PHENETIC CHARACTERIZATION, MOLECULAR PHYLOGENY AND BIO-PROSPECTING OF SELECTED SALINE MICROALGAE FROM INDIAN SUB-CONTINENT

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By

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Certificate

*This is to certify that the thesis entitled "**Phenetic characterization, molecular phylogeny and bio-prospecting of selected saline microalgae from Indian sub-continent**" is a bonafide record of research work carried out by **Ms. PREETHA. K (Reg. No. 3780)** under my supervision at Central Marine Fisheries Research Institute (CMFRI), Kochi for partial fulfillment of requirement for the degree of **Doctor of Philosophy** in **Marine Sciences,** Cochin University of Science and Technology, Kochi, and that no part of this work has previously formed the basis for the award of any degree, diploma, associate-ship, fellowship or any other similar title or recognition in any universities or institutes.*

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Declaration

*I hereby do declare that the thesis entitled "**Phenetic characterization, molecular phylogeny and bio-prospecting of selected saline microalgae from Indian sub-continent**", is an authentic record of research work done by me under the supervision of Dr. K. K. Vijayan, Director Central Institute of Brackish water Aquaculture, Chennai, (former Principal Scientist & Head, Marine Biotechnology Division, CMFRI, Kochi, 682018) and that no part of this work, has previously formed the basis for the award of any degree, diploma associate-ship, fellowship or any other similar title of any university or institution.*

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Preetha K

Contents

List of Abbreviations

AA	-	Aminoacid
ANOVA	-	Analysis of variance
ARA	-	Arachidonic acid
BGA	-	Blue green algae
BLAST	-	Basic local alignment search tool
CCAP	-	Culture collection of Algae and Protozoa
CCMP	-	Provasoli-Guillard National Center for Culture of Marine Phytoplankton
Chl	-	Chlorophyll
DDW	-	Double distilled water
DHA	-	Docosahexaenoic acid
DMSO/Me_2SO	-	Dimethyl sulfoxide
EPA	-	Eicosapentaenoic acid
ESNW	-	Enriched seawater natural medium
FAO	-	Food and agricultural organization
GLA	-	γ-linolenic acid
GLY	-	Glycerol
LA	-	Linoleic acid
LC- PUFA	-	Long chain poly unsaturated fatty acids
MeOH	-	Methanol
NFMC	-	National Facility for Marine Cyanobacteria
PCR	-	Polymerase chain reaction
PUFA	-	Poly unsaturated fatty acids
SCP	-	Single cell protein
UTEX	-	University of Texas at Austin Culture Collection of Algae
WHO	-	World health organization

Dedicate To My Family & Teachers

Chapter 1

INTRODUCTION AND SCOPE OF THESIS

Contents

1.1 What are Microalgae?

The term "Microalgae" implies to a group of microscopic photosynthetic organisms normally with cell size ranging from about 2 to 200 μm. Some common examples of 'the microalgae' are – *Spirulina*, *Anabaena*, *Chlamydomonas*, *Chlorella*, diatoms and dinoflagellates. Interestingly, microalgae are more common to us as pond scum or a nuisance which cause water eutrophication turning them blue/green, or as slimy green patches on wet places. In wet ecosystems especially in oceans, however, these microbes are the store houses of carbon which support the food web through photosynthesis. To the modern world of biotechnology, microalgae are more important as the suppliers of food or fuel and generally termed as "Future food" or "Green gold of future" (Day n.d.; Wolkers et al. 2011).

Taxonomically, all prokaryotic and eukaryotic single celled producers (oxygenic, photosynthetic forms) may fit in to the group of microalgae. The prokaryotic forms are generally called blue green algae or cyanobacteria. Protists (diatoms, dinoflagellates, golden & gloden-brown algae) and the single celled algae (green or red) which comprise the eukaryotic forms

(Dragone et al. 2010). Systematically different classes (with suffix – *phyceae*) are assigned to catalog microalgae, for example, Cyanophyceae (Cynaobacteria), Bacillariophycea (diatoms), Dinophyceae (dinopflagellates), Prymnesiophyceae (golden algae), Chlorophyceae (green algae) etc. There are approximately 2 to 8 lacks of species, of which only 10-30 thousands are described in literature (Wolkers et al. 2011). The systematic position of microalgae can be given as follows (Lee 2008).

Systematic classification of Prokaryotic and Eukaryotic microalgae (Examples *Arthrospira* and *Chlorella*)		
Kingdom	**Prokaryota**	**Eukaryota**
Phylum/Group	Cyanophyta	Chlorophyta
Class	Cyanophyceae	Trebouxiophyceae
Order	Oscillatoriales	Chlorellales
Family	Phormidiaceae	Chlorellaceae
Genus	*Arthrospira*	*Chlorella*
Species	*A. Platensis*	*C. vulgaris*

Microalgae have a simple body structure but with a large diversity in size and shape. All are single celled, with one or many locomotory flagella (*Chlamydomonas*) or without flagella (*Chlorella*). The shape of cell can vary from spherical, oval, elongated, spiral, rod etc. They can also exist in cell aggregations like chains (*Anabeana*, *Skeletonema*) and colonies (*Nostoc*, *Volvox*, *Botryococcus*). Prokaryotes have typical bacterial body plan without membrane boundaries in cytoplasm. Eukaryotes have all important cell organelles with nucleus and plastids. The chief photosythetic pigment is *Chl a* (some also have *Chl* b or *Chl* c) and accessory pigments are the carotenoids or phycobiliproteins. Reproduction is simple (no specialized reproductive

structures), but varied in different groups – vegetative (cell division), asexual (spore formation, cyst) or sexual methods (Barsanti and Gualtieri 2006).

In aquatic ecosystem microalgae are either free floating (phytoplankton e.g. Phytoflagellates - *Chlamydomonas*), benthic (on sand or rock in continental areas e.g. benthic diatoms like *Navicula*), epiphytic (on large weeds e.g. *Polysiphonia lanosa* on *Ascosphyllum*), symbiotic (with other higher organisms like corals and sponges e.g. Zoochlorella, Zooxanthella) or parasitic (rarely *Choreocolax* on *Polysiphonia*) (Lee 2008) in nature. Microalgae can also have wide range of tolerance and can inhabit normal (fresh, brackish, marine), extreme – (hyper saline lakes, salt pans, hot springs, acidic/alkaline pools, polar water) or even polluted water bodies. They are also present in/on soil, deserts, ice, salt beds etc. Their ability to adapt and tolerate extreme climatic conditions is unique and remarkable which make many of them a candidate for physiological studies (e.g. *Chlamydomonas* sp.) (Hema et al. 2007).

1.2 Ecological Perspective of Microalgae

In an evolutionary viewpoint, fossil records have shown that microalgae are the oldest life form on planet earth (e.g. Stromatolites – oldest fossils made by cyanobacteria) (Castro and Huber 2003). However, in the present world of global warming and pollution, rather than an old form of life, they are more significant as oxygenic organisms; which are presumed to contribute to a major portion of the biosphere's productivity. Latest reports say a maximum of 75% global Oxygen production with an equal CO_2 absorption by microalgae (Wolkers et.al 2011). Larger ecosystems like open ocean depends totally on microalgae for their primary production (as there are no larger plants) forming the base of food chain. Therefore a correlation between

microalgae and total fish production & fishery industry (directly or indirectly) cannot be denied ("Microalgae and Fisheries Production" 2008). Some evidences demonstrate a relationship between marine microalgae blooms and cloud formation (phytoplankton blooms → dimethyl sulfide + sunlight = sulfide aerosols + water vapour = clouds) which regulates the atmospheric temperature (Moran and Armbrust 2007). Taken as a whole, microalgae are the key components of the biosphere with an exceptional regulatory function and have a unique balancing role in the atmosphere.

1.3 Molecular Taxonomy of Microalgae - an Emerging Field:

Conventional methods of microalgae taxonomy are based on morphology, physiology and biochemical composition of the organism. Due to the small size and cellular plasticity, the most used microscopic examination for identification remains to be difficult, time consuming and tedious, which also claims expertise in morphological characterization (Juan et al. 2008). Molecular taxonomy is a robust approach in microalgal identification which remains to be free from environmental and developmental influences and does not insists on any previous experience in microalgae identification (Bornet et al. 2004). 'Polymerase chain reaction' (PCR) amplification of a desired gene and its sequence BLAST analysis against submissions present in the universal database, remains to be the easiest technique used in genetic characterization and thereby taxonomic elucidation.

1.4 Microalgae in Applied Biology

1.4.1 Food

Due to their exceptional nutritional value, microalgae have become dietary supplements or health promoters. The high protein content (50-70%) makes them an unconventional source of protein with complete amino acids,

hence providing all essential amino acids to the diets (Gouveia et.al. 2008). "*Spirulina*" alone is called as "The Super food" by WHO and "Space food" by NASA, FAO reports on malnutrition eradication programmes based on *Spirulina* (Habib et al. 2008; Sharma and Dunkwal 2012). *Spirulina (Arthrospira platensis* and *A. maxima)* is one among the most popular microalgae as an SCP (single cell proteins) and a health food for humans and animals. Other important species like *Aphanizomenon flos-aquae* and *Chlorella vulgaris* are also consumed as SCPs (Moore 2001).

The other macro-nutrients in microalgae include about 10-60% of carbohydrates and about 1- 70% of lipids (can be 90% in special cases) on dry weight basis (Spolaore et al. 2006). Microalgal carbohydrates include glucose, starch, sugars and other polysaccharides. The high digestibility of algal carbohydrates made them suitable for consumption as a whole (Becker 2004). Lipids have remarkably high 'poly unsaturated fatty acid' (PUFA) ratio, which include all the essential fatty acids (Arachidonic, Linoleic and Linolenic acids) and long chain poly unsaturated fatty acids (LC-PUFAs) like DHA (docosahexaenoic acid; 22:6(n −3)) and EPA (eicosapentaenoic acid; 20:5(n − 3)) (Mansour et al. 2005). Microalgae are also found rich in all essential and nonessential Vitamins including A, B_1, B_2, B_6, B_{12}, C, E, Nicotinate, Biotin, Folic acid and Pantothenic acid (Spolaore 2006). The pigments in microalgae include chlorophyll (0.5-1.5% of dry weight), carotenoids (0.1- 0.2%) and Phycobiliproteins (in Cyanobacteria and Red algae). The pigments from microalgae are natural colouring agents with antioxidant property.

1.4.2 Feed

Microalgae are utilized in aquaculture as live feeds for all growth stages of bivalve molluscs (e.g. oysters, scallops, clams and mussels), for the

larval/early juvenile stages of abalone, crustaceans and some fish species, and for zooplankton used in aquaculture food chains (Conceicao et al. 2010). PUFAs derived from microalgae, i.e. DHA, EPA and ARA are known to be essential for various larvae (Beelen et al. 2009). For salmonids microalgae impart colour to the flesh along with other nutritional benefits. *Chaetoceros calcitrans, Skeletonema costatum, Tetraselmis* sp., *Isochrysis galbana, Pavlova lutheri, Chlorella* sp. etc. are some of the commonly used microalgae in aquaculture. In rearing aquatic animals the "Green water" technique not only improves water quality by oxygen production, pH stabilization etc., but also regulates bacterial population, probiotic effect and stimulates immunity of the reared animals. Microalgae could also be used in the formulation of dry fish food for on-growing (Brown 2002).

Similarly, microalgae have also shown positive results in animal rearing as nutritional supplements. Incorporation of *Spirulina* in poultry diets showed high growth rates and low mortality. Algal diet showed an improvement in weight gain for pigs and similar positive effects were noticed for ruminants as well (Becker 2004). More over no adverse symptoms or unwanted side effects are reported so far in relation with microalgal consumption.

1.4.3 High Value Compounds

Microalgae are diverse in producing several primary and secondary metabolites with possible applications in food, feed, pharmaceutics and cosmetics (Yamaguchi 1997; Spolaore, Joannis-cassan, Duran, & Isambert, 2006). Pigments from microalgae – chlorophylls, carotenoids and phycobiliproteins have wide applications as natural colouring agents in dietary items as well as in cosmetics (Luísa Gouveia, Batista, et al. 2008). Similarly

microalgae are naturally rich in long chain polyunsaturated acids (LC-PUFAs) which are good antioxidants as well as required for the normal growth and functioning of nervous, cardio-vascular and endocrine systems. Major high value compounds from microalgae and their applications are given in table 1.1

Table: 1.1 List of high value compounds from microalgae and their applications (Guedes, Amaro, and Malcata 2011; Khozin-goldberg, Iskandarov, and Cohen 2011; Pulz and Gross 2004; Spolaore et al. 2006; Vílchez et al. 2011)

High value compound		Microalga source	Important use / activity
Pigments	Chlorophyll	*Chlorella* sp.	Natural colourant Vitamin A precursor (β-carotene) Antioxidant Prevent degenerative diseases Overall health promotion
	β-carotene	*Dunaliella salina*	
	Astaxanthin	*Haematococcus pluvialis*	
	Cantaxanthin	*H. pluvialis, C.vulgaris*	
	Lutein	*Scenedesmus almeriensis, H. pluvialis*	
	Phycocyanin	*Arthrospira platensis*	
	Phycoerythrin	*Porphyridium cruentum*	
LC-PUFAs	γ-linolenic acid (GLA)	*Arthrospira platensis*	Essential fatty acids Metabolic precursors Antioxidants Prevent degenerative diseases, Overall health promotion For nervous &cardiac function
	Arachidonic acid (ARA)	*Porphyridium cruentum*	
	Eicosapentaenoic acid (EPA)	*Nannochloropsis* sp., *Isochrysis galbana,*	
	Docosa hexaenoic acid (DHA)	*Thraustochytrium* sp. *Schizochytrium* sp. *Isochrysis galbana*	
Others	Vitamins (A, B complex, C and E)	*A. platensis, A. maxima*	Nutritional supplements Metabolic precursors Antioxidants Prevent degenerative diseases, Overall health promotion
	Aminoacids	*Arthrospira* spp., *Aphanizomenon* sp.	
	Antioxidants	Green and blue green algae	
	Antimicrobials	Blue green algae	
	Toxins	Blue green algae, brown algae, diatoms etc.	Developing assays Anti-tumour agents

1.4.4 Energy source

Microalgae are considered as the "Green gold of future" (Wolkers et al. 2011). Microalgae can be a suitable alternative feedstock for next generation biofuels because certain species (e.g. *Botryococcus braunii*) contain high amounts of oil, which could be extracted, processed and refined into

transportation fuels, using currently available technology (Gouveia and Oliveira 2009). The 'IIIrd generation' bio-fuel research based on microalgae continues with a search for, a) a new species, b) new techniques in harvesting & processing or c) a new genetic engineering technology to modify known species with high production rate. Microalgae can provide several different types of renewable, non-toxic and highly biodegradable biofuels, coupled with CO_2 sequestration - a CO_2-neutral fuel production (Schenk 2008). These include methane produced by anaerobic digestion of the algal biomass, biodiesel derived from microalgal oil and photobiologically produced biohydrogen (Yusuf Chisti 2007). Microalgae have fast growth rate, permit the use of non-arable land and non-potable water, use far less water and do not displace food crops cultures. Rather their production is not seasonal and they can be harvested daily. Furthermore the microalgal residue produced can be utilized either as manure or for the production of BTL (biomass to liquid), bio-ethanol and bio-methanol (Benemann 2000). However microalgal biodiesel has to be technically feasible and to be economically competitive with petrodiesel. For this, microalgal production, harvesting and extraction must be optimized, along with improvements to algal biology through genetic and metabolic engineering (Gouveia and Oliveira 2009).

1.4.5 Bioremediation:

The adaptability of the microalgae to polluted conditions is explored for bioremediation of bio/chemical pollutants (which is called phyco-remediation) (Olguı 2003). When combined with production of biofuel/feed/bioactive compounds the phycoremediation becomes an integrated biotechnological approach with less production cost and many byproducts.

Scope and Outline of Thesis

Microalgae biotechnology is still in its early life. As mentioned above, there are nearly 30,000 species in literature. However, only a few tens are characterized and explored for various applications. This gives a vast opportunity for a study and research in microalgae. Knowing the environmental and economic significance of this potential group, many institutions are now actively involved in microalgae research. All over the world, there are culture collections for microalgae (CCAP, CCMP, UTEX, etc.) who isolate, identify, characterize and preserve the algal diversity. In comparison with agriculture, algal culturing has got only 50 years of experience which denotes a great but unrealized potential (Peter Thompson, CSIRO). However in the last few decades the growth in algal biotechnology has shown a large progress, with novel strains, techniques and products.

In India having a good coastal line and inland water bodies, a research on microalgae was very important, especially when rarely an exclusive culture collection for microalgae was found in the country. Even though, many investigations have been published on the diversity aspects of phytoplankton, benthic algae etc. (Sanilkumar 2009; Sanilkumar et al. 2009; Subrahmanyan, Gopinathan, and Pillai 1971, Gopinathan 1983), a study on isolated cultures of microalgae from Indian coast remains novel. Further a combined approach of morphology and molecular phylogeny for taxonomic elucidation in microalgae was rarely found from the country. To taxonomically characterize the new isolates of microalgae, after morphological evaluation (Chapter 2), molecular identification and phylogeny were tried using different genetic markers (Chapter 3 Part I).

Current systematics recommends a polyphasic approach in taxonomic identification, which is more effective and accurate than conventional methods or a single way of approach (Bock, Krienitz, and Pröschold 2011; Margheri et al. 2003). Classic taxonomic treatments generally take account of, morphology, physiology and to some extend chemotaxonomy (Aslam et al. 2007). Whereas, modern system of molecular identification consider only genetic traits (gene sequence, size, orientation etc.), which are free from developmental and reproductive behaviors. Molecular tools are comparatively easy and do not demand expertise in microalgae morphology. However, wrong interpretations and incorrect submissions in Genebank raised a confusion in microalgae taxonomy (Borowitzka and Siva 2007), which can be resolved only by a polyphasic approach – a combination of different tools – morphology, physiology, biochemistry and genetics. Such an attempt was made in this study, to taxonomically characterize Indian strains of *Dunaliella* which got published in *Aquatic Biosystems* (Preetha et al., 2012). The original report is also reproduced in the thesis as Chapter 3 Part II.

Aquaculture is one of the fastest growing sectors in the world with about 6.6 x 10^7 tonnes production per year (FAO 2014). An aquaculture system always demands continuous supply of nutritionally rich microalgae as feed at primary level. Moreover, the growth, development and stability of the reared animals directly depend on the quality and quantity of these live feeds. Hence before feeding trials, it is necessary to know the nutrient values of the isolates. Many of the new isolates of microalgae were traditional live feed species, and nutrient profile of those strains was important before employing them in a system as larval diets. Hence, selected common strains were evaluated for important nutrients (protein, lipid, fatty acids and carotenoids) for possible use in aquatic animal feeding (Chapter 4).

Bioprospecting of microalgae for possible utilization in human diet is greatly upcoming (Luisa Gouveia et al. 2006; Luísa Gouveia, Batista, et al. 2008; Luísa Gouveia, Coutinho, et al. 2008). *Spirulina* and *Chlorella* have been there in common use for human consumption and animal growing across the world. Recent studies of Gouveia et al., (2006 & 2008) identified other possible strains like *Isochrysis galbana* for human dietary application. Owing to their high nutritional values and several supplementary heath promoting functions, microalgae can be a novel dietary supplement, convenient for all age groups. In this thesis, five nutritionally rich strains (*Isochrysis galbana, Nannochloropsis oceanica, Tetraselmis* sp., *Dunaliella salina* and *Chaetoceros gracilis*) were tried as an ingredient in normal butter cookies and evaluated the sensory as well as nutriotional qualities (Chapter 5).

Keeping in mind the requirment of present day society and the institute (CMFRI) the follwing objectives were under taken for the present study:

- To isolate native microalgae from Indian coast (marine, brackish and hyper saline) and characterize them morphologically and biochemically (phenetic characterization)
- To identify or standardize laboratory culture techniques (including purification and preservation) for specific microalgae
- To categorize microalgae using specific genetic markers (molecular taxonomy) and to describe their evolutionary relationship (molecular phylogeny) within a genus or class
- To identify a better genetic marker for microalgae identification
- To recognize potential strains for aquaculture feeding by nutrient (protein, fatty acids, carotenoids) profiling
- To mass culture, harvest and utilize the biomass in human diet as functional food.

Chapter 2

ISOLATION AND CULTURE PRESERVATION OF MICROALGAE FROM INDIAN COAST

Contents

Abstract

Microalgae, the tiny photosynthetic organisms fascinated the world in recent years with their commercial possibilities (food, feed, fuel, fertilizer, bioactive molecules etc.) along with an environmental balancing role (e.g. CO_2 sequestration). India having a vast coastline and immense inland water bodies (saline, hyper-saline, brackish and fresh water) harbouring a rich diversity of microalgae, however least explored for developing an exclusive culture collection of microalgae and for bioprospecting examine. Present chapter deals with the isolation, purification, culturing and preservation of microalgae from Indian coast, and development of a culture collection of marine microalgae for possible use at applied level. Microalgae were isolated using standard protocols (serial dilution, agar plating etc.) from water samples collected from selected 23 locations across Indian coast. After purification, all stable strains were maintained along with a few other pure species collected from various sources (culture collections, universities, hatcheries etc.). After preliminary morphology, isolates were assigned with strain code and maintained under standard culture conditions. Selected strains were attempted for antibiotic purification and for short term (in/on agar) and long term (cryo) preservation. From about 170 isolates obtained (isolated + procured), 136 pure stable strains are currently maintained in the collection. Strains belonging to 7 different classes were identified morphologically including diatoms, green algae, haptophytes and blue green algae. Out of 40 selected strains only 17 strains (including Isochrysis galbana, Dunaliella salina, Chlorella vulgaris, Picochlorum sp., Nannochloropsis oceanica, Synchocystis sp. and Chaetoceros sp.) got purified by single treatment of a cocktail of antibiotics (24/48h treatment). All short term and long term preservations were 100% successful for coccoid green and blue green algae. Agar embedding and agar plating were successful for up to 3 years in 40% and 88% of attempted 25 strains respectively (green & blue green algae and rarely diatoms). Cryopreservation with DMSO 10% was most stable for up to 1 year in <10% strains (total) majority being green & blue green algae only. Diversity aspects of the microalgae along Indian coast were also discussed on a preliminary basis.

2.1 Introduction

Value of marine microalgae is increasing day by day for an array of applications including human/animal nutrition and health (Spolaore et al. 2006), CO_2 sequestration (Cheng et al. 2006) and energy production (Kais, Chowdhury, and Shahriar 2011). Several microalgae culture collections function all over the world (e.g. CCAP, CCMP, UTEX, etc.) with an aim to identify and preserve the algal diversity as well as to support research on/in microalgae. In about 40 culture collections (16 countries) there are approximately 11,000 strains belonging to 3000 species are maintained (Watanabe 2005). However, this remains only less than 10% of the total microalgal global diversity and the balance is still there to be catalogued for various biological and biochemical applications (Medlin and Töbe 2011).

India has a long coastline of about 8129 km and has immense inland (fresh to hyper saline) water bodies with a huge biodiversity, of which microalgae share a larger fraction. Pioneers like Prof. T V Desikachari (1919-2005; Indian Council of Agricultural Research) have contributed much in cataloguing microalgal diversity of India (Sanilkumar 2009). However, at application side, only handfuls of microalgae are in use, particularly restricted in aqua-feeding. Hence, our focus was to have more number of isolates from native saline habitats, and to develop a collection of indigenous strains for particular use in food/feed or any other area where the organism may fit in.

Concepts on algal culturing technique was developed in late 19th and early 20th centuries (Preisig and Andersen 2005) and expanded a lot in last 50 years; still in search for better technological solutions in culturing and harvesting of microalgae. Around the world several small groups are working on finding solutions to such problems (Thompson, n.d.). In this chapter,

important aspects related to the culture protocols have been discussed, including the difficulties emerged during the development of native live microalgae collection.

The first important task is the isolation of single cell/species from a consortium of about million/ml (water sample) cells. Several methods can be applied for this like, a) traditional microbial techniques like dilution or plating, b) typical algal method like micropipette purification or c) advanced automated flow cytometer based single cell separation (Parvin, Zannat, and Habib 2007; Thompson, n.d.; Andersen 2005).

Identification and laboratory growing of the new isolates are the second and third steps involved. Morphological identification remains a most used identification tool. Lack of common taxonomic keys and morphological plasticity are the major constraints in phenotypic taxonomy of microalgae. Hence, presently molecular methods are also applied in par with the structural evaluations. In this chapter only morphological identification of the strains is discussed and molecular taxonomy in Chapter 3.

Once cultures are developed, removal of unwanted bacteria and other harmful microbes is necessary for the stability of the cultures. An axenic culture (a pure culture free from all kind of microbes) is particularly important when the high value compounds are aimed through mass culturing. Generally a treatment with antibiotics may help the removal of all problematic microbes (Jones, Rhodes, and Evans 1973). As can be expected, the antibiotics - their concentration and duration of treatment are significant, because many strains are susceptible to certain antibiotics in high doses. A handful of microalgae were attempted for axenisation by antibiotic treatments and the results are discussed in later half of this chapter.

Maintenance of pure cultures has always been a bottle neck (Andersen 2005). Serial periodic culturing is labour-intensive and also may change natural properties of the organism owing to genetic drift (Gwo et al. 2005; Abreu et al. 2012). Most of the repositories depend on long term (5-15 years) cryopreservation which is an emerging field and promise different freezing protocols with different types of cryoprotectants (DMSO, glycerol, methanol etc.) (Day & Brand 2005). For those strains which cannot survive cool-thaw stresses, short term (1-5 years) agar based methods are adopted. Agar plating is an old practice generally used for isolation and culture maintenance. Second half of the chapter also includes a description on different preservation protocols and their positive and negative phases on selected microalgae representing different classes.

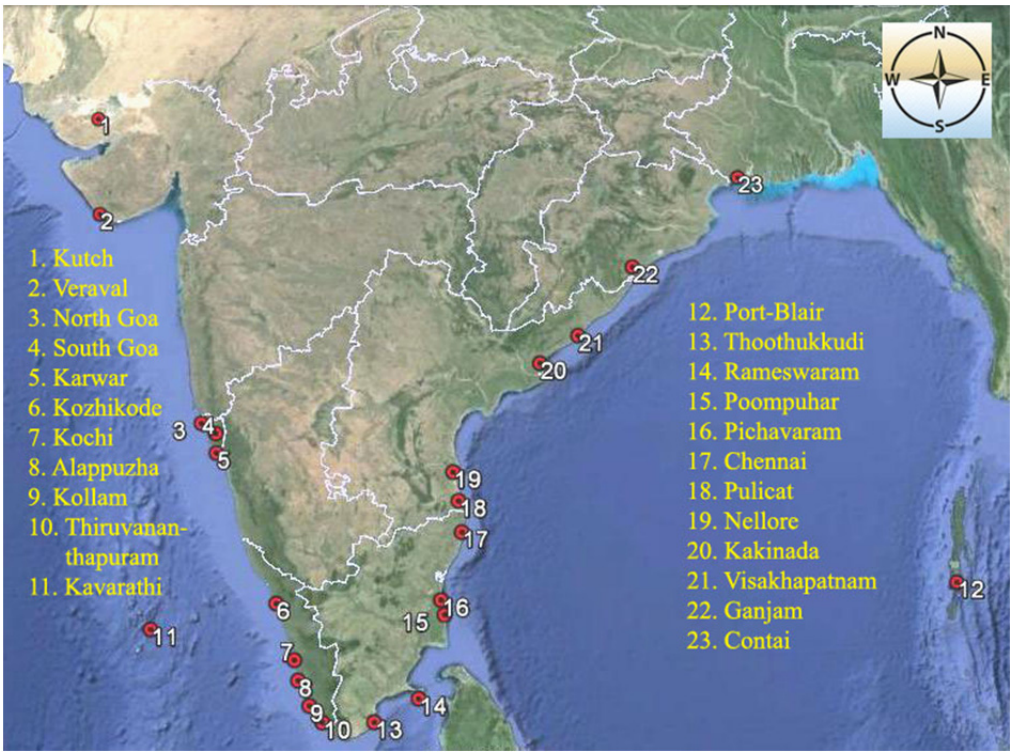


Figure 2.1: Image showing the sampling locations along west and east coasts of India

Initial aim of the work was to have a collection of indigenous strains of marine microalgae for possible use in aquaculture feeding, for the reason that this fast growing sector demands continuous supply of high quality live feeds. As the work progressed with isolation and characterisation, new isolates came out with probable use in diverse segments (food, fuel, nutraceutics, cosmetics etc.). The whole chapter corresponds to various segments of culture practices – isolation, laboratory culturing, morphological identification, purification and preservation of those microalgae retrieved from saline habitats.

2.2 Materials and Methods

All the protocols used for isolation, purification, culturing and preservation were widely adopted from Anderson's (2005) "Algal Culturing Techniques" and Coutteau (1996). Morphological identification was done referring the monographs and keys (e.g. Tomas 1997, Desikachari 1959, Lee 2008, Gopinathan 2002) etc.) or online databases (e.g. www.algaebase.com, Wikipedia – the free encyclopedia (https://en.wikipedia.org), Encyclopedia of life (http://eol.org/), http://www.boldsystems.org etc.).

2.2.1 Water Sample Collection and Isolation

Locations were selected covering almost entire coastal India (Figure 2.1, Table 2.1) and only water samples were collected for isolation of algae. These water samples were either concentrated (ten times) by filtration using 50-200 μm mesh size bolting silk filters or enriched (adding media) for a couple of weeks. Isolation was performed aseptically by (a) serial dilution, (b) agar plating or (c) micropipette purification (Figures 2.2 & 2.3) (Gopinathan 2005). Sterile solid (agar 1-1.5%) and liquid media were prepared with

suitable salinity. Agar plates were prepared in disposable petri-plates and after sample plating, these petri-plates were sealed with parafilm to prevent dehydration of agar. All dilution tubes and plates were incubated for 2-10 weeks for colour development or colony appearance on illuminated racks.

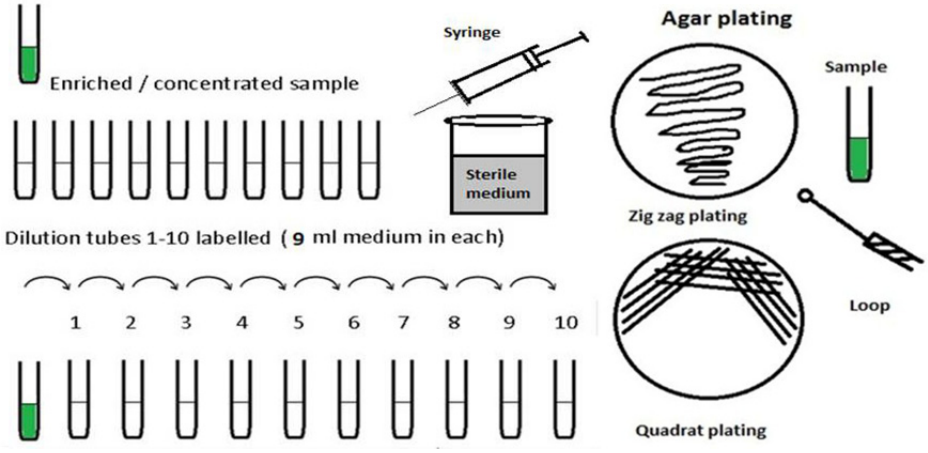


Figure 2.2 & 2.3: Diagrammatic representation of serial dilution – an isolation technique for microalgae. Diagrammatic representation of agar plating isolation

Other than isolation of new strains, a few pure monoalgal cultures (31 strains) were procured from CMFRI live feed cultures (Kochi, Chennai, Thoothukkudi and Kozhikode centres), Antenna trust (Madurai, Tamilnadu), Andhra University (Andhrapradesh), Aquaculture farms and CSIRO Microalgae culture collection (Australia).

Table 2.1 List of sampling sites with details of habitat, geographical co-ordinates and month of collection

Site of sample collection	Location	Habitat, salinity	Geographical Co-ordinates	Date of sample collection
		West coast		
Kutch salt pan	Kutch, Gujarat	Hyper saline, 180 ppt	23°50' N, 69°39' E	February and April, 2010
Veraval beach	Veraval, Gujarat	Marine, 33 ppt	20°90' N, 70°37' E	February and April, 2010
Ribandar salt pan	North Goa, Goa	Hyper saline, 280 ppt	15°30' N, 73°51' E	May, 2010
Pilar salt pan		Hyper saline, 260 ppt	15°26' N, 73°53' E	May, 2010
Vainguinin beach (Dona-paula)		Marine, 33 ppt	15°45' N, 73°80' E	May, 2010
Miramer beach		Marine, 34 ppt	15°48' N, 73°80' E	May, 2010
Betul Beach	South Goa	Marine, 34 ppt	15°14' N, 73°94' E	May, 2010
Karwar beach	Karwar, Karnataka	Marine, 32 ppt	14°80' N, 74°12' E	March, 2010
Kamburam (West hill) beach	Kozhikode, Kerala	Marine, 33-35 ppt	11°27' N, 75°76' E	May - December, 2010
Aquaculture pond		Brackish, 5-16 ppt	11°27' N, 75°76' E	January & February 2011
Vypeen Puthuvype (Harbour)	Kochi, Kerala	Marine, 28-30 ppt	9°98' N, 76°24' E	Feb - Nov, 2010
Marine jetty		Brackish, 8-20 ppt	9°96' N, 76°28' E	Feb - Nov, 2010
Satar Island		Brackish, 10-18 ppt	10°18' N, 76°17' E	Feb - Nov, 2010
Vypeen barmouth		Marine, 33-38 ppt	9°97' N, 76°24' E	Feb - Nov, 2010
Narakkal beach		Marine, 35-38 ppt	10°01' N, 76°20' E	Feb - Nov, 2010
Aquaculture farms, Malippuram		Brackish, 10-15 ppt	10°01' N, 76°22' E	Feb - Nov, 2010
Mangalavanam Mangroves		Brackish, 5-15 ppt	9°98' N, 76°27' E	Feb - Nov, 2010
Thattappally spill way	Alappuzha, Kerala	Brackish, 22 ppt	9°31' N, 76°38' E	Januaray, 2011
Andhakaranazhi beach		Marine, 35 ppt	9°74' N, 76°28' E	May, 2013
Ashtamudi lake	Kollam, Kerala	Brackish, <15 ppt	8°94' N, 76°55' E	February, 2010
Neendakara harbour		Marine, 32 -33 ppt	8°94' N, 76°53' E	February, 2010
Vizhinjam harbour	Thiruvananthapuram, Kerala	Marine, 32 -33 ppt	8°37' N, 76°99' E	November, 2010
Veli beach		Marine, 32 -35 ppt	8°51' N, 76°88' E	November, 2010
Kavarathi sea	Lakshadweep Islands	Marine, 37 ppt	10°57' N, 72°63' E	February, 2010

Site of sample collection	Location & State	Habitat, salinity	Geographical Co-ordinates	Date of sample collection
		East Coast		
Port-blair sea	Andaman & Nicobar Islands	Marine, 36 ppt	11°62' N, 92°75' E	October, 2010
Mullakkad salt pan	Thoothukkudi, Tamilnadu	Hyper saline, 300ppt	8°71' N, 78°12' E	February, 2010
Beach Road salt pan		Hyper saline, 150 ppt	8°77' N, 78°15' E	April, 2011
Tuticorin Bay		Marine, <25 ppt	8°78' N, 78°16' E	April, 2011
Pamban beach (near bridge)	Rameswaram, Tamilnadu	Marine, 34 ppt	9°28' N, 79°20' E	February, 2011
Thonidurai beach		Marine, 33 ppt	9°28' N, 79°12' E	February, 2011
Poompuhar beach	Poompuhar, Tamilnadu	Marine, 32 ppt	11°13' N, 79°85' E	Januaray, 2011
Pichavaram mangroves	Pichavaram, Tamilnadu	Brackish, >10 ppt	11°43' N, 79°79' E	Januaray, 2011
Adayar beach	Chennai, Tamilnadu	Marine, 30 ppt	13°01' N, 80°27' E	March, 2010
Kovalam beach		Marine, 32 ppt	12°79' N, 80°25' E	March, 2010
Kelambakkam salt pan		Hyper saline, >100 ppt	12°77' N, 80°22' E	March, 2010
Kovalam salt lake		Hyper saline, 50 ppt	12°79' N, 80°24' E	March, 2010
Pulicat hyper saline lake	Pulicat, Andhrapradesh	Hyper saline, 98-150 ppt	13°72' N, 80°13' E	March, 2010
Pulicat saline canal		Saline, 28 ppt	13°72' N, 80°16' E	March, 2010
Rice field		Fresh water, <5 ppt	13°70' N, 80°07' E	March, 2010
Krishnapatnam salt pan	Nellur, Andhrapradesh	Hyper saline, >100 ppt	14°26' N, 80°10' E	May and October, 2010
Muthukur salt pan		Hyper saline, >100 ppt	13°36' N, 78°62' E	May and October, 2010
Kakinada salt pan	Kakinada, Andhrapradesh	Hyper saline, 90 ppt	16°96' N, 82°27' E	May and October, 2010
Ramakrishna beach	Visakhapatanam, Andhrapradesh	Marine, 30 ppt	17°71' N, 83°32' E	May and October, 2010
Bheemili salt pan		Hyper saline, 90 ppt	17°89' N, 83°42' E	May and October, 2010
Ganjam salt pan	Ganjam, Orissa	Hyper saline, 65 ppt	19°38' N, 85°07' E	May, 2010
Kontai salt pan	Kontai, West bengal	Hyper saline, >70 ppt	21°77' N, 87°75' E	May, 2010

2.2.2 Culturing of Microalgae

The f/2 media (Appendix 2.1) was used both for enrichment, isolation and culturing of all saline isolates including brackish water strains, whereas for fresh water strains F media (f/2 in double concentration) was used. For growing

Arthrospira (*Spirulina*) spp. Paoletti media (Volkmann et al. 2008) (Appendix 2.2) was selected.

Figure 2.4: Microalgae culture room; pure cultures maintained on illuminated wooden racks with temperature adjusted to 24±1 °C (above); pure cultures in conical flasks (below)

Standard culture conditions were maintained for all cultures with 24±1°C temperature and circadian light:dark cycle (12:12 or 8:16 hrs) of 2000 - 3000 Lux light intensity (white fluorescent lamps) (Figure 2.4). Periodic sub culturing in 3 to 8 weeks interval (based on the species) was performed and purity checking was carried out regularly under microscope. All isolates were maintained in 100 ml conical flasks with 75ml culture volume corked with non-absorbent cotton plugs. Acclimated stable isolates were assigned with strain codes (e.g. *Isochrysis galbana* MBTD-CMFRI-S001, Table 2.2) after morphological examination for identification.

Table 2.2: List of microalgae present in MBTD-CMFRI Culture Collection of Marine Microalgae, strain code, name and isolation details are included.

Class - Bacillariophyceae			
Strain code	**Morphological identification**	**Isolated from**	**Isolation method**
MBTD-CMFRI-S005	*Chaetoceros calcitrans*	CMFRI old culture	.....
MBTD-CMFRI-S018	*Cyclotella* sp.	Vypeen barmouth (WC)	Agar plating
MBTD-CMFRI-S019	*Thalassiosira* sp.	Mangalavanam (WC)	Agar plating
MBTD-CMFRI-S021	*Nitzschia* sp.	Vypeen barmouth (WC)	Agar plating
MBTD-CMFRI-S024	*Chaetoceros* sp.	Tuticorin Bay (EC)	Serial dilution
MBTD-CMFRI-S031	*Chaetoceros* sp.	Satar Island (WC)	Serial dilution
MBTD-CMFRI-S033	*Thalassiosira* sp.	Mangalavanam (WC)	Serial dilution
MBTD-CMFRI-S037	Pennate Diatom	Malippuram (WC)	Agar plating
MBTD-CMFRI-S038	Pennate Diatom	Vypeen barmouth (WC)	Agar plating
MBTD-CMFRI-S039	Pennate Diatom	Malippuram (WC)	Agar plating
MBTD-CMFRI-S042	*Chaetoceros* sp.	Vypeen barmouth (WC)	Serial dilution
MBTD-CMFRI-S043	*Navicula transitans*	Vypeen barmouth (WC)	Serial dilution
MBTD-CMFRI-S044	*Cyclotella* sp.	Marine jetty (WC)	Agar plating
MBTD-CMFRI-S045	*Thalassiosira* sp.	Mangalavanam (WC)	Agar plating
MBTD-CMFRI-S049	*Skeletonema* sp.	Marine jetty (WC)	Micropipette purification
MBTD-CMFRI-S050	*Minutocellus* sp.	Vizhinjam beach (WC)	Agar plating
MBTD-CMFRI-S051	*Thalassiosira* sp.	Veli beach (WC)	Agar plating
MBTD-CMFRI-S052	*Cyclotella* sp.	Veli beach (WC)	Agar plating
MBTD-CMFRI-S058	Pennate Diatom	Veli beach (WC)	Agar plating
MBTD-CMFRI-S060	*Navicula* sp.	Veli beach (WC)	Agar plating
MBTD-CMFRI-S061	*Cylindrotheca* sp.	Betul beach (WC)	Micropipette purification
MBTD-CMFRI-S062	*Chaetoceros* sp.	CMFRI old culture	Serial dilution
MBTD-CMFRI-S065	*Chaetoceros* sp.	Narakkal beach (WC)	Serial dilution
MBTD-CMFRI-S069	*Thalassiosira* sp.	Neendakara beach (WC)	Serial dilution
MBTD-CMFRI-S079	*Cyclotella* sp.	Poompuhar beach (EC)	Agar plating
MBTD-CMFRI-S080	*Cyclotella* sp.	Poompuhar beach (EC)	Agar plating
MBTD-CMFRI-S084	*Thalassiosira* sp.	Kovalam, Chennai (WC)	Serial dilution
MBTD-CMFRI-S088	*Minutocellus* sp.	Kavarathi, (WC)	Agar plating
MBTD-CMFRI-S090	Pennate Diatom	Poompuhar beach (EC)	Agar plating
MBTD-CMFRI-S092	*Nitzschia* sp.	Pulicat canal (EC)	Agar plating
MBTD-CMFRI-S099	*Nitzschia* sp.	Kelambakkaom* (EC)	Agar plating
MBTD-CMFRI-S117	*Navicula* sp.	Vypeen barmouth (WC)	Agar plating
MBTD-CMFRI-S131	Pennate Diatom	Adayar beach (EC)	Agar plating
MBTD-CMFRI-S132	*Thalassiosira* sp.	Miramar beach (WC)	Agar plating
MBTD-CMFRI-S136	*Navicula* sp.	Kamburam beach (WC)	Agar plating
MBTD-CMFRI-S148	*Nitschia* sp.	Poompuhar beach (EC)	Agar plating
MBTD-CMFRI-S150	Pennate Diatom	Kamburam beach (WC)	Agar plating
MBTD-CMFRI-S158	*Thalssiosira* sp.	Thottappally (WC)	Serial dilution
MBTD-CMFRI-S167	*Bellerochea* sp.	Kamburam beach (WC)	Serial dilution
MBTD-CMFRI-S168	*Melosira* sp.	Pamban beach (EC)	Serial dilution
MBTD-CMFRI-S172	*Chaetoceros gracilis*	Andhakaranazhi (WC)	Agar plating
MBTD-CMFRI-S175	*Skeletonema* sp.	Abad Farm	**

Class – Eustigmatophyceae			
Strain code	**Morphological identification**	**Isolated from**	**Isolation method**
MBTD-CMFRI-S006	*Nanochloropsis* sp.	CMFRI old culture	…….
MBTD-CMFRI-S007	*Nanochloropsis* sp.	CMFRI old culture	…….
MBTD-CMFRI-S008	*Nanochloropsis* sp.	CMFRI old culture	…….
MBTD-CMFRI-S012	*Nanochloropsis* sp.	CMFRI old culture	…….
MBTD-CMFRI-S014	*Nanochloropsis* sp.	CMFRI old culture	…….
MBTD-CMFRI-S015	*Nanochloropsis* sp.	CMFRI old culture	…….
MBTD-CMFRI-S076	*Nanochloropsis* sp.	CMFRI old culture	…….
MBTD-CMFRI-S077	*Nanochloropsis* sp.	CMFRI old culture	…….
MBTD-CMFRI-S078	*Nanochloropsis* sp.	CMFRI old culture	…….

Class - Prymnesiophyceae			
Strain code	**Morphological identification**	**Isolated from**	**Isolation method**
MBTD-CMFRI-S001	*Isochrysis galbana*	CMFRI old culture	…….
MBTD-CMFRI-S002	*Isochrysis galbana*	CMFRI old culture	…….
MBTD-CMFRI-S003	*Ochrosphaera* sp.	CMFRI old culture	…….
MBTD-CMFRI-S004	*Dicrateria* sp.?	CMFRI old culture	…….
MBTD-CMFRI-S073	*Isochrysis galbana*	CMFRI old culture	…….
MBTD-CMFRI-S106	*Isochrysis galbana*	CMFRI old culture	…….
MBTD-CMFRI-S119	*Prymnesium parvum*	Vypeen Barmouth (WC)	Serial dilution
MBTD-CMFRI-S157	*Isochrysis galbana*	Andaman & Nicobar (EC)	Serial dilution
MBTD-CMFRI-S169	*Isochrysis galbana*	Vypeen Harbour (WC)	Serial dilution
MBTD-CMFRI-S173	*Isochrysis galbana*	Andhakaranazhi farm culture	**

Class - Prasinophyceae			
Strain code	**Morphological identification**	**Isolated from**	**Isolation method**
MBTD-CMFRI-S011	*Tetraselmis gracilis?*	CMFRI old cultue	…….
MBTD-CMFRI-S027	*Tetraselmis* sp.	Satar island (WC)	Agar plating
MBTD-CMFRI-S028	*Tetraselmis* sp.	Vypeen barmouth (WC)	Agar plating
MBTD-CMFRI-S057	*Tetraselmis* sp.	Vypeen harbour (WC)	Agar plating
MBTD-CMFRI-S075	*Tetraselmis gracilis?*	CMFRI old cultue	…….
MBTD-CMFRI-S081	*Tetraselmis* sp.	Ashtamudi lake (WC)	Agar plating
MBTD-CMFRI-S082	*Tetraselmis* sp.	Poompuhar baech (EC)	Agar plating
MBTD-CMFRI-S093	*Tetraselmis* sp. (H.S)	Pulicat lake (EC)	Serial dilution
MBTD-CMFRI-S094	*Tetraselmis* sp. (H.S)	Pulicat salt pan (EC)	Serial dilution
MBTD-CMFRI-S097	*Tetraselmis* sp. (H.S)	Krishnapatanam (EC)	Serial dilution
MBTD-CMFRI-S101	*Tetraselmis* sp.	Kelambakkam* (EC)	Serial dilution
MBTD-CMFRI-S126	*Tetraselmis* sp. (H.S)	Pilar salt pan (WC)	Serial dilution
MBTD-CMFRI-S127	*Tetraselmis* sp. (H.S)	Pilar salt pan (WC)	Serial dilution
MBTD-CMFRI-S142	*Tetraselmis* sp. (H.S)	Kakinada salt pan (EC)	Serial dilution
MBTD-CMFRI-S143	*Tetraselmis* sp. (H.S)	Bheemili salt pan (EC)	Serial dilution

Class - Trebouxiophyceae			
Strain code	**Morphological identification**	**Isolated from**	**Isolation method**
MBTD-CMFRI-S026	*Chlorella* sp.	Narakkal beach(WC)	Agar plating
MBTD-CMFRI-S029	Nanoplankton	Vypeen Barmouth (WC)	Agar plating
MBTD-CMFRI-S030	Nanoplankton	Vypeen Barmouth (WC)	Agar plating
MBTD-CMFRI-S048	Nanoplankton	Marine Jetty (WC)	Agar plating
MBTD-CMFRI-S056	Nanoplankton	Marine Jetty (WC)	Agar plating
MBTD-CMFRI-S070	Nanoplankton	Marine Jetty (WC)	Agar plating
MBTD-CMFRI-S071	*Chlorella* sp.	Marine Jetty (WC)	Agar plating
MBTD-CMFRI-S072	*Chlorella* sp.	Marine Jetty (WC)	Agar plating
MBTD-CMFRI-S083	Nanoplankton	Kamburam beach (WC)	Agar plating
MBTD-CMFRI-S095	*Chlorella* sp.	Pulicat canal (EC)	Agar plating
MBTD-CMFRI-S102	Nanoplankton	Narakkal (WC)	Agar plating
MBTD-CMFRI-S129	*Dictyosphaerium* sp. (FW)	Marine Jetty (WC)	Serial Dilution
MBTD-CMFRI-S134	Nanoplankton	Ramakrishna beach (EC)	Agar plating
MBTD-CMFRI-S138	Green Chlorophyte (BW)	Culture pond, Kozhikode (WC)	Agar plating
MBTD-CMFRI-S144	Nanoplankton	Dona-Paula beach (WC)	Agar plating
MBTD-CMFRI-S146	Nanoplankton	Vypeen Harbour (WC)	Agar plating
MBTD-CMFRI-S155	Nanoplankton	Ramakrishna beach (EC)	Agar plating
MBTD-CMFRI-S164	Nanoplankton	Pamban beach(EC)	Agar plating
MBTD-CMFRI-S170	Nanoplankton	Ramakrishna beach (EC)	Agar plating
MBTD-CMFRI-S171	*Chlorella vulgaris* (FW)	CMFRI campus pool (WC)	Agar plating

Class - Chlorophyceae			
Strain code	**Morphological identification**	**Isolated from**	**Isolation method**
MBTD-CMFRI-S086	*Dunaliella* sp. (H.S)	Mullakkad salt pan (EC)	Serial dilution
MBTD-CMFRI-S089	*Dunaliella* salina (H.S)	CMFRI old culture	.....
MBTD-CMFRI-S096	*Dunaliella* sp. (H.S)	Muthukur salt pan (EC)	Serial dilution
MBTD-CMFRI-S108	*Dunaliella* sp. (H.S)	Pure culture, Andhra University	**
MBTD-CMFRI-S109	*Dunaliella* sp. (H.S)	Pure culture, Andhra University	**
MBTD-CMFRI-S110	*Dunaliella* sp. (H.S)	Pure culture, Andhra University	**
MBTD-CMFRI-S111	*Dunaliella* sp. (H.S)	Pure culture, Andhra University	**
MBTD-CMFRI-S115	*Dunaliella* sp. (H.S)	Kelambakkom salt pan (EC)	Serial dilution
MBTD-CMFRI-S118	*Dunaliella* sp. (H.S)	Muthukur salt pan (EC)	Serial dilution
MBTD-CMFRI-S121	*Dunaliella* sp. (H.S)	Pulicat (EC)	Serial dilution
MBTD-CMFRI-S122	*Dunaliella* sp. (H.S)	Ribandar salt pan (WC)	Serial dilution
MBTD-CMFRI-S123	*Dunaliella* sp. (H.S)	Ribandar salt pan (WC)	Serial dilution
MBTD-CMFRI-S124	*Dunaliella* sp. (H.S)	Pilar salt pan (WC)	Serial dilution
MBTD-CMFRI-S125	*Dunaliella* sp. (H.S)	Pilar salt pan (WC)	Serial dilution
MBTD-CMFRI-S133	*Dunaliella* sp. (H.S)	Kutch salt pan (WC)	Serial dilution
MBTD-CMFRI-S135	*Dunaliella salina*	Kamburam beach (WC)	Serial dilution
MBTD-CMFRI-S147	*Dunaliella* sp. (H.S)	Kutch salt pan (WC)	Serial dilution
MBTD-CMFRI-S151 (CS-256)	*Dunaliella salina* (H.S)	CSIRO, Australia, pure culture	**
MBTD-CMFRI-S166	*Dunaliella* sp. (H.S)	Beach road salt pan (EC)	Serial dilution
MBTD-CMFRI-S130	*Chlorella* sp. (FW)	Pulicat rice field (EC)	Agar plating
MBTD-CMFRI-S139	*Monoraphidium* sp. (BW)	Culture pond, Kozhikode (WC)	Agar plating

Class - Cyanophyceae			
Strain code	**Morphological identification**	**Isolated from**	**Isolation method**
MBTD-CMFRI-S010	*Synechocystis* sp.	CMFRI old culture	…….
MBTD-CMFRI-S016	*Arthrospira platensis*	Antenna trust, Madurai	**
MBTD-CMFRI-S034	Unidentified BGA	CMFRI old culture	…….
MBTD-CMFRI-S041	Unidentified BGA	Vypeen barmouth (WC)	Agar plating
MBTD-CMFRI-S047	Unidentified BGA	Marine Jetty (WC)	Agar plating
MBTD-CMFRI-S091	Unidentified BGA	Pulicat canal (EC)	Agar plating
MBTD-CMFRI-S098	*Cyanothece* sp. (H.S)	Krishnapatanam (EC)	Serial dilution
MBTD-CMFRI-S100	*Oscillatoria* sp. (H.S)	Pulicat lake (EC)	Micropipette purification
MBTD-CMFRI-S107	*Synechocystis* sp	CMFRI old culture	…….
MBTD-CMFRI-S120	*Cyanothece* sp. (H.S)	Ganjam salt pan (EC)	Serial dilution
MBTD-CMFRI-S137	*Oscillatoria* sp.	Ganjam salt pan (EC)*	Micropipette purification
MBTD-CMFRI-S141	Unidentified BGA (BW)	Culture pond, Kozhikode (WC)	Agar plating
MBTD-CMFRI-S152 (CS-328)	*Arthrospira maxima*	CSIRO, Australia, pure culture	**

Unidentified green algae			
Strain code	**Morphological identification**	**Isolated from**	**Isolation method**
MBTD-CMFRI-S113	Unidentified green (BW)	Narakkal beach (WC)	Agar plating
MBTD-CMFRI-S145	Unidentified green (BW)	Narakkal beach (WC)	Agar plating
MBTD-CMFRI-S165	Unidentified green alga	Poompuhar beach (EC)	Agar plating
Unidentified			
MBTD-CMFRI-S055	Unidentified Pink	Vizhinjam beach (WC)	Serial dilution
MBTD-CMFRI-S159	Unidentified pink flagellate	Vypeen Harbour (WC)	Serial dilution

All strains are marine, otherwise mentioned in brackets. HS - Hyper saline; FW – Fresh water; BW – Brackish water; WC – West coast; EC – East coast
*Saline pool near salt pan with lower salinity (<45 ppt)
** Strains procured or collected from culture collections, universities or aquaculture farms.

2.2.3 Microscopy and Morphological Examination

Live algal cells were examined using a Nikon 80i Research microscope (Nikon, Japan) with DIC (differential interference contrast) optics and images were captured using Nikon DSFi 1e camera. Key taxonomic features like cell size, shape, and colour, cell coverings, length & number of flagella, characteristics of stigma, pyrenoid and chloroplast, etc. were studied. Scalar measurements such as cell length and width, were taken from each strain randomly, immediately after fixing the cells with 1% Lugol's iodine. Details of each strain were recorded separately on data sheets.

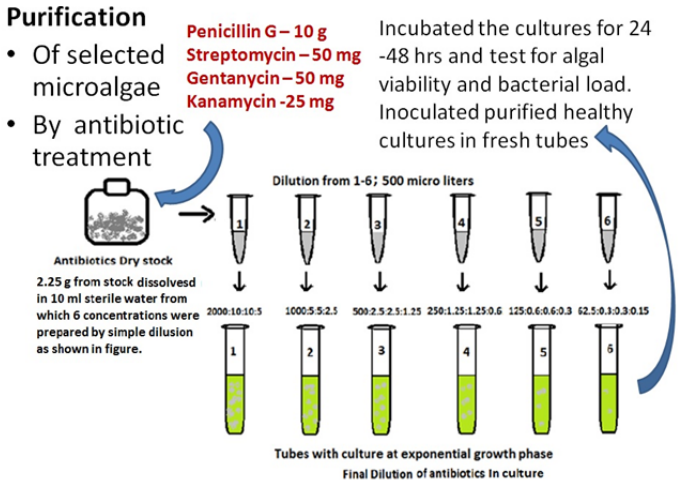


Figure 2.5: Antibiotic purification protocol - a diagrammatic representation

2.2.4 Antibiotic Treatment for Purification

Only 40 selected marine strains (Table 2.3) from different groups were attempted for purification using antibiotics. Protocol followed (Figure 2.5) was modified from Droop (1967) which is simple, convenient and suitable for flagellates and smaller species of microalgae (Guillard 2005). Dry stock of antibiotics – Penicillin G (10 g), Streptomycin (50 mg), Gentamycin (50 mg) and Kanamycin (25 mg) was weighed mixed and stored. To prepare cocktail of antibiotics, 2.25 g was weighed from stock and dissolved in 10 ml sterile water and refrigerated. Fully grown test cultures (duplicates) were treated with 6 concentrations of the antibiotics, which were prepared by simple dilution method (1, ½, ¼, ⅛, $^{1}/_{16}$ and $^{1}/_{32}$). Final concentration of the antibiotics (P:S:G:K) was 2000:10:10:5 / 1000:5:5:2.5 / 500:2.5:2.5:1.25 / 250:1.25:1.25:0.6 / 125:0.6:0.6:0.3 / 62.5:0.3:0.3:0.15 µg/ml respectively in culture tubes labeled 1 to 6. The tubes were then incubated for 24 to 48 h under normal culture conditions. When some strains (diatoms) were observed with high load of bacteria or cyanobacterial/fungal contamination those cultures were sonicated for 10 seconds for 90 KHz before antibiotic treatment.

Table 2.3: List of microalgae strains treated with antibiotics

Sl. No.	Strain Name	Treatment given	Final bacerial load after treatment	Successful treatment	Bacterial load after subculturing	Purified or not
Class - Prymesiophycae						
1	*Isochrysis galbana* S002	A	Nil	1 for 48 hrs	Nil	✓
2	*Isochrysis galbana* S157 *	A & S+A	Nil	1 for 24 hrs, A	Yes	×
3	*Isochrysis galbana* S169 *	A & S+A	Nil	1/8 for 24 & 48 hrs, A	Nil	✓
4	*Prymnesium* sp. S119*	A & S+A	in 48 hrs	---	---	×
Class - Eustigmatophyceae						
5	*Nannochloropsis* sp. S006	A	Nil	1/4 for 48 hrs	Yes	×
Class - Bacillariophyceae						
6	*Chaetoceros* sp. S005	A	in 72 hrs	---		×
7	*Cyclotella* sp. S018	A & S+A	Nil	1/4 for 24 hrs, A & S+A	Yes	×
8	*Thalassiosisra* sp. S019	A	Nil	1/2 for 24 hrs & 48 hrs	Yes	×
9	*Nitzschia* sp. S021	A	in 24 hrs	---		×
10	*Thalassiosisra* sp. S033	A & S+A	Nil	1/2 for 48 hrs, A	Yes	×
11	Pennate diatom S038	A & S+A	in 24 hrs	---		×
12	Pennate diatom S039	A & S+A	in 24 hrs	---		×
13	*Chaetoceros* sp. S042	A & S+A	Nil	1 for 48 hrs, A & S+A	Yes	×
14	*Navicula* sp. S060	A	Nil	1 for 48 hrs	Yes	×
15	*Cylindrotheca closterium* S061	A	Nil	1 for 24 hrs	Nil	✓
16	*Chaetoceros* sp. S065	A	in 24 hrs	---		×
17	*Thalassiosisra* sp. S069	A	Nil	1/2 for 48 hrs & 1 for 24 hrs	Nil	✓
18	*Thalassiosisra* sp. S084	A & S+A	in 24 hrs	---		×
19	*Minutocellus polymorphus* S088	A	Nil	1/2 for 48 hrs	Nil	✓
20	Pennate diatom S090	A	in 72 hrs	---		×
21	*Nitzschia longissima* S092	A	in 24 hrs	---		×
22	*Navicula* sp. S117	A	in 24 hrs	---		×
23	*Thalassiosira* sp. S132	A	Nil	1/4 for 24 hrs, A	Yes	×
24	*Navicula* sp. S136	A	Nil	1 for 48 hrs	Nil	✓
25	*Nitzschia* sp. S148	A	Nil	1/16 for 48 hrs	Nil**	×
26	*Bellerochea* sp. S169	A	Nil	1/8 for 24 & 1/16 48 hrs	Nil	✓
27	*Melosira* sp. S168	A	in 24 hrs	---	---	×
Class - Prasinophyceae						
28	*Tetraselmis* sp. S028	A & S+A	Nil	1 for 24 hrs, A & S+A	Yes	×
29	*Tetraselmis* sp. S081	A & S+A	Nil	1/32 for 48 hrs, S+A	Nil	✓
Class - Trebouxiophyceae						
30	*Chlorella* sp. S072	A	Nil	1/8 for 48 hrs	Nil	✓
31	*Chlorella* sp.S095 #	A & S+A	Nil	1/8 for 24 & 48 hrs, S+A	Nil	✓
32	*Didymogenes* sp. S026	A	Nil	1/16 for 24 hrs & 1/32 for 48 hrs	Nil	✓
33	Green alga S165	A	Nil	1/32 48 hrs	Nil	✓
34	*Picochlorum* sp. S170	A	Nil	1/2 for 48 hrs	No	✓
Class - Chlorophyceae						
35	*Dunaliella* sp. S096*	A & S+A	Nil	1/32 for 48 hrs, A & S+A	Yes	×
36	*Dunaliella salina* S089	A	Nil	1/32 for 24 hrs	Nil	✓
37	*Dunaliella* sp. S118	A & S+A	Nil	1/32 for 24 & hrs, A	Nil	✓
38	*Dunaliella salina* S135*	A & S+A	Nil	1 for 24 hrs, A	Yes	×
Class - Cyanophyceae						
39	*Synechocystis* sp. S107	A & S+A	Nil	1 for 24 hrs & 1/2 for 48 hrs, A	Nil	✓
40	*Oscillatoria* sp. S137	A & S+A	Nil	1 for 48 hrs, A	Nil	✓

*Sonication damaged the cells; **_Nitzschia_ sp. S148 strain became free from bacteria but fungal contamination pertained; # In _Chlorella_ sp. S095, sonication helped removal of cyanobacterial contamination. A- Antibiotic treatment; S+A – Sonication and antibiotic treatment

After these time intervals, viability of microalgae cells was checked microscopically by Evan's blue staining. Also these cultures (100 µl) were inoculated into 5 ml sterile Zobel Marine Broth (ZMB) and incubated at room temperature at 200 rpm, to know the bacterial load. From antibiotic treated healthy microalgal cultures 1 ml was inoculated into fresh sterile f/2 media (10 ml). In two weeks, when the inoculated (treated) microalgae were grown considerably, a sample was again inoculated in ZMB to confirm purity of the algal cultures. Growth of bacteria in ZMB was noted by turbidity after 24, 48 and 72 h. Once the strains were found completely free from culturable bacteria, they were maintained separately. All treatments as well as test were performed in duplicates and with a control.

2.2.5 Preservation of Microalgae

Only selected 25 strains of microalgae representing different classes and genera were employed for the preservation trials. Microalgae were grown in 500 ml f/2 marine medium with salinity 35 ppt and sampled required volume at late exponential growth phase for preservation.

2.2.5.1 Agar slants:

About 15 ml 1.5% f/2 agar medium was taken in wide mouth screw cap glass tube (30 ml), autoclaved and slants were prepared 24 h prior to plating. Microalgae (Table 2.4) centrifuged and the pellet was washed twice with fresh sterile media. From this a loop full of cells was zig-zag plated on the f/2 agar slant. The tubes were then incubated facing the slant toward light illumination for 20-60 days. When a good patch or lawn of cells was formed on the slant the tubes were shifted to growth chambers where temperature was maintained at 18 – 20°C without illumination (not absolute darkness). After a period of time (1-3 years) cells from the slant were looped out and mixed aseptically with sterile media. Viability of cultures was checked in 2-4 weeks incubation on culture racks.

Table 2.4: Results of different preservation methods of microalgae: Agar plating (AP), Agar embedding (AE) and Cryopreservation (Cryo) in different cryoprotectants DMSO (D – 5/10/20 %), Methanol (M-5%) and Glycerol (G-5%); Also given the results of preservation at 4, -20 and -80 °C. Period of survival is given in months (m) and year/s (yr/s). NG: no growth; LN: liquid nitrogen

Sl. No.	Strain	Preservation method						
		AP	AE	Cryo -D 5%	Cryo - D 10%	Cryo - D 20%	Cryo - M 5%	Cryo - G 5%
Class - Prynesiophyceae								
1	*I. galbana* S002	6 m	NG	….	NG.	….	NG	NG
2	*Isochrysis* sp. S157	3 m	NG				NG	NG
3	*Prymnesium* sp. S119	3 m	NG	…	NG	…	NG	NG
4	*Ochrosphaera* sp. S003	3 yrs	2 yrs	…	-80° C & LN, 3 m	…	NG	NG
Class - Eustigmatophyceae								
5	*Nannochloropsis* sp. S078	2-3 yrs	2 yrs	…	-80° C & LN, 3 m	…	NG	NG
Class - Raphidophyceae								
6	*Heterosigma* sp. S156	NG	NG	NG	NG	NG	NG	NG
Class - Bacillariophyceae								
7	*Chaetoceros* sp. S065	1 yr	3 m	-80° C, 6 m	-80° C, 6 m	-80° C, 6 m	NG	NG
8	*Chaetoceros* sp. S005	6 m	NG	-80° C, 6 m	-80° C, 6 m	NG	NG	NG
9	*Cyclotella* sp. S018	6 m	3 m	NG	NG	NG	NG	NG
10	*Bellerochea* sp. S167	6 m	3 m	NG	NG	NG	NG	NG
11	*Thalassiosira* sp. S019	1 yr	3 m	…	LN, 3 m	…	NG	NG
12	*Nitzschia* sp. S021	2 yrs	3 m	…	LN, 3 m	…	NG	NG
13	*Navicula* sp. S136	3 yrs	6 m		LN, 3 m	…	NG	NG
14	*Skeletonema ardens* S049	NG	NG	NG	NG	NG	NG	NG
Class - Prasinophyceae								
15	*Tetraselmis* sp. S082	3 yrs	6 m	NG	NG	NG	NG	NG
16	*Tetraselmis* sp. S075	3 yrs	1 yr	NG	NG	NG	NG	NG
Class - Trebouxiophyceae								
17	*Chlorella* sp. S072	3 yrs	3 yrs	-80° C & LN, 1 yr	-80° C & LN, 1 yr	-80° C & LN, 1 yr	LN, 1 yr	in LN, 6 m
18	*Picochlorum* sp. S170	3 yrs	3 yrs	-80° C & LN, 1 yr	-80° C & LN, 1 yr	-80° C & LN, 1 yr	-80° C & LN, 1 yr	-80° C & LN, 1 yr
19	*Picochlorum* sp. S030	3 yrs	3 yrs	-80° C & LN, 1 yr	-80° C & LN, 1 yr	-80° C & LN, 1 yr	-80° C & LN, 1 yr	NG
Class - Chlorophyceae								
20	*D. salina* S135	1 yr	NG	NG	NG	NG	NG	NG
21	*Dunaliella* sp. S147	1 yr	NG	NG	NG	NG	NG	NG
Class - Cyanophyceae								
22	*Oscillatoria* sp. S137	1 yr	NG	4° C, -80° C & LN, 1 yr	4° C, -80° C & LN, 1 yr	NG	4° C, -80° C & LN, 1 yr	NG
23	*Synechocystis* sp. S107	3 yrs	1 yr	4° C, -80° C & LN, 1 yr	4° C, -80° C & LN, 1 yr	4 °C, -80° C & LN, 1 yr	4° C, -80° C & LN, 1 yr	4° C, -80° C & LN, 1 yr
24	*Cyanothece* sp. S098	6 m	NG	…	LN, 3 m	…	NG	NG
25	*A. platensis* S016	NG	NG	NG	NG	NG	NG	NG

2.2.5.2 Agar Embedding

Sterile 2% f/2 agar media was autoclaved and allowed to cool. Just before solidification, with this molten agar media (10 ml), about 10 ml of dense microalgae cultures were mixed in 50 ml transparent, plastic, screw-cap vials and labeled. Once the agar got solidified, the vials were capped and shifted to illumination racks with normal culture temperature (25±1°C). Once the cultures got stabilized (in 2-4 weeks, noted colour change for healthy embedded cultures) the vials were shifted to culture chambers (18-20°C). For thawing, a scoop of agar with microalgae cells was mixed with 30 ml sterile media in a conical flask. In two to three days time, when cells from agar started growing out in liquid media, the broth was examined under microscope for healthy live cells.

