MARINE DIATOM *NAVICULA PHYLLEPTA* MACC8 AS POTENTIAL BIODIESEL FEEDSTOCK: ISOLATION, CHARACTERIZATION AND OPTIMIZATION FOR ENHANCED BIOMASS AND LIPID PRODUCTION

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By

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Ph.D. Thesis under the Faculty of Environmental Studies

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Certificate

This is to certify that research work presented in the thesis entitled "**Marine Diatom *Navicula phyllepta* MACC8 as Potential Biodiesel Feedstock: Isolation, Characterization and Optimization for Enhanced Biomass and Lipid Production"** is an authentic record of research work carried out by **Ms. Sanyo Sabu** under my guidance at National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Cochin- 682016, in partial fulfillment of the requirements for the Degree of Doctor of Philosophy and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition. All the relevant corrections and modifications suggested by the audience and recommended by the doctoral committee of the candidate during the pre-synopsis seminar have been incorporated in the thesis.

Cochin - 682016
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Declaration

I hereby do declare that the work presented in this thesis entitled "**Marine Diatom *Navicula phyllepta* MACC8 as Potential Biodiesel Feedstock: Isolation, Characterization and Optimization for Enhanced Biomass and Lipid Production"** is based on the original work done by me under the guidance of Dr. Valsamma Joseph, Associate Professor, National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Cochin- 682016, in partial fulfillment of the requirements for the award of the Degree of Doctor of Philosophy and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

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Sanyo Sabu

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You gave me your time, the most thoughtful gifts of all - Dan Zadra

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If we knew what it was we were doing, it would not be called research, would it?

- Albert Einstein

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Sanya Sabu

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Chapter 1

GENERAL INTRODUCTION

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Fossil fuels such as oil, coal and natural gas have been the greatest demanded energy sources since after industrial revolution. Fuels represent around 70% of the total global energy requirements, particularly in transportation, manufacturing and domestic sector (Gouveia and Cristina, 2009). These fuels are made up of hydrocarbons, which store chemical energy in atomic bonds of hydrogen and carbon, and subsequent breakage of these bonds upon combustion releases energy, which makes them valuable to our society. The increasing and continuous usage of fossil fuels is not sustainable as they are finite and non-renewable sources of energy

(Hook and Tang, 2013). In addition, high number of on-road diesel vehicle emissions significantly contribute to the atmospheric levels of the most important greenhouse gases, carbon dioxide (CO_2) and other urban pollutants, such as carbon monoxide (CO), nitrogen oxides (NOx), unburned hydrocarbons, particulate matters and aromatics, which significantly contribute to air pollution and global warming (Lam and Lee, 2012; Zhu and Ketola, 2012; Giakoumis et al., 2013).

To satisfy the demands with the supply of resources while simultaneously curbing the global carbon-dioxide (CO_2) emissions, it requires a transition away from fossil fuels towards renewable systems. To find clean and renewable energy sources is one of the most challenging problems faced by mankind in the near to long term future (Dresselhaus and Thomas, 2001; Armaroli and Balzani, 2007; Mata et al., 2010). The associated issues are intimately connected with economic development and prosperity, global stability, standard of living and environmental sustainability (Mata et al., 2010). Many countries around the world established targets for CO_2 reduction in order to meet the sustainability goals agreed under the Kyoto Protocol (Wigley, 1998; Santilli et al., 2005), and these were the major concerns raised also in the climate change conferences at Copenhagen (2009) and Paris (2015). A massive reduction in the ecological footprint of energy generation reside in a multi-faceted approach using alternative renewable sources of energy such as nuclear, solar, thermal or photovoltaic, hydrogen, wind, geothermal and biofuels (Lam and Lee, 2012). According to a recent study from International Energy Agency (IEA), energy produced from combustible renewables and waste has the highest potential among other renewable sources (Outlook, 2010). Hence, it was predicted that energy from combustible

sources such as biomass, i.e. biofuels will play a major role as an alternative renewable energy (Dewulf and Van Langenhove, 2006; Gilbert and Perl, 2008).

1.1 Microalgae- Third generation feedstock for biofuels

Biofuels can be a best alternative for renewable energy as it fixes the atmospheric carbon dioxide levels thereby contributing towards mitigation of global warming (Sivakumar et al., 2010; Zhu et al., 2014a). At the same time, the burning of these fuels does not have any impact on global carbon-dioxide concentrations as release is balanced by uptake through photosynthesis (Hall et al., 1991). Biofuels can be categorized based on the source of biomass as first, second and third generation of biofuels. First generation and second generation of biofuels comprised of edible and non-edible sources such as corn, sunflower, sugar cane, rapeseed, palm, animal fat, vegetable oil, Jatropha and waste cooking oil (Nigam and Singh, 2010). However, the first and second generation of biofuels were not much feasible as they resulted in an imbalance in world food market thereby increasing the cost of edible oil and biofuel. In addition, the use of such feed stocks would compete with agriculture for arable land used for food production (Singh et al., 2011a).

Third generation technology is based on microalgae, which are single cells with phototrophic, mixotrophic or heterotrophic mode of nutrition (Show et al., 2017). The biomass produces three major components namely carbohydrate, protein and lipid (oils). Microalgal oil and the spent biomass are considered as potential source of biofuel feedstocks (Pienkos and Darzins, 2009). Microalgae have many advantages over other crop based sources.

Algal productivity is many folds higher than crop plants on land area basis (Schenk et al., 2008; Rathmann et al., 2010). They have high growth rates compared to higher plants and can be grown throughout the year enabling continuous and multiple harvesting year round. Their photosynthetic energy conversion efficiency is very high resulting in superior lipid productivity. Microalgae are known to accumulate lipids more than half of their biomass (50- 80%) (Hu et al., 2008; Khan et al., 2009). Hence they are capable for producing higher oil yield than other food crops (Fig. 1.1). These tiny organisms can be cultivated in any form of water resources such fresh water, brackish water, sea water and even waste water.

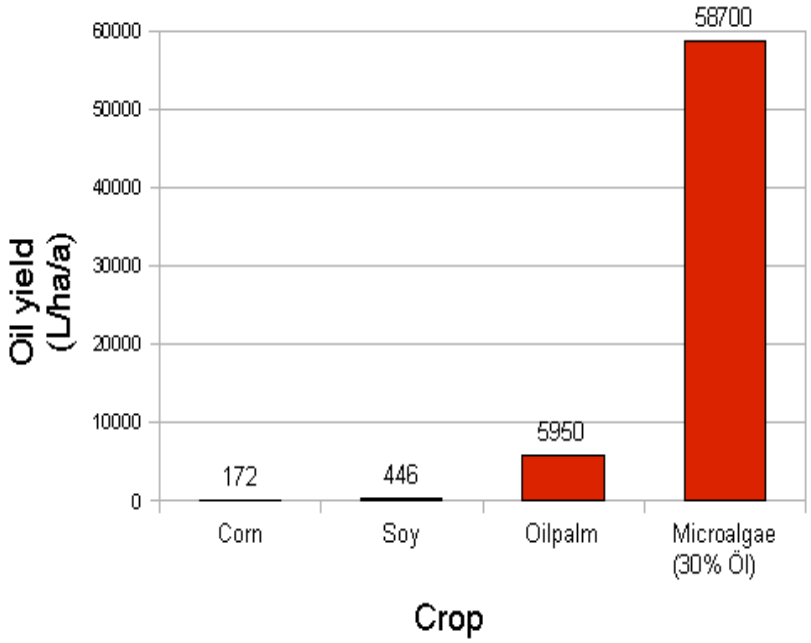


Fig. 1.1 Comparison of oil yield in agricultural plants in microalgae (Chisti, 2007)

Microalgal biodiesel is eco-friendly that it is a carbon neutral fuel due to the photosynthetic fixation of atmospheric carbon dioxide. This makes it advantageous to cultivate them in farms near industrial area by absorbing carbon dioxide from air. They are suitable to be grown in non-arable lands including deserts and sea shores lands (Hu et al., 2008; Singh et al., 2011b; Rawat et al., 2012). The cost associated with harvesting and transportation is relatively low as compared to that of oil crops. Residual biomass post extraction offers methods for improving economics by using it as a fertilizer or for producing other high energy products (Ahmad et al., 2011).

Microalgae have been identified as a versatile feedstock for various forms of biofuels such as bioethanol, biohydrogen, biodiesel, biogas and many other fuel types via thermal or biochemical conversions (Li et al., 2008; Zhu et al., 2014a). Bioethanol is produced by hydrolysis of sugar or carbohydrates and then fermentation by yeast (Liu et al., 2012a). Ethanol production from microalgal cellulose is beneficial compared to woody biomass as the cell wall of these single cells contain no or very less lignin or hemicellulose favoring the hydrolysis of cell walls (Harun et al., 2010; Ho et al., 2010; John et al., 2011). The compositions of microalgae are much more identical and consistent compared to woody biomass making pretreatment processes much easier. Finally, some of the microalgae contain starch or glycogen more than half of their cell biomass resulting in high bioethanol yield (John et al., 2011).

Biohydrogen is produced by microalgae in closed culture system utilizing sunlight and water in the absence of oxygen with the help of hydrogenase enzyme (Melis and Happe, 2001). Some microalgae, such as

Scenedesmus obliquus (Florin et al., 2001), *Scenedesmus* sp. (Winkler et al., 2002), *Platymonas subcordiformis* (Guan et al., 2004) and *Chlamydomonas reinhardtii* (Meuser et al., 2012) have been investigated to have substantial hydrogenase activity in biohydrogen production.

The other types of fuels include biogas, syngas, jet fuel, bio oil and bio-butanol produced by appropriate conversion technologies. Microalgae-derived jet fuel has also received much attention. Jet fuel blends (derived from a variety of oil-rich feedstock, including algae) have shown to be compatible for use in selected commercial demonstration jet test flights (Ghasemi et al., 2012).

1.2 Biodiesel

Biodiesel is mono-alkyl (usually methyl) esters (fatty acid methyl ester, or FAME) made by the transesterification of triacylglycerides from vegetable oils or animal fats or algal lipids (Knothe and Steidley, 2005). Transesterification of oil with alcohol in the presence of a catalyst produced biodiesel and glycerol (Fig. 1.2). There are several advantages when using biodiesel as a liquid fuel source. As the fuel is obtained from biomass, it contributes to less atmospheric CO_2 emissions, particulate matter, sulfur, and aromatic compounds than burning petroleum diesel (Aresta et al., 2005; Rakopoulos et al., 2006; Demirbas, 2007). Biodiesel has been experimentally shown to be less toxic to soil micro-biomes, easily volatile and contributes no net toxic gases such as sulphur dioxide or carbon dioxide to the atmosphere (Williams and Laurens, 2010).

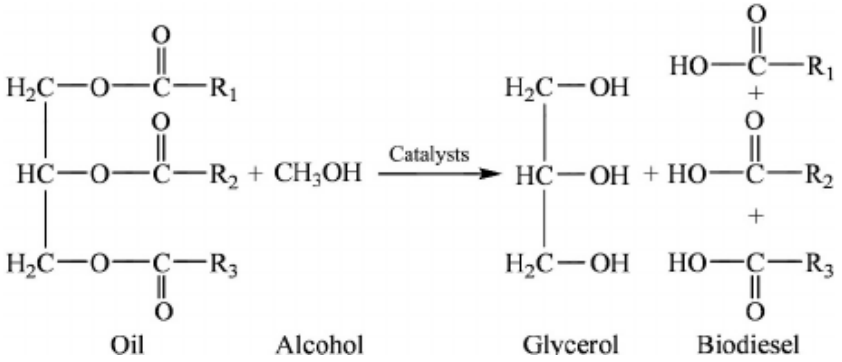


Fig. 1.2 Transesterification reaction of microalgal lipids resulting in biodiesel and byproduct of glycerin (Ahmed et al., 2015)

The infrastructure needed for biodiesel already exists. Biodiesel can be used in existing diesel engines blended with petroleum diesel (B20, B2, B5) or can be run unblended in engines with minor modifications (Bowman et al., 2006; Crookes, 2006; Rakopoulos et al., 2006). Biodiesel has twice the viscosity of petroleum diesel and hence its lubrication properties can actually improve engine life (Bowman et al., 2006). Biodiesel has low toxicity and is biodegradable (Aresta et al., 2005; Demirbas, 2007) and a more complete combustion than gasoline, giving a cleaner burn (Bowman et al., 2006). Furthermore, it has a higher flashpoint, allowing safer handling and storage and greater lubricity for engines than other fuels. According to biodiesel standard of American Society for Testing Materials (ASTM), biodiesel from microalgal oil is similar in properties to the standard diesel, and is also more stable according to their flash point values. Biodiesel has 25% higher energy than bioethanol and requires much less energy input in production (Yu et al., 2011).

Despite the many advantages, there are limitations hindering the complete replacement of petrodiesel (Knothe, 2006). Negative biodiesel

characteristics include poor cold-temperature properties such as solidifying property, which can lead to fuel starvation and engine failure. The presence of polyunsaturated fatty acids in biodiesel also makes it susceptible to oxidation or hydrolytic degradation by water, which decreases the stability of biodiesel during long-term storage. In addition, the emissions from biodiesel contain a higher concentration of nitrogen oxide (NOx) than normal petro-diesel which limits its usage in areas under strict air quality standards. One of biodiesel's biggest limitations is cost and supply. The higher production costs of biofuel along with the lack of successful industry examples to date further hinder industry-scale adoption of biodiesel (Yu et al., 2011).

1.3 Microalgae- characteristics and classification

Microalgae and cyanobacteria are major components of the plant kingdom and play a major role in building and maintaining the earth's atmosphere by producing oxygen and consuming carbon dioxide (Muller-Feuga et al., 2003). Microalgae are unicellular microscopic, polyphyletic and non-cohesive assemblage of CO_2 evolving, autotrophic organisms, which grow by photosynthesis (Greenwell et al., 2009). Algae are primitive plants (thallophytes) lacking roots, stems and leaves and have no sterile covering of cells around the reproductive cells, and have chlorophyll a as their photosynthetic pigment (Lee, 1980). According to Khan et al. (2009), the most important groups of algae in terms of abundance are: diatoms, green algae, blue–green algae (cyanobacteria) and golden algae. Algal structures are primarily for energy conversion without any development beyond cells and their simple development allows them to adapt to prevailing environmental conditions (Falkowski and Raven, 1997). Algae can be either autotrophic or heterotrophic. Some photosynthetic algae are

mixotrophic, i.e., they perform photosynthesis as well as acquire exogenous organic nutrients (Lee, 1980). The phylogenetic diversity of the microalgae is very broad and is reflected in an equally wide range of molecular and biochemical properties. The biochemical diversity of microalgae is seen in pigments, cell walls and mucilages, fatty acids and lipids, oils, sterols and hydrocarbons, photosynthetic storage products, and bioactive compounds, including secondary metabolites. Molecular and ultrastructural evidence of evolutionarily conserved features (eg. ribosomal RNA, rbcl, internal transcribed spacer gene sequencing, flagellar hairs and roots, plastid and mitochondrial structure, the mitotic apparatus) have made the classification of microalgae more structured and detailed (Metting, 1996).

Microalgae can be found in a large range of places where light and water are present including ocean, lake, soils, ice and rivers and also in extreme environments such as hot springs, halophilic waters and polar ice caps (Parker et al., 2008; Deng et al., 2009). Microalgae demonstrate a great biodiversity (between 200,000 to several millions of species) (Radmer and Parker, 1994; Norton et al., 1996). Bold and Wynne (1985) and Stevenson (1996) concluded that there are about 26, 000 species of algae all over the world and Norton et al. (1996) reported 37, 300 species of microalgae. Mann and Droop (1996) estimated the number of diatoms itself comes to more than 2, 00,000, with planktonic, periphytonic and benthic forms. The microscopic species are distributed as planktonic, which remain suspended in the water column, and as benthic, which live in association with some substrata. Some are mobile, swimming by means of flagella or gliding over the substrata. Others are non-motile-drifting in the plankton, fixing to a substratum or lying free. Several microalgae, in spite of their characteristic

adaptations to the specific habitat, have been found both in benthic and pelagic environments (Sanilkumar, 2009). Most of the microalgal species produce unique products like carotenoids, antioxidants, fatty acids, enzymes, polymers, peptides, toxins and sterols (Pulz and Gross, 2004; Koller et al., 2014).

