), microfiltration (0.1–10 μm), ultrafiltration (0.02–0.20 μm) and reverse osmosis (less than 0.001 μm) (Fig. 10).

Flotation

Flotation is a separation process performed based on the attachment of air bubbles to solid particles. This technique is classified as a physicochemical gravity separation process in which gas bubbles pass through a liquid–solid suspension causing the microalgae to float to the surface by adhering to the gaseous bubbles (Shelef et al. 1984). The flotation mechanisms are divided into four types based on their separation mechanisms, and the types are dispersed air flotation, dissolved air flotation, microbubble generation and electrolytic flotation (Fig. 11).

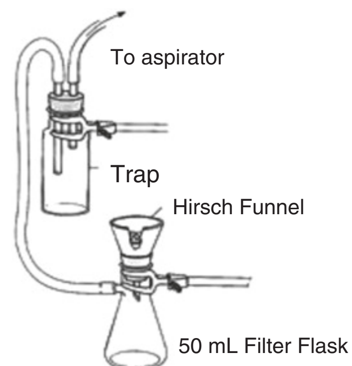


Fig. 10 The vacuum filtration. (Source: Brennan and Owende 2010)

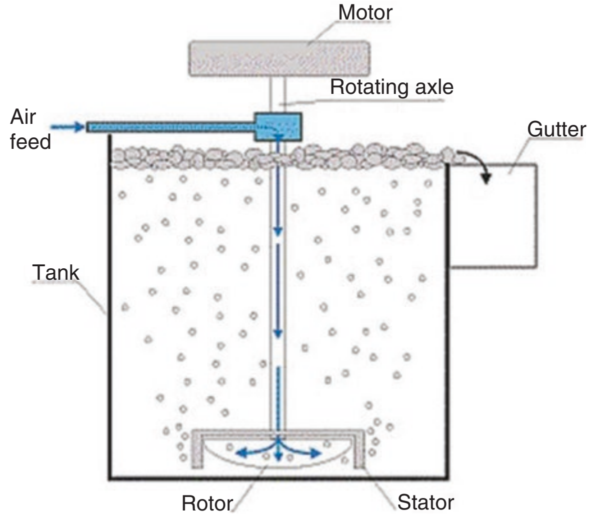


Fig. 11 The general flotation technique. (Source: Kaartinen and Koivo 2014)

Flocculation

Flocculation is used to separate microalgal cells from broth by the addition of one or more chemicals. Microalgal cell walls carry a negative charge that prevents self-aggregation within the suspension. This negative charge is countered by the addition of polyvalent ions called flocculants (Fig. 12).

To estimate the chemical flocculation, stationary-phase harvested microalgae will be stored in the jar or 1 L beaker at room temperature (25 °C). The beakers should have an internal diameter of 6.3 cm; the depth should be 6.4 cm; the impeller diameter is 5 cm; and the impeller distance should be 2.5 cm from the bottom of the beaker. Before adding chemical flocculants, the initial microalgae biomass concentration should be estimated as per the standard methods such as cell counts or optical density measurement. Two relatively nontoxic, readily available and inexpensive flocculants such as aluminium sulphate and ferric chloride can be used as flocculants. The stock solutions of the flocculants will be made at 20 gL^{-1} concentration. The 20 g of salt will be dissolved at deionized water and the volume is made up to 1 L. From the stock solutions, the flocculants can be used for microalgae separation with different concentration. As per the previous researchers, minimum range (0.5–2.5 g L^{-1}) of flocculants resulted maximum separation (95%) than maximum range from the medium (Chatsungnoen and Chist 2016).

Microalgal Paste Production

Microalgae will be stock cultured according to Perumal et al. (2015). The microalgae mass culture will be done adopting any of the above explained techniques. The cultured microalgae will be concentrated adopting various techniques such as

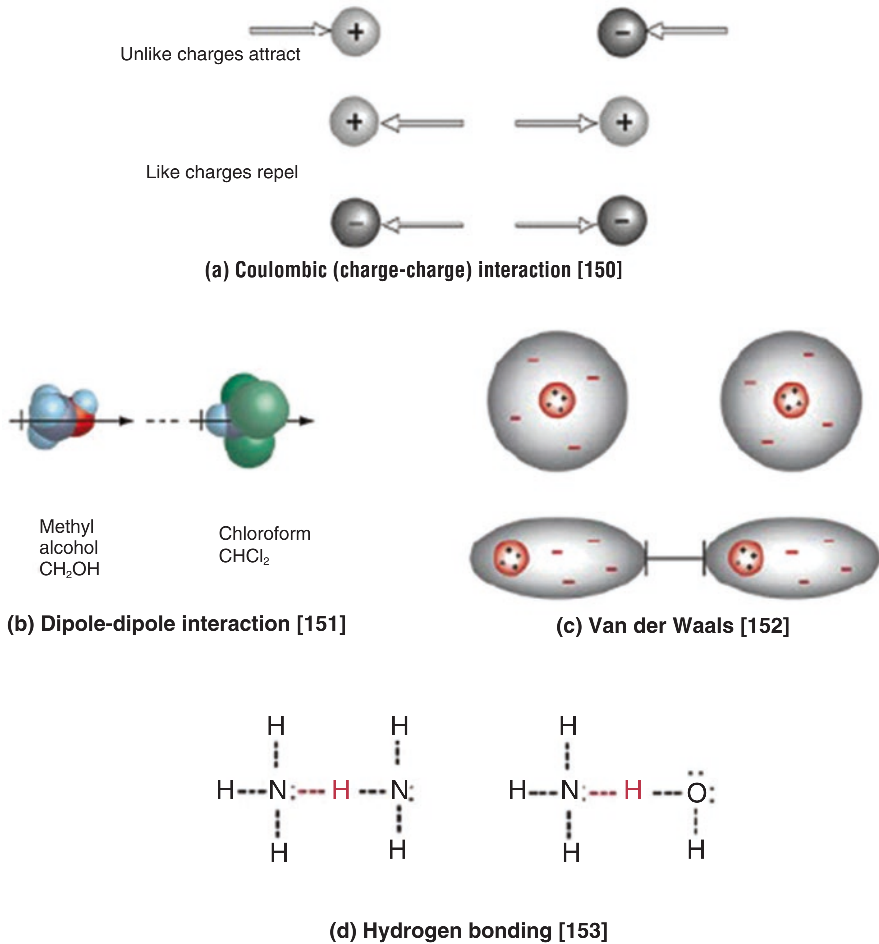


Fig. 12 Attachment mechanisms during chemical flocculation. (Source: Hattab et al. 2015)

ferric-induced flocculation and deflocculation, pH-induced flocculation and deflocculation and centrifugation according to Knuckey et al. (2006) and Nunes et al. (2009), respectively.

Ferric-Induced Flocculation and Deflocculation

Microalgae paste will be prepared adopting the ferric-induced flocculation and deflocculation technique according to Knuckey et al. (2006). In brief, the laboratory-scale flocculation will be done in 250 ml beakers using a magnetic stirrer for mixing with the stock solutions of ferric chloride and polyelectrolyte diluted with 1:5–1:10 with HCl and 1:10 with deionized water, respectively. For flocculation of larger volumes, the stock solutions will be used undiluted. For larger-scale (10–500 L)

flocculation, cultures will be mixed by aeration. The ferric chloride will be added slowly to the zone of maximal mixing. Mixing will be continued until small flocs will be visible, and then the polyelectrolyte will be added and the mixing reduced. When large flocs formed, the mixing will be stopped and the flocs will be allowed to settle under gravity. Surface water will then siphoned off and the settled floc will be collected and stored at 4 °C. After 24 h at 4 °C, the floc settled further and the supernatant will be again removed. Ferric flocs will be deflocculated using Na_2-EDTA. The floc will be mixed for 5 min before being centrifuged to recover cells. The yellow, iron-rich supernatant will be discarded and the collected cells resuspended in sterile seawater.

pH-Induced Flocculation and Deflocculation

The microalgae paste will also be prepared using the method of pH-induced flocculation and deflocculation according to Knuckey et al. (2006). In brief, lab-scale microalgae flocculate will be made in 250 mL beakers using a magnetic stirrer for mixing. Stock solutions of NaOH and polyelectrolyte will be used to flocculate algal cultures and cultures will be diluted 1:5–1:10 with deionized water. With larger volumes (e.g. 10–500 L cultures), the NaOH stock solution will be diluted 1:2 with potable water and the polyelectrolyte used undiluted. The NaOH will be added to the point of maximal mixing at a rate so that there will be a steady increase in culture pH: e.g. for 250 mL volumes, this took 5–10 s; for 500 L, 5–10 min. Between pH 10.0 and 10.6 (i.e. after addition of NaOH to 6–12 mM final concentration), there will be a shift in the clarity of the culture as a white precipitate formed. At this stage, NaOH addition ceased, mixing will be reduced and the polyelectrolyte will be added to give a final concentration of 0.5 mg L^{-1}. When large flocs formed, the mixing will be stopped and the flocs will be allowed to settle under gravity. Surface water will be siphoned off and the floc collected. The suspension will be deflocculated immediately by adjusting the pH of the floc slurry to pH 7.5–8.0 using concentrated HCl, with mixing. The floc slurry darkened as the bulk of the white precipitate dissolved leaving small aggregates of densely packed cells. These would be settled overnight at 4 °C and any supernatant will be removed. Harvested material will be readily disaggregated to single-cell suspensions by dilution in seawater and mild agitation.