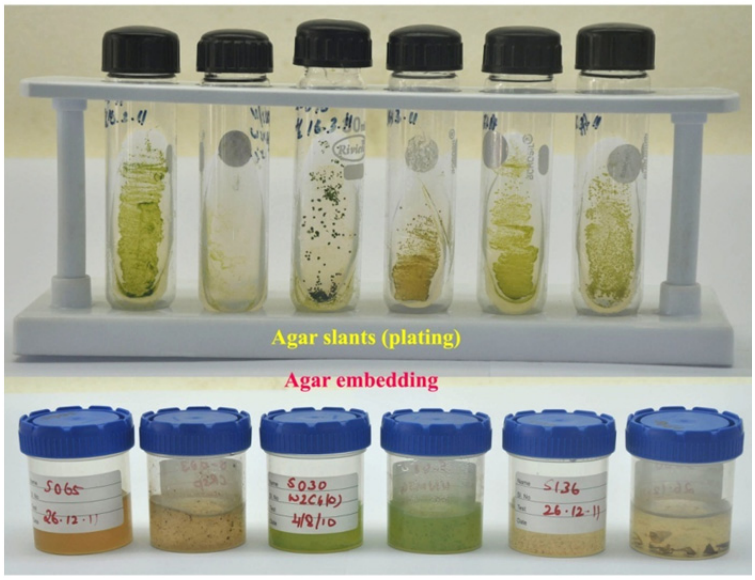


Figure 2.6: Images of agar based preservation; microalgae grown on agar slants and in embedded form

Table 2.5: Morphological descriptions of microalgae belonging to different classes, based on microscopic examination

Class - Bacillariophyceae	
Genus – Chaetoceros (Plate 1)	
Strain	**Cell characteristics**
MBTD-CMFRI-S005 *Chaetoceros calcitrans*	Cells solitary in culture; brown in colour; heterovalvate; upper valve convex. size: apical axis: 4.86-11.40 µm, prevalvar axis: 3.87-5.68 µm. setae: 4, 17-26um long, smooth, parallel or slightly curved chloroplast: single? yellow in colour. reproduction: by cell division; sexual reproduction and spore formation not noticed
MBTD-CMFRI-S024 *Chaetoceros* sp.	Cells solitary, brown, box shaped; size: apical axis – 3.5-5 µm, prevalvar axis – 3.4- 4.5 µm; setae: four, 16-23 µm long, straight; chromatophore: single, brown; reproduction: cell division
MBTD-CMFRI-S042 *Chaetoceros* sp.	Cells solitary rarely forms chain in culture, box shaped, homovalvate, brown; size: valve axis – ca. 6 µm, prevalvar axis – ca. 8 µm; setae: four; 20-24 µm long; smooth almost parallel; chloroplast: single, brown; reproduction: cell division
MBTD-CMFRI-S062 *Chaetoceros* sp.	Cells solitary in culture; box shaped; brown coloured, valves both convex; size: valve axis – 4.4-6 µm; prevalvar axis- 6.15- 10.56 µm; setae: very long parallel; 19.5 – 40 µm long; chloroplast: single? brownish; reproduction: cell division
MBTD-CMFRI-S065 *Chaetoceros* sp.	Cells solitary, brown, box shaped; size: valve axis – ca. 3 µm; prevalvar axis – 5-6 µm; setae: four, parallel, 8-12 µm long, smooth; chloroplast: single, brown; reproduction : cell division
MBTD-CMFRI-S172 *Chaetoceros gracilis*	Cells solitary, brown, oval cylindrical shaped; size: valve axis – ca. 3-4 µm; prevalvar axis – 6-8 µm; setae: four, curved slightly, 10-12 µm long, smooth; chloroplast: single?, brown; reproduction : cell division (**Plate 4**, S172)
Genus – Cyclotella (Plate 2)	
MBTD-CMFRI-S018 *Cyclotella* sp. (*C. cryptica*?)	Cells solitary; yellowish brown in colour; size: valve dia.- 6-7µm, prevalvar axis – 5-10 µm; chloroplasts: chromatophores 2 (or 1?), yellowish brown; marginal organic threads present but very lightly visible; reproduction: cell division
MBTD-CMFRI-S044 *Cyclotella* sp. (*C. atomus*?)	Cells small, solitary, discoid, yellow brown; size: valve dia. & prevalvar axis almost same – 4.8-5.57 µm; no organic threads observed from marginal strutted processes; chloroplast: two, yellow; reproduction: cell division
MBTD-CMFRI-S052 *Cyclotella* sp.	Solitary, cylindrical cells, brown in colour; size: valve dia. – 4-5 µm; prevalvar axis – 7.7-11.24; no organic threads o marginal strutted processes noticed; chloroplast: many? brownish; reproduction: by cell division
MBTD-CMFRI-S079 & S080 *Cyclotella* sp.	Cells solitary or colonial in mucilage layer, discoid box shaped, yellowish brown; size: valve dia. & prevalvar axi almost equal – 5-6 µm; no organic threads from strutted processes; chloroplast: two or many?, yellow; reproduction: cell division
Genus – Minutocellus (Plate 2)	
MBTD-CMFRI-S050 MBTD-CMFRI-S088 *Minutocellus* sp.	Cells small, solitary or in chain of 2-4 cells or colonial in mucilage, box shaped; brown; size: valve dia. – ca. 3 µm; prevalvar axis- 2.5- 4.3 µm; no setae or organic threads observed; chloroplast: single? yellow; reproduction: cell division
Genus – Bellerochea (Plate 4 S167)	
MBTD-CMFRI-S167 *Bellerochea* sp.	Cells in ribbons, loosely joined cells, weekly silicified, girdle view rectangular, short elevations at each corner of valve; brownish yellow; chloroplast – numerous, oval; size: ca. 20- 40 µm both valve and prevalvar axis; reproduction: not observed
Genus – Skeletonema (Plate 4 S032 & S049)	
MBTD-CMFRI-S032 *Skeletonema* sp. (*S. costatum*?)	Cells in chain, discoid ovate; reddish brown; size: valve dia. – ca. 7 µm; prevalvar axis – ca. 3.7 µm; chloroplasts: 1 or 2, reddish yellow; presence of typical external tubes of marginal strutted processes connecting the cells in chain with gap ca. 3.5 µm; reproduction: cell division

MBTD-CMFRI-S049 *Skeletonema* sp.	Cells in chain, discoid ovate; reddish brown; size: valve dia. – ca. 5 µm; prevalvar axis – ca. 3.7 µm; chloroplasts: 1 or 2, reddish yellow; presence of typical external tubes of marginal strutted processes connecting the cells in chain with gap ca. 3 µm; reproduction: cell division
Genus – Thalassiosira (Plate 3)	
MBTD-CMFRI-S019 *Thalassiosira* sp.	Cells are solitary in culture; discoid; yellowish brown; size: valve dia. – 13-14 µm, prevalvar axis – 9-10 µm; chloroplast: many, yellow in colour; marginal & central strutted processes present with organic threads clear; reproduction: cell division
MBTD-CMFRI-S045 *Thalassiosira* sp.	Large discoid cells, mucilaginous colony forming, brown; culture – viscous; size: valve dia.– 10-11 µm average up to 21.7 µm; prevalvar axis 6.8-13 µm; chloroplast: many, yellow, spherical; no organic threads from marginal strutted processes; many central strutted processes; reproduction by cell division
MBTD-CMFRI-S045 *Thalassiosira* sp.	Cells are solitary in culture; discoid; yellowish brown; size: valve dia. – 6.7-8.5 µm up to 13 µm, prevalvar axis – average 5 µm up to 8.2 µm; chloroplast: many, yellow in colour; marginal & central strutted processes present with organic threads clear; reproduction: cell division
MBTD-CMFRI-S069 *Thalassiosira* sp.	Cells solitary, yellowish brown, discoid; size: valve dia. – 18.2-19.9 µm ; prevalvar axis – 15 -17.2 µm; marginal and central strutted processes with organic threads present clearly; chloroplast: many, yellow; reproduction : cell division
MBTD-CMFRI-S084 *Thalassiosira* sp.	Cells large are solitary in culture; discoid; yellowish brown; size: valve dia. & prevalvar axis almost equal up to 12 µm; chloroplast: many, yellow in colour; marginal & central strutted processes present with clear organic threads; reproduction: cell division
MBTD-CMFRI-S132 *Thalassiosira* sp.	Large discoidal cells, mucilaginous colony forming, brown; culture – viscous; size: valve dia. – 4.5-32 µm; prevalvar axis 8-9 µm; chloroplast: many, yellow, spherical; no organic threads from marginal strutted processes; reproduction by cell division
MBTD-CMFRI-S158 *Thalassiosira* sp.	Discoidal cells, mucilaginous colony forming, brown; valve undulated on axial view; size: valve dia. – 5.7 - 27 µm; prevalvar axis ca. 8 µm; chloroplast: many, yellow; no organic threads from marginal strutted processes; strutted processes not clear; reproduction by cell division (**Plate 4**, S158)
Genus – unclear (Plate 4, S167)	
MBTD-CMFRI-S168 *Melosira* sp.?	Cells are in chain, attached, long, cylindrical or ovoid, brown; size: valve dia. 2-3 µm; prevalvar axis – 6-7 µm; no setae or organic threads / strutted processes; chloroplast – single? reproduction: cell division
Genus – Navicula (Plate 6)	
MBTD-CMFRI-S043 *Navicula* sp.	Valves linear lanceolate with slightly rostrate ends; striae radiate or parallel, brown, girdle; view rectangular; cells show sliding movement; size: valve width – ca. 5.88 µm; length – 12.85 µm; chloroplasts: two; asymmetrical covering the whole length of the girdle, yellow; reproduction: cell division
MBTD-CMFRI-S060 *Navicula* sp.	Valves linear lanceolate with slightly rostrate ends; striae radiate or parallel; brown; girdle view rectangular; cells show sliding movement; size: valve width – 5-6 µm; length – 18-21 µm; chloroplasts: two; asymmetrical covering the whole length of the girdle, yellow; reproduction: cell division
MBTD-CMFRI-S117 *Navicula* sp.	Cells solitary, benthic (attached) with sliding movements; brown colour; valves linear, lanceolate with tapering ends; rectangular in girdle view; size: valve width – ca. 3.6 µm, length – ca. 13.2 µm; chloroplasts : two, one at each side of the cell, ovoid, yellow; fill entire frustules; reproduction: cell division
MBTD-CMFRI-S136 *Navicula* sp.?	Cells solitary, benthic (attached) with sliding movements; brown colour; valves linear, lanceolate with tapering ends; rectangular in girdle view; size: valve width – ca. 5 µm, length – ca. 16-18 µm; chloroplasts : two, one at each side of the cell, ovoid, yellow; fill entire frustules; reproduction: cell division
Genus – Nitzschia/Cylindrotheca (Plate 5)	
MBTD-CMFRI-S021 *Nitzschia* sp.	Cells solitary, benthic (attached) with sliding movements; brown colour; valves linear, lanceolate. cells rectangular in girdle view; size: valve width – ca. 3 µm, length – ca. 9 µm; chloroplasts : two, one at each side of the cell, ovoid, yellow; reproduction: cell division

MBTD-CMFRI-S061 *Nitzschia/Cylindrotheca* sp.	Solitary; frustules (cell) cylindrical, fusiform with rounded poles, valves highly elongated and rotating when movement; brown; shows gliding movement; size: length – ca. 50 µm; chloroplast: two, yellowish brown; details of valve structures –raphae system unclear in LM.
MBTD-CMFRI-S092 *Nitzschia/Cylindrotheca* sp.	Solitary; frustules (cell) cylindrical, fusiform with rounded poles, valves highly elongated, and rotating when movement; brown colour; shows gliding movement; size: length – ca. 55 µm; chloroplast: two, yellowish brown; details of valve structures –raphae system unclear in LM.
MBTD-CMFRI-S099 *Nitzschia/Cylindrotheca* sp.	Solitary; frustules (cell) cylindrical, fusiform with rounded poles, valves highly elongated, and rotating when movement; brown colour; shows gliding movement; size: length – ca. 50 µm; chloroplast: two, yellowish brown; details of valve structures –raphae system unclear in LM.
MBTD-CMFRI-S148 *Nitzschia* sp.	Cells solitary, benthic, yellow brown, valves fusifirm, slightly asymmetrical; size: valve width ca. 2.5 µm, length – 7.8- 8.9 µm; chloroplast: two, yellow, ovoid, in either side of the cell; raphe / striae not viewed; reproduction by cell division
Genus – unclear (Plate 7)	
MBTD-CMFRI-S037 Pennate diatom	Cells solitary, brown, valve linear, lanceolate. rectangular in girdle view; subacute ends; size: small cells, valve length – 6-7 µm, width – 3-3.5 µm; chloroplast: one or two, yellow; reproduction- cell division
MBTD-CMFRI-S038 Pennate diatom	Cells solitary, brown valves oval rectangular in girdle view, raphe present; size: valve length – 5-6 µm, width ca. 2.7 µm; chloroplast: single centrally placed? yellow; striae not clear
MBTD-CMFRI-S039 Pennate diatom	Cells small, solitary, ovoid valve, rectangular in girdle view one side broader, yellowish; brown; raphe present, striae parallel; size: valve width – 2-3 µm, length – 5-6 µm; chloroplast: single, yellow
MBTD-CMFRI-S068 Pennate diatom	Cells fusiform with tapering ends in valve view; diamond or square shaped in girdle view; yellow brown, attached to substratum. no movement observed; raphea and striae unclear; size: valve width – ca. 3 µm, length – 4-5 µm; chloroplast: two on either side of the valve; yellow brown
MBTD-CMFRI-S090 Pennate diatom	Cells small fusiform, cylindrical in valve view; roughly rectangular in girdle view; weekly silicified; golden yellow; benthic attached to substratum; no movements observed; size: average length 5.5 µm, width of valve 2-3 µm; chloroplast: single in the centrally placed in cell; yellow; raphae / striae not clear in lm; reproduction: cell division (2.10 -S090)
MBTD-CMFRI-S150 Pennate diatom	Cells solitary, benthic, slightly asymmetric body with one face flat, and other face convex; size: valve length 6-7 µm; width ca. 2.5 µm; chloroplast: single? yellow, centrally placed; other features not clear

Class – Prymnesiophycea (Plate 8)	
Genus – Isochrysis Species *I. galbana*	
Strain	**Cell characteristics**
MBTD-CMFRI-S001 MBTD-CMFRI-S002 MBTD-CMFRI-S073 MBTD-CMFRI-S106 MBTD-CMFRI-S157 MBTD-CMFRI-S169	Cells naked; colour - golden yellow/brown; size - length: 5-6 µm; width : 3-5 µm; shape - ovoid or elongated with variable shape; flagella - two, smooth, approximately equal to the cell length; haptonema absent; chloroplast - single, yellow brown; reproduction by cell division
Genus – Ochrosphaera	
MBTD-CMFRI-S003 *Ochrosphaera* sp.	Unicellular, coccoid; cells in aggregation; cells have envelop harbouring carbonate of lime, colour –brown; size 8-10 µm in diameter; shape – spherical; flagella – absent, nonmotile cells; chloroplast – single, cup shaped?, yellow brown; pyrenoid – present, not visible in lm; reproduction – binary fission; sexual?
Genus – Prymnesium	
MBTD-CMFRI-S119 *Prymnesium* sp. (*P. parvum?*)	Cells covered by organic scales; colour - yellow-brown; size - length: 10-12 µm, width : 2-3 µm; shape - elongated compressed with variable shape; flagella - two, 1 1/2- 2 to the cell length; haptonema 1/3rd to cell length; chloroplast – two, golden yellow; vibrating granules in the posterior part of the cell. EM needed to differentiate from *P. patelliferum*

Class – Raphidophyceae (Plate 9)	
Genus – Heterosigma	
Strain	**Cell characteristics**
MBTD-CMFRI-S156	Unicellular, free swimming flagellate, brown in colour, ovoid, globular, with delicate cell membrane.
Heterosigma sp.	Identified by molecular method – 18S rDNA sequence blast analysis
Class – Eustigmatophyceae (Plate 9)	
Genus – Nannochloropsis	
Strain	**Cell characteristics**
MBTD-CMFRI-S006 MBTD-CMFRI-S007 MBTD-CMFRI-S012 MBTD-CMFRI-S015 MBTD-CMFRI-S076 MBTD-CMFRI-S077 MBTD-CMFRI-S078	Unicellular, free-floating; cells subspherical, 2-4 µm diam. or cylindrical, 3-4 x 1.5 µm. yellow-green parietal chloroplast; pigments typically eustigmatophycean (Antia & Cheng, 1982); stigma not observed; zoospores not produced; reproduction by cell division. (*Nannochloropsis oceanica* based on molecular identification -18S rRNA gene)

Class – Trebouxiophyceae (Plate 10-13)	
Genus – Chlorella	
Strain	**Cell characteristics**
MBTD-CMFRI-S071/S072 MBTD-CMFRI-S095 *Chlorella* sp.	Cells single, green, and spherical/subspherical; size 3-6 µm diameter; chloroplast cup shaped with parietal; pyrenoid clearly visible with starch granule covering; reproduction by autospores.
MBTD-CMFRI-S171 *Chlorella vulgaris.*	Cells single, green, spherical/ovoid; size 4-8µm diameter; chloroplast cup/saucer shaped with parietal; pyrenoid clearly visible with starch granule covering; reproduction by autospores.
Genus – Picochlorum/Nanochlorum/Nannochloris	
MBTD-CMFRI-S030 MBTD-CMFRI-S048 MBTD-CMFRI-S056 MBTD-CMFRI-S070 MBTD-CMFRI-S083	Cells oval/egg shaped, coccoid, single/colonial, with polar spine like projections, green; size – about 3x8 µm; chloroplast green single, lateral; pyrenoid clearly visible spherical with starch granules in the middle of chloroplast; stigma not present. reproduction by autosporulation *Nanochlorum* sp.?
MBTD-CMFRI-S029 *Picochorum* sp.	Cells single, green, spherical 2-6 µm diameter; chloroplast single lateral; pyrenoid and stigma not noticed; many vacuoles present; reproduction by cell division
MBTD-CMFRI-S102 MBTD-CMFRI-S144 MBTD-CMFRI-S155 MBTD-CMFRI-S134 MBTD-CMFRI-S164	Cells single or aggregated, green spherical with 1-2 µm size; chloroplast single cup shaped; pyrenoid and stigma not observed; reproduction by cell division. *Picochorum* sp.
MBTD-CMFRI-S170 *Picochorum* sp.	Cells spherical or elliptical, yellowish green; single, 1-2 µm size; chloroplast cup shaped; pyrenoid not present; red eye spot (stigma) prominently visible; reproduction by cell division
Genus – Dydimogenes	
MBTD-CMFRI-S026 *Didymogenes* sp.	Cells green, spherical, solitary; cells 2-10 µm size; parietal bean shaped chloroplast; pyrenoid present; reproduction by autospores
Genus – Dictyosphaerium	
MBTD-CMFRI-S129 *Dictyosphaerium* sp.	Cells green, spherical, colonial attached to the ends of thin stalks emerging from center of colony and branching dichotomously or tetrachotomously; cells 2-8 µm size; cup shaped chloroplast; pyrenoid present; reproduction by autospores
Genus –unclear	
MBTD-CMFRI-138 *Oocystidium* sp.?	Cells green, single, broadly spherical to oval, within a colourless mucilaginous envelop(contains remnants of ruptured cell walls); size 10-20 µm; chloroplast single (double in mature cells) and cup shaped with pyrenoid single or double per chloroplast, surrounded by separate starch granules; no stigma; reproduction by autospores
MBTD-CMFRI-165	Cells green, single or aggregated, broadly oval with about 6-15 µm size. chloroplast single with pyrenoid; stigma unclear; cytoplasm with large vacuoles in single cells; reproduction by autospores.

Class – Prasinophyceae (Plate 13 & 14)	
Genus – Tetraselmis	
Strain	**Cell characteristics**
MBTD-CMFRI-S011 MBTD-CMFRI-S075 MBTD-CMFRI-S105 *T. gracilis?*	Colour - yellow green; size - length: 6.02-12.37 µm, width : 6-9.9 µm; shape - elliptical, slightly compressed body with a depression; body covered with theca; flagella - four originate from the depression; ½ length to body, easily get detached; chloroplast - single; cup shaped; granular; lobular appearance; yellowish green with reddish tint; pyrenoid - large; basal/ axial; not clearly visible; stigma - one/ two/three? large; orange-red; medial; distinct; irregularly shaped; refractile granules - not seen; reproduction observed - cell division & cyst formation
MBTD-CMFRI-S027 MBTD-CMFRI-S028 *Tetraselmis* sp.	Colour - light olive green; size - length: 8.21-11.09 µm; width : 5.07-6.56 µm; flagella - equal to cell length; chloroplast -single; cup shaped; granular; yellowish green; pyrenoid - large; sub-basal; amylosphere - u shaped starch shield; stigma - one/ two; medium; red- orange; median; distinct; refractile granules – present; other characters same as above strains
MBTD-CMFRI-S057 *Tetraselmis* sp.	Colour: green; size- length: 8.22-11.47 µm, width : 4.75-7.94 µm, shape, elliptical ovoid, not so compressed body with a depression; body covered with theca, flagella: four originate from the depression; chloroplast: single; cup shaped; granular & globular; yellowish green; pyrenoid: large; not clear; stigma: one, sometimes 2; large; red- orange; median; diffuse; refractile granules; present, many; other features same as above
MBTD-CMFRI-S081 *Tetraselmis* sp.	Colour: yellow green; size- length: 7.35-8.93 µm, width : 5.2-6.28 µm; shape: elliptical, compressed body with a depression; body covered with theca; flagella: equal to body length; chloroplast: single, cup shaped, granular, yellowish green; pyrenoid: small, not clear; stigma: one, sometimes 2, large, red- orange, posterior, diffuse; refractile granules, longitudinal rows of granules present, many; other features same as above
MBTD-CMFRI-S082 *Tetraselmis* sp.	Colour: green; size- length: 6.5-12.45 µm, width : 3.75-7.2 µm, all other features same as S027 & S028
MBTD-CMFRI-S101 *Tetraselmis* sp.	Colour: green; size- length: 7-11.85 µm, width : 3.2-7.55 µm, flagella: half to body length; all other features same as S027 & S028
MBTD-CMFRI-S093/94 MBTD-CMFRI-126/127 MBTD-CMFRI-142/143 *Tetraselmis indica*	Morphology matching with *T. indica* (Arora, Anil, Leliaert, Delany, & Mesbahi, 2013). Cells green slightly compressed, oval with size – length 12-26 µm and width 8-19 µm. flagella four, about equal to body length; granular protoplasm, stigma diffuse orange, posterior; chloroplast cup shaped yellow green; theca present.

Class – Chlorophyceaea	
Genus – Dunaliella (Plate 15)	
Strain	**Cell characteristics (general, details of individual strains discussed in Chapter 3 Part II)**
Dunaliella sp.	Unicellular, biflagellate and uninucleate algae without cellulosic cell walls, cells fusiform, ellipsoid, ovoid, obovoid, globose or depressed-globose. chloroplast parietal and principally cup-shaped; pyrenoid and stigma present; reproduction by cell division sometimes giving rise to a palmelloid stage; occasionally cells become encysted; sexual reproduction by fusion of isogametes
Genus –Mychonastes & Monoraphidium (Plate 13)	
MBTD-CMFRI-S130 *Mychonastes* sp.	Cells solitary, green, spherical, size 1-5 µm; chloroplast: single, parietal; pyrenoid absent; stigma not observed; reproduction by autospores
MBTD-CMFRI-S139 *Monoraphidium* sp.	Cells green singular sigmoid in shape with rounded ends; size 6-10 x 2-5 µm; chloroplast single parietal; pyrenoid present; reproduction by autospores; sexual reproduction not noticed

Class – Ulvophyceae (identified by molecular taxonomy) (Plate 13)	
Genus – unclear	
Strain	**Cell characteristics**
MBTD-CMFRI-S113	Cells colonial 4-16 cells/colony, spherical, smooth walled, green and uninucleate; chloroplast single, parietal, cup shaped; pyrenoid and stigma present; reproduction by sporulation
MBTD-CMFRI-S145	Cells solitary, spherical, smooth walled, green and uninucleate; chloroplast single, saucer shaped with pyrenoid; stigma large, orange; vacuoles/lipid droplets present in cytoplasm; reproduction by sporulation

Class – Cyanophyceae (Prokaryotic) (Plate 16 & 17)	
Genus – Arthrospira	
Strain	**Cell characteristics**
MBTD-CMFRI-S016 *A. platensis*	Trichome spirally coiled, unbranched, free living and motile / floating; deep blue-green colour; length: 0.5 to 1mm, width: 10-12 µm; granulated cytoplasm; cross walls present; reproduction by fragmentation of trichomes.
MBTD-CMFRI-S152 *A. maxima*	Length: 0.5 to 2 mm, width: 11-14 µm; all other characters same as *A.platensis*. (Morphologically these two species are very similar. Distinguished based on culture morphology in higher salinity (20 ppt) – *A. platensis* cells aggregate to form clusters with an increase in salt concentration. *A. maxima* is much salt tolerant than *A. platensis*)
Other genera	
Strain	**Cell characteristics**
MBTD-CMFRI-S041 *Synechococcus* sp.?	Unicellular, cells solitary or in short chains , without mucilage covering; shape: oval, cylindrical with rounded ends; pale blue green colour; size: 1-2 x 2-3 µm; reproduction by binary fission –plane of division perpendicular to longer axis of the cell; after division, cells separate or remain arranged in short rows (pseudofilaments) of several cells
MBTD-CMFRI-S091 *Synechocystis* sp/ *Synechococcus* sp.?	Cells single, spherical, no mucilage covering, pale blue green; size 0.5-1 µm diameter; reproduction by cell division (binary fission) always in two perpendicular planes; cells remain arranged as long chain of cells (up to 12 cells) after cell division.
MBTD-CMFRI-S098 MBTD-CMFRI-S120 *Cyanothece* sp.	Unicellular, cells solitary, cylindrical, widely oval with rounded ends; pale blue green with brownish pigment granules in cytoplasm which is highly granular (culture colour is grayish blue green); size 5-10 x 10-17 µm; reproduction by binary fission at perpendicular to longitudinal plane of cell; highly viscous culture.
MBTD-CMFRI-S100 *Gietlerinema* sp.	Filamentous, unbranched, form mats over the substratum; size: 2-3 µm wide and > 2mm long trichome with rounded ends; deep blue green colour; cylindrical cells slightly constricted at cross walls (visible at 1000x); reproduction by fragmentation.
MBTD-CMFRI-S137 *Oscillatoria* sp.	Unbranched filamentous trichomes, isopolar and straight; deep blue green colour; free flowing, motile; size: 3-6 µm wide and several mm long (even up to 1 cm); cross walls prominent, cells poorly constricted at cross walls; end cells rounded; reproduction by filament fragmentation.

Unidentified microalga (Plate 9 – S055)	
MBTD-CMFRI-S055	Cells irregularly shaped, in colony, branched, attached to and spread on substratum; pink in colour; size 10-15 µm wide. Other features unclear

2.2.5.3 Cryopreservation

About 200 ml culture of each strain at late log phase was centrifuged, washed and resuspended in fresh sterile medium (20 ml). Cells were then left undisturbed for some time to recover from the shock of centrifugation for about

2 h, under dim light. About 500 µl of this concentrated culture was taken into labeled cryo-vials, in duplicates for each experiment (total 5 X 2 X 2 = 20 vials).

Cryoprotective agents (CPAs) used include dimethyl sulfoxide (DMSO/Me_2SO – 5%, 10% and 20%), methanol (MeOH - 5%) and glycerol (Gly - 5%). Stocks of the cryoprotectants were prepared in double strength (DMSO – 10, 20 and 40 % and methanol 10% and glycerol 10%) in sterile media. 500 µl of different CPAs (total 5, including different concentrations of DMSO) were mixed with the cultures in cryovials and capped and plunged into liquid nitrogen either directly or after a pre-cooling (for 1 h) in -80 °C. In the same method strains were also attempted to preserve at 4 °C,-20 °C and -80 °C.

Samples were thawed after 3, 6 and 12 months period of time. For thawing, samples from liquid nitrogen were removed and suspended in 30 °C water bath until the ice was completely melted. These tubes were then centrifuged (at 5000 rpm for 2-10 min) and supernatant decanted and the cells were re-suspended in 10 ml fresh sterile medium and incubated for one week under dim light and then for 2-4 weeks under normal culture illumination. Viability of cells was checked each time – after addition of cryoprotectant, immediately after thawing and then after final incubation, by Evan's blue staining under microscope.

2.3 Results

2.3.1 Isolation and culturing of microalgae:

Nearly 140 isolates of microalgae were isolated out of which 105 strains are preserved in the MBTD-CMFRI Culture Collection of Marine Microalgae, along with 30 procured pure cultures (Table 2.2). The culture collection named after the Marine Biotechnology Division (MBTD) of CMFRI, and all strains were given the strain code starting with 'MBTD-

CMFRI-', however for the ease of discussion only the final part of the strain code (e.g. S001) is included throughout the text as well as in many tables and figures (also in following chapters).

Among the isolates diatoms shared major percentage and were from saline habitats (mostly marine and brackish). Coccoid green algae generally got from fresh and brackish water habitats, while all prasinophytes (*Tetraselmis* spp.) and prymnesiopytes (golden algae) were from saline waters. Isolates of hyper saline and fresh water pools were mostly green algae.

Among the strains maximum number of isolates were from genus *Dunaliella* (12) and secondly from *Tetraselmis* (10) both segregated by simple dilution techniques, however further purified by agar plating. Among diatoms *Chaetoceros* spp. were commonly got isolated both by quadrate plating and serial dilutions. It was easy to obtain *Thalassiosira*, *Cyclotella*, pennate diatoms and coccoid green algae on agar plates. Flagellates like *Isochrysis* and *Prynesium* and chain forming *Skeletonema* were revived by serial dilution and further purified by micro-pipette method.

Some species like *Coscinodiscus*, *Leptocylindricus*, (diatoms) *Nephroselmis*, (green alga) *Anabaena*, *Anabaenopsis*, (cyanobacteria) etc. were lost after 5-8 subculturings. While species like *Skeletonema* (diatom), *Cyanothece* (blue green alga) and hyper saline *Tetraselmis* (Prasinophyceae) were found difficult or slow in growth in laboratory, most of the other strains were quite stable, especially the green algae (Trebouxiophyceae and Chlorophyceae).

2.3.2 Morphological identification:

Descriptions on morphological features of microalgal isolates are given in Table 2.5 and the microscopic images of the strains shown as Figures 2.7 to

plate 1-17. Genus level identification was quite easy for larger species and those with clear distinguishable phenotypic features e.g. *Skeletonema* (cells in chain appeared like beads connected by several short threads), *Chaetoceros* (presence of setae from corners of rectangular cells), *Prymnesium* (elongated compressed body, haptonema, flagella position and granular cytoplasm), *Tetraselmis* (slightly compressed cell with anterior notch, and four flagella), *Dunaliella* (ovoid thin walled cells with two flagella) etc. Nanno/picoplanktonic isolates were however having little phenotypic features, evident enough for discrimination under light microscope. Similarly, separation of species with similar morphology (shape, colour, size and cellular traits) was also not easy – for example, *Navicula* and smaller *Nitzschia*, *Cyclotella* from *Thalassiosira,* coccoid green algae – including *Nannochloropsis, Nanochlorum/Picochlorum/Nannochoris, Chlorella, Mychonastes*, *Dydimogenes* etc. This was further resolved by molecular methods which is discussed in the third chapter.

2.3.3 Antibiotic purification

Out of 40 strains selected 17 strains got purified by the treatment. The concentration, duration of treatment and final status of purity of the strains are detailed in Table 2.3. Different concentrations of the cocktail were used for 24 and 48 hrs duration, and none of the microalgal strains were sensitive to antibiotic as such. However, sonication was negative for delicate cells like *Isochrysis, Dunaliella* and *Prymnesium* where cells got ruptured, but was effective for many diatoms which reduced bacterial load considerably. Sonication before antibiotic treatment helped in complete removal of cyanobacteria from *Chlorella* sp. S095. There were strains which showed no bacteria immediately after antibiotic treatment, but developed bacterial contamination following few subculturing. Among the purified strains, major

percentage was shared by green algae which were having better resistance both to antibiotics and sonication.

Trial for antibiotics purification in this study, indicates need of modifications in treatments, to develop axenic cultures of microalgae, especially for diatoms. Diatoms almost certainly harbor intracellular bacteria, hence, even though immediately noticed to be axenic, turned non-axenic after several sub-culturing (Table 2.3). Only 5 out of 22 species of diatoms got purified after treatment, mostly by higher concentrations of antibiotics and/or longer duration of exposure. In contrast, only 3/11 green algae (23-38 in Table) remained impure. Most easily purified strains belonged to class Trebouxiophyceae, however were the least contaminated strains before using antibiotics.

2.3.4 Preservation of Microalgae

Only 25 selected strains representing the different classes were chosen for preservation trials. Results are depicted in Table 2.4. Simple traditional agar based plating in screw cap tubes (Figures 2.6) was the best among the three methods, however was useful only for those strains which normally grows on agar, e.g. pennate diatoms, green algae (including *Nannochloropsis*), coccoid cyanobacteria and tough walled *Ochrosphaera*. Among these, nonflagellate green algae, *Navicula*, *Synechocystis* and *Ochrosphaera* remained alive even after 3 years of preservation. *Isochrysis* sp. which normally poorly grows on agar was able to survive on agar for less than 6 months, but direct transfer form loan (agar) to another agar slant was however difficult.

Agar embedding (Figure 2.6) is a simple and effective technique of conservation, particularly for green nonflagellates. When *Chlorella* and related strains stayed alive for more than 3 years, others could survive for few months

to 2 years. Flagellates, filamentous blue green algae and *Skeletonema* sp. did not show any positive results and died even in two weeks period of incubation.

Results of cryopreservation at -80°C and liquid nitrogen (LN) were good enough only for thick walled coccoid green algae and the two cyanobacteria (Table 2.4), irrespective of type and concentration of cryoprotectants. *Oscillatoria* and *Synechocystis* lived also in refrigerated samples (4°C) but none of the studied strains survived in -20°C (freezer). *Dunaliella* and *Tetraselmis* cells remained intact for up to 3 months only in -80°C and in LN with 10% DMSO, however did not revive after thawing. *Ochrospaera*, *Nannochloropsis* and some diatoms survived only in DMSO (5 or 10%) at -80°C or LN for 3-6 months. Methanol and glycerol were proved poor cryoprotectants for wide range of microalgae.

2.4 Discussion

2.4.1 Isolation and Maintenance of Cultures:

New cultures of microalgae will advance our knowledge in taxonomy, physiology, genomics and biodiversity (Andersen 2005). Species richness in 'MBTD-CMFRI Culture Collection of Marine Microalgae' was good enough with regard to number of isolates, however did not reflect the original microalgae species diversity of coastal India. This was probably because of the simple sampling, random isolation and standard culture methods followed. Specific techniques are required to have exact aimed organism in isolation (Gopinathan 2005). Micropipette method is such a one but demands a steady hand and experience. In spite of all these, obtaining >100 pure isolates was not an easy task. More than that, perpetual maintenance of these cultures was another bottleneck.

Algal diversity of saline aquatic ecosystems always had diatoms as dominant in numbers and species, and the same replicated in isolation. Major

isolates from marine and brackish water habitats were diatoms (nearly 40%), which were the first to grow in dilution culture tubes and on agar. Most of the strains of this group were good to grow on agar surface, hence got purified easily by streak plating. On the other hand removal of diatoms was necessary for purifying many flagellates (e.g. *Isochrysis* sp.) for which Germanium dioxide (GeO_2, about 5 mg/L) was used (Barsanti and Gualtieri 2006). Problem faced with cyanobacteria as a contaminant was solved to some extend by agar plating and further by mild sonication and antibiotic treatment.

Different groups of microalgae responded in different ways to selected culture conditions which was interesting as well as challenging too. Within a 'standard' there were dissimilarities in the range used for physical conditions (light, temperature, salinity, nutrients etc.) and in the time intervals of culture transfer. Large number of isolates possessed problems, however similarities observed within classes helped to group the strains, for example, all diatoms were euryhaline, hence salinity of medium was adjusted to 35 ppt for all strains, which was also used for all other marine isolates.

In an artificial system, selection of media is vital for the stable growth of the organisms. A number of natural as well as artificial sea water media enrichments were available with nitrogen, phosphorus and iron as key ingredients which are essential for the growth of microalgae. For wide-range algae, among the natural SW media, f/2 medium, Erdschreiber medium (e.g., Plymouth Erdschreiber medium), and ESNW (enriched natural sea water) medium appear to dominate the references (Harrison & Berges 2005). Almost all isolated and procured strains were thriving well in f/2 media except *Arthrospira* spp. (grown in Paoletti media). But, concentration of f/2 medium was not sufficient for fresh and brackish water isolates of green algae; hence nutrient solutions were used in double strength (F medium) in distilled water

and 15-17 ppt sea water (½ ddw + ½ sw) for fresh and brackish water strains respectively. Even though many diatoms were brackish in origin, noted with a wide range of salinity tolerance from 5 to 40 ppt. Therefore all were maintained in normal sea water (salinity adjusted to 35 ppt) media for the ease of maintenance. *Dunaliella* spp. and other hyper saline strains were always retained in high salinity medium (prepared in sea water by addition of salt), to preserve their natural physiology and morphology, even though they had a wide range of salinity tolerance (about 30 – 200 ppt).

Light intensity was another important physical factor which affects the rate of photosynthesis and therefore growth of microalgae. Cyanobacteria with phycobilisomes preferred dim light (<1000 Lux), and showed longer life (more than three months) of cultures when maintained in dim light (Lorenz, Friedl and Day 2005). Diatoms and haptophytes however needed a higher light intensity (1500-2000 Lux) to survive for a longer period (2-3 moths). Green algae were the best to stay alive even in high light intensity or in very dim light (but not in complete darkness). The light:dark regimen was best between 12:12 to 16:8 for all the strains.

For temperature, <18°C was fatal, particularly because the strains are purely tropical. Prymnesiophytes (*Isochrysis* and *Prymnesium*) were the most responsive. *Skeletonema* (diatom) also showed sensitivity, both died when the temperature was decreased or increased by 2°C from the normal range (20-37°C). Green algae and cyanobacteria were able to get conserved at 18-20°C in culture tubes or on agar slops when provided with dim light.

Transfer interval of cultures was also different for different groups. When diatoms, brown flagellates and blue green algae had shorter transfer intervals (2-3 weeks) green algae and *Nannochloropsis* had longer (5 to 10

weeks). Understanding of safe transfer intervals of individual strains was important mainly to avoid the bacterial load, as dead microalgae normally provide a good medium for heterotrophic microbial growth. In addition, optimal post-transfer conditions are to be generally maintained to refresh the new inoculums and to ensure the healthy growth (Lorenz, Friedl and Day 2005).

In addition to the above mentioned aspects, cleanliness and sterility of culture media, equipments and the racks/room/chamber etc. were also significant. Proper labeling of culture vials, including stain code, name, medium, date of transfer and other important data, has to be guaranteed during the perpetual maintenance of the cultures.

2.4.2 Morphological Identification and Taxonomy

Algae are broadly classified into different groups – Cyanophyta (prokaryotes), Glaucophyta, Chlorophyta, Rhodophyta, Euglenophyta, Dinophyta, Cryptophyta, Heterokontophyta, Prymnesiophyta and Apicomplexa (Lee 2008), and majority of these groups also comprise microscopic algae – the microalgae. Further, groups like Dinophyta (dinoflagellates) and Heterokontophyta solely contain microbial unicellular forms. In this study we got species belonging to four groups – Cyanophyta (Class – Cyanophyceae), Chlorophyta (Chlorophyceae, Prasinophyceae, Trebouxiophyceae and Ulvophyceae), Prymnesiophyta (Prymnesiophyceae) and Heterokontophyta (Bacillariophyceae and Raphidophyceae). And about 30 genera were identified based on morphological features which were later confirmed or revised by employing genetic markers.

Microalgae are single celled simple live forms, but taxonomic identification of these microscopic organisms was not so simple and easy. Magnification of cells, even 200, 400 or 1000x, was weakly sufficient to

characterize them at species level. Basic features like colour, shape, size and presence and absence of flagella or other locomotory apparatuses (setae, haptonema, cellular processes etc.) helped the grouping of many strains into classes and to a larger extend into genera. Complexity arose when the cells were too similar or when there were no corresponding references available.

When the subject of 'species' identification came based on morphology, many characters were found overlapping among the strains. In addition several ultra-structural differences of flagella, cell organelles like pyrenoids, ornamentation of frustules in diatoms etc. have been significantly used in modern taxonomy (Yamaguchi et al. 2011; Nozaki, Onishi, and Morita 2002; Lundholm, Daugbjerg, and Moestrup 2002), insisted electron microscopic evaluation. Further, algal taxonomy is under constant and rapid revision based on advanced genetic and ultra-structural level appraisal (Štenclová 2013; Bock 2010; Sato, Matsumoto, and Medlin 2009). Hence molecular taxonomy was also employed before final conclusion of the species. In the table 2.5, to avoid confusion, the name of the strains given were the modified ones (based on molecular identification too which is detailed in Chapter 3).

2.4.3 Purification of Microalgae

Purification roughly means to have single species of microalga per culture (unialgal or monoalgal cuture), but may contain other associated microbes (e.g. bacteria). This is normally done by applying one or more of following methods - agar plating, centrifugation, filtration, sonication, single cell washing by micropipette, use of chemical agents (e.g. tellurite) etc. Agar plating is one of the most used method, however can be employed only for microalgae which can grow on the surface of agar (Guillard 2005).

Antibiotics are principally used to obtain 'axenic' cultures which contain only the microalga but no other microbial contaminants (bacteria, fungi or protozoa). Particular microalgae cultures may harbor different types of microbial impurities at different densities. Hence lethality of antibiotics mainly depends upon the type of antibiotic used, its concentration (intensity) and time of exposure (Jones, Rhodes, and Evans 1973; Guillard 2005). In addition, resistance of physically-delicate microalgae to antibiotics will be poor, which also has to be considered during the treatment (Cho et al. 2002).

Keeping the above factors in mind, for an assorted collection of microalgae (40 strains), a cocktail of 4 wide range antibiotics (Penicillin-G, Streptomycin, Gentamycin and Kanamycin) was used at variable concentrations with 24 and 48 h time exposures, following the protocol of Droop (1967). Even highest of the concentrations (Penicillin 2000, Streptomycin 10, Gentamycin 10 and Kanamycin 5 µg/ ml) did not harm any of the microalgae treated. But only 17 microalgae were got purified, however the antibiotics had considerably reduced bacterial load from the cultures. Many of the cultures which were heavily loaded with bacteria previously, after treatment, developed bacteria in broth only after 72 h incubation. A second treatment may thence clear the bacteria completely. Besides, for specific microalgae, based on their contamination rate, diverse techniques must be employed, of which only sonication was tried for selected strains. Sonication helps in releasing epiphytic microbes and those contaminants which live in mucus and extracellular polysaccharide secretions of microalgae (Azma et al. 2010). Complete removal of unwanted cyanobacteria from *Chlorella* sp. S095 was a good example for effectiveness of sonication.

A major difficulty observed in this study was that the strains were variedly loaded with bacteria both in numbers and types. Secondly, only

culturable bacteria were considered and treated, while many unculturable microbes may also harbor microalgal cultures. In short, more individual experimental trials are needed to develop a full proof culture system, incorporating different techniques for separate species based on their microhabitat (phycosphere).

2.4.4 Preservation of Live Microalgae

Microalgae are actively metabolizing organisms and hence demands periodic subculturing within appropriate time intervals. Routine maintenance of large numbers of cultures is often difficult, time consuming and costly. Moreover, when more cultures are handled, possibility of cross-contamination is higher. Another problem is the genetic drift and alteration in physiological and phenotypic traits (Day and Brand 2005, Acreman 2004). This can be exemplified by decrease of size in diatoms in artificial culture systems, due to lack of sexual reproduction (Evans and Mann 2009).

Cryopreservation is an advantageous method for preservation of microalgae. Selected strains were attempted in this study showed some positive results for green and blue green algae. Studies on different cryo-protocols with different cryoprotective agents (CPA) are available for both saline and fresh water microalgae (Abreu et al. 2012; Canavate and Lubinn 1995; Gwo et al. 2005; Nakanishi, Deuchi, and Kuwano 2012). Three CPAs glycerol (GLY), methanol (MeOH) and dimethyl sulfoxide (DMSO/Me_2SO) are the most commonly used, which can penetrate though the plasma membrane and equilibrate cytoplasm and avoid cryo-injury. Glycerol has been a successful cryoprotectant in the preservation of spermatozoa and bacteria and was reported also for a few microalgae like *Tetraselmis* and *Chlorella* (Day et.al. 1993, 1997). MeOH, with a faster equilibration rate, was described

as an effective CPA for fresh water microalgae but not for marine forms. For marine algae dimethyl sulfoxide (DMSO) was proven better than the other two CPAs. Day and Brand (2005) correlate this with the slow equilibration rate of DMSO. In this study, MeOH and Glycerol preserved marine isolates of *Chlorella* sp. S072 and *Picochlorum* spp. (S170 and S030) were found live. Response of *Ochrosphaera*, *Nannochloropsis*, coccoid green algae, *Oscillatoria*, *Synechocystis* and some diatoms was positive with DMSO preservation. When different concentrations of DMSO were tried, 10% was detected to be ideal for most successfully cryo-preserved strains and that too without any pre-cooling (direct plunging into LN).

Microalgae were also tried to preserve at 4°C, -20°C and -80°C other than LN. Interestingly, two blue green algae (*Oscillatoria* and *Synechocystis*) were found live in 4°C preserved samples. However, -20°C was good for none, which was in disparity with the results of Tzovenis, Triantaphyllidis, and Naihong (2004). Refrigeration or freezing of harvested (concentrated) microalgae is often employed in aquaculture feeding for storage purpose, however with or without any CPA (Palanichamy and Rani 2004; Seychelles et al. 2009). Further rarely any studies have shown viability status of the cells. Most the strains (including *Synechocystis*) experimented here are common live feeds (Pratoomyot, Srivilas, and Noiraksar 2005). Hence the results for *Synechocystis* sp. preservation at 4°C show possibility of refrigerating it for use as live feed.

Both freezing and thawing events may cause cell injuries (Tzovenis, Triantaphyllidis, and Naihong 2004). *Isochrysis* preserved in DMSO (5 & 10%) in -80 and LN were observed with only <40% intact cells immediately after thawing which got further reduced (<10%) after some time. Many species (*Dunaliella* and *Tetrasemis*) were noted viable and healthy (minimum

20% cells) immediately after thawing but post thaw culture was a failure due to unknown reasons. Rate of survival was also different in different CPAs, where 40 - 80% was there in DMSO preserved samples but < 40% with other CPAs. We got comparable results with previous reports for *Nannchloropsis*, *Chaetoceros*, *Chlorella*, and *Thalassiosira*, however not for *I. galbana, Skeletonema*, *Tetraselmis* and *Dunlaiella* (Abreu et al. 2012; Canavate and Lubinn 1995; Gwo et al. 2005; Nakanishi, Deuchi, and Kuwano 2012).

Recent report by Nakanishi, Deuchi, and Kuwano (2012) shows the success of cryopreservation of microalgae for up to 15 years. On the other hand, higher cost (of specialized equipments, continuous supply of LN, and training for handling), lack of generalized protocol (necessitate individual standardization) and low/un-predictable revival of cells remain as major disadvantages of cryopreservation (Day and Brand 2005). The data for cryopreservation in this study was however taken only for initial 1 year and only non-flagellate green algae were live for the full period, while others only for 3 or 6 months. This indicates requirement of more experimental investigations and thereby modifications in the procedure for better survival of individual species, because effectiveness of cryoprotectants and cooling protocols varies among the strains (Taylor and Fletcher 1998; Nakanishi, Deuchi, and Kuwano 2012).

Several simple, low cost and successful preservation methods can preserve microalgae for shorter periods, nearly 6 – 12 months. Immobilization or entrapment in natural polysaccharides is one of best methods available. Moreno-Garrido (2008) has reviewed on different immobilization techniques for various purposes including culture preservation. Many of the previous studies (Chen 2001, Hertzberg and Jensen 1989, Lukavsky 1988, Joo et.al 2001, Romo and Perez-Martinez 1997) were mostly on alginate beads or in

agar entrapments, and were found successful for many diatoms, green algae, blue green algae and some flagellates. In present study, many strains which failed in cryopreservation, effectively got preserved 'in' or 'on' agar (17/25 and 22/25 strains respectively) for a period of 3 months to 3 years. This procedure therefore can save time, effort and money spent on serial transfers of microalgae as well as on cryopreservation.

2.4.5 Remarks on Different Classes of Microalgae:

To get a clear perspective on different classes and genera of microalgae, some important aspects of each genera present in the collection are given, including their systematic position, general features and importance (isolation details and morphological descriptions of strains in Table 2.1., 2.2 and 2.5)

I. Kingdom : Prokaryota

Phylum : Cyanophyta

Class : Cyanophyceae:

Commonly called as 'blue green algae' or 'Cyanobacteria'; most primitive algae; pigments: chlorophyll a and phycobiliproteins; storage product – glycogen; simple morphology – single cells without nucleus and other cellular compartments; used as food, feed, fertilizer and in physiological and molecular studies.

a) Order : Oscillatoriales

1. **Genus :** ***Arthrospira*** (MBTD-CMFRI-S016 & S152)

Common name – 'Spirulina'; Filamentous blue green alga; grown in fresh or brackish waters with high alkalinity (pH 10-11); used as "single cell proteins" (SCPs); *A. platensis & A. maxima* are two economically important species used in malnutrition eradication; source of phycocyanin (Habib et al. FAO

2008). Culture very stable (6-12 months), however other preservation techniques failed; can easily be purified by micropipette washing.

2. **Genus :** ***Oscillatoria*** (MBTD-CMFRI-S137)

Filamentous blue green alga; grows in fresh, brackish, marine and hot spring waters. Several strains have antimicrobial property (Prabakaran 2011; Thajuddin and Subramanian 2005). S137 is a halo-tolerant species; can be preserved by agar plating and cryopreservation (with CPAs DMSO and MeOH)

3. **Genus :** ***Gietlerinema*** (MBTD-CMFRI-S100)

Filamentous blue green alga; grows in fresh, brackish, marine and thermal springs waters; toxic species present (Dogo et al. 2011). Strain S100 was identified as Gietlerinema by 16S rDNA sequence similarity (Chapter 3, Table 3.4.1)

b) Order : Chroococcales

4. **Genera :** ***Synechococcus* & *Synechocystis*** (MBTD-CMFRI-S041, S010, S034 & S107)

Unicellular coccoid cells; both have similar morphology, normally discriminated by nucleic acid sequence dissimilarities (Lee 2008); uses and importance: as aquaculture feed, in genetic engineering, antimicrobials (Pulz and Gross 2004; Martins et al. 2008; Cardoso et al. 2012). Preserved by agar and in LN, -80°C and 4°C

5. **Genera :** ***Cyanothece*** (MBTD-CMFRI-S098 & S120).

Unicellular ovoid cells, larger than above two genera (longer up to 10-17 µm); reported with significant roles in the nitrogen cycle in aquatic and terrestrial environments (Bandyopadhyay, Elvitigala, and Welsh 2011); bio-hydrogen (as fuel) production (Min and Sherman 2010) Our cultures – hyper

saline, highly viscous, with exo-polysaccharide production (Philippis et al. 1993); poorly preserved on agar and in LN with DMSO.

II. Kingdom : Eukaryota

Phylum : Heterokontophyta

Class : Bacillariophycea

Commonly called diatoms. Cells covered with silicicious frustules; chlorophyll a and c; fucoxanthin; storageproduct usually chrysolaminarin. Largest with about 200 living genera; uses: as aquaculture feed, in nanotechnology, in genetic engeneering, physiological studies, bio-fuel production etc. (Yadugiri 2009; Ying and Kangsen 2005; Scholz and Liebezeit 2012; Barsanti and Gualtieri 2006; Pulz and Gross 2004); fossil diatoms – diatomaceous earth – used for purification, filtration, etc.

a) Order : Chaetocerotales

6. **Genus : *Cheatoceros*** (MBTD-CMFRI-S005, S024, S031, S042, S062, S065 & S172)

Unicellular or colonial centric bipolar diatoms; brown; about 500 species described; commonly used aquaculture feed; other uses – bioactive compounds, bio-fuel etc. (Banerjee et al. 2011; Balamurugan et al. 2013; Schenk et al. 2008). Strains are euryhaline; some grow on agar; poorly preserved in LN.

b) Order : Thalassiosirales

7. **Genus : *Cyclotella*** (MBTD-CMFRI-S018, S044, S052, S079 & S080)

Centric diatom; drum shaped, free-living or chain forming cells; brown in colour; live in fresh, brackish or marine waters; used as an aquaculture feed,

model organism, bio-fuel etc. (Spolaore et al. 2006; Renaud, Thinh, and Parry 1999). Strains are euryhaline; grow on agar and weakly in agar.

8. **Genus : *Thalassiosira*** (MBTD-CMFRI-S019, S033, S045, S051, S069, S084, S132 & S158)

Centric diatom; discoid, cylindrical cells in chain or in mucilage; brown; live in fresh, brackish or marine waters; used as an aquaculture feed, model organism, bio-fuel, genetic engineering etc. (Alverson et al. 2011; Knuckey et al. 2006; Bowler et al. 2008; Borowitzka 1997). Strains are euryhaline; grow well on agar and weakly in agar; in LN for 3 months.

9. **Genus : *Skeletonema*** (MBTD-CMFRI-S032 & S049)

Centric diatom; cells cylindrical or drum shaped in chain; brown; mostly marine; used as aquaculture feed, in molecular research, bio-fuels (Popovich et al. 2011; Hwang et al. 1999; Gao, Smith, and Alberte 1993); Strains S032 & S049 morphologically similar but differ by 18S rDNA phylogeny, *S. costatum* and *S. ardens* respectively; negative results for all preservation techniques.

c) Order : Cymatocerales

10. **Genus : *Minutocellus*** (MBTD-CMFRI-S050 & S088)

Centric diatom; cells cylindrical; attached on substratum, colonial; brown; marine; grows well on agar

d) Order : Hemiaulales

11. **Genus : *Bellerochea*** (MBTD-CMFRI-S167)

Centric diatom, with delicate frustules; brown; unicellular; marine; used as aquaculture feed (Coutteau 1996); preservation poorly by agar.

e) Order : Bacillariales

12. **Genus : *Nitzschia*** (MBTD-CMFRI-S021, S092 & S148)

Pennate diatom; benthic; unicellular or colonial; euryhaline; used as live feed in aquaculture (Wen and Chen 2000; Ying and Kangsen 2005); preservation on agar was good, in LN and agar entrapment were not so effective.

13. **Genus : *Cylindrotheca*** (MBTD-CMFRI-S061 & S099)

Pennate diatom; benthic; unicellular; euryhaline; used as live feed in aquaculture (Brown & Jeffrey, 1995; Liang, Mai, & Sun, 2005); grows well on agar. *C. closterium* and *Nitzchia longissima* are morphologically indistinguishable.

f) Order : Naviculales

14. Genus : *Navicula* (MBTD-CMFRI-S043, S060, S117 & S136)

Unicellular pennate diatom; boat-shaped; euryhaline; benthic; considered as a keystone species; used as live feed, in bio- fuel (Brown & Jeffrey, 1995; Popovich et al., 2011); preserved well on agar and poorly in agar and in LN.

III. Kingdom : Eukaryota

Phylum : Heterokontophyta

Class : Eustigmatophyceae:

Yellow-green unicells; euryhaline; eye-spot outside chloroplast; chlorophyll a and c; fucoxanthin; storage product usually chrysolaminarin; flagellate or nonflagellate cells.

a) Order : Eustigmatales

1. **Genus : *Nannochloropsis*** (MBTD-CMFRI-S006, S012, S015, S076, S077 & S078)

Unicellular; nanoplanktonic; yellow-green, sub-spherical cells; marine; uses: common aquaculture feed with high EPA content, source of EPA, for

bio-fuels, genetic engineering, and in bioremediation. (Doan & Obbard, 2012; Hoshida, Ohira, Minematsu, Akada, & Nishizawa, 2005; Roncarati, Meluzzi, Acciarri, Tallarico, & Melotti, 2004; Yoshida, Ishii, Ishihara, Saito, & Okada, 2008). By molecular phylogeny all strains were identified as *N. oceanica.* Can be preserved by agar and in LN (with 10% DMSO).

IV. Kingdom : Eukaryota

Phylum : Heterokontophyta

Class : Raphidophyceae

Common name – 'chloromonads'; unicellular, biflagellate, heteroflagellate, ovoid cells; chlorophyll a & c; euryhaline and eurithermic; toxic species produce neurotoxin similar to brevitoxin.

a) Order : Chattonellales

1. **Genus : *Heterosigma*** (MBTD-CMFRI-S156)

Unicellular, biflagellate – anterior tinsel and posterior naked, ovoid yellowish cells; planktonic; cause toxic blooms in marine waters (Nagasaki & Yamaguchi, 1997); Very delicate cells, culture highly sensitive; failed in all preservation methods.

V. Kingdom : Eukaryota

Phylum : Prymnesiophyta

Class : Prymnesiophycea

Unicelluar yellow brown cells with two whiplash flagella; haptonema present; chlorophyll a and c; fucoxanthin; scales may present outside cell; storage product chrysolaminarin

a) Order : Isochrysidales

1. **Genus :** ***Isochrysis*** (MBTD-CMFRI-S001, S002, S073, S157, S169 & S173)

Unicellular, planktonic, flagellate (2 smooth flagella) and haptonema not prominent; uses: most commonl aquaculture live feed, with high EPA and DHA contents, source of PUFAs –DHA, and pigments (Kim, Kang, Kwon, Chung, & Pan, 2012; Liu, Sommerfeld, & Hu, 2013; Roncarati et al., 2004)

b) Order : Prymnesiales

2. **Genus :** ***Prymnesium*** (MBTD-CMFRI-S119)

Unicellular, planktonic, biflagellate, yellow-brown cells with haptonema; bloom forming *P. parvum* produces exotoxin prymnesin, cause fish mortalities (Katırcıoğlu, Akın, & Atıcı, 2004)

c) Order : Coccolithales

3. **Genus :** ***Ochrosphaera*** (MBTD-CMFRI-S003)

A coccolithophore; unicellular, found in cell aggregations; yellow-brown, cells covered with calcium carbonate scales (coccoliths), which also form microfossils. (Dashiell, 2010; Fresnel & Probert, 2005); our strain grows well on agar and in embedded form. Cryopreservation was effective with 10% DMSO.

VI. Kingdom : Eukaryota

Phylum : Chlorophyta

Class : Prasinophyceae

Primitive green algae; scaly or naked flagellates (nonflagellates also present); green with chlorophyll a & b; *Ostreococcus tauri* smallest eukaryote

(<1µm size); inhabit fresh, brackish, marine and hyper saline waters; uses: as aquaculture feed and in physiological and genetic research.

a) Order : Chlorodendrales

1. **Genus :** ***Tetraselmis*** (MBTD-CMFRI-S011, S027, S028, S057, S075, S081, S082, S101, S094,S126, S142)

Unicellular green tetra-flagellate; mostly marine, brackish water and hyper saline strains are also present; cells covered in theca; uses: a common aquaculture feed, and in bio-fuel research (Matos, Junior, Neto, Koening, & Leca, 2007; Moheimani, 2012; Pane, Feletti, Bertino, & Carli, 1998); last three strains S094, S126 and S142 are hyper saline strains identified as *T. indica* (a new species) by 18S rDNA phylogeny (Arora, Anil, Leliaert, Delany, & Mesbahi, 2013); can be preserved by agar plating, other methods were not successful.

VII. Kingdom : Eukaryota

Phylum : Chlorophyta

Class : Trebouxiophyceae

Encompass motile/nonmotile unicells, colonies and filaments; inhabit fresh, brackish and rarely marine waters; uses: as food, feed, nutritional supplement, bio-fuel, bioactive compounds, molecular research etc.

a) Order : Chlorellales

1. **Genus :** ***Chlorella*** (MBTD-CMFRI-S071, S072, S095 and S171)

Unicellular or colonial, planktonic nonflagellates, green cells; present in fresh, brackish and marine waters; uses: *C. vulgaris* as single cell protein (SCP), as food for human beings, as feed for animals and fishes, as bio-fuel (Elumalai,

Baskaran, Prakasam, & Kumar, 2011; Gouveia, Raymundo, Batista, Sousa, & Empis, 2006; Ördög et al., 2012; Vijayavel, Anbuselvam, & Balasubramanian, 2007); both agar based and LN preservation methods were good enough.

2. **Genus : *Picochlorum/Nannochlorum/Nanochloris*** (MBTD-CMFRI-S030, S056, S070, S102, S134, S164, S170)

Unicellular or colonial, planktonic or benthic, nonflagellate, green cells; marine or brackish water origin; uses: a live feed, for bio-fuel, (Zahn, 1994; Zhu & Dunford, 2013); all preservation methods were good (in/on agar & LN).

3. **Genus : *Didymogenes*** (MBTD-CMFRI-S026)

Unicellular coccoid, green, planktonic alga; brackish water origin (Pröschold, Bock, Luo, & Krienitz, 2010); Present strain was initially identified morphologically as *Chlorella* sp. but by 18S rDNA phylogeny matched with *Didymogenes* sp. Grows well on agar.

4. **Genus : *Oocystidium*** (MBTD-CMFRI-S138)

Unicellular or colonial, coccoid green cells; can easily be discriminated from other Chlorellales by colourless mucilaginous envelop of cells; lives in fresh or brackish waters; present strain was identified by 18S rDNA sequence similarities.

b) Order : Chlorococcales

5. **Genus : *Dictyosphaerium*** (MBTD-CMFRI-S129)

Colonial coccoid green alga, inhabit fresh water, but euryhaline; used as pollution detectors (Pena-Vazquez, Maneiro, Pérez-conde, Moreno-bondi, & Costas, 2009). Present strainS129 was a fresh water isolate but can tolerate salinity (up to 30 ppt) and acidity (up to pH 3); higher salinity will change morphology – turn to unicells, no colony formation; grows well on agar.

VIII. Kingdom : Eukaryota

Phylum : Chlorophyta

Class : Chlorophyceae

Unicelluar, colonial and chain forms chain; motile cells are biflagellate; pigments chlorophyll a & b and carotenoids; mostly fresh water; also found in brackish, marine, hyper saline, polar and thermal waters; green, yellow or red coloured cells; used as food, feed, source of bioactive molecules, pigments, biofuel and in molecular, physiological research and genetic engineering.

a) Order : Chlamydomonadales

1. **Genus :** ***Dunaliella*** (MBTD-CMFRI-S086, S089, S108, S109, S110, S111, S115, S118, S121, S122, S123, S124, S125, S133, S135, S147, S151, S166)

Unicellular, biflagellate, ovoid, green, yellow green or orange cells; usually inhabits hyper saline waters – halophilic and halotolerant. *D. salina* most halotolerant eukaryote (>300 ppt), accumulates beta-carotene by increase in salinity, light, temperature or on nutrient deprive conditions; used as live feed, and as source of natural β-carotene, antioxidants, bioactive compounds, biofuel etc.; used in physiological and molecular research. (Hadi, Shariati, & Afsharzadeh, 2008; Kleinegris, Janssen, Brandenburg, & Wijffels, 2010; Oren, 2005; Shariati & Hadi, 2011); all strains were hyper saline origin except S135 which was identified as *D. salina* (Preetha, John, Subin, & Vijayan, 2012)

b) Order : Sphaeropleales

2. **Genus :** ***Monoraphidium*** (MBTD-CMFRI-S139)

Unicellular, helically twisted or sigmoid body; planktonic; green; habitat: mostly fresh or brackish water; uses: as feed and source of bioactive

molecules (Ordog et al., 2004). Present strain was isolated from brackish water pool, identified by molecular phylogeny (*rbc*L gene).

3. **Genus : *Mychonastes*** (MBTD-CMFRI-S130)

Unicellular or colonial; green spherical cells; inhabit fresh waters mostly; planktonic; Strain S130 was morphologically similar to Chlorella (except lack of pyrenoid), and identified by 18S rDNA phylogeny.

IX. Kingdom : Eukaryota

Phylum : Chlorophyta

Class : Ulvophyceae

Include both micro and macroalgae. In the collection we got two strains S113 and S145 with sarcinoid (cells aggregated into 3 dimensional packets) and unicellular morphology respectively. The identification as ulvophycean strains was however done by 18S rDNA gene sequence similarities (more details in table 2.5 and Table 3.4.1 of Chapter 3)

2.5 Conclusion

Present study is comprised of an account on microalgal diversity of a culture collection developed from saline water bodies along the Indian coast, and describes essential culture practices involved. Even though, the sampling sites were selected in such way to cover almost entire coastal belt of the country, the original diversity did not reflected in the collection. Only culturable forms of microalgae originated and sustained in laboratory. However, in an experimental point of view, 9 classes, 30 genera and 140 strains were not too small. The perpetual maintenance of the cultures, retaining the purity and vigor of the strains, was the major challenge. Morphological identification was as well not so easy, particularly for nano/pico size

microalgae. Phenotypic plasticity of cells enlarged the difficulty, which was later on solved to some extend by molecular taxonomy (next chapter). The heterogeneous cultures also posed disputes in purification as well as preservation experiments; and results highlighted green algae as most stable in both aspects. This study is unique and important in a conservation point of view and this is an original one in such a manner from India. In addition the culture collection also opens new realms for research in microalgae biology and biotechnology. Further, this piece of document can also be a reference material for those who work in this area.