Microalgae can be divided into categories depending on their pigmentation, biological structure and metabolism:

a) *Size based classification*- Microalgae are small organisms, which can be divided into 4 size categories as the microplankton (20 to 1000 μm), the nanoplankton (2 to 100 μm), the ultraplankton (0.5 to 15 μm) and the picoplankton (0.2 to 2 μm) (Callieri and Stockner, 2002; Gopinathan, 2004). The small size of the organism allows them to do an effective photosynthesis to produce lipids, carbohydrates and proteins.

b) *Taxonomic groups*- Microalgae was classified into 11 classes by Fritsch (1948) based on the type of pigments, nature of reserve food material and the mode of reproduction. They are Chlorophyceae, Xanthophyceae, Chrysophyceae, Bacillariophyceae, Cryptophyceae, Dinophyceae, Chloromonodineae, Euglinineae, Phaeophyceae, Rhodophyceae and Cyanophyceae. However, all these species are not equally interesting for biodiesel production (William and Laurens, 2010).

c) *Metabolism based classification*- Microalgae can be separated into 4 main types based on metabolism called photoautotrophic, heterotrophic, mixotrophic and photoheterotrophic (Chen et al., 2011). Photoautotrophic microalgae convert inorganic carbon

(CO_2) and water to biomass by photosynthesis reaction in presence of light (Cadoret and Bernard, 2008). It is reported that photoautotrophic microalgae contain high levels of lipids, but their biomass productivity in cultivation systems such as photobioreactors or open ponds is generally lower than heterotrophic microalgae (Chisti, 2007). Heterotrophic microalgae need organic carbon as a source of carbon and energy. The mass production of such organisms is carried out in closed bioreactors such as fermentors. They are more promising than the photoautotrophic species for the production of biodiesel (Xu et al., 2006a; Martek, 2008; Xiong et al., 2008). However, they do not help in the mitigation of the emissions of CO_2, which is also one of the main goals of microalgal fuel production. Some microalgae species also have mixotrophic mode of nutrition as they grow in light or dark using both inorganic and organic carbon sources (Liang et al., 2009). The use of microalgae with mixotrophic metabolism is relatively rare for biodiesel production. Photoheterotrophic metabolism means that microalgae need light as a source of energy and a source of organic carbon (Chen et al., 2011). As these microalgae need an inexpensive source of organic carbon and need long periods of the sunlight, photoheterotrophic metabolism is less interesting for biodiesel production (Veillette et al., 2012). Vitamin auxotrophy is wide spread within algal kingdom with half of total species requiring vitamin as a growth supplement (Croft et al., 2006) as well as an important factor for lipid production (Hakalin et al., 2014).

1.4 Lipid and fatty acid profiles of microalgae

The microalgae produces lipids, which generally include neutral lipids, polar lipids, wax esters, sterols and hydrocarbons, chlorophylls as well as prenyl derivatives such as carotenoids, tocopherols, terpenes and quinines. Figure 1.3 shows the chemical structures of representatives of lipid classes (www.LipidMAPS.org). Lipids produced by microalgae can be grouped into two categories, non-polar lipids (storage lipids) and polar lipids (structural lipids). Storage lipids mainly in the form of triacylglycerides (TAGs), consists predominately of saturated fatty acids and some monounsaturated fatty acids (Siaut et al., 2011), which are found to be ideal for biodiesel production. Structural lipids typically have a high content of polyunsaturated fatty acids (PUFAs), which give essential nutrition for aquatic animals and humans when included in feed or food (Spolaore et al., 2006; Hemaiswarya et al., 2011). Polar lipids (phospholipids) and sterols are important structural components of cell membranes which act as a selective permeable membrane for cells. These lipids maintain specific membrane functions, providing the matrix for a wide variety of metabolic processes and participate directly in membrane fusion events (Guschina and Harwood, 2009). Some polar lipids may act as key intermediates in cell signaling pathways such as inositol lipids, sphingolipids, oxidative products and play a role in responding to changes in the environment (Sharma et al., 2012). Triacylglycerides are abundant storage products, which can be easily broken down to release metabolic energy (Gurr et al., 2002). They are mostly synthesized in the light, stored in cytosolic lipid bodies, and then re-utilized in the dark for polar lipid synthesis (Thompson, 1996). Microalgal triacylglycerides generally consists of saturated and monounsaturated fatty

acids. However, some oleaginous species have demonstrated a capacity to accumulate high levels of long-chain polyunsaturated fatty acids (PUFA) as TAG (Alonso et al., 1998; Bigogno et al., 2002a). A detailed study on both accumulation of TAG in *Parietochloris incisa* and its storage into chloroplastic lipids led to the inference that TAGs may play an additional role beyond being a storage product in this alga (Bigogno et al., 2002b; Khozin-Goldberg and Cohen, 2006). Hence, PUFA-rich TAGs are metabolically active and are suggested to act as a reservoir for specific fatty acids (Makewicz et al., 1997; Khozin-Goldberg and Cohen, 2006).

According to varying morphology and habitat, the algae comprises of diverse compositions of acyl lipids and their fatty acids. Compared to cyanobacteria, eukaryotic forms of microalgae, especially marine isolates, consists of more number of saturated and unsaturated fatty acids. In general, saturated and mono-unsaturated fatty acids are predominant in most algae examined (Borowitzka, 1988). The major saturated fatty acids found in microalgae are palmitic acid, whereas stearic acid is found in lesser amount than higher plants. Specifically, the major fatty acids present in various classes of microalgae are C16:0 (palmitic acid) and C16:1 (palmitoleic acid) in the Bacillariophyceae; C16:0 and C18:1(oleic acid) in the Chlorophyceae, Euglenophyceae, Eustigmatophyceae and Prasinophyceae; C16:0, C16:1 and C18:1 in the Chrysophyceae; C16:0 and C20:1 (eicosenoic acid) in the Cryptophyceae; C16:0 in the Dinophyceae and Rhodophyceae; C16:0, C16:1 and C18:1 in the Prymnesiophyceae; C14:0, C16:0 and C16:1 in the Xanthophyceae and C16:0, C16:1 and C18:1 in cyanobacteria (Cobelas and Lechado, 1989).

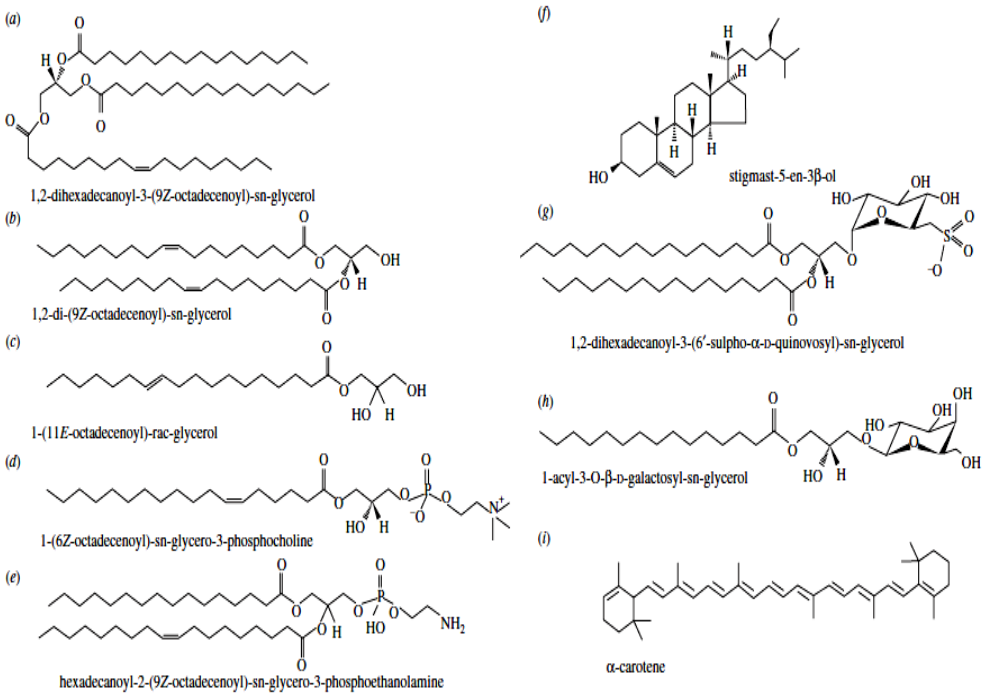


Fig. 1.3 Chemical structures of most common representatives from seven lipid classes: (a) triacylglycerides; (b) diacylglycerides; (c) monoglycerides; (d) phospholipids; (e) sterols; (f) sulpholopids; (g) glycolipids; (i) carotenoids (courtesy www. LipidMAPS.org)

Microalgae are reported to be rich in very long chain polyunsaturated fatty acids (PUFAs) as major components. This is a main characteristic of marine species. The major acids are arachidonic (AA) (C20:4n-6), eicosapentaenoic (EPA) (C20:5n-3) and docosahexaenoic (DHA) (C22:6n-3). The major PUFAs are C20:5n-3 (EPA) and C22:6n-3 (DHA) in Bacillariophyceae, C18:2 (linoleic acid) and C18:3n-3 (α-linolenic acid) in green algae, C18:2 and C18:3n-3 in Euglenophyceae, C20:5, C22:5 and C22:6 in Chrysophyceae, C18:3n-3, 18:4 (stearidonic acid) and C20:5 in Cryptophyceae, C20:3 and C20:4n-3 in Eustigmatophyceae, C18: 3n-3 and

C20:5 in Prasinophyceae, C18:3n-3 and C22:6n-3 in Dinophyceae, C18:2, C18:3n-3 and C22:6n-3 in Prymnesiophyceae, C18:2 and C20:5 in Rhodophyceae, C16:3 (Hexadecatrienoic acid) and C20:5 in Xanthophyceae, and C16:0, C18:2 and C18:3n-3 in cyanobacteria (Cobelas and Lechado, 1989; Basova, 2005).

Certain microalgae may contain other unusual lipids such as chlorosulpholipids (Haines, 1973) and halogenated fatty acids (Dembitsky and Srebnik, 2002). Other newly-discovered compounds such as long-chain (C35–C40) alkenones and their derivatives are described in Guschina and Harwood (2006).

1.5 Triacylglycerol (TAG) biosynthesis in microalgal systems

Microalgae are able to survive heterotrophically on exogenous carbon sources offering prefabricated chemical energy, which the cells often store as lipid droplets (Ratledge, 2004). Another natural mechanism through which microalgae can alter lipid mechanism is the stress mechanism owing to a lack of nutrients or alteration of any culture conditions (Tornabene et al., 1983). Under unfavorable growth conditions, many algae divert their metabolic pathways toward the biosynthesis of storage lipids or polysaccharides. TAG accumulation in response to environmental stresses such as nutrients, temperature, light, salinity and culture age likely occurs as a means of providing an energy deposit that can be readily catabolized in response to a more favorable environment to allow rapid growth (Harwood, 1998).

The lipid metabolism pathways are less understood in algae compared to higher plants. It is proposed that the general pathways of microalgae are analogous to that of higher plants on the basis of sequence homology and biochemical characteristics of certain enzymes. But unlike plants, where different classes of lipids are synthesized and stored in different parts of the organism, the whole process of carbon dioxide fixation to TAG synthesis takes place in a single algal cell and the lipid bodies are stored in chloroplast (Hu et al., 2008). Triacylglyceride synthesis in algae is proposed to occur via direct glycerol pathway which is also known as 'Kennedy pathway' after Professor Eugene Kennedy (Ratledge, 1988). The TAG pathway begins with the basic fatty acid precursor, acetyl- CoA and continues through fatty acid biosynthesis, complex lipid assembly, and saturated fatty acid modification, until finally reaching TAG formation and storage (Ohlrogge and Browse, 1995). The first committed step of fatty acid synthesis is catalyzed by a multifunctional enzyme complex, acetyl CoA carboxylase (ACCase), which produces malonyl-CoA from acetyl CoA and bicarbonate. The malonyl group is transferred from CoA to ACP (aceyl carrier protein) catalyzed by a malonyl-CoA: acyl carrier protein malonyl transferase. The common 16- or 18-carbon fatty acids are formed by a series of two-carbon chain-elongating reactions catalyzed by a multi-subunit enzyme, fatty acid synthase (FAS), which is a major player in *de novo* fatty acid synthesis (Harwood, 1998). In *de novo* fatty acid synthesis, either the newly synthesized fatty acid is hydrolysed by a thioesterase and further modified by desaturases or directly transferred to complex lipid formation using plastid acyltransferases (Murphy, 2005; Guschina and Harwood, 2007). After release from plastids, free fatty acids are exported to the cytosol and

converted to acyl-CoA esters by an acyl- CoA synthetase, which are later transferred to the endoplasmic reticulum (ER) for further elongation, modification, or participation in the synthesis of membrane lipids or storage TAGs (Schnurr et al., 2002; Koo et al., 2004). The fatty acids produced in the chloroplast transferred to *sn*-1 position of glycerol 3- phosphate by glycerol-3-phosphate acyltransferase (GPAT) to produce lysophosphatidic acid (LPA). LPA is then again acylated by lysophosphatidic acid acyltransferase (LPAT) to form phosphatidic acid (PA). Phosphatidic acid phosphatase (PAP) catalyzes the removal of the phosphate group from phosphatidic acid to generate 1, 2-diacylglycerol (DAG), the central intermediate of all glycerolipids. The last and committed step to oil synthesis is catalyzed by diacylglycerol acyltransferase (DGAT), where DAG is converted to TAG (Hu et al., 2008; Cagliari, 2011). Fig. 1.4 shows a simplified view of the metabolic pathways in microalgal lipid biosynthesis (Radakovits et al., 2010)

After certain amount of TAGs are formed in specific domain of endoplasmic reticulum (ER), droplets of oil bud off of the ER, forming distinct cellular organelles. Due to their highly hydrophobic nature, oils could not exist alone in the cell. Instead they are enclosed by a single-layer membrane made of phospholipids with the hydrophilic head groups on the surface. These subcellular compartments are called oil bodies, lipid droplets or oleosomes. Oil bodies consist of a neutral lipid core enclosed by a membrane lipid monolayer coated with proteins (Huang, 1992).

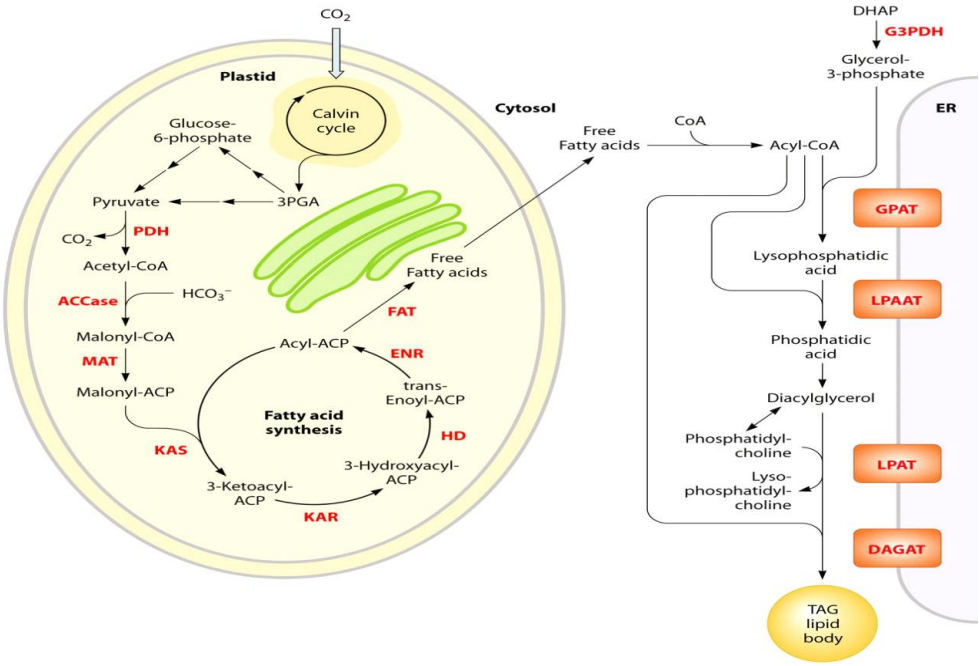


Fig. 1.4 Simplified overview of the metabolites and representative pathways in microalgal lipid biosynthesis shown in black and enzymes shown in red. Free fatty acids are synthesized in the chloroplast, while TAGs may be assembled at the ER. ACCase, acetyl-CoA carboxylase; ACP, acyl carrier protein; CoA, coenzyme A; DAGAT, diacylglycerol acyltransferase; DHAP, dihydroxyacetone phosphate; ENR, enoyl-ACP reductase; FAT, fatty acyl-ACP thioesterase; G3PDH, gycerol-3-phosphate dehydrogenase; GPAT, glycerol-3-phosphate acyltransferase; HD, 3-hydroxyacyl-ACP dehydratase; KAR, 3-ketoacyl-ACP reductase; KAS, 3-ketoacyl-ACP synthase; LPAAT, lyso-phosphatidic acid acyltransferase; LPAT, lyso-phosphatidylcholine acyltransferase; MAT, malonyl-CoA:ACP transacylase; PDH, pyruvate dehydrogenase complex; TAG, triacylglycerol (Radakovits et al., 2010)

1.6 Biomass and biodiesel production from microalgae

The whole process of biodiesel production from microalgae includes two phases: first is the upstream process, which consists of high density and high quality production of microalgal biomass and lipid, and second is the

downstream processes including harvesting, extraction and conversion into biodiesel (Medipally et al., 2015). The economic viability of microalgal biodiesel lies in the production of high density microalgal biomass in a limited period. In order to achieve this, two major approaches have been actively researched and developed: one is controlling the metabolic pathways and other is cultivation system designs (Wu et al., 2012).