Centrifugation, Preservation and Storage Techniques

Microalgae paste will be prepared according to Nunes et al. (2009) adopting centrifugation method. In brief, fresh algal cultures will be harvested at exponential phase and centrifuged at 2000 g, with inflow of 130 L/h, and adjusted to obtain mean cell retention rate of 80%. For each microalgae species, the volume centrifuged will be 2000 L, resulting in 4 L paste production. Following centrifugation, samples from

the concentrates will be diluted and counted to determine concentration. Pastes will be stored without addition of additives, and in some cases, pastes will also be stored with the addition of a 0.1% ascorbic acid (vitamin C) solution (Heasman et al. 2000). Pastes will be stored in capped plastic containers or zip lock plastic cover and kept in the dark and refrigerated at 4 °C in a domestic refrigerator.

Conclusion

Culturing of microalgae is very tough to employ in indoor and outdoor level. The success of microalgae culture is depending on various factors such as environment, water quality and other factors. In this chapter we discussed about the mass scale culture methods of microalgae and microalgae paste production for further use. The mass culture of microalgae and different harvesting techniques finally converted as a paste may contribute to the fish and shrimp culture in commercial-scale level in hatcheries and industries. Besides, these techniques may contribute to the Indian market as a novel and promising product to be used in multiple applications in aquaculture industries.

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An Estimation of Antimicrobial and Antioxidant Activity of Microalgae

C. Premkumar, M. Divya, N. Krishnaveni, P. Santhanam, and P. Pachiappan

Introduction

Algae include a large heterogeneous assemblage of relatively simple plants that have little in common except for their characteristic autotrophic mode of nutrition. Marine microalgae are the rich sources of structurally dynamic and biologically active compounds (Ely et al. 2004). Wide variety of bioactive secondary metabolites has been derived from marine algae for prospective development of novel drugs by the pharmaceutical industries. Marine sources are more active than the other natural sources. Algae have provided a source of inspiration for novel drug compounds for large contributions to human health and well-being. Algal proteins and lipoproteins are found to have antibacterial, antifungal, and antiviral activity (Burja et al. 2001). The compounds produced from algae are of greater interest as a source of safer or more effective substitutes than synthetically produced antimicrobial agent, and they are easily available and cheaper. The first generation of drugs was usually simple botanicals employed in more or less their crude form.

Marine organisms were reported to produce a wide range of bioactive secondary metabolites as antimicrobial, antioxidant, anthelmintic, and cytotoxic agents, and bioactive substances included alkaloids, polyketides, cyclic peptides, polysaccharides, phlorotannins, diterpenoids, sterols, quinones, lipids, and glycerols (Cabrita et al. 2010). Marine microalgae also produce a variety of compounds for pharmaceutical, food, and cosmetic applications including carotenoids, terpenoids, ste-

C. Premkumar · M. Divya · N. Krishnaveni · P. Santhanam (✉)
Marine Planktonology & Aquaculture Laboratory, Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

P. Pachiappan
Department of Biotechnology, School of Biosciences, Periyar University, Salem, Tamil Nadu, India

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roids, amino acids, phlorotannins, phenolic compounds, halogenated ketones, alkanes, and cyclic polysulfides (Taskin et al. 2007). This papers reveals the methods of estimation of antimicrobial and antioxidant activity of microalgae.

Materials and Methods

Collection, Isolation, and Preparation of Microalgae Samples

The microalgae are collected from coastal, salt pan, and surface lake water bodies using 48 μm plankton net. Then the single microalgae species is isolated from the field-collected mixed samples adopting standard methods, viz., plating methods, serial dilution, and centrifugation. Multiple media recipes are employed to isolate the colonies. The field samples are first diluted to aid in the isolation process. Sterilized Petri dishes containing approximately 20 ml of agar medium are used to plate these diluted samples. One milliliter of the diluted sample is transferred to a media plate and spread evenly across the surface. Inoculated plates are placed in a temperature-controlled area (20–25 °C, approximately 27 μE/m/s) where the algae are allowed to grow for about 14 days. Grown algae cultures are streaked using sterile technique onto additional sets of nutrient media plates and placed back in the culture room for isolation. This streaking method is repeated until isolation into axenic unialgal cultures is achieved. The number of colonies that are transferred from each dilution plate into other pure media plates depends on the amount of contamination. Identification of the microalgae is based on the morphology of each isolated colony. Following the isolation of individual microalgae colonies, each strain is initially labeled based on the sampling location and special media requirements. Isolated algae are maintained as stock cultures and are stored on a cool, low-light rack. These stock cultures are maintained by replating each onto new nutrient media at least once a month or more frequently depending on the nature of each isolated strain (Lee et al. 2014). The microscopic observation of cell morphology such as the size of the organism, presence or absence of chloroplast, flagella, paramylon bodies, etc. are taken into account and identified. The species are identified according to the morphological characters microscopically (Tomas 1997).

Growth Conditions

Large-scale cultivation on ASN III medium containing different concentrations of NaCl, $MgCl_2$, KCl, $NaNo_3$, $K2HPo_4{\cdot}3H_2O$, $MgSo_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$, citric acid, ferric ammonium citrate, EDTA, $Na_2CO_3{\cdot}H_2O$, and trace elements at pH 7.5 are done in five aquariums (16 L, each). The cultures are gassed with air containing 0.3% CO_2 (v/v) and continuously illuminated with two cool white fluorescent lamps (40 W each, Philips). The culture temperature is maintained at 28 ± 2 °C.

Growth Measurements

Algal growth is spectrophotometrically measured as described by Payer (1971). The calculated biomass (the average of three experiments) is used to obtain maximum specific growth rates (from the log phase of the growth curves by exponential regression). Productivities are calculated from the equation P = (Xi−X0)/ti, where *P* is the productivity (mg L^{-1} day^{-1}), *X*0 is the initial biomass density (mg L^{-1}), *Xi* is the biomass density at time i (mg L^{-1}), and ti is the time interval (h) between X0 and Xi17.

Microalgae Preparation

Pure cultures prior to the stationary phase of growth (10 days) are harvested and collected by centrifuging at 10,000 rpm for 3 min. The harvested biomass is rinsed with sterile water to remove any associated debris. The collected microalgal pellets are dried under shade and made into a coarse powder with mechanical grinder for further use.

Characterization of Microalgae

For examining the microalgae characterization using a scanning electron microscope, 2 ml of microalgae collected from the exponential phase is centrifuged at 6000 rpm for 5 min. Primary fixation of the algal cells is performed by incubating them in 2.5% glutaraldehyde at room temperature in the dark for at least 4 h followed by secondary fixation in 1% osmium tetroxide overnight at 4 °C. The algal cells are dehydrated gradually in increasing ethanol series (10%, 30%, 50%, 70%, 90%, and 100%), critical point dried, sputter coated with gold palladium using a polaron SC7640 auto/manual high-resolution sputter coater, and examined under a FEI Quanta 200 scanning electron microscope at 10 kV.

Drying

The harvested algal biomass is collected in aseptic plastic bags and subjected to drying under direct sunlight. After complete drying, the dried algal biomass is placed in hot air oven at 60 °C for 1 h. Until further use, the algal biomass is stored under refrigerated condition (4 °C). After harvesting through separation of the algae from the support media, the dewatered slurry is dried for stability, extraction, or other further processing.

Traditional Extraction Procedures

Simple extraction procedures such as decoction, maceration, liquid-liquid extraction, infusion, percolation, digestion, and hot continuous extraction (Soxhlet) are extensively studied for biomass processing (Han et al. 2007).

Preparation of Algal Extracts

Freshly dried algae are mixed with solvents (150 ml solvent/100 g of *Algae*) in Soxhlet apparatus and extracted for 60 min. The extracts are filtered and the solvent is removed by air-drying. The extracts are stored in an airtight glass bottles in a refrigerator for the analysis of phytonutrients and other findings.

Chemical Extraction of Algae

For extraction, 30 g of the dried algae is extracted using cold methanol, chloroform, and diethyl ether for 48 h, and then the extracts are concentrated to dryness in a rotary evaporator. The extracts are diluted using dimethyl sulfoxide (DMSO) (2 mg/ml) and then filtered for sterilization.