Figure 2.7 Microscopic images of pure (mono-algal) strains of microalgae - Plates 1-17 (morphological descriptions in Table 2.5)

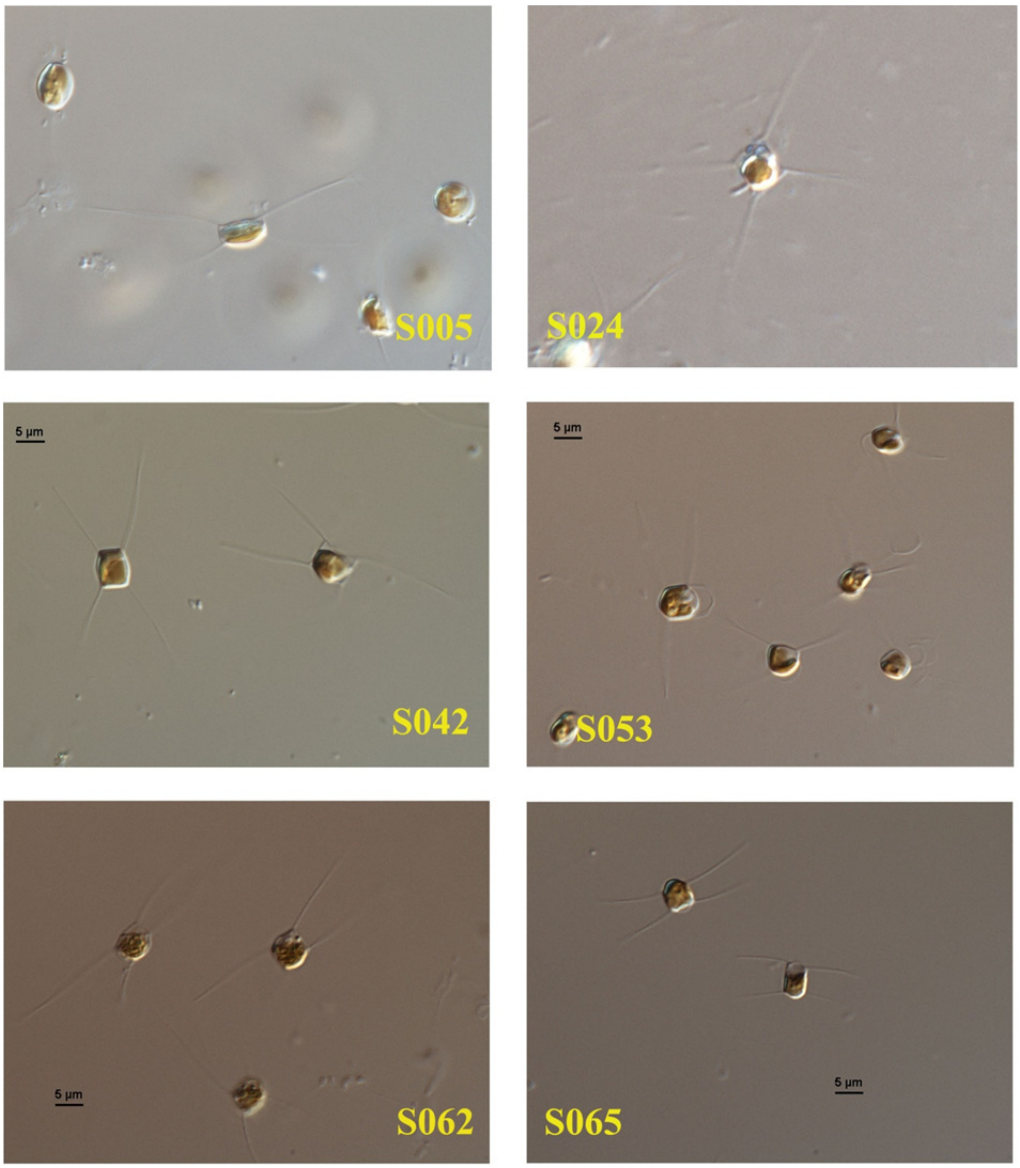


Plate 1: Bacillariophyceae – Centric diatoms – *Chaetoceros* spp. (Strains S005, S024, S042, S053, S062, & S065)

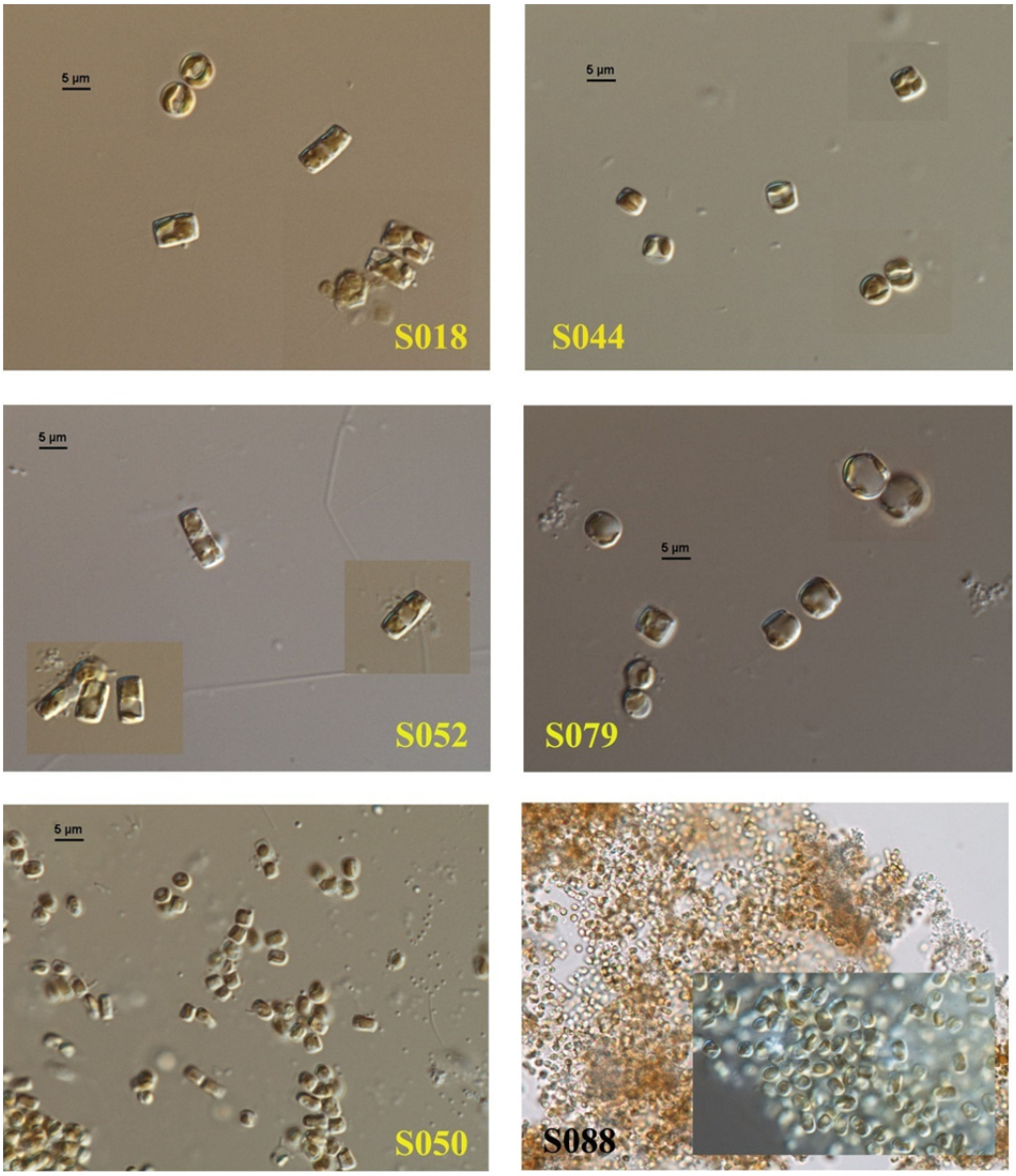


Plate 2: Bacillariophyceae – Centric diatoms – *Cyclotella* spp. (S018, S044, S052 & S079) and *Minutocellus polymorphus* (S050 & S088)

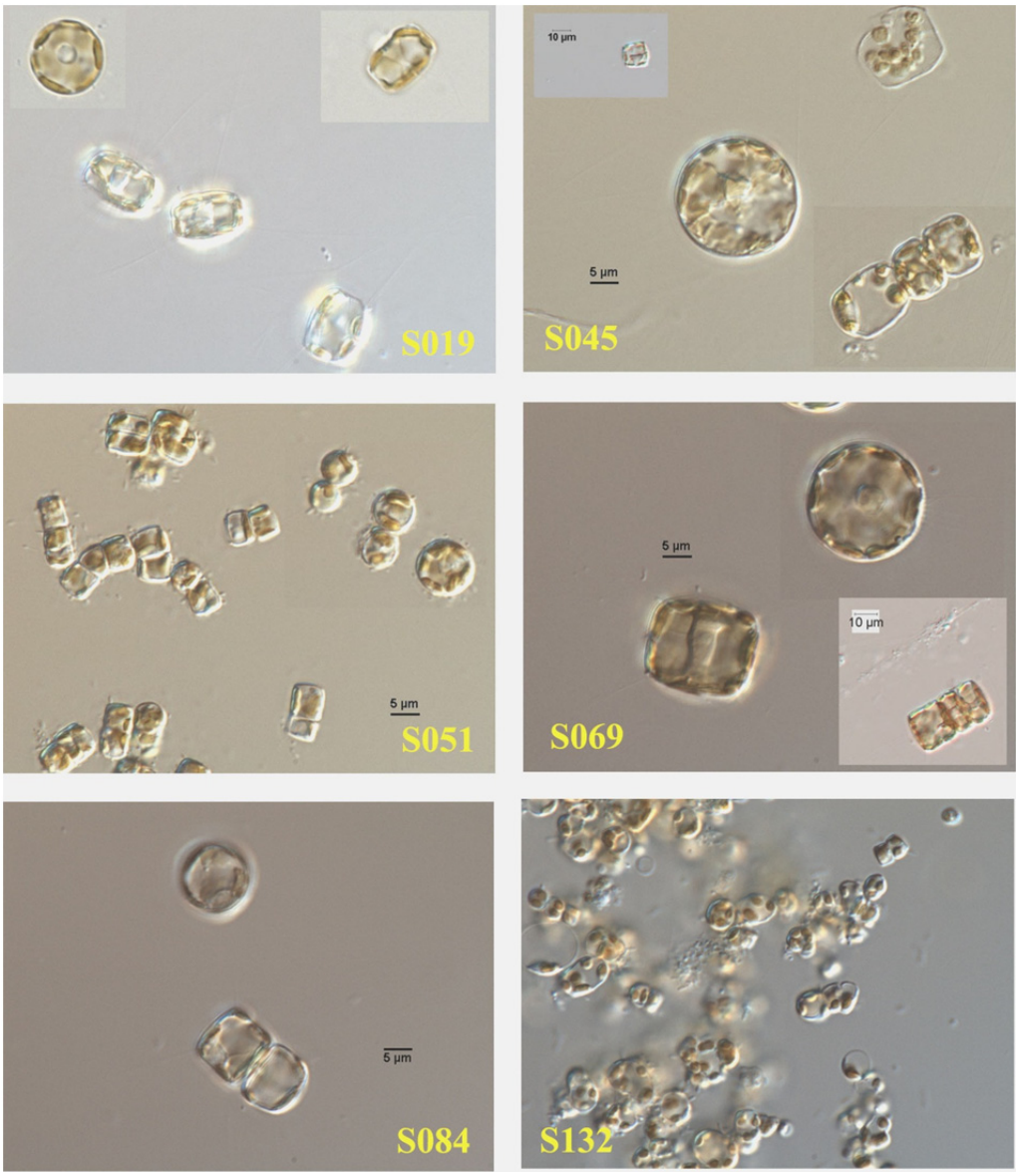


Plate 3: Bacillariophyceae – Centric diatoms – *Thalassiosira* spp. (S019, S045, S051, S069, S084 & S132)

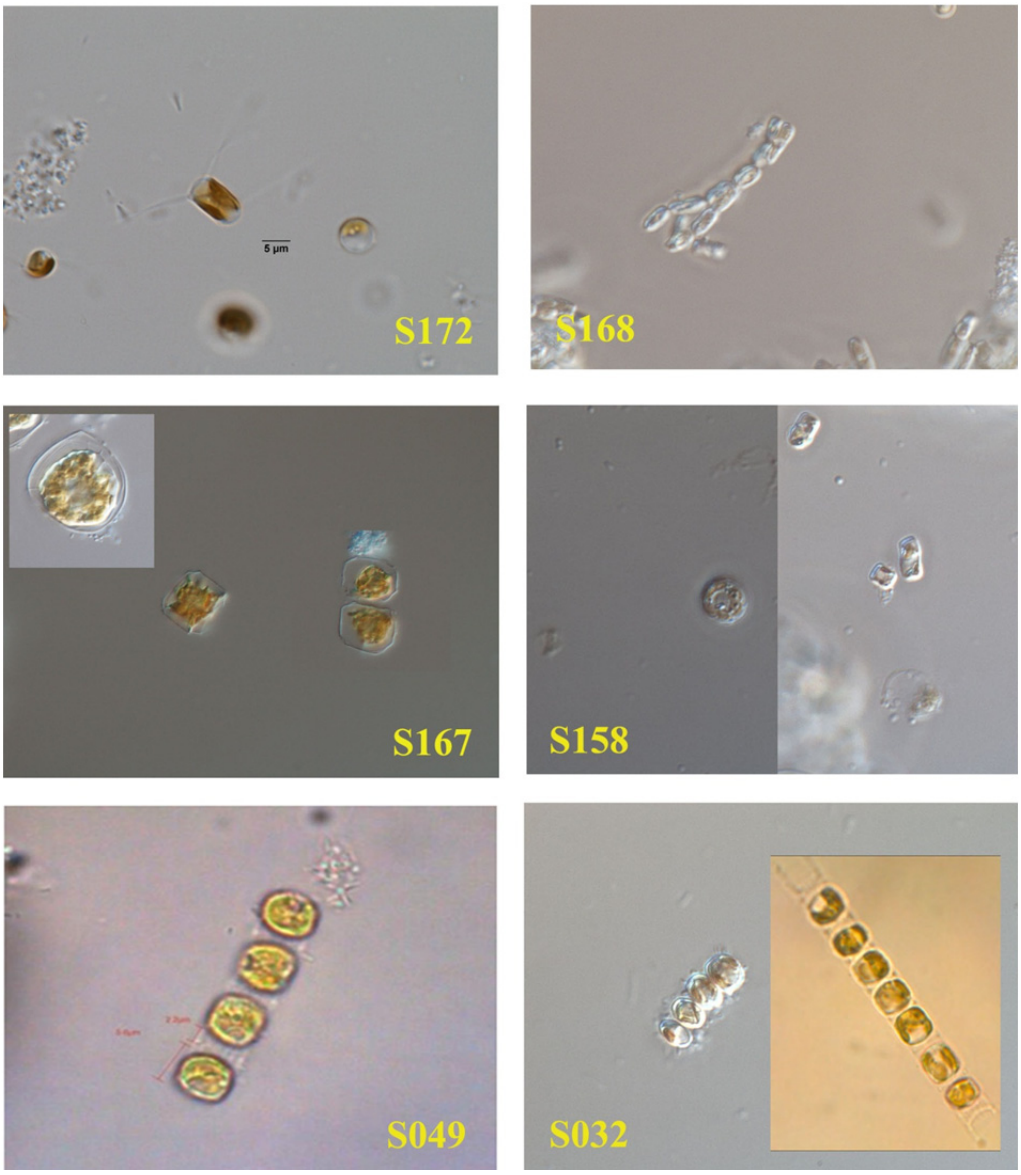


Plate 4: Bacillariophyceae – Centric diatoms – *Chaetoceros gracilis* (S172), *Melosira* sp.? (S168), *Bellerochea* sp. (S167), *Thalassiosira* sp. (S158) and *Skeletonema* spp. (S032 & S049)

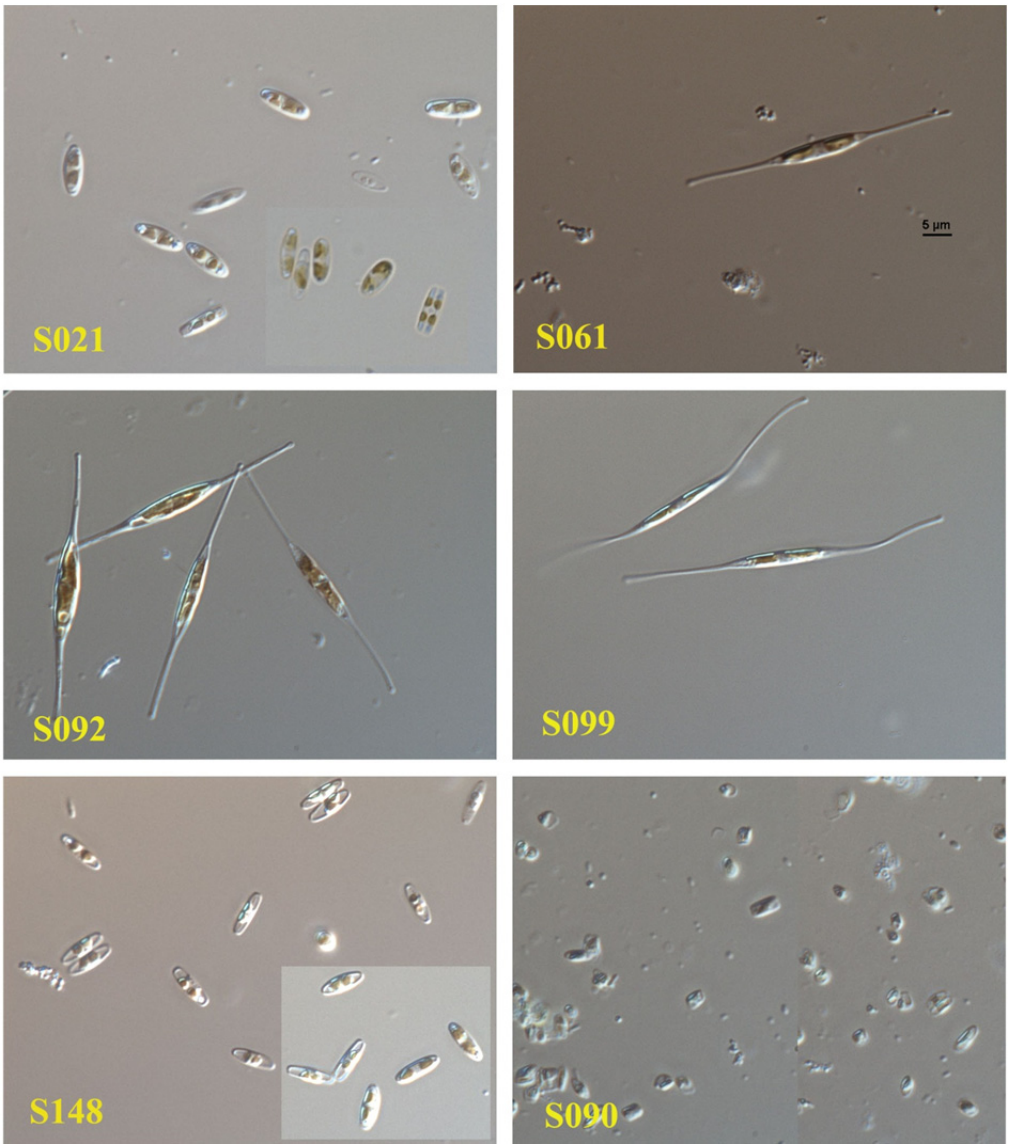


Plate 5: Bacillariophyceae – Pennate diatoms – *Nitzschia* sp. (S021 & S148), *Nitzschia/Cylindrotheca* spp. (S061, S092, S099) and an unidentified strain (S090)

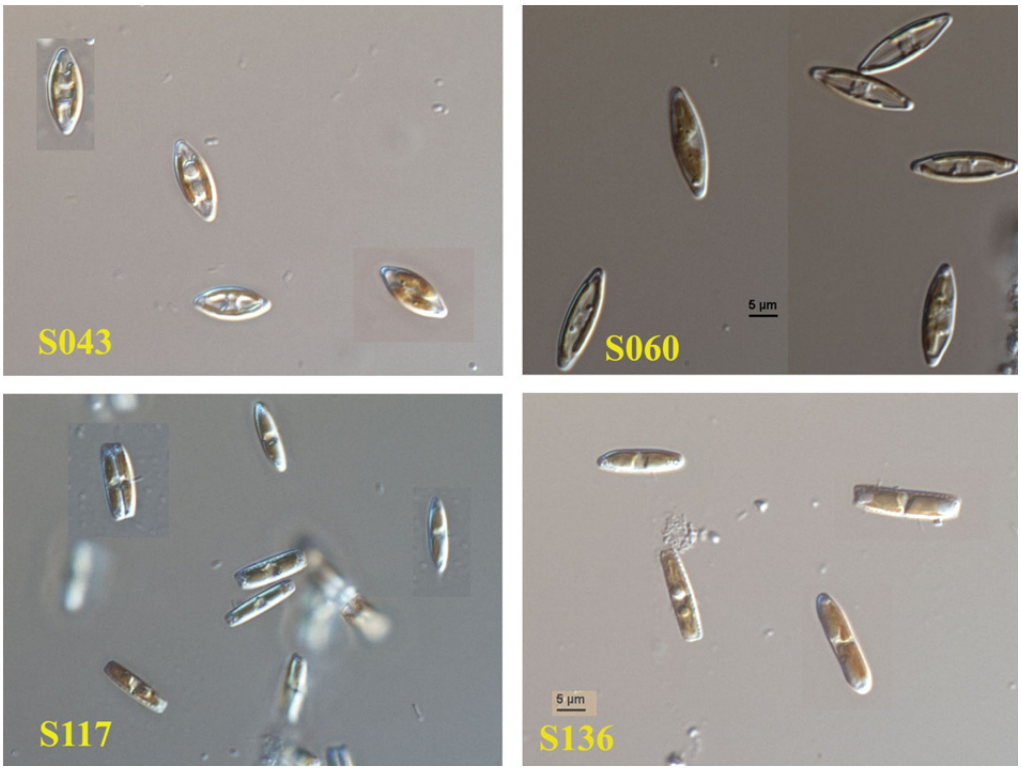


Plate 6: Bacillariophyceae – Pennate diatoms – *Navicula* spp. (S043, S060, S117 and S136)

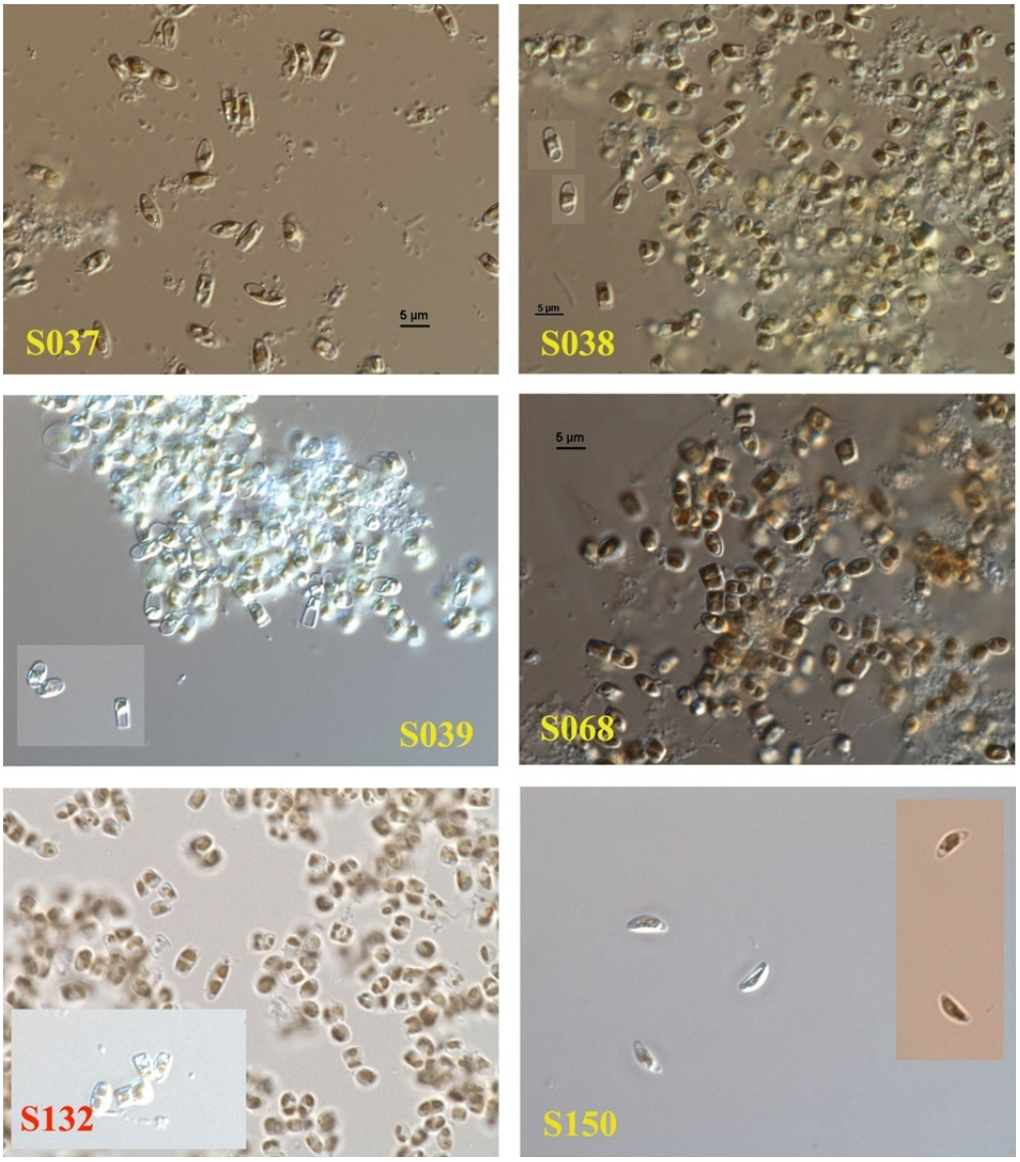


Plate 7: Bacillariophyceae – Pennate diatoms – unidentified isolates (S037, S038, S039, S068, S132 & S150)

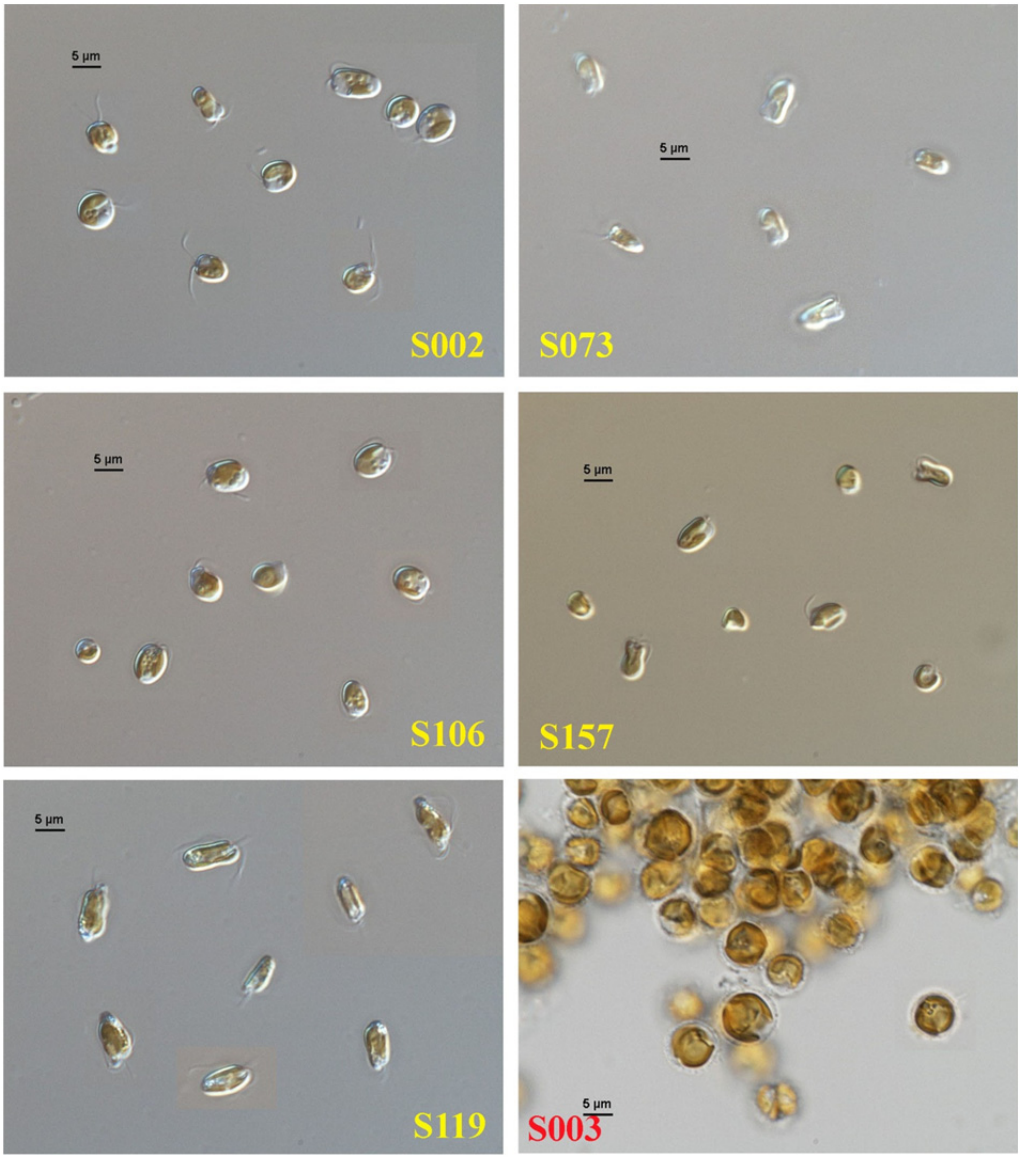


Plate 8: Prymnesiophyceae – *Isochrysis galbana* (S002, S073, S106 & S157), *Prymnesium* sp. (S119) and *Ochrosphaaera* sp. (S003)

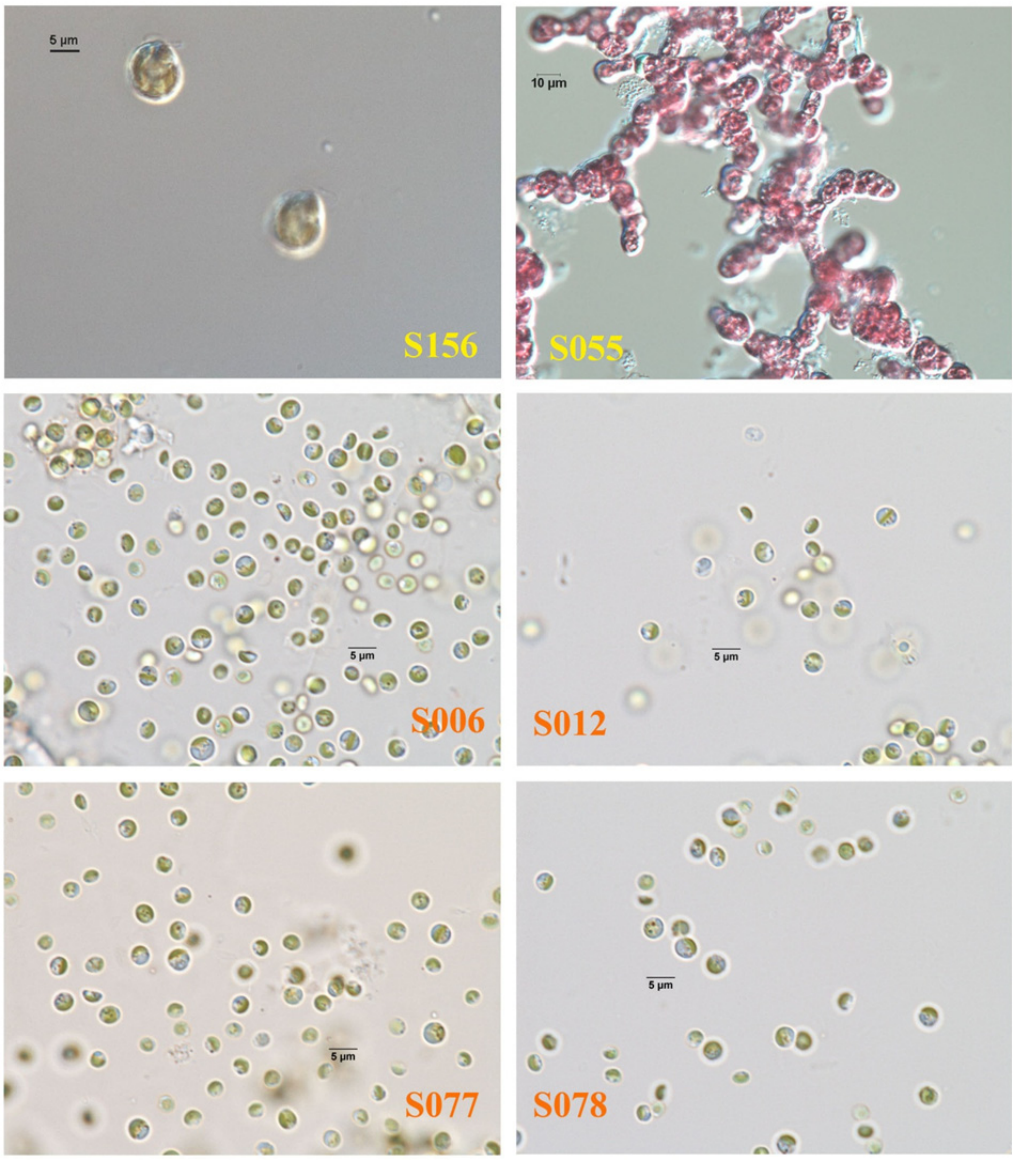


Plate 9: Raphidophyceaen *Heterosigma* sp. (S156), an unidentified benthic microalga (pink, S055) and Eustigmatophycean *Nannochloropsis oceanica* (S006, S012, S077 & S078)

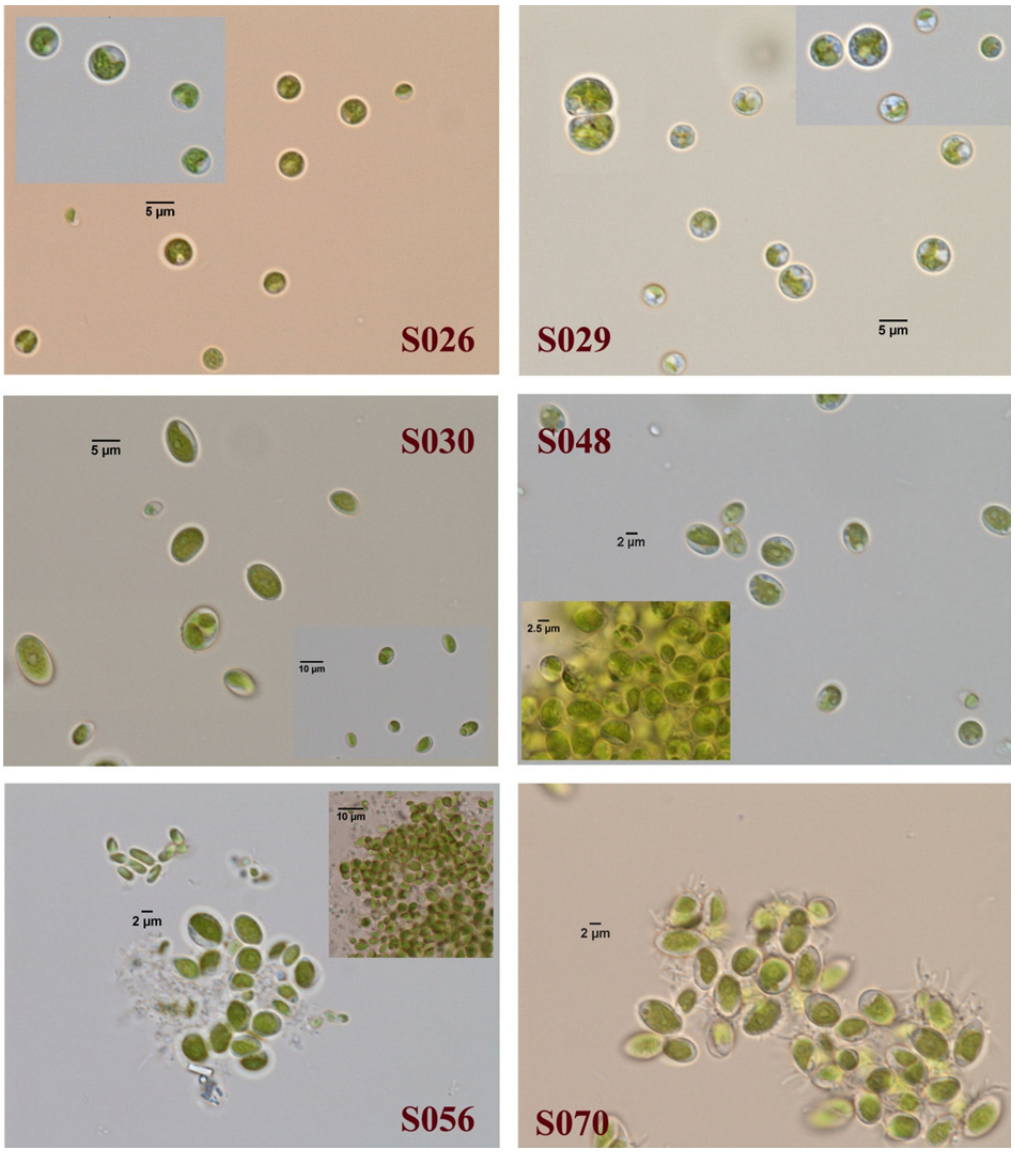


Plate 10: Trebouxiophyceae – *Didymogenes* sp. (S026) and *Nanochlorum/ Picochlorum/ Nannchloris* spp. (S029, S030, S048, S056 & S070)

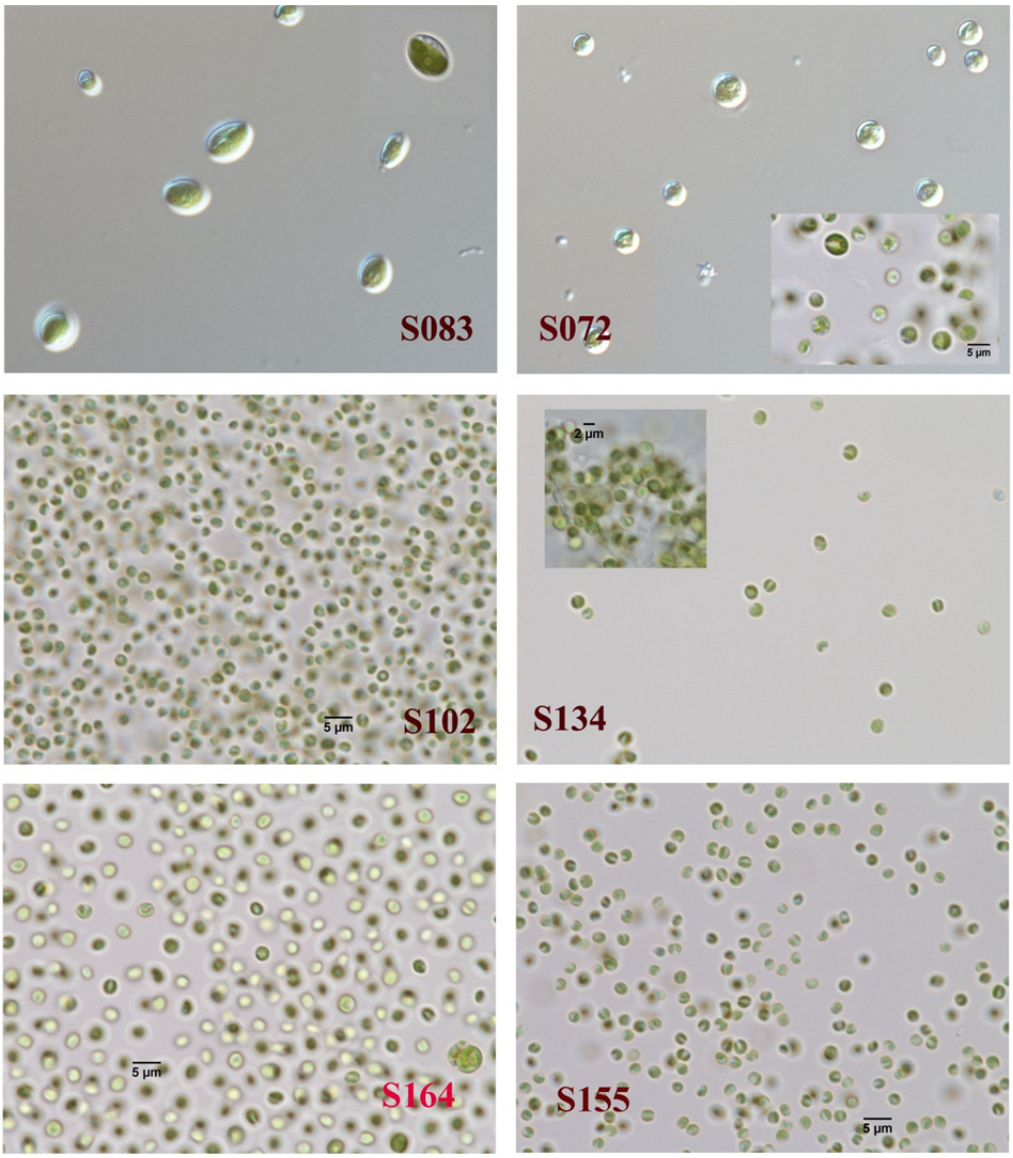


Plate 11: Trebouxiophyceae - *Nanochlorum/Picochlorum/Nannchloris* spp. (S083, S102, S134, S155 & S164) and *Chlorella* sp. (S072)

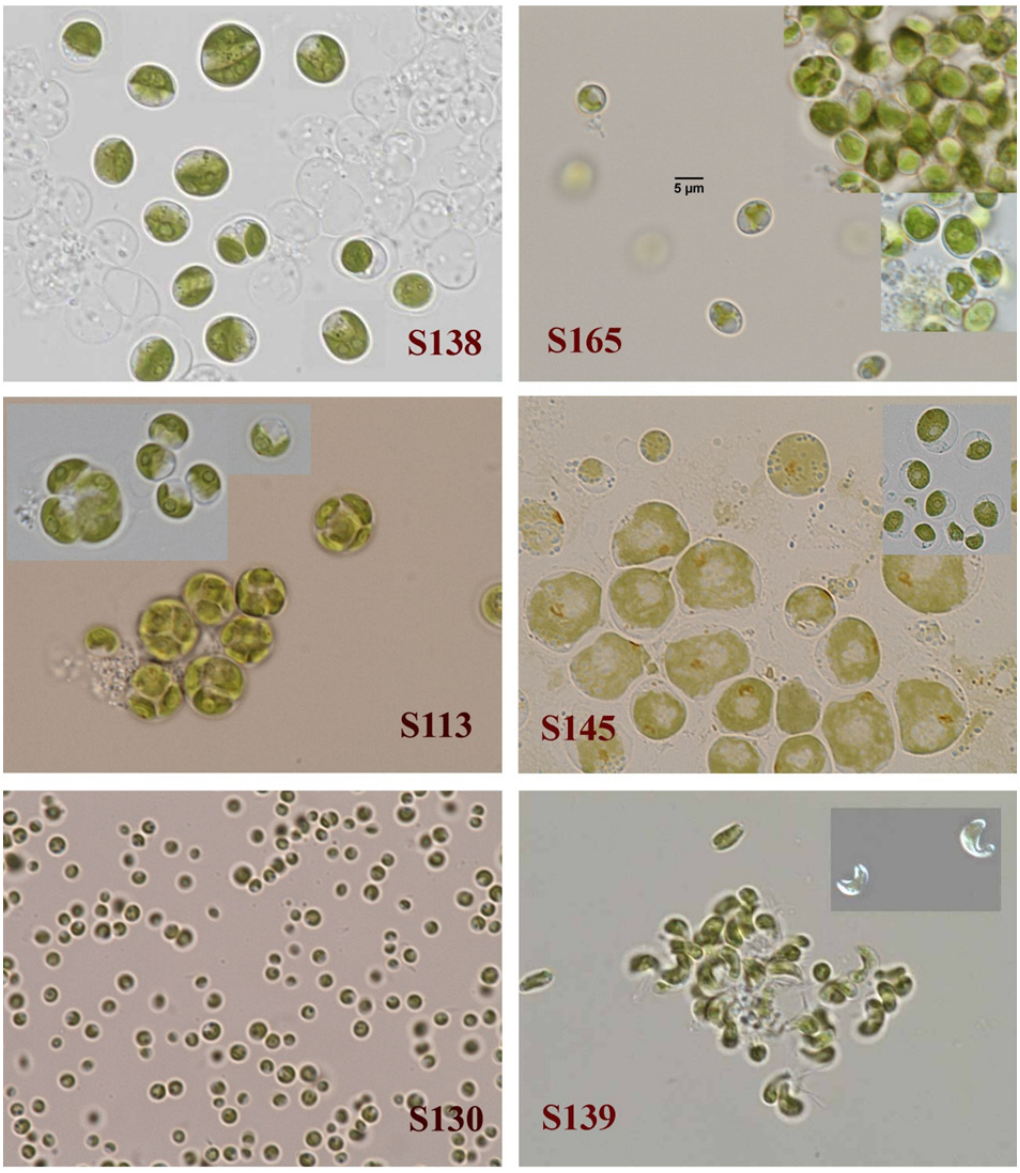
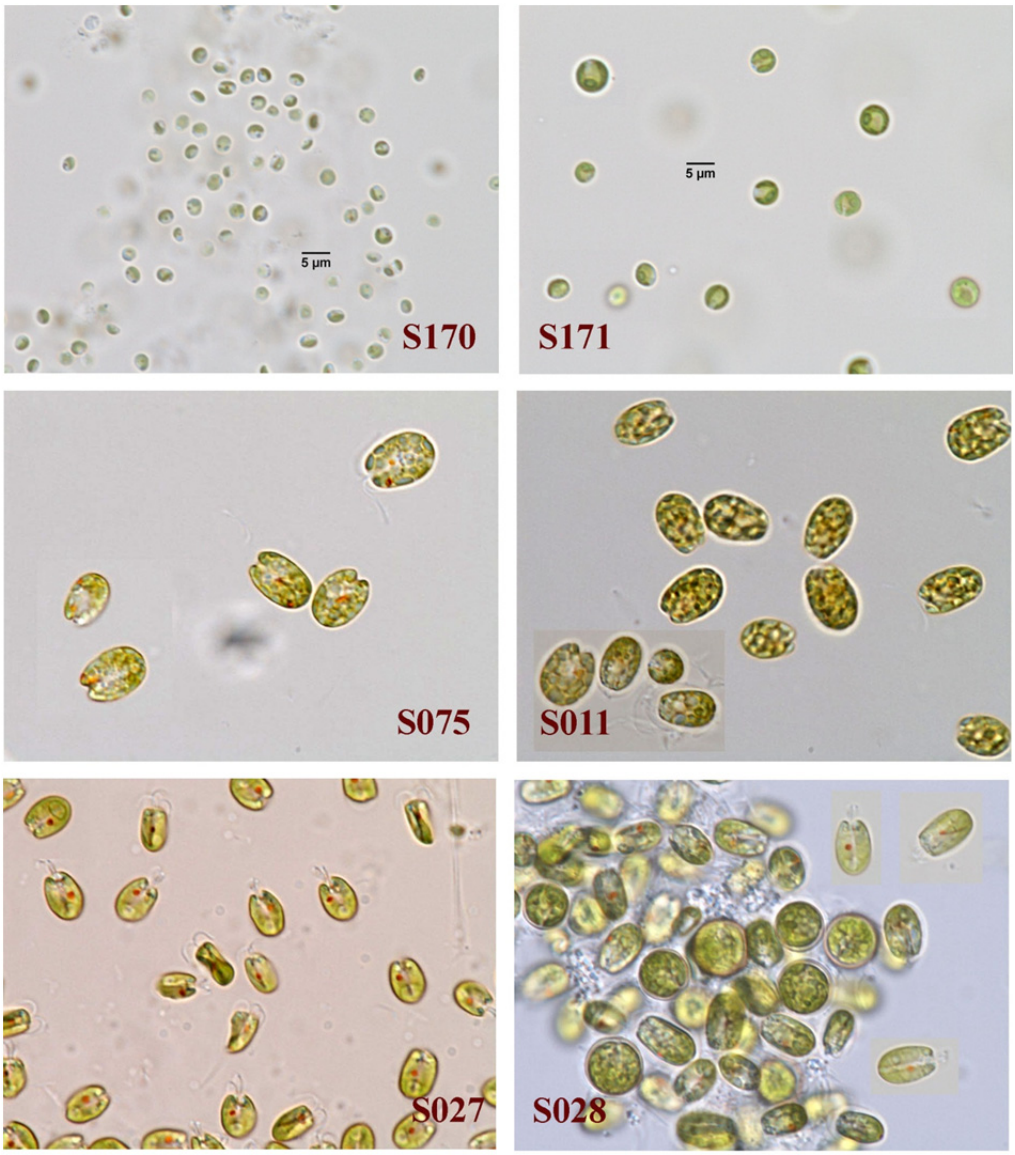


Plate 12: Trebouxiophyceae – *Oocystidium* sp. (S138) an unidentified strain (S165); Ulvophyceae - (S113 & S145) Chlorophyceae –*Mychonastes* sp. (S130) and *Monoraphidium* sp. (S139)



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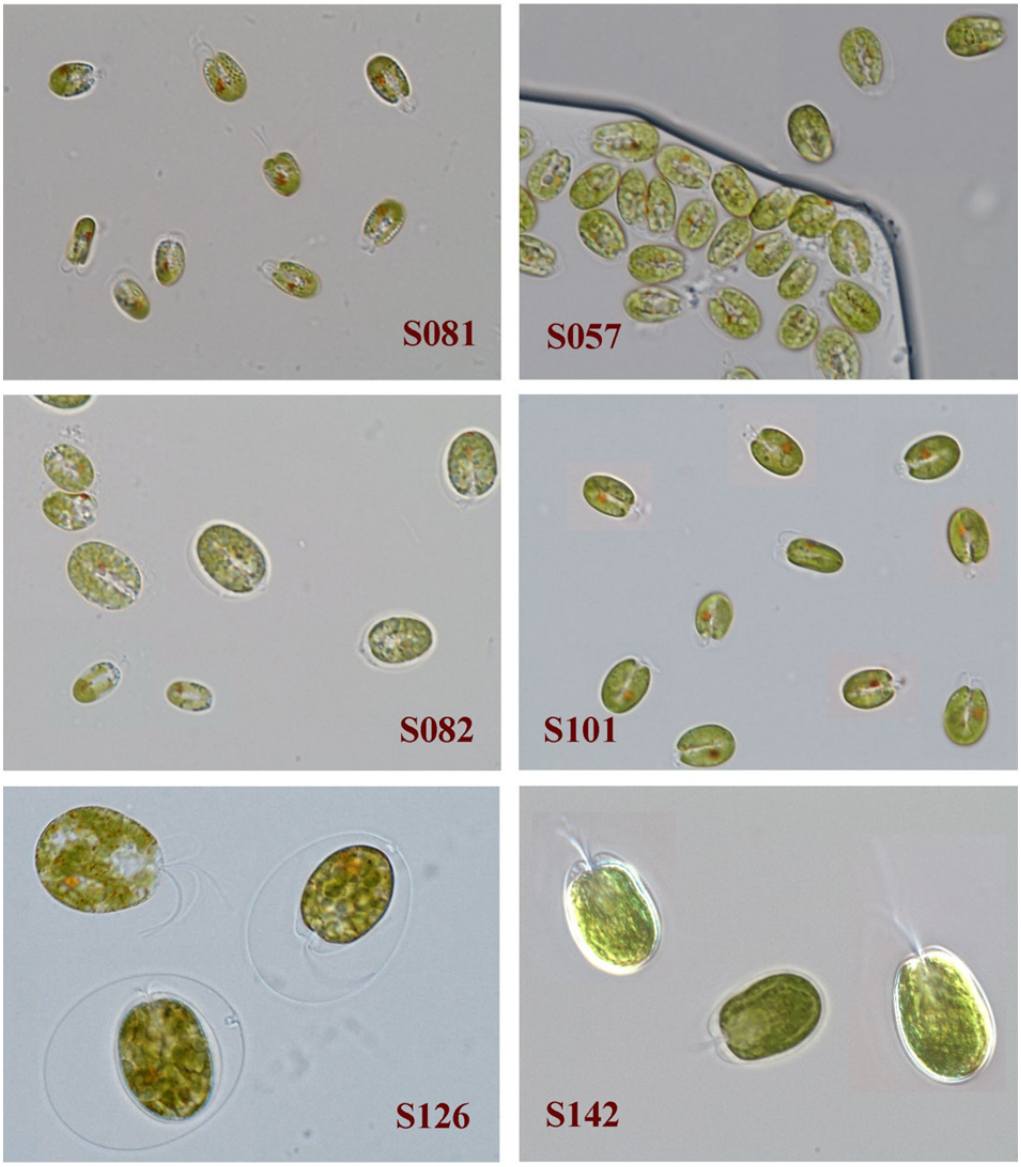


Plate 14: Prasinophyceae – *Tetraselmis* sp. (S057, S081, S082, S101) and *Tetrasemis indica* (S126 & S142) all 400X

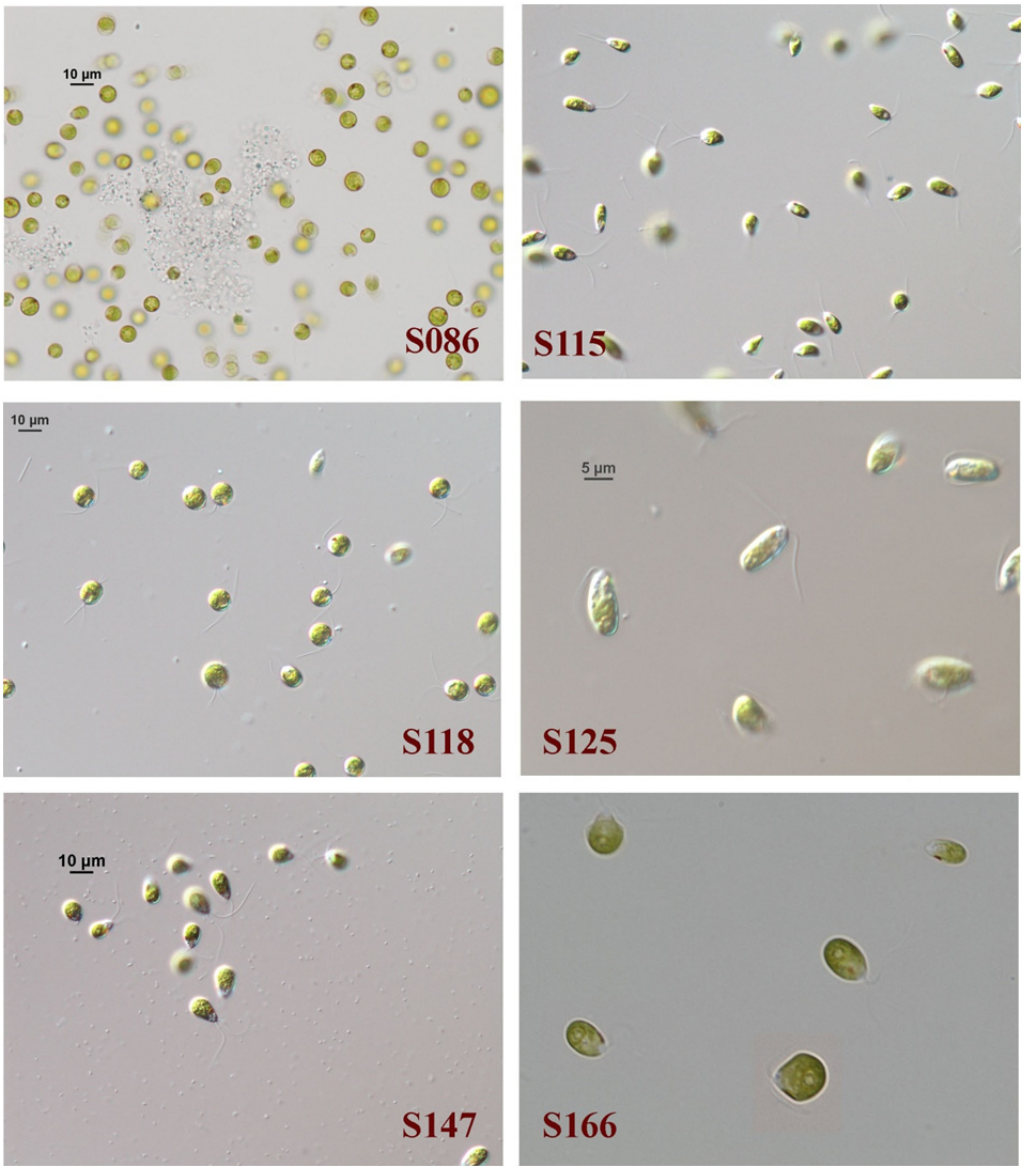


Plate 15: Chlorophyceae – *Dunaliella* spp. (S086, S115, S118, S125, S147 & S166) all 400X

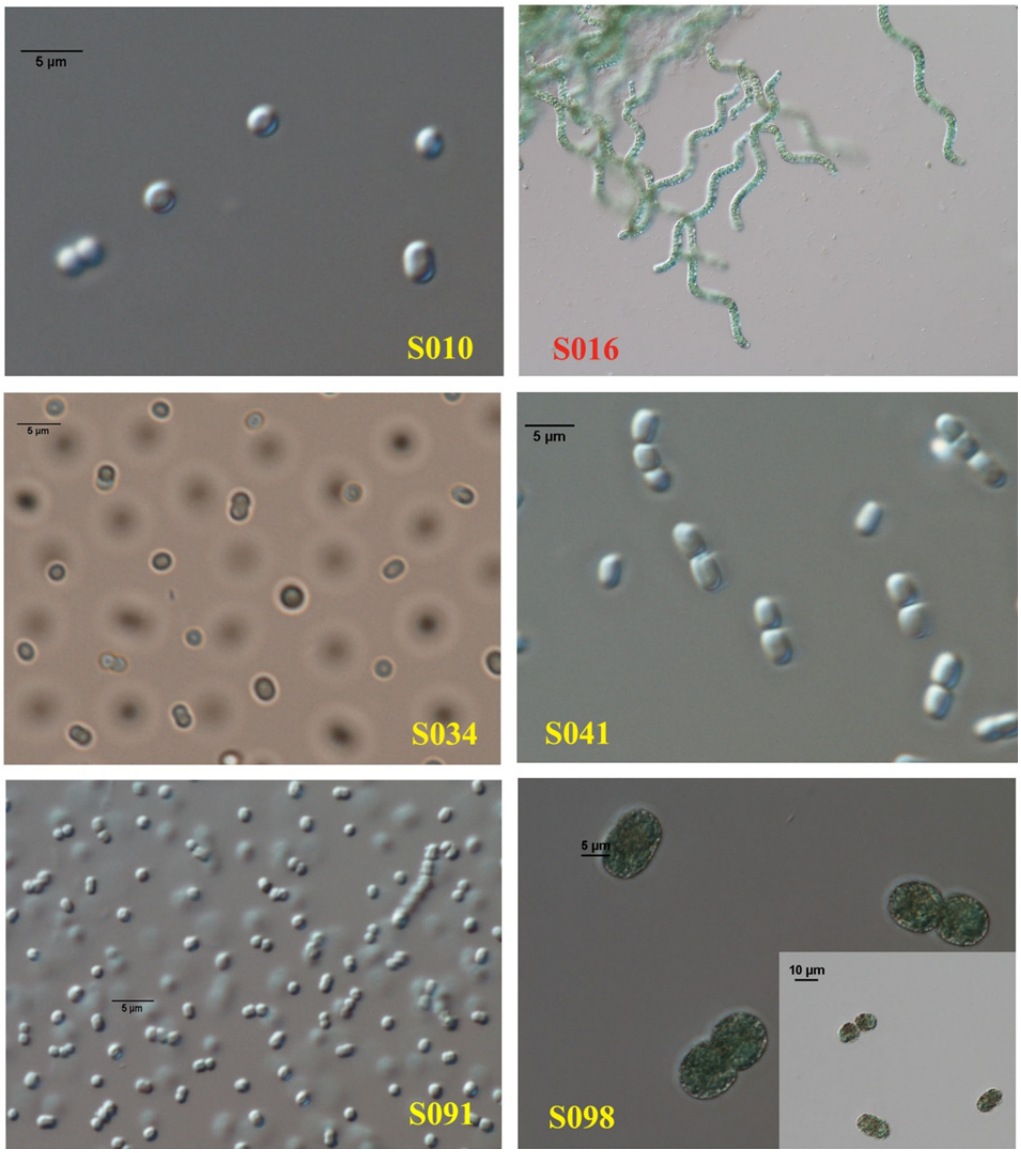


Plate 16: Cyanophyceae – *Synechocystis* sp. (S010 & S034), *Arthrospira platensis* (S016), Unidentified (S041 and S091) and Cyanothece sp. (S098)

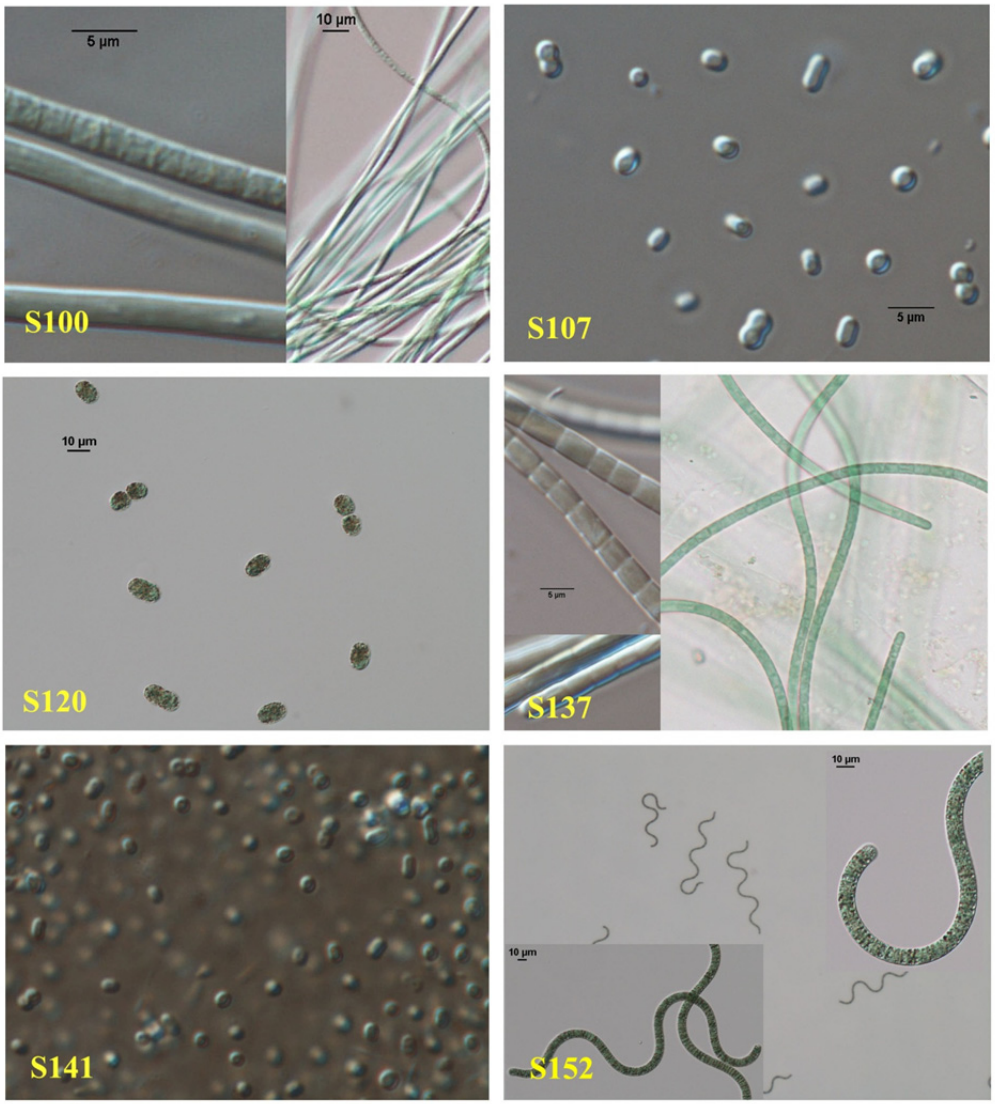


Plate 17: Cyanophyceae – *Gietlerinema* sp. (S100), *Synechocystis* sp. (S107), *Cyanothece* sp. (S120), *Oscillatoria* sp. (S137), Unidentified (S141), *Arthrospira maxima* (S152)

Chapter 3

Part-I

MOLECULAR PHYLOGENY AND TAXONOMIC CHARACTERIZATION OF MICROALGAE FROM INDIAN COAST

Contents

Abstract

Smaller size and phenotypic plasticity make the taxonomic identification of microalgae hard and time consuming. Molecular taxonomy is an advanced tool, unbiased by morphological and environmental factors. Preliminary morphology (chapter 2) of the isolates of 'MBTD-CMFRI-Culture Collection of Microalgae' was not sufficient in designating taxonomic names. Selected strains were attempted for molecular taxonomy based on small subunit (SSU) rRNA gene (18S for eukaryotes and 16S for prokaryotes) and were classified up to genus level. Other molecular markers - internal transcribed spacer (ITS) region, Rubisco large subunit (rbcL) gene and Cytochrome oxidase I (COI) gene were used as supporting tools, however they having only 50-60% success rate in PCR amplification and only <30% significant matches in database. Out of 120 strains, 98 were identified by BLAST analysis. Results confirmed the presence of a minimum of 34 genera from 9 different classes belonging to4 groups (Heterokontophyta, Haptophyta, Chlorophyta and Cyanophyta). When 18 strains were failed either in amplifying or in reviving sequences,6 strains (four diatoms– S036, S037,S090& S131 and 2 green algae – S113 & S165) were poorly identified.18S rDNA sequences of about 75 isolates were analyzed for their phylogeny (Maximum Likelihood and Neighbour Joining) and supplementary information regarding their origin and relationships was derived. Among monophyletic hetrokontophytes, dominant isolates were diatoms (monophyletic). In chlorophytes, both Chlorophycaea and Prasinophycea were paraphylectic. Haptophyta was represented by only two genera – Isochrysis (Prymnesiophyceae) and Ochrosphaera (Raphidophyceae), and both got clearly discriminated from others. Present study reveals the applicability of 18S rDNA as an introductory tool in microalgae taxonomy and also discusses the difficulties confronted with other regions (ITS, COI and rbcL).

3.1 Background

The traditional methods of identification of the living beings based on morphology, physiology and biochemical analysis were most practiced, and inevitable for all organisms including microalgae. However, smaller size and flexible morphology of microalgae makes the study tedious (Juan et al. 2008). For maximum clarity, a detailed and time consuming examination based on advanced microscopic tools like SEM or TEM (Scanning and Transmission Electron Microscopy) are also emphasized. Moreover, the conventional methods are normally laborious and time consuming which necessitates pecialized in-depth knowledge in taxonomy coupled with and technical skills (Bornet et al. 2004; Jahn et al. 2007; Radha et al. 2012). In addition, for many microalgae (e.g. *Chlorella* and *Dunaliella*), the cell size and shape are highly variable and depend on biotic and abiotic factors such as environmental, age and nutrition (Aslam et al. 2007; Gonzalez and Bashan 2000; Polle n.d.; Wu, Hseu, and Lin 2001). Some of the well studied examples for morphology changes are, increase in colony size in *Scenedesmus* and bristle formation in *Micractinium* (in presence of predators, which were called "*Daphnia*-factors" and "*Barchionus*-factors") (Bock 2010). This makes the identification processfurther complex and, consequently, deep knowledge of the species is crucial.

Molecular taxonomy is an advanced and reliable technique and has been utilized to discriminate extremely similar organisms. It is independent from environmental and growth factors, and hence emerged as a faster and potent device in identification (Bornet et al. 2004). Lewis and McCourt (2004) in their review on green algal systematics opined that - 'this is the "Age of molecules", while it was the "Age of Ultrastructure" previously for taxonomy'. This might be because, after molecular phylogenetic studies, considerable rearrangements

have occurred in taxonomic positioning (e.g. *Chlorella* was put in Trebouxiophycea from Chlorophyceae). The molecular data also brought in a better clarity to the 'ultra-structure based cataloging' (Lewis and McCourt 2004). The attractiveness of this advanced tool is that, by analyzing a part of a small conserved gene (e.g. ribosomal RNA) we get precision about the species and its relationship with other related organisms. It has been well proven as a supplementary tool leading to significant revision of old determinative classification of algae (Rehnstam-Holm and Godhe n.d.). Studies of Muller (2005) on *Chlorella vulgaris* based on ITS sequence & AFLP genotyping and that of Assuncao et al. (2012) on *Dunaliella salina* using ITS2 secondary structure are good examples where considerable genetic variations were described among the strains of same species.

Several DNA based methods based using polymerase chain reaction (PCR), gene size and gene sequences have been recognized in taxonomy investigations. The commonly used genetic markers include - ribosomal RNA operon (18S, 28S& ITS for eukaryotes, 16S for prokaryotes); plastidal RNA operon (16S in eukaryotes); mitochondrial (COI), chloroplast (*rbc*L, *atp*B, *psa*B) and nuclear (actin) protein coding genes; microsatellite DNA sequences etc. (Buchheim et al. 2010; Caisová 2011; Lundholm, Daugbjerg, and Moestrup 2002; Nozaki, Onishi, and Morita 2002; Rehnstam-Holm and Godhe n.d.). Several barcoding techniques like RAPD (Chandra paramanik and Chikkaswamy 2014; Medlin, Groben, and Valentin 2002; Mostafa et al. 2011; Prabakaran et al. 2011), RFLP (Gonzalez, Gomez, and Montoya 1999), AFLP (Gaebler, Hayes, and Medlin 2007; Muller et al. 2007), ISSR (Bornet et al. 2004; Mostafa et al. 2011), HMA (Oldach et al. 2000) etc. were also employed. However, in microalgae taxonomic characterization, SSU rRNA gene remains the preferred marker especially due to, a) the presence of

hundreds of copies of the gene in the genome, b) large available data base, c) universal primers and high amplification rate, d) absence of lateral gene transfer, and e) ease in analysis owing to suitable number and position of nucleotides(Jung, Han, and Ki 2009).

Last two decades have witnessed high frequency of phylogeny based on ITS region along with 18S rDNA in eukaryotic micro-algal taxonomy as well as biodiversity studies (Aslam et al. 2007; Berglund et al. 2005; Jahn et al. 2007; Jung et al. 2009; Moro et al. 2009; Pocock et al. 2004; Sakata et al. 2005; Wu et al. 2001).Taxonomy and species separation based on ITS region, especially ITS2 was viewed as most efficient due to its highly conserved nature within the species and divergence between species (Hoshina et.al 2010, Hoshina & Fujiwara 2013). Coleman and his coworkers (1997, 2007, 2009) have developed the "CBC Species Concept" in eukaryotes which is based on the compensatory base changes (CBC) present in the conservative regions of ITS2 secondary structure which was also correlated with mating ability. Most recently Hoshina (2014) successfully studied CBC in ITS 2 secondary structure in combination with SSU rRNA gene phylogeny to discriminate microbial fresh water green algae of classes Trebouxiophyceae and Chlorophyceae. Moniz and Kaczmarska (2009, 2010), in their study recommend 5.8S + ITS2 transcript secondary structure as a robust candidate in diatom barcoding. However ITS2 based studies are in their infancy and remains limited to the above mentioned groups.

Among the other recommended genes, COI remains restricted to certain genera of diatoms including *Sellaphora*, *Pinnularia* and *Nitzschia* (Evans and Mann 2009; Evans, Wortley, and Mann 2007) and red & brown algae (Kucera and Saunders 2008; Saunders 2005). The plastidal *rbc*L gene, even being highly diverse within a genus, is typically confined to green micro

and macro-algae (Nozaki et al. 2002; Saunders and Kucera 2010) with a very few trials in other groups (Daugbjerg and Andersen 1997). Moreover, most of these investigations, for a better barcode for microalgae, followed a poly-phasic approach (comprehensive morphology and multiple gene phylogenies) and were confined to species resolution of closely related or semi-cryptic species. Hence, whenever a common barcode for a particular group was examined (for example Moniz and Kaczmarska (2009) in diatoms; Caisová (2011) in Chaetophoralean algae), 18S was recorded to be a basic as well as easy tool in taxonomic delineation.

In a culture collection of microalgae, taxonomic characterization of the strains is essential for comparison and future investigation (Radha et al. 2012). After a preliminary phenotypic discrimination (provided in the 2nd Chapter of this thesis), taxonomic inference of the isolates was not yet confirmed. Hence for auxiliary verification of taxonomic position, molecular tools like nucleotide BLAST analysis and phylogeny have been done. In the present study SSU rRNA gene (18S for protist microalgae and 16S for Cyanobacteria) sequence blasting was used for genus/species level discrimination of nearly 120 strains of microalgae. To support the data, 3 other genetic markers - ITS region, Rubisco large subunit (*rbc*L) gene and COI gene were also employed. For cyanobacteria 16S rDNA was used, which remains to be the most applied genetic marker for blue green algae(Moreira, Vasconcelos, and Antunes 2013). Phylogenetic clusters of 18S rDNA, derived from Maximum Likelihood (ML) and Neighbour Joining(NJ),werecompared and discussed for selected 75 strains, belonging to groups - Chlorophyta (Chlorophyceae, Prasinophyceae and Trebouxiophyceae) Heterokontophyta (Bacillariophyceae, Raphidiophyceae and Eustigmatophyceae) and Haptophyta (Prymnesiophyceae). To our knowledge, this is the first

molecular phylogenetic study conducted for tropical Indian isolates of microalgae in a collective approach.

3.2 Materials and Methods

3.2.1 Strain Selection

Total of 120 pure and stable strains of microalgae were chosenfrom the MBTD-CMFRI culture collection developed through a world bank funded through ICAR-NAIP project, which included 21 old and 99 new strains, isolated from diverse habitats of Indian coast (Chapter 2). Along with saline (marine, brackish and hyper saline) strains a few fresh water and hot spring strains were also included in this study. Descriptions regarding the isolates are given in Table 3.1. Standard culture conditions were maintained for strains from different habitats (details in Chapter 2).

Table 3.1: List of microalgae from MBTD-CMFRI culture collection studied for molecular identification.