1.6.1 Modes of metabolism

Microalgae utilizes three major nutrition mode based on which three types of cultivation methods are developed: phototrophic, heterotrophic and mixotrophic. Phototrophic mode of cultivation is considered to be most economical method for microlagal biofuel production as it utilizes only light and carbon dioxide, and is the most commonly used cultivation condition for microalgal growth (Illman et al., 2000; Gouveia et al., 2009; Gouveia and Oliveira, 2009; Mandal and Mallick, 2009; Yoo et al., 2010). Heterotrophic cultivation is based on organic carbon sources and limited light. Microalgae can adapt to different organic compounds such as sucrose, glycerol, xylan and organic acids in slurry. Heterotrophic cultivation of *Chlorella pyrenoidosa* resulted in high biomass productivity (Wu and Shi, 2006). But from an environmental point of view it cannot help in curbing atmospheric CO_2. In mixotrophic cultivation, there is combined utilization of light and organic carbons, which can appreciably boost the biomass and lipid productivities in microalgae (Park et al., 2012). Microalgae such as *Spirulina platensis* (cyanobacteria) and *Chlamydomonas reinhardtii* (green algae) were reported to exhibit mixotrophic metabolism (Chen, 1996).

1.6.2 Cultivation designs

The two major cultivation systems for all the types of cultivation methods are open pond and closed bioreactors (Borowitzka, 1999; Ugwu et al., 2008). Cultivation of algae in open ponds has been extensively studied (Hase et al., 2000; Moreno et al., 2003; Moheimani and Borowitzka, 2006; Radmann et al., 2007). Some common types are raceways stirred by a paddle wheel, extensive shallow unmixed ponds, circular ponds mixed with a rotating arm, and sloping thin-layer cascade systems, of which raceways are the most commonly used artificial system (Chisti, 2007). Raceways are easy to be maintained, but they are expensive to be constructed and there is high risk of contamination (Ugwu et al., 2008; Deng et al., 2009). Closed type bioreactors have the advantages of saving water, energy and chemicals and high productivity, which make them the most desired choice for biofuel production (Barbosa et al., 2003; Schenk et al., 2008). Some types of photobioreactors (PBRs) are flat panel (Hu et al., 1996), horizontal tubular air lift (Rubio, 1999), bubble column (Degen et al., 2001; Ogbonna et al., 2002; Chini Zittelli et al., 2003), stirred tank (Ogbonna et al., 1999) and helical tubular (Hall et al., 2003). In an interesting adaptation of mass microalgal culture, Rodolfi et al. (2009) suggested a hybrid-system by combining the use of open ponds with PBRs in a two-stage strategy, whereby cell yield in an enclosed photobioreactor is maximized under optimal conditions followed by transfer to a second, stressing condition carried out in raceway ponds, whereby lipid-accumulation is provoked. A hybrid cultivation system such as this has been used for the successful production of oil and astaxanthin from *Haematococcus pluvialis* (Huntley and Redalje, 2006).

1.6.3 Harvesting

Harvesting is very important and crucial process in microalgal biofuel production as it needs to be cost effective and energy efficient. There is no single best method for harvesting microalgae as microalgae cells are very small (typically in the range of 2–70 μm) and the cell densities in culture broth are low (usually in the range of 0.3-5 g L^{-1}) (Danquah et al., 2009, Wu et al., 2012). The choice of harvesting technique depends on the characteristics of microalgae such as density, size and the value of the target products (Brennen and Owende, 2010). Strain selection is a very important criterion, as some colonial and filamentous microalgae make harvesting much easier. The most commonly adopted harvesting techniques are centrifugation (Beneman and Oswald, 1996), gravity settling, filtration, flocculation, floatation and electrophoresis (Lee et al., 1998; Grima et al., 2003; Azarian et al., 2007; Uduman, 2010). Bioflocculation is likely the cheapest harvesting process. Certain species naturally flocculate (auto flocculation by biofilm formation), while others flocculate in response to some specific environmental stimuli, nutrient stress, pH and the level of dissolved oxygen (Beneman and Oswald, 1996). An interesting variation of this method known as co-bio-flocculation was presented by Ami Ben-Amotz (Pienkos, 2007), where naturally flocculating alga like *Skeletonema* was used to form flocs with high lipid varieties of *Nannochloropsis*.

1.6.4 Extraction and conversion

In order to obtain biodiesel, lipids and fatty acids have to be extracted from wet or dry microalgae. Intracellular lipid components can be extracted through variety of ways such as mechanical crushing or pressing, chemical

extraction, ultrasonication, microwave assisted extraction (Koberg et al., 2011), super critical carbon dioxide extraction (Halim et al., 2011) and enzymatic extraction (Mercer and Armenta, 2011; Halim et al., 2012). Cravotto et al. (2008) reported that ultrasonication and microwave extraction gave higher yield than other conventional methods.

Microalgal lipids in the form of triglycerides or fatty acids can be converted to biodiesel through esterification of fatty acids in presence of a catalyst (Chisti, 2007; Johnson and Wen, 2009). Transesterification is a multiple step reaction, including three reversible steps in series, where triglycerides are converted to diglycerides, then to monoglycerides, and monoglycerides are then converted to esters (biodiesel) and glycerol (by-product) (Mata et al., 2010). The reaction takes place using super critical fluids and via enzymatic and acid/base catalysis (Fukuda et al., 2001, Borges and Diaz, 2012, Medipally et al., 2015). Krohn et al. (2011) used supercritical methanol and porous Titania microspheres in a fixed bed reactor to catalyze the transesterification of triglycerides and free fatty acids to biodiesel, which reached conversion efficiencies of up to 85%. Patil et al. (2011b) reported a process involving simultaneous extraction and transesterification of wet algal biomass containing about 90% of water under supercritical methanol conditions.

1.7 Biochemical and molecular approaches for enhanced biofuel yield in microalgae

Low lipid productivity of fatty acid synthesizing microalgae resulting in high capital cost is a major bottleneck hindering the commercial production of microalgal biodiesel (Sharma et al., 2012). Involvement of engineering aspects

at the biochemical, genetic, metabolic or genomic levels can be considered as a potential strategy for enhanced lipid profile in microalgae. Biochemical engineering refers to increasing the lipid productivity in microalgae by modulating the nutritional or cultivation conditions (Courchesne et al., 2009). Synthesis and accumulation of large amounts of TAGs accompanied by considerable alterations in lipid and fatty acid composition can occur in microalgae when placed under stress conditions caused by chemical or physical environmental stimuli, either individually or in multiple combinations (Hu et al., 2008). Nutrient-starvation has so far been the most commonly employed approach for directing metabolic fluxes to lipid biosynthesis of microalgae (Courchesne et al., 2009). A number of studies have been conducted on nutrient starvation to enhance lipid yield in microalgae. The growth of diatom *Stephanodiscus minutulus* under silicon, nitrogen or phosphorus limitation resulted in an increase in TAG accumulation and a decrease of polar lipids (Lynn et al., 2000). Similarly, in *Chlamydomonas* sp. under nutrient limitation, an increase in palmitoleic acid and oleic acid was detected, whereas PUFA content reduced (Arisz et al., 2000). Nitrogen is the main nutrient component targeted for altering the lipid metabolism. Various reports have been published showcasing the increase in lipid yield upon nitrogen starvation (Kawata et al., 1998; Yamaberi et al., 1998; Illman et al., 2000; Li et al., 2008, Rudolfi et al., 2009). Other lipid induction factors studied include phosphorus (Reitan et al., 1994; Khozin-Goldberg and Cohen, 2006), iron (Liu et al., 2008), temperature (Renaud et al., 2002; Sushchik et al., 2003), salinity (Xu and Beardall, 1997; Takagi et al., 2006; Chen et al., 2008), pH (Guckert and Cooksey, 1990; Tatsuzawa et al., 1996) and light (Napolitano, 1994; Brown et al., 1996; Khotimchenko et al., 2005).

Nutrient limitation has undesirable effect on microalgal cell biomass such as lowering of biomass and reduction in total lipid content. In order to overcome such hurdles, it is essential to have an insight and understanding of various rate limiting steps in lipid biosynthetic pathway (Courchesne et al., 2009), expression and regulatory analysis of genes and enzymes involved in triacylglycerol formation and of those enzymes that influence TAG biosynthesis directly or indirectly (Verma et al., 2010). The integrated use of lipidomics, genomics, proteomics, and metabolomics provides deep insight into the cellular processes and helps in algal strain improvement (Schenk et al., 2008). The approaches, especially transcriptomics and proteomics, offer the possibility of identifying differentially expressed genes and proteins, which are directly or indirectly involved in lipid biosynthesis and degradation. The identification and expression of key regulatory genes and their proteins, such as transcription factors, kinases and phosphatases in transgenic cells can efficiently alter whole physiological pathways leading to higher production of the targeted metabolite (Anderson et al. 2004; Mc Grath et al., 2005; Dombrecht et al., 2007). The various techniques employed to study the metabolic flux include monitoring of consumption and production of key compounds (Dong et al., 2006, Righelato and Spracklen, 2007) or the isotopic labelling of key metabolite precursors or intermediates and the monitoring of these isotopes in a time-dependent manner (Fernie et al., 2005) using radioactive isotopes (Conklin et al. 1997; Yang et al., 2002; Schwender et al., 2004) or stable isotopes for NMR spectroscopy (Schwender et al., 1996; Sriram et al., 2004) and mass spectrometry (Yang et al., 2002; Schwender et al., 2004; Shastri and Morgan, 2007).

After identification of the pathways and key enzymes involved in the biosynthetic pathways, genetic engineering has the potential to improve algal productivity. The complete genomes of *Anabaena* (cyanobacteria), *C. reinhardtii* and *Volvox carteri* (green algae), *P. tricornutum* and *T. pseudonana* (diatoms) *Cyanidioschizon merolae* (red alga), *Osteococcus lucimarinus* and *Osteococcus tauri* (pico-eukaryotes) and *Aureococcus annophageferrens* (a harmful alga belonging to Chrysophyceae) (Armbrust et al., 2004; Derelle et al., 2006; Bowler et al., 2008; Beer et al., 2009; Radakovits et al., 2010) have been already sequenced. The sequencing projects for some other microalgal species such as *Fragilariopsis cylindrus*, *Pseudonitzschia, Thalassiosira rotula*, *Botryococcus braunii*, *Chlorella vulgaris*, *Dunaliella salina*, *Galdieria sulphuraria* and *Porphyra purpurea* are under progress (Liolios et al., 2008; Sasso et al., 2012). The genetic transformations of microalgal species for various purposes have been actively researched. *Chlamydomonas* sp. has emerged as a model eukaryotic microbe for the study of many processes at nuclear and chloroplast level, including photosynthesis, phototaxis, nutrient acquisition, and the biosynthesis and functions of lipids and proteins (Lumbreras et al., 1998; Fuhrmann et al., 2005; Mayfield et al., 2007; Hu et al., 2008).

Genetic engineering approaches have been adopted to increase the biomass production in microalgae by improving photosynthetic efficiency. The number of light harvesting complexes or chlorophyll antenna size was reduced to increase the photosynthetic efficiency by 50% in *C. reinhardtii* (Mussgnug et al., 2007). In another study conducted by Huesemann et al. (2009), no growth improvement was observed in algal antenna mutants cultured in outdoor ponds and also in laboratory conditions. With the aim of

engineering an algal strain with high lipid content for biodiesel production, acetyl-CoA carboxylase (ACCase) was first isolated from the microalga *Cyclotella cryptica* by Roessler (1988) and then successfully transformed into the diatoms *C. cryptica* and *Navicula saprophila* (Dunahay et al., 1995, 1996; Sheehan et al., 1998). Though ACCase gene was successfully expressed with 2-3 folds increase in the enzyme activity, no significant increase in lipid accumulation was observed in the transgenic diatoms (Dunahay et al., 1995; 1996). In expressing recombinant thioesterases to enhance the expression of shorter chain length fatty acids, Radakovits et al. (2010) were able to improve the level of lauric and myristic acids in the diatom *Phaeodactylum tricornutum,* proving advantageous for biofuel feedstock because biodiesel made from saturated short or medium chain length fatty acids has a relatively low cloud point and resistance to oxidation. In addition, several studies have shown metabolic shifts in starchless mutants of *Chlamydomonas reinhardtii* in favour of an overexpression of TAG (Moellering et al., 2009; Wang et al., 2009; Li et al., 2010b). In a starchless mutant, Moellering et al. (2009) inhibited the expression of major lipid droplet proteins by RNA interference (RNAi), which not only increased the size of the lipid globules (Moellering and Benning, 2010), but also resulted in decreased growth. In contrast, the fatty acid content of a starchless selected mutant of *Chlorella pyrenoidosa* was doubled without detriment to its growth characteristics (Ramazanov and Ramazanov, 2006). Wang et al. (2009) studied on the lipid productivity of wild type and starch-less mutant strain of *Chlamydomonas reinhardtii* and found that under nitrogen stress, the mutant strain produced 30 fold increase in lipid content. This suggests that it is possible to improve the productivity

of microalgae using lipid selection strategies. The application of genetic engineering in the improvement of microalgal biofuel production is still in its infancy stage. The current molecular strategies required to improve microalgal biodiesel production include blocking energy rich compounds producing metabolic pathways, elimination of fatty acid β-oxidation that consumes TAGs; modification of lipid characteristics; direct biological synthesis of fatty acids; and secretion of TAGs, free fatty acids and wax esters directly into the medium (Radakovits et al., 2010). Targeted metabolic manipulations via gene knockdown can also be used to increase accumulation of fuel-relevant molecules in eukaryotic microalgae without comprising on growth (Trentacoste et al., 2013). The emerging tools of genome engineering are going to play a pivatol role in biofuel production from marine microalgae in the future.

1.8 Multivariate statistical techniques for process optimization in biofuel production

In any bioprocess technology, it is very much necessary to improve the performance of the biochemical processes with minimum investment of time and cost. Optimization refers to improving the performance of any systems by discovering the best conditions to attain maximum benefit out of it (Araujo and Brereton, 1996). Traditionally, optimization of an experiment/ process was done by changing one factor at time (one-variable-at-time) and monitoring the responses while keeping other factors constant. This technique is disadvantageous as it is much time and material consuming; and not depicting the interactive effects of different parameters on the responses (Lundstedt et al., 1998; Bas and Boyaci, 2007). Development of rigorous models for a given biological system with many complex metabolic processes

and variables is still a big challenge (Franco-Lara et al., 2006). The optimization of analytical procedures has been carried out by multivariate statistic techniques such as Response Surface Methodology (RSM), Artificial Neural Network (ANN) and Uniform design (UD) (Nunez et al., 2013).

Response surface methodology (RSM) is a collection of statistical and mathematical techniques used for optimizing and improving processes in which a response of interest is influenced by several variables. It defines the effect of the individual parameters, alone or in combination, on the processes/systems and also generates a mathematical model which describes the whole processes (Anjum et al., 1997; Myers et al., 2016). The most commonly used designs to determine response surfaces are the full and fractional factorial designs and the more complex central composite (CCD), Box-Behnken (three level design), Doehlert and mixture designs (Box et al., 2005; Bruns et al., 2006; Ferreira et al., 2007). Response surface methodology has been widely adopted in various steps of biofuel production starting from optimizing the culture media and conditions for high yield production till the final transesterification process of converting oil to biodiesel (Mandal and Mallick 2009; Patil et al., 2011a, b; Chen et al., 2014; Skorupskaite et al., 2015).

Artificial neural network (ANN) is another popular tool progressively applied in a number of optimization works (Karim et al., 2003), and have been utilized with high success for system design, modeling, optimization and control mainly due to their capacity to learn, filter noisy signals and generalize information through a training procedure (Gasteiger and Zupan, 1993; Montague and Morris, 1994; Kim and Lewis, 1998). Contrary to the

conventional model that requires various order (second, third or fourth) needs to be provided, ANN is more flexible and does not impose any restriction on the type of relationship governing the dependence of output parameters on the various running conditions (Garcıa-Gimeno et al., 2003). Mohammed et al. (2013) compared the efficiency of RSM and ANN models in increasing the lipid productivity in microalga, *Tetraselmis* sp. FTC 209 and concluded that though ANN appeared to be more accurate and dynamic in simulating the true behaviour of dataset over RSM, both methodologies complemented each other in interpreting the results.

Another popular design model is Uniform Design (UD), which was developed by Fang at the end of 70's (Fang, 1980). This experimental plan produces uniform scattering of the design points over the experimental domain. One of the most important advantages of uniform design over traditional experimental design is that the experiment can be performed in a relatively small number of runs even when the number and levels of the factors are large (Fang, 1998; Fang and Qin, 2003; Fang and Chan, 2006). Uniform designs do not have a mathematical model associated and hence it is suitable for studying systems with an unknown underlying model. Artificial neural network and multiple linear regressions could be used to model systems after experimentation and data collection with uniform designs. Uniform design has been successfully applied in various fields such as chemical engineering, chemistry (Liang et al., 2000; Chen et al., 2003), and biotechnology (Cao et al., 2004; 2006). It has been used for optimizing concentrations of culture medium components and process variables related to biochemical process (Xu et al., 2006; Wei et al., 2009; Hua and Xu, 2011).