Twenty grams of the dried powdered algal sample is successively extracted by Soxhlet apparatus, according to the method adopted by Sadasivam and Manickam (1996) using different organic solvents with analytical reagent (AR) quality. These solvents are petroleum ether (40–60 °C), methylene chloride (39.6 °C), chloroform (61.15 °C), acetone (56 °C), and finally methanol (64.7 °C). To ensure the complete extraction process, exhaustive extraction is applied with each solvent for 10 h. Extracts of different organic solvents are collected separately into dry clean beakers, after that they are recovered from the solvents by evaporation in a rotary evaporator at 60 °C and then dried in desiccators for 1 h, and finally the extracts are weighted and the percentage of each extract is determined as follows:

$$\text{Extract\%} = \text{Weight of extract in grams} / \text{Weight of sample in grams} \times 100$$

The results are expressed as percentage of DW, where DW = algal dry weight. The extracts are kept under vacuum desiccators until used for further work.

Phytochemical Analysis

Preparation of Sample for Qualitative Phytochemicals Analysis

For the phytochemical screening, fresh microalgae samples are used. Five grams of fresh sample is taken and homogenized with 50 ml of water, ethanol, methanol, benzene, and petroleum ether solution separately. The extract is boiled for 1 h, cooled, and filtered. The filtrate is used for screening of phytochemicals by using standard procedure (Harborne 1973).

Phytochemical Analysis with TLC

Phytochemical analysis of the microalgal extract is carried out using chemical methods, and the phytochemicals are confirmed by thin-layer chromatography according to the proposed methodology (Harborne 1998).

Test for Carbohydrates

A small quantity of algal extract is dissolved separately in 5 ml of distilled water and filtered. The filtrate is tested to detect the presence of carbohydrates.

Molisch's Test To 2 ml of algal extract, 2 ml of Molisch's reagent is added. Then, 2 ml of concentrated sulfuric acid is added along the sides of the test tubes. Disappearance in color on the addition of excess solution indicated the presence of carbohydrates.

Benedict's Test To 0.5 ml of algal extract, 5 ml of Benedict's reagent is added. The mixture is then boiled for 5 minutes. Presence of a bluish green precipitate indicated the presence of carbohydrates.

Test for Glycosides To 2 ml of algal extract, 1 ml of aqueous NaOH solution is added. The appearance of a yellow color indicated the presence of glycosides.

Test for Proteins and Amino Acids

Ninhydrin Test A small quantity of algal extract solution is boiled with 0.2% solution of ninhydrin. Purple color indicated the presence of free amino acids.

Test for Phytosterols and Triterpenoids

Salkowski Test To 2 ml of the algal extract, 1 ml of concentrated sulfuric acid is added. Chloroform is added along the sides of the test tube. A red color produced in the chloroform layer indicated the presence of phytosterols or if it is yellow in color at the lower layer indicated the presence of triterpenoids.

Test for Flavanoids

Zinc Hydrochloride Reduction Test The algal extract is treated with mixture of zinc dust and concentrated hydrochloric acid. Red color indicated the presence of flavanoids.

Test for Alkaloids A small portion of the solvent-free algal extract is stirred separately with a few drops of diluted hydrochloric acid and filtered. The filtrate is tested with Mayer's reagent (potassium mercuric iodide solution). The cream precipitate indicates the presence of alkaloids.

Test for Tannins

Gelatin Test To 5 ml of algal extract, few drops of 1% lead acetate is added. Absence of a yellow or red precipitate indicated the absence of tannins.

Test for Saponins To 5 ml of the algal extract, a drop of sodium bicarbonate is added. It is then shaken vigorously and kept undisturbed for 3 min. Appearance of a honeycomb-like froth indicated the presence of saponins.

Antimicrobial Activity of Crude Algal Extract

Test Organisms

The potential microbial pathogens are collected and stored as inoculums. The 18 hrs incubated nutrient broth of clinical isolated cultures are prepared and used for the antimicrobial activity test (equilibrated to McFarland standard turbidity scale 105- 06 cfu/ml for bacteria).

Preparation of the Crude Algal Extract

The algal culture is filtered and the filtrate is used as the extracellular extract. The biomass obtained is grinded and used as the intracellular extract.

Agar Well Diffusion Technique

Antimicrobial activity of microalgal extract is determined by Agar well diffusion technique as described by Srinivasan et al. (2001). In brief, different concentrations of methanol, chloroform, acetone, and hexane extracts of microalgae are tested against selected bacterial pathogens. Sterile Mueller-Hinton agar plates are prepared and swabbed with the clinical isolated cultures. Using a sterile cork borer, wells are cut in the Mueller-Hinton agar plates. Intracellular extract and extracellular extract are added into their respective wells. The appropriate standard antibiotic discs for each organism are placed on the plates. The plates are subjected to incubation at 37 °C for 24 h. The zone of inhibition around the well is observed and examined.

Disc Diffusion Method

Antimicrobial activity of microalgae is performed by Disc Diffusion Method (Bradshaw 1992). In short, sterile 6 mm diameter filter paper discs are impregnated with different concentrations, viz., 10, 20, and 30 µl, of methanol, chloroform, and hexane extracts of the selected microalgae extract (0.5, 1, and 2 mg $disc^{-1}$), and the overnight bacteria culture was swabbed on Mueller-Hinton agar medium using sterile cotton swabs. The different concentrations of microalgal extract were placed on the plates and incubated at 37 °C for 24 h. Tobramycin discs (Bioanalyse, 10 µg $disc^{-1}$) and nystatin discs (Oxoid, 30 µg $disc^{-1}$) are used as positive controls. The inhibitory effects are evaluated by the diameter size of inhibition zone.

A 25 µl of each extract is loaded on sterile filter paper discs with 5 mm in diameter and air-dried and then deposited on the surface of agar medium (Mueller-Hinton agar, pH 7.4 ± 0.2 at 25 °C). The plates containing Mueller-Hinton agar are inoculated by immersing a swab in the inoculums and spreading it onto the entire surface of the media and then incubated at 37 °C for 24 h (Ballantine et al. 1987). After incubation for 24 h, whether a clear zone around a disc appears or not is observed in each Petri plate. The presence of clear zone around a disc is evidence of antibacterial activity. Diameters of the zones of inhibition are measured in millimeters. Each test is prepared in triplicate. Discs loaded with each extracting solvent are considered as control.

The algae samples are cleaned and then rinsed with sterile water to remove any associated debris. These cleaned fresh materials are allowed to air-dry and then pulverized with the help of a blender. The powder (5 g) is filled in sterile tubes and extracted with methanol, ethanol, chloroform, and diethyl ether by using a rotary evaporator apparatus at 40 °C for 12 hrs. From the solvent extracts, 5 ml is collected separately, allowed to dry at room temperature and weighed to estimate the concentration in 1 ml. The dry extracts are completely dissolved in 5 ml of 0.5% Tween 80 and preserved at 5 °C in bottles with airtight screw cap until further use (Lin et al. 1999), and the extracts are used for further antimicrobial studies. Tween 80 is mixed with double distilled water and served as control for all the experiments. The experiments are carried out in triplicates. Antimicrobial study is carried out by disc diffusion method.

Antioxidant Assay

Several studies have focused on physiological properties of some valuable antiviral or antioxidant compounds in algae. The presence of compounds which possess antioxidant activity in biological systems in higher plants is well documented, while in microalgae is less documented.

Preparation of Organic Extracts of Sample for Antioxidant Activities

The dried algal sample is ground to coarse powder, weighed and wrapped in Whatman No.1 filter paper, and successively extracted with 200 ml of different solvents such as benzene, chloroform, ethanol, ethyl acetate, methanol, and petroleum ether with their increasing order of polarity by soxhlation for 12–24 h. The extract is analyzed for the presence of antioxidant activities by referring standard procedure (Thoudam et al. 2011).

DPPH Stable Free Radical Scavenging Activity

A total of 50 μl solution of the microalgae extracts (10 mg/ml) is placed in cuvettes and 2 ml of methanolic DPPH (Sigma, Germany) solution (6×10^{-5} M) is added. The absorbance is determined at 515 nm using spectrophotometer. Measurements are recorded immediately as the absorbance at t = 0. Then the test solution is incubated in the dark for 30 min before reading and recording the absorbance at t = 30. The determinations are performed in three replicates. The radical scavenging

activity of the tested sample is expressed as inhibition percentage of the DPPH radical scavenging activity, calculated using the following formula: % inhibition = [(Abs t = 0−Abs t = 30)/Abs t = 0] × 100, where Abs t = 0 is the absorbance at t = 0 and Abs t = 30 is the absorbance at t = 30.

DPPH Assay

1 mg of algal extract powder is dissolved in 1 ml of 50% ethanol solution to obtain 1000 μg/ml of sample solution. 1000 μg/ml solutions are serially diluted into 1 μg/ml, 5 μg/ml, 10 μg/ml, 20 μg/ml, 50 μg/ml, 100 μg/ml, 200 μg/ml, 500 μg/ml, and 1000 μg/ml with 50% ethanol. In each reaction, the solutions are mixed with 1 ml of 0.1 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH), 0.45 ml of 50 mM Tris-HCl buffer (pH 7.4), and 0.05 ml samples at room temperature for 30 min. 50% ethanol solution is used as control. The reduction of the DPPH free radical is measured by reading the absorbance at 517 nm. DPPH, a purple-colored, stable free radical, is reduced to the yellow-colored diphenylpicrylhydrazine when antioxidants are added. L-Ascorbic acid and (+)-catechin are used as positive controls. The inhibition ratio (percent) is calculated from the following equation: % inhibition = [(absorbance of control − absorbance of test sample)/absorbance of control] × 100%. The antioxidant activity of each sample is expressed in terms of IC_{50} (micromolar concentration required to inhibit DPPH radical formation by 50%), calculated from the inhibition curve.