Sl. No	Morphological identification	Reference Code	Origin	18S rDNA	ITS	*rbcL*	CO I	Molecular identification (95-100% similarity)
			Haptophyta					
1	*Isochrysis galbana*	MBTD-CMFRI-S001	M, Pure culture from CMFRI culture collection - Kochi & Tuthukudi	✓	×	×	×	*Isochrysis galbana*
2	*Isochrysis galbana*	MBTD-CMFRI-S002		✓	×	×	×	*Isochrysis galbana*
3	*Isochrysis galbana*	MBTD-CMFRI-S073		✓	—	—	—	*Isochrysis galbana*
4	*Isochrysis galbana*	MBTD-CMFRI-S157	M, Andaman Nicobar Island	✓	—	—	—	*Isochrysis galbana*
5	*Ochrosphaera* sp.	MBTD-CMFRI-S003	M, Pure culture from CMFRI culture collection - Kochi & Tuthukudi	✓	×	×	×	*Ochrosphaera* sp.
6	*Dicrateria* sp.?	MBTD-CMFRI-S004		×	—	—	—	—
7	*Prymnesium parvum*	MBTD-CMFRI-S119	M, Vypeen barmouth, Kochi	×	×	×	×	—
			Heterokontophyta					
8	*Heterosigma* sp.	MBTD-CMFRI-S156	M, Alappuzha Thottappily sea	✓	×	×	×	*Heterosigma akashiwo*
9	*Chaetoceros calcitrans*	MBTD-CMFRI-S005	M, Pure culture from CMFRI, Kochi	✓	×	×	✓	*Chaetoceros* sp.
10	*Cyclotella* sp.	MBTD-CMFRI-S018	M, Bar mouth, Fort Kochi	✓	×	×	✓	*Cyclotella cryptica*
11	*Thalassiosira* sp.	MBTD-CMFRI-S019	BW, Mangalavanam mangrove	✓	×	×	×	*Thalassiosira weissflogii*
12	*Nitzschia* sp.	MBTD-CMFRI-S021	M, Bar mouth - Fort Kochi	✓	×	×	×	*Nitzschia sp.*
13	*Chaetoceros* sp.	MBTD-CMFRI-S022		✓	×	×	×	*Chaetoceros* sp.
14	*Coscinodiscus* sp.	MBTD-CMFRI-S025	BW, Cochin back waters - Sathar island	×	—	×	✓	—
15	*Cyclotella* sp.	MBTD-CMFRI-S031		✓	×	×	×	*Cyclotella atomus*

16	*Skeletonema* sp.	MBTD-CMFRI-S032	BW, Cochin back waters - CUSAT Jetty	✓	×	×	✓	*S. costatum/subsalsum*
17	*Thalassiosira* sp.	MBTD-CMFRI-S033	BW, Mangrove, Mangalavanam	✓	×	×	×	*Thalassiosira* sp.
18	*Nitzschia* sp.	MBTD-CMFRI-S036	M, Vypeen Bar mouth	✓	×	×	×	*Cylindrotheca closterium*
19	Pennate diatom	MBTD-CMFRI-S037	BW, Cochin back waters - Malippuram	✓	×	×	×	Bacillariophyta sp.
20	Pennate diatom	MBTD-CMFRI-S038	M, Vypeen Bar mouth	×	—	×	×	—
21	Pennate diatom	MBTD-CMFRI-S039	BW, Cochin back waters - Malippuram	×	—	×	✓	—
22	*Chaetoceros* sp.	MBTD-CMFRI-S042	M, Bar mouth, Fort Kochi	✓	×	—	×	*Chaetoceros* sp.
23	*Navicula transitans*	MBTD-CMFRI-S043		✓	×	—	✓	*Navicula* sp.
24	*Cyclotella* sp.	MBTD-CMFRI-S044	BW, Cochin back waters - M jetty	✓	×	—	✓	*Cyclotella atomus*
25	*Thalassiosira* sp.	MBTD-CMFRI-S045	BW, Mangrove, Mangalavanam	×	×	—	✓	*Thalassiosira* sp.
26	*Skeletonema* sp.	MBTD-CMFRI-S049	BW, Cochin back waters - M jetty	✓	×	—	✓	*Skeletonema ardens*
27	*Minutocellus* sp.	MBTD-CMFRI-S050	BW, Vizhinjam,, TVM, Kerala	✓	×	—	×	*Minutocellus polymorphus*
28	*Cyclotella* sp.	MBTD-CMFRI-S052		✓	×	—	✓	*Cyclotella cryptica*
29	*Chaetoceros* sp.	MBTD-CMFRI-S053	BW, Veli, TVM, Kerala	✓	×	—	✓	*Chaetoceros* sp.
30	*Navicula* sp.	MBTD-CMFRI-S060		✓	×	—	×	*N. gregaria/pseudaccepta*
31	*Nitzschia* sp.	MBTD-CMFRI-S061	M, Betul beech, Goa	✓	×	—	×	*Fallacia/Psammodictyon* sp.
32	*Chaetoceros gracilis*	MBTD-CMFRI-S062	M, CMFRI, Tuthukudi culture collection	✓	×	—	✓	*Chaetoceros* sp.
33	*Chaetoceros* sp.	MBTD-CMFRI-S065	BW, Cochin back waters - Njarackal	✓	×	—	×	*Chaetoceros* sp.
34	*Thalassiosira* sp.	MBTD-CMFRI-S069	M, Neendakara beech, TVM, Kerala	✓	×	—	×	*Thalassiosira weissflogii*
35	*Chaetoceros dacitrans*	MBTD-CMFRI-S074	M, Pure culture from CMFRI, Kozhikode	✓	—	—	×	*Chaetoceros* sp.
36	*Cyclotella* sp.	MBTD-CMFRI-S079	M, Poompuhar beach, TN	✓	×	—	✓	*Cyclotella atomus*
37	*Cyclotella* sp.	MBTD-CMFRI-S080	M, Poompuhar beach, TN	✓	×	—	✓	*Cyclotella atomus*
38	*Thalassiosira* sp.	MBTD-CMFRI-S084	M, Kovalam beach, Chennai, TN	×	×	—	✓	*Thalassiosira* sp.
39	Pennate diatom	MBTD-CMFRI-S088	M, Lakshadweep	✓	—	—	×	*Minutocellus polymorphus*
40	Pennate diatom	MBTD-CMFRI-S090	M, Poompuhar beach, TN	✓	—	—	×	*Navicula* sp.
41	*Nitzschia sp.*	MBTD-CMFRI-S092	Pulikat hyper saline Pulikat lake 3, AP	×	—	—	×	—
42	*Nitzschia sp.*	MBTD-CMFRI-S099	Kelambakom saline pool (81), Chennai, TN	✓	—	—	×	*Cylindrotheca closterium*
43	Pennate diatom	MBTD-CMFRI-S117	M, Vypeen barmouth, Kochi, Kerala	×	—	—	×	—
44	Pennate diatom	MBTD-CMFRI-S131	Adayar lake, M, Chennai, TN	✓	—	—	×	Bacillariophyta sp.
45	*Thalassiosira* sp.	MBTD-CMFRI-S132	M, Goa beach, Goa	✓	—	—	×	*Thalassiosira profunda*
46	*Chaetoceros* sp.	MBTD-CMFRI-S136	M, Kozhikode beach, Kerala	✓	✓	—	×	*Chaetoceros tenuissimus*
47	*Nitzschia* sp.	MBTD-CMFRI-S148	M, Poompuhar beach, TN	✓	—	—	×	*N. microcephala/ovalis*
48	Diatom	MBTD-CMFRI-S150	M, Kozhikode beach, Kerala	×	—	—	—	—
49	*Nitzschia* sp.	MBTD-CMFRI-S153	Manikaran hot spring, HP	✓	×	—	—	*Nitzschia palea*
50	Diatom	MBTD-CMFRI-S158	M, Thottappily sea, Alappuzha , Kerala	✓	×	—	—	*Thalassiosira profunda*
51	*Bellerochea* sp.	MBTD-CMFRI-S167	M, Kozhikode beach, Kerala	✓	×	—	—	*Bellerochea* sp.
52	*Chaetoceros* sp.	MBTD-CMFRI-S172	M, Andhakaranazhi beach, Alappuzha	✓	—	—	—	*Cheatoceros gracilis*
53	*Skeletonema* sp.	MBTD-CMFRI-S175	M, From private aquafarm, Alappuzha	—	—	—	×	*Skeletonema* sp.
54	*Nannochloropsis* sp.	MBTD-CMFRI-S006		✓	×	×	✓	*N. oceanica*
55	*Nannochloropsis* sp.	MBTD-CMFRI-S012	M, Pure culture from CMFRI, Kochi, Tutukodi & Kozhikode	✓	—	—	—	*N. oceanica*
56	*Nannochloropsis* sp.	MBTD-CMFRI-S076		✓	—	—	—	*N. oceanica*
57	*Nannochloropsis* sp.	MBTD-CMFRI-S077		✓	×	×	✓	*N. oceanica*

58	*Nannochloropsis* sp.	MBTD-CMFRI-S078		✓	×	×	✓	*N. oceanica*
			Chlorophyta					
59	*Chlorella* sp.	MBTD-CMFRI-S026	BW, Cochin back waters, Njarackal	✓	×	×	✓	*Didymogenes* sp.
60	Green alga	MBTD-CMFRI-S029		✓	×	✓	×	*Nannochlorum* sp.
61	Green alga	MBTD-CMFRI-S030	M, Vypeen Bar mouth	✓	×	✓	×	*Picochlorum/Nannochlorum* sp.
62	Green alga	MBTD-CMFRI-S035		✓	×	×	×	*Nannochloris* sp.
63	Green alga	MBTD-CMFRI-S048	BW, Cochin back waters - M jetty	×	×	✓	✓	—
64	Green alga	MBTD-CMFRI-S056	Brackish water, Veli, TVM	✓	×	✓	×	*Picochlorum/Nanochlorum*sp.
65	Green alga	MBTD-CMFRI-S070	BW, Cochin back waters - M jetty	✓	×	✓	×	*Picochlorum/Nanochlorum* sp.
66	*Chlorella* sp.	MBTD-CMFRI-S071	M, Contamination in S016 culture-lab	✓	×	×	×	*Chlorella vulgaris*
67	*Chlorella* sp.	MBTD-CMFRI-S072	M , Contamination, Hatchery	✓	×	×	×	*Chlorella vulgaris*
68	Green alga	MBTD-CMFRI-S083	M, Kozhikode beach	✓	✓	×	✓	*Picochlorum/Nanochlorum*sp sp.
69	*Chlorella* sp.	MBTD-CMFRI-S095	HS, Pulikat lake 3, AP	✓	—	✓	×	*Chlorella vulgaris*
70	Green alga	MBTD-CMFRI-S102	M, Njarakal beach, Kochi	✓	—	×	×	*Picochlorum/Nanochlorum* sp.
71	Green alga	MBTD-CMFRI-S113	BW, Pulikat lake 1, AP	✓	✓	×	×	*Halochlorococcum/Desmochloris* sp.
72	*Dictyosphaerium* sp.	MBTD-CMFRI-S129	Fresh water pool near CMFRI, Kochi	✓	—	×	×	*D. ehrenbergianum*
73	*Chlorella* sp.	MBTD-CMFRI-S130	Fresh water, Pulicat pool - 6, AP	✓	✓	✓	×	*Mychonastes* sp.
74	Green alga	MBTD-CMFRI-S134	M, RK beach, Vishakhapattanam	✓	—	×	×	*Picochlorum/Nanochloris* sp.
75	Green alga	MBTD-CMFRI-S138	BW, pond, near Kozhikode beach	✓	×	×	×	*Oocystidium* sp.
76	*Selenastrum* sp.	MBTD-CMFRI-S139		✓	×	✓	×	*Selenastrum/Monoraphidium* sp.
77	Green alga	MBTD-CMFRI-S144	M, Dona pola beach, Goa	✓	×	×	×	*Picochlorum/Nanochlorum* sp.
78	Green alga	MBTD-CMFRI-S145	BW, Njarackal aquaculture pond, Kochi	✓	×	×	×	*Pseudoneochloris* sp.
79	Green alga	MBTD-CMFRI-S146	M, Vypeen harbour, Kochi	×	×	×	—	—
80	*Scenedesmus* sp.	MBTD-CMFRI-S154	Hot spring, Manikaran, AP	✓	×	×	—	*Scenedesmus* sp.
81	Green alga	MBTD-CMFRI-S155	M, RK beach, Visakhapattanam , AP	✓	×	×	—	*Nanochloris* sp.
82	Green alga	MBTD-CMFRI-S164	M, Pamban sea, Rameswaram, TN	×	×	×	—	—
83	Green alga	MBTD-CMFRI-S165	M, Poompuhar Beach, TN	✓	×	×	—	Trebouxiophycean alga
84	Green alga	MBTD-CMFRI-S170	M, Pamban sea, Rameswaram, TN	✓	✓	✓	—	*Picochlorum/Nanochlorum*sp.
85	*Chlorella vulgaris*	MBTD-CMFRI-S171	FW, CMFRI hatchery pond, Kochi	✓	✓	✓	—	*Chlorella vulgaris*
86	*Dunaliella* sp.	MBTD-CMFRI-S086	HS, Tuthukudi salt pan, TN	✓	✓	✓	—	*Dunaliella* sp.
87	*Dunaliella salina*	MBTD-CMFRI-S089	M, CMFRI Cochin culture collection	✓	✓	✓	—	*Dunaliella salina*
88	*Dunaliella* sp.	MBTD-CMFRI-S096	HS, Nellore Krishnapatanam salt pan, AP	✓	—	—	—	*Dunaliella* sp.
89	*Dunaliella* sp.	MBTD-CMFRI-S115	HS, Kelambakkom saltpan, Chennai, TN	✓	✓	✓	—	*Dunaliella* sp.
90	*Dunaliella* sp.	MBTD-CMFRI-S118	HS, Nellore salt pan, AP	✓	✓	✓	—	*Dunaliella* sp.
91	*Dunaliella* sp.	MBTD-CMFRI-S121	HS, Pulicat lake, AP	✓	✓	✓	—	*Dunaliella* sp.
92	*Dunaliella* sp.	MBTD-CMFRI-S122	HS, Ribandar salt pan, Goa	✓	✓	✓	—	*Dunaliella* sp.
93	*Dunaliella* sp.	MBTD-CMFRI-S124	HS, Pilar salt pan - 1, Goa	✓	✓	✓	—	*Dunaliella* sp.
94	*Dunaliella* sp.	MBTD-CMFRI-S125	HS, Pilar salt pan - 3, Goa	✓	✓	✓	—	*Dunaliella* sp.
95	*Dunaliella* sp.	MBTD-CMFRI-S133	HS, Kutch salt pan, Gujrat	✓	✓	✓	—	*Dunaliella* sp.
96	*Dunaliella salina*	MBTD-CMFRI-S135	M, Kozhikode sea (sampling 1)	✓	✓	✓	—	*Dunaliella salina*
97	*Dunaliella* sp.	MBTD-CMFRI-S147	HS, Varaval salt pan, Gujrat	✓	✓	✓	—	*Dunaliella* sp.
98	*Dunaliella salina*	MBTD-CMFRI-S151	HS, Pure culture from CSIRO, Australia	✓	✓	✓	—	*Dunaliella salina*
99	*Tetraselmis striata*	MBTD-CMFRI-S011	M, Pure culture from CMFRI, Kochi	×	—	×	✓	*Tetraselmis* sp.

100	*Tetraselmis* sp.	MBTD-CMFRI-S027	BW, Cochin back waters - Sathar island	×	✓	×	×	*Tetraselmis* sp.
101	*Tetraselmis* sp.	MBTD-CMFRI-S028	BW, Cochin back waters - Fort Kochi	✓	✓	✓	×	*Tetraselmis apiculata*
102	*Tetraselmis* sp.	MBTD-CMFRI-S057	M, Vypeen Harbour, Kochi	✓	✓	✓	✓	*Tetraselmis* sp.
103	*Tetraselmis gracilis*	MBTD-CMFRI-S075	M, Pure culture from CMFRI, Tutukudi	✓	✓	✓	—	*Tetraselmis astigmatica*
104	*Tetraselmis* sp.	MBTD-CMFRI-S081	BW, Ashtamudi lake, Kollam	✓	✓	✓	—	*Tetraselmis apiculata/striata*
105	*Tetraselmis* sp.	MBTD-CMFRI-S082	M, Poompuhar beach, TN	✓	✓	✓	—	*Tetraselmis apiculata/striata*
106	*Tetraselmis* sp.	MBTD-CMFRI-S087	HS, Tuthukudi salt pan Pink bloom	✓	—	—	—	*Tetraselmis indica*
107	*Tetraselmis* sp.	MBTD-CMFRI-S094	HS, Pulikat lake 4, AP	✓	×	×	×	*Tetraselmis indica*
108	*Tetraselmis* sp.	MBTD-CMFRI-S101	M, Kelambakom pool near beach, TN	✓	✓	✓	—	*Tetraselmis apiculata/striata*
109	*Tetraselmis* sp.	MBTD-CMFRI-S105	M, Pure culture from CMFRI, Kochi	×	✓	×	—	*Tetraselmis astigmatica*
110	*Tetraselmis* sp.	MBTD-CMFRI-S127	HS, Pilar salt pan - 2, Goa	✓	×	×	×	*Tetraselmis indica*
111	*Tetraselmis* sp.	MBTD-CMFRI-S142	HS, Kakinada salt pan, AP	✓	×	×	—	*Tetraselmis indica*
112	*Tetraselmis* sp.	MBTD-CMFRI-S143	HS, Bheemli saltpan, AP	✓	×	×	—	*Tetraselmis indica*
	Cyanophyta			**16S rDNA**				
113	*Arthrospira platensis*	MBTD-CMFRI-S016	BW, Antenna trust, Madurai, TN	✓	—	—	—	*Arthrospira platensis*
114	*Synechococcus* sp.	MBTD-CMFRI-S041	M, Fort Kochi beach, Kochi	✓	—	—	—	*Synechococcus* sp.
115	*Oscillatoria*sp.	MBTD-CMFRI-S100	HS, Pulikat lake 3, AP	✓	—	—	—	*Glietlerinema*sp.
116	*Synechocystis salina*	MBTD-CMFRI-S107	M, Pure culture CMFRI, Kochi	✓	—	—	—	*Synechocystis* sp.
117	*Cyanothece* sp.	MBTD-CMFRI-S120	HS, Orissa -Ganjam (4)	✓	—	—	—	*Cyanothece* sp.
118	*Anabaenopsis* sp.	MBTD-CMFRI-S128	BW, Malippuram brackish farm, Kochi	✓	—	—	—	*Anabaenopsis* sp.
119	*Oscillatoria* sp.	MBTD-CMFRI-S137	M, Kozhikode beach	✓	—	—	—	*Oscillatoria* sp.
	Unidentified							
120	Unknown alga in pink	MBTD-CMFRI-S055	BW, Vizhinjam, TVM	×	×	×	×	—

3.2.2 DNA isolation

Isolation of DNA was done based on a method modified from Wu, Zarka, and Boussiba (2000). Briefly, from mono-algal stock cultures 10ml of the culture was taken at exponential growth phase and was centrifuged at 6000 rpm, for 10 min. and the pellet was washed twice with TE buffer (70mM Tris:30mM Na2 EDTA, pH 8.6). The cells were then ground with acid washed glass beads using a homogenizer, after adding 450 µl TEG (25 mM TrisHCl; 10 mM EDTA; 50 mM glucose) buffer with lysozyme (5mg of lysozyme prepared in 1 ml buffer). The homogenate was vortexed and about 50 µl 10% SDS was added, mixed well and incubated - on ice, at room temperature (RT) and at 60

°C for 15:10:15 min respectively. Finally about 5 µl proteinase K was added, mixed and again incubated for overnight at 37 °C (in water bath).

After cell digestion (which can be observed by change of natural colour of the culture), the released DNA was extracted from suspension by phenol-chloroform method (Sambrook et.al 1989). To the above suspension equal volume of neutral phenol (phenol-chloroform-isoamyl alcohol) was added, mixed gently, incubated at RT for 10 min., centrifuged and the supernatant (aqueous phase) was saved. Neutral phenol extraction was repeated twice, and to the final aqueous phase equal volume of Chloroform- isoamyl alcohol (24:1) was added, mixed gently, centrifuged and saved the aqueous phase. From this DNA were precipitated using $1/10^{th}$ volume of 3M sodium acetate and approximately 400µl isopropanol/ethanol. To enhance the precipitation the tubes were kept at 4°C on ice for about 30 min to several hours (until precipitation appeared). Pelletized DNA was separated by centrifugation, washed (twice) using 70% ethanol and air dried. The DNA was then dissolved in 30-50 µl TE buffer and stored for further use. All centrifugations (Microcentrifuge, Prism R, Labnet International, USA) during the procedure were done for 10 min at 4°C. Purity of DNA was checked by gel electrophoresis and DNA was quantified by photometry (Eppendorf Biophotometer, Germany).

3.2.3 Polymerase Chain Reaction (PCR)

PCR reactions were carried out in Veriti Thermal Cycler (Applied Biosystems, US). Different primers (18S/16S rDNA, ITS region, *rbc*L gene and COI gene) tried are listed in Table 3.2, used for the amplification of genes. Standardized thermal cycles for each successful primer sets used are given in

Table 3.3. PCR mix with a total volume of 25 µl contained - PCR buffer at 1X concentration with 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 1.5 U of Taq polymerase (Sigma, USA) 5 picomoles of each primer and 25 ng of genomic DNA. PCR products were checked for purity by agarose gel (1.5 %) electrophoresis. Further for purification of amplified products GenElute PCR Cleanup kit was used following the manufacturer's instruction. Cycle sequencing was carried out using forward and/or reverse primers for each gene. After BLAST analysis of similarity search with available database, all sequences of DNA fragments were submitted to NCBI GenBank (Acc. Nos. in Tables 3.4.1 -3.4.4).

3.2.4 Phylogeny based on 18S rRNA Gene:

All sequences with ≥400 bp length were aligned with available reference sequences collected from NCBI GenBank for a) Heterokontophyta and Haptophyta and b) Chlorophyta using the CLUSTAL-W (Thompson, Higgins, and Gibson 1994) algorithm in DNA Sequence Analysis Software Package (Bioedit 7.0). Introns present in some strains (e.g. *Bellerochea* sp.) were detected and removed and joined exons were used in dendrogram construction. Phylogenic tree was drawn using MEGA 6 (Tamura et al. 2013) with Kimura 2 parameter model based on Maximum Likelihood (ML) and Neighbour Joining (NJ) with 1000 bootstrap replications. Separate trees were constructed for each sub-group or genus for a closer look.

Table 3.2: Details of primers tried for molecular phylogeny of microalgae

Sl. No.	Primer name	Primer Sequence (5' – 3')	Gene/region amplified	Reference
1	18S Univ F	TGGTTGATCCTGCCAGT	18S ribosomal RNA gene (SSU)	Mallatt et al. (2003)
2	18S Univ R	TAATGATCCTTCCGCAGGTTCACCT		
3	MA 1 (F)	CGGGATCCGTAGTCATATGCTTGTCTC		Olmos et.al 2000, 2002, 2009
4	MA 2 (R)	GGAATTCCTTCTGCAGGTTCACC		
5	ss5 (F)	GGTGATCCTGCCAGTAGTCATATGCTTG		Matsunaga et.al, 2009
6	ss3 (R)	AGTCAAATTAAGCCGCAGGC		
7	18F1	AGCTCGTAGTTGGATTTCTG		
8	18R1	AGTCAAATTAAGCCGCAGGC		
9	18SU467F	ATCCAAGGAAGGCAGCAGGC		
10	18SU1310R	CTCCACCAACTAAGAACGGC		
11	ITS 1 (F)	TCCGTAGGTGGACCTGCGG	Internal Transcribed Spacer 1, 5.8S rDNA Internal Transcribed Spacer 2, partial 28S rDNA	Polle et.al 2008
12	ITS 2 (R)	GCTGCGTTCTTCATCGATGC		
13	ITS 3 (F)	GCATCGATGAAGAACGCAGC		
14	ITS 4 (R)	TCCTCCGCTTATTGATATGC		
15	rbcL 475-479	CGTGACAAACTAAACAAATATGG	Rubisco gene large subunit	Assuncao et.al 2011
16	rbcL 1181–1160	AAGATTTCAACTAAAGCTGGCA		
17	rbcLfor1	TGCWGGNTTYAAAGCHGG		Buchheim et.al 2010
18	rbcLrev1a	GCRTTMCCCCAAGGRTG		
19	rbcLrev1b	GGCATRTGCCAHACRTG		
20	GazF2	CAACCAYAAAGATATWGGTAC	Cytochrome oxidase I (*Cox*1 gene)	Evans et.al 2007
21	GazR2	GGATGACCAAARAACCAAAA		
22	KEintF	GAGAGCAAAAAGTTTACCATTTCA		
23	KEint2F	GAAGCWGGWGTWGGTACW		
24	KEdtmR	AAACTTCWGGRTGACCAAAAAGGWTG		
25	KEintR	CAAATAAAATTRATWGCWCCTAA		
26	CYA106F	CGGACGGGTGAGTAACGCGTGA	16S rRNA gene (SSU)	Nubel et.al 1997
27	CYA359F	GGGGAATYTTCCGCAATGGG		
28	CYA781R(a)	GACTACTGGGGTATCTAATCCCATT		
29	CYA781R(b)	GACTACAGGGGTATCTAATCCCTTT		

Table 3.3: List of successful primers, with standardized PCR programme (for each primer set) and product size details

Gene targetted	16S rRNA*	18S rRNA	CO I gene	ITS region	*rbcL* gene
Primers	CYA 106F and CYA 781R (Nubel et.al, 1997)	18S UnivF and 18S UnivR (Fawley et.al, 1999)	KEint 2F/ GazF2and KEdt mR (Evans et.al, 2007)	ITS 1F/ITS3F and ITS 4R (Polle et.al, 2008)	*rbcL*475-497 and 1181-1160 (Nozaki et.al., 1995)
Product size	**Ca. 700 bp**	**Ca. 1800 bp**	**Ca. 400 bp**	**Ca. 700 bp**	**Ca. 700 bp**
Thermal Cycling steps	Initiation 94°C -3 min; 35 cycles of 94°C -30 sec 60°C -30 sec 72°C -45 sec; Final extension 72°C -5 min	Initiation 95°C -3 min; 35 cycles of 95°C -30 sec 52°C -30 sec 72°C -1.5 min; Final extension 72°C -10 min	Initiation 95°C -3 min; 35 cycles of 95°C -30 sec 52°C -30 sec 72°C -35 sec; Final extension 72°C -5 min	Initiation 95°C -3 min; 35 cycles of 95°C -30 sec 55°C -10 sec 72°C -45 sec; Final extension 72°C -7min	Initiation 95°C -3 min; 35 cycles of 95°C -30 sec 55°C -30 sec 72°C -1 min; Final extension 72°C -10 min

3.3 Results

3.3.1 DNA isolation

In the present study, the protocol for DNA isolation of Cyanobacteria by Wu et al. (2000) was modified and successfully used for diverse groups of microalgae. Grinding with glass beads(Radha et al. 2012) was useful for almost all strains of microalgae, which was not mentioned in the original protocol. Yield of DNA was superior (up to 600 - 800 ng/ml) from delicate cells (e.g. *Isochrysis*),when compared to thick walled cells (e.g. *Ochrosphaera*, green algae, *Nannochloropsis* and some diatoms – about 100-200 ng/ml) probably due to a difference in cell digestion.

3.3.2 PCR amplification

Amplification rate was highest for the ribosomal gene - about 98% for 18S and 100% for 16S. Not all primers were successful for different genes in different groups. 18S rDNA was amplified by Univ F& Univ R (Mallatt, Garey, and Shultz 2004) in almost all strains with ca. 1800 bp amplicon (Fig. 3.1.1). MA1-MA2 primers were principally selected for the genus *Dunaliella* based on previous studies (Olmos et al. 2002, 2009) and was found 100% successful for

the genus. A deviation in PCR product size from normal 1760 bp (Caisová 2011) (Figure 3.1.2) was observed in some strains (particularly in chlorophytes) indicating the presence of introns (Wilcox et al. 1992). Almost all strains of *Dunaliella* were having larger product than 1800 bp (exceptS147). Other green algae S138 (*Oocystidium* sp.), S139 (*Monoraphidium* sp.) and S145 (*Pseudoneochloris* sp.) had produced c.a. 2500, 2200 and 2200 bp respectively. Only one diatom, *Bellerochea* sp.-S167, had about 2000 bp and upon sequencing presence of one intron of size 109 nucleotides (approximately 500 bp away from 5' end of the gene) was detected. Rarely in some strains (e.g. S039 and S048) 18S gene was not amplified whereas other genes (*rbc*L, ITS or COI) did. The 16S rRNA gene was amplified using primers CYA106F and CYA781R (Nubel et al. 1997), with product size of ca. 700 nucleotides in all selected blue green algae (Figure 3.1.3).

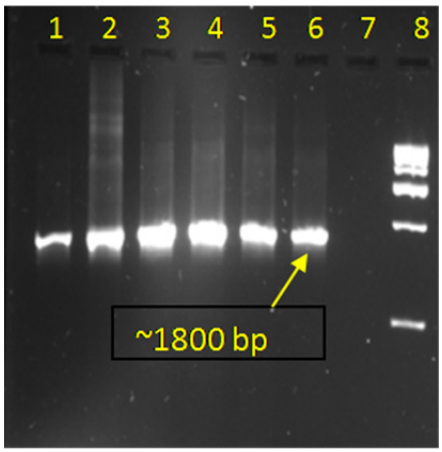


Figure 3.1.1 Image showing 18S rDNA amplified products by primers UNiv 18SF and Univ 18SR, on agarose gel. Lanes - 1: S001, 2: S002, 3: S005, 4: S006, 5: S012, 6: S018, 7: Negative control, 8: 1 kb ladder.

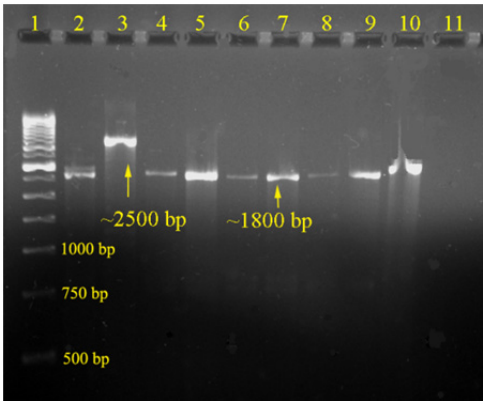


Figure 3.1.2 Image showing 18S rDNA amplified products on agarose gel by primers UNiv 18SF and Univ 18SR. In lane 3 size ca. 2500 bp by strain S138, a deviation from normal 18S rRNA gene. Other lanes - 1: 250 bp ladder, 2: S134, 4: S144, 5: 146, 6: S154, 7: S164, 8: S170, 9: S171, 10: S172, 11: –ve control.

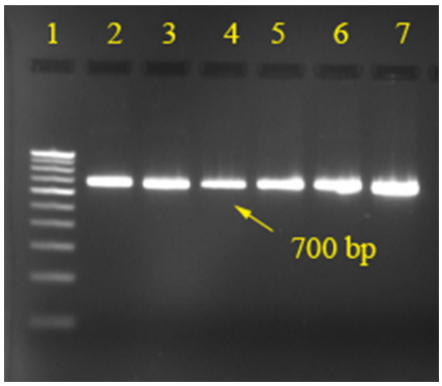


Figure 3.1.3 Image showing 16S rDNA amplified products of Cyanobacteria on agarose gel by primers CYA106F & CYA781R. Lanes – 1: 100 bp ladder, 2: S016, 3: S041, 4: S100, 5: S107, 6: S120, 7: S137

Only in 50% isolates (mostly green algae) ITS region was amplified by 2 sets of primers – ITS1 & ITS4 and ITS3 & ITS4 (Polle et.al 2008) with product size of ca. 800 bp and 400 bp respectively (Figure 3.1.4 & 3.1.5). However, sequencing was failed in 20% strains (<50 bp). In *Tetraselmis* spp. (marine isolates only), *Dunaliella* spp. and *Mychonastes* sp., about 400 - 600 nucleotides were recovered, where as in other strains it was <200 bp (both sets

of primers were tried). In remaining algae, either amplification or sequencing was not successful.

Rubisco large subunit gene got amplified in ca. 52% attempted strains (typically green algae) but successfully sequenced only in 37% (Table 3.1). Out of 5 different primers (Table 3.2) only one set - *rbc*L 475-479 & *rbc*L 1181–1160 (Assunção et al. 2011) was amplified with product size ca. 700bp (Figure 3.1.3).The gene was supportive only in green algae (Table 3.1.6).

COI gene was attempted particularly in diatoms and in chosen other strains, with 6 primers. Combination of GAzF2 & KEdtmR and KEint2F & KEdtmR (Evans et al. 2007), produced ca. 700 & 400 bp products respectively (Figure 3.1.7 & 3.1.8), however with a less percentage of amplification (only in 29% strains). The microalgae, for which the sequences got recovered, were mostly centric diatoms.

Table 3.4.1: NCBI BLAST analysis results for 18S rDNA (protists) and 16S rDNA (Cyanobacteria) partial sequences

Sl. No.	Strain code	Strain name	Acc. No.	Maximum Similar spicies (strain)	NCBI Acc. No. of matching strains	Coverage/identity % (no. of base pairs)
Class - Prymnesiophyceae						
1	MBTD-CMFRI-S001	*Isochrysis galbana*	JF708123	*Isochrysis galbana* AL & *I. galbana* DB	HM246242, GQ118682	100/100 (403)
2	MBTD-CMFRI-S002	*Isochrysis galbana*	JF708124	Do	HM246242, GQ118682	100/100 (696)
3	MBTD-CMFRI-S073	*Isochrysis galbana*	JF708158	Do	HM246242, GQ118682	100/100 (815)
4	MBTD-CMFRI-S157	*Isochrysis* sp.	KM087982	*Isochrysis galbana* Ifremer-Argenton 98	HM149543	100/100 (734)
5	MBTD-CMFRI-S003	*Ochrosphaera* sp.	JF708125	*Ochrosphaera verrucosa* ALGO HAP82 & *O. neapolitana* CCAP 932/1	AM490980, FR865767	100/100 (811)
Class - Raphidophyceae						
6	MBTD-CMFRI-S156	*Heterosigma* sp.	Not submitted	*Heterosigma akashiwo* CCMP1870, etc. and HH 200907-2	Q250796, AY788936	100/100 (741)
Class - Bacillariophyceae						
7	MBTD-CMFRI-S005	*Chaetoceros* sp.	JF708126	*Chaetoceros gracilis* UTEX LB 2375, & *C. debilis*	AY265895, AY229896	100/96 (669/698)
8	MBTD-CMFRI-S018	*Cyclotella* sp.	JF708130	*Cyclotellacryptica* CCMP 332	JX437397	100/99 (630/631)
9	MBTD-CMFRI-S019	*Thalassiosira* sp.	Not submitted	*Thalassiosira weissflogi* L1296, CCAP1085/1	DQ514889, FJ600728	100/100 (149)

10	MBTD-CMFRI-S021	*Nitzschia* sp.	JF708131	*Nitzschia microcephala* BA 100, 85, 32 etc &*N. ovalis* CCAP 1052/12	KC759159, FR865500	100/99 (779/785)
11	MBTD-CMFRI-S022	*Chaetoceros* sp.	JF708133	*Chaetoceros gracilis* UTEX LB 2375 &*C. debilis*	AY229897, AY229896	100/99 (695/696)
12	MBTD-CMFRI-S031	*Cyclotella* sp.	JF708138	*Cyclotella choctawhatcheeana* clone 1132 &*C. atomus* ROR01-04	JF790990, DQ514858	100/100 (526)
13	MBTD-CMFRI-S032	*Skeletonema* sp.	Not submitted	*Skeletonema costatum* FAR 001, FTY 034 etc. &*S. subsalsum* CCAP 1077/8	AB948141, AJ535166	100/100 (654)
14	MBTD-CMFRI-S033	*Thalassiosira* sp.	JF708139	*Thalassiosiraweissflogii* L1296, CCAP1085/1, CCAM 1010	DQ514889, FJ600728	100/98 (325/330)
15	MBTD-CMFRI-S036	*Nitzschia* sp.	JF708141	*Cylindrothecaclosterium* KMMCC:B-353, *C. closterium* MGB0501	GQ468541, DQ019446	100/98 (555/568)
16	MBTD-CMFRI-S037	Pennate diatom	JF708142	Bacillariophyta sp. 1 MAB 2013 isolate GSP 162-1	KF177730	100/98 (388/395)
17	MBTD-CMFRI-S042	*Chaetoceros* sp.	JF708143	*Chaetoceros gracilis, C. debilis, C. muellerii, Chaetoceros curvisetus*	AY265895, AY229896, HQ912558, AY229895	100/96 (743/773)
18	MBTD-CMFRI-S043	*Navicula transitans*	JF708144	*Navicula* sp. Nav. 30 & Nav. 1	AY485502	100/99 (801/802)
19	MBTD-CMFRI-S044	*Cyclotella* sp.	JF708145	*Cyclotella atomus* ROR01-04	DQ514858	100/99 (801/802)
20	MBTD-CMFRI-S049	*Skeletonema* sp.	JF708146	*Skeletonema ardens* FDK005, FDK003S&2S, CCMP 794	AB948137	100/100 (556)
21	MBTD-CMFRI-S050	Diatom	JF708147	*Minutocellus polymorphus* CCMP 497	HQ912568	100/100 (608)
22	MBTD-CMFRI-S052	*Cyclotella* sp.	JF708148	*Cyclotella cryptica* CCMP 331, CCAP 1070/2	FR865514	100/100 (507)
23	MBTD-CMFRI-S053	*Chaetoceros* sp.	JF708149	*Chaetoceros gracilis, C. debilis, C. muellerii*	AY265895, AY229896	100/96 (743/773)
24	MBTD-CMFRI-S060	*Nitzschia* sp.	JF708152	*Navicula gregaria* BA102 &*N. pseudaccepta* MBCCC 8	JN674064	100/100 (477)
25	MBTD-CMFRI-S061	*Nitzschia* sp.	Not submitted	*Fallacia/Psammodictyon/Nitzschia* sp.	KJ961671, AB430617, HM805036	100/100 (149)
26	MBTD-CMFRI-S062	*Chaetoceros* sp.	JF708153	*Chaetoceros muellerii*	AY485453	100/97 (560/583)
27	MBTD-CMFRI-S065	*Chaetoceros* sp.	JF708154	*Chaetoceros calcitrans/muellerii/gracilis/debilis*	AY265895, AY229896, HQ912558, AY229895	100/99 (364/365)
28	MBTD-CMFRI-S069	*Thalassiosira* sp.	JF708155	*Thalassiosira weissflogii* L1296 & CCAP 1085/1	DQ514889, FJ600728	100/99 (789/794)
29	MBTD-CMFRI-S074	*Chaetoceros clacitrans*	JF708159	*Chaetoceros gracilis* UTEX LB 2375, &*C. debilis*	AY265895, AY229896	100/96 (800/830)
30	MBTD-CMFRI-S079	*Cyclotella* sp.	JF708166	*Cyclotella atomus* ROR01-04	DQ514858	100/99 (860/861)
31	MBTD-CMFRI-S080	*Cyclotella* sp.	Not submitted	*Cyclotella atomus* ROR01-04	DQ514858	100/99 (860/861)
32	MBTD-CMFRI-S088	Pennate diatom	JF708171	*Minutocellus polymorphus* CCMP 497	HQ912568	100/100 (812)
33	MBTD-CMFRI-S090	Pennate diatom	JF708172	*Navicula cryptotenella* AT212Gel01	AM502011.	100/92 (297/324)
34	MBTD-CMFRI-S099	*Nitzschia* sp.	JF708177	*Cylindrotheca closterium* KMMCC:B-353, *C. closterium* MGB0501	GQ468541, DQ019446	100/99 (662/663)
35	MBTD-CMFRI-S131	Pennate diatom	JF708181	Bacillariophyta sp. MBIC 10816	AB183643	100/99 (577/578)
36	MBTD-CMFRI-S132	*Thalassiosira* sp.	JF708182	*Thalassiosira profunda* X9III 2	KC284713	100/99 (579/583)
37	MBTD-CMFRI-S136	*Chaetoceros* sp.	JF708184	*Chaetoceros tenuissimus*	AB847417	100/100 (707)
38	MBTD-CMFRI-S148	*Nitzschia* sp.	KM087970	*Nitzschia microcephala* BA 100, 85, 32 etc &*N. ovalis* CCAP 1052/12	KC759159, FR865500	100/99 (533/534)
39	MBTD-CMFRI-S153	*Nitzschia* sp.	KM087976	*N. palea* TCC139-2/TCC 583/TCC 570	KF959653	100/100 (623)
40	MBTD-CMFRI-S158	Diatom	KM087968	*Thalassiosira profunda* X9III 2	KC284713	100/98 (745/751)

41	MBTD-CMFRI-S161	Diatom	Not submitted	*Psammodictyon* sp. MS 2012	JQ885984	100/99 (701/702)
42	MBTD-CMFRI-S167	*Bellerochea* sp.	KM087977	*Bellerochea malleus* CCMP 173	DQ514845	84/99 (515/518)-intron- (81/84)
43	MBTD-CMFRI-S172	*Chaetoceros* sp.	KM087981	*Cheatoceros gracilis* UTEX LB 2375	AY625895	100/99 (690/692)
				Class - Eustigmatophyceae		
44	MBTD-CMFRI-S006	*Nannochloropsis* sp.	JF708127	*Nannochloropsis ocanica* CCAP 849/10,9,8	KJ756836	100/100 (373)
45	MBTD-CMFRI-S007	Do	JF708128	Do	KJ756836	100/100 (217)
46	MBTD-CMFRI-S012	Do	JF708129	Do	KJ756836	100/100 (801)
47	MBTD-CMFRI-S076	Do.	JF708163	Do	KJ756836	100/100 (846)
48	MBTD-CMFRI-S077	Do	JF708164	Do	KJ756836	100/100 (846)
49	MBTD-CMFRI-S078	Do	JF708165	Do	KJ756836	100/100 (841)
				Class - Trebouxiophyceae		
50	MBTD-CMFRI-S026	*Chlorella* sp.	JF708134	*Didymogenes solialla, D. palatina* & *Chlorellasorokiniana* Yco Ju 1 & 4	AB731605, M205840, KF864477	100/99 (780/781)
51	MBTD-CMFRI-S029	Green alga 1	JF708136	*Nannochlorum* sp. MBIC10091	AB058309	100/99 (689/690)
52	MBTD-CMFRI-S030	Green alga 2	JF708137	*Prasinoderma* sp. MBIC 10059, &*Nannochlorum* sp. MBIC10053	AB183584, AB058304	100/100 (441)
53	MBTD-CMFRI-S035	Green alga 3	JF708140	*Nannochloris* sp. KMMCC C-93, C-184	GQ122341, JQ315642	100/99 (498/490)
54	MBTD-CMFRI-S056	Green alga 2	JF708150	*Picochlorum maculatum* PSBDU-003, *Prasinoderma* sp. MBIC 10059, *Nanochlorum* sp. MBIC10053	KJ754560, AB183584, AB058304	100/100 (505)
55	MBTD-CMFRI-S070	Green alga 2	JF708156	*Picochlorummaculatum* PSBDU-003, *Prasinoderma* sp. MBIC 10059, *Nanochlorum* sp. MBIC10053	KJ754560, AB183584, AB058304	100/100 (787)
56	MBTD-CMFRI-S071	*Chlorella* sp.	JF708157	*Chlorella vulgaris* UMT-MI	KJ561358	100/100 (782)
57	MBTD-CMFRI-S083	Green alga 2	KM087974	*Nannochloris* sp. MBIC 10062, 10055 &*Nannochlorum* sp. MBIC 10096	AB183583, AB058312	100/100 (551 Reverse)
58	MBTD-CMFRI-S095	*Chlorella* sp.	JF708175	*Chlorella vulgaris* UMT-MI	KJ561358	100/99 (597/604)
59	MBTD-CMFRI-S102	Green alga 3	Not submitted	*Picochlorum oculatum* &*Nannochlorum eukaryotum*	AY422075, X06425	100/99 (672/674)
60	MBTD-CMFRI-S129	*Dictyosphaerium* sp.	JF708180	*Dictyosphaerium* sp. CCAP 222/5, *D. ehrenbergianum* CCAP 222/28	GQ487242, GQ477063	100/100 (566)
61	MBTD-CMFRI-S134	Green alga 3	JF708132	*Picochlorum maculatum, P. oklahomensis, Nannochloris* sp. MBIC 10596, *N. atomus* CCAP 251/7	AY422073	100/100 (563)
62	MBTD-CMFRI-S138	Green alga 5	KM087980	*Oocystidium* sp. CCAP 222/49	HQ008711	100/99 (596/605, Reverse)
63	MBTD-CMFRI-S144	Green alga 3	JF708162	*Picochlorum oculatum*&*Nannochlorum eukaryotum*	AY422075, X06425	100/99 (760/761)
64	MBTD-CMFRI-S155	Green alga 3	Not submitted	*Nannochloris* sp. MBIC 10596	AB183620	100/100 (732)
65	MBTD-CMFRI-S165	Green alga 7	KM087978	*Phyllosiphon arisari* PY9a1	JF304471	99/91 (691
66	MBTD-CMFRI-S170	Green alga 3	KM087973	*Picochlorum* sp. S16. UTEX 2378 &*Nannochlorum* sp. MBIC10208	AY422076, AB058331	100/99 (701/702)
67	MBTD-CMFRI-S171	*Chlorella vulgaris*	KM087975	*Chlorella vulgaris* KMMCC: C-105, C-88, C-27	GQ122343, GQ122334, GQ122336	100/100 (553 Reverse)
				Class - Chlorophyceae		
68	MBTD-CMFRI-S086	*Dunaliella* sp.	JF708169	*Dunaliella* sp.	Discussed in Part II of this chapter	1246 bp
69	MBTD-CMFRI-S089	*Dunaliella salina*	JF708173	*Dunaliella salina*		1575 bp
70	MBTD-CMFRI-S096	*Dunaliella* sp.	JF708176	*Dunaliella* sp.		805 bp

71	MBTD-CMFRI-S115	*Dunaliella* sp.	JN807315	*Dunaliella* sp.		2490 bp
72	MBTD-CMFRI-S118	*Dunaliella* sp.	JN807316	*Dunaliella* sp.		2160 bp
73	MBTD-CMFRI-S121	*Dunaliella* sp.	JN807317	*Dunaliella* sp.		2121 bp
74	MBTD-CMFRI-S122	*Dunaliella* sp.	JN807318	*Dunaliella* sp.		2491 bp
75	MBTD-CMFRI-S125	*Dunaliella* sp.	JN807319	*Dunaliella* sp.		2630 bp
76	MBTD-CMFRI-S133	*Dunaliella* sp.	JF708183	*Dunaliella* sp.		2481 bp
77	MBTD-CMFRI-S135	*Dunaliella salina*	JF708161	*D. salina*		2066 bp
78	MBTD-CMFRI-S147	*Dunaliella* sp.	JN807320	*Dunaliella* sp.		1687 bp
79	MBTD-CMFRI-S151	*Dunaliella salina*	JN807321	*D. salina*		1575 bp
80	MBTD-CMFRI-S130	*Chlorella* sp.	KM087983	*Mychonastes* sp., *M. ovalimbae* CCAP 260/13, *M. racemoses* CCAP 222/52 &*M. Pushpae* CCAP 260/10	JN617908, GQ477052, GQ477051	100/99 (499/501)
81	MBTD-CMFRI-S154	*Scenedesmus* sp.	KM087971	*Scenedesmus* sp. Pk1	KF569755	100/100 (721)
				Class - Prasinophyceae		
82	MBTD-CMFRI-S028	*Tetraselmis* sp.	JF708135	*Tetraselmis apiculata* CCAP 66/15 & *T. striata* SAG 41.85	KJ756817, JN904000	99/99 (341/345)
83	MBTD-CMFRI-S057	*Tetraselmis* sp.	JF708151	*Tetraselmis* sp. NTI 8	AY954899	100/100 (403)
84	MBTD-CMFRI-S075	*Tetraselmis gracilis*	JF708160	*Tetraselmis astigmatica* CCMP 880/	JN376804	100/98 (795/815)
85	MBTD-CMFRI-S081	*Tetraselmis* sp.	JF708167	*Tetraselmis apiculata* CCAP 66/15 & *T. striata* SAG 41.85	KJ756817, JN904000	100/99 (842/847)
86	MBTD-CMFRI-S082	*Tetraselmis* sp.	JF708168	*Tetraselmis apiculata* CCAP 66/15 & *T. striata* SAG 41.85	KJ756817, JN904000	100/99 (680/682)
87	MBTD-CMFRI-S087	*Tetraselmis* sp.	JF708170	*Tetraselmis* sp. MA 2011 (*T. indica*)	HQ651184	99/100 (489)
88	MBTD-CMFRI-S094	*Tetraselmis* sp.	JF708174	*Tetraselmis* sp. MA 2011 (*T. indica*)	HQ651184	90/100 (504)
89	MBTD-CMFRI-S101	*Tetraselmis* sp.	JF708178	*Tetraselmis apiculata* CCAP 66/15 & *T. striata* SAG 41.85	KJ756817, JN904000	100/99 (545/546)
90	MBTD-CMFRI-S127	*Tetraselmis* sp.	JF708179	*Tetraselmis* sp. MA 2011 (*T. indica*)	HQ651184	89/100 (574)
91	MBTD-CMFRI-S143	*Tetraselmis* sp.	KM087972	*T. indica* MA-20011	HQ651184	90/100 (636)
				Class - Ulvophyceae		
92	MBTD-CMFRI-S113	Green alga 4	KM087979	*Halochlorococcum* sp. KMMCC 151 &*Desmochloris* sp. TP-2008	JQ315540, FM882217	100/97 (535/554)
93	MBTD-CMFRI-S145	Green alga 6	KM087969	*Pseudoneochloris marina*	U41102 (PMU41102)	100/98 (579/589)
				Class - Cyanophyceae (16S rDNA)		
94	MBTD-CMFRI-S016	*Arthrospira platensis*	KM087984	*A. platensis* SAG85.79, SAG86.79, SAG22.99	KM019968, KM019966, KM019967	100/100 (585)
95	MBTD-CMFRI-S041	*Synechococcus* sp.	KM087985	*Rhabdoderma* cf. rubrum Kopara-CH	AJ621833	98/100 (578/578)
96	MBTD-CMFRI-S100	*Oscillatoria*sp.	KM087986	*Glietlerinema* sp. CNP-4003	KC404068	95/99 (480/481)
97	MBTD-CMFRI-S107	*Synechocystis salina*	KM087987	*Synechococcus elongatus* BDU-30312, 70542, 130192 & *Synechocystis* sp. NN	GU186900, KM061377	100/99 (471/472)
98	MBTD-CMFRI-S120	*Cyanothece* sp.	KM087988	Uncultured *Cyanothece* clone	HQ914226	100/100 (361)
99	MBTD-CMFRI-S128	*Anabaenopsis* sp.	KM087989	*Anabaenopsis* sp.	KC912784.	100/99 (556/557)
100	MBTD-CMFRI-S137	*Oscillatoria* sp.	KM087990	*Oscillatoria* sp. BDU-142191	GU186897	100/100 (528)

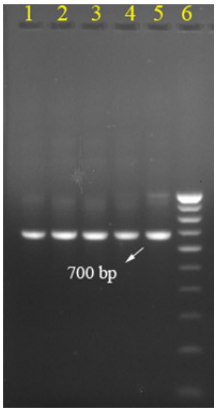


Figure 3.1.4 Image showing ITS region PCR products by primers ITS1 and ITS4, on agarose gel. Lanes - 1: S027, 2: S028, 3: S057, 4: S081, 5: S082, 6: 100 kb ladder.

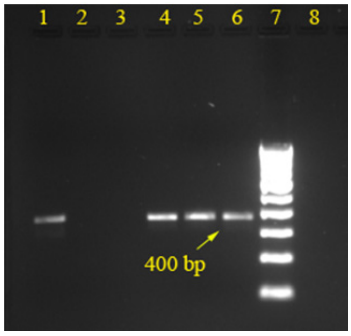


Figure 3.1.5 Image showing ITS region amplified products by primers ITS3 and ITS4, on agarose gel. Lanes – 1: S075, 4: S083, 5: S113, 6: S130, 7: 100 bp ladder, 8: -ve control. Lanes 2 & 3 – no amplification for S077 and S078

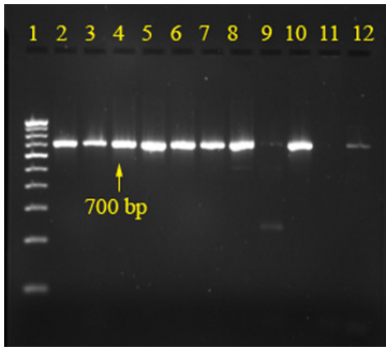


Figure 3.1.6 Image showing *rbc*L gene amplified products by primers *rbc*L 475-479 & *rbc*L 1181–1160, on agarose gel. Lanes – 1: 100 bp ladder, 2: S028, 3: S029, 4: S030, 5: S048, 6: S057, 7: S070, 8: S075, 10: S095, 12: S130. Lanes 9 & 11 no amplification for S078 and S102 respectively.

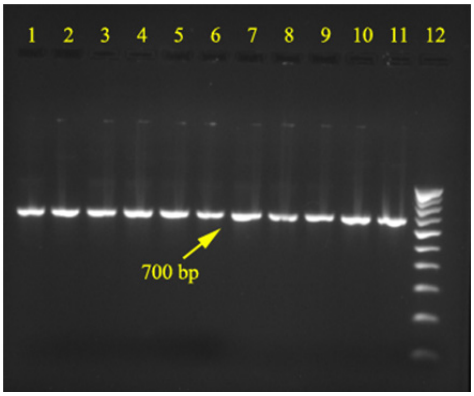


Figure 3.1.7 Image showing COI gene amplified products by primers KEint2F & KEdtmR, on agarose gel. Lanes – 1: S005, 2: S042, 3: S062, 4: S039, 5: S044, 6: S049, 7: S052, 8: S079, 9: S080, 10: S026, 11: S048, 12: 100 kb ladder

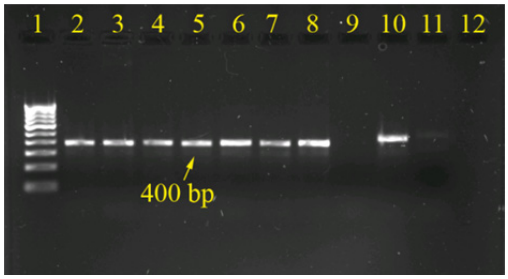


Figure 3.1.8 Image showing COI gene amplified products by primers GAzF2 & KEdtmR, on agarose gel. Lanes – 1: 100 kb ladder, 2: S025, 3: S053, 4: S077, 5: S078, 6: S084, 7: S001, 8: S079, 9: S019 (not amplified), 10: S018, 11: S021 (not amplified), 12: -ve control.

3.3.3 Blast Analysis

Similarity search of partial gene sequences in NCBI database showed presence of 34 genera belonging to 9 different classes (Tables 3.1 & 3.4.1). In case of 18S gene, for some strains sequencing was done from reverse side (3' end), when forward (5' end) sequencing failed. The results of blasting for genes 18S, 16S, ITS, *rbc*L and COI are depicted in Table 3.4.1 to 3.4.4 respectively. Similarity search was successful up to genus level identification (rarely at species level), based on nuclear (18S, 16S and ITS) and platsidal

(*rbc*L) genes. The method was particularly useful for morphologically closer and smaller (nannoplanktonic) green algae and diatoms.

16S rRNA blast results for the prokaryotic microalgae were satisfactory to infer generic identity. All 7 strains examined, showed 99-100% matching with the counterpart organism. When combined with morphology, *Arthrospira platensis* S016, *Cyanothece* sp. S120, *Anabaenopsis* sp. S128 and *Oscillatoria* sp. S137 confirmed their taxonomic identification. Out of the remaining three, the coccoid unidentified strain S041 got identified as *Rhabdoderma cf. rubrum*and S100 was recognized as *Glieterinema* sp.with only one nucleotide substitution. But the *Synechocystis* sp. S107 showed affiliation to two Indian species - *Synechococcus elongates* BDU30312 and one *Synechocystis* sp. NN.

In case of protist microalgae, 18S gene was most successful. Almost 94% of amplified strains got classified with 98-100% available identities in database (Table 3.4.1).Some of the most difficult coccoid green algae were grouped into different classes. For example, S130 and S026 were identified morphologically as *Chlorella* sp. but SSU sequences identified them as *Mychonastes* sp. and *Didymogenes* sp. respectively. In the same way, discrimination of morphologically related diatoms - *Nitzschia*&*Navicula, Thalassiosira* & *Cyclotella,*and the two *Skeletonema* spp. was made possible. Many unidentified strains got discriminated, like the picoplanktonic diatoms S050 &S088 as *Minutocellus polymorphus* (100% similarity) and S148 as *Nitzschia* sp. (99% similarity). For strain S139 (morphologically identified as *Selenastrum* sp.), even 63 out of 64 bp (sequenced) were matching with *Monoraphidium* sp. which was also matched with same species by *rbc*L gene 95% similarity (605/638 bp) (Table 3.4.3).

Table 3.4.2: NCBI BLAST analysis results for ITS region partial sequences (results for *Dunaliella* are in Part II)

Sl. No.	Strain code	Strain name	Acc. No.	Maximum Similar spicies (strain)	NCBI Acc. No. of matching strains	Coverage/identity % (no. of base pairs)
1	MBTD-CMFRI-S027	*Tetraselmis* sp.	KM087991	*T.inconspicua* strain CCAP 66/19C	KJ756818	91/98 (438/473)
2	MBTD-CMFRI-S028	*Tetraselmis* sp.	KM087992	Do	Do	91/98 (438/473)
3	MBTD-CMFRI-S057	*Tetraselmis* sp.	KM087993	*T. inconspicua* strain CCAP 66/19C	KJ756818	100/87 (573/669)
4	MBTD-CMFRI-S075	*Tetraselmis* sp.	KM087994	*T. cordiformis* SAG 26.82	HE610130	48/96 (171/179)
5	MBTD-CMFRI-S081	*Tetraselmis* sp.	KM087995	*Tetraselmis suecica*KMMCC 1158	JQ315738	93/91 (441/495)
6	MBTD-CMFRI-S082	*Tetraselmis* sp.	KM087996	*T. inconspicua* strain CCAP 66/19C	KJ756818	91/98 (438/473)
7	MBTD-CMFRI-S101	*Tetraselmis* sp.	KM087997	*T. inconspicua* strain CCAP 66/19C	KJ756818	91/98 (438/473)
8	MBTD-CMFRI-S083	Green alga	Not submitted	no significant matches	…..	<60 base pairs.
9	MBTD-CMFRI-S113	Green alga	Not submitted	*Pseudoneochloris marina* CCMP257	HE575897	100/100 (161)
10	MBTD-CMFRI-S130	Green alga	Not submitted	*Mychonastes* afer CCAP 211/406	GQ477049	100/83 (687/829)
11	MBTD-CMFRI-S170	Green alga	Not submitted	no significant matches	…....	<200 bp
12	MBTD-CMFRI-S171	*C. vulgaris*	Not submitted	*Chlorella* sp.	…....	99/95 (280/300)

BLAST results of ITS region and *rbc*L gene are given in tables 3.4.2 and 3.4.3 respectively, and were supportive only for some of the green algae. Both ITS and *rbc*L were successful for *Dunaliella* spp. (which is discussed in Part II of this chapter). In case of *Tetraselmis* spp. obtained sequences of these regions were between 400 – 600 bp. However matching (87–98% for ITS and 89-97% for *rbc*L) was different from that of each other and 18S rDNA. Rubisco gene was also amplified in some Trebouxiophycean (*Chlorella*sp.) and the Chlorophycean strains (*Monoraphidium* and *Mychonastes*) with 95-100% similarity with concerned genus (Table 3.4.3).The picoplanktonic *Picochlorum* like strains (S029, S030, S048, S056, S070 and S170) had no respective matches in the database for the plastidal gene, all corresponding with 'uncultured microorganism'.

Table 3.4.3: NCBI BLAST analysis results for *rbc*Lgene partial sequences (results for *Dunaliella* are in Part II)

Sl. No.	Strain code	Strain name	Acc. No.	Maximum Similar spicies (strain)	NCBI Acc. No. of matching strains	Coverage/identity % (no. of base pairs)
1	MBTD-CMFRI-S028	*Tetraselmis* sp.	KM202123	*Tetraselmis* sp. NT18, *T. seucica*	AY954897, EU555175	82/97 (510/526), 98/95 (597/626)
2	MBTD-CMFRI-S057	*Tetraselmis* sp.	KM202124	Do	Do	Do
3	MBTD-CMFRI-S075	*Tetraselmis* sp.	KM202125	*T. seucica*	EU555175	98/89 (556/626)
4	MBTD-CMFRI-S081	*Tetraselmis* sp.	KM202126	*T. seucica*	EU555175	98/95 (592/626)
5	MBTD-CMFRI-S082	*Tetraselmis* sp.	KM202127	*Tetraselmis* sp. NT18, *T. seucica*	AY954897, EU555175	82/97 (510/526), 98/95 (597/626)
6	MBTD-CMFRI-S101	*Tetraselmis* sp.	KM202128	Do	Do	Do
7	MBTD-CMFRI-S029	Green alga	KM202129	Uncultured marine microorganism	FJ981923	90/98 (599/613)
8	MBTD-CMFRI-S030	Green alga	KM202130	Uncultured marine microorganism	FJ981923	90/97 (584/602)
9	MBTD-CMFRI-S048	Green alga	KM202131	Uncultured marine microorganism	FJ981923	90/98 (599/613)
10	MBTD-CMFRI-S056	Green alga	KM202132	Uncultured marine microorganism	FJ981923	90/97 (599/613)
11	MBTD-CMFRI-S070	Green alga	KM202133	Uncultured marine microorganism	FJ981923	90/97 (599/613)
12	MBTD-CMFRI-S095	Chlorella sp.	KM202134	*Chlorella vulgaris* Cvq	EU038286	100/100 (671)
13	MBTD-CMFRI-S130	Green alga	KM202135	*Mychonastes* sp., *M. homosphaera* CAUP H6502 ,	EF113452, KC145515	99/98 (653/665)
14	MBTD-CMFRI-S139	Green alga	KM202136	*Monoraphidium* sp. LUCC 004, *M.circinale* NIES 480	KC810300, AB175934	99/95 (605/638)
15	MBTD-CMFRI-S171	*Chlorlla vulgaris*	KM202137	*Chlorella vulgaris* Cvq	EU038286	100/100 (671)
16	MBTD-CMFRI-S170	Green alga	KM202138	Uncultured marine microorganism	FJ981923	91/94 (577/613)

COI gene sequences of *Skeletonema* sp. S049 matched with *Skeletonema ardens* with 99% similarity (18S rDNA showed 100% similarity with same species) and helped in confirmation of the species (Table 3.4.4). For remaining diatoms COI partial sequences had no specific matches (less than 90% similarity). The two *Nannochloropsis* isolates S077 & S078 got 99% matches (one mismatch for 286 & 254 bp) with *N. oceanica* and confirmed 18S identification (100% similarity) of the strains. In remaining strains sequence recovery was poor and was not useful in species discrimination.

Table 3.4.4: NCBI BLAST analysis results for COIgene partial sequences

Sl. No	Strain code	Strain name	Acc. No.	Maximum Similar spicies (strain)	NCBI Acc. No. of matching strains	Coverage/identity % (no. of base pairs)
1	MBTD-CMFRI-S005	*Chaetoceros* sp.	KM202108	*T. pseudonana*	DQ186202	99/83 (521/630)
2	MBTD-CMFRI-S045	*Chaetoceros* sp.	KM202109	*T. pseudonana*	DQ186202	99/83 (553/618)
3	MBTD-CMFRI-S053	*Chaetoceros* sp.	KM202110	*Sellaphora cf minima*	EF164929	81/86 (267/310)
4	MBTD-CMFRI-S062	*Chaetoceros* sp.	KM202111	*T. pseudonana*	DQ186202	99/83 (515/624)
5	MBTD-CMFRI-S039	Pennate diatom	KM202112	*Pinnularia subanglica* Pin 650	JN418698	98/85 (485/568)
6	MBTD-CMFRI-S044	*Cyclotella* sp.	KM202113	*Skeletonemapseudocostatum*CCMP2472	AB706245	98/86 (501/581)
7	MBTD-CMFRI-S049	*Skeletonema*sp.	KM202114	*S.ardens* CCMP794	AB706216	100/99 (614/620)
8	MBTD-CMFRI-S052	*Cyclotella* sp.	KM202115	With brown alga *Alaria crassifolia* KU1165	AB775220	89/79 (408/514)
9	MBTD-CMFRI-S079	*Cyclotella* sp.	KM202116	*Skeletonema potamos* FCH024	AB706249	99/86 (517/603)
10	MBTD-CMFRI-S080	*Cyclotella* sp.	KM202117	*Skeletonema potamos* FCH024	AB706249	100/87 (502/579)
11	MBTD-CMFRI-S084	*Cyclotella* sp.	KM202118	*Skeletonemapseudocostatum* CCMP2472	AB706245	100/90 (280/310)
12	MBTD-CMFRI-S026	*Chlorella* sp.	KM202119	*Chlorella* sp. ArM0029B	KF554428	99/87 (557/634)
13	MBTD-CMFRI-S048	green alga	KM202120	*Pythiummiddletonii* voucher CBS52874	HQ708738	98/85 (502/592)
14	MBTD-CMFRI-S083	green alga	KM202121	*Pythium splendens* voucher CBS46248	HQ708836	86/82 (432/527)
15	MBTD-CMFRI-S025	*Coscinodiscus* sp.	KM202122	*Pythium irregulare* strain STE-U6752	GU071835	100/79 (280/356)
16	MBTD-CMFRI-S011	*Tetraselmis* sp.	not submitted	*Tetraselmisaff maculata*	AF116777	99/81 (467/570)
17	MBTD-CMFRI-S018	*Cyclotella* sp.	not submitted	No significant matches	....	< 100 bp
18	MBTD-CMFRI-S043	*Navicula* sp.	not submitted	No significant matches	....	< 100 bp
19	MBTD-CMFRI-S057	*Tetraselmis* sp.	Not submitted	*Tetraselmis maculata*	AF116777	99/86 (537/6270)
20	MBTD-CMFRI-S077	*Nannochloropsis* sp.	not submitted	*Nannochloropsis oceanica* CCMP 531	KC598090	100/99 (285/286)
21	MBTD-CMFRI-S078	*Nannochloropsis* sp.	not submitted	*Nannochloropsis oceanica* CCMP 531	KC598090	100/99 (253/254)
22	MBTD-CMFRI-S022	*Chaetoceros* sp.	not submitted	No significant matches	....	< 100 bp
23	MBTD-CMFRI-S023	*Chaetoceros* sp.	not submitted	No significant matches	....	< 100 bp
23	MBTD-CMFRI-S002	*Isochrysisgalbana*	not submitted	*Skeletonema* spp.	....	99/88 (311/355)
24	MBTD-CMFRI-S007	*Nannochloropsis* sp.	not submitted	No significant matches	....	< 100 bp
25	MBTD-CMFRI-S008	*Nannochloropsis* sp.	not submitted	No significant matches	....	< 100 bp
26	MBTD-CMFRI-S031	*Cyclotella* sp.	not submitted	No significant matches	....	< 100 bp

3.3.4 18S rDNA Phylogeny

Separate phylogenetic analyses were conducted based on Maximum Likelihood and Neighbour Joining methods for Heterokontophytes, Haptophytes, and Chlorophytes, out-grouped by members of complementary groups. Boot strap values given throughout the figures and the text are in the form of ML/NJ, derived from ML and NJ phylogenies.

3.3.4.1 Heterokontophyta and Haptophyta

The dendrogram of the two groupswere produced with Chlorophyte algae as an out group. Figures 3.2.1 (ML) and 3.2.2 (NJ) depict overall cladistic separation of different genera. Topology of both the trees was same in separation of clades, and monophyly of heterokontopytes (major clade H), haptophytes (clade A) and diatoms (class Bacillariophyceae, clade G) (except that of *Bellerocheae*). Further detailed inter/intra-generic relationships within each group are illustrated in subsequent figures (3.2.3– 3.2.10).

Haptophyta (Class Prymnesiophyceae; Fig. 3.2.3) was represented by two species, *Isochrysis* and *Ochrosphaera*. All the *Isochrysis* isolates got clustered with *I. galbana* and the single isolate of *Ochrosphaera* was allied to the Japanese strain *O. verrucosa* ALGO HAP82 (similarity 100%).

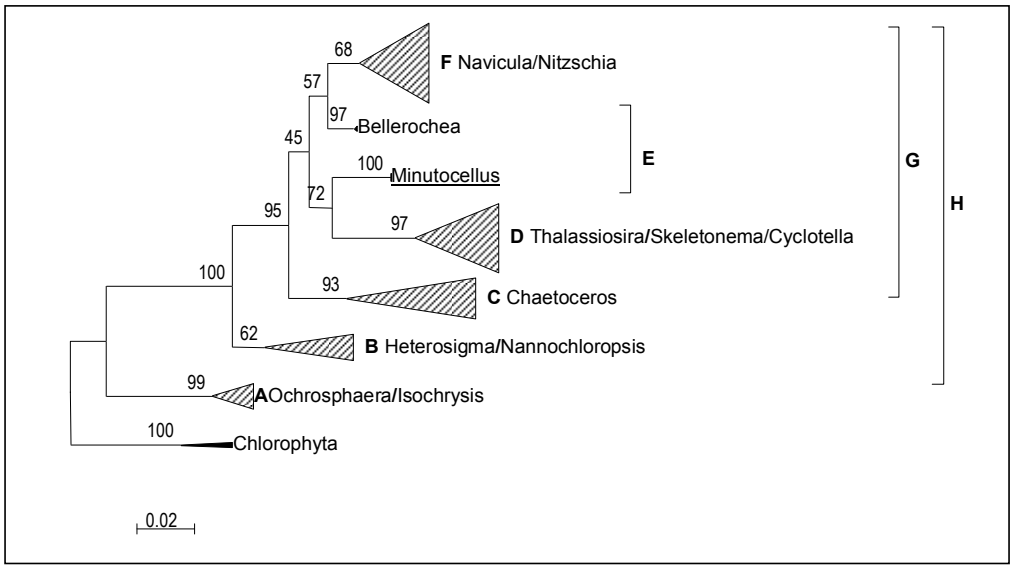


Figure 3.2.1 Maximum Likelihood tree inferred from 18S rRNA gene partial sequences showing the outline of clustering of major species with their counter strains; Height of triangles reflect relative number of strains in that branch.

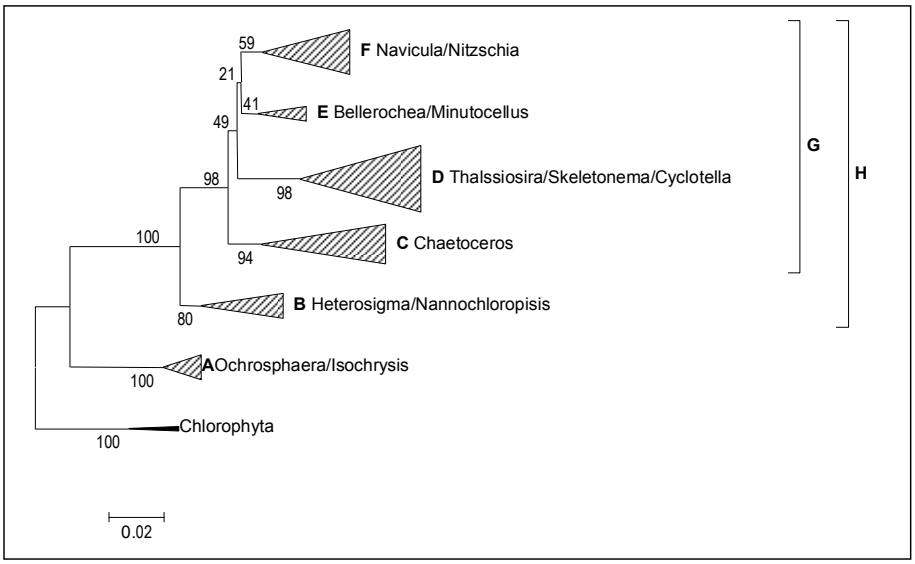


Figure 3.2.2 Neighbour Joining tree inferred from 18S rRNA gene partial sequences showing the outline of clustering of major species with their counter strains; Height of triangles reflect relative number of strains in that branch.