1.9 Challenges in the commercialization of algal biodiesel

Microalgae have gained economic and industrial interests for their uniqueness in producing many valuable products ranging from therapeutic proteins to biofuels. The goal of microalgal biotechnology is to improve the productivity of these organisms in order to meet the demands of the rapidly growing market (Spolaore et al., 2006). Although there is high potential for such biofuels in world market, there are many bottlenecks that limit the successful commercialization of microalgae based biodiesel. Insufficient production of algal dry mass along with low lipid yield is the major limiting factor in biofuel production (Medipally et al., 2015). Choices of appropriate bioreactors, harvesting and processing procedures are the major challenges in this field (Vasudevan and Briggs, 2008). The other drawbacks of the current state of biofuel production from microalga are the high investments costs and the high demand on auxiliary energy for biomass production and for lipid processing to biodiesel (Schlagermann et al., 2012).

There are some approaches, which will help to reduce costs and accelerate the commercialization of algal biodiesel (Thurmond, 2009; Wu et al., 2012). Some of them are:

a) *Selection of high performance strains*- Strain selection can be performed with a special focus not only on high lipid content and areal lipid productivity, but also on the fatty acid profiles matching biodiesel requirements. Excellent strains of microalgae can be obtained by screening of a wide range of naturally available isolates and the efficiency of those can be improved by selection and genetic transformation (Schlagermann et al., 2012; Wu et al., 2012).

b) *Development of cost effective cultivation and harvesting systems*- The various large-scale culture systems also need to be compared on their fundamental properties such as their light utilization efficiency, temperature control, the hydrodynamic stress on the organism, the ability to maintain axenic monocultures and their scalability (Borowitzka, 1999).The coupling of waste heat from power plants and other industrial sources might help to scale up the productivity of large scale open ponds at low night temperature areas (Packer, 2009). Photobioreactors with a real time smart on-line monitoring system for maintaining optimal growth conditions are very promising (Meireles et al., 2002) The complex harvesting and processing procedures combined with insufficient production of algal dry mass are limiting factors for algal biofuel production (Ahrens and Sander, 2010).

c) *CO_2 fixation, wastewater treatment and microalgal cultivation*- Integrating intensive, large-scale microalgal cultivation with traditional municipal or industrial wastewater treatment may provide the means for generation of significant quantities of bioenergy. Most sources of municipal wastewater are rich in ammonia, phosphate and other essential nutrients required supporting microalgal biomass production thereby limiting the requirement of external inputs (McGinn et al., 2011). Various feasibility studies have been reported in wastewater based microalgal cultivation and nutrient removal (Chinnasamy et al., 2010; Kong et al., 2010; Li et al., 2010a). Development of high CO_2 tolerant strains will help to curb the CO_2 pollution and also boost the microalgal biomass productivity. De

Morais and Costa (2007a; 2007b; 2007c) could isolate microalgae with high CO_2 utilisation ability from waste treatment ponds of coal fired thermal power plants.

d) *Co-product fraction marketing strategies*- This includes utilization of biomass fractions left after extraction of lipid for nutritional and commercially valuable chemical production purposes. Microalgae have good prospects in the food, feed, pharmaceutical, and cosmetic industries. A bio-refinery approach that converts microalgae to wide range of products including biodiesel and value-added products will be critical to the success for aspiring algal biodiesel based industries (Huntley and Redalje, 2007; Wu et al., 2012; Medipally et al., 2015)

1.10 Bio-refinery concept for marine microalgal biofuels

Though the microalgal fuel industry is gaining huge interest, a large scale commercial production is still questionable due to its high investments in capital and operations. The capital costs involved in the biodiesel production account for approximately 50% of the total costs, whereas biomass harvesting and drying will contribute to 20-30% (Mata et al., 2010). The reality is that most of the studies available in literature are based on lab-scale experimental facilities with a low capacity of biofuel production and there is no scaled up set ups (Bahadar and Khan, 2013). An integrated algal bio-refinery for multiple high-value products can bring in the financial sustainability to the algal biofuel production units. The term bio-refinery applies to a diverse range of raw materials to sustainably and simultaneously generate a wide spectrum of intermediates and products including food and

feed (Zhu, 2015). Microalgae contain abundant lipids, carbohydrates (starch and/or cellulose), proteins, fats and a variety of inorganic and complex organic molecules. Some of the ingredients such as carbohydrates and lipids can be converted into biofuels, while others can be extracted and produced into different valuable byproducts or co-products, such as cosmetics, pharmaceuticals and nutritious feed (Fig. 1.5) (Ree and Annevelink, 2007; Zhu, 2015).

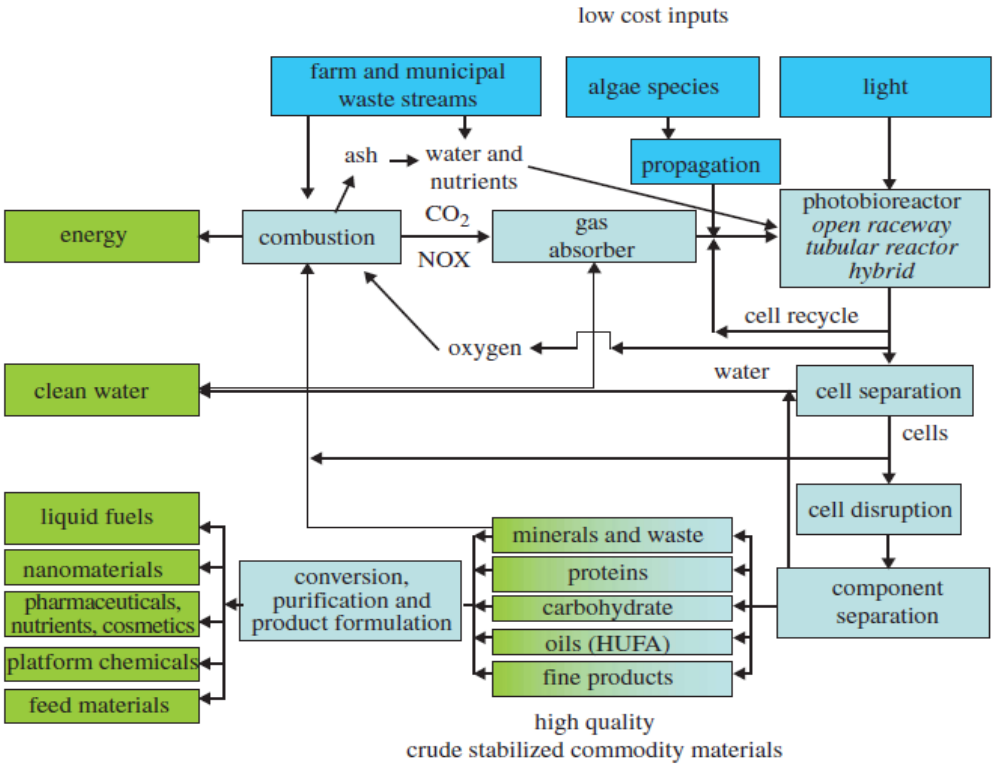


Fig. 1.5 Marine algal bio-refinery (Greenwell et al., 2009)

The high contents of antioxidants and pigments (carotenoids such as fucoxanthin, lutein, betacarotene, astaxanthin and phycobilliproteins) (Orosa et al., 1997; Rodrigues et al., 2014), and the presence of long-chain

polyunsaturated fatty acids (PUFAs) and essential amino acids such as methionine, threonine and tryptophan makes microalgae an excellent source of nutritional compounds (Brown, 1991; Becker, 2004). Co-extraction of high-value products enhances the nutritional or nutraceutical value of the microalgal oil (Becker 2004). Microalgae have also been screened for new pharmaceutical compounds with biological activity, such as antibiotics, antiviral, anticancer, enzyme inhibitory agents and other therapeutic applications (Mata et al., 2010). It is reported that *Chlorella* sp. has been used as food additive and also against infant malnutrition and neurosis (Yamaguchi, 1996). Furthermore, algae are believed to have a positive effect on the reduction of cardio-circulatory and coronary diseases, atherosclerosis, gastric ulcers, wounds, constipation, anaemia, hypertension, and diabetes (Fabregas et al., 1994, Yamaguchi, 1996; Nuno et al., 2013). Besides these, microalgae can also synthesize polysaccharides that can be used as an emulsion stabilizer or as bio-flocculants and polyhydroxyalkanoate (PHA) used in the production of bio-plastics. Microalgae can also produce isoprene, which is a key intermediate compound for the production of synthetic rubber; adhesives and elastomers in chemical industries (Matos et al., 2013, Gouveia, 2014). Furthermore, the amino acids produced by microalgae can be used as biofertilizers and therefore, assist higher plant growth (Dey, 2011; Sahu et al., 2012). Finally, the microalgal biomass leftovers after the extraction of added-value compounds can be used for the production of liquid biofuels (bioethanol, biodiesel, biobutano and bio-oil) (Gouveia and Oliveira, 2009; Miranda et al., 2012) or gaseous biofuels (biomethane, biohydrogen and syngas) (Marques et al., 2011; Ferreira et al., 2013; Batista et al., 2014). In addition, leftovers after anaerobic digestion, CO_2 released

from anaerobic digestion and harvest water can also be recycled to cultivate microalgae and also used as the substrate for biofuel and fertilizer generation (Uggetti et al., 2014; Zhu et al., 2014b).

1.11 Rationale and purpose of the present study

There are various challenges and opportunities hidden in the field of algal based technology. Out of hundreds of microalgal strains reported, only few are capable of producing high contents of lipid, most of which are marine microalgal strains. The vast biodiversity of marine microalgae of Indian waters is yet to be explored fully for biotechnological applications. Therefore, the key technical challenge includes identifying native strains with highest growth rates and oil contents with adequate composition. The isolation and characterization of algae from unique aquatic environments should be a continuing effort. The application of suitable and efficient technology for sampling, isolation and screening and culture maintenance is important. Identification of selected strains using novel molecular techniques is more accurate than current conventional methods. New sets of species specific primers need to be designed for the accurate identification of microalgal species form their population. A clear understanding of the nutritional requirement of members of various classes of micro-algae and also the different culture conditions is a prerequisite for determining the technique of culturing and maintaining the algae for a long period. The relationship between the physiology of the cultured organisms and the bioreactors in which they are grown offer many possibilities for improvement and optimization. The culture conditions of the selected promising species need to be optimized individually to provide a measure of

maximum biological productivity expected. The success for microalgal fuel depends upon developing algal culture systems that will increase the production of oil-rich biomass without compromising growth. An active research is needed to solve the contradiction of lipid content and lipid productivity.

1.12 Objectives

Based on the scope and relevance of the study area, the following were the identified objectives:

1) Isolation, identification and phylogenetic analyses of marine microalgae from the west coast of India
2) Screening for the most potent microalgal strain for biofuel production
3) Optimization of culture conditions and media compositions for high biomass and lipid production in *Navicula phyllepta* MACC8 using response surface methodology
4) Enhancement of lipid production in *Navicula phyllepta* MACC8 under selected stress conditions using two stage cultivation method.

Chapter 2

ISOLATION, IDENTIFICATION AND PHYLOGENETIC ANALYSES OF MARINE MICROALGAE FROM WEST COAST OF INDIA

Contents

2.1 Introduction

Microalgae cover a heterogeneous group of single celled photosynthetic organisms, which include both prokaryotic and eukaryotic. They are free living, pelagic/ benthic found in marine, brackish and fresh waters. Microalgae have tremendous diversity, which offers a great potential that has to be explored to any great extent. In a major maritime country like India, with a coastline of approximately 7,500 km, there is immense scope for exploring the indigenous microalgal flora in the vast open sea for biotechnological applications, especially for cleaner fuel production (Hytonen et al., 2014). However, most of the studies on marine microalgae from Indian Ocean have mainly focused on harmful algal blooms and phytoplankton ecology. Algal blooms caused by diatoms, dinoflagellates, cyanobacteria, raphidophytes and haptophytes (Bhat and Matondkar, 2004) have been reported in literature. With respect to the spatial distribution of algal blooms along the western continental shelf from Gujarat to Kerala, the

highest prevalence of bloom incidents were reported from Kerala coast (Jugnu, 2006) followed by Mangalore and Goa (D'silva et al., 2012; Pereira and Almeida, 2014). Gopinathan (1972) and Sanilkumar (2009) studied the qualitative and quantitative analysis on phytoplankton of the Cochin estuary and described 120 species. Some marine phytoplankton diversity studies also have been done on the east coast of India (Lakshmi and Rao, 2009; Srinivasakumar and Rajashekhar, 2009). There are many studies on the distribution, abundance and productivity of phytoplankton (Devassy and Goes, 1989; Perumal et al., 1999; Gomes et al., 2000), but the isolation and culturing of the microalgae are limited to a few representatives.

Isolation is a necessary process to obtain pure cultures and presents the first step towards the selection of microalgae strains with potential for biodiesel production. Traditional isolation techniques include the use of a micropipette for isolation under a microscope or cell dilution followed by cultivation in liquid media or agar plates. Single cell isolation using micropipettes (e.g., a glass capillary) carried out under inverted microscope is time-consuming, but results in identifiable pure cultures (Andersen, 2005). Gravity separation is perhaps more frequently used to concentrate the target organisms rather than to establish unialgal cultures. Another approach in the laboratory includes the enrichment of some microalgae strains by adding nutrients for algal growth. The most important nutrient sources for algal growth are nitrogen and phosphate. Some particular algae species may require trace minerals for their growth (e.g., silicon for diatoms) (Andersen, 2005). An automated single cell isolation method that has been developed and widely used for cell sorting is flow cytometry (Davey and Kell, 1996). This technique has been successfully used for microalgae cell sorting from

water with many different algae strains (Reckermann, 2000), primarily based on properties of chlorophyll autofluorescence (CAF) and green autofluorescence (GAF) to distinguish algae species such as diatoms, dinoflagellates or prokaryotic phytoplankton. Another advanced method is to use micromanipulator which is stipulated in literature as the ideal tool for algal screening and isolation (Kacka and Donmez, 2008; Moreno-Garrido, 2008).

Algal taxonomy is a key discipline in phycology and is critical for physiology, ecology, algal genetics, applied phycology, and bio-assessment (Manoylov, 2014). According to the records, the estimated number of described species in microalgae ranges between 40,000 and 60,000, but the number of un-described species ranges from hundreds of thousands to millions of species spread over the globe (Norton et al., 1996, Sastre and Posten, 2010). It is important to mention that the effective use of an algal strain relies on its correct identification. Microalgal species have traditionally been discriminated by morphological observations and pigment profiles (Bast, 2012). Most of them are distinguished based upon their plastid and flagellar characteristics. Microscope-based microalgal cell identification methods are usually the standard procedures used in laboratories for the rapid screening of algal samples. Conventional microscopy techniques used to analyse microalgae can give misleading results since they lack morphological markers for precise identification (Godhe et al., 2002; Bertozzini et al., 2005). With the advent of electron microscopy (EM), the study of ultrastructure of cells became possible and such details were widely accepted as a reliable character for cladistics analysis of various algal taxa, because the characteristics were considered as

evolutionarily conserved (Friedl, 1997). The relatively high magnification range (approximately 10 to 1000 times of their original size) allows phycologists to detect microalgal specimens in much greater detail than in those examined by light microscopy.

Molecular approaches for taxonomic identification of algae have proved to augment or even replace morphological identification (Deans et al., 2012). The information of the genetic makeup of the organism helps to have a clear understanding on the identification of pure isolates. Molecular methods are very effective for identification of pico-sized fractions that have very few morphological features available for identification (Not et al., 2007). DNA-based methods have previously been used to identify diatoms (Diez et al., 2001; Zeidner et al., 2003; Armbrust et al., 2004; Fuller et al., 2006), and polymerase chain reaction (PCR) methods using specific primers designed based on specific molecular markers were adopted to identify genera or species of phytoplankton (Gray et al., 2003; Skovhus et al., 2004; Galluzzi et al., 2005). Bott et al. (2010) and Kudela et al. (2010) extensively studied different tools of DNA-based detection methods for harmful algal blooms focusing on cell enumeration and identification, molecular phylogenetics and applications of high-throughput sequencing methods.

The molecular markers are the genes that are shared by all organisms or a group of organisms irrespective of their nutritional classes (Doelle et al., 2009). The most common DNA regions analysed for phylogenetic analysis are ribosomal RNA genes (rRNA), mitochondria genes, plastid genes (rbcL), ITS (Internal Transcribed Spacer), and microsatellite DNA sequences. Ribosomal RNA genes (rDNA) have high number of copies and

the presence of conserved and variable regions have made possible a new method of species identification, classification and phylogenetic relationship determination (Mullis et al., 1986; Mullis and Faloona 1987; Saiki et al., 1988). Algal cells have two varieties of SSU rDNA, nuclear-encoded and chloroplast-encoded, which have been considered for analysing algal taxonomy and phylogenetic relationships (Huss and Sogin, 1990; Wilcox et al., 1992; Steinkotter et al., 1994; Schreiner, 1995). Class-specific and genus-specific primers are generally designed to target the mature rRNA sequences of 18S, 5.8S, or 28S (Medlin et al., 2000). In contrast, the ITS sequences that are more divergent are usually used for species or strain identification (Senapin et al., 2011). The analyses of microsatellites and internal short sequence repeat (ISSR) also have been applied to microalgae (Nagai et al., 2007; Evans et al., 2009; Casteleyn et al., 2010).