FRAP Assay

The procedure described by Collins and Lyne (1987) is followed for FRAP assay. The principle of this method is based on the reduction of a ferric-tripyridyltriazine complex to its ferrous-colored form in the presence of antioxidants. The FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue-colored Fe(II)-tripyridyltriazine compound from the colorless oxidized Fe(III) form by the action of electron-donating antioxidants. The FRAP reagent consists of 300 mM acetate buffer (3.1 g sodium acetate +16 ml glacial acetic acid, made up to 1 L with distilled water; pH = 3.6), 10 mM TPTZ in 40 mMHCl, and 20 mM $FeCl_3.6H_2O$ in the ratio of 10:1:1. Briefly, 50 μl of sample supernatant is added to 1.5 ml of freshly prepared and prewarmed (37 °C) FRAP reagent in a test tube and incubated at 37 °C for 10 min. The absorbance of the blue-colored complex is read against reagent blank (1.5 ml FRAP reagent+50 μl distilled water) at 593 nm. For construction of the calibration curve, five concentrations of $FeSO_4$ $7H_2O$ (1000, 750, 500, 250, and 125 μmol L^{-1}) are used, and the absorbencies are measured as sample solution. The data is expressed as mole ferric ions reduced to ferrous form per liter (FRAP value).

Antioxidant Property Assay

DPPH (2,2-Diphenyl-1-2-Picrylhydrazyl) Assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity is determined for the extracts (glass bead and steel ball method). 1 ml of methanolic solution of DPPH radicals (3×10^{-4} mol/l) is added to 2.5 ml of sample and measured immediately (without incubation) after 20 minutes of incubation period. The absorbance recorded spectrophotometrically at 517 nm. A decrease by 50% of the initial DPPH concentration is defined as the IC_{50} (inhibitory concentration). The IC_{50} value (μg/ml) is determined for all the extracts. BHT is used as the reference compound (IC_{50} = 0.021 μg/ml). The capacity of the scavenging free radicals is calculated as follows:

$$\text{DPPH} = \left(\frac{1001 - \text{As} - A\text{b}}{A\text{c}} \right)$$

As = Sample absorbance of the algal extract in the DPPH solution
Ab = Blank absorbance of the sample extract without DPPH solution
Ac = Absorbance of the control solution containing only DPPH (1 ml of DPPH radical of 3×10^{-4} mol/l concentration + 2.5 ml of methanol)

H_2O_2 Radical Scavenging Assay

The H_2O_2 radical scavenging assay in the microalgae extract is determined by using BHA (butylated hydroxyanisole) according to Collins and Lyne (1987) as a standard. Samples at different concentration in ethanol is added to H_2O_2 solution (0.6 ml, 40 mM) and allowed to stand for 10 min. The absorbance is measured at 230 nm and a blank solution containing phosphate buffer without H_2O_2 is used. The percentage of H_2O_2 scavenging of both the extracts and standard compounds is calculated (IC_{50} = 0.011 μg/ml). The H_2O_2 radical scavenging activity is calculated as follows:

$$\text{Scavenging effect} = \left(\frac{\text{Control} - \text{Sample}}{\text{Sample}} \right)$$

Total Phenolic Content

The total phenolic content in the microalgae extracts is determined spectrophotometrically according to the Folin-Ciocalteu 18 method using gallic acid as a standard (the concentration range, 0.025–0.5 μg/ml). 200 μl of the sample is mixed with 9 ml of distilled water, and 1 ml of Folin-Ciocalteu reagent and 10 ml of 7% sodium carbonate are added. After 90 minutes of incubation, the absorbance is determined at 765 nm. The total phenolic content is expressed as GAE (gallic acid equivalent) in milligram per gram extract 19. The standard absorbance at 765 nm = 0.431 C gallic acid (μg/ml) − 9.33×10^{-3}, R2 = 0.9992.

Toxicity Test Using Brine Shrimp Lethality Test (BSLT) Method

The brine shrimp *Artemia* cysts are put into containers filled with seawater for hatching and aerated under 40–60 W in candescent lamp. The hatching temperature is maintained in the range of 25–30 °C with light for 48 h. After the cysts hatched, *Artemia nauplii* are taken to be tested (Weerapreyakul et al. 2012).

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A Method of Extraction, Purification, Characterization, and Application of Bioactive Compounds from Phytoplankton

C. Premkumar, S. Dinesh Kumar, S. Ananth, and P. Santhanam

Introduction

Microalgae are an immense group of unicellular prokaryotic and eukaryotic organisms that are mainly autotrophic (Tartar et al. 2003; Ueno et al. 2003). Capability of microalgae as simple growth requirements and photoautotrophic and capacity to modulate their metabolism, make them attractive for demand by the pharmaceutical, food, cosmetic or biodiesel industries. Microalgae can be used as a feed for fish larvae in aquaculture and as animal fodder, due to their rich content of fatty acids, protein, antioxidant pigments and polysaccharides (Yaakob et al. 2014). Microalgae produce a wide variety of bioactive products with potential commercial values such as antibacterial, antifungal, antiviral, antiplasmodial, enzyme-inhibiting, immunostimulant, and cytotoxic activities (Ghasemi et al. 2004). Even though the microalgae has high potential and comprise more and more bioactive substances, only β-carotene and astaxanthin have been produced at an industrial scale (Dominguez et al. 2005). This paper explains the methods involving extraction, purification, characterization, and application of bioactive compounds from phytoplankton.

C. Premkumar · S. Dinesh Kumar · P. Santhanam (✉)
Marine Planktonology & Aquaculture Laboratory, Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

S. Ananth
Marine Planktonology & Aquaculture Laboratory, Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

Department of Animal Husbandry, Dairying and Fisheries, Ministry of Agriculture and Farmers Welfare, Krishi Bhavan, New Delhi, India

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Materials and Methods

Isolation of Microalgae

The isolation of the required species of microalgae will be done by one of the following methods as described by Perumal et al. (2015). (1) Washing method or centrifugation: The individual species of microalgae will be isolated by repeated washing or centrifugation. (2) By exploiting the phototactic movement: Providing light in one place by preventing light at all other sides, the phytoflagellates will move to one direction where light is available, and with a micropipette, microalgae will be isolated. (3) By agar-plating method: For preparing the agar medium, 1.5g agar will be added to 1 liter of suitable medium or even natural freshwater, brackish water, or seawater, and this agar solution will be sterilized in an autoclave for 15 min under 150 lbs pressure and 120 °C temperature. Then this medium will be poured in sterilized Petri dishes and left for 24 h. In case of culture tubes, the medium will be poured in 1/3 part in tubes and properly plugged with cotton before autoclaving. After autoclaving, mediums will be cooled and a loop full of microalgae culture will be streaked in agar plate. After incubation of 24–36 h at incubator, the grown microalgae will be subcultured in test tube or another agar plate for axenic culture. (4) Micromanipulation: The algal cell will be isolated in drop of enrichment sample. While observing, the cell will be sucked up into micropipette. Then the cell will be transferred to a drop of sterile medium on agar plate. This process will be repeated to "wash" the cell. After repeated washing, the cell will be transferred to dilute medium in a tissue culture plate, Petri dish, or culture tube. Culture vessel will be placed under low light at appropriate constant temperature. Growth will be checked under microscopically. A colonial unialgal culture results from this method. (5) Serial dilution: Tubes will be labeled as 10^{-1}–10^{-10} to indicate dilution factor. Aseptically 1 ml of enrichment sample will be added to the test tube (10^{-1}) and mixed gently. 1 ml of this dilution will be taken and added to the next tube (10^{-2}) and then mixed gently. This procedure will be repeated for the remaining tubes (10^{-3}–10^{-10}). Test tubes will be incubated under controlled temperature and light conditions. The cultures will be examined microscopically after 2–4 weeks by withdrawing a small sample aseptically from each dilution tube. A unialgal culture will grow in one of the higher dilution tubes, e.g., 10^{-6}–10^{-10}. If tubes contain two or three different species, then micromanipulation will be used to obtain unialgal cultures.