In heterokontophytes, the two classes Raphidophycea and Eustigmatophyceae, represented by single species each (*Heterosigma* and *Nannochloropsis*), appeared as sister clades with bootstrap value 62/75 % (ML/NJ) (Figure 3.2.4). The major group of diatoms (clade G of Fig. 3.2.1) included different subclades with considerable separation of centric and pennate diatoms (Fig. 3.2.5). Isolates of genera *Thalassiosira*, *Cyclotella* and *Skeletonema* appeared in the same clade (D) in both phylogenies, but a difference was there in the arrangement of *Skeletonema* clade (Figure 3.2.5, in the circle NJ arrangement is shown).All *Chaetoceros* spp. clearly got separated (95/98%) from remaining diatoms in clade C. Clade F grouped the pennate diatoms together, however with only 68/59% bootstrap value.

When each cluster was separately elaborated (Figures 3.2.6 – 3.2.10), the existing diversity (in the culture collection) within each genera was observed. There were genetically different strains of 6 C*haetoceros*, 2 C*yclotella*, 2 *Skeletonema*, 3 *Thalassiosira,* 1 *Bellerocheae,* 1 *Minutocellus* and 13 pennate diatoms (*Nitzschia* 7, *Navicula* 2, others 4), with a totalof 28 diverse strains of diatoms. Among these,

maximum divergence (8-11 %) was shown by anunidentified pennate diatom S090 (allied to *Navicula*) and *Chaetoceros tenuissimus* S136.

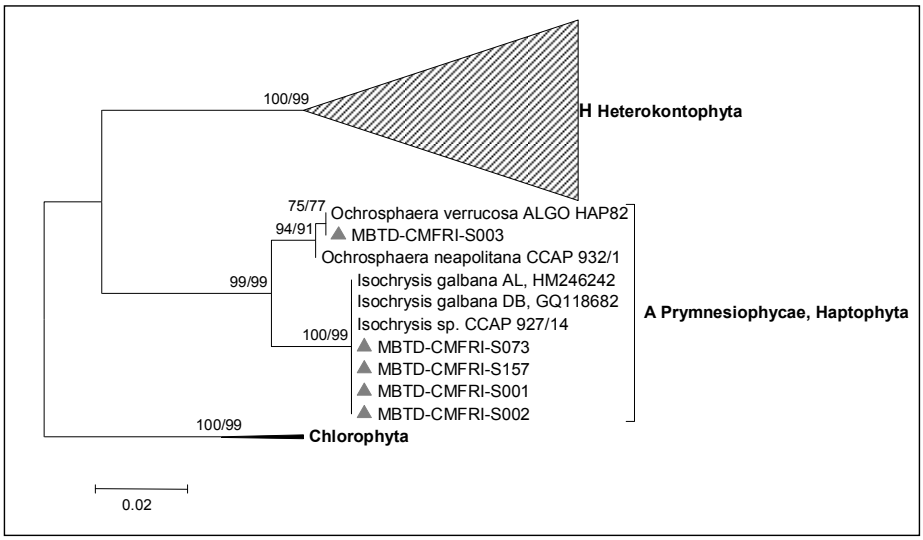


Figure 3.2.3: Combined Maximum Likelihood and Neighbour Joining tree inferred from 18S rRNA gene partial sequences to indicate clustering of Haptophyte strains; BS values shown for nodes – ML/NJ; Height of triangles reflects relative number of strains in that branch.

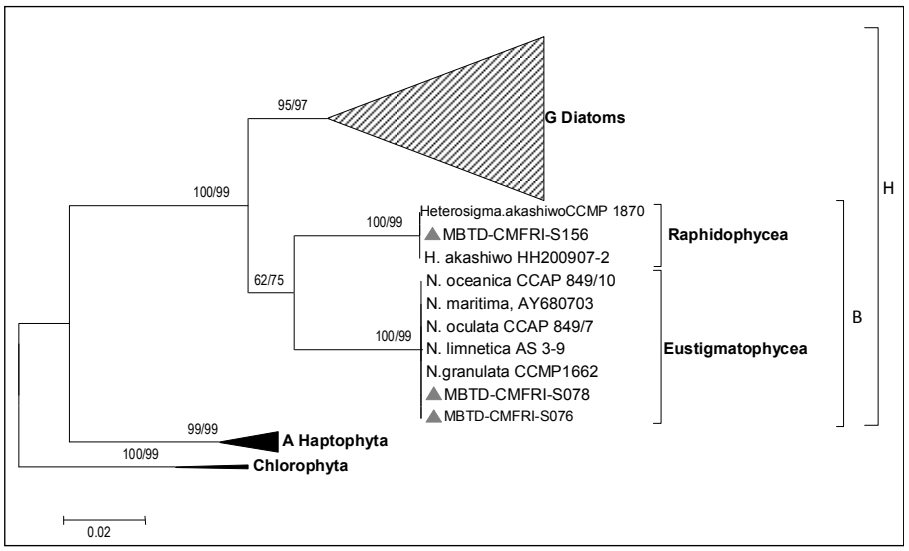


Figure 3.2.4 Combined Maximum Likelihood and Neighbour Joining tree inferred from 18S rRNA gene partial sequences to indicate clustering of Raphidophycean and Eustigmatoophycean isolates. BS values shown for nodes – ML/NJ. Height of triangles reflects relative number of strains in that branch.

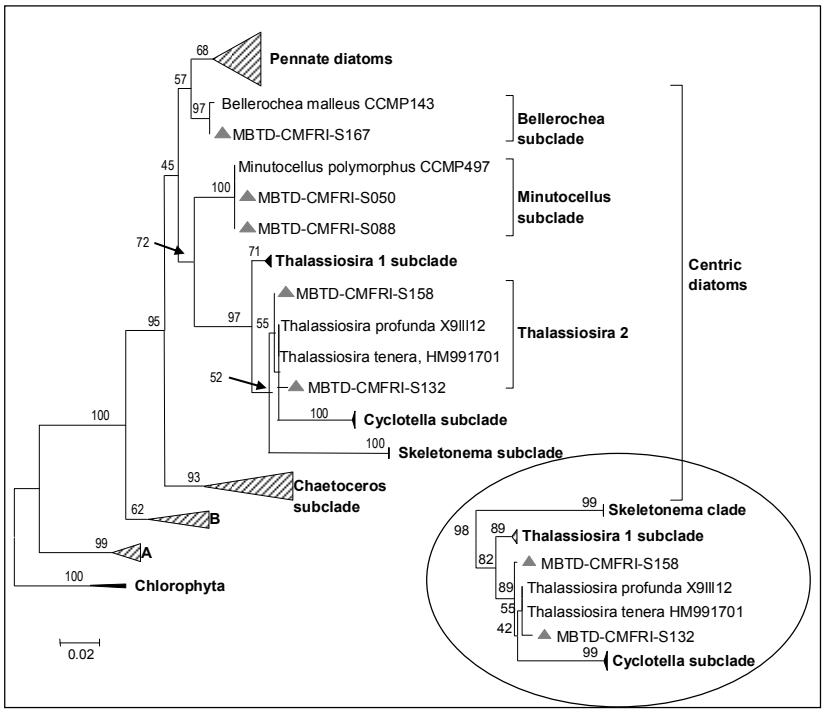
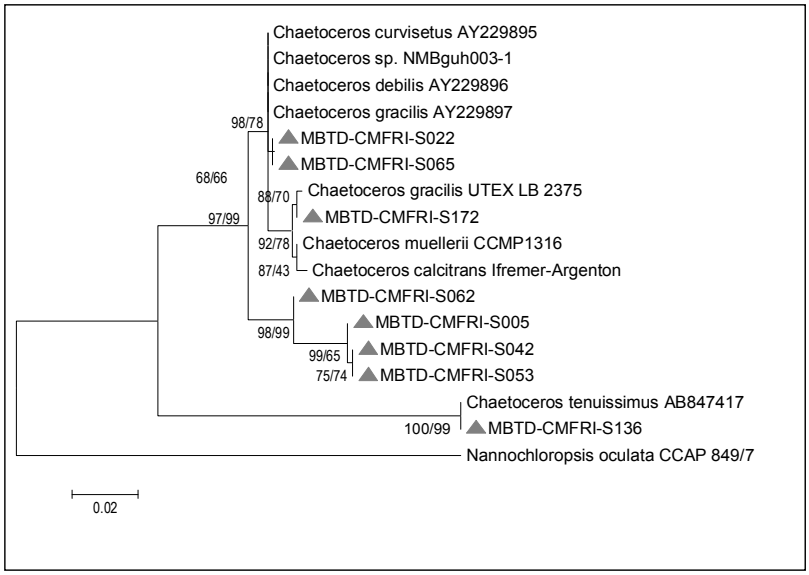


Figure 3.2.5: Maximum Likelihood and Neighbour Joining (in shape) phylogenies inferred from 18S rRNA gene partial sequences depicting *Skeletonema/Thalassiosira/Cyclotella* clustering. Also shows the relationships between different genera of centric diatoms



3.2.6 Combined Maximum Likelihood and Neighbour Joining tree inferred from 18S rRNA gene partial sequences viewing clustering of Chaetoceros isolates. BS values shown for nodes – ML/NJ.

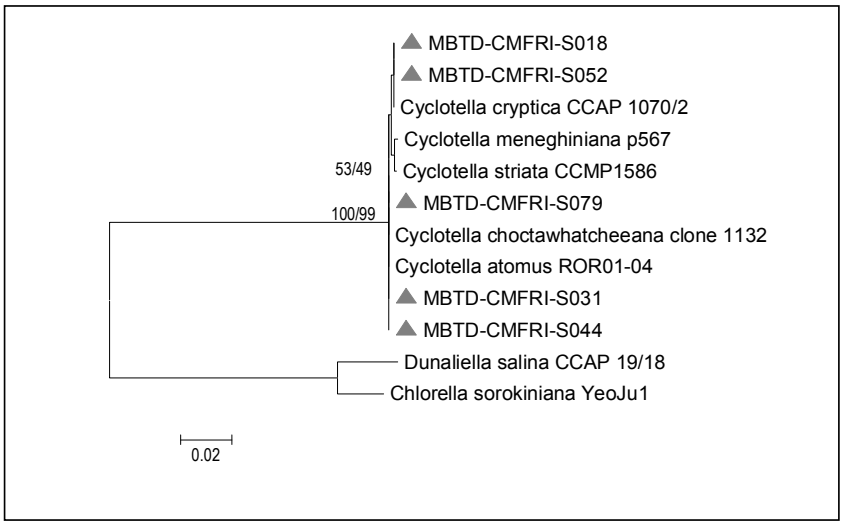


Figure 3.2.7 Combined Maximum Likelihood and Neighbour Joining tree inferred from 18S rRNA gene partial sequences viewing clustering of *Chaetoceros* isolates. BS values shown for nodes – ML/NJ.

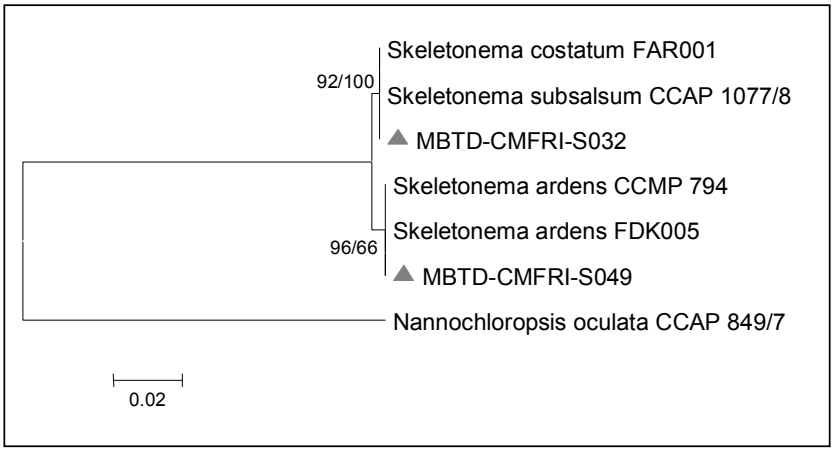


Figure 3.2.8 Combined Maximum Likelihood and Neighbour Joining tree inferred from 18S rRNA gene partial sequences viewing clustering of *Skeletonema* isolates. BS values shown for nodes – ML/NJ.

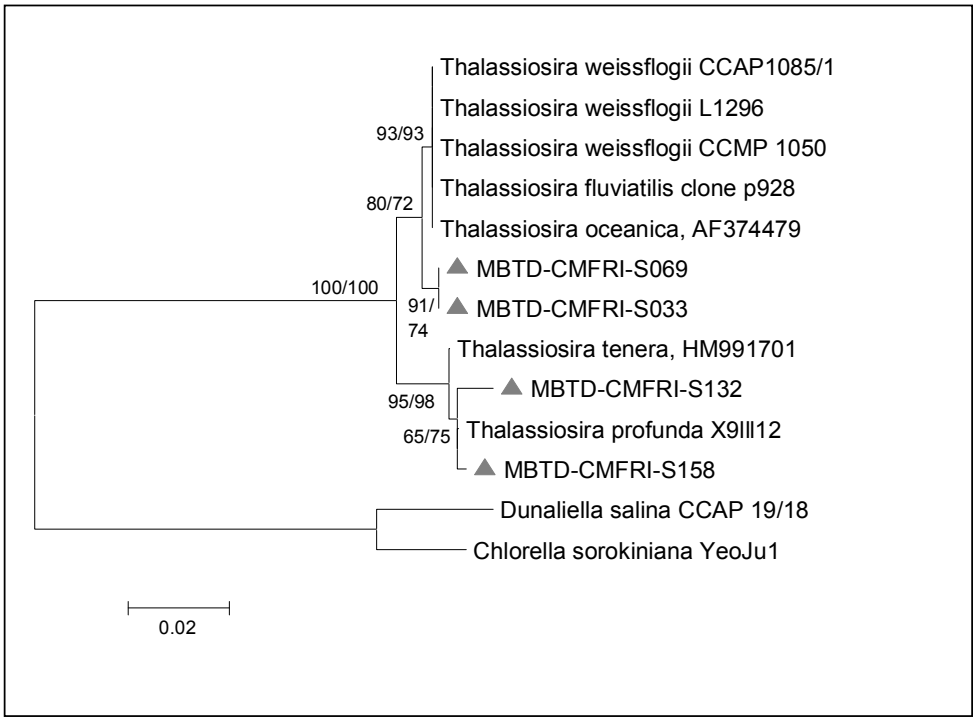


Figure 3.2.9 Combined Maximum Likelihood and Neighbour Joining tree inferred from 18S rRNA gene partial sequences viewing clustering of *Thalassiosira* isolates. BS values shown for nodes – ML/NJ.

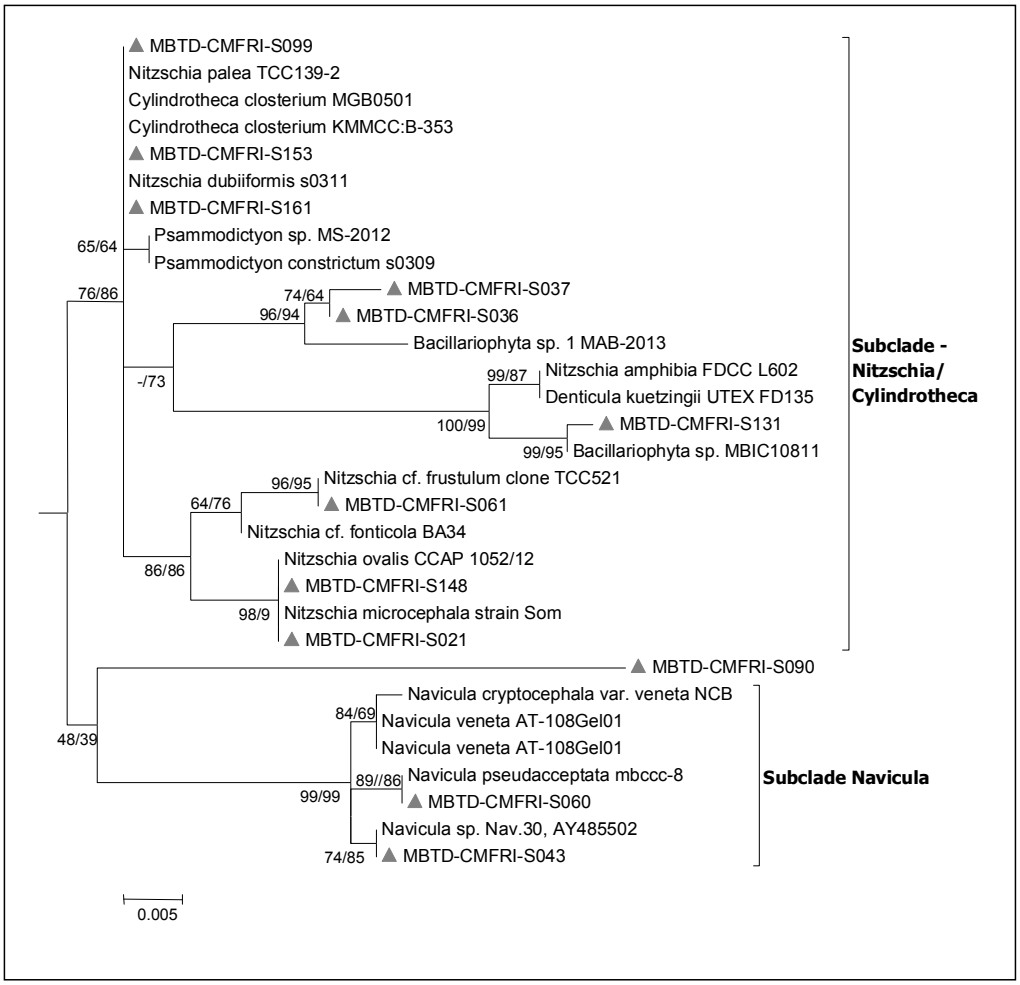


Figure 3.2.10 Combined Maximum Likelihood and Neighbour Joining subtree inferred from 18S rRNA gene partial sequences viewing clustering of pennate diatoms. BS values shown for nodes – ML/NJ

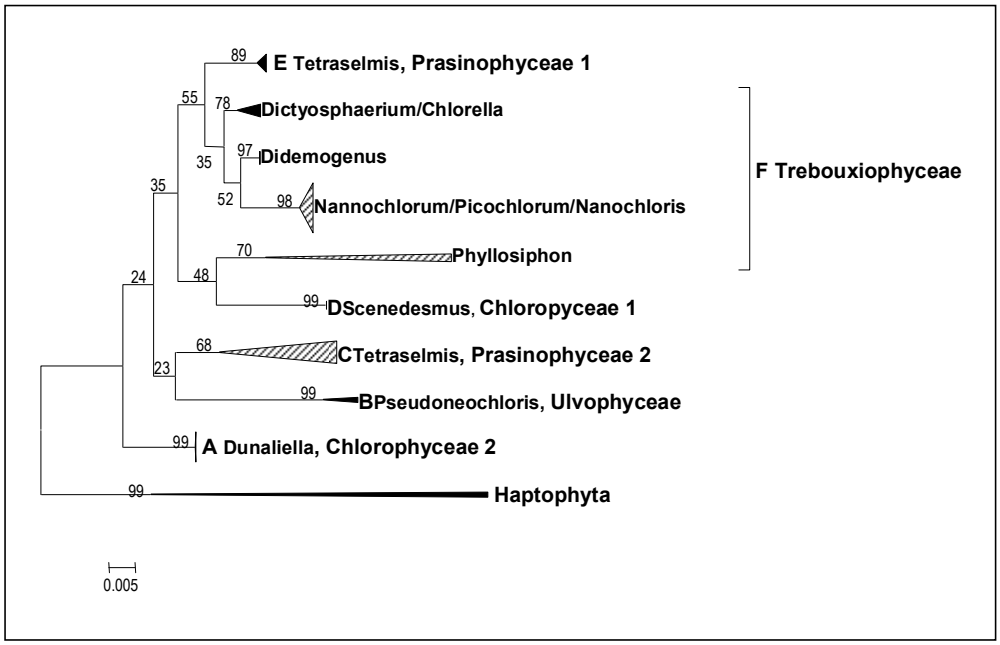


Figure 3.3.1 Maximum Likelihood tree inferred from 18S rRNA gene partial sequences showing the outline of clustering of major Chlorophytemicroalgae with their counter strains; Height of triangles reflect relative number of strains in that branch.

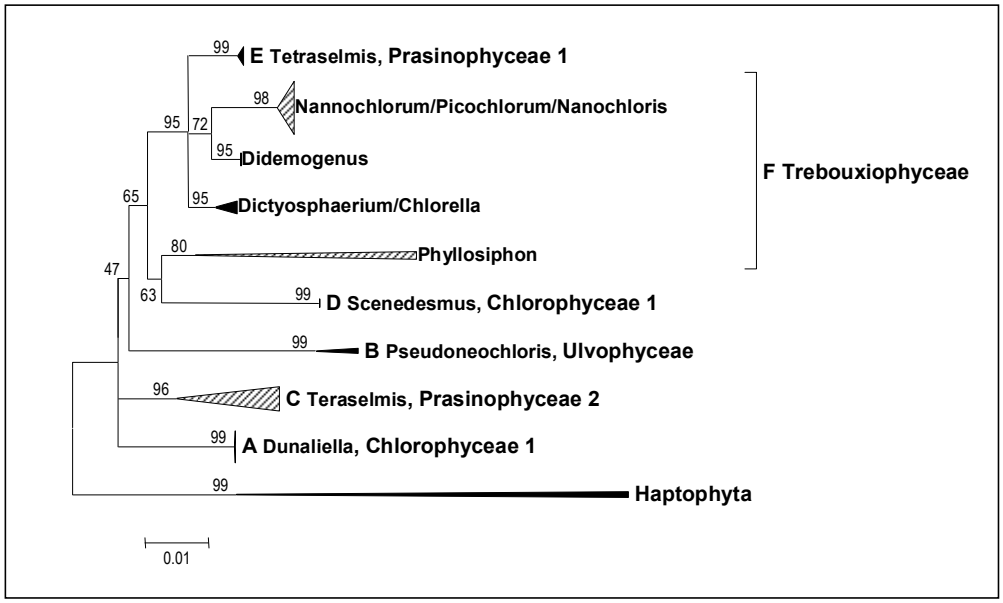


Figure 3.3.2 Neighbour Joining tree inferred from 18S rRNA gene partial sequences showing the outline of clustering of major Chlorophytemicroalgae with their counter strains; Height of triangles reflect relative number of strains in that branch.

3.3.4.2 Chlorophyta

Topology of ML (Figure 3.3.1) and NJ (Figure3.3.2) trees of green algae was slightly different in cladistic arrangement of different green algae, and NJ tree was supported with higher percentage of bootstrap values. In both dendrographs separation of Trebouxiophyceae (F), Chlorophyceae (*Scenedesmus* and *Dunaliella* spp. in 2 clades - D& A), Prasinophyceae (*Tetraselmis* spp. in 2 cladesE&C) and Ulvophycea (B) were the same. All Trebouxiophycean strains, except *Phyllosiphon arisari* (a siphonous parasitic green alga),were monophyletic and the latter along with strain S165 (unidentified) was the most divergedclade (divergence >10%) in the group.

In figures 3.3.3–3.3.5, similarity and divergence within each classare depicted. Phylogeny of *Tetraselmis* spp., indicate origin of the genus in two separate clades (paraphyletic, Figure 3.4.1&2) and presence of at least 4 different strains/species in collection (3.3.5). Our hyper saline *Tetraselmis* isolates (of clade C) got clustered with another salt tolerant Indian *Tetraselmis* sp. MA-2011 (*T. indica*). Major clade of Trebouxiophyceae was comprised of picoplanktonic *Picochlorum*, *Nanochlorum*, *Nannochloris* and *Prasinoderma* isolates clustered overall in a mixed fashion including isolates from Indian waters. Members of *Chlorella* sp., *Didymogenes* sp. and *Dictyosphaerium* sp. were the others in the clade. The unidentified S145 was found associated to *Pseudoneochloiris* sp. (Ulvophyceae) (Fig. 3.3.4). *Scenedesmus* (D) and *Dunaliella* (A),the two genera of Class Chlorophyceae, were paraphyletic in origin. Phylogeny and diversity of Indian isolates of *Dunaliella*are described in part II of this chapter.

3.4 Discussion

3.4.1 Molecular Taxonomy, Phylogeny and Diversity of Microalgae

For the first time, a detailed study on the microalgal diversity study has been taken up using a combined phenetic and molecular approach, from the Indian subcontinent. In general, molecular taxonomy proved its utility as tool, sufficiently variable to differentiate organisms at genus/species level. Among the several methods reported (Ebenezer, Medlin, and Ki 2012; Medlin et al. 2002), DNA sequence similarity search against a molecular reference database (e.g. NCBI) remains to be most conservative for microbes (Moniz and Kaczmarska 2009). In microalgae, even though several molecular loci were attempted and many of them were shortlisted including nuclear, plastidal and mitochondrial genes, none of them were perfect as a barcode. Among the nuclear genes, the small subunit ribosomal RNA (SSU rRNA) was advantageous in being extensively used for diverse groups of prokaryotic (16S) and eukaryotic (18S) microalgae, with a large available database for similarity search (Auinger, Pfandl, and Boenigk 2008; Jahn et al. 2007).

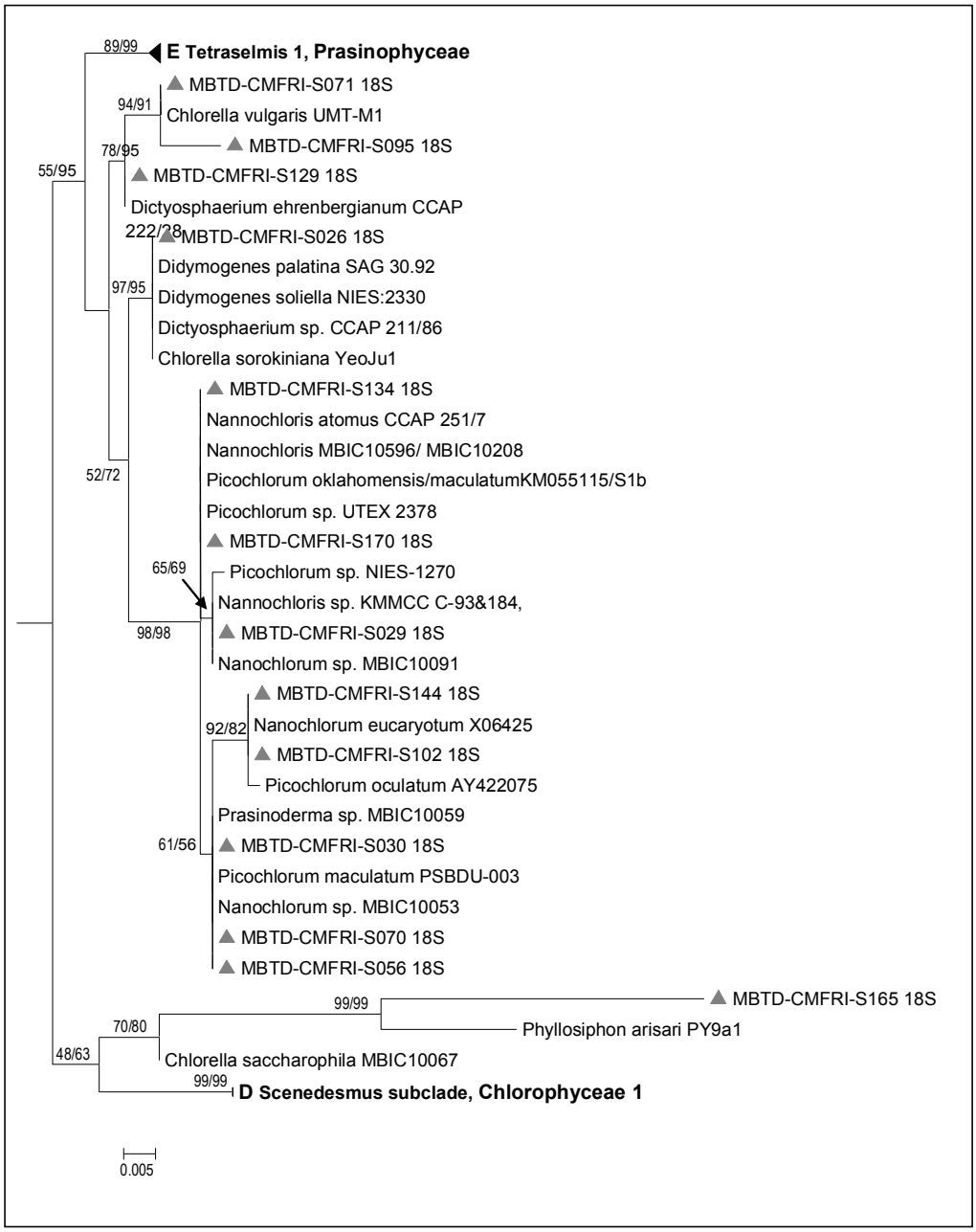


Figure 3.3.3 CombinedMaximum Likelihood - Neighbour Joiningsub tree inferred from 18S rRNA gene partial sequences showing the clustering of Trebouxiophyceanmicroalgae; BS values shown for nodes – ML/NJ.

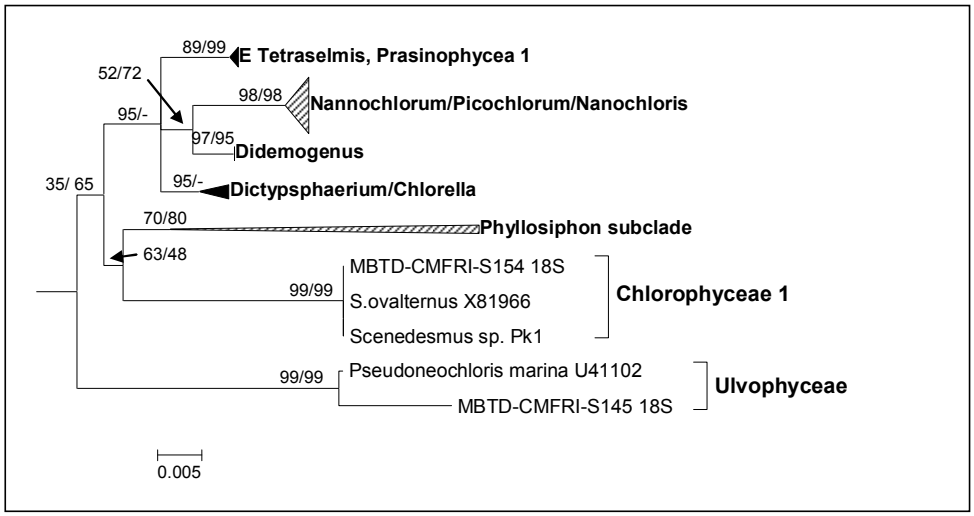


Figure 3.3.4 CombinedMaximum Likelihood - Neighbour Joiningsub tree inferred from 18S rRNA gene partial sequences showing the clustering of *Scenedesmus* and *Pseudoneochloris*; BS values shown for nodes – ML/NJ.

As mentioned in the results, by the analysis of a small stretch (even <200bp) of SSU ribosomal RNA gene, almost all isolates inspected in this study got discriminated comparatively well at genus level, and to some extend at species level. Those strains which were so look-alike externally also got categorized by BLAST analysis. This was exemplified best by morphologically un-differentiated *Chlorella* (S095, S072 & S171) and other coccoid green algae (S026, S029, S030, S048, S056, S070, S102, S113, S130, S145, S154, S165 and S170). Green alga S170 was initially identified as *Nannochloropsis* sp. (Eustigmatophyceae), especially due to the yellowish green colour of culture and its pico size. But 18S ribosomal gene analysis grouped it with *Picochlorum* sp.

Among the above mentioned strains, dilemma was there for the "*Nanochloris/ Nannochlorum/ Picochlorum*" strains (S029, S030, S056, S070, S102, S134, S154 and S170). The 18S gene analysis clearly resolved the issue and these isolates were specifically matched with either one or the other above

mentioned species. It was noted that the identity of the reference strains used (Fig. 3.3.3) - *Nanochloris* (UTEX 2378 & 2491, CCAP 251/7), and *Nannochlorum* (MBIC 10091, 10096, 10208 & 10053), had been rechecked by Henley et al. (2004) based on phenotypic and phylogenetic features and classified them into *Picochlorum* genus. Further the subclade of above strains (Trebouxiophyceae) had a Prasinophycean - *Prasinoderma* sp. MBIC10059, which indicates a wrong identity of the strain and suggests revision. In this way all of the above halophilic isolates, must be members of *Picochlorum* genus, where as *Nannochloris* was referred mostly as a fresh water species. Different species names can be assigned to our strains based on morphology (cell size, presence/absence of pyreniod etc.) and molecular similarity. For example, S134 was affiliated to *P. atomus* (CCAP 251/7), S030/S070/S056 to *P. maculatum* (PSBDU-003), S170 to *P. oklahomensis* (KM055115) and S102/S144 to *P. eukaryotum* (X06425). Nevertheless, a more detailed multifaceted (morphological, biochemical and phylogentic) investigation is suggested for a final conclusion.

In case of *Nannochloropsis* spp., all were old isolates and were recorded and maintained as either *N. oculata* or *N. salina*. Molecular analysis in the present study clearly defines these strains as *N. oceanica* by 18S phylogeny (100%) and by COI gene sequences (99%). In the same way old strains of *Chaetoceros* (S005, S062 and S074, maintained as *C. calcitrans*) and *Tetraselmis* (S011, *T. striata* & S075, *T. gracilis*) were also modified as *Chaetoceros* sp. and *Tetraselmis* sp. For previously identified old algae, at one place molecular taxonomy helped in confirmation of the identification (e.g. *Isochrysis galbana*) and in another place corrected the wrong designations (e.g. *Ochrosphaera* sp. S003 which was maintained as *Chromulina* sp.). About S003 *Ochrosphaera* sp., the transformation of species might have occurred by

a culture contamination, which therefore insists periodic checking of cultures for purity. Suitability of SSU rRNA gene in species characterization of microalgae is further demonstrated by the identification of unknown diatoms S088 & S050 as *Minutocellus polymorphus,* and discrimination of blue green algae S100 & S137 as *Glietlerinema* sp. &*Oscillatoria* sp. respectively.

In some strains, different genes were observed to match with same species and confirmed their identity. Partial sequences of 18S, ITS and *rbc*L of a coccoid green alga S130 had shown 99%, 98% and 83% similarity with *Mychonastes* sp. Other examples are *S. ardens* S049 (18S and COI) and *C. vulgaris* S171 (18S and *rbc*L). However, those supplementary marker regions were having significantly poor hits in database for most of the remaining strains (Tables 3.3 - B, C & D). These outcomes further confirm the use of 18S rRNA gene for basic or first level identification of microalgae. Even after sequencing of three genes (18S, ITS and *rbc*L) none of the marine *Teraselmis* isolates were recognized properly (different genes showed similarity with different species - Table 3.3A, B & C). These problems confronted can be overruled by a polyphasic approach(Aslam et al. 2007; Bock 2010) – study based on a combination of phenotypic and molecular traits, which we have done for the isolates of *Dunaliella* and discussed in part B of this chapter.

Group I introns of ribosomal RNA gene are relatively common in green algae (Wilcox et.al 1992). Presence of introns and thereby the increase in gene size have been used effectively in taxonomy of genus *Dunaliella* (Hejazi et al. 2010; Olmos et al. 2002 and Chapter 3B). In this study it was noticed that many green algae and one diatom to have intron/s inflating the 18S gene. Three brackish water chlorophyte strains - S138 (*Oocystidium*), S139 (*Monoraphidium*) and S145 (*Pseudoneochloris*), were expected to have introns because of a larger 18S gene (>2000 bp). However, partial sequences

did not reveal their occurrence, probably due to their presence on the other end of the gene. In diatoms, *rbc*L and COI genes were accounted for the presence of introns (Evans 2007). Here, an Indian diatom *Bellerochea* sp. S167 was observed with an intron in 18S rDNA, of 109 nucleotides long, and this is a first report for the genus to our knowledge. For similarity search the intron was excised, and the gene got blasted against *B. malleus* CCMP173 with 99% identity. It remains a question that in taxonomic delineation, whether presence of introns have to be included or not (discussed in Part II of chapter).

In phylogeney, clustering of *Thalassiosira* spp. was in accordance with Alverson et al. (2011) where the strains were paraphyletic in origin and the two sister clades *Skeletonema* and *Cyclotella*were found to share the same major clade (Fig. 3.2.5). Monophyly of pennates was further confirmed(Medlin 2010), with clear separation of *Nitzschia* and *Navicula* subclades (Fig. 3.2.10). Within this, four nanno-size pennate diatoms S036, S037, S131 and S090 had ambiguous relatives phylogenetically, and hence remained unidentified. The latter (S090) was the most diverged (8 – 11%) among the diatoms and propose the chance of a new species, probably a *Navicula* (because of closeness both by 18S gene – *N. cryptotenalla* 92% and by morphology).

Among Chlorophytes, the clustering of different classes (Fig. 3.3.1 & 3.3.2) can be compared to previous studies (Lewis and McCourt 2004; Watanabe et al. 2000), where poly/paraphyly of most of the classes (Trebouxiophycea, Chlorophyceae and Prasinophycae) were reported. All Trebouxiophyceans got clustered in a major clade except *Phyllosiphonarisari*showing the paraphyly of the class. Similarly *Tetraselmis* isolates (Prasinophyceae) grouped in to two distant clusters (C & E) and Chlorophyceae the two subclades were the A and D. The green single celled

marine isolate S165 (from south west coast) which was clustered with a siphonous parasitic green alga - *Phyllosiphonarisari* (with identity 91%), was the most diverged (7-10 %) among chlorophytes and possibly could be a new species.

The taxon diversity present in the collection illustrates less number of genera than expected from such a varied regional sampling from major coastal India. This could be probably resulted from the random isolation and common culture protocols followed than specific techniques for specific algae. However, when some genera were considered (Figures 3.2.1 to 3.2.10 & 3.3.5),more genetically dissimilar strains were found. For example in *Chaetoceros*, out of 9 isolates 6 genetically different strains were present. Similarly, 3 out of 6 strains of *Thalassiosira*, 2/5 of *Cyclotella,* 2/2 of *Skeletonema*, 3/6 of *Nitzschia*, 2/2 of *Navicula*, 4/7 of *Tetraselmis* were genetically dissimilar.

Most of the similar strains (same species) were originates of diverse locations showing their adaptability to a range of salinities. This can also be correlated with the descriptions provided by the algal websites www.Algaebase.org. and World register for Marine organisms (www.marinespecies.org) for the species. For e.g. the two *T. wiessflogii* S033 and S069 were the isolates of mangrove and marine ecosystems respectively and a cosmopolitan distribution is reported for the species. The case of other diatoms was not much different, all originated from a varying saline conditions. Among green algae, there were *Chlorella vulgaris* from fresh water (S171) and marine (S095) environment. Other coccoid green algal generic variability was obtained from stagnant pools – *Dictyosphaerium* sp.-S129, *Oocystidium* sp.-S138,*Monoraphidium* sp.-S139,*Pseudoneochloris* sp.-S145, *Mychonastes* sp.-S130 and *Desmochloris* like S113 were all isolated

from slightly eury-haline (0-40 ppt) water bodies. *Picochlorum* spp. was isolated both from both sea and brackish waters. Two hyper saline *Tetraselmis* isolates (*T. indica*) S094 and S127 were from south east (AP) and south west (Goa) coasts of India in order. *T. indica* from Goa was first reported by Arora et al. (2013), and present study substantiates the presence of the same species in other hyper saline habitats of the country (out of 5 isolates only one was from Goa while others were from AP and Tamilnadu).

3.5 Conclusion

The focus of the present study was the taxonomic identification of microalgae and mapping the phylogenetic relationships between them. When a large set (>100) of microalgae from a culture collection was studied, detailed morphological examination of each strain was monotonous, laborious as well as lengthy. SSU rDNA BLAST analysis alone helped in the discrimination of more than 80% strains (nearly 34 genera), with a revision of many already identified strains (morphologically). This shows the high range of applicability of the gene in microalgae taxonomy. Further 18S phylogeny confirmsthe presence of two un-described species (probably new) in the collection – one pennate diatom MBTD-CMFRI-S090 and a chlorophyte MBTD-CMFRI-S165. Present study revealed the versatile nature and use of the 18S gene in microalgal atxonomy, where the maximum rate of amplification was obtained using a single set of primers (Univ F & Univ R) along with a large sequence bank accessible for the appraisal. Consequently, we got restrained in using 18S rDNA as a core taxonomic region (for protist algae) mainly due to the limitations confronted with other genes (COI gene, ITS region and *rbc*L gene) - like non-specificity of primers, weak sequence recovery and limited database (Table 3.1). However, many studies (Daugbjerg and Andersen 1997; Evans et al. 2007; Hoshina 2014; Lundholm et al. 2002; Mills and Kaczmarska 2006;

Nozaki et al. 2002; Saunders and Kucera 2010) validate the prospect of COI gene and ITS2 secondary structure to become a better barcode in future, if provided with a strong supporting phenetic and genetic record. Present study combining the morphology and molecular tool in the taxonomy has provided new approach in resolving the identification and taxonomic issues among microalgae, which could be further refined in future studies to have a more specific profiles.

Chapter 3

Part-II

PHENOTYPIC AND GENETIC CHARACTERIZATION OF DUNALIELLA (CHLOROPHYTA) FROM INDIAN SALINAS AND THEIR DIVERSITY

Contents

Abstract

The genus Dunaliella (Class – Chlorophyceae) is widely studied for its tolerance to extreme habitat conditions, physiological aspects and many biotechnological applications, like a source of carotenoids and many other bioactive compounds. Biochemical and molecular characterization is very essential to fully explore the properties and possibilities of the new isolates of Dunaliella. In India, hyper saline lakes and manmade salt pans were reported to bloom with Dunaliella spp. However, except for the economically important D. salina, other species are rarely characterized taxonomically from India. Present study was conducted to describe Dunaliella strains from Indian salinas using a combined morphological, physiological and molecular approach with an aim to produce better understanding on taxonomy and diversity aspects of this genus from India. Comparative phenotypic and genetic studies revealed high level of diversity within the Indian Dunaliella isolates. Species level identification using morphological characteristics clearly delineated two strains as D. salina with considerable β-carotene content (>20 pg/cell) under stress. The variation in 18S rRNA gene size, amplified with MA1-MA2 primers, ranged between

3.6 Introduction

Dunaliella, the unicellular microalga, is one of the best studied organisms in both general and applied phycology for its higher tolerance to extreme conditions of salinity, light, temperature and pH as well as for its richness in natural carotenoids, glycerol, lipids and many other bioactive compounds (Avron and Ben-Amtoz 1992; Ben-Amtoz 2004; Borowitzka and Siva 2007; Tafreshi and Shariati 2009). *Dunaliella salina* is reported as the most halo-tolerant photosynthetic eukaryote with a remarkable degree of tolerance from 0.5 to 5 M salt concentrations (30-300 ppt) (Ben-Amtoz 2004). This genus naturally inhabits saline and hyper saline waters and has a cosmopolitan distribution (Olmos et al. 2009). Among the 28 different species of *Dunaliella*, 23 are saline or hyper saline (Borowitzka and Siva 2007, Oren 2005; Preisig 1992; Gonzalez et al. 2009; Buchheim 2010).

Many countries, including India (Parry Agro Industries Ltd., Murugappa group), use the alga *D. salina* for the industrial production of β-carotene with wide range of applications (Tafreshi and Shariati 2009; Borowitzka and Borowitzka 1998; Spolaore et al 2006; Kleinegris et al. 2010). Apart from *D. salina, D. tertiolecta* is used in aquaculture, and many other species were found promising for the production of biofuel and for bioprospecting of antioxidants, bioactive compounds etc. (Tafreshi and Shariati 2009, Oren 2005). Considering the economic importance, most of the studies were mainly focused on the taxonomic, physiological and biotechnological aspects of the halophilic species *D. salina* (Olmos et al. 2009; Gomez and Gonzaléz 2001 & 2004; Raja et al. 2007a & 2007b; Polle et al. 2008; Mishra & Jha 2009 & 2011) (especially from Indian subcontinent) and on the marine species *D. tertiolecta*. But similar exclusive or comparative studies are rarely available for other species (Eyden 1975; Hoshaw & Malouf

1981; Uriarte et al. 1993; Gonzalez et al. 2001), probably due to their lesser importance and/or limited distribution.

Typically the taxonomy of *Dunaliella* anchors on the morphological and physiological features of the organism. Apart from the general morphology, salinity tolerance and carotenoid (especially β-carotene) production are the two commonly studied physiological attributes of *Dunaliella*, where considerable variations have been accounted at inter and intra-species levels (Teodoresco 1905; Massyuk 1973). Recently, Borowitzka and Siva (2007) have given a detailed account of taxonomic revision of the genus *Dunaliella* with special emphasis on saline species bringing more clarity in classification. *Dunaliella* are unique in having a thin plasma membrane instead of a rigid cell wall (Oliveira et al. 1980) and are able to change their cell shape and volume in response to changes in osmolarity and other growth conditions (Polle et al. 2008; Gonzalez et al. 1999; Chen and Jiang 2009; Hajezi et al. 2010). Due to this high plasticity of cell morphology, the traditional practice of species differentiation, merely based on light microscopic observations becomes difficult and time consuming. Consequently many misidentifications arose in the literature which brought in controversies and confusions in the taxonomic organization of the genus *Dunaliella* (Borowitzka and Siva 2007; Olmos et al. 2009).

Molecular taxonomy emerged as a faster and powerful tool as it is consistent and independent from environmental factors and growth stages (Bornet et al. 2004). It seems to be an advanced and reliable device for the characterization and differentiation of morphologically plastic organisms. Since 1999, molecular characterization has been found promising in the taxonomy of *Dunaliella* (Oren 2005; Hajezi et al. 2010). Currently 18S rRNA gene (Olmos et al. 2000 & 2009), Internal Transcribed Spacer (ITS) region

(Gomez and Gonzalez 2004; Gonzalez et al. 1999 & 2001) and large subunit of the ribulose-bisphosphate carboxylase (*rbc*L) gene (Buchheim et al. 2010) are being widely used as effective molecular tools in *Dunaliella* characterization and biodiversity studies. Use of these molecular markers has resulted in the suggestion for re-designation of many species (Gonzalez et al. 2009; Ramos et al. 2011). Nevertheless the confusion regarding the taxonomy still persists due to the mis-identifications, and will be there until a revision is made, combining the robust molecular phylogeny, supported by morphological and physiological attributes. Owing to these conditions many authors opined to have a combined approach rather than a single system of taxonomic identification (Borowitzka and Siva 2007, Ramos et al. 2011).

In India, *Dunaliella* are found in salt pans, saline and hyper saline ponds, lakes, pools etc. as a major primary producer. Many species including *D. salina* have been reported (Jayapriyan et al. 2010) to form blooms in artificial salt pans. Documentation of Indian *Dunaliella* strains describing a comprehensive characterization based on phenotypic and molecular traits is rarely found. In this background, we conducted a study on characterization of *Dunaliella* strains isolated from the Indian salinas, using morphological, physiological and molecular tools and have made an attempt to produce the best possible information about Indian *Dunaliella*. Based on the results obtained, taxonomic position and diversity aspects of the Indian *Dunaliella* isolates and the genus are discussed.

3.7 Materials and Methods

3.7.1 Sampling, Isolation and Culture Conditions: (Refer sections 2.2.1 and 2.2.2 of Chapter 2)

3.7.2 Microscopy and Morphological Study (Refer section 2.2.3)

Major taxonomic features observed include size, shape and colour of the cell, length of flagella, characteristics of stigma, pyrenoid and chloroplast and other cytoplasmic inclusions like refractile granules. Scalar measurements such as cell length and width, were taken from a minimum of 30 cells from each strain randomly during mid growth phase immediately after fixing the cells with 1% Lugol's iodine. The descriptive statistics such as minimum, maximum, mean and standard deviation were estimated for the above scalar measurements. One way analysis of variance (ANOVA) was performed using SPSS (Version 10.0) to identify whether there is any statistically significant difference among different *Dunaliella* strains for each character.

3.7.3 Salinity Tolerance Study

For salinity tolerance study, different *Dunaliella* strains were cultured in five salinity concentrations *viz*., 0.5, 1.5, 2.5, 3.5 and 4.5 M NaCl in 150 ml (250 ml conical flasks) modified Johnson (J/I) medium (Borowitzka and Borowitzka 1988) (Appendix). Other culture conditions like temperature and light were kept constant as given for normal culture maintenance. Cell characteristics like cell size and colour were examined at late growth phase under DIC microscope (Nikon, Japan). Cell count was taken on every third day using a Neubauer haemocytometer. Cell density was calculated and plotted against days of growth to obtain optimum salinity for each strain.

3.7.4 β-Carotene Analysis

Beta carotene was estimated under normal (1.5M NaCl, irradiance of 40-50 µmol photons m^{-2} s^{-1}) and stressed (3.5M NaCl, irradiance of 100-150

μmol photons m^{-2} s^{-1}) growth conditions. Total pigment was extracted from 4 ml culture at late growth phase (25^{th} day) in 4 ml ice cold 100 % acetone. Liquid cultures were centrifuged (8000 rpm, 10 min.), the pellet washed with distilled water and re-suspended in ice cold acetone and left overnight at -20°C until the pellet became colourless. The extract was centrifuged at 5000 rpm for 5 min and absorbance was taken for the supernatant at 454 nm wavelength. Readings were compared with standard curve prepared with synthetic β-carotene (Type 1, Sigma, USA) in 100% acetone as described by Hajezi et al. (2010). Cell density was calculated for the same day of extraction and *β*-carotene was calculated per cell in picograms.

3.7.5 DNA Isolation & PCR Amplification, Sequencing and Phylogeny

The procedures followed were described in detail in sections 3.2.2, 3.2.3 and 3.2.4. A gene fragment of 18S rRNA was amplified using conserved primers MA1 & MA2 (Olmos et al. 2002). Internal transcribed spacer (ITS) region (~700 bp), including ITS1, 5.8 S rRNA and ITS2, was amplified using the primers ITS1 and ITS4 (17). The sequence were aligned with the various available sequences (Table 3.5) of *Dunaliella* spp. and, *Chlamydomonas reinhardtii* (ITS) and *Paulschulzia pseudovolvox* (*rbc*L) as out group using the CLUSTAL-W algorithm (Thompson et al. 1995) in Bioedit 7.0 (DNA Sequence Analysis Software package). To evaluate diversity within Indian *Dunaliella* isolates, *D. tertiolecta* and *D. acidophila* were used as out groups in ITS and *rbc*L phylogenies respectively. Pair wise genetic distances among different *Dunaliella* species and between the present isolates were calculated based on Kimura 2 parameter model for ITS region and Tamura 3 parameter for *rbc*L gene. The best nucleotide substitution model selection and

phylogenetic analysis based on maximum likelihood was carried out using MEGA 5 (Tamura et al. 2011) with 1000 boot strap replications. All the sequence information generated in the present study was deposited in the NCBI database (Table 3.6).

Table 3.5: Geographical origin and gene sequence accession details of *Dunaliella* strains studied in the present work. Indian strains were grouped into subsets based on the 18S rDNA size obtained by PCR amplification with MA1-MA2 primers

Groups	Strain code	Isolated from	Geographic co-ordinates	Month of collection	Salinity of the sampled water	18S rDNA product size	Genebank accession No.		
							18S rDNA	ITS region	*rbc*L gene
	CS265	*Dunaliella salina*; Reference strain from CSIRO collection of living microalgae, Australia				2210 bp	JN807321	JN797804	JN797820
	MBTD-CMFRI-S135	Sea water, Calicut, Kerala (WC)	11°15' N 75°46' E	May 2009	33 ppt	2230 bp	JF708161	JN797802	JN797818
I	MBTD-CMFRI-S089	Saltpan, Kelambakkom, Chennai, TN (EC)	Culture maintained in CMFRI phytoplankton culture collection, isolated from Chennai salt pan.			2210 bp	JF708173	JN797806	JN797811
	MBTD-CMFRI-S118	Salt pan, Nellore, AP (EC)	14°16' N 80°07' E	March 2009	300 ppt	2290 bp	JN807316	JN797808	JN797813
II	MBTD-CMFRI-S086	Salt pan, Tuticorin, TN, (EC)	08°47' N 78°09' E	February 2009	300 ppt	2290 bp	JF708169	JN797805	JN797810
	MBTD-CMFRI-S121	Pulicat salt lake, AP (EC)	13°40' N 80°11' E	March 2009	150 ppt	2250 bp	JN807317	JN797809	JN797814
	MBTD-CMFRI-S115	Kelambakkom saltpan, Chennai, TN (EC)	12°45' N 80°12' E	March 2009	380 ppt	2550 bp	JN807315	JN797807	JN797812
III	MBTD-CMFRI-S122	Salt pan, Ribandar, Goa (WC)	15°30' N 73°51' E	May 2009	280 ppt	2550 bp	JN807318	JN797799	JN797815
	MBTD-CMFRI-S133	Salt pan, Kutch, Gujarat (WC)	23°50' N 69°39' E	July 2009	320 ppt	2530 bp	JF708183	JN797801	JN797817
IV	MBTD-CMFRI-S125	Salt pan, Pilar, Goa (WC)	15°26' N 73°53' E	May 2009	260 ppt	2640 bp	JN807319	JN797800	JN797816
V	MBTD-CMFRI-S147	Salt pan, Kutch, Gujarat (WC)	23°50' N 69°39' E	April 2009	180 ppt	1820 bp	JN807320	JN797803	JN797819

NB: For convenience strain codes used in text included only third part of full strain code (e.g., S086). AP– Andhra Pradesh; TN-Tamil Nadu; WC-west coast; EC – east coast.

Table 3.6: Morphological and physiological characteristics of 10 Indian *Dunaliella* strains: Grouping of the subsets was formed based on common morphological features including cell size and β-carotene accumulation at high salinity and light (stress).

Groups	Strain Code	Cell colour	Cell shape	Flagella length	Stigma	Pyrenoid	Refractile granules	Mode of reproduction observed	β Carotene normal/stress (pg/cell)	Salinity optimum	Identified as
I	MBTD-CMFRI-S135	Green to red	Ovoid, spherical, cylindrical	1.3 or 1.5 to cell length	Not clearly visible or diffuse	Large with distinct amylosphere	Absent	Cell division	8.68/22.94	1.5M NaCl	*D. salina*
	MBTD-CMFRI-S089	Green to red	Ovoid, spherical	1.3 to cell length	Not visible/ Diffuse large	Large with distinct amylosphere	Absent	Cell division	6.53/23.36	1.5M NaCl	*D. salina*
II	MBTD-CMFRI-S118	Green to orange	Ovoid spherical	1.5 to cell length	One; large, red, median, diffuse	Small with amylosphere	Absent	Sexual, cell division	2.11/3.47	2.5M NaCl	*Dunaliella sp.*
	MBTD-CMFRI-S121	Green	Ovoid pyriform	1.5-2 to cell length	One; large, red, median, distinct	Large with amylosphere	Absent	Sexual, cell division	1.59/2.17	1.5M NaCl	*Dunaliella sp.*
	MBTD-CMFRI-S086	Green	Ovoid, oval or pyriform	1.5- 2 to cell length	One; Small, red, median, distinct	Small with amylosphere	Present	Sexual, cell division	2.68/3.41	0.5 M NaCl	*Dunaliella sp.*
III	MBTD-CMFRI-S115	Green	Ovoid, oval or fusiform	1.3 to cell length	One; small, red, anterior, distinct	Small with amylosphere	Absent	Palmella, aplanospores	1.05/1.99	1.5M NaCl	*D. viridis?*
	MBTD-CMFRI-S122	Green	Oval, cylindrical,	1.3 to cell length	One; large, red, anterior, distinct,	Large, Amylosphere	Present	Palmella stage	0.67/1.78	1.5M NaCl	*D. viridis?*
	MBTD-CMFRI-S133	Yellow green	Fusiform, Elliptical	1.3 to cell length	One, Two at lower salinity; small, red, median, distinct	Small, with amylosphere	Absent	Cell division, Palmella, aplanospores	0.51/1.26	1.5M NaCl	*D.viridis/D. bioculata?*
IV	MBTD-CMFRI-S125	Green	Cylindrical fusiform	Equal or 1.3 to cell length	One, large, red, anterior, distinct	Small, with distinct separate starch grains	Absent	Cell division	0.70/1.8	1.5M NaCl	*D. minuta?*
V	MBTD-CMFRI-S147	Green	Oval, fusiform	1.5 or 2 to cell length	One, large, red, median distinct	Large with amylosphere	Present	Palmella (dominant stage), Cell division	0.89/6.7	1.5M NaCl	*Dunaliella sp.*

3.8 Results

3.8.1 Morphological & Physiological Parameters

Morphologically all ten strains of the green biflagellate chlorophytes isolated from 7 different locations along the Indian coast (Table 3.6) were identified as *Dunaliella* (Figure 3.4) following the revision of the genus by Borowitzka and Siva (2007). Of the ten strains, nine were isolated from hyper saline water bodies and one strain (S135) was marine. Though purified by agar plating, the cultures were not axenic. All morphological characteristics of different geographical Indian isolates of *Dunaliella* are summarized in Table 3.7.

Table 3.7: Descriptive statistics of cell size variables and F-values (derived from the analysis of variance) of different *Dunaliella* isolates from Indian coast. Grouping of subsets was statistically formed based on the average length/width of the *Dunaliella* cells.

Groups	I		II			III			IV	V	
Strain code	MBTD-CMFRI-S135	MBTD-CMFRI-S089	MBTD-CMFRI-S086	MBTD-CMFRI-S118	MBTD-CMFRI-S121	MBTD-CMFRI-S115	MBTD-CMFRI-S122	MBTD-CMFRI-S133	MBTD-CMFRI-S125	MBTD-CMFRI-S147	F value
Length µm	17.51±1.78 (12.30-21.17)	14.12±2.25 (10.01-18.82)	9.15±1.02 (6.44-10.68)	9.51±1.09 (7.96-12.25)	9.37±1.30 (6.45-11.77)	9.02±0.96 (6.79-12.12)	8.46±1.12 (5.62-10.55)	7.91±0.93 (6.54-9.78)	9.89±1.37 (8.38-12.99)	11.17±1.50 (8.02-13.83)	138.33*
Width µm	10.30±1.96 (8.61-19.79)	9.57± 1.35 (7.46-12.58)	6.14±0.92 (3.52-8.08)	6.91±0.74 (5.84-8.76)	5.94±0.96 (4.14-7.54)	5.09±0.77 (3.02-6.98)	4.74±0.48 (3.91-5.76)	3.89±0.60 (3.11-5.10)	4.34±0.69 (3.27-5.95)	7.23±1.15 (5.40-10.07)	125.85*

Measurements are presented as, Mean ± SD (min. - max.); *Significant at the 1% level; *SD* is standard deviation.

High level of morphological plasticity with cell shape and size was observed among all the 10 *Dunaliella*, strains, but a general consistency in cell size was noticed within the range given (Table 3.6) (Massyuk 1973b; Ginzburg and Ginzburg 1985). Among the 10 strains, S135 (Calicut, marine isolate), S089 (Chennai) and S147 (Kutch) were considerably larger while strain S133 (Kutch) was the smallest.

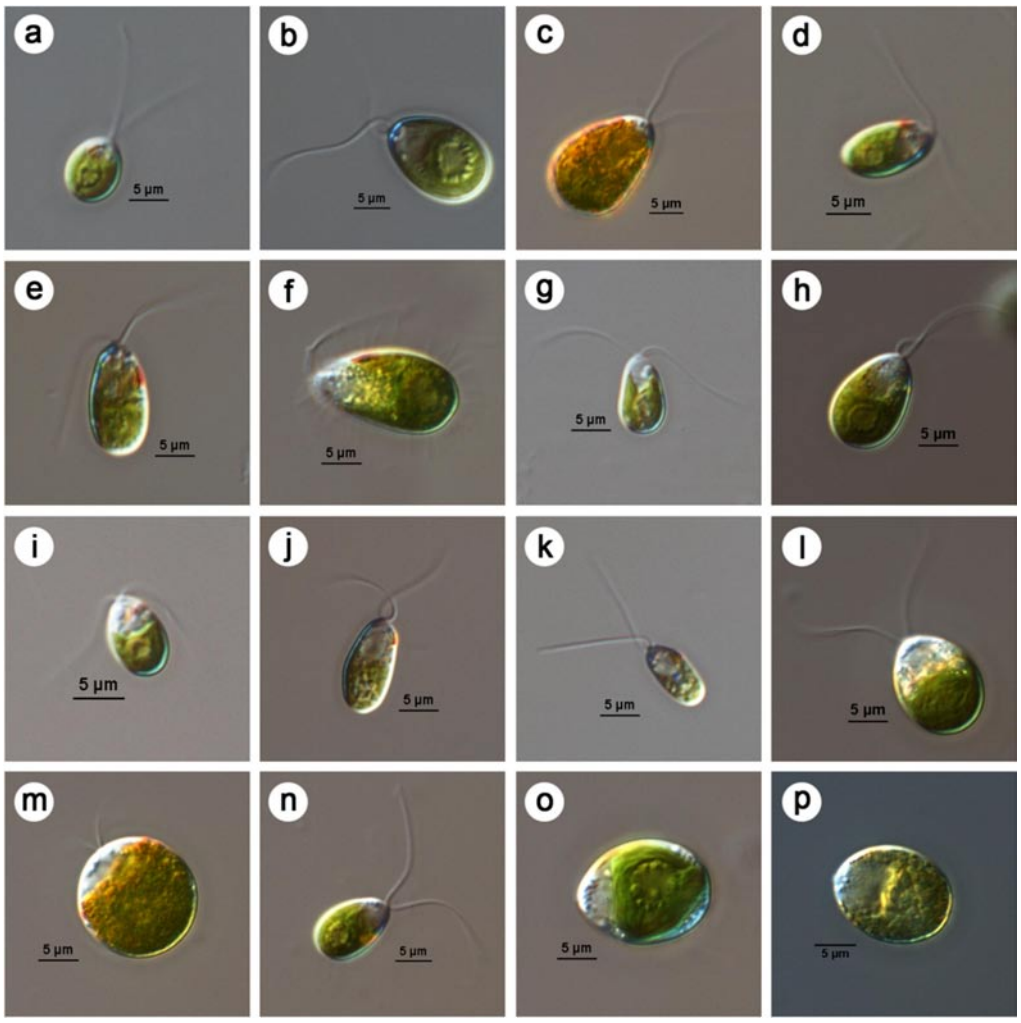


Figure 3.4 DIC microscopic images of different *Dunaliella* isolates (a) *Dunaliella sp.* S086 (Tuticorin salt pan), (b) & (c) *D. salina* S089 (CMFRI old strain), (d) *D. viridis*? S115 (Chennai salt pan), (e) & (f) *Dunaliella* sp. S118 (Nellore salt pan), (g) & (h) *Dunaliella sp.* S121 (Pulicat lake), (i) *D. viridis*? S122 (Goa salt pan), (j) *Dunaliella* sp. S125 (Goa salt pan), (k) *Dunaliella* sp. S133 (Kutch salt pan), (l) & (m) *D. salina* S135 (Calicut marine isolate), (n) *Dunaliella* sp. S147 (Kutch salt pan), (o) & (p) *D. salina* CS265 (Australian reference strain). In brackets given the origin of isolates. (c) & (m) orange red cells of Indian isolates of *D. salina* (S089 & S135) grown at 4.5 M NaCl concentration. (f) & (h) large yellow green cells of S118 and S121 at 4.5 M NaCl. (p) Reference strain *D. salina* CS265 at 2.5 M NaCl turning orange. Scale bar given – 5 µm

In salinity tolerance study (0.5 – 4.5 M NaCl), sufficient growth (approximately 5 - 20 million cells/ml in 28 days from an initial cell density of 15-60 thousand cells/ml) was obtained for each strain in different salinities with optimum growth at 1.5 or 2.5 M salt concentrations (growth rate was 0.1±0.05 div.d^{-1} during exponential growth period), emphasizing that all the strains (including the marine isolate S135) are halophilic in nature. Beta carotene was quantified in all the isolates (Table 3.7) at 'normal' and stressed growth conditions. Under stress (3.5M NaCl, irradiance of 100-150 µmol photons m^{-2} s^{-1}) higher level of the pigment (23.4 & 22.9 pg/cell) was recorded in the two Indian strains S089 and S135 respectively while for the Australian reference strain *D. salina* CS265, it was nearly 36 pg/cell. For the three strains cells turned orange/red at high salinity ((Figure 3.4, c & m). Lower quantities of the pigment (<2 pg/cell) were observed in the strain S133 from Kutch and the two Goa strains S122 and S125. For the remaining strains it was around 2-7 pg/cell, under stress.

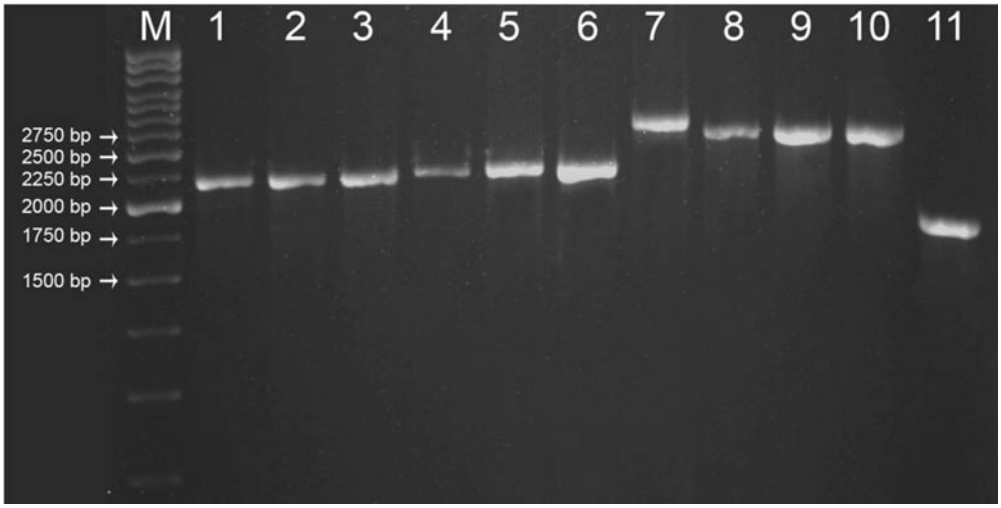


Figure 3.5 18S rDNA amplification with MA1 & MA2 primers in 1% Agarose gel. Lane 1–11 CS265, MBTD-CMFRI-S089, S135, S086, S118, S121, S125, S115, S122, S133 and S147 respectively & Lane M -250bp ladder (Genie, India)

3.8.2 Molecular Characterization Based on 18S rRNA Gene Size

Amplification of 18S rRNA gene with primers MA1 & MA2 from different *Dunaliella* isolates in the present study gave products with size ranging from ~1820 - 2640 bp (Table 3.6 & Figure 3.5). In the present study, based on the 18S rDNA gene size clear grouping of the 10 Indian *Dunaliella* strains was possible. Out of the ten strains only one strain, S147 (Kutch) produced the shortest band (~1820 bp) showing similarity to that reported for *D. tertiolecta* (~ 1770 bp) probably due to the absence of any introns (Group V). The two Indian strains S089 (CMFRI strain) and S135 (Calicut marine isolate) and the reference strain CS265 (*D. salina*) produced ~2200 bp size band (Table 3.6) closer to the reported *D. salina* (~2170bp). This further supported phenotypic identification of the above two Indian strains as *D. salina* (Group I). Studies using the 18S PCR products revealed a clear separation of morphologically similar strains (*D. viridis?*), into two groups - (Group II & Group III in Table 3.6 & Figure 3.5). The 18S rDNA size (~2300 bp) of Group II strains (S086 (Tuticorin), S118 (Nellore) & S121 (Pulicat)) was showing an indication that these strains are closer to *D. salina* than *D. viridis*. While group III strains (S122 (Goa), S115 (Chennai) & S133 (Kutch)) gave a band size of ~2550/2530 bp which could be compared to the reported *D. viridis* (~2495 bp) or *D. parva* (~2570 bp) probably with one or two introns. The band size of the Goa strain S125 (~2640 bp, Group IV) was however not in accordance with any of the reported species of *Dunaliella* (Olmos et al. 2009). Partial (~600 bp) sequencing of 5' terminus region of the PCR products could not confirm the presence of any introns, as well as the generated partial sequence information (refer Table 3.6 for GenBank accessions) were found to be highly conserved across species and therefore could not specify the species delineation. At this context further characterization was carried out based on

molecular phylogeny of a more variable ITS region and a conserved *rbc*L gene for more clarification about species link of Indian *Dunaliella.*

3.8.3 ITS Phylogeny

The phylogenetic analysis based on ITS region (~700 bp) using maximum likelihood confirmed high level of genetic diversity within Indian *Dunaliella* isolates All *Dunaliella* spp. (including the sequences from NCBI, Table 3.5) were found to be separated in 3 major clusters, with *Chlamydomonas reinhardtii* forming an out group as expected (Figure 3.6a). When out grouped with *D. tertiolecta* (Figure 3.6b), the ITS tree branching was found well supporting the morphology and 18S rRNA gene size based grouping (Group I-V) of the ten new isolates of *Dunaliella*.