The most efficient technique for identification of microalgal isolates based on their DNA is by polymerase chain reaction (PCR). Moro et al. (2009) developed new PCR primer sets which were able to amplify microalgal rRNA genes from environmental samples with accurate specificity. When using microalgae for PCR studies, there are great chances of potential inhibitors that often cause PCR inhibition and low yields (Godhe et al., 2002; Bertozzini et al., 2005). Direct PCR helps to overcome this issue by amplification from a few cells without the need for DNA extractions (Godhe et al., 2002; Auinger et al., 2008). Quantitative analyses of microalgae from preserved field samples combining morphological and small-subunit (SSU) rRNA gene sequence using single cell PCR has been reported by Auinger et al. (2008). The detection of individual cells using fluorescently-labelled rRNA-targeted oligonucleotide probes have been

successfully demonstrated (DeLong et al., 1989). Galluzzi et al. (2005) developed a real-time PCR-based assay for rapid detection of all toxic species of the *Alexandrium* genus from environmental samples and cultures. A further promising molecular approach is the application of DNA microarrays, which are applied generally for gene expression and have been used with oligonucleotide probes of conserved genes for species identification at all taxonomic levels (Gescher et al., 2008). A combination of molecular probes/primers and DNA microarrays could serve as a rapid and reliable tool for rapid screening of microalgae. The application of novel molecular techniques has the potential to revolutionise microalgal classification, especially for identifying hyper- lipid producing microalgae.

In the present study, marine microalgal strains were isolated from the west coast of India, and identified using taxonomic and molecular tools to understand the phylogenetic relatedness to already investigated oil producing strains.

2.2 Materials and methods

2.2.1 Sample collection

Water samples were collected from Trivandrum to Mumbai along the west coast of India by cruises (numbers 269, 270, 284, 285 and 302) on-board FORV Sagar Sampada, Ministry of Earth Sciences, Government of India. The collections were made from the top and 10m depth of the water column at each location. The microalgal cells were concentrated from sea water by filtering 50 L sea water using 20 µm nylon mesh, and the pico-phytoplankton were filtered using 0.2 µm cellulose acetate filter paper. The samples from 7 stations of Cochin Estuary (9° N 76 ° E) were collected by

cruise on a boat and phytoplankton samples were concentrated as above. The concentrated algal samples were inoculated on board into enriched sea water and transported to the laboratory and incubated under white fluorescent light (20 μmol m^{-2} s^{-1}) at 25 °C with 16:8h light: dark.

2.2.2 Isolation, enrichment and purification of microalgal cells

In order to isolate single microalgal species from the field water samples, the mixed cultures were first enriched with multiple natural sea water based media recipes. The most commonly used media recipes were F/2 (Guillard, 1975), Walne's (Walne, 1970) and Keller's K medium (Keller et al., 1987) and BG11 medium (Allen and Stanier, 1968) specifically for cyanobacteria. The standard plating and serial dilution methods were used to separate algal populations. Sterilised dry glass petridishes containing approximately 25 mL of 1-1.5% agar medium were used to plate these diluted samples. 100 μL of the diluted sample was transferred to a media plate and streaked onto the agar. Inoculated plates were placed at 25-28 °C, approximately 27 μmol m^{-2} s^{-1} where the algae were allowed to grow for about 14 days. Grown algae cultures were picked using sterile tips and inoculated into sterile nutrient media and placed under light. This streaking method was repeated until isolation into unialgal cultures was achieved. Serial dilution of the mixed cultures to many folds was also followed to isolates single cells of most dominating microalgae species in the field samples. Inverted microscopy (Zeiss Axiovert 25 CFL) was also employed to isolate small cells from the water samples using sterile glass pasteur pipettes. Following the isolation of individual microalgae colonies, each strain was initially labelled based on the sampling location and media used. The isolated algae were maintained as stock cultures and were stored on a

cool, low light shelf. These stock cultures were maintained by sub-culturing into fresh nutrient media at least once in a month, or more frequently depending on the nature of each isolated strain.

The monocultures were purified or made free of bacteria/or contaminating cyanobacteria using a cocktail of antibiotics. The basic antibiotic preparation recommended consisted of 100 mg of penicillin G (sodium or potassium salt), 25 mg of dihydrostreptomycin sulfate and 25 mg of gentamycin sulfate in 10 mL of distilled (or deionized) water, and sterilized by membrane filtration. The standard dose of 0.5 mL of antibiotic mix was added to 50 mL of algal medium. Subsequent subculturing was done within 24 h of initial treatment to get completely axenic monocultures. In order to remove contaminating diatoms from monocultures of other groups, 10 mg L^{-1} of germanium dioxide in 1N NaOH was added to the culture medium.

2.2.3 Morphological characterization

2.2.3.1 Microscopic examination

The monocultures were examined under light microscopy by taking a small drop of the culture on glass slide with a cover slide over it. The cells are allowed to settle for 3-5 min. Proper care was taken to not to dry up the specimen preparation. The morphology of the cell was observed carefully under 100 X oil immersion of light microscope (Olympus CH20iBIMF, India).

2.2.3.2 Scanning electron microscopy

The cultures were observed under scanning electron microscope for a detailed understanding of the morphology. 5 mL microalgal cells in their exponential phase (8-10 days) were harvested and washed in 1X phosphate

buffer solution (PBS) for 2-3 times. The cells were centrifuged at 8000 × g and supernatant was discarded. 500 µL of 2.5% gluteraldehyde was added and incubated at 4 °C. The cells were harvested after 12h and washed in 1X PBS for 2-3 times. 200 µL of 2% osmium tetroxide was added and incubated at 4°C for 4 h or more till the culture go stained black/dark brown. The cells were harvested and washed with 1X PBS for 2-3 times. The obtained cells were dehydrated in a series of 10%, 25%, 50%, 75%, 100% acetone and dried in vacuum. A few drops of hexamethyldisilazane (HMDS) were immediately dispensed onto the slides mounted with delicate cells of dinoflagellates or any other flagellated species. The samples were mounted on SEM stubs, and then sputter coated with gold and viewed with VEGA3 TESCAN SEM (Botes et al., 2002; Grant, 2008). The cultures were identified using the standard identification keys (Thomas, 1997; Joseph and Saramma, 2011).

2.2.4 Molecular identification

2.2.4.1 DNA extraction

Three different DNA isolation methods were performed for diatoms and green algae in this study. They are described as mentioned below:

(a) Method A (Lee et al., 2003):

Microalgal culture (10-12 mL) was centrifuged at 6000 ×g for 5 min. The supernatant was discarded and resuspended the pellet in 1mL TE buffer (10 Mm Tris-Cl, 1 Mm EDTA, pH. 8) and mixed thoroughly. The cells were harvested by centrifugation and resuspended in 1 mL lysis buffer (50 mM Tris–Cl pH 8.0, 100 mM NaCl, 50 mM EDTA pH 8.0, 2% sodium dodecyl sulphae, 0.2% polyvinylpyrrolidone, 0.1% ß mercapto ethanol) and 10 µL proteinase K (20 mg mL^{-1}) and

incubated at 37 °C for 1 h and then at 55°C for 2h. Further extraction was carried out by phenol- chloroform method (Sambrook and Russell, 2001). One volume of phenol: chloroform: isoamyl alcohol (25:24:1 v/v) was added and the tube was mixed by inverting slowly 5-6 times and kept for 5 min. The tubes were centrifuged at 14500 ×g for 15 min at 4 °C for phase separation. The upper aqueous layer was transferred to a new tube and equal volume of chloroform: isoamyl alcohol (24:1v/v). The tubes were centrifuged at 14500 ×g for 15 min at 4 °C. The step was repeated twice and the aqueous layer was transferred to a new 1.5 mL microcentrifuge tube to which one volume ice cold absolute ethanol was added and incubated in -20 °C overnight. The tubes were centrifuged at 14500 × g for 15 min. The DNA pellet was washed twice by adding 200 µL ice cold 70% ethanol. The DNA was pelleted by centrifugation at 14500 × g at 4 °C and air dried. 50-100 µL of double distilled water (Milli Q) or 10 mM Tris-Cl buffer was used for dissolving the DNA and stored at -20 °C for further use.

(b) Method B (modified method of Wu et al., 2000):

Exponentially growing algal culture (10-12 mL) was pelletized by centrifugation at 6000 × g for 5 min. The supernatant was removed; the pellets were washed with distilled water and suspended in 450 µL of TEG (25 mM Tris HCl, 10mM EDTA, 50mM glucose) at pH 8. 100 µL of lysozyme (5 mg mL^{-1}) and 50 mg glass beads were added to the tubes and vortexed for 3-5 min. To this solution, 50 µL of 10% SDS was added and incubated in ice for 10 min.8 µL of proteinase K (20 mg mL^{-1}) was added and further incubated at 60 °C for 60 min in

a water bath. The solution was vortexed in intervals of 15 min. Once the cells get lysed completely, 1mL of phenol: chloroform: isoamyl alcohol (25:24:1 v/v) was added and gently inverted for 5-6 times. After that the sample was centrifuged at 13000 ×g for 10 min at 4 °C and the aqueous phase was separated to a new microcentrifuge tube. The sample was then washed with chloroform: isoamyl alcohol (24:1 v/v) twice till the pink colour disappeared. The aqueous phase was separated to a new 1.5mL microcentrifuge tube. Double the volume of ice cold absolute ethanol was added and kept for overnight incubation at-20 °C. After incubation, the solution was directly centrifuged at 13000 ×g for 15 min at 4 °C. The supernatant was discarded and the pellet was washed with 75% ethanol twice and air dried. The pellet, free of ethanol smell, was dissolved in 100 µL double distilled water or Tris-EDTA buffer.

(c) **Method C** (Cheng and Jiang, 2006):

In this method, 8-10 mL algal culture was centrifuged at 8000 × g for 2 min. After removing the supernatant, the cells were washed with 400 µL STE Buffer (100 mM NaCl, 10 mM Tris/ HCl, 1 mM EDTA, pH 8.0) twice. Then the cells were centrifuged at 8000×g for 2 min. The pellets were re-suspended in 200 µL TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0). 50 mg of 425–600 µm size-fractionated glass beads (Sigma, USA) were added to the cell suspension. Then 100 µL Tris-saturated phenol (pH 8.0) was added to these tubes, followed by a vortex-mixing step of 120s to lyse cells. The samples were subsequently centrifuged at 13000×g for 5 min at 4 °C to separate the aqueous phase from the organic phase. 160 µL of upper aqueous phase was transferred

to a clean 1.5 mL tube. To this, 40 µL TE buffer was added to make 200 µL and mixed with 100µL chloroform and centrifuged for 5 min at 13000×g at 4 °C. The lysate was purified by chloroform extraction until a white interface was no longer present; this procedure might have to be repeated two to three times. The upper aqueous phase (160 µL) was transferred to a clean 1.5 mL tube. Then 40 µL TE and 5 µL RNase (at 10 mg mL^{-1}) were added and incubated at 37 °C for 10 min to digest RNA. Then 100L chloroform was added to the tube, mixed well and centrifuged for 5 min at 13000×g at 4 °C. The upper aqueous phase (150 µL) was transferred to a clean 1.5 mL tube. The aqueous phase contained purified DNA and was directly used for the subsequent experiments or stored at -20 °C.

The concentration and purity of the isolated DNA were checked spectrophotometrically (Hitachi, Tokyo, Japan) by taking the ratio of absorbance at 260 nm for DNA and 280nm for proteins.

Concentration of DNA (ng μL^{-1}) = Abs_{260}× 50× dilution factor

2.2.4.2 PCR amplification using different molecular markers

The extracted genomic DNA was used as a template for amplification of the class specific and genus specific conserved genes as listed in Table 2.1. The PCR reactions were carried out using 2.5µL of 10X Taq buffer (with added 2.5 mM $MgCl_2$), 2.5 µL of 2.5 mM dNTPs, 1 µL each of forward and reverse primer (10 pmol), 1µL of template DNA and 0.5 µL 0.25U Taq polymerase and made up to 25 µL using sterilised double distilled water. The PCR was performed in gradient Thermal cycler (Eppendorf, Germany) with the following programme: 95 °C for 5 min, 94 °C for 30 s, annealing

temperature varying from 52-56 °C, extension at 72 °C for 1-2 min, and 72 °C for 10 min. The whole reaction was standardized for each primer set. The PCR products were confirmed by running 5 µL of PCR reaction mixed with 3 µL of loading buffer (75 mM EDTA, 3% glycerol, 0.02% bromophenol blue) on the 1 % agarose (Sigma Aldrich, USA) in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA) gel (ethidium bromide 0.1 µg mL^{-1} of gel) with 110 V applied current. DNA bands on the gel were visualized under UV light in Gel documentation system (BIORAD Gel Doc™ XR^{+}, USA) with Image lab™ software.

2.2.4.3 Transformation into *Escherichia coli* (*E. coli* DH5α)

For cloning, 3.5 µL of PCR product was mixed with 5µL of 2X rapid ligation buffer, 0.5 µL of pGEM-T Easy vector and 1µl of T4 DNA ligase. The ligation reaction was incubated at 4 °C for 18 h. Transformation was performed with competent *Escherichia coli* (*E.coli* DH5α) cells by mixing the 100 µL of competent cells with the ligation reaction product in a 1.5 mL microcentrifuge tube. The tube was gently flicked and placed on ice for 20 min. The cells were subjected to a heat shock for 90 s in a water bath at exactly 42 °C. The tubes were again transferred to ice for 2 min. An aliquot of 600 µL of the culture was transferred to S.O.C. medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) at 37°C. After the incubation, 200 µL aliquots were spread on pre-warmed plates with selective LB agar medium (1% tryptone, 0.5% yeast extract, 1% NaCl, 15% agar, pH 7.0; ampicillin 100 mg mL^{-1}) and cells were allowed to grow overnight at 37 °C. The colonies were selected using blue/white screening. White colonies were isolated and grown overnight at 37 °C in 10 mL Luria Broth (LB) containing 100 mg mL^{-1} of ampicillin.

2.2.4.4 PCR confirmation of gene insert in the selected clones

To confirm the insert, colony PCR of the white colonies were carried out using vector primers T7 F (5´- taatacgactcactatagg-3´) and SP6 R (5´-gatttaggtgacactatag-3´). White colonies (template) were picked up from the plate and dispensed into PCR mix (25 µL) containing 2.5 µL 10X PCR buffer, 2.0 µL of 2.5 mM dNTPs, 1µL of 10 pmol μL^{-1} of T7 and SP6 primers, 0.5U of Taq polymerase and the total volume was made up with sterile double distilled water. The amplification profile consisted of initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 15 s, annealing of primers at 57 °C for 20 s, primer extension at 72 °C for 60 s followed by a final extension at 72 °C for 10 min. An aliquot of 10 µL of PCR product was analysed by 1% agarose gel electrophoresis and visualized under ultra violet light, documented using gel documentation system.

2.2.4.5 Plasmid extraction and purification

Plasmids from the positive clones were extracted using the GenElute Plasmid miniprep kit (Promega, WI, USA). An aliquot of 2 mL culture of recombinant *E.coli* after overnight incubation was pelletised at 12000 × g for 1 min and subjected to a modified alkaline cell lysis procedure followed by adsorption of the plasmid DNA onto a Miniprep binding column. The binding column was inserted into a microcentrifuge tube and centrifuged at 12000 × g for 1 min. Plasmid DNA bound to the column was washed twice with wash solution to remove the endotoxins, salt and other contaminants. The column was transferred to a fresh collection tube to elute the plasmid DNA, 100 µL 10Mm Tris–Cl was added and centrifuged at 12000 × g for 1 min the plasmid DNA was stored at -20 °C.

Purified plasmids were analysed by E*co*RI restriction enzyme digestion to determinate correct inserts. The reaction mix (20 µL) consisted of 2 µL of 10X buffer (NEB- E*co*R1 buffer), 5µL of plasmid DNA, 0.5 µL of E*co*R1 enzyme (20,000U mL^{-1}), and the final volume was made up using sterile double distilled water (MilliQ). The reaction was incubated at 37 °C for 1 h and the enzyme was heat inactivated at 65 °C for 20 min. Positive clones were selected for sequencing.

2.2.4.6 Sequencing and analyses

The plasmids, or the PCR products in some cases, were sent for sequencing based on Sanger dideoxy method using ABI 3730xl DNA Analyzer. After the removal of vector contamination using VecScreen at NCBI, the sequences were assembled and alignments were checked using GeneTool Lite 1.0 software (Wishart et al., 2000). The aligned sequences were analysed using nucleotide BLAST (Basic Local Alignment Search Tool) in which the query sequences were searched within nucleotide collection database (nr/nt) using Megablast for finding highly similar sequences. The main algorithm parameters used consisted for E value set at 10. The maximum target sequences were kept at 100. In the scoring matrix, a reward/ penalty ratio of 0.5(1/-2) was preferred for sequences that are 95% conserved. Low complexity regions were filtered in the search for finer tuning. The species showing the highest similarity with query sequence was submitted in the NCBI GenBank database.