Culture of Microalgae

After isolation of the required species in culture tubes, it may be subcultured again in few 50 ml test tubes. The test tube culture can be transferred to small culture flasks and to bigger flasks by adding 3–5 ml of the stock culture. Therefore every

2 weeks, a new set of ten test tubes for each species should be inoculated from the previous set. The filtration of water and medium enrichment should be done not earlier than 3 days, prior to inoculation. The algal stock culture will be maintained in special air conditioning room. Stock cultures will be kept in 1 and 2 l culture flasks and 5 and 15 l plastic containers. The water will be filtered by using filter bag (1 micron), sterilized by using autoclave, and after cooling water will be transferred to the culture flask. Culture flasks will be plugged with cotton or covered by aluminum foil. All vessels used for algal culture will be sterilized properly and dried in an oven before use. The culture medium used for indoor stock culture of microalgae will be selected depending on the species. About 10 ml of the inoculum in the growing phase will be transferred to the culture flasks and the culture will be provided with 1000 lux light and 12:12 h light and dark cycle. After 8-10 days, the maximum exponential phase is obtained. The temperature and salinity will be maintained between 23 and 25 °C and 28 and 30 ppt, respectively, for the entire culture period. The continuous aeration will be provided for culture. After 8–10 days, when the maximum exponential phase has been reached, light is reduced to 500 lux for further growth. The time required for the maximum growth varies depending on the species, under controlled conditions of light and temperature, with or without aeration. At the time of maximum exponential phase of growth, the color of the culture turns into dark green. A maximum of five culture flasks will be kept for each species as stock culture. The microalgae will be harvested following the standard procedures like centrifugation and filtration as shown in Chapter 16. The harvested algal biomass is rinsed with sterile water to remove adhering debris, and algal pellets are dried in hot air oven at 60 °C for 12 h. The dried biomass is made into a coarse powder with mechanical grinder for further use.

Antibacterial Screening

The microalgae extracts are tested for antibacterial activity for screening the metabolites producing algae species by using different organic solvent followed by solvent extraction. The algal extracts are centrifuged at 5000 rpm. The supernatant is twice extracted with solvent and then the extract condensate to the total volume of 50 μg by using evaporator. The extracts are tested against fish and human pathogens following agar diffusion method. In this method, Mueller-Hinton agar plates are prepared and swabbed with the fish and clinical isolated cultures. Using a sterile cork borer, wells are cut in the Mueller-Hinton agar plates and extracts are added into their respective wells. The appropriate standard antibiotic for each organism is placed on the plates. The plates are subjected to incubation at 37 °C for 24 hrs. The zone of inhibition around the well is observed and examined (Baur et al, 1966). Based on the results obtained in antibacterial activity, the potential microalgae and promising solvent are selected for the extraction of bioactive compounds from microalgae.

Extraction of Bioactive Compounds

The algal culture is harvested after 12–18 days by centrifugation at 5000 rpm for 10 min. Algal pellet is extracted with 100 ml of ethyl acetate or ethanol as solvent system by Soxhlet apparatus for 24 h. The extracts are filtered and then concentrated in a rotary evaporator under vacuum conditions at 45 °C. The extract is dried under reduced pressure and stored in −20 °C for further studies. The filtrated crude extract is collected, and purification process is done by using thin-layer chromatography and column chromatography.

Purification

Thin-Layer Chromatography

Thin-layer chromatography is a physical separation method based on the distribution of the biocomponents in solvent extracts; TLC has two stages, a fixed one, called stationary phase, and the mobile phase, which passes through the stationary phase. The crude extracts are applied for the thin-layer chromatography (TLC) for the isolation of bioactive metabolite by scraping the spots of the chromatoplates (Hong and Kim 2013). The active fraction noticed by Rf value calculation. Purification of the extract is made on silica gel column chromatography (GF243 Merck, Germany). A further purification of fraction compounds is made by a column chromatography method.

Column Chromatography

In column chromatography purification, the silica gel is used as absorbent stationary phase, and mobile phase is prepared into slurry using hexane and *n*-hexane/acetone. The concentrated extracts are loaded onto a silica gel with 200–300 mesh size packed into a glass column (2 × 30 cm) and equilibrated with a mixture of *n*-hexane/acetone (6:4) and then eluted with the same solvent. The active compounds containing fraction is collected and concentrated into a small volume. Further purification of active compound is carried out by HPLC.

HPLC

HPLC is a chromatographic technique that is used to identify, quantify, and separate a mixture of compounds. The isolated crude extracted compound is subjected to HPLC 75-4 C18 column. An aliquot of the filtrate (20 μL) is injected into the HPLC

column and eluted with a linear gradient of mobile phase containing solvent A (0.1% acetic acid) and solvent B (methanol). The solvent gradient is programmed from 0% to 100% solvent B in 30 min. with a flow rate of 1 mL/min. The peaks in the chromatogram are detected by a photodiode array detector at 254 nm.

Characterization of Bioactive Compounds from Microalgae

UV Spectrometer

The purified bioactive compounds are subjected to ultraviolet-visible (UV-Vis) spectroscopy, which provides information on the chromophores in the compound and the types of compound, based on the type of bonding formed. For example, unsaturated organic compounds have a wide absorbance range from the UV to the visible region, but longer conjugated compounds will shift the absorbance peak to a longer wavelength (Skoog 2004). Saturated compounds have a lower absorbance range of 170–250 nm, but the presence of lone-pair electron atoms (oxygen or nitrogen) shifts the absorbance to a high wavelength. This is because less energy is required for excitation of the molecules, and therefore lower-frequency energy is absorbed.

FTIR

Sample Preparation for FTIR Spectroscopy

The preparation of sample for FTIR spectroscopy is carried out as described by Naumann et al. (1991b). Briefly, known weight of dry algal sample (1 mg) is taken in a smooth agate mortar and mixed thoroughly with 2.5 mg of dry potassium bromide (KBr) using a pestle. The powder is filled in the micro-cup of 2 mm internal diameter to obtain the diffuse reflectance infrared spectrum for replicate samples. All IR spectra are recorded at room temperature (26 °C ± 1 °C) in the mid-infrared range (4000–400 cm^{-1}) using FTIR 8201PC, Fourier transform infrared spectrometer (Shimadzu). Typically, 20 scans are signal-averaged for a single spectrum. Each spectrum is displayed in terms of absorbance as calculated from the reflectance-absorbance spectrum using the Hyper-IR software. To minimize the difficulties arising from unavoidable shifts, baseline correction is applied. Each spectrum is normalized as normalization produces a spectrum in which maximum value of absorbance becomes 2 and minimum value 0. To improve the resolution of complex bands, the digitized original spectrum is smoothed on noisy spectrum using Kubelka Munk algorithm and converted into its second derivative using the Savitzky-Golay algorithm (Savitzky and Golay 1964) using 21-point smoothing.

IR spectroscopy can also be used to show the types of bonding between atoms in the molecule of interest, based on the vibration between the bonds. Significant regions of absorption include peaks from hydrogen bonding between –OH groups detected at 3100–3600 cm^{-1}, depending on the attachment of other groups, and from carbonyl groups with strong signals between 1630 and 1850 cm^{-1} (Field et al. 2008).

GCMS

GC-MS is a chromatographic technique that can identify different volatile compounds within a test sample. The standard nonpolar capillary column, with a dimension of 30 m X 0.25 mm X 0.25 μm, is used, and the injection volume is one microliter. The extract is diluted in hexane and injected in the split mode. The carrier gas is helium and the flow rate is 1.0 ml/min. The temperature is programmed to an oven temperature of 80 °C raised to 260 °C at 5 °C/min.

Mass spectroscopy (MS) or GCMS gives information on the molecular mass, molecular formula, and fragmentation pattern of the compound. Gas chromatography-mass spectrometry (GC-MS) utilizes electron (or chemical) ionization to create various fragments, which can be used for comparison with library mass spectral databases. However, the most natural products are too large for GC-MS analysis, so liquid chromatography-mass spectroscopy (LC-MS) is used instead. In addition, the compounds must be thermally stable and have a low boiling point if GC-MS is to be used. LC-MS provides better fragmentation of large molecules, thus providing a better mass spectrum. However, the mass spectrum will differ between instruments because fragments of the molecules are produced due to the low-energy collision of the inert gas (Hill et al. 2009).

NMR

The pure active fractions are subjected to nuclear magnetic resonance (NMR), 1H-nuclear magnetic resonance (NMR), and 13C NMR for structural elucidation. According to Xia et al. (2013), purified active fraction from GCMS is dissolved in 1 mL CD3OD and used for NMR spectroscopy. The 1H and 13C NMR signals are recorded on a NMR system with a carbon-enhanced cold probe (1H with 500 MHz, 13C with 126 MHz). Chemical shifts are adjusted with δ (ppm) referring to the solvents peaks δH 3.31 and δC 49.2 for CD3OD. Data are processed with the MestReNova program and compared with data in the literature.

Application

Microalgae are a vast, largely untapped resource for a variety of natural products. These products may be used for human health supplements to animal feeds to biofuels. Some of the most valuable compounds derived from marine algae are the polyunsaturated fatty acids, carotenoids, antibiotic compounds, antifungal compounds, antifouling compounds, and oils for biofuels. Fortunately, they are plentiful and offer very little chance for overexploitation. Their potential for production in an aquaculture setting is also a huge benefit in addition to their valuable secondary actions and products including carbon dioxide fixation and nitrogen waste production for fertilizer. When production becomes more economically feasible and more efficient, marine microalgae may represent the biggest breakthrough in marine natural product development for medicine and other biotechnological products.