The genetic divergence values observed among clade 2 isolates (Group I & II) ranged up to 9.1% (between S089 & CCAP 19/3), which was comparable to that observed between different species of the genus *Dunaliella* (Gomez and Gonzalez 2004; Polle et al. 2008). The two Indian *D. salina* strains (Group I) S089 (CMFRI strain) and S135 (Calicut marine isolate) got clustered with the Australian *D. salina* strains CS265 and CCAP 19/18 with divergence value ranging from 1.9% (between CS 265 & CCAP 19/18) to 5.6% (between S089 & S135). Whereas, the strains S086 (Tuticorin), S121 (Pulicat) and S118 (Nellore) were found closer to *D. salina/D. viridis* CCAP 19/3. The much higher divergence (>8%) of the latter three Indian strains from 'high profile' *D. salina* strains was in agreement with the grouping of the three strains in Group II based on the morphological, physiological and 18S rDNA size based analysis.

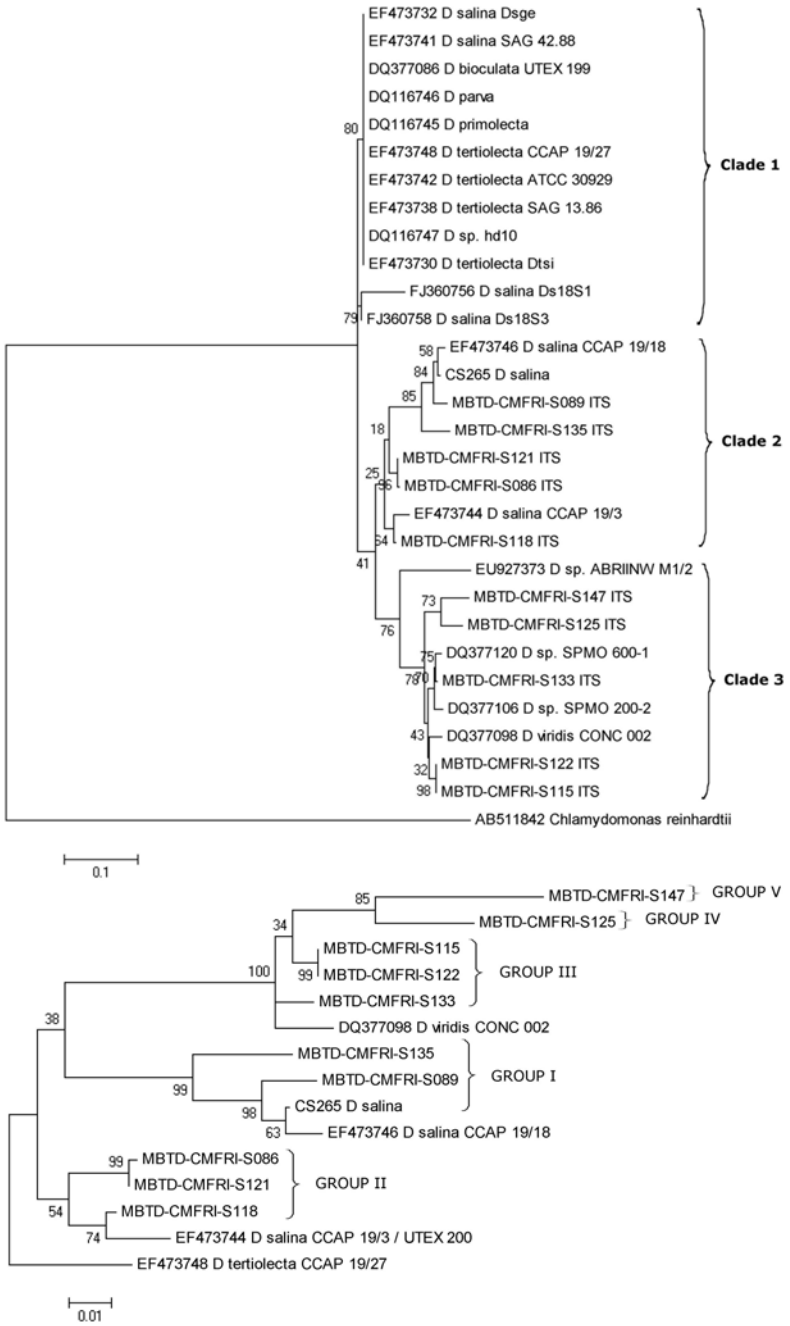


Figure 3.6a & 3.6b Phylogenetic tree of the Maximum Likelihood (ML) analysis inferred from the nuclear encoded ITS regions including 5.8S rDNA of *Dunaliella.* Bootstrap values for 1000 replicates are given at the internal nodes. In 3.6b, by out-grouping *D. tertiolecta,* clear assemblage of Indian *Dunaliella* isolates into five groups, is depicted.

The remaining five Indian *Dunaliella* strains (S115, S122, S125, S133 and S147) along with *D. viridis* CONC 002 formed a separate cluster (clade 3 of Figure 3.6). The strains showed divergence range from 0% (between S115 & S122) to 7.6 % (between S147 and *D. viridis* CONC 002). The two *D. viridis(?)* strains S115 (Chennai) & S122 (Goa) and the *D. viridis/D. biocuata(?)* strain S133 (Kutch) were found in close proximity (mean divergence of 2.22%) with CONC 002 *D. viridis* (Group III of Figure 3.7). The other two strains S125 and S147 (Group IV & V of Figure 3.7) were found to be well separated from the above group with divergence values of 4.98% and 6.42% respectively with the reference strain *D. viridis* CONC 002.

The mean pair wise genetic distance values observed among the Indian isolates of the two major clades (5.35% for clade 2 and 5.12% for clade 3) were comparatively higher than that observed among the named species of *Dunaliella* (1.14% clade 1). Further, the genetic divergence values observed among the Indian *Dunaliella* isolates based on ITS sequence variations were considerably higher than that reported in *Chlamydomonas* spp. (a minimum of 3.5% between two species) by Coleman & Mai (1997). Thus, the pattern of genetic divergence, along with the phylogenetic divergence pattern, clearly indicates the presence of at least five or more number of species/sub-species among the 10 Indian strains (including *D. salina* and *D. viridis*).

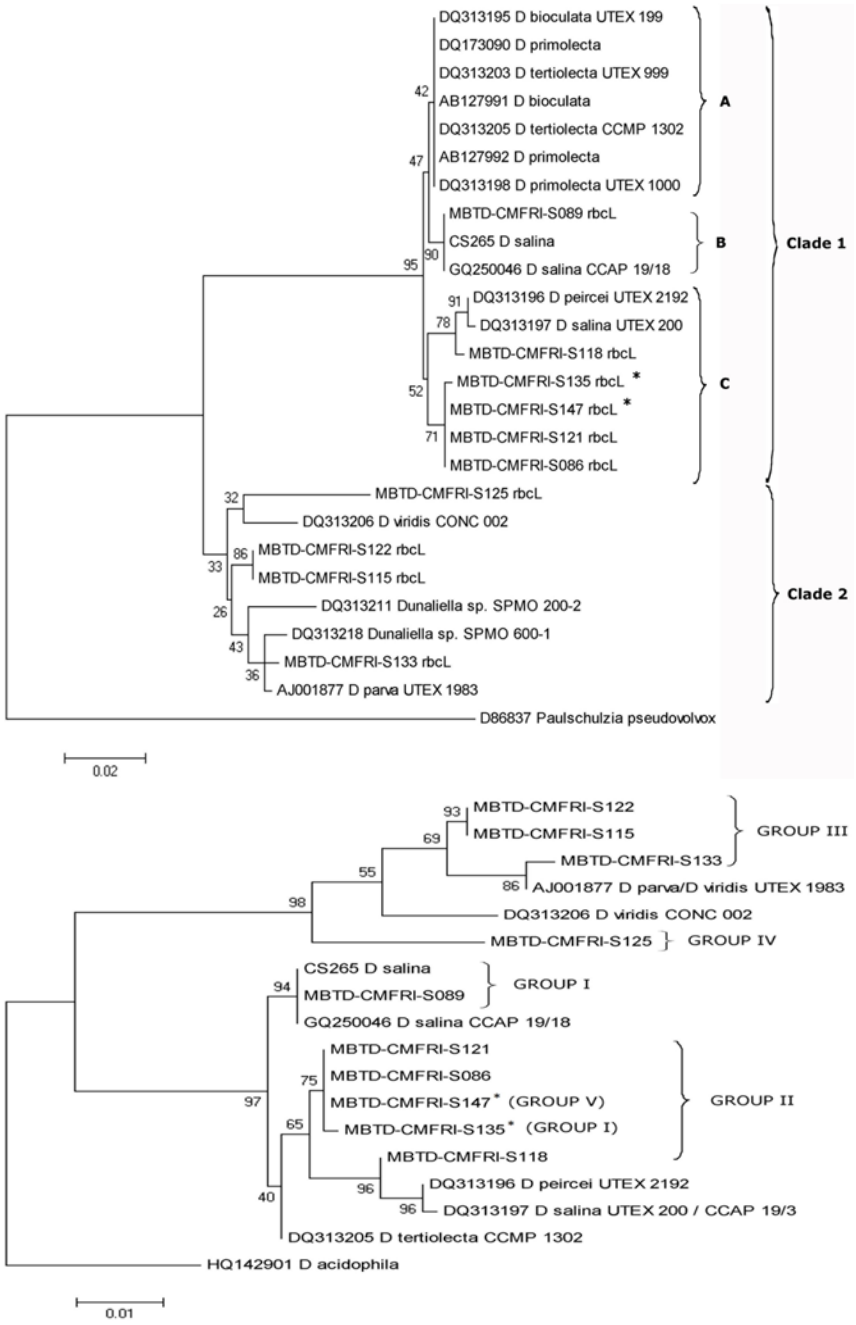


Fig. 3.7a & 3.7b: Phylogenetic tree of the Maximum Likelihood (ML) analysis inferred from the *rbc*L plastid gene partial sequences. Bootstrap values for 1000 replicates are given at the internal nodes. In 3.7b, asterisk (*) markings show the position changes of S147 (Group V) and S135 (Group I) strains.

3.8.4 *rbc*L gene phylogeny

The pattern of genetic diversity observed among the Indian *Dunaliella* strains based on *rbc*L gene sequence variations was in accordance with the above observations based on 18S rDNA and ITS analysis except for the positioning of S147 and S135. Topologies of the phylogenetic trees constructed using both maximum likelihood (Figure 3.8) analysis with *rbc*L gene sequence data were similar forming two major clusters with *Paulschulzia pseudovolvox* as out group. The mean genetic divergence value observed between the two clades was 5.89% and that observed among different isolates of *Dunaliella* ranged from 0.16 % to 7.73 %.

Being a protein coding gene, the pairwise genetic divergence (Tamura 3 parameter) values observed among *Dunaliella* isolates based on *rbc*L gene sequences were found to be less in comparison with that observed in ITS (a non coding region) sequences. The independent phylogenetic analyses using ITS (Figure 3.6) and rbcL gene (Figure 3.8) sequences (Kimura 2 and Tamura 3 parameters respectively) were found to be taxonomically incongruent especially in Clade 1. The major topological change observed was the change in the positioning of the isolate S147. Within *rbc*L phylogeny, this strain from Kutch was found closely allied with clade 1 (Figure 3.8), *w*hereas, with ITS data it was close to *D. viridis* CONC 002 and other Indian isolates (S125, S133, S115 and S122) in clade 3 (Figure 3.6). Similarly the marine *D. salina* strain S135 was appeared not close to 'high profile' *D. salina* isolates in sub-clade B, instead clustered in sub-clade C (with divergence of 1.15%).

Clustering of all remaining eight strains in both ITS and *rbc*L phylogenies was more or less similar. As expected S089 (*D. salina*, CMFRI) clustered with 'high profile' *D. salina* species CS265 and CCAP 19/18 in

clade 1, (sub-clade B, with 100% similarity). The positioning of three strains S086, S118 & S121 (sub-clade C, in Figure 3.8) along with *D. salina/D. viridis* UTEX 200/ CCAP 19/3 and *D. peircei/D. viridis* UTEX 2192 (with <1% divergence value) strongly indicates further taxonomic revision which was also emphasized in ITS phylogeny discussion. Similarly, in clade 2, the positioning of the Goa isolate S125 (with maximum divergence 7.33%) and the clustering of strains S133, S122 & S115 with *D. viridis* CONC 002 and *D. parva/D. viridis* UTEX 1983 (with divergence values 3.15% & 0.33% respectively) was in concordance with ITS phylogeny.

3.9 Discussion

Among the many listed attributes, cell size, colour, stigma and β-carotene accumulation are the major traits used to discriminate 'high profile' *Dunaliella* spp. like *D. salina* and *D. salina/bardawil*. Red *D. salina* (especially at high salinity) was reported to have significantly large cell size than other common strains like *D. parva*, *D. viridis* and *D. tertiolecta* (Borowitzka and Siva 2007). Limited carotenogenic capacity also discriminated other strains from *D. salina* where the latter can accumulate >20 pg β-carotene/cell (Borowitzka and Siva 2007). Coesel et al. (2008) and Olmos et al. (2009) obtained ~10 pg/cell of β-carotene under non-stressful growth conditions for the two hyper producing strains of *D. salina*, CCAP 19/30 and 19/18 respectively. In the present study, morphological and physiological observations of the two strains, S089 and S135, revealed that they are Indian strains of *D. salina*. Discrimination derived from basic morphology (taxonomic key), characterized the remaining strains as *D. viridis* except S125 (*D. minuta*?), S133 (*D. viridis/D. bioculata?*) and S147 (*Dunaliella sp.)* (Table 3.7). Detailed morphology and physiology based study illustrated considerable diversity in the Indian strains of *Dunaliella* but a little confusion prevailed due to overlapping features with more than one reported

species (like the cell size increase and β-carotene content of strains S121 and S118 at higher salinity, 2 stigmata of S133 at lower salinity and pyrenoid characteristics of S125) (Table 3.7 and 3.8). In the present investigation, molecular characterization was used as a supporting tool to resolve the confusion.

The banding pattern observed for ribosomal RNA gene in the present isolates was found matching with the reported gene sizes of 18S rDNA using MA1-MA2 primers (Olmos et al. 2009; Hajezi et al. 2010). The dissimilarity in product size observed among different isolates could be explained based on the presence/absence or difference in the size of introns across different species of *Dunaliella* (Olmos et al. 2009; Hajezi et al. 2010). Wilcox et al. (1992), have reported about the presence of 3 types of Group I introns in 18S rRNA gene of *Dunaliella*. With regard to this, Olmos et al., (2000, 2002) designed a set of conserved primers (MA1, MA2 & MA3) and a set of species specific primers (DSs, DPs, DBs). They used the conserved primers (MA1 & MA2) for preliminary differentiation of various known species of *Dunaliella* based on the size of the PCR product. Subsequently, morphologically very identical *Dunaliella* strains (for e.g., *D. salina* and *D. bardawil*) got discriminated by position and number of the introns Olmos et al. 2009; Hajezi et al. 2010). Based on these reports 18S rDNA of *D. tertiolecta* (~1770 bp) lacks an intron, *D. salina* (~2170 bp) has only one intron at 5' terminus, *D. viridis* (~2495 bp) has one longer intron again at 5' terminus and *D. parva* and *D. bardawil* have two introns (~2570 bp) one each at 5' and 3' terminus. Other than these important strains *D. peircei* having ~2088 bp (one intron at 5' terminus) was also reported.

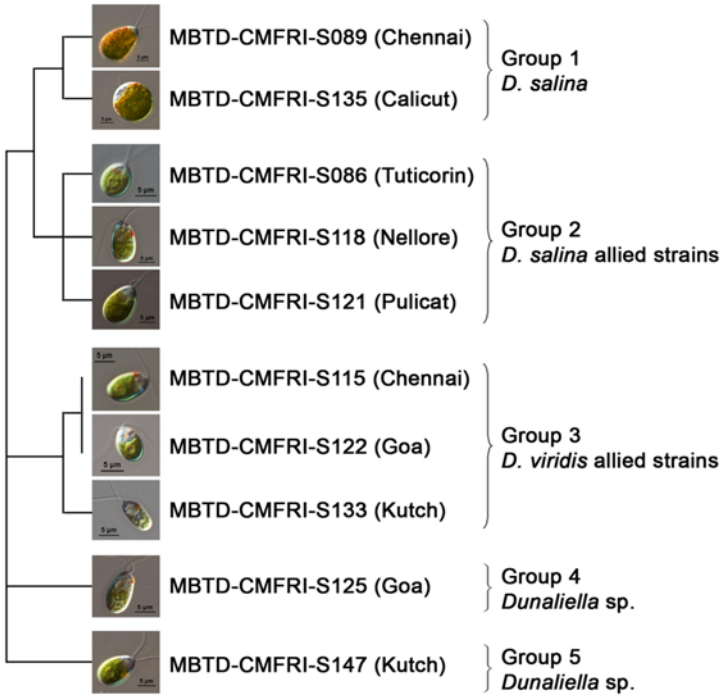


Fig. 3.8: Schematic representation of diversity of Indian *Dunaliella*. Grouping was done based on the morphology, 18S rDNA size variation (Fig. 3.5) and ITS and *rbc*L gene phylogenies (Fig. 3.6 & 3.7)

3.9.1 Grouping of Indian *Dunaliella* Strains:

After morphological, physiological and genetic analysis considerable correlation was observed in morphology (especially in cell size), 18S rDNA size and ITS phylogeny of the isolates and all the 10 Indian *Dunaliella* strains were got clearly grouped into 5 groups (Tables 3.6-3.8). A schematic representation of diversity in Indian *Dunaliella* is given in Figure 3.9.

The two larger carotenogenic strains (>20 pg/cell β-carotene content) S135 and S089 forming the GROUP I, produced 18S rDNA size ~2200 and clustered with *D. salina* CCAP 19/18 and CS265 in ITS phylogeny. These results confirmed the taxonomical identity of the two strains as *D. salina* (Section Dunaliella). But closeness of S135 to the two morphologically

dissimilar, lower β-carotene content strains, S121 (Pulicat) and S086 (Tunticorin) in *rbc*L phylogeny has to be noted, may be due to its marine origin.

GROUP II included the strains S086 (Tuticorin), S118 (Nellore) and S121 (Pulicat), which clustered with *D. salina/D.viridis* (CCAP 19/3) in ITS phylogeny and had ~2300 bp band for 18S rDNA. The present study shows the closeness of these three strains to *D. salina* by molecular analysis (18S rDNA size and ITS & *rbc*L phylogenies) rather than by morphological features. These strains were with lesser β-carotene content (~2 - 4 pg/cell) and cells were always green (only S118 turned slightly orange at higher salinity), smaller and with a clear stigma, which were not corresponding with hyper β-carotene producer strain of *D. salina* and more or less are the characters of *D. viridis* (Borowitzka and Siva 2007). However, there is a description of a greener *D. salina* (KCTC10654BP) from Korea (Polle et al. 2008) with low cellular β-carotene. But 18S rDNA size details are not available for the above Korean strain for comparison. All these factors along with the appearance of *D. viridis/D. peircei* UTEX 2192 close to S118 in *rbc*L phylogeny (clade 1, group D) and 18S intron phylogeny of *D. peircei* UTEX 2192 by Hajezi et al. (2010), emphasizes a need of revisiting the taxonomic identity of all the above reported strains along with the three Indian strains using molecular approaches.

GROUP III was formed by three strains, S115 (Chennai), S122 (Goa) and S133 (Kutch) allied to *D. viridis*. This further confirms the possibility of the former two strains to be Indian isolates of *D. viridis,* while the presence of two stigmata in the latter isolate S133 (only at lower salinity) has to be considered for re-examination and for final taxonomic identification.

The remaining two strains S125 and S147 were placed into two GROUPs (IV and V) as they were more clearly separated from other

Dunaliella strains on the basis of genetic characters than morpho-physiological traits. Based on the taxonomic key (Borowitzka and Siva 2007) the strain S125 was identified as *D. minuta* (longer pyriform cells) but with clear separate starch granules in pyrenoid differing from *D. minuta*. This strain from Goa salt pan appeared in the major clade of *D. viridis* (Figure 3.6 and Figure 3.7), but with larger divergence values in both (ITS 4.98%, *rbc*L 3.84%) the phylogenies. Further due to lack of molecular similarity with reported *D. minuta* (NCBI-BLAST analysis of ITS 2, results not given), the identity of the strain was kept in question and placed it in GROUP IV. The identity of the Kutch strain S147 was a little confusing but interesting. It resembled *D. tertiolecta* in general morphology and in 18S rDNA size (~1820 bp), while grouped with *D. viridis* in ITS phylogeny (Figure 3.6) and with *D. salina* in rbcL phylogeny (Figure 3.7). It was isolated from a salt pan, was having some ability to accumulate β-carotene (6.7 pg/cell) under stress and was having a dominant palmella stage, where *D. tertiolecta* was reported as a marine species without a palmella stage in its life cycle (Borowitzka and Siva 2007). These observations impelled in the grouping of S147 separately as GROUP V and are showing a probability for a new species in the group.

3.9.2 Diversity in Indian *Dunaliella* strains:

Buchheim et al. (2010) have reported diverse community formation of *Dunaliella* in heteroclimatic hypersaline soils than in purely aquatic habitats. They hypothesized that external factors like temperature and salinity can enhance diversification and apparently got supporting results from the phylogenetic study of about 30 different isolates of *Dunaliella* (where 3 different morphotypes were characterized), based on four genes (18S, 26S, ITS & rbcL). Subsequently, Azua-Bustos et al. (2010) reported a morphologically distinct new *Dunaliella* species, *D. atacamensis*, well adapted

for sub-aerial life and with higher genetic divergence from its sister species. Our isolates are purely from aquatic habitats, but with high level of environmental fluctuations, especially in salt pans, and showed high divergence when compared to the reported *Dunaliella* species (Clade1 of Figure 3.6 and 3.7) from NCBI. The geographic distance and isolation of the locations from where the strains were obtained could be proposed as a reason for the divergence among the above Indian *Dunaliella* isolates. However 100% sequence similarity and morphological resemblance observed between the two isolates S115 and S122 (isolated from Chennai - East coast and Goa - West coast respectively) need to be taken into account.

Grouping pattern observed in the reported *Dunaliella* strains from NCBI in the cladistic studies (Gonzalez et al. 1999; Hajezi et al. 2010; present study) suggests taxonomic revision of the strains especially when there are comments on confusion regarding the taxonomic status of many reported species. Consequently, Borowitzka and Siva (2007) have already proposed for an elaborate morphology/physiology based examination of each strain in conjunction with molecular biology. However, among the 28 morphologically differentiated species (Borowitzka and Siva 2007, Gonzalez et al. 2009), molecular aspects of only few important ones have been extensively studied and reported, and a very large percentage still remains unexplored genetically. Hence, even after a detailed study based on morphology, physiology and molecular aspects, particularly to avoid misnaming, strain codes were assigned to our isolates which are more appropriate for comparative studies as well as for future communications. Morphological and physiological study precisely groups six Indian strains into two sections – the carotenogenic Section Dunaliella (S089 and S135) and the non-carotenogenic Section Viridis (S115, S122, S133 and S125) (Borowitzka and Siva 2007, Preisig 1992). The

probability of the remaining four strains (S086, S118, S121 and S147) to come under Section Dunaliella is much higher as they are more carotenogenic (especially S147) and closer to *D. salina* in molecular analysis.

The sequence diversity within the Indian *Dunaliella* strains was distinct when compared to the listed species of *Dunaliella* (ITS region & *rbc*L gene), and shows possibility of presence of multiple species in the group. Without the knowledge of sexual compatibility between the genotypes, it is not possible to determine whether this diversity is really representing a biological species or evolutionary species or merely an intraspecific diversity (Buchheim et. al 2010). However, Coleman et al. (2000) have demonstrated a concurrence between ITS sequences and mating ability in *Dunaliella* spp. (Gomez and Gonzalez 2004). Since, high level of sequence divergence observed among Indian *Dunaliella* strains, could be correlated with sexual incompatibility, chance of more species/subspecies with respect to ITS phylogeny seems to be a realistic possibility.

3.10 Conclusion

Present study clearly shows high diversity within the Indian *Dunaliella* and highlights the reliability of 18S rDNA, ITS region and *rbc*L gene sequencing as a molecular tool in species identification and genetic diversity studies. In a recent study, based on morphological parameters Jayapriyan et al. (2010) have denoted the presence of five species of *Dunaliella* (*D. bioculata*, *D. tertiolecta*, *D. viridis*, *D. minuta* and *D. maritima*) from India (east coast). However in the same study, 18S rDNA species specific fingerprinting using primers of Olmos et al. (2000, 2002) have illustrated the same isolates as completely different species of *Dunaliella* (*D. parva*, *D. bardawil* and an unidentified *Dunaliella* sp.). Hence in the present study, for more clarity on

the species links, along with morphology and 18S rDNA size, phylogenies based on a more diverse ITS region and a more conserved *rbc*L gene were also included, which otherwise are not available for Indian *Dunaliella*. Consequently, presence of five or more species (or sub species), including two promising strains of *D. salina* (Section Dunaliella) and two *D. viridis*? (Section Viridis) strains, has got confirmed. The genetic characterization further helped in the separation of morphologically similar strains and in the clustering of Indian strains of *Dunaliella* into five groups. In this study considerable and consistent variation in ITS phylogeny among the new strains was well supporting the morphological, physiological and 18S rDNA based grouping (Figure 3.5 & 3.6, Table 3.7) and it is evidently shown that ITS region is more dependable as a molecular marker for taxonomic delineation of genus *Dunaliella*. Similarly, clustering of the reported species in a single clade clearly emphasizes most careful recording of species names (Borowitzka and Siva 2007). Hence, it is stressed to have a detailed molecular assessment coupled with additional examination of morphological (based on electron microscopy) and biological traits such as reproductive behavior (asexual-palmella, aplanospores etc.) and sexual compatibility, for further elucidation of taxonomic species lineation of unknown Indian strains as well as for resolving the issue of confusion prevailing in *Dunaliella* taxonomy.

Chapter 4

NUTRIENT PROFILING OF SELECTED MARINE MICRO-ALGAL STRAINS USED IN LARVICULTURE OF FINFISH AND SHELLFISH SPECIES USED IN AQUA-FARMING

Contents

Abstract

Microalgae are the primary producers in the aquatic habitats. They are the major and critical food source for larval stages of organisms used in aquaculture systems, as growth and proper larval development of cultured organism is typically dependent on their initial diet – the microalgae. The nutritional value of microalgae is therefore the important factor, in the selection and use in the larval culture phase of aquaculture. Twenty (16 new and 4 old) isolates of microalgae from the MBTD-CMFRI-Culture Collection of Marine Microalgae, were analyzed for their protein, lipid, fatty acid and pigment profiles. The protein and fatty acids are the two major key nutrients which promote growth and development and also bring stability to cultured organisms. Carotenoids are pigments which impart colour as well as provide immunity to higher organisms. The study revealed that average protein and lipid contents ranged between 11–54% and 10-56% respectively. When compared to Spirulina (A. platensis S016 with 45.26% protein) higher % of protein was observed in two new isolates of Chaetoceros sp. (S065 - 54.17% and S172 - 50.98%), one Tetraselmis sp. (S075 - 46.71%), and one Isochrysis galbana (S157 - 50.29%). Maximum lipid content (56%) was recorded in Picochlorum sp.S170. The total pigment content ranged from 0.6-1.6 mg/g of carotenoids and 1.5 – 3.6 mg/g of chlorophyll. Tetraselmis sp.S075 had higher content of pigments –chlorophyll (3.41 mg/g) and carotenoids (1.63 mg/g). In fatty acid (FA) profile, total saturated fatty acids (ΣSFAs) varied from 13.45 to 47.54%, ΣMUFA (mono unsaturated FA) from 6.3 to 50.44% and ΣPUFA (poly unsaturated FA) revealed a wide range from 17.34 to 69.51%. When compared to diatoms, present study showed higher levels of PUFA% (>46%) in all the selected green algae. Amongst them Tetraselmis strains were noted to have good ecosapentaenoic acid (EPA, 20:5n-3 up to 15.07%) and

docosahexaenoic acid (DHA, 22:6n-3 up to 10.36%). Nannochloropsis sp.-S078 was having good EPA (15.87%) and I. galbana-S157 with good DHA (7.85%). Other major PUFAs arachidonic acid (ARA), γ-linolenic acid (GLA) and linoleic acid (LA) were also noted in higher rates in diverse strains – maximum of 5.95% (Chaetoceros sp.-S172), 10.23% (Tetraselmis sp.-S075) and 15.67% (Tetraselmis sp.-S057). Present study reveals that nutrient profile varied significantly among the strains, indicating that, feeding of larvae with single species or strains, may not meet the individual requirement of the larval stages of the candidate animals used in aquaculture. Instead, use of multiple strains with compatible PUFA profile of the target species could be a better and preferable option for the production of healthy and quality larvae in hatchery rearing.

4.1 Introduction

Aquaculture is one of the fastest growing sectors with 6.6 x 10^7tons of global and India is the second largest country contributing to about 6% of the total produce (FAO 2014). The success of an aquaculture system depends mainly on the health and growth of the candidate animal cultured, in the larval stages and then in the farm grow-out; conversely these features are based on the balanced nutrition provided to the organism, as live/fresh and artificial feeds. Along with the growth in aquaculture industry, demand for quality live feeds and formulated feeds which could provide nutritional needs of the candidate species has also increased. Microalgae are the traditional source of nutrition in all kind of aquatic rearing systems of shell and fin fishes, particularly due to their high nutritional value and also because they are the primary link in the food chain of natural systems. They are utilized as live feeds for mollusks - in all growth stages of bivalves, oysters, scallops, clams and mussels and in the larval/early juvenile stages of Abalone; for larval/juvenile phases of crustaceans and some fishes; and for the zooplankton (e.g. rotifers, which are fed to adult fin/shellfishes) (Borowitzka 1997; Brown 2002). Other than live feeds, microalgae are also used in processed form - powder (dry), paste (wet) or in extracts for aquaculture feeding (D'Souza et al. 2002; Nunes et al. 2009). Though many studies have been conducted for alternate/artificial feeds or blends, a complete substitute has not been identified yet for live feeds, especially for larvae (Muller-feuga 2000). The superiority of live organisms relies typically on their motility in water column, where the target organism can easily detect and capture the feed (Conceicao et al. 2010).

In case of 'microalgae live feeds', continuous supply is obtained either a) by simple enrichment of raw (sea/brackish) water (which allows the blooming of all natural inhabitant phytoplankton) or b) as uni-algal cultures. The uncontaminated unialgal cultures particularly fulfill the requirement of high quality feed source where the nutritional quality of the alga is previously identified (Borowitzka 1997). To determine the nutritional values of microalgae, various extensive studies have been conducted (Brown and Jeffrey 1995; Renaud, Thinh, and Parry 1999; Martínez-Fernández, Acosta-Salmón, and Southgate 2006; Patil et al. 2007; Moura Junior et al. 2007) which provides a good record for the selection of microalgae before feeding trials.

Aquaculture rearing systems, especially the hatcheries, where the micro-size larvae requires specialized microalgae, normally have their own microalgae culturing facility, which will fulfill the requisite of continuous supply of unialgal cultures (Borowitzka 1997; Conceicao et al. 2010). Here, owing to the constant sub-culturing of the candidate microalgae, there may be (a) a change in the biochemical property of the species (due to mutation and lack of variety), (b) a decrease in the health or complete loss of the culture, or (c) a contamination of the culture with unwanted, nutritionally poor or toxic microbes. Therefore, in a hatchery system it becomes a basic constraint to have fresh or new isolates every time. Having a bank of a couple of good local isolates is always beneficial, which can thrive and preserve their nutrients in the local geo-climatic conditions. Usually it is an algal culture collection, which fulfills the requirement of larval rearing systems (Canavate and Lubinn 1995). As a prerequisite, these cultures should also be characterized for their taxonomic position, biochemical content and growth under controlled conditions. The data so generated would be of use by the farmers and entrepreneurs alike for the selection of appropriate strains for aquaculture activities.

Although many studies on comparative analysis of nutrient composition of microalgae belonging to different classes are widely available (Thinh, Renaud, and Parry 1999; Renaud, Thinh, and Parry 1999; Mansour et

al. 2005; Patil et al. 2007; Moura Junior et al. 2007; Brown and Jeffrey 1995), hardly any scientifically evaluated information is available from the whole Indian subcontinent. Here, most of the studies were focused either on the media formulation and optimization (light, salinity, pH etc.); yield, or on feeding/enrichment trials, using commonly available strains of microalgae (Ramakrishna et al. 2011; Raghavan, Haridevi, and Gopinathan 2008; Raghavan Gireesh 2009; R Gireesh et al. 2001; Gami, Naik, and Patel 2011; Vikas et al. 2012; Vijayagopal et al. 2012).

Present study is different in the sense that, it has been conducted with an aim to identify promising strains for larviculture, from a newly established culture collection of marine microalgae, comprised of strains from diverse habitats. Twenty selected strains of microalgae were examined for their protein, lipid, fatty acids and pigments for application in aquaculture nutrition of fin/shell fishes.

4.2 Materials and Methods

4.2.1 Strain Selection, Culturing and Sample Preparation:

From the MBTD-CMFRI Culture Collection of Microalgae (Chapter 2), 20 strains (16 new and 4 old) - 11 genera belonging to seven different classes, were selected for the study (Table 4.1). Selection was made based on their growth, stability and previous reports for use in aquaculture. All the strains, except *Spirulina*, were cultured in sterile f/2 sea water media (35±1ppt) in 5L (4L culture volume) conical flasks without aeration under light 6000-6500 Lux for 8:16/light:dark cycle and at 25±2°C. *Spirulina* was grown in Paoletti media (Volkmann et al. 2008) with salinity 10 ppt, while keeping all other culture conditions same. After 15-20 days growth (late exponential growth phase), the cells were harvested by centrifugation at 5000-8000 rpm for 5-10 min. The biomass was washed twice with sterile water, and then freeze-dried. The powdered microalgae were stored in air tight plastic containers at 4°C until use.

Table 4.1: Details of strains included in the nutrient profiling study

Species name	Strain Code#	Class	Source/ isolation details	Size in µm	Maximum similarity with / % of similarity*	NCBI Acc. No.
Isochrysisgalbana	S002	PRY	Old culture CMFRI live feed collection	3-6	*I. galbana* (HM246242, GQ118682)/ 100%	JF708123
Isochrysisgalbana	S157		Andaman Nicobar Islands sea	3-6	*I. galbana*(Ifremer-Argenton 98)/ 100%	KM087982
Cyclotella sp.	S018	Bacillariophycea	Cochin backwaters, Kerala	5-10	*C. cryptica* (CCMP 332)/ 99%	JF708131
Cyclotella sp.	(=S079) S080		Poompuharbeach, Tamil Nadu	4-12	*C. atomus*(ROR01-04)/ 99%	JF708166
Thalassiosira sp.	S019		Mangalavanam mangroves, Cochin, Kerala	9-14	*T. weissflogii, T. oceanica& T. fluviatilis* /100% (<200bp only)	Not submitted
Skeletonemaardens	S049		Cochin backwaters	3-5	*S. ardens* (CCMP 794)/ 100%	JF708146
Chaetoceros sp.	S065		Njarackal beach, Cochin, Kerala	3-6	*C. gracilis, C. debilis, C.calcitrans*/ 99%	JF708154
Chaetoceros sp.	S172		Andhakaranazhi beach, Alappuzha, Kerala		*C. gracilis, C. debilis, C.calcitrans, C. muellerii*/ 99%	KM087981
Navicula sp.	S060		Veli lake (saline), Thiruvananthapuram	8-21 (length)	*N. gregaria* (BA102) *& N. pseudaccepta* (MBCCC 8)/ 100%	JF708152
Navicula sp.?	S136A		Calicut Beach, Kerala	18-22 (length)	Not done	-
Tetraselmis sp.	S028	Prasinophyceae	Fort Kochi beach, Cochin	4-11	*T. apiculata* (CCAP 66/15) & *T. striata* (SAG 41.85)/ 99%	JF708135
Tetraselmis sp.	S057		Vypeen bar mouth, Cochin, Kerala	5-12	*Tetraselmis* sp. (NTI 8)/ 100%	JF708151
Tetraselmis sp.	S075		Old culture CMFRI live feed collection	6-13	*T. astigmatica*(CCMP 880)/ 98%	JF708160
Tetraselmis sp.	S081		Ashtamudi lake, Kerala	5-9	*T. apiculata*(CCAP 66/15)&*T. striata*(SAG 41.85)/ 99%	JF708167
Tetraselmis sp.	S082		Poompuhar beach, Tamil Nadu	6-12	Do	JF708168
Tetraselmis sp.	S101		Kelambakom saline pool, Chennai, TN	5-10	Do	JF708178
Dunaliellasalina	S135	CHL	Marine, Calicut Beach, Kerala	10-17	*D. salina* (CCAP 19/18)/ 100%	JF708161
Picochlorum sp./ *Nannochlorum* sp.	S170	TRE	Visakhapattanam beach, Andhra Pradesh	2-3	*Picochlorum* sp. (UTEX 2378)&*Nannochlorum* sp. (MBIC10208)/ 99%	KM087973
Nannochlorospsis sp.	S078	EUS	Do	2-5	*N. oceanica*(CCAP 849)/ 100%	JF708165
Arthrospiraplatensis (Spirulina)	S016	CYA	Pure culture, Antenna Trust, Madurai, Tamil Nadu	100-200 (trichome)	*A. Platensis***(SAG 85.79)/ 100%	KM087984

Strain code is prefixed with MBTD-CMFRI (e.g. MBTD-CMFRI-S002). *Molecular similarity based on SSU rRNA gene (18S/16SrDNA); sequences submitted in NCBI & Accession No.s in last column; Strain code or reference strains in brackets.**16S rRNA gene based. PRY: Prymnesiophyceae, EUS: Eustigmatophyceae, CYA: Cyanophyceae., CHL: Chlorophyceae, TRE: Trebouxiophyceae.

4.2.2 Chemical analysis

4.2.2.1 Protein:

Chemicals and Reagents (Sigma-Aldrich, USA)

1. Lysis Buffer

   Triton X-100 – 5ml/L,

   EDTA Na salt – 0.3722 g/L,

   Phenyl Methyl Sulphonyl Fluoride – 0.0348 g/L

2. Sodium Dodesyl sulphate – 0.005% in DDW

3. Complex reagent

   Prepared with reagents A, B and C in 100:1:1 ratio

   Reagent A – 0.4 g/L NaOH and 20 g/L NA_2CO_3

   Reagent B – 0.5% $CuSO_4$ and

   Reagent C - 1% Sodium Potassium Tartrate

4. Folin C reagent 1: 1 prepared with DDW

About 20 mg of dry algal sample was used for protein estimation in duplicate. Total protein was extracted and estimated by Lowry's method (Lowry et al. 1951) following the modified protocol of López et al. (2010). The microalgae samples were ground with lysis buffer and allowed to stand for 20 min. One ml of this suspension was mixed (in vortex) with 1 ml SDS. To the mix 2 ml complex reagent was added, vortexed and incubated at room temperature. After 10 min of incubation, 0.2 ml of F.C reagent (1:1 V/V) was added, mixed well, and the suspension was kept in dark for 30 min. After centrifugation for 2 min, at 5000 rpm absorbance of the supernatant was

recorded at 750 nm in a UV-spectrophotometer (Hitachi). The blank was prepared in the same manner as the sample using double distilled water. Total % of protein was calculated using the following equation

Protein % (W/W) = (CVD/m) * 100

where, C - Protein conc. (from calibration curve prepared using standard BSA) mg/ml

V - Volume of lysis buffer (used to re-suspend biomass) in ml

D - Dilution factor (if only further diluted the sample)

M - Biomass in mg

4.2.2.2 Lipid Extraction & Fatty Acid Analysis

Lipid extraction and fatty acid analysis were done based on the protocol of Bligh and Dyer (1959). From the lyophilized sample, 50-100 mg was weighed and lipids were extracted in 2:4:1 (v/v/v) chloroform/methanol/water mix. The chloroform with triglycerides was separated, concentrated in a rotor evaporator (45 °C) and weighed. The % of lipid per dry weight of sample was calculated.

Fatty acid methyl esters were prepared from the above lipid samples as described by Vikas et al. (2012). The lipid sample was saponified with 10 ml 0.5N KOH in CH_3OH in presence of nitrogen gas. To trans-esterify the saponifiable materials and to yield fatty acid methyl esters (FAME) the above material was allowed to react with 14% BF_3/CH_3OH. Then FAME was extracted with n-hexane/H_2O (1:2, v/v). The n-hexane layer (after removing the aqueous layer) passed through Na_2SO_4, concentrated in vacuum, reconstituted in petroleum ether (40-60 °C) and then stored at -20 °C for GC analysis. The FAME samples were analyzed by gas liquid chromatography (GLC) (Vijayagopal et al. 2010) with FID detector using fatty acid methyl ester standard (Supelco FAME 37 standard).

4.2.2.3 Pigment extraction and quantification

Total pigments extraction and quantification was done in 100% methanol as per the modified protocol of Lichtenthaler and Buschmann (2001) and Wellburn (1994). 10 mg of dry mass was ground with ice cold methanol and clear extract was separated by centrifugation. The extraction was repeated several times until the pellet became colourless. All extracted samples were pooled and absorbance was noted (spectophotometer) at 470 and 666 nm. Total chlorophyll and carotenoids were calculated using following equations

$$\text{Chlorophyll} = \frac{15.56 \times A666 \times Volumeofextract}{Volumeofculture} \mu g / mg$$

$$\text{Total carotenoids} = \frac{(1000 \times A470) - (25.36 \times A666)}{221} \times \frac{Volume\,of\,extract}{colume\,of\,culture} \mu g / mg$$

For algae which contain Chlorophyll b,

$$\text{Total carotenoids} = \frac{(1000 \times A470) - (44.76 \times A666)}{221} \times \frac{Volume\,of\,extract}{colume\,of\,culture} \mu g / mg$$

4.3 Results

A great diversity and variation in biochemical composition was observed among different strains of microalgae. However, when considered as a group certain similarity was also observed. For example all diatoms were having considerable EPA (3-13%), where as both of the Chlorophycean green algae, *Dunaliella salina* and *Picochlorum* sp. were poor in both EPA and DHA (<2%) but high ALA (>26%). In total fatty acids, all green algae had higher total PUFA (>40%), where as silico-flagellates and diatoms had higher saturated or monounsaturated fatty acids. Protein and lipid contents (Figure

4.1) were better in most of the new isolates when compared to old traditional strains of *Isochrysis galbana*-S002 and *Nannochlorospsis* sp.-S078. There were considerable amounts of Chlorophyll and carotenoids present in each strain (Figure 4.2) used in the present study.

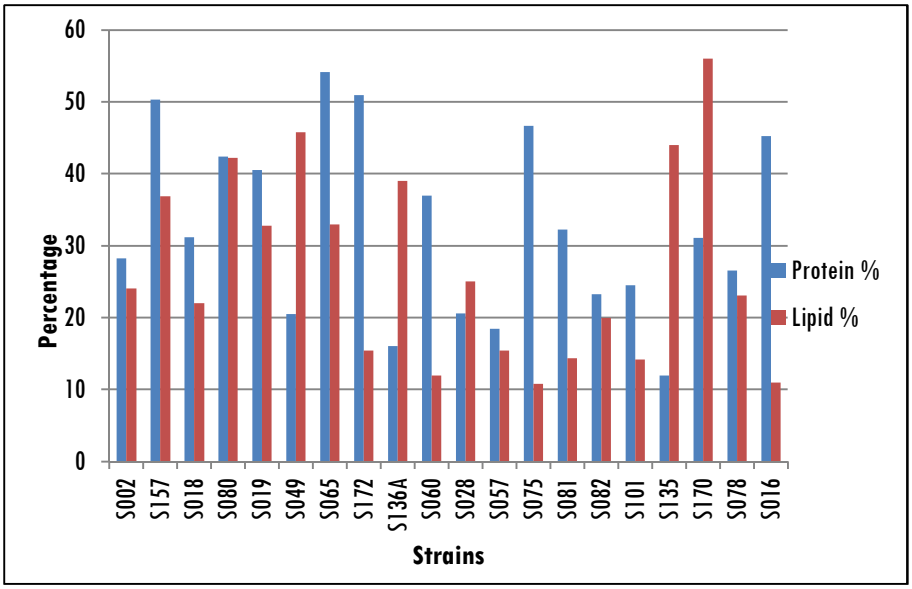


Figure 4.1. Protein and lipid percentage

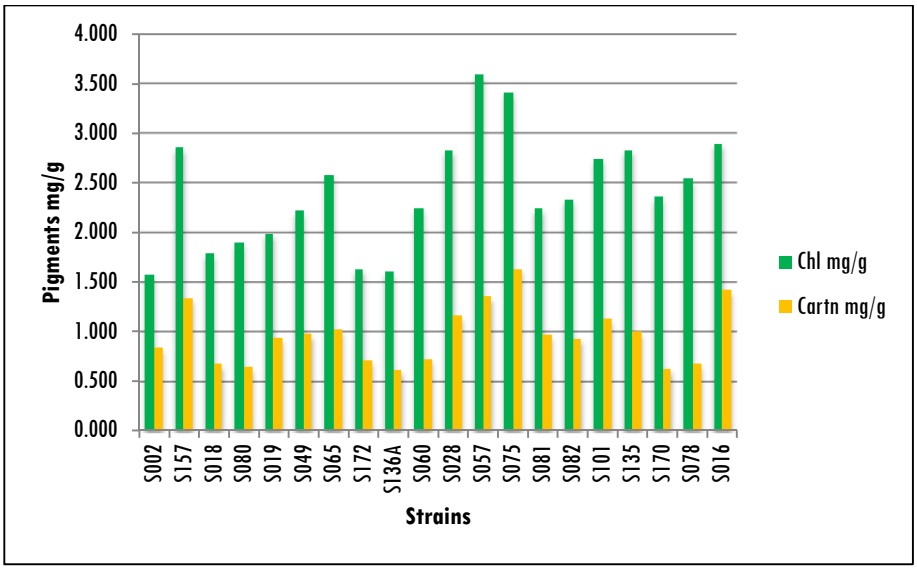


Figure 4.2. Pigments

Higher % of protein was observed in *Chaetoceros* spp. (S065 - 54.17% & S172 - 50.98%,), in a new isolate of *Isochrysis galbana* (S157 - 50.29%) and in *Tetraselmis* sp.S075 (46.71%), respectively than the *Spirulina* (*A. platensis*– 45.26%). While, two diatoms *Thalassiosira* sp.S019 and *Cyclotella* sp.S080 were found to have good protein content (40-42 %), and least protein was recorded in *Dunaliella salina* (11%). Lipid was highest in the green alga *Picochhorum* sp.S170 (56%). Percentage of lipid in remaining strains was about 30-50% except in *Tetraselmis* spp. and the old strain of *I. galbana* S002 (lipid 10-25%).

Fatty acid profile (Table 4.2) was quite excellent and interesting in many microalgae with good % of eicosapentaenoic acid (EPA – 20:5n-3), docosahexaenoic acid (DHA - 22:6n-3) and total poly unsaturated fatty acids. Large % of total PUFAs were present in *A. platenis* (69%) and all green algae (41-58%) whereas diatoms were good in total MUFAs (relative % in Figure 4.3). Both of the *I. galbana* strains and *Nannochloropsis* sp. have shown balanced saturated (SFA - 38, 33, 38 %), monounsaturated (MUFA - 21, 28, 31%) and PUFA (34, 30, 22%) rates respectively. *Cyclotella* spp., *Thalassiosira* sp. and *Skeletonema* sp. were having more SFAs (38-43%) than MUFAs (20-32%) and PUFAs (18-31%). *Navicula* spp. contained higher MUFAs (~42%) than others. In *Chaetoceros* sp.-S065 the SFA: MUFA: PUFA formula was 25:50:19 % while that of second *Chaetoceros* sp.-S172 it was 47:24:27. Relative % of high value PUFAs are depicted in Figure 4.3.

Table 4.2: Fatty acid profile of selected microalgae strains

Fatty acids (%)	I. galbana S002	I. galbana S157	Cyclotella sp. S018	Cycotella sp. S080	Thalassiosira sp. S019	S. ardens-S049	Chaetoceros sp. S065	Chaetoceros sp. S172	Navicula sp. S060	Navicula sp. S136A	Tetraselmis sp. S028	Tetraselmis sp. S057	Tetraselmis sp. S075	Tetraselmis sp. S081	Tetraselmis sp. S082	Tetraselmis sp. S101	D. salina S135	Green alga-S170	Nannochlo-ropsis S078	A. platensis-S016
12:00	0.37	1.86	1.15	0.26	2.56	1.5	0.14	9.29	1	0.19	1.31	-	-	0.14	6.99	-	1.6	2.14	0.84	0.18
13:00	-	-	0.43	-	-	-	-	-	-	-	0.26	-	-	-	-	-	-	-	-	-
14:00	15.68	13	12.5	6.64	11.44	6.09	5.04	4.51	2.37	2.57	1.13	1.08	2.67	0.65	6.19	1.49	1.05	3.68	8.53	0.49
15:00	0.52	4.5	1.83	1.86	1.55	0.6	0.36	10.27	0.38	0.47	0.44	1.42	1.74	0.14	4.93	3.44	2.39	3.65	0.69	0.15
16:00	15.42	12.82	13.69	29.67	15	18.09	15.23	4.91	25.34	31.6	6.09	15.15	6.98	10.29	9.29	14.6	17.02	8.61	23.35	7.13
17:00	-	0.51	1.24	1.03	0.85	21.18	-	0.95	1.95	0.25	0.7	0.6	1.28	0.22	1.72	0.86	1.68	1.14	0.63	0.28
18:00	0.12	2.97	5.28	3.98	7.09	4.66	4.66	3.1	1.42	0.81	2.26	1.46	2.67	4.71	5.16	3.04	3.11	3.99	3.29	1.6
20:00	0.71	1.71	1.89	0.1	1.95	0.97	0.22	3.77	0.54	0.47	8.18	2.15	4.53	1.23	2.06	4.59	11.81	3.15	0.6	2.63
22:00	-	0.51	0.22	0.17	0.57	0.2	-	10.73	0.08	1.23	13.93	3.31	7.91	14.2	0.23	2.64	1.51	1.37	0.14	-
24:00	0.23	0.75	0.43	-	0.86	0.33	0.14	-	0.34	0.1	0.17	0.56	0.58	0.58	1.6	0.34	1.09	0.57	-	0.83
∑ **SFA**	33.11	38.63	39.02	**43.81**	**41.88**	**53.62**	25.87	**47.54**	33.42	37.7	34.47	26.08	28.36	32.23	38.17	30.46	**41.26**	28.3	38.06	13.45
14:1n7	0.41	0.45	1.29	-	1.55	0.43	0.25	0.8	0.15	-	0.26	0.22	0.58	0.22	1.15	0.52	2.23	0.74	2.13	-
16:1n7	6.54	4.32	7.49	25.93	10.19	3.4	43.02	8.59	34.99	39.81	1.13	2.24	0.81	1.01	2.29	1.26	1.47	2.04	16.76	0.96
17:01	-	0.45	9.43	1.16	0.32	4.06	-	-	0.65	0.1	0.17	0.56	1.51	-	3.78	0.57	0.17	0.27	-	0.05
18:1n9	17.2	15.55	3.93	4.4	13.41	11.89	7.13	8.65	5.21	2.87	5.48	3.36	3.84	1.81	4.59	6.37	2.02	14.64	6.66	8.29
20:1n11	0.54	-	0.43	-	-	-	-	2.88	-	-	13.41	1.21	0.23	13.55	-	0.69	-	-	3.53	-
24:1n	1.26	0.45	2.89	0.64	0.48	0.7	-	1.72	0.77	0.1	-	-	-	-	0.11	-	0.42	0.4	1.25	7.31
∑ **MUFA**	28.51	21.22	26.23	32.2	25.95	20.48	50.44	24.04	**41.76**	**42.97**	20.45	8.72	9.41	16.66	11.92	13.6	6.3	18.09	31.01	16.6
18:2n6 **LA**	**3.38**	**3.18**	2.53	2.81	**3.66**	1.9	**3.42**	**7.3**	1.99	1.84	2.96	**15.67**	**8.72**	**4.78**	2.75	**11.13**	**7.14**	**10.18**	2.5	**12.86**
18:3n6 **GLA**	**5.94**	**4.53**	2.8	0.12	1.53	0.43	0.33	1.13	0.5	0.35	0.35	2.58	**10.23**	**7.32**	**5.27**	0.8	2.18	0.47	1.75	**9.63**
18:3n3 **ALA**	**14.79**	**7.07**	1.35	-	1.81	2.13	0.13	0.77	0.92	0.54	**11.14**	**15.24**	**6.51**	**10.8**	**5.27**	**12.91**	**29.2**	**26.93**	0.29	**11.88**
20:2n6	0.55	**6.11**	1.67	0.67	0.75	0.93	0.16	2.24	0.38	2.2	0.96	**11.92**	0.81	0.58	2.98	3.16	1.13	5.53	0.53	0.41
20:3n6	0.18	0.51	2.86	0.47	0.53	0.23	0.16	1.69	0.84	**3.68**	0.78	0.3	0.47	-	**8.25**	**4.76**	1.81	0.87	0.65	**10.87**
20:4n6 **ARA**	0.18	0.96	0.38	0.64	0.35	0.63	0.08	**5.95**	1	0.14	0.52	0.13	1.86	0.29	1.6	0.92	1.81	0.74	0.38	**11**
20:5n3 **EPA**	0.66	**3.53**	**12.72**	**12.37**	**11.42**	**10.86**	**7.98**	**3.53**	**13.97**	**6.23**	**14.89**	**8.39**	**12.44**	**15.07**	**7.68**	**8.67**	1.85	0.77	**15.87**	**8.62**
22:5n3	-	0.69	1.99	0.86	1.79	0.37	-	-	0.65	0.44	1.04	0.39	1.05	0.43	5.5	0.57	0.63	0.37	-	0.41
22:6n3 **DHA**	**4.78**	**7.85**	2.75	1.16	3.36	1.3	**7.22**	**4.14**	2.03	1.91	**8.45**	1.55	**6.16**	**10.36**	**6.99**	**3.1**	1.18	0.44	0.91	**3.74**
∑ **PUFA**	30.47	34.43	31.96	19.16	25.21	18.78	19.51	26.74	22.28	17.34	**41.09**	**58.75**	**56.62**	**49.7**	**46.31**	**50.38**	**46.93**	**46.28**	22.88	**69.51**

The '-' sign indicates <0.1% or negligible %.

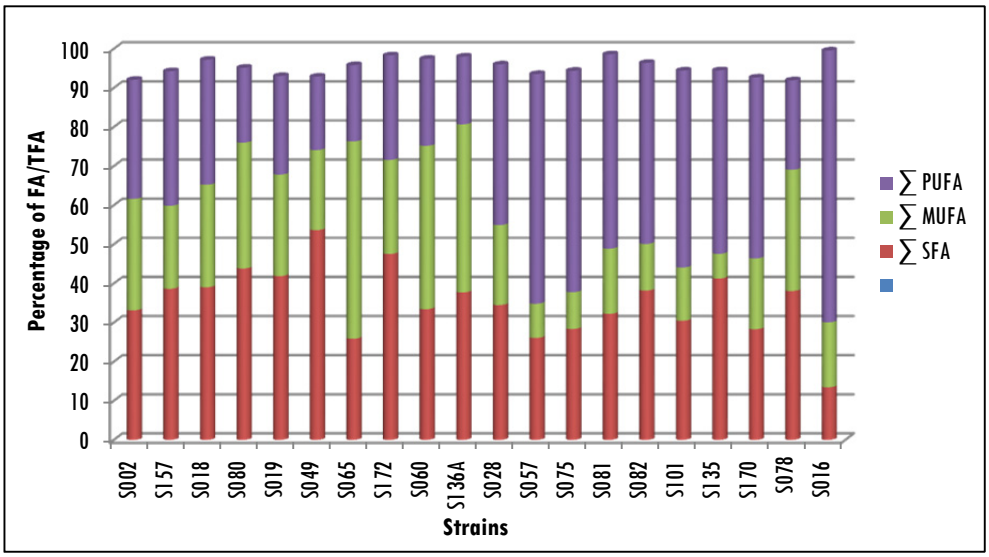


Figure 4.3: Fatty acid relative %

The ω-3 fatty acids – EPA and DHA were the maximum in *Nannochloropsis* sp.S078 (15.87%) and *Tetraselmis* sp.S081 (10.36%) respectively. Other *Tetraselmis* strains were also equally high in EPA (7.69-14.89%) and DHA (1.55-8.45%) ratios. Similarly two strains of *Tetraselmis* spp. – S075 and S057 contained highest γ-Linolenic acid (GLA – 18:3n-6, 10%) and Linoleic acid (LA - 18:2n-6, 15%) respectively. *A platensis* and all bacillariophycean algae were having significant EPA content (3.53-13.97%). Only one isolate of *I. galnana* (S157) had both EPA (3.53) and DHA (7.85), while the second strain of *I. galbana* (S002) had 4.78% of DHA but only 0.66% of EPA. Arachidonic acid (ARA – 20:4n-6) was present only in *A. platensis* (11%) and *Chaetoceros* sp.S172 (5.96%). All other strains were having <2% or negligible ARA. The two green algae *D. salina* S135 and *Picochlorum* sp.S170, had least EPA and DHA (≤1%), which was compensated by higher α-Linolenic acid (ALA - 18:3n-3) level - 29.2 & 26.93% (respectively). ALA content in diatoms was poor (0-2%) while remaining all the species had 5-15%.

Pigments were quantified in mg/g dry weight (Figure 4.2). There was uniformity in chlorophyll and carotenoid contents within a group. For example, diatoms had 1.6–2.5 mg/g & 0.6-1 mg/g respectively. The 'green' algae (including *Nannochloropsis* and *Arthrospira*) were better than diatoms for pigment values, which had chlorophyll 2.2–3.8 mg/g and carotenoids 0.6-1.6 mg/g. In both *Isochrysis* spp. chlorophyll content was almost double than carotenoids.

4.4 Discussion

Present study investigated nutrient profile of 20 selected strains of microalgae, comprising 16 pure isolates which were originally isolated from tropical Indian saline waters, identified by morphological and molecular methods (small subunit – 18S/16S rRNA gene sequence) and maintained in MBTD-CMFRI-culture collection (Table 4.1) and four old strains taken from the CMFRI Mariculture division. This report is novel in the sense that all major saline microalgal strains from the Indian coast are used for the nutrient profiling. The nutrient profile of many of the strains of microalgae in the present study was comparable with previous reports (Mansour et al. 2005; Martínez-Fernández, Acosta-Salmón, and Southgate 2006; Lang et al. 2011; Patil et al. 2007; Renaud, Thinh, and Parry 1999; Thinh, Renaud, and Parry 1999). As per the earlier reports (Conceicao et al. 2010; Brown 2002; Coutteau 1996) size, digestibility, biochemical composition, stability of the culture and non-toxic nature of the algae are the factors considered for short listing microalgae in larval feeding. When size (c.a. 2-100 µm) and the nutrient profile (high protein and good PUFAs), were taken in to consideration, all the strains showed their potential to be used as a larval feed. The nutrient diversity observed in the isolates can be further explored for their use in larviculture, individually or in a combination, to meet the necessary

dietary requirements of the aquaculture species of choice. The study revealed the potential of two benthic *Navicula* spp. (S060 and S136) as live feed for abalone larvae (de Viçose et al. 2012) in both size and nutrient composition, while all remaining strains were found suitable for feeding the larvae of finfish and shellfish used in aquafarming. One of the new isolate, picoplanktonic green alga, S170 (*Picochlorum* sp.) with highest lipid content (56%) higher PUFA (about 46%) and 31% protein, proved as a new candidate species ideal for larviculture nutrition.

Among the nutritional characteristics, protein and fatty acids are the two major key components which affect the growth and development of the species used in aquaculture. Studies (Flaak and Epifanio 1978; Utting 1986) have shown that in bivalves higher levels of dietary protein catered good larval growth. Similarly in larval stages of all species used in brackish and marine farming, availability of required levels of PUFAs (EPA, DHA and AA) were found essential for growth as well as development (Volkman et al. 1989; Reitan et al. 1997; Mansour et al. 2005). Poor survival rate and higher rate of mortalities and quality problems have been reported in case or larval and juvenile forms when fed with a PUFA deficient diet (Conceicao et al. 2010). Some Caroteniods (e.g. β-carotene) act as pro-vitamin or antioxidants and some (e.g. Astaxanthin) impart colour to the flesh (salmonids) and exoskeletal pigmentation (crustaceans) (Muller-feuga 2000; Vílchez et al. 2011). Presence of high protein (up to 54%), lipid (upto56%) and PUFAs (maximum EPA-15.87%, DHA – 10.36%, ARA -11%, GLA – 10.23%, ALA - 29.2%, LA – 15.57%) among the strains studied, promises a novel feeding regime using plurispecific microalgae diet for larvae, enabling the larvae with superior quality of vital nutrients (Ponis et al. 2006). Spolaore et al. (2006) and Aranda-burgos et al. (2014) have pointed towards the advantages of multispecies

microalgae feeding, for obtaining improved growth and survival rates in larval and juvenile forms of aquacultured species of finfish and shellfish.

Interestingly, differences and resemblances in profiles were detected within the same genus/species like in the case of the two *I. galbana* strains. *I. galbana* S157 was superior to the old strain *I. galbana* S002 in protein, lipid, pigments and PUFAs (EPA & DHA) content. A similar difference was observed among the two *Chaetoceros* spp. (S065 and S172), and the six *Tetrasemis* spp. (S028, S057, S075, S081, S082 and S101). However, the two *Cyclotella* spp. (*C. cryptica*-S018 and *C. atomus*-S080) were similar except in case of lipid content. The difference observed in *I. galbana* strains could be a consequence of repeated sub-culturing of the old strain (S002), resulting in the loss of some original properties (Canavate and Lubinn 1995). In case of *Tetraselmis* spp. the result was quite different - the old strain (S075) was equal or better in quality than the new strains. However the strains had considerable morphological differences and by 18S rRNA gene sequences strain S075 was corresponded as *T. astigmatica* while others as *T. apiculata*. (Chapter 3, Table. 3.4.1)

The high protein level observed in *Chaetoceros* spp. (54.17 & 50.98) was in agreement with Raghavan et.al (2008) who got up to 60% protein content at 20 ppt salinity for *Chaetoceros calcitrans*. In case of *I. galbana* S157 and *Navicula* sp.S060 the protein content (50%& 37% respectively) was higher than previously reported for *Isochrysis* sp. (Thinh, Renaud, and Parry 1999; Renaud, Thinh, and Parry 1999) and *Navicula* sp. (de Viçose et al. 2012; Scholz and Liebezeit 2012). For all other strains the protein content was almost comparable with earlier studies (Thinh, Renaud, and Parry 1999; Renaud, Thinh, and Parry 1999), except *D. salina* (only 11% protein) which was expected to have protein content ca.57% (Spolaore et al. 2006; Sánchez, Mart, and Espinola 2000; Becker 1986). Similarly the lipid content of all the

algal strains was in the ranges reported earlier (Thinh, Renaud, and Parry 1999; Renaud, Thinh, and Parry 1999; Liang, Mai, and Sun 2005; Popovich et al. 2011; Mansour et al. 2005; Huang, Huang, and Wen 2012), other than new isolate of *I. galbana* (S157), *Cyclotella* sp. (S080) *S. aredens* (S049) which were having 36, 42 & 45 % respectively.

Fatty acid profiles within groups were analogous with the results reviewed by Stansell et.al. (2011). Total PUFAs were higher (>40%) in Chlorophytes (*Tetraselmis* spp., *D. salina* and *Picochlorum* sp.) whereas lower (<31%) in diatoms (Bacillariphyceae). In *I. galbana* (Prymnesiophyceae) and the *Nannochloropsis* sp. (Eustigamatophyceae) total saturated and unsaturated FAs were more or less 20-40 % range. Except the pinnate diatoms (S060 and S136) and one *Chaetoceros* sp.S065, all remaining diatoms were having higher % of SFAs (39-53%) than MUFAs (20-32%). *Navicula* sp.S060 recorded FA composition very similar to the marine *Navicula incerta* studied by De Vicos et.al. (2012). The relatively high composition of MUFAs (>40%) than PUFAs with good lipid % in *Chaetoceros* sp.S065 and the pinnate diatom S136A shows their prospective use in biodiesel production (Stansell, Gray, and Sym 2011).

Nutritionally significant PUFAs (essential fatty acids) were relatively higher in the studied strains as compared to previous studies (Thinh, Renaud, and Parry 1999; Renaud, Thinh, and Parry 1999; Patil et al. 2007; Pratoomyot, Srivilas, and Noiraksar 2005; Mansour et al. 2005). Interestingly, large % of DHA in *Tetraselmis* spp. (up to 10%) and *Chaetoceros* spp. (4 and 7%) was not reported before. The EPA content however was slightly lesser in diatoms and *Nannochloropsis* sp. than previous studies. As expected ARA in all eukaryotic strains were less significant, except in *Chaetoceros* sp.-S172 (Liang, Mai, and Sun 2005). *A. platensis*, *I. galbana* and all green algae were superior in total 18C PUFAs (LA, GLA and ALA) than diatoms similar to

earlier studies. High % of ALA (18-35%) in *D. salina* validate results of Mendoza Guzmán et al. (2012). In case of *Picochlorum* there were seldom any studies on FA profile for comparison.

In case of pigments, *D. salina* is one of the best considered organisms for higher carotenoid production, under high salinity, temperature and light conditions (Kleinegris et al. 2010; Rad, Aksoz, and Hejazi 2011). Other microalgae rich in carotenoids include *Nannochloropsis*, *Isochrysis, Thalassiosira, Tetraselmis* and *A. platensis* (Vílchez et al. 2011). The native marine *D. salina*-S135 was analyzed for its carotenoid production in our previous study (Preetha et al. 2012) and was found carotenogenic in hyper saline condition.

Overall evaluation of the new strains emphasizes nutrient richness in many of the native isolates revealing their potential as live feed in aquaculture larviculture. Use of endemic species in aqua-farms and hatcheries is greatly demanded particularly because of their adaptability to local conditions and therefore the stability in mass culture (Mansour et al. 2005). The varied biochemical profile observed in the studied strains again emphasizes on the need of multispecies diet for larvae to ensure the balance in essential nutrients. A preference for certain species of microalgae (both quantity and quality) by certain candidate animals can be observed. For example a combination of *Chaetoceros* spp., *Thalassiosira pseudonana, Tetraselmis suecica* and *Isochrysis galbana* for bivalve larvae; *Navicula* spp. and *Nitzschia* spp. for gastropod (abalone) larvae; *Skeletonema costatum*, *C. gracilis* and *T. chuii* for penaeid shrimp larvae etc. were selected and used for better performance of growth and metamorphosis (Coutteau 1996). In addition, the ratio in 20C and 22C PUFAs (DHA, EPA, ARA) were noted critical for larvae of marine fishes where as in freshwater fish larvae ratio in 18C essential fatty acids (EFAs –

GLA and LA) are important (Tocher 2010). For bivalve larvae, relatively higher EPA (than DHA) and good AA contents in microalgae were desirable (Aranda-burgos et al. 2014). All these factors points on the need of selection, and use of combinations of microalgae in larval feeding, for which the novel data generated in the present study could be used.

Furthermore, the high protein, lipid and PUFA % in the studied isolates opens an area for research in processed feed formulations using microalgae. For formulated feed production, fish meal and fish oil are the two ingredients used as sources of complete protein and quality lipids (with PUFAs). Current scenario witnessing the spiraling cost of fishmeal and fish oils necessitates alternatives for fishmeal and fish oil, mainly because of an increase in aquaculture production (c.a. 10^8 tonnes). In the coming years, a demand for PUFAs may enhance to about 10^7 tonnes not only for aquaculture feeding but also for human as well as livestock consumption. A probable solution in commercial production of PUFAs, pigments and protein could be the microalgae (Becker, 2004). This opens up new avenues for microalgae based research at applied level.

4.5 Conclusion

This study was able to generate nutrient profile data of 20 selected tropical microalgae from the culture collection for possible application in larval feeding. It identified new isolates with better protein and PUFA profile than the conventional old live feeds and reveals the need for fresh native strains. In addition, a comparative similarity in composition of the nutrients among the species of same class was also released. A major variation in fatty acid profile was observed in different species and pronounced variation was noted in abundance of different PUFAs. Single strains, for example

Tetraselmis sp. S075, *A. platenis* S016, *I. galbana* S157 and *Chaetoceros* sp. S065, were superior among the isolates for multiple factors (protein, PUFAs and carotenoids). However, the variations in composition of nutrients put emphasis on feeding of larvae with plurispecific microalgae diet with compatible PUFA profile and protein content, rather than depending on a single species. It could be a better choice to assure better balance in essential nutrients and thereby for the production of healthy and quality larvae. By taking advantage of the data divulged here, indigenous strains of microalgae can also be identified and explored for the production of high value compounds like bio-fuel, which will be a supplementary outcome of this study. Further, each new isolate will be sufficiently good for examination for food, nutritional supplements, pigments or bioactive compounds.

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Chapter 5

MICROALGAE BUTTER COOKIES – SENSORY EVALUATION & PROSPECTS AS A FUNCTIONAL FOOD

Contents

Abstract

Marine microalgae are the conventional suppliers of nutrients in aquatic systems. Owing to their dietary values, use of microalgae as a functional ingredient in traditional or modern food items has become a recent trend. Microalgae are potential sources of several bioactive molecules like poly unsaturated fatty acids which improve human health by being basic metabolic precursors as well as free radical scavengers. Chlorophylls and carotenoids present in microalgae are powerful antioxidants with healing and preventive qualities for several chronic ailments. Present study was aimed to develop microalgae based 'functional cookies' and investigated applicability of 5 marine strains of aqua-culturally important, nutritionally promising (for PUFAs and pigments) microalgae in three concentrations (0.5, 1.5 and 2.5%). Sensory properties were analyzed to see the acceptance among the consumers and results were statistically evaluated. The proximate composition and overall fatty acid profile did not vary much between cookies, but sensory qualities and pigment contents significantly varied. Presence of omega-3 PUFA -Eicosapentaenoic acid (EPA – 0.12% of total fatty acids) and about 108 µg/g carotenoids & 265 µg/g chlorophylls, shortlist Nannochloropsis oceanica 1.5% cookies as one of the best with overall scoring of 7.7±0.9 in organoleptic analysis. At highest concentration (2.5%), N. oceanica cookies had 0.19% EPA with maximum chlorophylls (461±10 µg/g) and I. galbana highest carotenoids (263±1 µg/g). Tetraselmis cookies (all three) had higher concentrations of pigments and significant sensory scores. In short, nutritive and sensory values of microalgae cookies describe their prospective use as a health food.