2.2.5 Phylogenetic analysis

For phylogenetic analyses, a set of homologous sequences of the microalgal isolates acquired from NCBI were aligned in MEGA 5 software

using CLUSTAL W 1.6 algorithm (Thompson et al., 1994). The parameters set for pair wise and multiple alignments included a gap opening penalty of 15 and gap extension penalty of 6.66. The transition weight was assigned a value of 0.5 and delay divergent cut off was set at 30%. These are usually default settings. Finally, the tree was constructed using Neighbour Joining algorithm using Maximum Composite Likelihood model and gene distance was calculated using two parameter method of Kimura with bootstrap analyses value of 1000 using MEGA5 software (Tamura et al., 2011).

2.3 Results and discussion

2.3.1 Microalgal isolation, purification and culturing

The selection of fast-growing, productive microalgal strains, optimized for the local climatic conditions are of fundamental importance to the success of any algal mass culture (Mutanda et al., 2011). The seasonal variation in ambient water temperature in the tropical waters of Indian subcontinent facilitates the growth of different species of microalgae. The ecological and physico-chemical parameters in brackish habitats due to mixing of both saline and fresh water bodies make it a favorite place for survival by diverse microalgal strains. A total of 14 unialgal cultures were isolated by sampling across stations along the west coast of India (from Gujarat to Trivandrum), as well as 7 stations in backwaters of Cochin Estuary. Figure 2.1 shows the source location of the different microalgal cultures isolated along west coast of India and Cochin estuary. All the strains attained a stable growth in F/2 medium, except for *Amphidinium* sp. which grew well in K medium. A strain isolated from tropical waters would likely adapt better to local changing environmental conditions and provide a more stable and productive culture with the perspective of outdoor mass cultivation (Lim et al., 2012).

The purification of microalgae from bacterial contamination in the present study resulted in the diminished growth of some of the microalgal strains within a few days of treatment and further subculturing. The same was reported by Riquelme et al. (1988) that axenic cultures of *Asterionella glacialis* did not grow, but it was stimulated by the addition of bacteria. This is a clear evidence for the role of bacteria on the growth of the marine microalgal isolates. Microalgae, both fresh and marine, are always found to be associated with bacteria and the relationship can be either mutualistic or parasitic (Ramanan et al., 2016). Bacteria are inherent part of the physical environment of the microalgae both in laboratory and natural environment influencing the physiology of each other. For example, bacteria isolated from the deep sea water stimulated the growth of the diatom *Chaetoceros ceratosporum* and *Chaetoceros gracilis* (Natrah et al., 2014). Film of *Alcaligenes* on the surface of substratum stimulated the growth of *Nitzschia* sp. (Fukami et al., 1996). Algae supply fixed organic carbon to an artificial consortium of mutualistic bacteria and bacteria in return, supply dissolved inorganic carbon and low molecular organic carbon for algal consumption (Cho et al., 2015). Bacteria loosely or tightly associated with phytoplankton may efficiently increase sedimentation and the removal of organic matter from the water column (Grossart et al., 2005). Studies show micro-nutrients like vitamins (Croft et al., 2005; Kuo and Lin, 2013; Teplitski and Rajamani, 2011), macronutrients like nitrogen and carbon (Bolch et al., 2011; Kazamia et al., 2012; Kim et al., 2014) and phytohormones (Teplitski and Rajamani, 2011) are usually exchanged between algae and bacteria. On understanding the importance of bacteria on the growth of some of the strains especially *Biddulphia* sp. resulted in the maintenance and further study of the isolates in their xenic state itself.

2.3.2 Morphological identification of the isolates

The high diversity and plasticity makes microalgal identification very complex. With the aid of microalgal identification keys in some literatures, the isolates were identified and categorized based on the morphology of the culture and the cellular appearance of the isolated cells viewed under both light microscopy and scanning electron microscopy. Figure 2.2 shows the light micrographs of the 14 strains isolated in this study. Based on the light microscopy images and scanning electron micrographs (Fig. 2.3), two strains MACC2, MACC6 were morphologically identified as centric diatom *Biddulphia* sp. of phylum Bacillariophyta. The cells were solitary or united by spines to form short chains. The frustules in girdle view appeared rectangular or sub octagonal with a wide girdle as simple band. The valves were elliptical in shape, convex having apices drawn out to form narrow tubular weakly capitates processes directed outwards diagonally. The valves were spinous with numerous small chloroplasts lying against wall. The centre of the valve was almost flat and furnished with two slender straight spines directed outwards. The frustules were weakly siliceous. The length ranged from 35-40 µm and breadth 27-30 µm. The rest of the brown algae were pennate diatoms, initially identified as *Amphora* sp. MACC4, *Amphora* sp. MACC9, *Navicula sp.* MACC8 and *Nitzschia* sp. MACC11 sp. *Amphora* sp. cells were broad, oval, inflated in the median region with subordicular apices. The cell length was 10-15 µm and breadth ranged 3-5 µm. Its raphe was inflexed and biarcuate. The central and ventral areas were distinct. The cell's ventral margin was slightly concave and dorsal side convex. Ventral side comprised with arrow of short striae and dorsal side with indistinct longitudinal line. *Navicula* sp. on the other hand, had lanceolate valves,

tapering to narrow rounded ends. The raphe was filiform, straight, and had the external proximal ends terminating very close to one another. The striae radiated about the center of the valve, becoming parallel to nearly convergent at the apices. The cell size was 18-23 µm length and 3-6 µm width. *Nitzschia* sp. cells had dimensions of 6-8 µm length and 2-4 µm width. The valves of *Nitzschia* sp. were linear with rounded apices. Fibulas were relatively large and distinct and raphe eccentric within a kneel. MACC 1 was solitary cells, but formed loose aggregates of thick mucilaginous sheath of each cell. Cells were spherical and green in colour. MACC 10 cells were globular or ovoid and slightly dorso-ventrally flattened. The epitheca was nearly or slightly larger than hypotheca. The sulcus was narrow and extended. There was a red eye spot, which was very faintly visible near the sulcus. The cells were identified as a dinoflagellate *Durinskia baltica* with a size of 2 µm. The highly aggregated cells of MACC7 resembled the cyanobacteria *Pleurocapsa* sp. The cells were arranged in irregular groups or rows with pseudofilaments. Cells variable in size sometimes with granular content enveloped with a thick cell membrane. Cell division happened by irregular binary fission or multiple fission in the enlarged cells of numerous endospores. The green cells of MACC13 and MACC14 were spherical or slightly ellipsoidal in shape with a diameter of 1.7 µm and 1.4 µm respectively. They were mostly isolated or diplococcus. They found similarity to Chlorophyceae group showing maximum closeness to *Nannochloris* sp. or *Chlorella* sp. *Prymnesium* sp. MACC15 and MACC 16 was unicellular flagellate with oval or ellipsoidal shape. The cell ranged from 6-9 µm in length. Each cell carried two flagella of equal length and a short haptonema. Flagellae length ranges from 10-12 µm and highly flexible, non-coiling haptonema ranges from 3-5 µm. *Amphidinium* sp. MACC17 appeared

oval in shape, dorso-ventrally compressed with a size of 10-12 µm. The epicone was small, stretching to a length of 3 µm, bent in a tongue shaped form with left deflection arising from the ventral side of the hypocone. Hypocone was asymmetric with a truncated and oblique anterior, rounded antapex and convex sides. There was presence of transverse and longitudinal flagellum with a length of 18-20 µm (Thomas, 1997; Joseph and Saramma, 2011).

2.3.3 DNA extraction and molecular identification

The traditional method of differentiating the isolates based on the preliminary information on the morphological details can be sometimes misleading, especially in the case of pico-sized phytoplankton with limited morphological features. In such cases, molecular identification can be an effective tool for differentiating the pure isolates (Not et al., 2007). Extraction of genomic DNA from microalgae that is free of contaminants is very crucial for PCR based molecular characterisation. The morphological complexity of the cell wall and other structures in various groups of microalgae make it difficult to extract a good quality of nucleic acids (Varela-Alvarez et al., 2006). Hence it is important to adopt a good protocol modified accordingly for isolation of DNA from morphologically diverse group of microalgae. In this present study, three different protocols were compared for isolating a good quality of nucleic acid from diatom and green microalga. The method A, which was generally used for gram positive bacteria, could not extract enough nucleic acid from the microalgal cells, especially green cells. The methods B, a simplified protocol of Wu et al. (2000) with modifications, were followed for isolation of genomic DNA from the all of the microalgal isolates. Cell breakage is very critical step in the effective DNA isolation method. The very rigid cell wall coupled with small size of coccoid

microalgae especially green algae, makes it difficult to cause cell lysis. In such situations, microbead beating is found to be very effective way for cell breakage along with the treatment of lysozyme and proteinase K. The concentration of the lysozyme was reduced compared to the original protocol (i.e. from 50 mg mL^{-1} to 5 mg mL^{-1}). The use of salt solution for cell lysis was completely avoided in this work. The overnight wash with 70% ethanol was essential only for removal of polysaccharides and for high quantity of DNA. Method C is the fastest and easiest method for successful isolation of genomic DNA. Compared with all the other protocols, which required laborious steps, such as four to six changes of micro centrifuge tubes, incubation, precipitation, elution or washing and drying thereby resulting in poor quality of DNA, method C minimised the steps into to a simple phenol/chloroform extraction, and a precipitation was not necessary. The DNA could be isolated within a short time of approximately 50 min yielding a high molecular weight nucleic acid. In addition, the whole process proved to be cost effective too (Cheng and Jiang, 2006). In term s of nucleic acid concentration, a quiet good concentration of DNA (~300-700 ng μL^{-1}) was obtained using method B and C isolation method whereas method A could result only in range of 60-175 ng μL^{-1}. The purity of the DNA extracted was in the range of 1.5- 1.7 with slight protein contamination found in method B. Figure 2.4 shows the different gel images with the genomic DNA extracted from diatom and green algae with three different protocols. DNA extractions with a 260/280 ratio of above 1.80 are deemed to be of high quality and suitable for use in downstream applications (Eland et al., 2012). In all extraction procedures, comparatively good quantity of microalgae was isolated from diatom as their cell wall is actually easier to

break open than the wall of green algae (Hildebrand and Dahlin, 2000). Table 2.2 shows the efficiency of different DNA extraction methods on two types of microalgae.

For molecular identification of the isolates, different types of molecular markers have been used as it was difficult to achieve expected amplification of the genomic DNA using a single universal primer set (Fig. 2.3 c-j and Fig. 2.4 a, b). The genes targeted in the present study were SS rRNA (18S rRNA, 23S rRNA), LSU rRNA (28S rRNA), ITS and 5.8S rRNA. Some of the amplified products were cloned and transformed into *E.coli* cells. The inserts were confirmed by colony PCR, followed by plasmid extraction (Fig. 2.5 c -e). The purified plasmid/ amplified PCR products were sequenced, compared with other sequences deposited in gene database NCBI by BLAST and identified based on highest similarity to the species (Table 2.3). The sequences were deposited in GenBank with accession numbers (Table 2.3).

2.3.4 Phylogenetic analyses and relatedness to lipogenic species

Till now around 30,000 species have already been identified (Mata et al., 2010) and many microalgal species belonging to various classes have been investigated for their lipid content (McGinnis et al., 1997; Aguirre and Bassi, 2013; Cheng et al., 2013b; Saha et al., 2013). Significant differences in lipid contents are found within various species, but how the lipid-rich strains distribute among clades of microalgae is unknown, the clarification of which would benefit further isolation of new microalgae having a high lipid content. Besides, lipid content, growth rate and biomass production should also be considered when isolating candidate strains for biofuel production. In this study, 14 strains were isolated and tested for their lipid content, and a phylogenetic tree was constructed to determine if there were

taxonomic patterns of lipid production. Phylogenetic tree was constructed to reconfirm the identification through taxonomic position and also to get an understanding about the taxonomic pattern of lipid accumulation. The trees were constructed based on the data on different conserved region genes such as 18S rRNA, ITS, LSU used for the molecular based identification (Fig. 2.7-2.11). *Dixoniella* MACC1, positioned as outgroup, showed its highest similarity to the rhodophyte *Dixoniella grisea*, known for its sulphated exo-polysaccharide production, is lesser explored with respect to lipid production as there is only one report showing the presence of high lipid content in these cells (Pekarkova et al., 1988). *Biddulphia* sp. MACC2 and MACC6 belonged to the same clad confirming their similarity and this species have been already characterised by Islam et al. (2013) for its high lipid production and have got most suitable biodiesel properties. All pennate diatoms like *Amphora* sp. MACC4, *Amphora* sp. MACC9, *Navicula phyllepta* MACC8, *and Nitzschia* sp. MACC11 confirmed their identification with the respective phyla in the phylogram based on the 18S rRNA and ITS data. This class of isolates have already been explored intensively for their biodiesel production potential (Csavina et al., 2011; Abdel Hamid et al., 2013; Chtourou et al., 2015). *Pleurocapsa* sp. MACC7, the cyanobacterial isolate, is reported to have high content of saturated and unsaturated fatty acids according to Caudales et al. (2000). *Durinskia baltica* MACC10, a dinoflagellate, identified after its close relativeness to *Durinskia baltica* CS-38 strain got no records of high lipid content and is found to be phylogenetically in close relation with *Phaeodactylum tricornutum*, which is considered a potential candidate for biodiesel production (Acien et al., 1998; Eizadora et al., 2009). Chlorophytes have

always been the most appropriate choice for large scale biodiesel production because of their stable nature and greater lipid product. MACC13 and MACC14, the green cells among the isolates showed similarity to the genus *Picochlorum* sp. and *Nannochloris* sp. or *Nannochlorum* sp. respectively, several of which have been reported for their increased lipid producing capability (De la Vega et al., 2011; Pereira et al., 2011; El-Kassas, 2013). The genetic closeness to *Chlorella* sp. confirms the potential of the strains to be candidates for biodiesel production (Xu et al., 2006). The isolates MACC15, MACC16 (*Prymnesium* sp.), MACC17 (*Amphidinium* sp.) are less studied in terms of lipid accumulating activity with a few reports on *Prymnesium parvum* (Becker, 1994; Kazuhisa, 1997).

2.4 Conclusions

In the present study, about fourteen monocultures of microalgae were isolated from the marine water samples collected from west coast of India and some parts of Cochin Estuary. The 14 isolates belonged to various microalgal phyla such as Rodophyta, Bacillariophyta, Chlorophyta, Dinoflagellata, Haptophyta and Cyanobacteria. The strains were identified using morphological and molecular methods. Three DNA extraction methods were compared in which methods B and C were identified to be the most effective ones in terms of quantity and quality. Different molecular markers were studied for the molecular level identification of the different groups of microalgae in which 18SrRNA proved to be the universal marker for microalgal identification. Phylogenetic analysis of the monocultures with the related strains depicted the closeness to high lipid producing species which were already reported in most of the cases whereas there was no earlier information regarding a few.