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Potential Harmful Microalgae in Muttukadu Backwater, Southeast Coast of India

B. Balaji Prasath, P. Santhanam, R. Nandakumar, and T. Jayalakshmi

Introduction

Phytoplankters are the primary producers of organic matter and the nourishment of many food webs in aquatic surroundings (Moestrup et al. 2010), and furthermore phytoplankton are good indicators of environmental change, hence contributing to the assessment of eutrophication stages in aquatic ecosystems (Tas and Yilmaz 2015). High phytoplankton production can also affect the water quality, producing toxins in the aquatic system. This may cause massive kills of living marine resources as well as terrestrial animals (Anderson et al. 2012). Eutrophication triggers various physicochemical alternations in the aquatic ecosystem, especially in marine and freshwater environment, and dramatically increased the microalgal cell abundance; due to these proliferations, some toxin-producing species or harmful algal blooms may create problems in the formation of the public fitness and wellbeing. These blooms are referred to as harmful algal blooms (HABs). The numerous numbers of toxic species are found among dinoflagellates, but evidence has been offered for several species of other taxa like diatoms, flagellates and cyanobacteria suggesting

B. Balaji Prasath
Beijing Normal University-Hong Kong Baptist University, United International College, Zhuhai, Guangdong, China

Marine Planktonology & Aquaculture Lab., Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

P. Santhanam (✉) · R. Nandakumar
Marine Planktonology & Aquaculture Lab., Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

T. Jayalakshmi
Marine Planktonology & Aquaculture Lab., Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

National Centre for Sustainable Coastal Management, Ministry of Environment, Forest and Climate Change (MoEF&CC), Chennai, Tamil Nadu, India

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that they belong in this HAB category (Vershinin and Orlova 2008; Moestrup et al. 2010). Over the last one decade, increasing attention has been devoted to surveys of HABs in both aquatic ecosystems, because of the health hazard and their negative influence on aquaculture, recreation and tourism (Paerl and Huisman 2008). Studies of these microorganisms are commonly supported on the microscopic identification and enumeration of the cells of interest. Likewise, evaluation of the potential toxic hazard of discrimination specimen is based on direct identification and quantification of the toxins through immunochemical, chemical or biological methods. Potentially harmful microalgae are commonly found to appear in moderate numbers but under certain conditions may figure large glow that can have a devastating expression of the environment (Moestrup and Larsen 1992). The present paper deals with the surveying and documenting of the occurrence of harmful microalgae in Muttukadu backwater, southeast coast of India.

Materials and Methods

Phytoplankton samples were collected at each month for a period of 2 years (2010–2012) from Muttukadu coastal water (Fig. 1). Microalgal sampling was conducted with a plankton net (No. 30; size, 48 µm) for half an hour. After collection, samples were preserved in a 5% buffered formalin for further analysis. For the quantitative and qualitative analysis of phytoplankton, the settlement method (Sukhanova 1978) was adopted. Numerical plankton concentrates were then carefully transferred to a 1 mL capacity Sedgewick-Rafter and were analysed under an inverted microscope. Phytoplankton was identified using the standard taxonomic keys (Subrahmanyan 1946; Steidinger and Williams 1970; and Taylor 1976). Along this, sampling was done at locations where a visual observation of the bloom patches and fish's

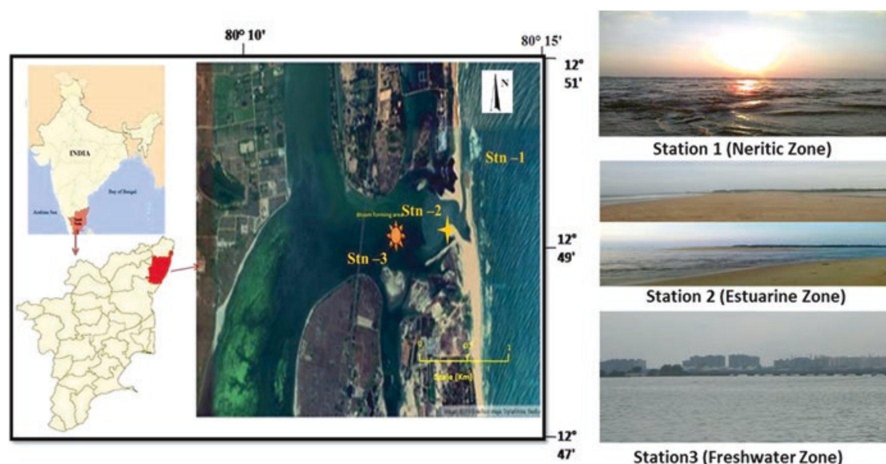

Fig. 1 Description of the sampling area (Muttukadu coast)

mortality if any was reported. The harmful algal floras analysed were assigned to major groups, viz., Bacillariophyceae, Dinophyceae, Cyanophyceae and Chlorophyceae. A specific phytoplankton had been taken into consideration to be in the bloom situation, while its cellular densities multiplied at least ten times the normal conditions. Additionally, increased densities of phytoplankton need no longer constantly bring about the visible shade. A toxic species can motive a damaging reaction; even though its densities are comparatively decreased, both those elements had been taken into consideration in identifying the bloom at those stations.

Results

Muttukadu backwater (Lat. 12° 49′ N; Long. 80° 15′ E) is located on the east coast of the Bay of Bengal, India. This backwater is normally not connected with the sea during May–September and leads to sandbar formation. However during monsoon season due to the high inflow of freshwater from land runoff that can erode the sand bar and the backwater can be connected with the sea. This estuary is shallow, with an average depth of 2 m, in most of the area. The Muttukadu backwater is dominated by tourism and recreational boating activities and, as with many different tropical estuaries, is threatened with the aid of the various uses to which people put into this water body and the land areas that drain into it. Therefore, the estuary has a multitude of environmental problems (Fig. 1). Based on the above-mentioned factors, three sampling stations were selected for the present investigation as follows: Station 1 represents the open sea and generally has a depth of about 8 m. The bottom is characterized by fine silt. Station 2 is located about 200 m away from station 1. The depth of this station ranged from 0.3 to 1 m. The depth of this station varies frequently due to dredging and bar formation. Station 3 can be characterized as freshwater zone, as it remains mostly unconnected with other stations. The station received the bulk of fresh water during monsoon time. The depth of the station ranged from 0.5 to 2.0 m. The bottom of the station is silty clay.

Phytoplankton samples were collected and analysed in a 2-year study period (November 2010–October 2012) from three stations which revealed the presence of toxic bloom-forming species. An account of this is offered to toxic bloom-forming species which passed off during the observed period, and the environmental variations during their bloom period are described. Qualitative analysis is a prerequisite while preparing a database of the phytoplankton resource of the location. Documenting the presence of a harmful algal species in the coastal waters is important, even if it is a rare member of the microalgal community of the region, as there exists a possibility of the sudden proliferation of these species resulting in toxic blooms when the right conditions prevail. In addition to this, some dinoflagellate genera like *Dinophysis* sp. have members which are toxic even at very low densities. The qualitative and quantitative analysis of phytoplankton collected from Muttukadu coastal waters revealed the presence of eight phytoplankton species, viz., probably; it is the first report of the occurrence of potential harmful algae in Muttukadu