Further, presence of more than one bioactive molecule in a single microalga or in a group of microalgae may function synergistically to improve the health through a 'functional food'.

5.1 Introduction

Microalgae are a diverse group (about 40,000 identified species) (Guzman et al. 2009) and are unique for incredible phytochemical production. These microbial plants play a crucial role in animal and fish nourishment, particularly due to their well-balanced nutrient composition. Their nutritional values include high protein content with balanced amino acid composition, lipids including essential fatty acids and PUFAs, carbohydrates, pigments like carotenoids and phycobilins, sterols, tocopherol, polyphenols, vitamins, minerals and other known/unknown bioactive compounds (Gouveia, Batista, et al. 2008; Pulz & Gross 2004; Spolaore et al. 2006).

Recently, Gouveia et al. (2010) and Lordan et al. (2011) have reviewed the potential of microalgae as source of functional molecules with respect to nutraceutical applications. They have evaluated on all the above mentioned chemicals from microalgae and their present day use and prospects. Among them, the long chain poly unsaturated fatty acids (LC-PUFAs), EPA and DHA are particularly important for the growth and development of nervous system and for smooth functioning of cardiovascular system. Marine fish is the primary dietary source of these ω-3 LC-PUFAs, whereas, marine fishes acquire these fatty acids from microalgae by feeding them (Rasoul-amini et al. 2009). However, declining fish resources, bioaccumulation of toxic substances, fishy odour and non-vegetarian origin etc. make fish fatty acids less venerable (Tonon et al. 2002). This makes microalgae significant as a candidate source for PUFAs.

Similarly microalgae produce a number of pigments – chlorophylls and carotenoids (β carotene, astaxanthine, Lutein, phycobili-proteins etc.).

Carotenoids are exceptionally good antioxidants as well as natural colouring agents (Paiva & Russell 1999; Guedes et al. 2011). Chlorophylls are commonly used to colour food and beverages also along with their derivatives were reported as anti-carcinogenic due to their ability to bind to carcinogenic molecules (Lordan et al. 2011). *Dunaliella salina, Heamatococcus pluvialis*, *Chlorella* sp. and *Arthrospira* spp. are some of the species which are already been explored for economical production of these pigments (Spolaore et al. 2006).

Fast and busy way of present-day life has augmented several habitual health problems like cardiovascular ailments, obesity and immune disorders. Extensive scientific research in this area, confirm a relationship between the diet and the prevalence and progression of these diseases. Meanwhile, in last two decades, people have started shifting to "functional foods", which will promote well-being and reduce risk of illness rather than simply satisfying hunger (Anonymous 2009). Number of natural products, traditional as well as modern, have been identified, explored and used for physical well-being, and microalgae being one among them.

Reports say, simply consumption of microalgae (approx. 3 g/day) will make feel better, probably due to its antioxidant and immune-stimulant functions (Moore 2001). Due to these functional properties microalgae are currently cultured in large scale, marketed and consumed a) in the form of powder, tablet and capsule, or b) incorporated with normal food stuffs like biscuits, pastas, beverages etc. (Pulz & Gross 2004; Schulz-friedrich n.d.). In the last decade, experiments on microalgae based functional foods (biscuits, emulsions, cookies, candies, salads, drinks, etc.) have shown great prospects to form a new food market niche (Gouveia, Coutinho, et al. 2008; Gouveia et al. 2006; Batista et al. 2010; Sharma & Dunkwal 2012; Kingman 2011).

Cookies are one of the most common snacks consumed over the world including India. Traditional as well as modern varieties including homemade biscuits are all-time favorite which provide nourishment as well as refreshment. In the present study an attempt was made to find opportunities for aquaculturally important marine microalgae as functional ingredients in normal butter cookies. Five biochemically promising Indian strains – *Isochrysis galbana*, *Nannochloropsis oceanica*, *Tetraselmis* sp., *Dunaliella salina* and *Chaetoceros gracilis* were selected for the study.

Generally marine microalgae (not all species) were observed to have a characteristic fishy or funky odor (like that of seaweeds) and not so palatable taste. Hence it was utmost important to know the acceptance of a food product based on microalgae, even if nutritional benefits is the primary concern, which previous studies has proven (Gouveia, Batista, et al. 2008; Gouveia et al. 2006; Gouveia, Coutinho, et al. 2008; Kingman 2011). Sensory properties of the products were evaluated with an aim to know the consumer acceptance. Along with other nutritional qualities, special emphasis was given to PUFAs and carotenoids. This remains the first attempt on *Nannochloropsis*, *Chaetoceros* and *Tetraselmis* in this aspect to our knowledge.

5.2 Materials and Methods

5.2.1 Microalgae Culturing

Five selected marine microalgae – *Isochrysis galbana*-S002, *Nannochloropsi oceanica*-S078, *Tetraselmis* sp.-S082, *Dunaliella salina*-S135, *Chaetoceros gracilis*-S172 were grown in 20 L jar (15L culture volume, Figure 5.1) in f/2 medium under standard culture conditions and harvested the fully grown microalgae by centrifugation. The biomass was lyosphilized, powdered and stored in cool dry place.

Figure 5.1:

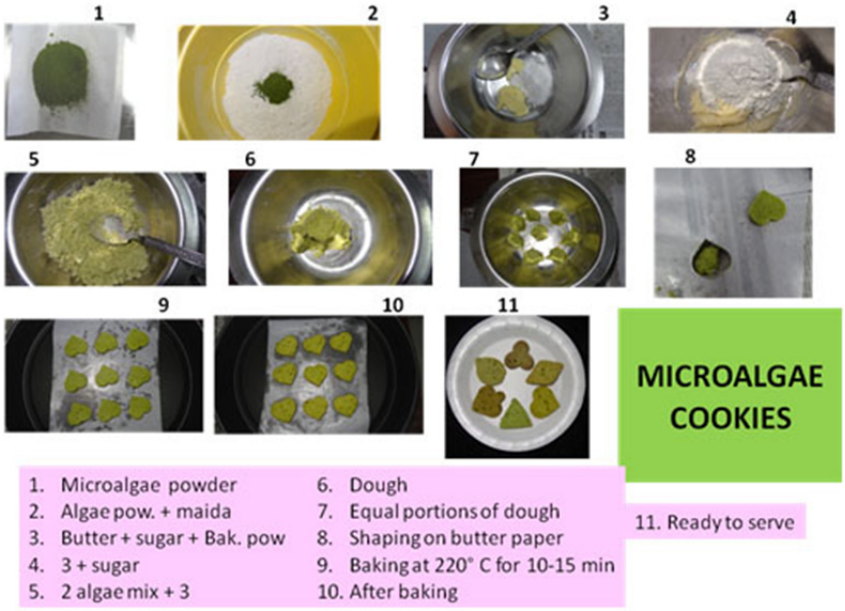


Figure 5.2: Recipe of microalgae cookies

5.2.2 Preparation of Microalgae Cookies

Normal butter cookies were prepared based on the recipe given (Figure 5.2), using 50% flour, 25% powdered sugar, 25% butter and 0.5% baking

powder and used as standard during analysis. For microalgae cookies each microalga and one mix (of the 5 strains 1:1:1:1:1, w/w) were also added in 0, 0.5, 1.5 and 2.5% (w/w) concentration. After cooling the cookies were stored in airtight plastic containers and all analyzes were done within 10 days of preparation. Each cookie was weighed approximated 5 gm. For chemical analysis, powdered cookies were used.

5.2.2.1 Recipe for Microalgae Butter Cookies

Ingredients:

1. Flour (Maida) – 100 gm (50%)
2. Powdered sugar – 50 gm (25%)
3. Butter – 50 gm (25%)
4. Baking soda – 1 gm (0.5%)
5. Microalgae powder – 1 gm, 3 gm and 5 gm each (0.5%, 1.5% and 2.5% w/w in concentration for dough). Sample cookies were coded as follows based on their quantity used respectively

Isochrysis galbana	–	ISO-1, ISO-3, ISO-5
Nannochloropsis oceanica	–	NAN-1, NAN-3, NAN-5
Tetraselmis sp.	–	TET-1, TET-3, TET-5
Dunaliella salina	–	DUN-1, DUN-3, DUN-5
Chaetoceros gracilis	–	CHE-1, CHE-3, CHE-5
Mix of five strains (1:1:1:1:1)	–	MIX-1, MIX-3, MIX-5
Normal cookies (Standard)	–	STD

Preparation

Step 1 : Ingredients 1 and 4 were mixed and sieved in to a bowl

Step 2 : Powdered microalgae were added mixed thoroughly and kept separately

Step 3 : In another bowl 2 and 3 three were mixed to form a soft cream

Step 4 : Into this first prepared flour was added, kneeded and made the dough

Step 5 : Small portions from the dough were taken and made into shapes (as shown in figure 5.3) on butter paper

Step 6 : The cookies were baked in a preheated oven at 180°C for 15 to 20 min or until the cookies were done

Step 7 : After cooling the cookies were stored in airtight containers in cool dry and dark place

NB: For normal (standard) cookies, step 2 was omitted.

5.2.3 Sensory Evaluation

A thirty member panel in 3 batches (10 each) with 18 females and 12 males between age group 25 and 48 years from the institute (CMFRI, Kochi) evaluated the samples using the 9-point hedonic scale test (Lim 2011; Lim et al. 2009; Nicolas et al. 2010). Each panelist was presented with samples (coded) in random order, individually and scores were recorded from excellent (9 points) to very poor (1 point) on a score card (Appendix 5). Scores were analyzed statistically using SPSS software.

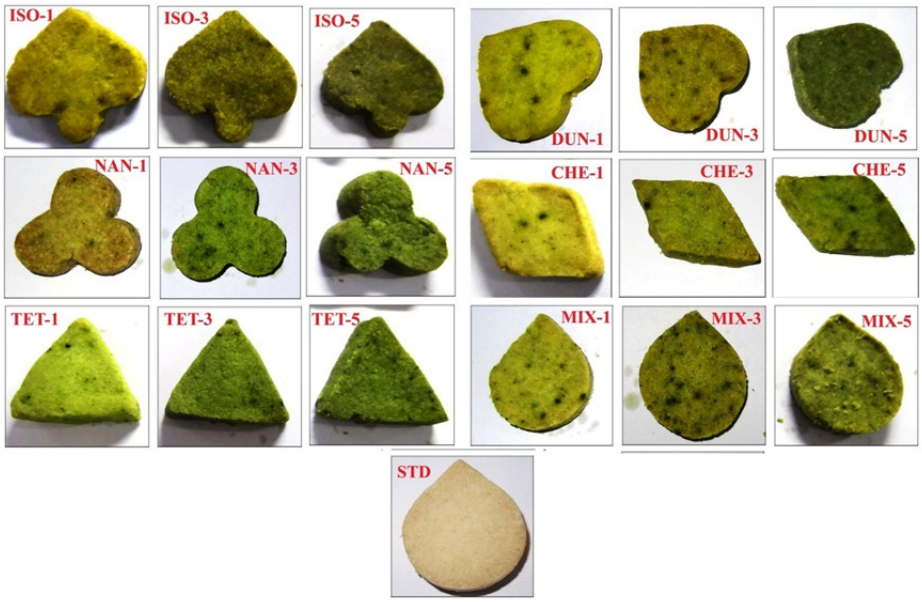


Figure 5.2 Images of different microalgae cookies at 0.5, 1.5 and 2.5% concentration and their comparison with normal cookies (STD)

5.2.4 Fatty acid analysis:

Bleigh & Dyer method – Gas chromatography

Fatty acids were analyzed following the protocol same as described in Chapter 4, section 4.2.2.2

5.2.5 Pigment extraction and quantification (Lichtenthalem 1983, 1987, 2001 & Wellburn 1994)

From powdered samples pigments were extracted in ice cold Methanol (100%) until the sample and extract became colourless. Extracts were separated by centrifugation (8000 rpm, 10 min), pooled and spectro-metrically recorded the absorbance at 470 nm and 666 nm for carotenoids and chlorophylls respectively. For calculation refer Chapter 4, section 4.2.2.3

5.2.6 Proximate Chemical Analysis

Prepared cookies were analyzed for dry matter, moisture, crude protein, crude fat, crude fibre, crude ash, acid insoluble ash and nitrogen free extract (carbohydrates) (AOAC 1990). Calorific value of the cookies was calculated by the Atwater method (protein x 4; fat x 9; carbohydrate x 4) (Osborne and Voogt 1978)

5.2.6.1 Moisture Determination

About 4-5 gm of powdered sample taken in an aluminium dish, covered and dried at 100 °C to constant weight. Moisture content was calculated in %.

$$\text{Moisture content \%} = \frac{\text{Wt. of fresh sample} - \text{Wt. of dry sample}}{\text{Wt. of fresh sample}} \times 100$$

5.2.6.2 Crude Protein

Crude protein content was measured as total nitrogen content multiplied with 6.25 by Kjeldahl method

Weighed approximately 0.25gm of dried powdered sample noting the exact weight, 'W' gms, into clean dry digestion tubes. Into each tube added approximately 1gm of digestion mixture (potassium sulphate & copper sulphate, 9:1 by weight). Then 12ml of con.H_2SO_4 was added into each tube and placed on the digester (Kjeltec) assembly and digested at 400^0C for $1^1/_2$ hrs. Sample was then cooled down to room temperature.

Each sample was then placed on the distillation unit (Kjeltec) and the program was set with water-70 ml, alkali-70 ml, receiver-30 ml, tube drain and distillation was made with steam in the unit. The instrument estimates the crude protein on entering the weight of sample W as,

$$\% \text{ crude protein} = \frac{\text{Volume of 0.1N HCL} \times 0.014 \times 6.25 \times 100}{W}$$

5.2.6.3 Crude Fat

Weighed 2-3 g of dried sample into an extraction thimble (residue from dry matter was used) and placed into the soxhlet apparatus, (Soxtec fat analyzer). Placed a dry, pre-weighed and marked aluminium cup in position beneath, and added 60 ml petroleum ether and connected to condenser. The temperature was adjusted to reach 100°C and boiling cycle was done for 15 minutes, by dipping the thimbles in solvent. The thimbles were raised and rinsed with condensed ether in the rinsing cycle for 30 minutes. This was followed by 10 minutes of recovery cycle where pure unsaturated ether was collected back and recovered. The fat containing cups with residual ether was then dried in hot air at 100°C for 1hr and then cooled in desiccator and weighed. The crude fat was calculated as

$$\text{Crude fat \% of dry mass} = \frac{\text{Weight of fat}}{\text{Weight of sample}} \times 100$$

5.2.6.4 Crude Ash

Weighed about 3 g powdered sample into a dry, pre-weighed porcelain dish and then ignited in a muffle furnace at 600°C for 3 hrs. Allowed to cool overnight deciccator and weighed.

Calculation:

$$\text{Crude ash } (\text{CA}) \text{ \%} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100$$

5.2.6.5 Acid Insoluble Ash

The residue obtained from ash determination was boiled with 25 ml 5N HCl and filtered through ash-less paper and washed with hot water until acid free. The paper with residue was transferred to respective crucible and dried in hot air

oven. It was then ignited in the muffle furnace at 600°C for 3 hrs. Cooled overnight and taken the weight. Percentage of acid insoluble ash was calculated as

$$\text{Acid insoluble ash \%} = \frac{\text{Weight of AIA}}{\text{Weight of sample}} \times 100$$

5.2.6.6 Crude Fibre

The thimbles containing fat free extract from the forgoing estimation of crude fat were dried in hot air oven at 50°C for overnight. Approximately 0.8 gms of fat free sample was weighed into gooch crucibles provided with fibretec extraction assembly. They were set on the assembly and two digestions, acid & alkali digestions in 1.25% H_2SO_4 and 1.25% NaOH were done one after the other for 30 minutes. Draining of acid and alkali and flushing of hot distilled water were done in between each digestion. The residue containing crucibles were removed, over dried at 60°C for overnight and weighed. They were ashed at 600°C for 3 hours in muffle furnace overnight, cooled and weighed again. Then percentage of crude fibre was calculated as

$$\text{\% crude fibre} = \frac{\text{Weight of crude fibre}}{\text{Weight of } \textit{extract}} \times 100$$

5.2.6.7 Nitrogen-free extract (NFE)

Calculated as,

100 – (% crude protein + % crude fat + % crude fibre + % crude ash + % moisture)

5.2.6. Statistical Analysis

The data of sensory evaluation were analyzed using '3 – way analysis of variance' (ANOVA) and further comparison was made by 'Student's t test' between different microalgae cookies at different concentration, (Snedecor and Cochran, 1967; Jayalakshmi, 1998)

5.3 Results

5.3.1 Sensory Evaluation

Results of organoleptic analysis are given in Tables 5.1, 5.2 and 5.3. The 3-way ANOVA table showed significant difference ($P<0.05$) within and between different attributes for each sensory quality. Difference was insignificant ($P>0.05$) only in 'species x respondents' for taste and 'concentration of microalgae x respondents' for crispiness (highlighted in grey – Table 5.1 C&D). This indicates that different respondents had almost similar opinion for taste with respect to species and for crispiness with respect to concentration of algae. Distribution parameters (Table 5.2) indicate a decrease in sensory qualities and significant difference between the cookies (Table 5.3) corresponding to an increase in concentration of microalgae.

Tables 5.1 A-E: 3 way ANOVA of microalgae cookies

A. Colour

Source	Sum of Squares	dof	Mean S-S	F ratio	dof of F	Remarks
Concentration of algae (A)	328.658	2	164.32	8.6465*	(2, 348)	$P < 0.05$
Species (B)	188.908	6	31.48	37.86*	(6, 348)	$P < 0.05$
Respondents (C)	212.324	29	7.32	8.80*	(29, 348)	$P < 0.05$
A x B		12	9.02	10.8*	(12, 348)	$P < 0.05$
B x C		174	1.50	1.81*	(174, 348)	$P < 0.05$
A x C		58	1.70	2.05*	(58, 348)	$P < 0.05$
Error	289.395	348	0.83			
Total	32696	629				

B. Aroma

Source	Sum of Squares	dof	Mean S-S	F ratio	dof of F	Remarks
Concentration of algae (A)	287.184	2	143.59	6.67*	(2, 348)	$P < 0.05$
Species (B)	50.373	6	8.39	8.72*	(6, 348)	$P < 0.05$
Respondents (C)	201.213	29	6.93	7.21*	(29, 348)	$P < 0.05$
A x B		12	8.91	9.27*	(12, 348)	$P < 0.05$
B x C		174	2.02	2.11*	(174, 348)	$P < 0.05$
A x C		58	1.43	1.48*	(58, 348)	$P < 0.05$
Error	334.697	348	0.96			
Total	32934	629				

C. Taste						
Source	**Sum of Squares**	**dof**	**Mean S-S**	**F ratio**	**dof of F**	**Remarks**
Concentration of algae (A)	522.641	2	261.32	9.243*	(2, 348)	$P < 0.05$
Species (B)	119.15	6	19.85	16.18*	(6, 348)	$P < 0.05$
Respondents (C)	329.178	29	11.35	9.25*	(29, 348	$P < 0.05$
A x B		12	20.66	16.83*	(12, 348)	$P < 0.05$
B x C		174	1.45	1.18	(174, 348)	$P > 0.05$
A x C		58	2.72	2.21*	(58, 348)	$P < 0.05$
Error	427.037	348	1.22			
Total	30499	629				

D. Crispiness						
Source	**Sum of Squares**	**dof**	**Mean S-S**	**F ratio**	**dof of F**	**Remarks**
Concentration of algae (A)	51.457	2	25.72	3.11*	(2, 348)	$P < 0.05$
Species (B)	11.621	6	1.93	4.59*	(6, 348)	$P < 0.05$
Respondents (C)	40.773	29	1.41	3.33*	(29, 348)	$P < 0.05$
A x B		12	2.76	6.45*	(12, 348)	$P < 0.05$
B x C		174	0.83	1.98*	(174, 348)	$P < 0.05$
A x C		58	0.42	1.01	(58, 348)	$P > 0.05$
Error	146.785	348	0.42			
Total	37807	629				

E. Overall						
Source	**Sum of Squares**	**dof**	**Mean S-S**	**F ratio**	**dof of F**	**Remarks**
Concentration of algae (A)	386.86	2	193.43	11.17*	(2, 348)	$P < 0.05$
Species (B)	72.96	6	12.16	16.13*	(6, 348)	$P < 0.05$
Respondents (C)	243.06	29	8.38	11.12*	(29, 348)	$P < 0.05$
A x B		12	12.05	16.00*	(12, 348)	$P < 0.05$
B x C		174	0.97	1.29*	(174, 348)	$P < 0.05$
A x C		58	2.06	2.73*	(58, 348)	$P < 0.05$
Error	262.24	348	0.75			
Total	31311	629				

*Calculated F is significant at 5% level ($P<0.05$)

Tables 5.2 A-E: Distribution parameters for microalgae cookies based on sensory qualities (colour, aroma, taste, crispiness and overall likeliness) at 1 g (0.5%), 3 g (1.5%) and 5 g (2.5%) microalgae concentrations

A. Colour

LEVEL		MEAN	VARIANCE	STDEV	COEV. %
Concentration 1 g (0.5%)					
Control		8.3	0.54	0.73	8.88
Isochrysis		7.56	0.71	0.84	11.15
Nannochloropsis		7.6	0.84	0.91	12.05
Tetraselmis		8.26	0.72	0.85	10.32
Dunaliella		8.26	0.46	0.68	8.22
Chaetoceros		7.6	0.57	0.75	9.96
Mix		8.23	0.37	0.61	7.47
Concentration 3 g (1.5%)					
Control		8.3	0.54	0.73	8.88
	0.989	6.43	0.97	0	15.37
Nannochloropsis		7.5	1.25	1.11	14.90
Tetraselmis		6.6	1.24	1.11	16.87
Dunaliella		7.2	1.89	1.37	19.11
Chaetoceros		6.33	1.35	1.1	18.38
Mix		6.06	0.92	0.96	15.88
Concentration 5 g (2.5%)					
Control		8.3	0.54	0.73	8.88
Isochrysis		6.13	2.58	1.60	26.2
Nannochloropsis		6.13	2.51	1.58	25.86
Tetraselmis		5.6	2.97	1.72	30.79
Dunaliella		5.86	3.71	1.92	32.85
Chaetoceros		5.96	1.89	1.37	23.09
Mix		5.53	2.11	1.45	26.28

B. Aroma

LEVEL	MEAN	VARIANCE	STDEV	COEV. %
Concentration 1 g (0.5%)				
Control	7.2	3.16	1.77	24.68
Isochrysis	7.86	0.58	0.76	9.7
Nannochloropsis	7.93	0.71	0.86	10.75
Tetraselmis	8.26	0.39	0.62	7.60
Dunaliella	8.4	0.37	0.61	7.27
Chaetoceros	7.76	0.71	0.84	10.86
Mix	8.2	0.56	0.74	9.12
Concentration 3 g (1.5%)				
Control	7.2	3.16	1.77	24.68
Isochrysis	6.56	1.31	1.14	17.44
Nannochloropsis	7.86	0.71	0.84	10.75
Tetraselmis	6.33	2.62	1.61	25.56
Dunaliella	7.46	1.11	1.05	14.14
Chaetoceros	6.36	1.43	1.19	18.79
Mix	7.13	1.44	1.20	16.87
Concentration 5 g (2.5%)				
Control	7.2	3.16	1.77	24.68
Isochrysis	5.33	2.88	1.7	31.86
Nannochloropsis	6.5	1.11	1.05	16.25
Tetraselmis	6.8	1.42	1.19	17.56
Dunaliella	6.03	1.09	1.04	17.37
Chaetoceros	6	1.66	1.29	21.51
Mix	6.16	2.73	1.65	26.83

C. Taste

Concentration 1 g (0.5%)				
LEVEL	**MEAN**	**VARIANCE**	**STDEV**	**COEV.%**
Control	7.06	1.39	1.18	16.71
Isochrysis	8.3	0.34	0.58	7.06
Nannochloropsis	6.73	2.06	1.43	21.32
Tetraselmis	8.8	0.16	0.4	4.54
Dunaliella	8.13	0.58	0.76	9.38
Chaetoceros	7.76	1.44	1.20	15.48
Mix	8.46	0.31	0.56	6.63
Concentration 3 g (1.5%)				
Control	7.06	1.39	1.18	16.71
Isochrysis	5.93	1.79	1.34	22.58
Nannochloropsis	7.56	1.17	1.08	14.34
Tetraselmis	6.26	3.99	1.99	31.89
Dunaliella	7.46	1.31	1.14	15.36
Chaetoceros	5.16	3.33	1.82	35.36
Mix	6.63	1.29	1.14	17.18
Concentration 5 g (2.5%)				
Control	7.06	1.39	1.18	16.71
Isochrysis	4.63	5.09	2.25	48.73
Nannochloropsis	5.33	3.55	1.88	35.35
Tetraselmis	6.33	1.75	1.32	20.92
Dunaliella	5.56	2.97	1.72	31.01
Chaetoceros	4.86	0.91	0.95	19.66
Mix	5.93	2.59	1.61	27.15

D. Crispiness

Concentration 1 g (0.5%)				
LEVEL	**MEAN**	**VARIANCE**	**STDEV**	**COEV. %**
Control	7.66	0.75	0.86	11.33
Isochrysis	7.93	0.72	0.85	10.76
Nannochloropsis	7.56	0.97	0.98	13.07
Tetraselmis	8.66	0.22	0.47	5.43
Dunaliella	8.3	0.41	0.64	7.71
Chaetoceros	7.83	0.67	0.82	10.46
Mix	8.36	0.49	0.70	8.44
Concentration 3 g (1.5%)				
Control	7.66	0.75	0.86	11.33
Isochrysis	7.53	0.78	0.88	11.74
Nannochloropsis	7.96	0.56	0.75	9.44
Tetraselmis	7.66	0.75	0.86	11.33
Dunaliella	7.9	0.62	0.79	9.99
Chaetoceros	7.53	0.44	0.67	8.89
Mix	7.66	0.35	0.59	7.77
Concentration 5 g (2.5%)				
Control	7.66	0.75	0.86	11.33
Isochrysis	7.53	0.38	0.61	8.20
Nannochloropsis	7.4	0.64	0.8	10.81
Tetraselmis	7.46	0.71	0.84	11.32
Dunaliella	7.26	0.26	0.51	7.04
Chaetoceros	7.03	0.36	0.60	8.59
Mix	7.06	0.26	0.51	7.24

E. Overall

Concentration 1 g (0.5%)				
LEVEL	**MEAN**	**VARIANCE**	**STDEV**	**COEV. %**
Control	7.26	1.06	1.03	14.18
Isochrysis	8	0.46	0.68	8.53
Nannochloropsis	7.16	0.40	0.63	8.88
Tetraselmis	8.46	0.24	0.49	5.89
Dunaliella	8.3	0.41	0.64	7.71
Chaetoceros	7.53	0.71	0.84	11.22
Mix	8.3	0.21	0.458	5.521
Concentration 3 g (1.5%)				
Control	7.26	1.06	1.03	14.18
Isochrysis	6.33	1.55	1.24	19.69
Nannochloropsis	7.73	0.79	0.89	11.53
Tetraselmis	6.33	2.95	1.71	27.14
Dunaliella	7.46	0.65	0.80	10.78
Chaetoceros	6.1	1.29	1.13	18.61
Mix	6.83	1.01	1.00	14.67
Concentration 5 g (2.5%)				
Control	7.26	1.06	1.03	14.18
Isochrysis	5.	2.89	1.7	33.33
Nannochloropsis	5.76	1.84	1.35	23.55
Tetraselmis	6.33	1.22	1.10	17.45
Dunaliella	5.86	2.84	1.68	28.77
Chaetoceros	5.26	1.59	1.26	23.98
Mix	6	2.2	1.48	24.72

The colour of cookies was appealing at 0.5% (score -7.6 to 8.3) & 1.5% (score - 6 to 7.5) concentrations with slight greenish/yellowish and green hue respectively (Figures 5.3, Table 5.2 A). The pictorial representation of Students t test results (Table 5.3 A) illustrates no significant difference (at 5% level) between control cookies and cookies with least concentration of *Dunaliella*, *Tetraselmis* and Mix. At 2.5% concentration the cookies were dark green in colour and less

attractive (Figure 5.3) and there was a significant difference (symbolized as "❍" for P<0.05 and "○" for P<0.01) between the control and algal cookies but all algal cookies resembled were alike with P value >0.05 ("●").

Aroma of microalgae cookies at lower concentration was buttery-fruity and was better than normal cookies. *Dunaliella* cookies smelled best at 0.5% concentration with 8.4±0.61 points, while *Nannochloropsis* scored highest (7.86±0.85) at 1.5%. All algal cookies with 0.5% algal mass gained higher than the control. Table 5.3 B evidently portrays negligible difference (at 5% level) in smell between the cookies, even at highest concentration of microalgae (level of significance if present was at 1% except *Isochrysis*). However some of the vegetarian respondents remarked algal cookies (*Isochrysis* and *Chaetoceros*) with "fishy" smell at concentration 2.5%.

On addition of 0.5 and 1.5% algae, the cookies tasted excellent to good, and at many places better than normal cookies (Table 5.2 C). The best were *Tetraselmis* (8.8±0.4) and mix (8.47±0.56) at minimum concentration, and *Nannochloropsis* (7.57±1.09) and *Dunaliella* (7.47±1.15) at medium concentration. At maximum concentration (2.5%) of microalgae, slight bitterness was a noted for *Isochrysis*, *Dunaliella* and *Chaetoceros*. Level of significance for taste was more or less varying between the microalgae cookies at all concentrations, but at highest concentration considerable divergence was there for microalgae from control.

As such differentiation was not possible in crispiness between the microalgae and normal cookies especially at the lower and middle concentrations (Tables 5.2 D and 5.3 D). Crispiness showed significance neither in different concentrations nor in different microalgae species. The rating was not less than 'good' (7.03±0.61 – 8.67±0.47) for all cookies.

Results for overall likeliness were almost the same as that of taste and average scores were not less than 6 at 0.5% and 1.5% concentrations (Table

5.2 E). Overall likeliness of 0.5% microalgae cookies (except *Nannochloropsis*) was better than standard. In 1.5% microalgae cookies, *Dunaliella* and *Nannochloropsis* scored higher than control. Level of significance was considerable ($P<0.05$) between normal and microalgae cookies especially at least and highest concentrations (Table 5.3 E).

Table 5.3 A-E Significance of Student's t statistics for comparison between microalgae cookies at different concentrations based on:

A. Colour

	Control	*Isochrysis*	*Nannochloropsis*	*Tetraselmis*	*Dunaliella*	*Chaetoceros*	Mix
Concentration 1 g (0.5%)							
Control	...	◉	◉	○	○	◉	○
Isochrysis	3.52	...	○	◉	◉	○	◉
Nannochloropsis	3.20	0.14	...	◉	◉	○	◉
Tetraselmis	0.15	3.14	2.86	...	○	◉	○
Dunaliella	0.17	3.47	3.14	...	...	◉	○
Chaetoceros	3.56	0.15	0	3.14	3.52	...	◉
Mix	0.37	3.43	3.08	0.17	0.19	3.49	...
Concentration 3 g (1.5%)							
Control	...	◉	◉	◉	◉	◉	◉
Isochrysis	8.14	...	◉	○	●	○	○
Nannochloropsis	3.21	3.84	...	◉	○	◉	◉
Tetraselmis	6.85	0.60	3.07	...	○	○	○
Dunaliella	3.79	2.43	0.91	1.82	...	●	◉
Chaetoceros	7.68	0.35	3.89	0.89	2.58	...	●
Mix	9.91	1.42	5.22	1.95	3.63	0.95	...
Concentration 5 g (2.5%)							
Control	...	◉	◉	◉	◉	◉	◉
Isochrysis	6.59	...	○	○	○	○	○
Nannochloropsis	6.67	0	...	○	○	○	○
Tetraselmis	7.75	1.21	1.22	...	○	○	○
Dunaliella	6.34	0.57	0.57	0.55	...	○	○
Chaetoceros	8.04	0.4	0.42	0.89	0.22	...	○
Mix	9.13	1.49	1.50	0.15	0.74	1.16	...

B. Aroma

Concentration 1 g (0.5%)							
	Control	***Isochrysis***	***Nannochloropsis***	***Tetraselmis***	***Dunaliella***	***Chaetoceros***	**Mix**
Control	...	○	○	◎	◎	○	●
Isochrysis	1.85	...	○	●	◎	○	○
Nannochloropsis	1.82	0	...	●	◎	○	○
Tetraselmis	3.04	2.17	2.04	...	○	●	○
Dunaliella	3.43	2.93	2.75	0.81	...	◎	○
Chaetoceros	1.55	0.47	0.45	2.55	3.27	...	●
Mix	2.79	1.67	1.58	0.36	1.11	2.06	...
Concentration 3 g (1.5%)							
Control	...	○	○	○	○	○	○
Isochrysis	1.61	...	◎	○	◎	○	○
Nannochloropsis	1.82	4.91	...	◎	○	◎	◎
Tetraselmis	1.94	0.63	4.51	...	◎	◎	●
Dunaliella	0.69	3.11	1.59	3.15	...	◎	○
Chaetoceros	2.09	0.65	5.51	0.08	3.71	...	●
Mix	0.16	1.83	2.68	2.13	1.12	2.43	...
Concentration 5 g (2.5%)							
Control	...	●	○	○	●	●	●
Isochrysis	4.08	...	◎	◎	○	○	○
Nannochloropsis	1.82	3.13	...	○	○	○	○
Tetraselmis	1.00	3.80	1.01	...	●	●	○
Dunaliella	3.04	1.88	1.68	2.59	...	●	○
Chaetoceros	2.94	1.68	1.61	2.44	0.10	...	○
Mix	2.29	1.89	0.91	1.67	0.36	0.42	...

C. Taste

	Control	*Isochrysis*	*Nannochloropsis*	*Tetrselmis*	*Dunaliella*	*Chaetoceros*	Mix
Concentration 1 g (0.5%)							
Control	...	⊙	○	⊙	⊙	●	⊙
Isochrysis	5.03	...	⊙	⊙	○	●	○
Nannochloropsis	0.96	5.43	...	⊙	⊙	⊙	⊙
Tetraselmis	7.48	3.79	7.46	...	⊙	⊙	○
Dunaliella	4.08	0.93	4.63	4.16	...	○	○
Chaetoceros	2.23	2.14	2.97	4.39	1.38	...	⊙
Mix	5.76	1.10	6.05	2.6	1.89	2.84	...
Concentration 3 g (1.5%)							
Control	...	⊙	○	○	○	⊙	○
Isochrysis	3.41	...	⊙	○	⊙	○	●
Nannochloropsis	1.67	5.1	...	⊙	○	⊙	⊙
Tetraselmis	1.85	0.74	3.07	...	⊙	●	○
Dunaliella	1.30	4.68	0.34	2.80	...	⊙	⊙
Chaetoceros	4.70	1.82	6.08	2.18	5.74	...	⊙
Mix	1.42	2.14	3.19	0.85	2.77	3.66	...
Concentration 5 g (2.5%)							
Control	...	⊙	⊙	●	⊙	⊙	⊙
Isochrysis	5.14	...	○	⊙	○	○	●
Nannochloropsis	4.19	1.28	...	●	○	○	○
Tetraselmis	2.22	3.49	2.33	...	○	⊙	○
Dunaliella	3.86	1.76	0.49	1.89	...	○	○
Chaetoceros	7.79	0.51	1.18	4.83	1.91	...	⊙
Mix	3.05	2.52	1.30	1.03	0.83	3.06	...

D. Crispiness

	Control	*Isochrysis*	*Nannochloropsis*	*Tetraselmis*	*Dunaliella*	*Chaetoceros*	Mix
Concentration 1 g (0.5%)							
Control	...	○	○	◎	◎	○	◎
Isochrysis	1.17	...	○	◎	○	○	●
Nannochloropsis	0.40	1.51	...	◎	◎	○	◎
Tetraselmis	5.44	4.04	5.40	...	●	◎	○
Dunaliella	3.15	1.85	3.35	2.48	...	●	○
Chaetoceros	0.75	0.45	1.11	4.74	2.41	...	●
Mix	3.36	2.10	3.54	1.90	0.37	2.65	...
Concentration 3 g (1.5%)							
Control	...	○	○	○	○	○	○
Isochrysis	0.57	...	●	○	○	○	○
Nannochloropsis	1.40	2.01	...	○	○	●	○
Tetraselmis	0	0.57	1.40	...	○	○	○
Dunaliella	1.07	1.66	0.32	1.07	...	○	○
Chaetoceros	0.65	0	2.31	0.65	1.90	...	○
Mix	0	0.67	1.68	0	1.27	0.80	...
Concentration 5 g (2.5%)							
Control	...	○	○	○	●	◎	◎
Isochrysis	0.67	...	○	○	○	◎	◎
Nannochloropsis	1.21	0.71	...	○	○	○	○
Tetraselmis	0.88	0.34	0.30	...	○	●	●
Dunaliella	2.14	1.78	0.75	1.08	...	○	○
Chaetoceros	3.22	3.11	1.96	2.24	1.58	...	○
Mix	3.20	3.13	1.88	2.17	1.48	0.22	...

E. Overall

	Control	*Isochrysis*	*Nannochloropsis*	*Tetraselmis*	*Dunaliella*	*Chaetoceros*	Mix
Concentration 1 g (0.5%)							
Control	…	⊙	○	⊙	⊙	⊙	⊙
Isochrysis	3.19	…	⊙	⊙	○	●	○
Nannochloropsis	0.44	4.80	…	⊙	⊙	○	⊙
Tetraselmis	5.64	2.97	8.65	…	●	⊙	○
Dunaliella	4.58	1.73	6.75	1.10	…	⊙	○
Chaetoceros	1.07	2.31	1.86	5.11	3.89	…	⊙
Mix	4.93	1.96	7.77	1.32	0	4.29	…
Concentration 3 g (1.5%)							
Control	…	⊙	○	●	○	⊙	○
Isochrysis	3.10	…	⊙	○	⊙	○	○
Nannochloropsis	1.84	4.91	…	⊙	○	⊙	⊙
Tetraselmis	2.50	0	3.89	…	⊙	○	○
Dunaliella	0.82	4.11	1.19	3.21	…	⊙	●
Chaetoceros	4.09	0.74	6.09	0.60	5.28	…	●
Mix	1.62	1.68	3.61	1.35	2.65	2.61	…
Concentration 5 g (1.5%)							
Control	…	⊙	⊙	⊙	⊙	⊙	⊙
Isochrysis	5.87	…	○	⊙	○	○	●
Nannochloropsis	4.74	1.65	…	○	○	○	○
Tetraselmis	3.33	3.28	1.74	…	○	⊙	○
Dunaliella	3.81	1.72	0.24	1.25	…	○	○
Chaetoceros	6.61	0.42	1.45	3.42	1.53	…	●
Mix	3.78	2.15	0.62	0.97	0.32	2.03	…

"●" - significant at 1% level; "⊙"- significant at 5% level; "○"- no significance at 5% level

5.3.2 Fatty acid analysis

In the cookies, normal butter was used for shortening, and hence fatty acid profile was close to the butter (Table 5.4) with maximum saturated fatty acids (SFA) and minimum polyunsaturated fatty acids (PUFAs). Chromatographs of some of the samples are shown in Figures 5.4.1 – 5.4.4. Palmitic acid (C16:0) was the principal FA with not <34% and >39%. When

Stearic and Myristic acids were the other two major SFAs (about 10 - 12%), Oleic acid (between 14 – 17%) was the dominant MUFA. All other important fatty acids were <6%. Among the PUFAs Only Linolenic (0.3-0.5%) and Linoleic (2 – 3%) acids were present in all cookies including standard. Only *Nannochloropsis* cookies (with 1.5 & 2.5% concentrations) were detected with presence of EPA (0.12 & 0.19% of total FAs respectively, Figures 5.4.3 and 5.4.4), while DHA was below detection level in all.

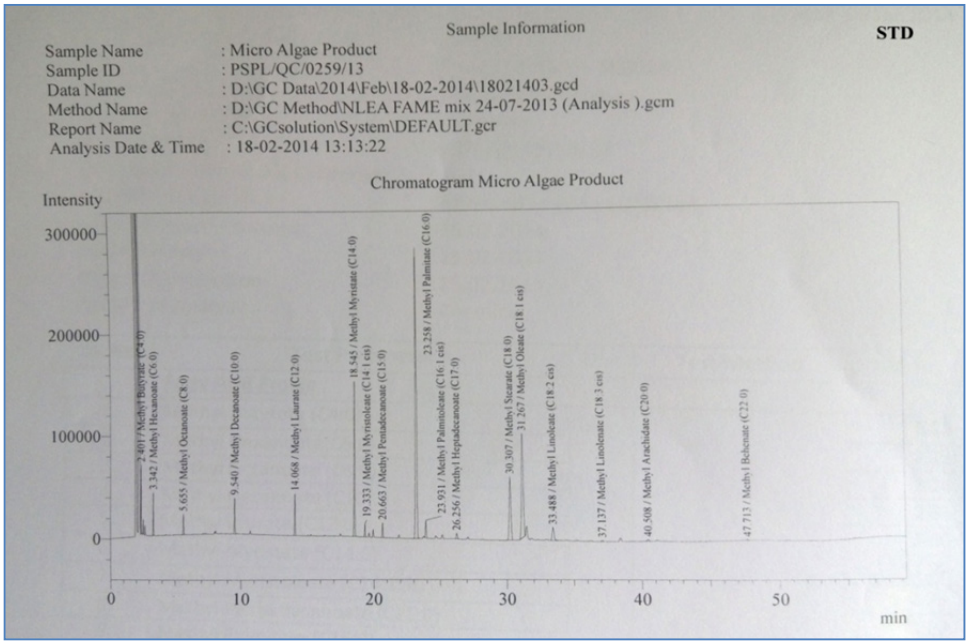
Irrespective of microalgae type and concentration the fatty acid composition was more or less varied with a minimum range. There was no particular increase or decrease in any of the fatty acids, between or among the different microalgae cookies, except Eicosapentaenoic acid (EPA). When calculated in mg/100 mg of sample, the EPA content was observed to be 29.046 and 46.835 in 1.5% and 2.5% *Nannochloropsis* cookies respectively. Even though, similar results were also expected from other microalgae cookies, none were found promising in contrast to previous studies (Gouveia, Coutinho, et al. 2008)

Table 5.4: Fatty acid composition (%) of microalgae cookies. Yellow highlighted – dominant fatty acids; Blue highlighted – EPA in NAN-3 & NAN-5

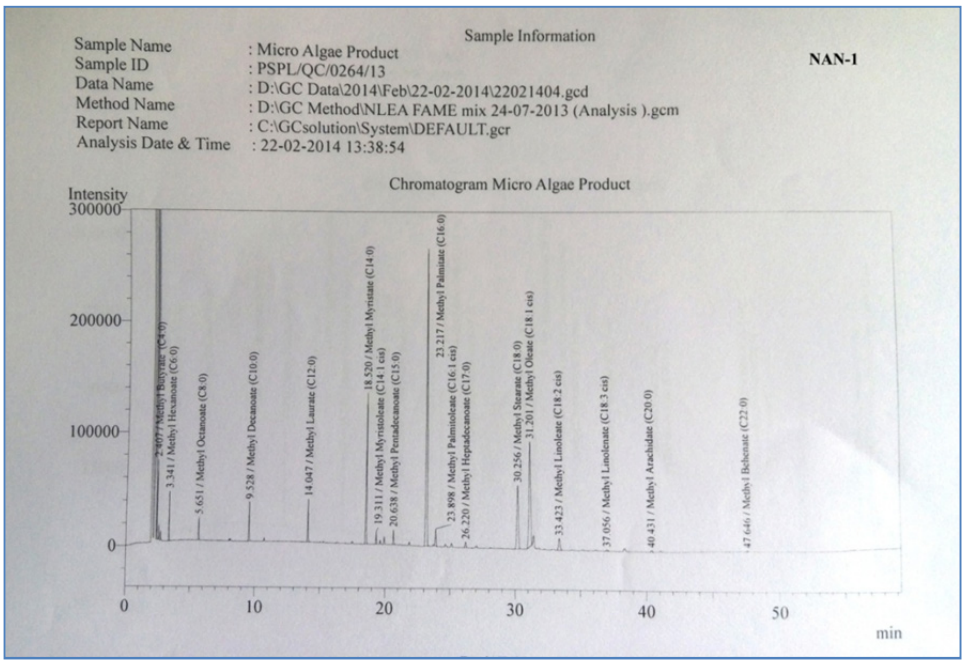
Compound	Fatty acid	Sample ID									
		Std	ISO-1	ISO-3	ISO-5	NAN-1	NAN-3	NAN-5	TET-1	TET-3	TET-5
C4:0	Butyric	5.13	5.53	5.20	5.68	5.75	5.29	5.21	5.71	5.34	5.32
C6:0	Hexanoic	2.13	2.47	2.31	2.44	2.41	2.29	2.26	2.52	2.40	2.38
C8:0	Octanoic	1.24	1.32	1.29	1.32	1.30	1.28	1.25	1.32	1.30	1.26
C10:0	Decanoic	2.58	2.55	2.63	2.62	2.49	2.52	2.50	2.56	2.52	2.54
C11:0	Undecanoic	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
C12:0	Lauric	3.00	2.86	3.07	2.99	2.75	2.86	2.82	2.89	2.99	2.98
C13:0	Tridecanoic	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
C14:0	Myristic	11.97	11.25	12.10	11.86	10.80	11.97	11.54	11.39	11.66	11.77
C15:0	Pentadecanoic	1.23	1.15	1.25	1.20	1.08	1.21	1.15	1.15	1.17	1.19
C16:0	Palmitic	37.37	36.64	37.43	37.09	34.15	38.39	36.71	37.84	37.68	37.99
C17:0	Heptadecanoic	0.72	0.70	0.72	0.70	0.64	0.72	0.67	0.73	0.71	0.73
C18:0	Stearic	11.38	11.09	11.44	11.12	10.36	11.37	10.79	11.64	11.74	11.57
C20:0	Arachidic	0.31	0.31	0.31	0.30	0.28	0.29	0.29	0.27	0.34	0.32
C22:0	Behenic	0.12	0.13	0.12	0.12	0.12	0.12	0.12	0.13	0.13	0.13
C23:0	Tricosanoic	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL

C24:0	Lignoceric	BDL	0.11	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
ΣSFA Total saturated FA		77.18	76.11	77.87	77.44	72.13	78.31	75.31	78.15	77.98	78.18
C14:1	Myristoleic	0.98	0.90	1.00	0.97	0.87	0.94	0.92	0.92	0.95	0.96
C16:1	Palmitoleic	1.67	1.64	1.68	1.70	1.53	1.72	1.67	1.64	1.65	1.67
C18:1	Oleic *cis*	15.97	16.09	15.86	15.67	14.59	15.98	15.30	16.33	16.14	16.08
C20:1	Eicosenoic *cis*	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
C22:1	Erucic *cis*	BDL	0.64	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
ΣMUFA Total monounsaturated FA		18.62	19.27	18.54	18.34	16.99	18.64	17.89	18.89	18.74	18.71
C18:2	Linoleic *cis*	2.53	2.86	2.50	2.53	2.16	2.45	2.33	2.46	2.55	2.52
C18:2	Linoelaidic *trans*	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
C18:3	Linolenic *cis*	0.33	0.54	0.39	0.39	0.28	0.32	0.29	0.27	0.35	0.38
C20:5	Eicosapentaenoic	BDL	BDL	BDL	BDL	BDL	0.12	0.19	BDL	BDL	BDL
C22:6	Docosahexaenoic	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
ΣPUFA Total polyunsaturated FA		2.86	3.40	2.89	2.92	2.44	2.89	2.81	2.73	2.90	2.90
Total fatty acids (ΣFA)		98.66	98.78	99.30	98.70	91.56	99.84	96.01	99.77	99.62	99.79

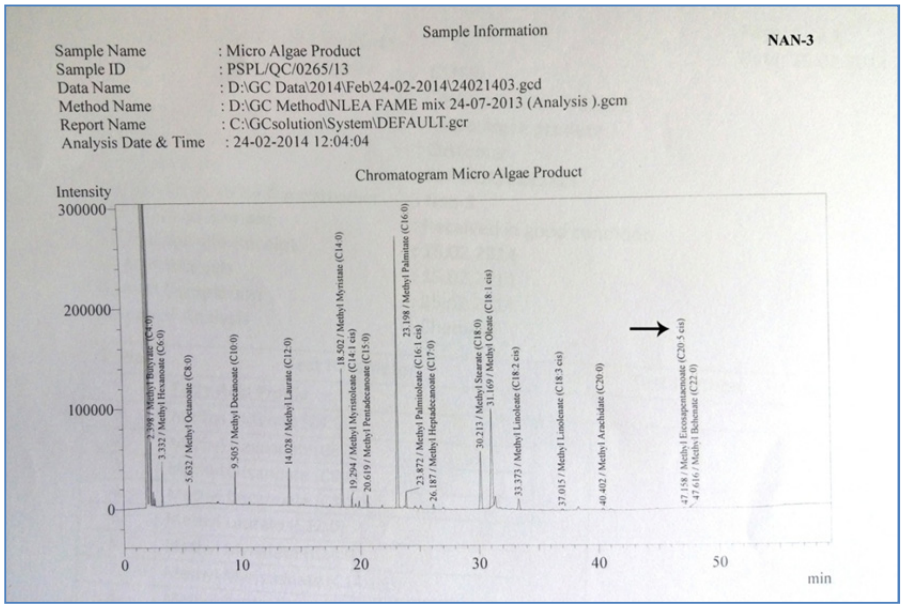
Compound	Fatty acid	Sample ID								
		DUN1	DUN3	DUN5	CHE1	CHE3	CHE5	M1	M3	M5
C4:0	Butyric	5.48	5.13	4.99	5.49	5.58	5.17	4.64	5.09	5.88
C6:0	Hexanoic	2.43	2.37	2.35	2.47	2.46	2.44	2.14	2.33	2.50
C8:0	Octanoic	1.29	1.29	1.33	1.35	1.37	1.33	1.22	1.32	1.35
C10:0	Decanoic	2.52	2.54	2.61	2.66	2.60	2.52	2.53	2.68	2.70
C11:0	Undecanoic	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
C12:0	Lauric	2.89	2.97	2.95	3.04	3.02	2.93	2.98	3.06	2.98
C13:0	Tridecanoic	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
C14:0	Myristic	12.00	12.17	12.03	12.00	12.16	11.73	11.91	12.02	11.70
C15:0	Pentadecanoic	1.19	1.23	1.21	1.20	1.22	1.19	1.24	1.22	1.18
C16:0	Palmitic	38.16	37.96	38.36	37.74	38.10	38.14	37.75	37.43	37.26
C17:0	Heptadecanoic	0.71	0.71	0.72	0.71	0.69	0.69	0.71	0.73	0.71
C18:0	Stearic	11.31	11.40	11.53	11.32	11.09	11.52	11.53	11.36	11.03
C20:0	Arachidic	0.29	0.29	0.31	0.30	0.30	0.32	0.27	0.30	0.29
C22:0	Behenic	0.13	0.11	0.13	0.13	0.10	0.13	0.12	0.12	0.12
C23:0	Tricosanoic	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
C24:0	Lignoceric	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
ΣSFA Total saturated FA		78.40	78.17	78.52	78.41	78.69	78.11	77.04	77.66	77.70
C14:1	Myristoleic	0.94	0.97	0.95	0.96	0.97	0.94	0.98	0.97	0.96
C16:1	Palmitoleic	1.65	1.66	1.65	1.66	1.73	1.75	1.67	1.70	1.72
C18:1	Oleic *cis*	16.02	15.86	15.97	15.78	15.64	16.15	16.11	15.90	15.69
C20:1	Eicosenoic *cis*	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
C22:1	Erucic *cis*	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
ΣMUFA Total monounsaturated FA		18.61	18.49	18.57	18.40	18.34	18.84	18.76	18.57	18.37
C18:2	Linoleic *cis*	2.52	2.55	2.47	2.44	2.47	2.42	2.56	2.47	2.48
C18:2	Linoelaidic *trans*	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
C18:3	Linolenic *cis*	0.32	0.38	0.33	0.31	0.35	0.34	0.34	0.28	0.36
C20:5	Eicosapentaenoic	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
C22:6	Docosahexaenoic	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
ΣPUFA Total polyunsaturated FA		2.84	2.93	2.80	2.75	2.82	2.76	2.90	2.75	2.84
Total fatty acids (ΣFA)		99.85	99.59	99.89	99.56	99.85	99.71	98.70	98.98	98.91



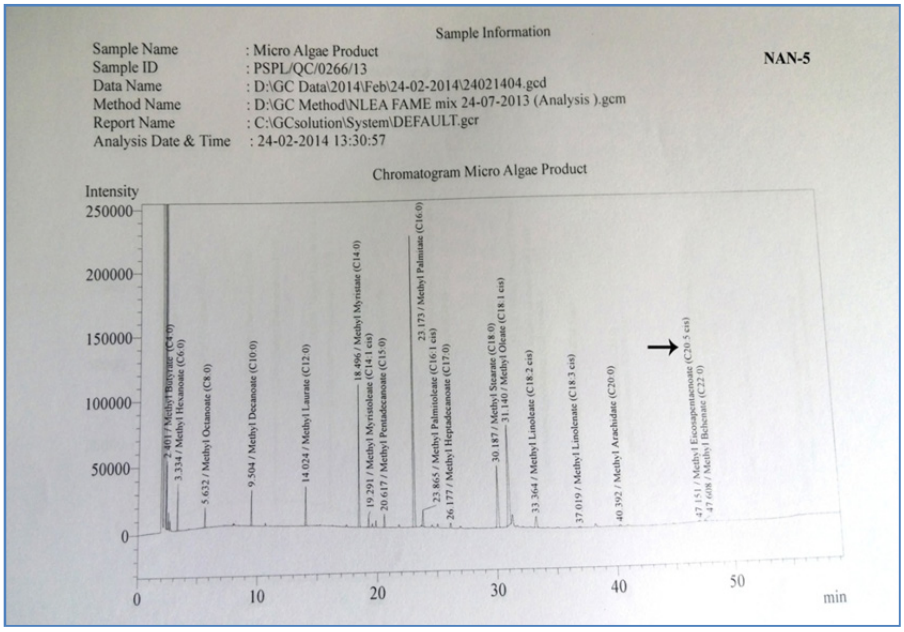
Figures 5.3.1



Figures 5.3.2



Figures 5.3.3



Figures 5.3.4

Figures 5.3.1 - 5.3.4: Chromatogram of cookies showing fatty acid profile; 5.3.1: Normal butter cookies; 5.3.2-4: *Nannochloropsis* cookies at 0.5, 1.5 and 2.5% concentrations respectively. Note (arrow mark) detection of EPA in 1.5% and 2.5% cookies, which is absent in STD and 0.5% cookies.

5.3.3. Total chlorophyll and Carotenoids in Cookies

Both Chlorophylls and Carotenoids are food colourants . Unlike other nutrients, pigment profile (% of Chlorophyll and carotenoids) of microalgal cookies depicted clear increase with regard to an increase in concentration of algae (Figure 5.2 And table 5.5). Chlorophyll content, at both 1.5 and 2.5% concentration, was highest in *Nannochloropsis* cookies (c.a. 0.265 & 0.461 mg/g respectively) and lowest in *Chaetoceros* (c.a. 0.117 & 0.150 mg/g respectively). *Isochrysis* products, were best for Carotenoids (0.115, 0.212 and 0.263 mg/g) at all concentrations. *Nannochloropsis*, *Chaetoceros* and Mix gave almost equal quantity of carotenoids within a range 77-88, 107-113 and 119-134 μg/g of samples with 1, 3 and 5 g of microalgae contents respectively.

Table 5.5: Pigment composition (mg/g) of microalgae cookies. Average values from triplicates are given with standard deviation.

Samp Name	Average Chlorophyll mg/g	Average Carotenoids mg/g
ISO-1	0.097 ± 0.035	0.115 ± 0.009
ISO-3	0.264 ± 0.007	0.212 ± 0.001
ISO-5	0.318 ± 0.004	0.263 ± 0.001
NAN-1	0.083 ± 0.003	0.077 ± 0.001
NAN-3	0.265 ± 0.001	0.108 ± 0.0002
NAN-5	0.461 ± 0.010	0.134 ± 0.002
TET-1	0.097 ± 0.020	0.095 ± 0.005
TET-3	0.262 ± 0.002	0.156 ± 0.001
TET-5	0.406 ± 0.002	0.206 ± 0.0004
DUN-1	0.106 ± 0.003	0.106 ± 0.0004
DUN-3	0.180 ± 0.000	0.142 ± 0.002
DUN-5	0.260 ± 0.010	0.172 ± 0.002
CHE-1	0.081 ± 0.003	0.088 ± 0.000
CHE-3	0.117 ± 0.001	0.107 ± 0.0002
CHE-5	0.150 ± 0.001	0.119 ± 0.0004
MIX-1	0.064 ± 0.001	0.079 ± 0.0002
MIX-3	0.244 ± 0.006	0.113 ± 0.001
MIX-5	0.251 ± 0.002	0.125 ± 0.0003
STD	0.027 ± 0.001	0.072 ± 0.000

5.3.4 Proximate Analysis

Table 5.6 corresponds to the results of proximate analysis on as such basis. With respect to an increase in microalgae only a little difference in protein, lipid and carbohydrates were detected. An increase in crude ash was noted on increase of microalgae concentration. Crude fibre and acid insoluble ash were negligible and zero respectively in all samples. Protein was highest in *Tetraselmis* sp. (TET-5, 6.11%) and *D. salina* (DUN-5, 5.92%) at 2.5% concentration. Percentage of fat content in different samples was more or less same, with lowest in Mix-1 (22.49%) and highest in NAN-1 (24.63%). When the calorific value was calculated, it was in a range between 500-515 kcal/100 g, irrespective of species or concentration of microalgae.

Table 5.6: Proximate Composition (in %) of Microalgae cookies (wet weight basis)

Sample Name	Dry Matter %	Moisture %	Crude Protein %	Crude Fat %	Crude Ash %	Crude Fibre %	Acid Insoluble Ash %	Carbohydrate (NFE) %	Calorie (kcal/ 100 g)
ISO-1	99.06	0.94	4.80	23.94	0.34	NEG	0	69.98	514.60
ISO-3	99.22	0.78	5.62	23.84	0.42	NEG	0	69.34	514.42
ISO-5	98.70	1.30	5.63	22.97	0.68	NEG	0	69.42	506.92
NAN-1	97.24	2.76	5.21	24.63	1.14	NEG	0	66.26	507.56
NAN-3	98.23	1.77	5.70	24.35	0.88	NEG	0	67.30	511.17
NAN-5	98.11	1.89	5.77	24.02	1.38	NEG	0	66.94	507.00
TET-1	98.22	1.78	5.42	23.77	0.22	NEG	0	68.81	510.81
TET-3	98.45	1.55	5.63	23.05	0.78	NEG	0	68.99	505.97
TET-5	99.29	0.71	6.11	23.77	1.07	NEG	0	68.35	511.76
DUN-1	98.70	1.30	5.12	22.93	0.55	NEG	0	70.11	507.24
DUN-3	98.98	1.02	5.51	23.07	0.58	NEG	0	69.81	508.94
DUN-5	97.94	2.06	5.92	23.41	0.77	NEG	0	67.84	505.74
CHE-1	99.21	0.79	5.24	23.94	0.69	NEG	0	69.34	513.78
CHE-3	98.59	1.41	5.41	23.11	1.07	NEG	0	68.99	505.60
CHE-5	98.40	1.60	5.25	22.97	1.68	NEG	0	68.50	501.75
MIX-1	98.94	1.06	5.33	22.49	0.53	NEG	0	70.59	506.09
MIX-3	99.60	0.40	5.67	23.19	0.80	NEG	0	69.94	511.14
MIX-5	99.39	0.61	5.79	22.79	1.12	NEG	0	69.68	507.02
STD	97.45	2.55	5.19	23.22	0.87	NEG	0	68.17	502.43

NEG – negligible.

5.4 Discussion

Plain butter cookies were prepared using microalgae as an ingredient without additional flavoring particularly to avoid masking the natural essence of microalgae. Different microalgae species were found promising for different attributes (sensory and nutritional) in cookies. Organoleptic analysis short listed *Tetraselmis* (0.5% concentration, with score >8) and *Nannochloropis* (1.5%, score >7.5) as best in all sensory qualities. Fatty acid profile promised only NAN-3 & NAN-5 (with EPA) whiles all others were more or less close to normal butter cookies. Proximate composition could not differentiate cookies much, either by concentration or by species, signify as 'functional' in case of pigment contents, as expected.

The results of sensory evaluation verify microalgae cookies superior to normal cookies in 'Aroma', 'Taste', 'Crispiness' and 'Overall' acceptance at 0.5% concentration. Colour was appealing with a greenish/yellow tint (Figure 5.3). Even at higher concentrations none had a sensory score (average) less than 4 (slightly poor) for any of the attributes. Lower scores for taste in highest concentration was due to a bitterness which was predominant in *Isochrysis*, *Dunaliella* and *Chaetoceros* samples. Similarly *Isochrysis* and *Chaetoceros* were also having a slight fishy smell (only in 2.5% concentration) which was also not so appealing, especially for vegetarians. This was contradictory to Gouveia, Coutinho, et al. (2008) who have reported no negative impact on odour when *Isochrysis galbana* was used in short-biscuits. Crispiness was the least diverged quality within and between different cookies (score 7-8.6), pointing towards little role of microalgae in modifying this property. On a whole, the microalgae cookies, *Tetraselmis* at 0.5% concentration and *Nannochloropsis* at 1.5% concentration were the best for all sensory qualities with scores more than 8 and 7.5 respectively.

Protein and total fatty acid compositions were neither differentiated much nor up to the mark as expected for algal cookies. The results in proximate

composition were less comparable probably for the reason that the cookies were prepared independently, while major % of lipid, protein and carbohydrates were contributed by key ingredients like floor, butter and sugar compared to the minor component microalgae. The results emphasize on uniformity concerns which should be considered during preparation (mixing & baking).

When coming to PUFAs, except *Nannochloropsis* cookies, none were detected with EPA. This can be correlated with the fatty acid composition (Chapter 4 Table 4.1), of *N. oceanica* S078 with highest EPA content (15.87%) among the five. Gouveia, Coutinho, et al. (2008) had studied *Isochrysis galbana* as a functional ingredient for PUFAs in biscuits and got positive results with 6-9% PUFAs of which a bigger percentage was contributed by Linoleic acid (LA) and EPA along with traces of DHA. However, in the present study, *I. galbana* incorporation was found not so beneficial, except a slight increase in total PUFAs (2.89 - 3.4%) than control (2.86%). This signifies that a good % of targeted biomolecule has to be ensured in the microalgal biomass before inclusion in food products. As all the biomass of studied strains have been previously analyzed (Chapter 4) and were found rich in one or more omega-3 PUFAs (LA, GLA, ARA, EPA and DHA), their presence in microalgae cookies was certain, however less than detection limit (0.1%).

The presence of pigments made microalgae cookies attractive with a green colouration. Rather than natural colouring agents microalgae pigments are also accounted for good antioxidant properties. Chlorophylls and their derivatives have shown anti-carcinogenic activity where they bind to carcinogenic compounds like PAHs (poly cyclic aromatic hydrocarbons) (Lordan et al. 2011). Functional values of 1.5% and 2.5% *Nannochloropsis* cookies were thus supplemented with chlorophylls (265 and 461 µg/g respectively) along with EPA (omega-3 fatty acid).

Carotenoids are another wonderful group of pigments which contribute to the nutritional values as well as oxidation stability of a food product in

addition to many therapeutic effects (Gouveia et al. 2006). In human beings consumption of 2-10 mg of carotenoids per day was observed with improvements in immune functions and overall health. When calculated, about 100 g of microalgae cookies were assumed to provide 1-2 mg of carotenoids and 1-4 mg of chlorophylls on an average. Among microalgal cookies, *I. galbana* had highest of carotenoids (115, 212, 263 μg/g) while others had about 100 - 200 μg/g in various concentrations. Fucoxanthine is the chief carotenoid reported in *I. galbana* which has strong antioxidant, anti-inflammatory, anti-obesity, anti-diabetic, anticancer, and antihypertensive activities (Kim et al. 2012; Xia et al. 2013). In addition, when current demand for natural colouring agents is estimated to be 15000 MT in 2015 for food alone (Lakshmi 2014), microalgae with high carotenoid contents and its additional health promoting features can create a better position in functional food ingredients.

Major concern in making a novel food items using uncommon microalgae was the safety of the food. Nevertheless, the commonly consumed *Spirulina* and *Chlorella* were 'generally recognized as safe' (GRAS), while others (including the 5 species used in this study) are accepted as 'non-toxic' (NT) (Enzing et al. 2014). The loss of functional molecules upon cooking was the second issue where most of the molecules (e.g. pigments) are thermo-labile. However, our results and previous studies (Gouveia, Coutinho, et al. 2008; Gouveia et al. 2006) confirm the stability of PUFAs (including ω-3s) and pigments in these food items probably because they are safely encapsulated in microalgal cells (Gouveia, Batista, et al. 2008).

Finally, the quality of microalgae depends up on the species/strain, its growth environment (physical and chemical) and time of harvest (Sánchez et al. 2000; Borowitzka et al. 1990; Lourenqo et al. 1997; Walker et al. 2005). Many microalgae were observed with an ability to accumulate certain chemicals upon stress (light, temperature or nutrient limitation). For example *Dunaliella salina* synthesizes and store β-carotene in stress (Lele 2005;

Kleinegris et al. 2010), whereas most others accumulate carotenoids as well as PUFAs (Hejazi & Wijffels 2004; Forján et al. 2007). Metabolically engineered microalgae, either by manipulation of culture conditions or by mutagenesis, can be employed for the production of preferred bio-molecules (Rosenberg et al. 2008). Therefore, a proper design starting from strain selection to culturing and harvesting is emphasized for superior quality microalgae and therefore the food products.

5.5 Conclusion

It was appreciable that microalgae incorporation not only improved nutritional quality, but enhanced the sensory attributes of the food. Evaluated five strains of marine microalgae were promising as functional ingredients mainly for carotenoids than fatty acids. As these microalgae are purveyors of many known/unknown bio-molecules (Gouveia et al. 2010; Garrido et al. 2009; Tafreshi & Shariati 2009; Gouveia, Coutinho, et al. 2008; Matos et al. 2007), further assessment (in vitro and in vivo) of the products is needed to explore more. To improve the flavour (or to mask the repulsive sensory properties) of high concentration microalgal cookies, inclusion of taste/odour modifiers (like spices) in recipes are proposed. Besides, to assure the targeted nutrients in the food product, microalgal strain selection and metabolic engineering of the species are significant. In addition, to get better quality, a healthier base like wheat, ragi, oats, etc. can be incorporated, rather than using plain floor (maida). To sum up, there is a long way yet to go for commercialization of these microalgae cookies. But the prospects are particularly high because of an increasing demand for more 'natural' products and as for microalgae - a natural consortium of countless bioactive molecules, can deliver multiple or synergistic functions in proper maintenance of good health.

Chapter 6

SUMMERY AND CONCLUSION

Contents

6.1 Summary

Microalgae are one of the most diverse and ancient groups of life on planet earth. Current scenario in microalgae research is mainly centered on the high value molecules (bioactive compounds), algal bio-fuels and CO_2 sequestration. However demand for this group as a nutritional supplement for aquaculture and for other animals (including human beings) keeps on ever increasing. Taking into account their biological significance, commercial value and the industrial require, microalgae were selected as a subject for this PhD study.

Present work entitled "Phenetic characterization, molecular phylogeny and bio-prospecting of selected saline microalgae from Indian subcontinent" was originated from a thought to have a couple of isolates of marine microalgae for 'bio-prospecting of bioactive compounds', but ended with a culture collection of microalgae, their characterization and bio-prospecting for dietary function. In the beginning of the work, it was observed that, even being an emerging high value commodity, microalgae pure cultures are less

available in India. This was the first motive for starting isolation of new strains, which gave rise to a reserve culture collection of microalgae for research and further exploitation.

More than 130 isolates, including 30+ genera belonging to 9 different classes, was the first and prime most achievement of this work. However, it doesn't touch the outcome expected for such a long and diverse sampling which was carried out during the initial two years and continued intermittently in the following years. Specific isolation, purification and culturing techniques are required for obtaining exact aiming species; even so only random procedures were followed here. Similarly, culture media and laboratory conditions were more or less the same for all strains by reason of easy maintenance.

Antibiotic purification done for selected strains was only the preliminary one. The heterogeneous nature of cultures demanded special purification methods other than simple dilution antibiotic treatment. Sonication, the only additional technique, was however employed only for highly contaminated strains and was fruitful too in reducing the bacterial load considerably. Another positive aspect was that none of the strains were sensitive to the type or concentration of antibiotics used. In spite of all, only <50% strains tried were purified by this method and hence recommend further studies in this area which are meticulous for specific strains.

Stability of green and blue green algae (especially the coccoid forms) was really good in both immobilization and freezing preservation experiments. Agar based preservations were found superior to cooling/freezing preservations both by quality and quantity. When agar plating could save 22 strains from 25 tried ones, embedding was successful for 17 strains, whereas cryopreservation (combining

both -80 and LN) was successful only for 13 strains with very less survival rates of cells (<40%). The results show a great prospect for on/in agar immobilization of cultures as a preservation tool for not less than 1-3 years period of time. The study also advocates additional experiments for developing exact protocols with ideal CPA and temperature ranges.