Table 2.1 Primers used in this study for identification of the marine microalgal isolates

Strain(with code)	Gene	Primers (5'-3') F-forward R-reverse	Reference
Dixoniella sp. MACC1 *Picochlorum* sp. MACC13	18S rRNA	F-ggtgatcctgccagtagtcatatgcttg R-gatccttccgcaggttcacctacggaaacc	Matsumoto and Sugiyama(2010)
Biddulphia sp. MACC2 *Nitzschia* sp. MACC11	18S rRNA	F-tgtaaaacgacggccagtattccagctccaatagcg R-caggaaacagctatgacgactacgatggtatctaatc	Zimmermann et al. (2011)
Amphora sp. MACC4	18S rRNA	F-agattgcccaggcctctcg R-ccatcgtagtcttaaccataaac	Moro et al. (2009)
Biddulphia sp. MACC6 *Navicula phyllepta* MACC8	18S rRNA	F-aacctggttgatcctgccagt R-tgatccttctgcaggttcacctac	Bruderand Medlin (2007)
Nannochloris sp. MACC14	18S rRNA	F- ggttgatcctgccagtagtcatatgcttg R-gatccttccgcaggttcacctacggaaacc	Dams et al. (1988)
Amphora sp. MACC9	ITS,5.8 rRNA	F-tccgtaggtgaacctgcgg R-tcctccgcttattgatatgc	Ferrer et al. (2001)
Pleurocapsa sp. MACC7 *Durinskia baltica* MACC10	23S rRNA	F-aggggtaaagcactgttt R -ccttctcccgaagttacg	Del Campo et al. (2010)
Prymnesium sp. MACC15 *Prymnesium* sp. MACC16 *Amphidinium* sp. MACC17	LSU rRNA	F- amaagtaccrygagggaaag R-scwctaatcattcgctttacc	Hamsher et al. (2011)

Table 2.2 Efficiency of three different methods of DNA extraction on two types of microalgae

Method	Microalgae	Purity ($A_{260/280}$)	DNA concentration (ng μL^{-1})
A	Diatom	1.7	190
	Green alga	1.8	80
B	Diatom	1.6	750
	Green alga	1.5	680
C	Diatom	2.1	594
	Green alga	1.9	300

Table 2.3 Microalgae species with similarity percentage and accession numbers

Phylum	Species with strain code (MACC)**	Similarity (%)	GenBank Accession Number
Rodophyta	*Dixoniella sp.* MACC1	91%	JF428838
Bacillariophyte	*Biddulphia* sp. MACC2	100%	JX524545
Bacillariophyta	*Amphora* sp. MACC4	94%	JX896689
Bacillariophyta	*Biddulphia* sp. MACC6	99%	KR007589
Cyanobacteriae	*Pleurocapsa* sp. MACC7	90%	KJ845342
Bacillariophyta	**Navicula phyllepta* MACC8	99%	KC178569
Bacillariophyta	*Amphora* sp. MACC9	98%	KJ845340
Dinoflagellata	*Durinskia baltica* MACC10	98%	KC161251
Bacillariophyta	*Nitzschia* sp. MACC11	99%	KT279819
Chlorophyta	*Picochlorum sp.* MACC13	100%	KP098569
Chlorophyta	*Nannochloris* sp. MACC14	99%	KJ845339
Haptophyta	**Prymnesium* sp. MACC15	97%	KJ845343
Haptophyta	*Prymnesium* sp. MACC16	98%	KJ845344
Dinoflagellata	*Amphidinium* sp. MACC17	96%	KJ845341

** Micro Algal Culture Collection * Isolated from brackish water

(a)

(b)

Fig. 2.1 Source point of different microalgal cultures isolated along (a) the west coast of India (b) Cochin Estuary

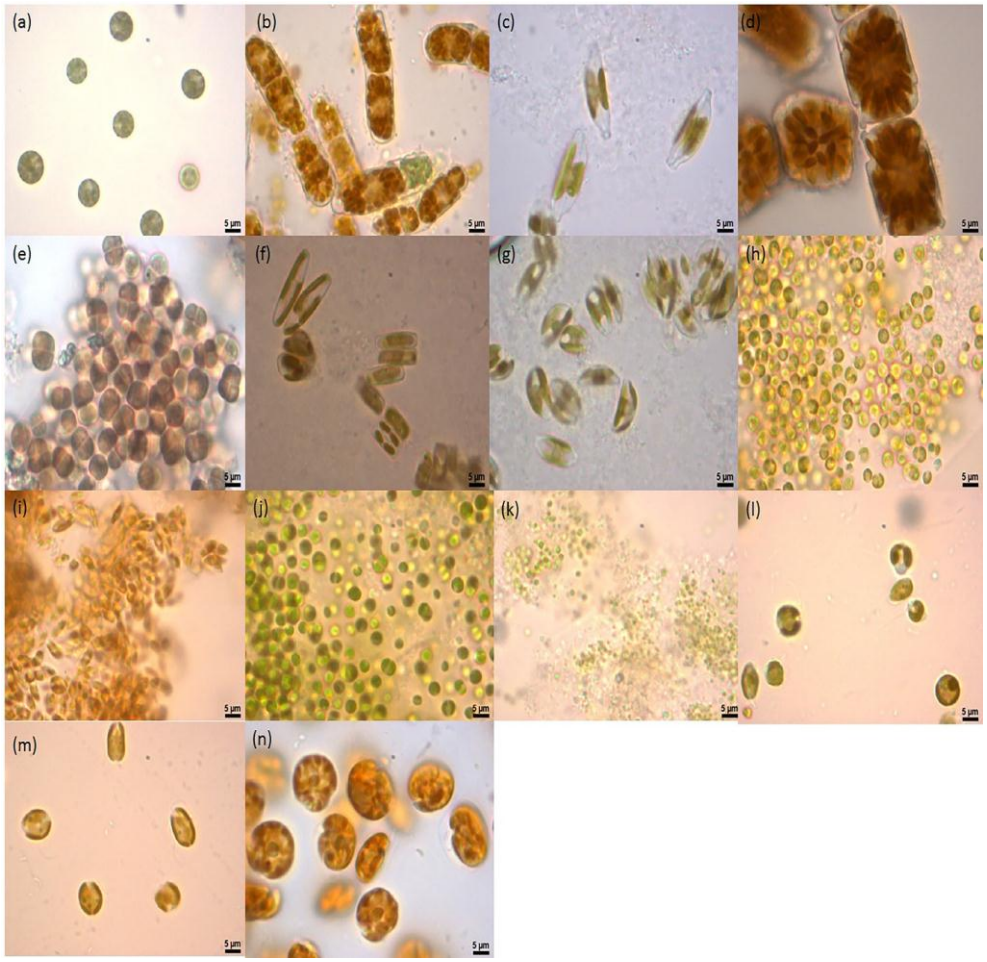


Fig. 2.2 Light micrographs of (a) *Dixoniella* sp. MACC1 (b) *Biddulphia* sp. MACC2 (c) *Amphora* sp. MACC4 (d) *Biddulphia* sp. MACC6 (e) *Pleurocapsa* sp. MACC7 (f) *Navicula phyllepta* MACC8 (g) *Amphora* sp. MACC9 (h) *Durinskia baltica* MACC10 (i) *Nitzschia* sp. MACC11 (j) *Picochlorum* sp. MACC13 (k) *Nannochloris* sp. MACC14 (l) *Prymnesium* sp. MACC15 (m) *Prymnesium* sp. MACC16 (n) *Amphidinium* sp. MACC17. Scale bar = 5µm

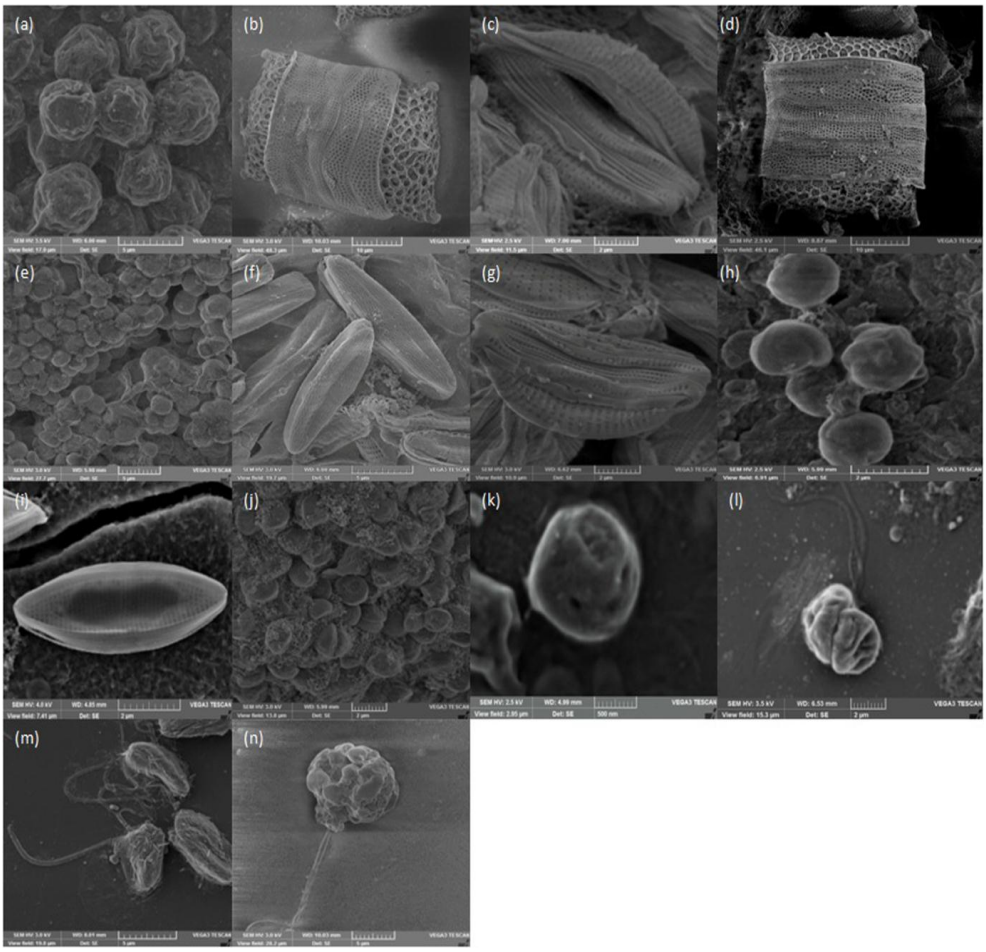


Fig. 2.3 Scanning electron micrographs of (a) *Dixoniella* sp. MACC1 (7.7kx) (b) *Biddulphia* sp. MACC2 (2.8kx) (c) *Amphora* sp. MACC4 (12kx) (d) *Biddulphia* sp. MACC6 (3kx) (e) *Pleurocapsa* sp. MACC7 (5kx) (f) *Navicula phyllepta* MACC8 (7kx) (g) *Amphora* sp. MACC9 (12.7kx) (h) *Durinskia baltica* MACC10 (32kx) (i) *Nitzschia* sp. MACC11 (28kx) (j) *Picochlorum* sp. MACC13 (30kx) (k) *Nannochloris* sp. MACC14 (70.3kx) (l) *Prymnesium* sp. MACC15 (13.5kx) (m) *Prymnesium* sp. MACC16 (7kx) (n) *Amphidinium*sp.MACC17 (4.9kx).

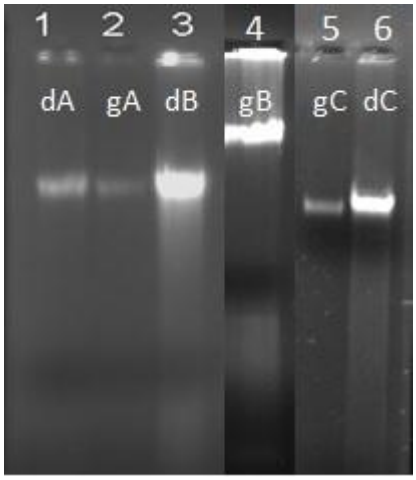


Fig. 2.4 Gel images showing genomic DNA isolated using Method A for diatom (dA, lane 1) and green alga (gA, lane 2); Method B for diatom (dB, lane 3) and green alga (gB, lane 4); Method C for green alga (gC, lane 5) and diatom (dC, lane 6)

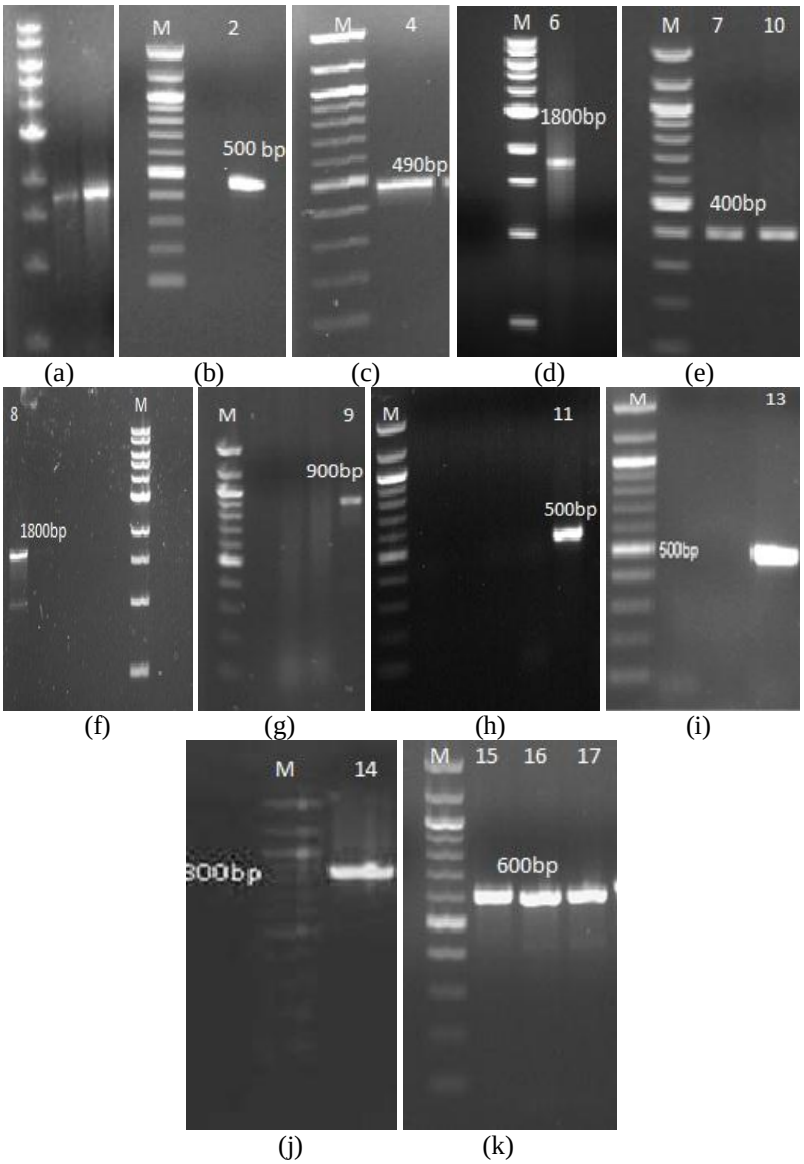
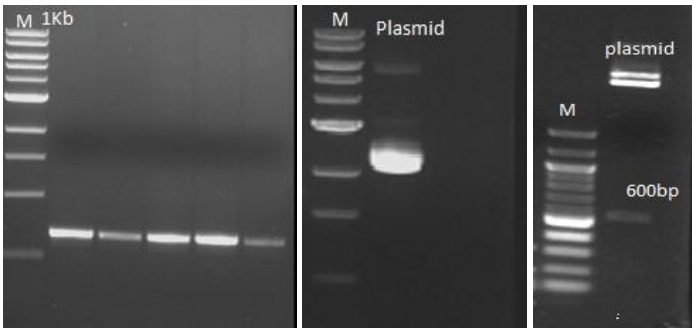


Fig. 2.5 PCR amplification products of (a) *Dixoniella* sp. MACC1 (lane 1 and 2); (b) *Biddulphia* sp. MACC2 (lane 3); (c) *Amphora* sp. MACC4 (lane 2); (d) *Biddulphia* sp. MACC6 (lanel 2); (e) *Pleurocapsa* sp. MACC7 (lane 2), *Durinskia baltica* MACC10 (lane 3); (f) *Navicula phyllepta* MACC8 (lane 1); (g) *Amphora* sp. MACC9 (lane 5); (h) *Nitzschia* sp. MACC11 (lane 6) (i) *Picochlorum* sp. MACC13 (lane 4); (j) *Nannochloris* sp. MACC14 (lane 2); (k) *Prymnesium* sp. MACC15 (lane 2), *Prymnesium* sp. MACC16 (lane 3), *Amphidinium* sp. MACC17 (lane 4); 1kb and 100 bp markers were used.



(a) (b) (c)

Fig. 2.6 Representative gel pictures showing (c) colony PCR of clones with insert (600 bp) (lane 2-6) (d) plasmid isolation (lane 2) (e) release of insert (lane 2). 1kb and 100 bp markers were used.