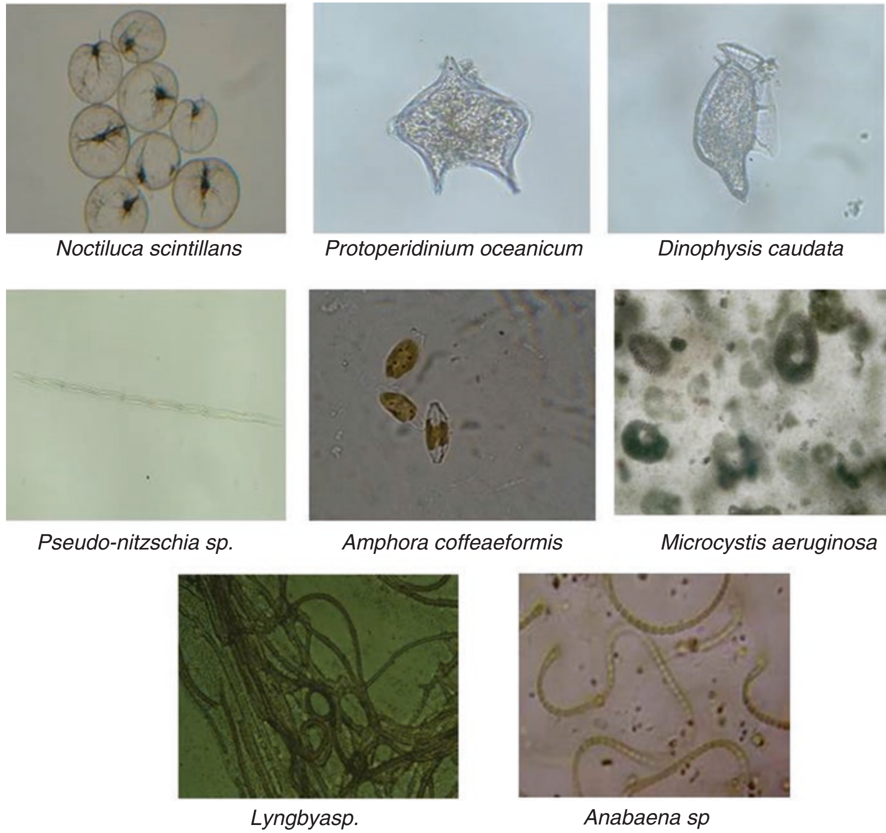

Fig. 2 Harmful microalgae reported from Muttukadu, southeast coast of India

backwater. These include the *Amphora coffeaeformis*, *Pseudo-nitzschia* sp., *Dinophysis caudata*, *Protoperidinium oceanicum*, *Noctiluca scintillans*, *Microcystis aeruginosa*, *Lyngbya* sp. and *Anabaena* sp. (Fig. 2) identified as harmful algae. Table 1 shows the months of occurrence and abundances of these harmful species at Muttukadu backwater.

A. Dinoflagellates

1. ***Noctiluca scintillans*** **(Macartney) Koroid and Swezy 1921**

Classification and Cell structure

Division	Pyrrophyta
Class	Dinophyceae
Order	Noctilucales
Family	Noctilucaceae
Genus	*Noctiluca*
Species	*N. scintillans*

Table 1 Toxic (TX) and potentially toxic (PT) nuisance microalgae species in Muttukadu coastal waters, southeast coast of India

Sl. No.	Species	Season/year of max. abundance (cells L^{-1})	Toxins	Category	Source
1.	*Amphora coffeaeformis*	March 2011 (8250)	Domoic acid	PT	Spatharis et al. (2009)
2.	*Pseudo-nitzschia* sp.	Jan 2011 (9000)	Domoic acid	PT	Spatharis et al. (2009)
3.	*Dinophysis caudata*	March 2012 (5250)	Okadaic acid	PT	Nikolaidis et al. (2005)
4.	*Protoperidinium oceanicum*	July 2012 (5250)	Azaspiracid toxins	PT	Spatharis et al. (2009)
5.	*Noctiluca scintillans*	June 2012 (6750)	Ammonia	PT	Escalera et al. (2007)
6.	*Microcystis aeruginosa*	June 2012 (6×10^6)	Microcystin	T	Metaxatos and Panagiotopoulos (2003)
7.	*Lyngbya majuscula*	Sep 2012 (45,000)	Antillatoxin A and B	PT	Metaxatos and Panagiotopoulos (2003)
8.	*Anabaena* sp.	April 2011 (60,000)	Antillatoxin A	PT	Metaxatos and Panagiotopoulos (2003)

Unarmoured dinoflagellate is a large (200 to >1 mm) sub-spherical inflated vegetative cell, with one flagellum and a striated tentacle. A ventral groove contains the flagellum, a tooth and a tentacle and is connected to a cystostome. The cytoplasm is vacuolated and can contain photosynthetic symbionts. The vegetative cells are exist with eukaryotic nucleus. Gametes are gymnodinioid, with dinokaryotic nucleus. Chloroplasts are absent. The cells are colourless but may attain a green colour because of the presence of endosymbiotic flagellates. It is phagotrophic in nature.

Occurrence

N. scintillans was recorded in the month of June 2012, and the cell densities were 6750 cells L^{-1} at station 1.

Toxin Chemistry and Toxicology

N. scintillans accumulates massive amounts of ammonia within the vacuoles which may be harmful to fish. It's found to feed heavily on fish eggs and fauna that end up in disruption within the food cycle. Blooms' area unit is believed to provide high concentrations of ammonia, which can be toxic to fish (Horner 2002). Blooms have additionally been related to mortality events in several marine invertebrates (Smithsonian 2012). Although, it doesn't turn out to be toxins, it's registered as a

harmful protoctist species (HAB) owing to its ability to provide toxic concentrations of ammonia, expend element levels within the water and clog alternative organisms gills (Escalera et al. 2007).

2. ***Protoperidinium oceanicum*** **(Vanhöffen) Balech 1974**
Classification and cell structure

Phylum	Dinophyta
Class	Dinophyceae
Order	Peridiniales
Family	Protoperidiniaceae
Genus	*Protoperidinium*
Species	*P. oceanicum*

The length of the cell is 220–300 µm; cell is star-shaped with one long apical horn and two long antapical horns (Horner 2002). The centre of the cell theca is round with a protrusion on each side. The cell cingulum is narrow and has wide lists. The two antapical horns are long, tubular, pointed and divergent. The left antapical horn is a bit shorter and thinner than the right. Cells have a deep sulcus that forms a strong indentation between the horns. The theca is reticulated with spiny junctions, making it look very ornate under SEM (Evagelopoulos 2002). It is similar to *P. claudicans. P. oceanicum* is larger than *P. claudicans* and has a longer, thinner apical horn and divergent antapical horns.

Occurrence

P. oceanicum was recorded during the month of July 2012 with cell density of 5250 cells L^{-1} at station 1.

Toxin Chemistry and Toxicology

P. oceanicum accumulates massive quantities of azaspiracid toxins inside the vacuoles which can be poisonous to finfish and shellfishes and can cause disruption within the food web.

3. ***Dinophysis caudata*** **(Saville-Kent 1881)**
Classification and cell structure

Division	Pyrrophyta
Class	Dinophyceae
Order	Dinophysiales
Family	Dinophysiaceae
Genus	*Dinophysis*
Species	*D. caudata*

The cells are large and irregularly subovate with fairly distinctive long ventral projection. The ventral sides of hypotheca are undulate or straight, dorsal side straight or slightly concave in the anterior half and straight or convex, running parallel with the ventral side in the posterior half. The anterior cingular lists are wide, supported by many posterior ribs, forming a wide and deep funnel-like structure with very low epitheca on the bottom. Left sulcal list is almost half of total length, supported by three ribs. Thecal plates are thick areolated.

Occurrence

D. caudata was recorded in the month of March 2012, and the recorded cell densities were 5250 cells L^{-1} at station 1.

Toxin Chemistry and Toxicology

D. caudata is suspected to motive diarrhetic shellfish poisoning in human beings and observed to have close mortality on mussels, scallops, clams and gastropods which ends up in disruption inside the food web.

B. Diatoms

1. ***Pseudo-nitzschia* sp. (H. Pergallo)**

Classification and cell structure

Division	Chrysophyta
Class	Bacillariophyceae
Order	Bacillariales
Family	Bacillariaceae
Genus	*Pseudo-nitzschia*
Species	*Pseudo-nitzschia* sp.

Cells of *Pseudo-nitzschia* can be easily distinguished from that of *Nitzschia* sp. by the stepped chains formed by the overlap of valve ends. Cells strongly elongate, are rectangular or have longer overlap of valve ends. Chains are motile, raphe is strongly eccentric, and raphe is not raised above the general level of the valve. Valve face interstriae often more than one to each fibula central larger interspace in most species. Valve face is slightly curved or flattened, not undulated. Valves are narrowly lanceolate to fusiform and linear with round or pointed ends. Transapical axes are heteropolar in some species. Striae structure is usually too delicate to resolve with LM. Chloroplasts have two plates, lying along the girdle one on either side of the median transapical plane. Resting spores are unknown. Species of this genus are morphologically very similar making its identification up to species level very difficult.

Occurrence

Pseudo-nitzschia sp. with amnesic shellfish poisoning shown by many of its members showed a constant presence in the phytoplankton sample in January 2011. The cell density was 9000 cells L^{-1} at station 1.

Toxin Chemistry and Toxicology

Amnesic shellfish poisoning or domoic acid poisoning has been proved to be as a result of participants of the genera *Pseudo-nitzschia* sp. Domoic acid binds to the kainate type of glutamate receptors and in the presence of endogenous glutamate causes huge depolarization of the neurons with a subsequent growth in cellular Ca2+ leading to neuronal swelling and loss of life.

2. ***Amphora coffeaeformis* (C. Agardh) Kützing, 1844**
Classification and cell structure

Phylum	Ochrophyta
Class	Bacillariophyceae
Order	Thalassiophysales
Family	Catenulaceae
Genus	*Amphora*
Species	*A. coffeaeformis*

Valves are semi-lanceolate to semi-elliptical and strongly dorsiventral. The dorsal margin is smoothly to flatly arched. The ventral margin is straight to slightly concave. The valve ends are protracted, narrowly rostrate to subcapitate, and slightly ventrally deflected. The raphe is positioned close to the ventral margin. The raphe is straight or may have a gradual dorsal bend near the central valve. The proximal raphe ends are weakly inflated with a slight dorsal deflection. The dorsal axial area is narrow, while the ventral side is wider. A dorsal fascia is absent; a ventral fascia is present but difficult to resolve in the light microscope due to the indistinct ventral striae. The dorsal striae are uninterrupted, slightly radiate and are becoming more radiate at the poles. The ventral striae are often difficult to resolve and radiate near the centre and convergent at the poles.