Isolation of microalgae was not so difficult when compared to taxonomic characterization and the culture maintenance. Morphological evaluation is a requisite for the systematic categorization; but was helpful only for those larger strains or those with distinctive cellular arrangements. The structural plasticity and overlapping phenotypes created difficulty in final cataloging, which was later cleared by gene sequence BLAST analysis to a larger extend. Molecular evaluation assisted in final confirmation of genus and even the species for some (e.g. *Isochrysis galbana*, *Nannochloropsis oceanica*, *Dunaliella salina*, *Minutocellus polymorphus*, *Chaetoceros gracilis*), while some strains got revised their morphological identification (e.g. Chlorllales). Among genetic markers, SSU rRNA (18S and 16S) turned to be most effective, particularly because of its larger available database, and better amplification and sequencing rate, compared to others (ITS region, *rbc*L gene and COI gene). COI gene, the universal barcode of animals, however was little useful in microalgal characterization. ITS and COI were the two shortlisted possible superior markers for diatoms and green algae. Still, due to lack of common primers (most of the primer sets were and group-specific), poor amplification and sequence recovery rate and lesser data for similarity search, these markers didn't contribute much in classifying the isolates.

When a combination of many genes along with phenetic traits was used for delineating strains within a genus, it turned to be effective in developing the systematic position, evolutionary lineage and grouping of Indian isolates of

Dunaliella. Based on the size of 18S rDNA amplicon alone it was possible to categorize the isolates into five groups which was further confirmed by ITS and *rbc*L phylogeny. Level of halo-tolerance, β-carotene accumulation property and morphology together with molecular data clearly delineated *D. salina* (S089 & S135) from others. Interestingly three Indian strains (S086, S118 and S121) which were close to *D. salina* by genetic makeup, poorly had the ability to accumulate carotenoids, hence can be considered as a subspecies of *D. salina*. Even though, the study confirmed presence of considerable diversity for *Dunaliella* in Indian salinas, discrepancy between genetic and phenotypic results held back confirmation of species name of strains other than *D. salina.* Here, to develop better understanding of the genus, it was stressed to have a revision of many species (previous Genebank submissions) adopting systematic molecular studies in tandem with strong morphology & physiology.

To fulfill the objective of identifying new strains for larval feeding, it was necessary to know the nutrient profile. For this 20 fast growing & stable isolates were selected, separately grown and the harvested dry biomass (lyophilized) was analyzed for protein, lipid, fatty acid and pigment contents. Individual strains were observed to contain varied levels of chemicals. Nutritional quality of *Chaetoceros* sp. S065 (protein 54%; EPA & DHA 7% each; size 3-6 μm), was superior among the strains on an overall basis, but individual strains were detected with higher values of single nutrients. PUFA profile alone of *Tetraselmis* spp. with higher % of ω-3 LC PUFAs proposes these strains for live feed (Artemia, rotifers etc.) enrichment. Many results were comparable to previous studies (e.g. for *I. galbana, Nannochloropsis* sp.*, Chaetoceros* sp.*, D. salina* etc.) but EPA and DHA in indigenous *Tetraselmis* spp. was better than the earlier reports. Looking into the diverse nutrient profile, a pluerispecific diet of microalgae (rather than using single species)

for feeding can be recommended as a better choice for compatible supply of protein, lipid, PUFAs and carotenoids to the larvae, and thereby ensuring superior growth and survival of the animal. However, final conclusion can be drawn only after feeding trials because the requirement of the animal differs according to species, age, and the environment.

Bioprospecting of microalgae for human diet improvement is a robust area of research and in India other than *Spirulina* and *Chlorella* no other strains were tried in human recipes. India is one of the most potential markets for functional /dietary supplements as major % of population in the country is becoming increasingly health conscious and with a trend in the movement from curative to preventive medicines. Due to a fast and unstable lifestyle dietary intake of nutritive food is progressively decreasing, resulting in deficiencies and health issues. One of the finest nutritional sources can be microalgae which are rich in all essential nutrients. Compared to any other agricultural crop, being a primary producer, the potential of microalgae particularly lies in its high nutritional value, faster growth, high ability to assimilate CO2 and adaptability to diverse climatic conditions. Microalgae are the richest sources of carotenoids and very long chain (VLC) PUFAs. Carotenoids and PUFAs have antioxidant property and have tremendous health benefits including preventive and curative power of cancer and coronary diseases. The idea of using the selected microalgae in biscuits was conceived from previous studies (Gouveia et.al 2008). The trial of microalgae butter cookies had positive results – good sensory qualities and nutritional value. Results show, about 50 gm (1-3% algae) of biscuit may fulfill the daily requirement of nutrients (as per FDA regulations). Hence considering the increasing demand for 'natural' products, microalgae could be a candidate for exploring in food sector.

The salient outcomes of this PhD work are given as follows:

- The most important outcome of this study is the culture collection named as "MBTD-CMFRI- Culture Collection of Marine Microalgae". Presently about 136 strains from marine, brackish water, fresh water and hyper saline habitats are maintained. This collection is a conservatory centre of microalgae bio-diversity and can be a resource for future research, biotechnological developments, bioprospecting and other various projects.
- The collection includes economically important *Dunaliella salina*, *Chlorella vulgaris*, *Arthrospira platensis*, *A. maxima* and common aquatic live feeds – *Isochrysis galbana*, *Nannochloropsis* sp., *Tetraselmis* spp., *Chaetoceros* spp., *Thalassiosira* spp., *Skeletonema* spp., *Navicula* spp., etc.
- Marine isolate of *D. salina* MBTD-CMFRI-S135, which is genetically different from the reference strains (S151 and S089) however with β-carotene production potential (22.92 pgram cell under stress), is a novel finding.
- *Tetraselmis indica* is a new halophilic species of genus *Tetraselmis* was originally reported from Goa (west coast) by Arora et al (2013). Present study reports the same species from other hyper saline waters of India – Pulicat, Kakinada, Bheemli, Kelambakkom, and Thoothukkudi all situated along the east coast.
- For preservation of wide range microalgae, agar plating and agar embedding were identified as easy and superior tools compared to cryopreservation. In cropreservation, DMSO (5 & 10%) was recognized as a suitable CPA than MeOH and GLY.

- The non toxicity of antibiotics (penicillin, streptomycin, gentamycin and kanamycin), was proven even at the highest concentration (2000:10:10:5 μg/ml) for microalgae. Sonication before antibiotic treatment was observed with positive effects in reducing microbial contamination.
- Among the molecular markers, small subunit (SSU) ribosomal RNA gene was realized as more suitable than other (COI, ITS and *rbc*L) genes for preliminary taxonomic identification of microalgae. Based on 18S gene sequences phylogenetic relationship of nearly 100 strains was got described. The study also generated enormous molecular data - 152 sequence submissions in NCBI GenBank.
- Present study also suggests polyphasic approach for taxonomic delineation of microalgae especially for those organisms with high phenotypic plasticity. Such an attempt for Indian isolates of *Dunaliella* was conducted involving morphology, physiology and molecular phylogeny, which had grouped 10 strains into 5 groups, confirming presence of *D. salina* and minimum of 5 other species in the collection.
- Biochemical profile of 20 selected strains for possible use in aquaculture feeding was done, and the data can be utilized for selective larval feeding and for zooplankton enrichments. Several new isolates (e.g. *Tetraselmis*) showed better nutrient profile than old traditional isolates.
- The lipid and fatty acid profile results also shortlists many potential strains in bio-fuel production, which is a supplementary outcome.
- Use of marine microalgae in normal butter cookies shows their prospects as novel nutritional supplements and as an ingredient in

human food items. The sensory evaluation results decode the safety as well as acceptance level of the microalgae product, and hence further exploration of microalgae in modern as well as traditional recipes.

- *Nannochloropsis* sp. was recorded with potential in enriching the cookies with EPA at 1.5% and 2.5% concentration of the alga. Further all microalgae incorporated cookies were appeared with much appealing colour than normal cookies. Hence algae can be used as natural colouring agents in food stuffs with prospective health benefits.

6.2 Conclusion

Present study clearly illustrates the methodology involved in microalgae isolation, identification, culturing and preservation and their prospects for further application in animal and human nutrition. It also describes the role of molecular methods in microalgae taxonomy and characterization; further points out the merits and demerits of different genetic markers over and above directs towards the solutions to improve the traditional taxonomic tools. The bio-prospecting aspects of the microalgae were defined in terms of their nutrient profile for appropriate use in aqua-feeding and in human food. This piece of work also opens a new area of research on microalgae as a supplementary dietary ingredient in traditional as well as contemporary culinary.

A tropical country like India, microalgae cultivation has great prospects, owing to a coastline of 8129 km, availability of sea water, adequate sunlight and stable climate with an average temperature of 30°C. A cultivation technique without compromising any natural resources, but utilizing unused coastal land masses by providing livelihood to fisherwomen and SHGs adds on the advantage. Currently, the cost of biomass production (after culturing,

harvesting and processing final powdered product may cost Rs. 2-20/gm) and lack of appropriate harvesting systems are the major challenges. However a cost effective program with Govt. funding/ private public participation/ voluntary, social organization initiatives supported by a strong R&D can be a solution to enhance the production. Similarly a well planned, executable, cost effective awareness program can be initiated for the acceptance of the products. Production and exploitation of microalgae can also be used as one of the methods in the mitigation of recent issues such as climate change, where freshwater and energy costs are minimal, while the biomass is an important outcome, to cater the needs of ever increasing population – alternative source of quality food and energy.

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ANNEXURES

Annexure 1: f/2 Media composition (Anderson 2005)

Sl. No.	Name	Nutrients	Stock Solution g/L	Quantity used	Conc. In final medium
1	F1 solution	NaNO3	75	1ml	8.82×10^{-4}
2	F2 solution	NaH2PO4	5	1ml	3.62×10^{-5}
3	F3 Solution*	Na2SiO3	30	1ml	1.06×10^{-4}
4	F4 trace metal soln.	Following recipe/1L		1ml	
	Into 950 ml of DDW Dissolve EDTA and other components, make up to 1L				
	1	FeCl3.6H2O	….	3.15gm/L	1.17×10^{-5}
	2	Na2EDTA.2H2O	…..	4.36gm/L	1.27×10^{-5}
	3	MnCl2.4H2O	180.0gm	1ml/L	9.10×10^{-7}
	4	ZnSO4.7H2O	22.0gm	1ml/L	7.65×10^{-8}
	5	CoCl2.6H2O	10.ogm	1ml/L	4.20×10^{-8}
	6	CuSO4.5H2O	9.8gm	1ml/L	3.93×10^{-8}
	7	Na2MoO4.2H2O	6.3gm	1ml/L	2.60×10^{-8}
5	F5 Vit. Soln.	Following recipe/1L		0.5ml	
	1	Thiamine.HCl (B1)	……	200mg/L	2.96×10^{-7}
	2	Biotin (Vit.H)	1.0gm/L	1ml/L	4.05×10^{-9}
	3	Cyanocobalamin (B12)	1.0gm/L	1ml/L	3.69×10^{-10}

Annexure 2: Paoletti medium (For *Spirulina*, Volkmann et al. 2008)

Macronutrients (weigh and add to 1L DDW)		
Sl. No.	**Nutrients**	**Quantity g/L**
1	KNO3	2.5
2	K2SO4	1.9
3	MgSO4.7H2O	0.25
4	CaCl2.2H2O	0.05
5	K2HPO4	0.5
6	NaHCO3	15.15
7	Na2CO3	8.9
8	NaCl	0.92
Micronutrients Stock in 1L (to the above medium add 1 ml from this)		
9	H3BO3	2.86
10	MnCl4.H2O	1.81
11	ZnSO4.7H2O	0.22
12	Na2MoO4.2H2O	0.39
13	CuSO4.5H2O	0.079
14	Co(NO3).6H2O	0.049
Fe.EDTA solution Stock in 1L (to the above medium add 1 ml from this)		
15	EDTA	29.8
16	FeSO4.7H2O	24.9

NB: If Paoletti medium was prepared in sea water (~25 ppt) medim only half strength nutrients were used.

Annexure 3: Modified Johnsons Medium (J/l) (Borowitzka, M.A., 1988).

To 980 ml of distilled water add:	
NaCl	**as needed to obtain desired salinity**
$MgCl_2 \cdot 6H_2O$	1.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
KCl	0.2 g
$CaCl_2 \cdot 2H_2O$	0.2 g
KNO_3	1.0 g
$NaHCO_3$	0.043 g
KH_2PO_4	0.035 g
Fe-solution	10 ml
Trace-element solution	10 ml
Fe solution (for 1 litre)	
Na_2EDTA	189 mg
$FeCl_3 \cdot 6H_2O$	244 mg
Trace-element solution (for 1 litre)	
H_3BO_3	61.0 mg
$(NH4)_6Mo_7O_{24} \cdot 4H_2O$	38.0 mg
$CuSO_4 \cdot 5H_2O$	6.0 mg
$CoCl_2 \cdot 6H_2O$	5.1 mg
$ZnCl_2$	4.1 mg
$MnCl_2 \cdot 4H_2O$	4.1 mg
Adjust pH to 7.5 with HCl	

Annexure 4: Published Article

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Phenotypic and genetic characterization of *Dunaliella* (Chlorophyta) from Indian salinas and their diversity

Krishna Preetha, Lijo John, Cherampillil Sukumaran Subin and Koyadan Kizhakkedath Vijayan*

Abstract

Background: The genus *Dunaliella* (Class – Chlorophyceae) is widely studied for its tolerance to extreme habitat conditions, physiological aspects and many biotechnological applications, such as a source of carotenoids and many other bioactive compounds. Biochemical and molecular characterization is very much essential to fully explore the properties and possibilities of the new isolates of *Dunaliella*. In India, hyper saline lakes and salt pans were reported to bloom with *Dunaliella* spp. However, except for the economically important *D. salina,* other species are rarely characterized taxonomically from India. Present study was conducted to describe *Dunaliella* strains from Indian salinas using a combined morphological, physiological and molecular approach with an aim to have a better understanding on the taxonomy and diversity of this genus from India.

Results: Comparative phenotypic and genetic studies revealed high level of diversity within the Indian *Dunaliella* isolates. Species level identification using morphological characteristics clearly delineated two strains of *D. salina* with considerable β-carotene content (>20 pg/cell). The variation in 18S rRNA gene size, amplified with MA1-MA2 primers, ranged between ~1800 and ~2650 base pairs, and together with the phylogeny based on ITS gene sequence provided a pattern, forming five different groups within Indian *Dunaliella* isolates. Superficial congruency was observed between ITS and *rbc*L gene phylogenetic trees with consistent formation of major clades separating Indian isolates into two distinct clusters, one with *D. salina* and allied strains, and another one with *D. viridis* and allied strains. Further in both the trees, few isolates showed high level of genetic divergence than reported previously for *Dunaliella* spp. This indicates the scope of more numbers of clearly defined/unidentified species/sub-species within Indian *Dunaliella* isolates.

Conclusion: Present work illustrates Indian *Dunaliella* strains phenotypically and genetically, and confirms the presence of not less than five different species (or sub-species) in Indian saline waters, including *D. salina* and *D. viridis*. The study emphasizes the need for a combined morphological, physiological and molecular approach in the taxonomic studies of *Dunaliella*.

Keywords: Dunaliella, Diversity, India, 18S rDNA, ITS, *rbc*L gene

Background

Dunaliella, the unicellular microalga, is one of the best studied organisms in both general and applied phycology for its higher tolerance to extreme conditions of salinity, light, temperature and pH, as well as for its richness in natural carotenoids, glycerol, lipids and many other bioactive compounds [1-4]. *Dunaliella salina* is reported as the most halotolerant photosynthetic eukaryote with a remarkable degree of tolerance from 0.5 to 5 M salt concentrations (30–300 ppt) [2]. This genus naturally inhabits saline and hypersaline waters and has a cosmopolitan distribution [5] and of the 28 species of *Dunaliella*, 23 are saline or hypersaline [3,6-9].

Many countries, including India, use *D. salina* for the industrial production of β-carotene with wide range of applications [4,10-12]. Apart from *D. salina*, *D. tertiolecta* is used in aquaculture, while many other species were found promising for the production of biofuel,

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bioprospecting of antioxidants, bioactive compounds etc. [4,6]. Considering the economic importance, most of the studies were mainly focused on the taxonomic, physiological and biotechnological aspects of the halophilic species *D. salina* [5,13-19] (especially from Indian subcontinent) and on the marine species *D. tertiolecta*. But similar exclusive or comparative studies are rarely available for other species [20-23], probably due to their lesser importance and/or limited distribution.

Typically the taxonomy of *Dunaliella* anchors on the morphological and physiological features of the organism. Apart from the general morphology, salinity tolerance and carotenoid (especially β-carotene) production are the two commonly studied physiological attributes of *Dunaliella*, where considerable variations have been accounted at inter and intra-species levels [24,25]. Recently, Borowitzka and Siva [3] have given a detailed account of taxonomic revision of the genus *Dunaliella* with special emphasis on saline species bringing more clarity in classification. *Dunaliella* are unique in having a thin plasma membrane instead of a rigid cell wall [26] and are able to change their cell shape and volume in response to changes in osmolarity and other growth conditions [17,27-29]. Due to this high plasticity of cell morphology, the traditional practice of species differentiation merely based on light microscopic observations becomes difficult and time consuming. Consequently many misidentifications arose in the literature which brought in controversies and confusions in the taxonomic organization of the genus *Dunaliella* [3,5].

Molecular taxonomy emerged as a faster and powerful tool as it is consistent and independent from environmental factors and growth stages [30]. It seems to be an advanced and reliable device for the characterization and differentiation of morphologically plastic organisms. Since 1999, molecular characterization has been found promising in the taxonomy of *Dunaliella* [6,29]. Currently 18S rRNA gene [5,31], Internal Transcribed Spacer (ITS) region [14,23,27] and large subunit of the ribulose-bisphosphate carboxylase (*rbc*L) gene [9] are being widely used as effective molecular tools in *Dunaliella* characterization and biodiversity studies. Use of these molecular markers has resulted in the redesignation of many species [8,32]. Nevertheless, the confusion regarding the taxonomy still persists due to the misidentifications, and will be there until a complete revision is made, using an integrated approach with molecular phylogeny, supported by morphological and physiological attributes. Many researchers opined to have such an integrated approach rather than a single system of taxonomic identification [3,32].

In India, *Dunaliella* are found in salt pans, saline and hypersaline ponds, lakes, pools etc. as a major primary producer. Many species including *D. salina* have been reported [33] to form blooms in salt pans. However, taxonomic characterization of Indian *Dunaliella* strains based on phenotypic and molecular traits is rarely available. In this background, we conducted a study on the characterization of *Dunaliella* strains isolated from the Indian salinas, using morphological, physiological and molecular tools and have made an attempt to depict the best possible description on Indian *Dunaliella* spp. Based on the results obtained, taxonomic position and diversity aspects of the Genus *Dunaliella* from Indian salinas are discussed.

Results and discussion

Morphological & physiological parameters

Morphologically all ten strains of the green biflagellate chlorophytes, isolated from 7 different locations along the Indian coast (Figure 1 & Table 1), were identified as *Dunaliella* (Figure 2) following the revision of the genus by Borowitzka and Siva [3]. Of the 10 strains, 9 were isolated from hypersaline water bodies and 1 (S135) was marine. Though purified by agar plating, the cultures were not axenic. All morphological characteristics of different geographical Indian isolates of *Dunaliella* are summarized in Table 2.

High levels of morphological plasticity in cell shape and size was observed among all the 10 *Dunaliella*, strains, but a general consistency in cell size was noticed within the range given (Table 3) [34,35]. Among the 10 strains, S135 (Calicut, marine isolate), S089 (CMFRI old strain isolated from Chennai) and S147 (Kutch) were considerably larger while strain S133 (Kutch) was the smallest.

In salinity tolerance study (0.5 – 4.5 M NaCl), sufficient growth (approximately 5–20 million cells/ml in 28 days from an initial cell density of 15–60 thousand cells/ml) was obtained for each strain in different salinities with optimum growth at 1.5 or 2.5 M salt concentrations (growth rate was 0.1±0.05 div.d^{-1} during exponential growth period), emphasizing that all the strains (including the marine isolate S135) are halophilic in nature. Beta carotene was quantified in all the isolates (Table 2) at 'normal' and stressed growth conditions. Under stress (3.5M NaCl, irradiance of 100–150 μmol photons m^{-2} s^{-1}) higher level of the pigment (23.4 & 22.9 pg/cell) was recorded in the 2 Indian strains, S089 and S135 respectively while for the Australian reference strain *D. salina* CS265, it was nearly 36 pg/cell. The 3 strains turned orange/red at high salinity (Figure 2, c, m & p). Lower quantities of the pigment (<2 pg/cell) were observed in the strain S133 from Kutch and the 2 Goa strains, S122 and S125. For the remaining strains it was around 2–7 pg/cell, under stress.

Among the many listed attributes, cell size, colour, stigma and β-carotene accumulation are the major traits used to discriminate carotenogenic *Dunaliella* spp. like

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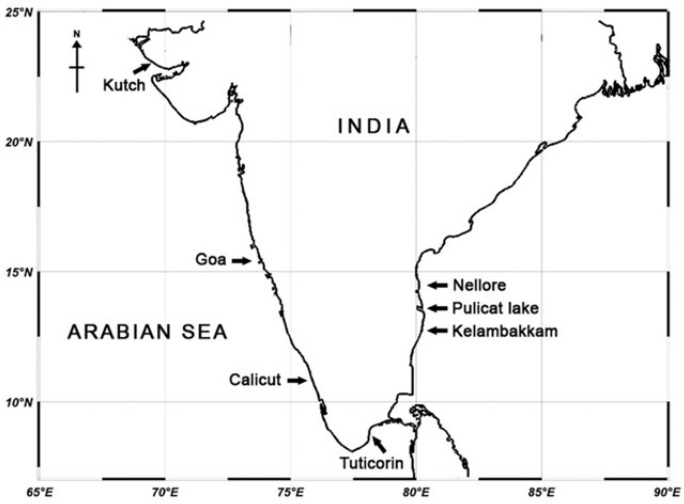

Figure 1 Sampling locations of *Dunaliella* strains used in the present study along the Indian coast.

D. salina and *D. bardawil/salina*. Red *D. salina* (especially at high salinity) was reported to have significantly large cell size than other common strains like *D. parva*, *D. viridis* and *D. tertiolecta* [3]. Limited carotenogenic capacity also discriminated other strains from *D. salina* where the latter can accumulate >20 pg β-carotene/cell [3]. Coesel et al. [36] and Olmos et al. [5] obtained 10 pg/cell of β-carotene under non-stressful growth conditions for the two hyper producing strains of *D. salina*, CCAP 19/30 and 19/18 respectively. In the present study, morphological and physiological observations of the 2 strains, S089 and S135, revealed that they are Indian strains of *D. salina*. Discrimination derived from basic morphology (taxonomic key), characterized the remaining strains as *D. viridis* except S125 (*D. minuta*?), S133 (*D. viridis/D. bioculata*?) and S147 (*Dunaliella sp.)* (Table 2). Detailed morphology and physiology based studies illustrated considerable diversity within the Indian strains of *Dunaliella* but a little confusion prevailed due to overlapping features with more than one reported species (like the cell size increase and β-carotene content of strains S121 and S118 at higher salinity, two stigmata of S133 at lower salinity and pyrenoid characteristics of S125) (Tables 2 and 3). In the present investigation, molecular characterization was used as a tool to resolve the confusion.

Molecular characterization based on 18S rRNA gene size

Amplification of 18S rRNA gene with primers MA1 & MA2 from different *Dunaliella* isolates in the present study gave products with size ranging from ~1820–2640 bp (Table 1 & Figure 3). The banding pattern observed among the present isolates was found matching with the reported gene sizes of 18S rDNA using MA1-MA2 primers [5,29]. The dissimilarity in product size observed among the different isolates could be explained based on the presence/absence or difference in the size of introns across different species of *Dunaliella* [5,29]. With regard to the presence of 3 types of Group I introns in 18S rRNA gene [37], Olmos et al., [31,38] designed a set of conserved primers (MA1, MA2 & MA3) and a set of species specific primers (DSs, DPs, DBs). They used the conserved primers (MA1 & MA2) for preliminary differentiation of various known species of *Dunaliella* based on the size of the PCR product. Subsequently, morphologically identical *Dunaliella* strains (e.g., *D. salina* and *D. bardawil*) got discriminated by position and number of the introns [5,29]. Based on these reports 18S rDNA of *D. tertiolecta* (~1770 bp) lacks an intron, *D. salina* (~2170 bp) has only one intron at 5' terminus, *D. viridis* (~2495 bp) has one longer intron again at 5' terminus and *D. parva* and *D. bardawil* have two introns (~2570 bp) one each at 5' and 3' terminus. Other than these strains, *D. peircei* having ~2088 bp (one intron at 5' terminus) was also reported.

In the present study, based on the 18S rDNA gene size, clear grouping of all the 10 Indian *Dunaliella* strains was possible (Figure 3 & Table 1). Out of the 10 strains only 1 strain, S147 (Kutch) produced the shortest band (~1820 bp) showing similarity to that reported for

Table 1 Geographical origin and gene sequence accession details of *Dunaliella* strains studied in the present work

Groups	Strain code	Isolated from	Geographic co-ordinates	Month of collection	Salinity of the sampled water	18S rDNA product size	Genebank accession No.		
							18S rDNA	ITS region	*rbcL* gene
	CS265	*Dunaliella salina*; Reference strain from CSIRO collection of living microalgae, Australia				2210 bp	JN807321	JN797804	JN797820
I	MBTD-CMFRI-S135	Sea water, Calicut, Kerala (WC)	11°15′ N 75°46′ E	May 2009	33 ppt	2230 bp	JF708161	JN797802	JN797818
	MBTD-CMFRI-S089	Kelambakkom salt pan, Chennai, TN (EC)	Culture maintained in CMFRI phytoplankton culture collection, isolated from Chennai salt pan.			2210 bp	JF708173	JN797806	JN797811
II	MBTD-CMFRI-S118	Salt pan, Nellore, AP (EC)	14°16′ N 80°07′ E	March 2009	300 ppt	2290 bp	JN807316	JN797808	JN797813
	MBTD-CMFRI-S086	Salt pan, Tuticorin, TN, (EC)	08°47′ N 78°09′ E	February 2009	300 ppt	2290 bp	JF708169	JN797805	JN797810
	MBTD-CMFRI-S121	Pulicat salt lake, AP (EC)	13°40′ N 80°11′ E	March 2009	150 ppt	2250 bp	JN807317	JN797809	JN797814
III	MBTD-CMFRI-S115	Kelambakkom salt pan, Chennai, TN (EC)	12°45′ N 80°12′ E	March 2009	380 ppt	2550 bp	JN807315	JN797807	JN797812
	MBTD-CMFRI-S122	Salt pan, Ribandar, Goa (WC)	15°30′ N 73°51′ E	May 2009	280 ppt	2550 bp	JN807318	JN797799	JN797815
	MBTD-CMFRI-S133	Salt pan, Kutch, Gujarat (WC)	23°50′ N 69°39′ E	July 2009	320 ppt	2530 bp	JF708183	JN797801	JN797817
IV	MBTD-CMFRI-S125	Salt pan, Pilar, Goa (WC)	15°26′ N 73°53′ E	May 2009	260 ppt	2640 bp	JN807319	JN797800	JN797816
V	MBTD-CMFRI-S147	Salt pan, Kutch, Gujarat (WC)	23°50′ N 69°39′ E	April 2009	180 ppt	1820 bp	JN807320	JN797803	JN797819

NB: For convenience strain codes used in text included only third part of full strain code (e.g., 5086). *AP*, Andhra Pradesh; *TN*, Tamil Nadu; *WC*-west coast; *EC*, east coast.
Indian strains were grouped into subsets based on the 18S rDNA size obtained by PCR amplification with MA1-MA2 primers.

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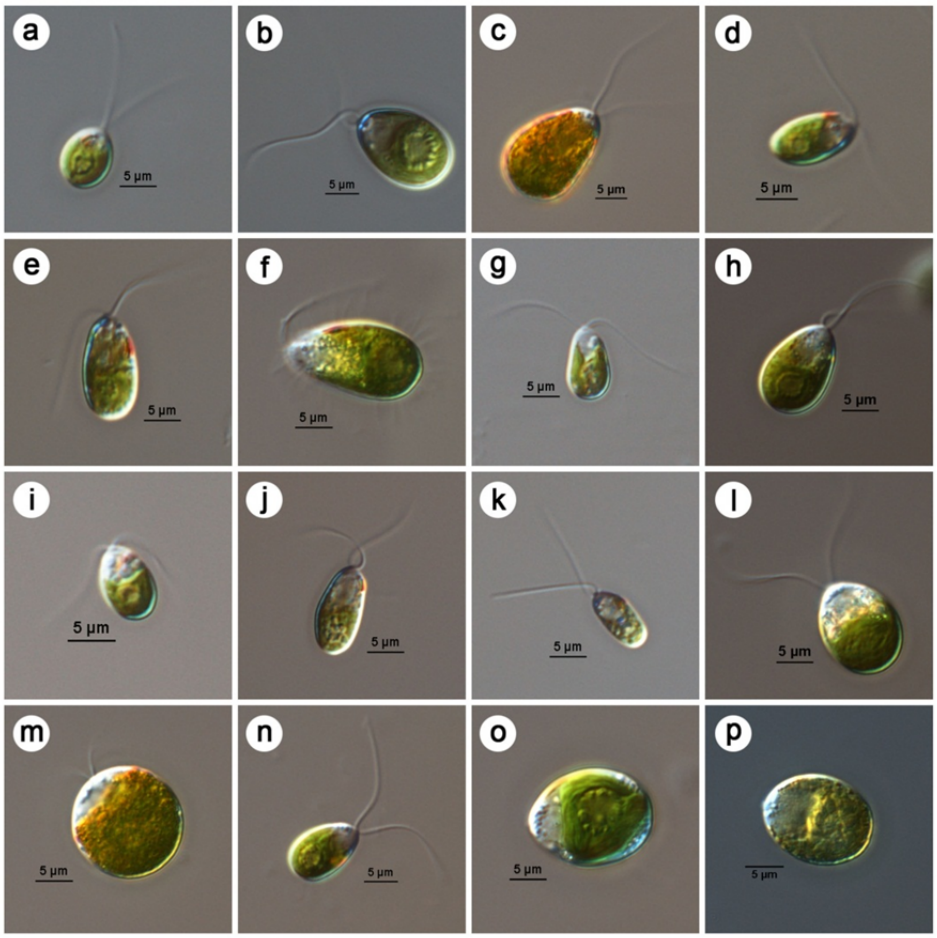

Figure 2 DIC microscopic images of different *Dunaliella* isolates. (**a**) *Dunaliella sp.* S086 (Tuticorin salt pan), (**b**) & (**c**) *D. salina* S089 (CMFRI old strain), (**d**) *D. viridis?* S115 (Chennai salt pan), (**e**) & (**f**) *Dunaliella* sp. S118 (Nellore salt pan), (**g**) & (**h**) *Dunaliella sp.* S121 (Pulicat lake), (**i**) *D. viridis?* S122 (Goa salt pan), (**j**) *Dunaliella* sp. S125 (Goa salt pan), (**k**) *Dunaliella* sp. S133 (Kutch salt pan), (**l**) & (**m**) *D. salina* S135 (Calicut marine isolate), (**n**) *Dunaliella* sp. S147 (Kutch salt pan), (**o**) & (**p**) *D. salina* CS265 (Australian reference strain). In brackets given the origin of isolates. (**c**) & (**m**) orange red cells of Indian isolates of *D. salina* (S089 & S135) grown at 4.5 M NaCl concentration. (**f**) & (**h**) large yellow green cells of S118 and S121 at 4.5 M NaCl. (**p**) Reference strain *D. salina* CS265 at 2.5 M NaCl turning orange. Scale bar given – 5 µm.

D. tertiolecta (~1770 bp) probably due to the absence of any introns (Group V). The 2 Indian strains S089 (CMFRI old strain, Chennai) and S135 (Calicut marine isolate) and the reference strain CS265 (*D. salina*) produced ~2200 bp size band (Table 1) closer to the reported *D. salina* (~2170 bp). This further supported phenotypic identification of the above 2 Indian strains as *D. salina* (Group I). Studies using the 18S PCR products revealed a clear separation of morphologically similar strains (*D. viridis?*), into 2 groups - (Group II & Group III in Table 1 & Figure 3). The 18S rDNA size (~2300 bp) of Group II strains (S086 (Tuticorin), S118 (Nellore) & S121 (Pulicat)) was showing an indication that these strains are more close to *D. salina* than *D. viridis*. While

Table 2 Morphological and physiological characteristics of 10 Indian *Dunaliella* strains

Groups	Strain Code	Cell colour	Cell shape	Flagella length	Stigma	Pyrenoid	Refractile granules	Mode of reproduction observed	β Carotene normal/stress (pg/cell)	Salinity optimum	Identified as
I	MBTD-CMFRI-S135	Green to red	Ovoid, spherical, cylindrical	1.3 or 1.5 to cell length	Not clearly visible or diffuse	Large with distinct amylosphere	Absent	Cell division	8.68/22.94	1.5M NaCl	*D. salina*
	MBTD-CMFRI-S089	Green to red	Ovoid, spherical	1.3 to cell length	Not visible/ Diffuse large	Large with distinct amylosphere	Absent	Cell division	6.53/23.36	1.5M NaCl	*D. salina*
II	MBTD-CMFRI-S118	Green to orange	Ovoid spherical	1.5 to cell length	One; large, red, median, diffuse	Small with amylosphere	Absent	Sexual, cell division	2.11/3.47	2.5M NaCl	*Dunaliella sp.*
	MBTD-CMFRI-S121	Green	Ovoid pyriform	1.5-2 to cell length	One; large, red, median, distinct	Large with amylosphere	Absent	Sexual, cell division	1.59/2.17	1.5M NaCl	*Dunaliella sp.*
	MBTD-CMFRI-S086	Green	Ovoid, oval or pyriform	1.5- 2 to cell length	One; Small, red, median, distinct	Small with amylosphere	Present	Sexual, cell division	2.68/3.41	0.5 M NaCl	*Dunaliella sp.*
III	MBTD-CMFRI-S115	Green	Ovoid, oval or fusiform	1.3 to cell length	One; small, red, anterior, distinct	Small with amylosphere	Absent	Palmella, aplanospores	1.05/1.99	1.5M NaCl	*D. viridis?*
	MBTD-CMFRI-S122	Green	Oval, cylindrical,	1.3 to cell length	One; large, red, anterior, distinct,	Large, Amylosphere	Present	Palmella stage	0.67/1.78	1.5M NaCl	*D. viridis?*
	MBTD-CMFRI-S133	Yellow green	Fusiform, Elliptical	1.3 to cell length	One, Two at lower salinity; small, red, median, distinct	Small, with amylosphere	Absent	Cell division, Palmella, aplanospores	0.51/1.26	1.5M NaCl	*D.viridis/ D. bioculata?*
IV	MBTD-CMFRI-S125	Green	Cylindrical fusiform	Equal or 1.3 to cell length	One, large, red, anterior, distinct	Small, with distinct separate starch grains	Absent	Cell division	0.70/1.8	1.5M NaCl	*D. minuta?*
V	MBTD-CMFRI-S147	Green	Oval, fusiform	1.5 or 2 to cell length	One, large, red, median distinct	Large with amylosphere	Present	Palmella (dominant stage), Cell division	0.89/6.7	1.5M NaCl	*Dunaliella sp.*

Grouping of the subsets was formed based on common morphological features including cell size and β-carotene accumulation at high salinity and light (stress).

Table 3 Descriptive statistics of cell size variables and F-values (derived from the analysis of variance) of different *Dunaliella* isolates from Indian coast

Groups	I		II		III			IV	V	
Strain code	MBTD-CMFRI-S135	MBTD-CMFRI-S089	MBTD-CMFRI-S086	MBTD-CMFRI-S118	MBTD-CMFRI-S121	MBTD-CMFRI-S115	MBTD-CMFRI-S122	MBTD-CMFRI-S133	MBTD-CMFRI-S125	MBTD-CMFRI-S147	F value
Length µm	17.51±1.78 (12.30-21.17)	14.12±2.25 (10.01-18.82)	9.15±1.02 (6.44-10.68)	9.51±1.09 (7.96-12.25)	9.37±1.30 (6.45-11.77)	9.02±0.96 (6.79-12.12)	8.46±1.12 (5.62-10.55)	7.91±0.93 (6.54-9.78)	9.89±1.37 (8.38-12.99)	11.17±1.50 (8.02-13.83)	138.33*
Width µm	10.30±1.96 (8.61-19.79)	9.57± 1.35 (7.46-12.58)	6.14±0.92 (3.52-8.08)	6.91±0.74 (5.84-8.76)	5.94±0.96 (4.14-7.54)	5.09±0.77 (3.02-6.98)	4.74±0.48 (3.91-5.76)	3.89±0.60 (3.11-5.10)	4.34±0.69 (3.27-5.95)	7.23±1.15 (5.40-10.07)	125.85*

Measurements are presented as, Mean ± SD (min. - max.); *Significant at the 1% level; *SD* is standard deviation.
Grouping of subsets was statistically formed based on the average length/width of the *Dunaliella* cells.

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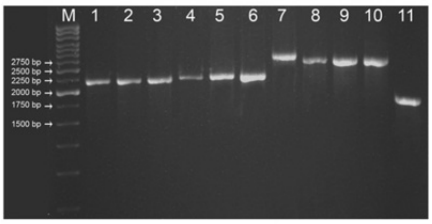

Figure 3 18S rDNA amplification with MA1 & MA2 primers in 1% Agarose gel. Lane 1–11 CS265, MBTD-CMFRI-S089, S135, S086, S118, S121, S125, S115, S122, S133 and S147 respectively & Lane M -250 bp ladder (Genie, India).

group III strains (S122 (Goa), S115 (Chennai) & S133 (Kutch)) gave a band size of ~2550/2530 bp which could be compared to the reported *D. viridis* (2495 bp) or *D. parva* (~2570 bp) probably with 1 or 2 introns. The band size of the Goa strain S125 (~2640 bp, Group IV) was however not in accordance with any of the reported species of *Dunaliella* [5]. Partial (~600 bp) sequencing of 5' terminus region of the PCR products could not confirm the presence of any introns, while the generated partial sequence information (refer Table 1 for GenBank accessions) was found to be highly conserved across species and therefore could not specify the species delineation. Further characterization was carried out based on molecular phylogeny of a more variable ITS region and a conserved *rbc*L gene for more clarification about species lineages of Indian *Dunaliella*.

ITS phylogeny

The phylogenetic analysis based on ITS region (~700 bp) using maximum likelihood confirmed high level of genetic diversity within Indian *Dunaliella* isolates. All *Dunaliella* spp. (including the sequences from NCBI, Table 4) were found to be separated into 3 major clusters, with *Chlamydomonas reinhardtii* forming an out group as expected (Figure 4). Majority of the named species of *Dunaliella* from NCBI were found to be grouped under a single cluster (clade 1) except *D. salina* CCAP 19/18, *D. salina/D. viridis* CCAP 19/3, *D. viridis* CONC 002 and *Dunaliella* spp. ABRIINW M1/2, SPMO 200–2, SPMO 600–1 and the reference strain of *D. salina* CS265. When out grouped with *D. tertiolecta* (Figure 5), the ITS tree branching was found well supporting the morphology and 18S rRNA gene size based grouping (Group I-V) of the 10 new isolates of *Dunaliella*.

The genetic divergence values observed among clade 2 (Figure 4) isolates ranged up to 9.1% (between S089 & CCAP 19/3), which was comparable to that observed between different species of the genus *Dunaliella* [14,17]. The 2 Indian *D. salina* strains (Group I, Figure 5) S089 (CMFRI old strain) and S135 (Calicut marine isolate) got clustered with the Australian *D. salina* strains CS265 and CCAP 19/18 with divergence values ranging from 1.9% (between CS 265 & CCAP 19/18) to 5.6% (between S089 & S135). Whereas, the strains S086 (Tuticorin), S121 (Pulicat) and S118 (Nellore) were found closer to *D. salina/D. viridis* CCAP 19/3. The much higher divergence (>8%) of the 3 Indian strains from the carotenogenic *D. salina* strains (CCAP19/18 and CS265) was in agreement with the grouping of the 3 strains in Group II (Figure 5) based on the morphological, physiological and 18S rDNA size based analyses.

The remaining 5 Indian *Dunaliella* strains (S115, S122, S125, S133 and S147) along with *D. viridis* CONC 002 formed a separate cluster (clade 3 of Figure 4). The strains showed divergence range from 0% (between S115 & S122) to 7.6% (between S147 and *D. viridis* CONC

Table 4 Sequence accession no.s of *Dunaliella* and other strains from NCBI database included in present study

Strain	ITS Accession No.	rbcL Accession No.
D tertiolecta CCAP 19/27	EF473748	
D tertiolecta ATCC 30929	EF473742	
D tertiolecta SAG 13.86	EF473738	
D tertiolecta Dtsi	EF473730	
D tertiolecta CCMP 1302		DQ313205
D tertiolecta UTEX 999		DQ313203
D peircei/D viridis UTEX 2192		DQ313196
D primolecta	DQ116745	DQ173090
D primolecta		AB127992
D primolecta UTEX 1000		DQ313198
D parva	DQ116746	
D parva/D viridis UTEX 1983		AJ001877
D bioculata UTEX 199	DQ377086	DQ313195
D bioculata		AB127991
D salina Dsge	EF473732	
D salina SAG 42.88	EF473741	
Dunaliella sp. hd10	DQ116747	
D salina Ds18S3	FJ360758	
D salina Ds18S1	FJ360756	
D salina/D viridis CCAP 19/3 (UTEX 200)	EF473744	DQ313197
D salina CCAP 19/18	EF473746	GQ250046
D viridis CONC 002	DQ377098	DQ313206
Dunaliella sp. ABRIINW M1/2	EU927373	
Dunaliella sp. SPMO 200-2	DQ377106	DQ313211
Dunaliella sp. SPMO 600-1	DQ377120	DQ313218
Chlamydomonas reinhardtii	AB511842	
Paulschulzia pseudovolvox		D86837

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002). The 2 *D. viridis(?)* strains S115 (Chennai) & S122 (Goa) and the *D. viridis/D. biocuata(?)* strain S133 (Kutch) were found in close proximity (mean divergence of 2.22%) with CONC 002 *D. viridis* (Group III of Figure 5). The other 2 strains S125 and S147 (Group IV & V of Figure 5) were found to be well separated from the above group with divergence values of 4.98% and 6.42% respectively with *D. viridis* CONC 002.

The mean pair wise genetic distance values observed among the Indian isolates of the 2 major clades (5.35% for clade 2 and 5.12% for clade 3) were comparatively higher than that observed among the named species of *Dunaliella* (1.14% clade 1). Further, the genetic divergence values observed among the Indian *Dunaliella* isolates based on ITS sequence variations were considerably higher than that reported in *Chlamydomonas* spp. (a minimum of 3.5% between 2 species) by Coleman & Mai (1997) [39]. Thus, the pattern of genetic divergence, along with the phylogenetic divergence pattern, clearly indicates the presence of at least 5 or more number of species/sub-species among the 10 Indian strains (including *D. salina* and *D. viridis*).

*rbc*L gene phylogeny

The pattern of genetic diversity observed among the Indian *Dunaliella* strains based on *rbc*L gene sequence variations was in accordance with the above observations based on 18S rDNA and ITS analysis except for the positioning of S147 and S135. The phylogenetic tree constructed, using maximum likelihood (Figure 6) analysis with *rbc*L gene sequence data, was forming 2 major clusters with *Paulschulzia pseudovolvox* as out group. The mean genetic divergence value observed between the 2 clades was 5.89% and that observed among different isolates of *Dunaliella* ranged from 0.16% to 7.73%.

Being a protein coding gene, the pairwise genetic divergence (Tamura 3 parameter) values observed among *Dunaliella* isolates based on *rbc*L gene sequences were found to be less in comparison with that observed in ITS (a non coding region) sequences. The independent phylogenetic analyses using ITS (Figure 4) and rbcL gene (Figure 6) sequences (Kimura 2 and Tamura 3 parameters respectively) were found to be taxonomically incongruent especially in clade 1. The major topological change observed was the change in the positioning of the isolate S147. Within *rbc*L phylogeny, this strain from Kutch was found closely allied with clade 1 (Figure 6), *w*hereas, with ITS data it was close to *D. viridis* CONC 002 and other Indian isolates (S125, S133, S115 and S122) in clade 3 (Figure 4). Similarly, the marine *D. salina* strain S135, got clustered with non-carotenogenic strains in sub-clade C (with divergence of 1.15%) instead of carotenogenic *D. salina* (sub-clade B).

Clustering of all the remaining 8 strains in both ITS and *rbc*L phylogenies was more or less similar. As expected S089 (*D. salina*, CMFRI old strain) clustered with carotenogenic *D. salina* species CS265 and CCAP 19/18 in clade 1, (sub-clade B, with 100% similarity). The positioning of 3 strains S086, S118 & S121 (sub-clade C, in Figure 6) along with *D. salina/D. viridis* UTEX 200/ CCAP 19/3 and *D. peircei/D. viridis* UTEX 2192 (with <1% divergence value) strongly suggests further taxonomic revision. Similarly, in clade 2, the positioning of the Goa isolate S125 (with maximum divergence 7.33%) and the clustering of strains S133, S122 & S115 with *D. viridis* CONC 002 and *D. parva/D. viridis* UTEX 1983 (with divergence values 3.15% & 0.33% respectively) was in concordance with ITS phylogeny.

Grouping of Indian *Dunaliella* strains

The 2 larger carotenogenic strains (>20 pg/cell β-carotene content) S135 and S089 forming the GROUP I, produced 18S rDNA size ~2200 bp and clustered with *D. salina* CCAP 19/18 and CS265 in ITS phylogeny. These results confirmed the taxonomical identity of the 2 strains as *D. salina* (Section Dunaliella). But closeness of S135 to the 2 morphologically dissimilar, lower β-carotene content strains, S121 (Pulicat) and S086 (Tuticorin) in *rbc*L phylogeny has to be noted, which may be due to its marine origin.

GROUP II included the strains S086 (Tuticorin), S118 (Nellore) and S121 (Pulicat), which clustered with *D. salina/D.viridis* (CCAP 19/3) in ITS phylogeny and had ~2300 bp band for 18S rDNA. The present study shows the closeness of these 3 strains to *D. salina* by molecular analysis (18S rDNA size and ITS & *rbc*L phylogenies) rather than by morphological features. These strains were with lesser β-carotene content (~2–4 pg/cell) and cells were always green (only S118 turned slightly orange at higher salinity), smaller and with a clear stigma, which were not corresponding with hyper β-carotene producer strain of *D. salina* and are more or less characters of *D. viridis* [3]. However, there is a description of a greener *D. salina* (KCTC10654BP) from Korea [17] with low cellular β-carotene. But 18S rDNA size details are not available for the above Korean strain for comparison. All these factors along with the appearance of *D. viridis/D. peircei* UTEX 2192 close to S118 in *rbc*L phylogeny (clade 1, sub-clade C) and 18S intron phylogeny of *D. peircei* UTEX 2192 by Hajezi et al. [29], emphasizes a need of revisiting the taxonomic identity of all the above reported strains along with the 3 Indian strains using molecular approaches.

GROUP III was formed by 3 strains, S115 (Chennai), S122 (Goa) and S133 (Kutch) allied to *D. viridis*. This further confirms the possibility of the former 2 strains to be

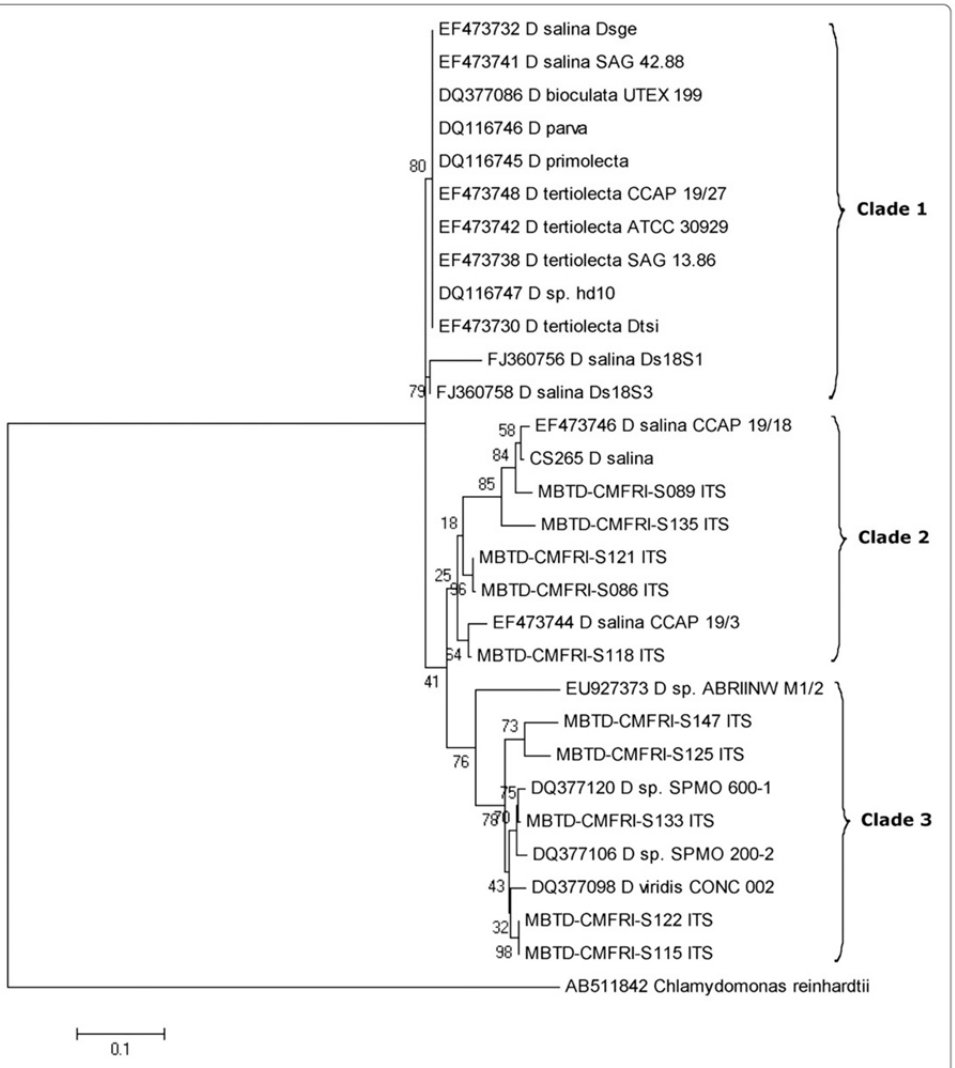

Figure 4 Phylogenetic tree of the Maximum Likelihood (ML) analysis inferred from the nuclear encoded ITS regions including 5.8S rDNA of *Dunaliella*. Bootstrap values for 1000 replicates are given at the internal nodes.

Indian isolates of *D. viridis*, while the presence of 2 stigmata in S133 (only at lower salinity) has to be considered for re-examination and for final taxonomic identification.

The remaining 2 strains S125 and S147 were placed in 2 GROUPs (IV and V) as they were more clearly separated from other *Dunaliella* strains on the basis of genetic characters than morpho-physiological traits. Based on the taxonomic key [3] the strain S125 was identified as *D. minuta* (longer pyriform cells) but with clear separate starch granules in pyrenoid differing from *D. minuta*. This strain from Goa salt pan clustered with *D. viridis* (Figures 4 and 6), but with larger divergence

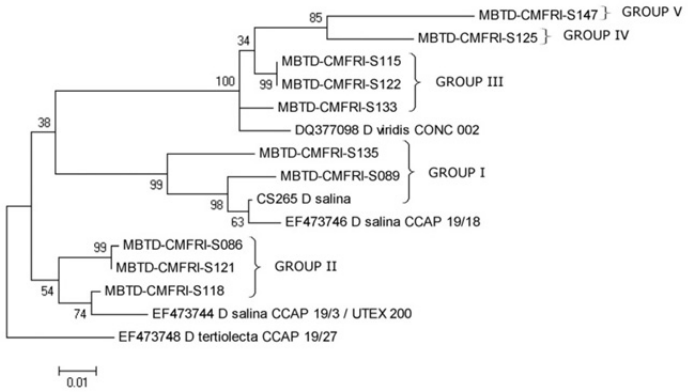

Figure 5 Phylogenetic tree (ML) of ITS region out grouped with *D. tertiolecta*; illustrated groups of 10 Indian *Dunaliella* strains. Bootstrap values for 1000 replicates are given at the internal nodes.

values in both (ITS 4.98%, *rbc*L 3.84%) the phylogenies. Further due to the lack of molecular similarity with reported *D. minuta* (NCBI-BLAST analysis of ITS 2, results not given), the identity of the strain was kept in question and placed in GROUP IV. The identity of the Kutch strain S147 was a little confusing but interesting. It resembled *D. tertiolecta* in general morphology and 18S rDNA size (~1820 bp), while grouped with *D. viridis* in ITS phylogeny (Figures 4 & 5) and with *D. salina* in *rbc*L phylogeny (Figure 6). It was isolated from a salt pan, having a dominant palmella stage and with a little higher β-carotene content (6.7 pg.cell^{-1}) in stress, while *D. tertiolecta* was reported as a marine species without a palmella stage in its life cycle [3]. These observations resulted in the grouping of S147 separately as GROUP V and are showing a probability for a new species in the group.

Diversity in Indian *Dunaliella* strains

Buchheim et al. [9] have reported diverse community formation of *Dunaliella* in heteroclimatic hypersaline soils than in purely aquatic habitats. They hypothesized that external factors like temperature and salinity can enhance diversification and apparently got supporting results from the phylogenetic study of about 30 different isolates of *Dunaliella* (where 3 different morphotypes were characterized), based on 4 genes (18S, 26S, ITS & *rbc*L). Subsequently, Azua-Bustos et al. [40] reported a morphologically distinct, new *Dunaliella* species, *D. atacamensis*, well adapted for sub-aerial life and with higher genetic divergence from its sister species. Our isolates are purely from aquatic habitats, but with high level of environmental fluctuations, especially in salt pans, and showed high divergence when compared to the reported *Dunaliella* species (Clade1 of Figures 4 & 6) from NCBI. The geographic distance and isolation of the locations (Figure 1), from where the strains were obtained could be proposed as a reason for the divergence among the above Indian *Dunaliella* isolates. However 100% sequence similarity and morphological resemblance observed between the two isolates S115 and S122 (isolated from Chennai - East coast and Goa - West coast respectively) need to be taken into account.

Grouping pattern observed in the reported *Dunaliella* strains from NCBI in the cladistic studies ([27,29], present study) suggests a taxonomic revision of the strains especially when there are comments on confusion regarding the taxonomic status of many reported species. Consequently, Browitzka and Siva [3] have proposed an elaborate morphology/physiology based examination of each strain in conjunction with molecular biology. However, among the 28 morphologically differentiated species [3,8], molecular aspects of only few important ones have been extensively studied and reported, and a very large percentage still remains unexplored genetically. Hence, even after a detailed study based on morphology, physiology and molecular aspects, particularly to avoid misnaming, strain codes were assigned to our isolates which are more appropriate for comparative studies as well as for future communications. Morphological and physiological study precisely groups 6 Indian strains into 2 sections – the carotenogenic Section Dunaliella (S089 and S135) and the non-carotenogenic Section Viridis (S115, S122, S133 and S125) [3,7]. The probability of the remaining 4 strains (S086, S118, S121 and S147) to come under Section Dunaliella is much

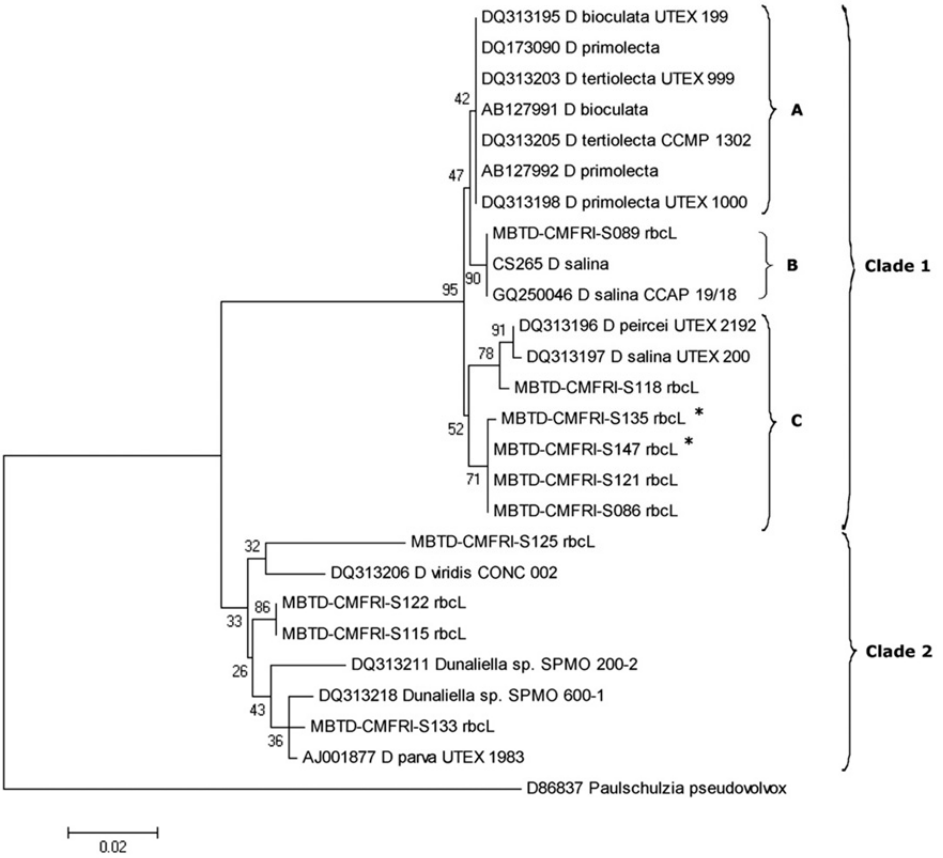

Figure 6 Phylogenetic tree of the Maximum Likelihood (ML) analysis inferred from the *rbc*L plastid gene partial sequences. Bootstrap values for 1000 replicates are given at the internal nodes.* show the position changes of S147 and S135 strains.

higher as they are more carotenogenic (S147) and closer to *D. salina* (S086, S118, S121) in molecular analysis. A schematic representation of the diversity in Indian *Dunaliella* is given in Figure 7.

The sequence diversity within the Indian *Dunaliella* strains was distinct when compared to the listed species of *Dunaliella* (ITS region & *rbc*L gene), and shows possibility of the presence of multiple species in the group. Without the knowledge of sexual compatibility between the genotypes, it is not possible to determine whether this diversity is really representing a biological/evolutionary species or merely an intra-specific diversity (9). However, Coleman et al. [41] have demonstrated a concurrence between ITS sequences and mating ability in *Dunaliella* spp. [14]. Since, high level of sequence divergence observed among the Indian *Dunaliella* strains, could be correlated with sexual incompatibility, chances of more species/subspecies with respect to ITS phylogeny seems to be a realistic possibility. Accordingly, the present study proposes the ITS region to be selected as a molecular marker in taxonomic delineation, which is smaller than 18S rRNA gene (with introns) and more diverged than *rbc*L gene.

Conclusion

The present study clearly shows high diversity within the Indian *Dunaliella* and reliability of 18S rDNA, ITS region and *rbc*L gene sequencing as a molecular tool in

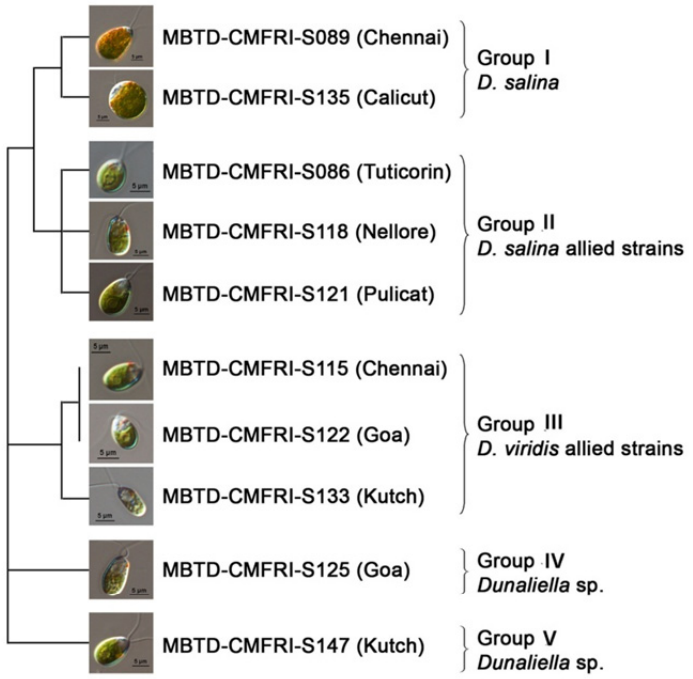

Figure 7 Schematic representation of diversity of Indian *Dunaliella*. Grouping was done based on the morphology, 18S rDNA size variation (Figure 3) and ITS and *rbc*L gene phylogenies (Figures 4 & 5).

species identification and genetic diversity studies. In a recent study, based on morphological parameters Jayapriyan et al. [33] have denoted the presence of 5 species of *Dunaliella* (*D. bioculata*, *D. tertiolecta*, *D. viridis*, *D. minuta* and *D. maritima*) from India (east coast). However in the same study, 18S rDNA species specific fingerprinting using primers of Olmos et al. [31,38] have illustrated the same isolates as completely different species of *Dunaliella* (*D. parva*, *D. bardawil* and an unidentified *Dunaliella* sp.). Hence in the present study, for more clarity on the species lineages, along with morphology and 18S rDNA size, phylogenies based on a more diverse ITS region and a more conserved *rbc*L gene were also included, which otherwise are not available for Indian *Dunaliella*. Consequently, presence of 5 or more species (or sub species), including 2 promising strains of *D. salina* (Section Dunaliella) and 2 *D. viridis*? (Section Viridis) strains, has got confirmed. The genetic characterization further helped in the separation of morphologically similar strains and in the clustering of Indian strains of *Dunaliella* into 5 groups. Similarly, clustering of the reported species in a single clade (Clade 1 with 100% similarity) in a both the phylogenies clearly emphasizes most careful recording of species names [3]. Hence, for resolving the issue prevailing in *Dunaliella* taxonomy and for elucidation of taxonomic species lineation of unknown Indian isolates, it is stressed to have further detailed molecular assessment coupled with additional examination of morphological (based on electron microscopy) and biological traits such as reproductive behavior (asexual- palmella, aplanospores etc.) and sexual compatibility.

Materials and methods

Sampling, isolation and culture conditions

Water samples were collected from selected water bodies along the Indian coast during the months of February to July 2009 (Figure 1 & Table 1). Strains were isolated by serial dilution directly or after enrichment for a period of 1 or 2 weeks. Further purification was done with agar plating and picking the single colony to obtain unialgal cultures. *Dunaliella salina* CS265 was purchased from Collection of Living Microalgae, CSIRO, Australia and used as a reference strain. The strain S089

(*D. salina*?, an old isolate from Chennai salt pan) was collected from Phytoplankton (aquaculture live feed) Culture Collection at CMFRI, Kochi. After purification, all the cultures were maintained in 75 ml modified Johnson (J/I) medium [10] in 100 ml flasks with 1.5 M (~200 ppt) NaCl, at temperature 25±1°C and at an irradiance of 40–50 μmol photons m^{-2} s^{-1} supplied by cool white fluorescent lamps on a 12 h light: 12 h dark cycle. All cultures were sub-cultured once in a month basis.

Microscopy and morphological study

Live culture samples were examined using a Nikon 80i Research microscope (Nikon, Japan) with DIC (Differential Interference Contrast) optics and images were captured using Nikon DSFi 1e camera. Major taxonomic features observed include size, shape and colour of the cell, length of flagella, characteristics of stigma, pyrenoid and chloroplast and other cytoplasmic inclusions like refractile granules. Scalar measurements such as cell length and width, were taken from a minimum of 30 cells from each strain randomly during mid growth phase immediately after fixing the cells with 1% Lugol's iodine. The descriptive statistics such as minimum, maximum, mean and standard deviation were estimated for the above scalar measurements. One way analysis of variance (ANOVA) was performed using SPSS (Version 10.0) to identify whether there is any statistically significant difference among different *Dunaliella* strains for each character.

Salinity tolerance study

For salinity tolerance study, different *Dunaliella* strains were cultured in 5 salinity concentrations *viz*., 0.5, 1.5, 2.5, 3.5 and 4.5 M NaCl in 150 ml (250 ml conical flasks) modified Johnson (J/I) medium. Other culture conditions like temperature and light were kept constant as given for normal culture maintenance. Cell characteristics like cell size and colour were examined at late growth phase under DIC microscope (Nikon, Japan). Cell count was taken on every third day using a Neubauer haemocytometer. Cell density was calculated and plotted against days of growth to obtain optimum salinity for each strain.

β-carotene analysis

Beta carotene was estimated under normal (1.5M NaCl, irradiance of 40–50 μmol photons m^{-2} s^{-1}) and stressed (3.5M NaCl, irradiance of 100–150 μmol photons m^{-2} s^{-1}) growth conditions. Total pigment was extracted from 4 ml culture at late growth phase (25^{th} day) in 4 ml ice cold 100% acetone. Liquid cultures were centrifuged (8000 rpm, 10 min.), the pellet washed with distilled water and re-suspended in ice cold acetone and left overnight at −20°C until the pellet became colourless. The extract was centrifuged at 5000 rpm for 5 min and absorbance was taken for the supernatant at 454 nm wavelength. Readings were compared with standard curve prepared with synthetic β-carotene (Type 1, Sigma, USA) in 100% acetone as described by Hajezi et al. [42]. Cell density was calculated for the same day of extraction and β-carotene was calculated per cell in picograms.

DNA isolation

DNA was isolated from 10 ml liquid culture at late growth phase following modified phenol–chloroform method of Wu et al. [43]. Cells were pelletized by centrifugation at 6000 rpm for 5 minutes, washed in distilled water and re-suspended in 450 μl TEG (25 mM TrisHCl; 10 mM EDTA; 50 mM glucose) buffer (pH 8) with Lysozyme (5 mg/ml) and vortexed with glass beads and then added 50 μl 10% SDS. The tubes were then incubated on ice for 10 min. and added 8 μl Proteinase K (20 mg/ml) and further incubated at 60°C for 60 min in a water bath. Once the cells were lysed completely, the DNA was purified following standard phenol/chloroform extraction and ethanol precipitation [44].

PCR amplification, sequencing and phylogeny

A gene fragment of 18S rRNA was amplified using conserved primers MA1 & MA2 [5,31]. Reactions were carried out in Veriti Thermal Cycler (Applied Biosystems, US) with a total volume of 25 μl containing PCR buffer at 1× concentration with 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 1.5 U of Taq polymerase (Sigma, USA) 5 picomoles of each primer and 25 ng of genomic DNA. Thermal cycling initiated with 3 min at 95°C and then 35 cycles of 30 sec at 95°C, 30 sec at 52°C and 2 min at 72°C. Final extension was for 10 min at 72°C. Amplified products were checked by electrophoresis in 1% agarose gel. The size (bp) of the amplified product was calculated by comparing it with standard molecular weight DNA marker (Step up 100 bp DNA ladder, Merck, India) using the software Image Lab version 3 (Biorad, USA).

Internal transcribed spacer (ITS) region (700 bp), including ITS1, 5.8 S rRNA and ITS2, was amplified using the primers ITS1 and ITS4 (17). Thermal cycling followed an initial denaturation for 3 min at 95°C and 35 cycles of 30 sec at 95°C, 10 sec at 55°C and 45 sec at 72°C followed by a final extension at 72°C for 7 min. Partial (700 bp) region of *rbc*L gene was amplified using the primers *rbc*L 475–497 and *rbc*L 1181–1160 following Nozaki et al. [45] and Assuncao et al. [46]. The reaction mix composition for ITS and rbcL gene were the same as in the case of 18S rRNA gene amplification (given above). Amplified products were tested on 1.5% agarose gel.

All PCR products were purified using *Gen*Elute PCR Cleanup Kit (Sigma, USA) following manufacturer's

instruction. Cycle sequencing was carried out using forward primers (MA1 and *rbc*L 475–497) for 18S rRNA and *rbc*L genes respectively. Whereas the ITS region was sequenced using both forward (ITS1) and reverse (ITS4) primers. Sequences of DNA fragments were imported to BLAST [47] for similarity searches with available database at NCBI GenBank. The sequence was further aligned with the various available sequences (Table 4) of *Dunaliella* spp. and, *Chlamydomonas reinhardtii* (ITS) and *Paulschulzia pseudovolvox* (*rbc*L) as out groups using the CLUSTAL-W algorithm [48] in Bioedit 7.0 (DNA Sequence Analysis Software package). To clearly illustrate grouping pattern in Indian *Dunaliella* isolates, *D. tertiolecta* was out grouped in ITS (Figure 5) phylogeny. Pair wise genetic distances among the different *Dunaliella* species and between the present isolates were calculated based on Kimura 2 parameter model for ITS region and Tamura 3 parameter for *rbc*L gene. The best nucleotide substitution model selection and phylogenetic analysis based on maximum likelihood (with 1000 boot strap replications) were carried out using MEGA 5 [49]. All the sequence information generated in the present study were deposited in the NCBI database (Table 1).

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
KKV and KP conceived the study and wrote the manuscript. KP and CSS did isolation and maintenance of cultures and performed experimental work and data analysis. Molecular data analysis and interpretations were done by LJ. All authors have read and approved the final manuscript.

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Annexure 5

Sensory (organoleptic) analysis – Score Card No:……

Method: Nine points Hedonic scale method

Name: ……..... Age: ……. Gender: Male / Female

Food habit: Vegetarian / Non-vegetarian Date: ……/……/……..

NB: Make Tick (✓) mark against your choice. Write special remarks if any (e.g. Aroma – buttery or fishy, Taste – Fishy or biscuit)

Sample Code: …… Signature: ……..

	Excellent	Very good	Good	Moderately good	Not good/ bad	Slightly poor	Moderately Poor	Poor	Very poor	Other Remarks
Colour										
Aroma										
Taste										
Crispi-ness										
Over-all										

Excellent – 9; Very good – 8; Good – 7; Mod. good – 6; Not good/bad – 5; Slightly poor – 4; Mod. poor – 3; Poor – 2; Very poor- 1

……