Occurrence

Amphora coffeaeformis was recorded in the month of March 2011. The cell density was 8250 cells L^{-1} at station 1.

Toxin Chemistry and Toxicology

The species *Amphora coffeaeformis* is known to produce domoic acid which binds to the kainate type of glutamate receptors. Due to the presence of endogenous glutamate, large depolarization of the neurons with a subsequent growth in cellular Ca^{2+} leading to neuronal swelling and death.

C. Cyanophyta
1. ***Anabaena* sp.**
Classification and cell structure

Phylum	Cyanobacteria
Order	Nostocales
Family	Nostocaceae
Genus	*Anabaena*
Species	*Anabaena* sp.

Colonies grow in filamentous clumps of multicell chains. Cells are cylindrical or barrel-shaped. The end cells are often much longer than mid-chain cells and may be hyaline (having a glass-like appearance). Anabaena is one of the four cyanobacteria genera that can produce toxins. The cell size is 4–50um and varies with the type of cell (vegetative smallest, akinetes largest).

Occurrence

Anabaena sp. was observed during the month of April 2011. The recorded cell density was 60,000 cells L^{-1} at station 3.

Toxin Chemistry and Toxicology

Anabaena sp. was determined to provide anatoxin-a which evolved with the blockage of sodium and potassium channels, leads to decrease in neural movement potentials, flaccid paralysis, breathing arrest, and subsequent death.

2. ***Lyngbya majuscula* (Harvey ex Gomont 1892)**
Classification and cell structure

Phylum	Cyanobacteria
Class	Cyanophyceae
Order	Oscillatoriales
Family	Oscillatoriaceae
Genus	*Lyngbya*
Species	*L. majuscula*

Lyngbya is a doubtlessly toxic, marine cyanobacterium (blue-green algae). It grows connected to rock, wood, seagrass, seaweed (macroalgae), coral and the sediment in clumps or mats of first-rate, darkish, cotton wool-like strands 10 to 30 centimetres lengthy. While *Lyngbya* is found in large quantities, it accumulates fuel bubbles (from excessive rates of photosynthesis) across the filaments inflicting the clumps to upward push to the surface and shape huge conspicuous floating mats. Floating *Lyngbya* may wash up on beaches, frequently combined with seagrass.

Occurrence

L. majuscula was recorded in Muttukadu coastal waters during the month of September 2012. The cell density was 45,000 cells L^{-1} at station 1.

Toxin Chemistry and Toxicology

L. majuscula was found to produce antillatoxin A and B. They disrupt normal signaling between neurons and muscles in several ways, leading to death by causing paralysis of respiratory muscles followed by suffocation.

3. ***Microcystis aeruginosa* (Kützing) Kützing 1846.**
Classification and cell structure

Phylum	Cyanobacteria
Class	Cyanophyceae
Order	Chroococcales
Family	Microcystaceae
Genus	*Microcystis*
Species	*M. aeruginosa*

The species are colonial. Colonies tend to float near the surface in nutrient-rich fresh water and other low-salinity waters. Cells have no individual mucilaginous envelopes, but the colony is encased in fine colourless mucus. The colony can produce toxins (microcystins), and dense blooms can be dangerous to aquatic ecosystems as well as to animal and human health. The cell size is 2–3um.

Occurrence

M. aeruginosa was reported in Muttukadu waters in the month of June 2012. The cell density was 6 x 106 cells L^{-1} at station 1.

Toxin Chemistry and Toxicology

M. aeruginosa was found to produce microcystin-LR and caused extensive liver damage. Microcystin-LR is a naturally occurring toxin produced by cyanobacteria. It is one of a large family of microcystins. It is considered the most toxic compound of this family. Microcystin-LR inhibits protein phosphatase type 1 and type 2A (PP1 and PP2A) activities in the cytoplasm of liver cells. This leads to an increase in phosphorylation of proteins in liver cells.

Several phytoplanktonic species were identified during the 24 months sampling period. The available data indicate that eight identified HAB species have spread across the Muttukadu coastline during 2 years. Among these, certain microalgae were associated with the occurrence of important HAB incidents causing damage in the marine biota and the water quality. There is a strong indication that these incidents were eutrophication-induced phenomena but sporadic in time, space and recurrence of the causative species.

Discussion

Several earlier researchers have describe the information on the characteristics, causative factors, and effects of HABs species in coastal ecosystems (Cembella et al. 2005; Ranston et al. 2006). In present study, it was noticed that all the eight microalgae species are identified as potentially harmful. Among these, 2 species of Bacillariophyceae, 3 species of Dinophyceae, 3 species of Cyanophyceae are found to be proliferate, especially during the summer season and late summer due to the excessive nutrients because Muttukadu backwater receiving a bulk of freshwater influx from the catchment area. It is clearly understood that the prevailing environmental conditions might be inducing the growth and proliferation of microalgae species exist in the Muttukadu coast particularly, the species *Microcystis aeruginosa* bloomed in this backwater when favourable environmental and water quality conditions exist besides the availability of excessive nutrients discharged from domestic resources and effluents from factories domestically located (Balaji Prasath et al. 2014).

The massive blooms and scums of cyanobacteria, like the ones of *M. aeruginosa* and *Anabaena* sp., can be stimulated with the aid of land disturbances which include construction and filling of reservoirs, which cause eutrophication in the catchment water body (Harper 1992). In the present study, *Pseudo-nitzschia* species had been found to be more abundant in the open sea in January, 2011, as located on the Muttukadu coast. *Pseudo-nitzschia* sp. is commonly pronounced to occur at low temperatures in early spring (Liefer et al. 2009). This indicates the result compared with the previous study species is determined to be more abundant at low temperatures. In addition, studies on identification and toxicity of *Pseudo-nitzschia* species in the Muttukadu coast are necessary as a way to better understand their potentially

harmful impact (Ruggiero et al. 2015). Dinoflagellate blooms were reported from many coastal regions from all over the world (Campbell et al. 2008). *Dinophysis caudata* had been found in March 2012 (5250 cells L^{-1}) in Muttukadu water. *Dinophysis* sp. reached the abundance levels up to a thousand cells L^{-1} which have to be cautiously monitored in this location (Tas et al. 2009), and it has been mentioned that even these low abundance levels of *Dinophysis* sp. are enough to toxify mussels (Hoppenrath et al. 2009).

Monitoring programs want to be deliberate for stopping human intoxication in destiny. Muttukadu coastal ecosystems are actually experiencing outstanding interference from human activities which include uncontrolled dumping of untreated sewage in coastal waters. Most of these ought to boost up the rise of toxic microalgae including *Amphora coffeaeformis, Pseudo-nitzschia* sp., *Dinophysis caudata*, *Protoperidinium* sp., *Noctiluca scintillans, Microcystis aeruginosa, Lyngbya* sp. and *Anabaena* sp. The accelerated influx of tourists additionally imparts a tremendous stress in these coastal surroundings. This stress ought to once more result in modifications of water quality, and consequently the related biota. Therefore, it is not sudden that numerous species of doubtlessly dangerous microalgae have been found in Muttukadu backwaters. The existing study displays definitely that potentially harmful microalgae do exist in Muttukadu backwaters. To what volume do they affect the seafood, the customers and the financial system? What are the biomass, productivity and seasonality of those species? What causes them to arise? Those are a number of the questions that want to be responded. As such, this requires a detailed examination to evaluate their spatial and temporal distribution. Instead, a monitoring programme can be installed in the area. This would lead to better information on factors that manage the abundance and distribution of harmful microalgae, with the intention of controlling the feasible effects on the fishery and seafood consumers. A continuous monitoring of potential harmful microalgae in the observed areas on an extended-time period might help in understanding the exact reasons and effects of blooms within the coastal ecosystems. Statistics suggest that eight diagnosed HAB species have unfolded throughout the Muttukadu shoreline during 2011–2012. Among these, *M. aeruginosa* have been associated with the prevalence of HAB incidents inflicting damage within the marine biota and the water.

Conclusion

The study concluded that totally 8 microalgae belonging to 3 dinoflagellates, 2 diatoms, and 3 cyanobacteria were identified as bloom forming species from the Muttukadu Backwater, Southeast coast of India. Further, the study inferred that all the 8 species are potentially harmful. Among these, cyanobacteria *Microcystis aeruginosa* was found to be a dominant taxa forming dense and successive blooms inflicting water discoloration, and fish mortality had been reported. The findings of the present study suggested that the continuous monitoring of this water body to be given high priority as this backwater is biodiversity rich and ecotourism spot.

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