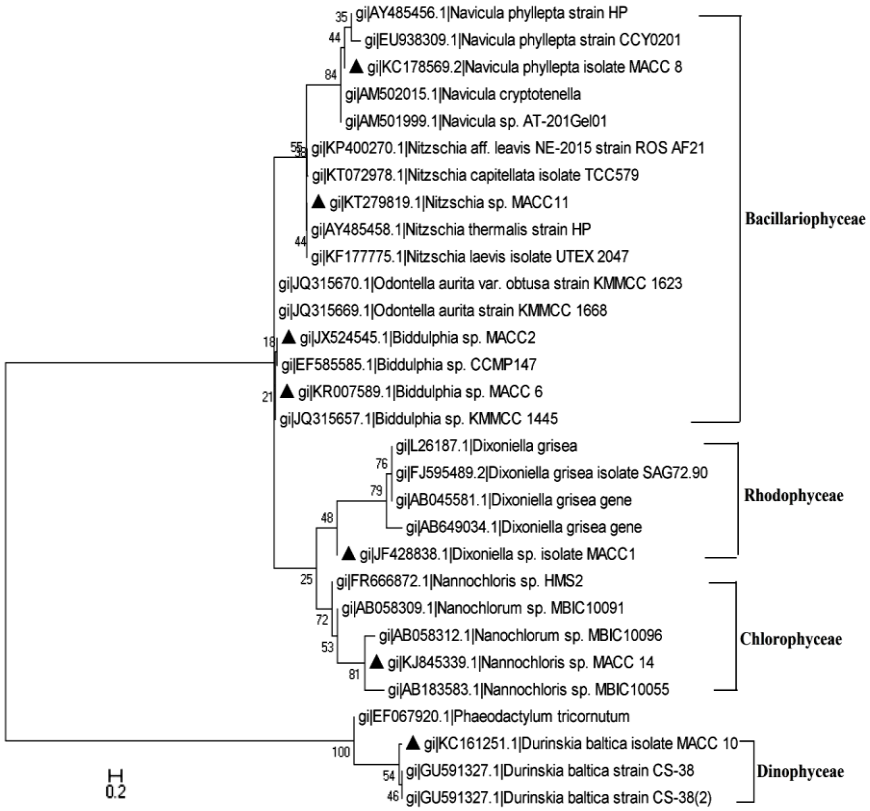


Fig. 2.7 Phylogenetic tree of *Dixoniella* sp. MACC1, *Biddulphia* sp.MACC2, *Biddulphia* sp. MACC6, *N. phyllepta* MACC8, *Durinskia baltica* MACC10, *Nitzschia* sp. MACC11, *Nannochloris* sp. MACC14 based on 18S rRNA gene sequences

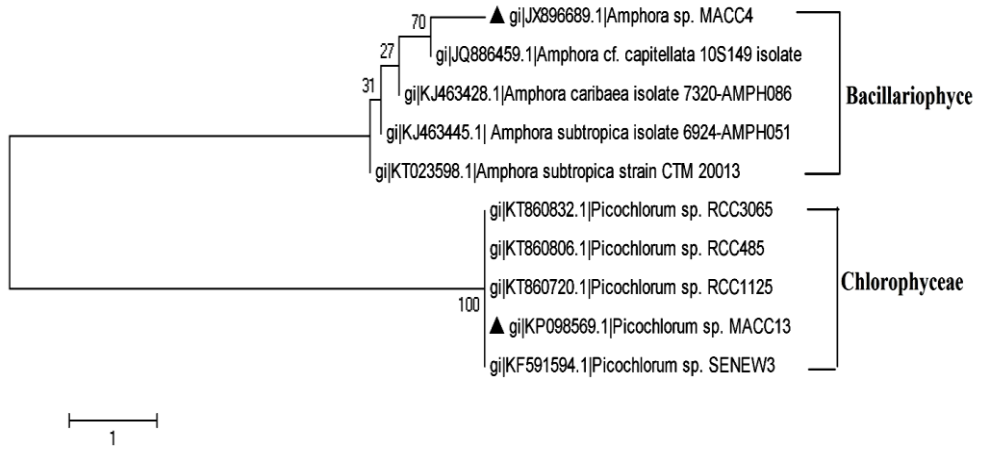


Fig. 2.8 Phylogenetic tree of *Amphora* sp. MACC4, *Picochlorum* sp. MACC13 based on 18S rRNA gene sequences

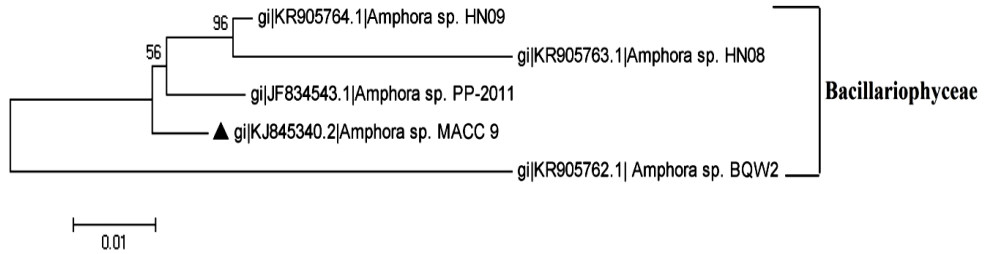


Fig. 2.9 Phylogenetic tree of *Amphora* sp. MACC9 based on 5.8 S and ITS gene sequences

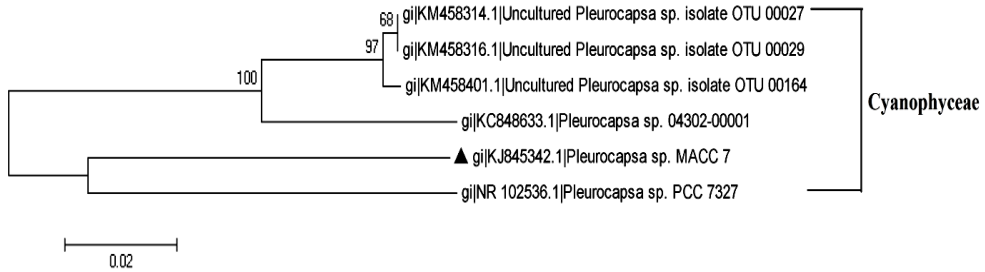


Fig. 2.10 Phylogenetic tree of *Pleurocapsa* sp. MACC7 based on 23S rRNA gene sequence

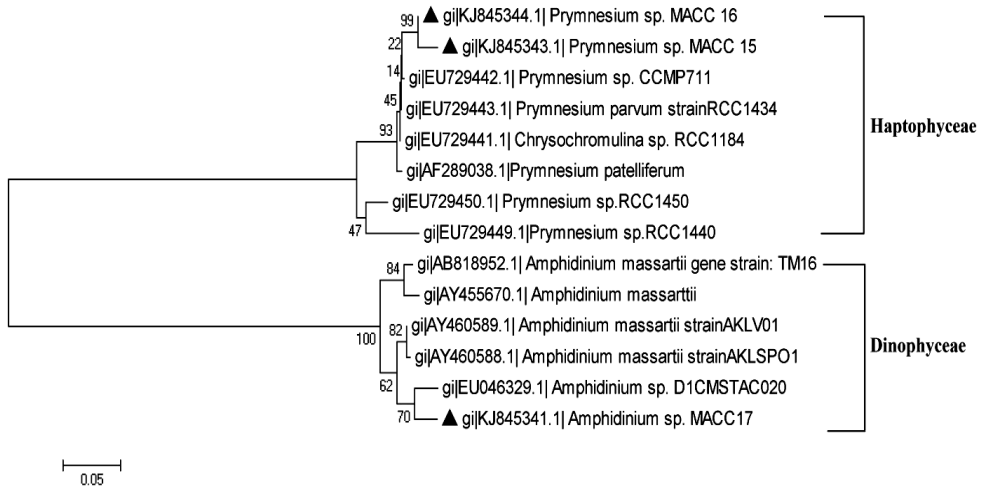


Fig. 2.11 Phylogenetic tree of *Prymnesium* sp. MACC15, *Prymnesium* sp. MACC16, *Amphidinium* sp. MACC17 based on LSU rRNA gene sequences

Chapter 3

SCREENING FOR THE MOST POTENT MICROALGAL STRAIN FOR BIOFUEL PRODUCTION

Contents

3.1 Introduction

Microalgal bioprospecting is the isolation of exceptional microalgal strains from aquatic environments for potential value added products (Olaizola, 2003; Spolaore et al., 2006). A great deal of literatures emphasizing on mass cultivation and sustainable use of microalgae for biofuels are available (Brennan and Owende, 2010; Mata et al., 2010; Ahmad et al., 2011, but not on bio-prospecting and establishment of microalgal culture collection for biofuel application. To maintain the cost effectiveness of commercial biodiesel production, selection of appropriate strains according to the site of cultivation is imperative. Screening of native microalgal strains with desirable traits provides a robust platform for large scale biomass production (Araujo et al., 2011). A multicriteria-based strategy has to be considered toward successful selection of a specific wild microalgal strain for biodiesel production. It includes growth rate; lipid quantity and quality, especially the fatty acid profile; response to processing conditions such as

temperature, nutrient input and light, and competition with other microalga and/or bacterial species; nutrient requirements and rate of uptake of CO_2, and nitrogen and phosphorus, ease of biomass harvesting, oil extraction and further processing; and possibility of obtaining high added-value co products (Chisti, 2007, Brennan and Owende, 2010; Amaro et al., 2012). The best performing microalgal strain can be obtained by screening of a wide range of naturally available isolates and the efficiency of those can be improved by selection, adaptation and genetic engineering (Schenk et al., 2008). To determine the suitability of algal biomass as lipid feedstock for algal biofuel production, lipid content and its fatty acid composition are the important criteria (Knothe, 2008).

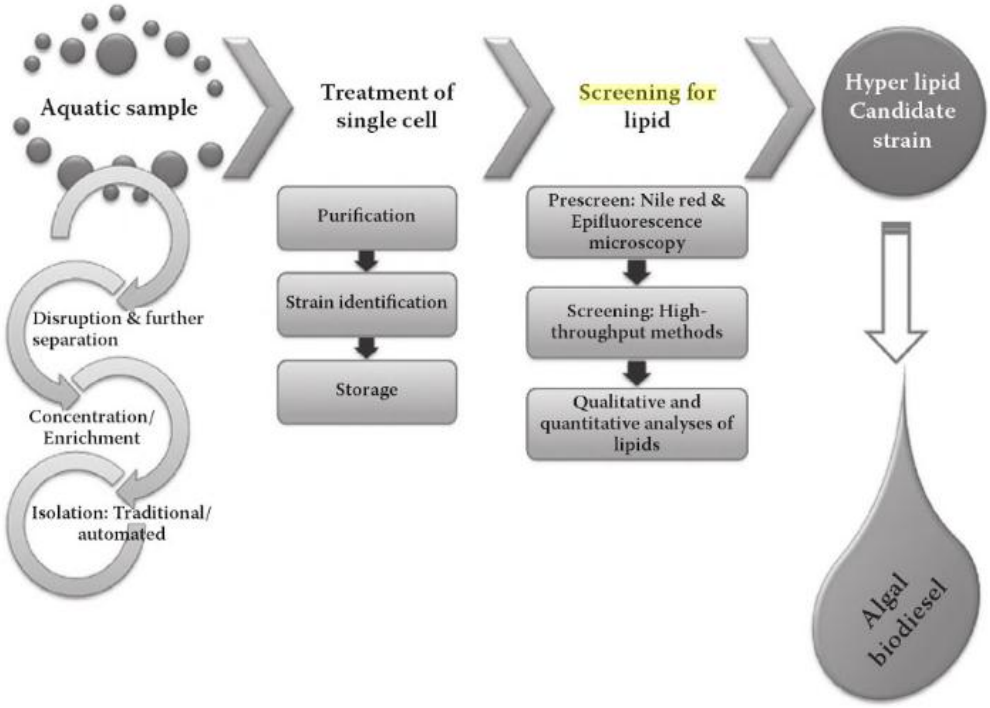


Fig. 3.1 Schematic outline for procedures in bio prospecting of microalgae for biodiesel production (Karthikeyan, 2013)

The identification of lipid composition in selected algal strains is essential for determining the suitability for biodiesel and fuel quality. According to the Solar Energy Research Institute (SERI) report, the most promising species for fuel production are *Botryococcus braunii* due to its important quantities of hydrocarbons, *Nannochloropsis salina* with its high quantities of ester fuel production, and *Dunaliella salina* due to its high quantities of fatty acids (Feinberg, 1984). Fig 3.1 shows the stepwise protocol for screening of microalgae for biodiesel production.

Lipid analyses, both quantitative and qualitative, are crucial for selecting suitable strains for biodiesel production. Conventional methods for total lipid estimation consist of solvent extraction or gravimetric methods used by Bligh and Dyer (1959). Separation and profiling of lipid components require elaborate techniques in order to satisfy criteria of biodiesel quality, which include thin layer chromatography (TLC), gas chromatography-mass spectroscopy (GC/MS) and/or high pressure liquid chromatography (HPLC) (Eltgroth et al., 2005). The major disadvantages of the conventional methods are that they are time consuming, labor-intensive and havaea low throughput screening rate. High throughput method using Nile red (9-diethylamino-5H-benzo[α]phenoxazine-5-one), a lipid- soluble fluorescent dye, has been commonly used to evaluate the lipid content of microalgae (Cooksey et al., 1987; Elsey et al., 2007). It is relatively photostable and allows one to differentiate between neutral and polar lipids. Another alternative to this is the lipophilic fluorescent dye BODIPY 505/515 (4, 4-difluoro-1, 3, 5, 7-tetramethyl-4-bora-3a, 4a diaza-s-indacene), which has recently been used as a vital stain to detect oil storage within viable microalgal cells. Lipid bodies get stained in green and chloroplasts in red in live oleaginous algal

cells (Cooper et al., 2010). The advantage of BODIPY is that high lipid yielding cells may be identified and isolated microscopically using a flow cytometry or a fluorescence-activated cell sorter. A microwave-assisted Nile red staining method for microalgal lipid determination was developed for those strains with thick, rigid cell walls that prevent penetration of the fluorescence dye into the cell (Chen et al., 2011). Montero et al. (2010) showed that by using cell sorting capabilities of flow cytometer, in combination with the lipid-soluble fluorescent dye Nile red (NR), isolation and selection of cells with high and stable lipid content can be done. Various studies demonstrated the use of Fourier transform infrared micro-spectroscopy (FTIR) to determine lipid and carbohydrate accumulation of microalgae (Dean et al., 2010; Laurens and Wolfrum, 2011).

In this present chapter, the lipid content of the fourteen marine microalgal strains were determined as a first step of screening and further only seven isolates with high lipid content were selected and characterized by various screening procedures for the selection of most potential candidate as biodiesel feedstock.

3.2 Materials and methods

3.2.1 Analyses of biochemical composition

Total lipids, protein and carbohydrate contents of the microalgal species were estimated in order to determine the highest lipid producing strain along with their nutritional composition. An aliquot of 1 mL cell suspension from each of the 14 algal cultures in liquid media at an absorbance of 0.2 measured at 750 nm was inoculated into F/2 medium and K medium (for *Amphidinium* sp.), incubated under conditions mentioned earlier for a period of 15 days and

the cells were harvested by centrifugation at 8944 × *g* at 4 °C for 10 min. The supernatants were decanted and cell pellets were washed with 0.5 M ammonium bicarbonate and then frozen at -20 °C and lyophilized at -72 °C. The total lipids were extracted from microalgal biomass following the modified method of Bligh and Dyer (1959). Another set of 50 mg of lyophilized sample was treated with 4 mL 5% trichloroacetic acid (TCA), vortexed for 30 s, heated at 90 °C for 30 min, cooled in an ice bath for 30 min, and centrifuged at 1500 x *g* for 20 min. The supernatant was withdrawn and 1 mL used for spectrophotometric determination of carbohydrate using the procedure described by Dubois et al. (1956). The pellet was further treated with 3 mL 0.5 M NaOH, vortexed for 10 s, heated at 90 °C for 30 min, and allowed to stand overnight at room temperature. The samples were then centrifuged (1500×*g*, 10 min), and the protein concentration of the supernatant determined spectrophotometrically using a procedure of Lowry et al. (1951). The data presented represent the mean ± standard error of the mean (SEM) or standard deviation (SD) as noted with n = 3.

3.2.1.1 Total lipid estimation (Bligh and Dyer, 1959)

Fifty milligram of lyophilized microalgal biomass in 10 mL culture tube was taken, to which 1.6 mL water; 4 mL methanol; and 2 mL chloroform were added. The solution was mixed for 30 s and an additional 2 mL chloroform and 2 mL water were added and the contents of the culture tube were mixed for 30 s. The tubes were centrifuged at 3105 × *g* for 10 min. The upper layer was withdrawn using a pipette and the lower chloroform phase containing the extracted lipids was transferred into another culture tube, the residue was extracted twice as above and the chloroform phases were pooled together and dried under nitrogen. Thereafter, the total lipids were

measured gravimetrically in an electronic weighing balance [Sartorius, Germany; d= 0.01 mg (80 g), 0.1 mg (220 g)] and the lipid content was calculated.

3.2.1.2 Total carbohydrate estimation (Dubois et al., 1956)

Different concentrations of glucose solutions (0.02- 0.14 mg mL^{-1}) were prepared from stock solution of glucose (1mg mL^{-1}). The sample solution of volumes 0.1 and 0.2 mL were taken in two different test tubes and the volumes of all solutions were made upto 1mL. One mL of 5% phenol solution was added followed by 5 mL of 96% sulphuric acid and the tubes were shaken well. After 10 min of incubation, the tubes were placed in a water bath at 25-30 °C for 20 min and the absorbance was read at 490 nm. The reading of a blank prepared in the same method using distilled water was also measured. The concentration of the total carbohydrate in the samples were calculated from the slope of the graph plotted using various glucose standards.

3.2.1.3 Total protein estimation (Lowry et al., 1951)

Different dilutions of bovine serum albumin (BSA) solutions were prepared by mixing stock of BSA solution (1mg mL^{-1}) and the final volume was made up to 5 mL. The BSA range was from 0.1 to 0.8 mg mL^{-1}. From these different dilutions, 0.2 mL was pipetted in to different test tubes and 2mL of alkaline copper sulphate reagent was added. The alkaline copper sulphate reagent was prepared by mixing 2 mL of reagent B (equal amount of 1.56% copper sulphate solution in 2.37% sodium potassium tartarate solution) in 100 mL of reagent A (2% sodium carbonate in 0.1 N NaOH). The solutions were mixed well and incubated at room temperature for 10 min

followed by addition of 0.2 mL of Folin Ciocalteau reagent (1N). The solutions were further incubated for 30 min and the absorbances were read at 660 nm while taking a zero absorbance using a blank. The concentrations of the total protein in the microalgal samples were determined from the slope of the standard curve plotted against known concentrations.

3.2.2 Growth rate, biomass and lipid productivity estimation

The cultures were grown till their exponential phase (up to 8 days approximately for most of the algal cultures) and 1mL inoculum of each having an absorbance (Abs) of 0.2 at 750 nm was added to experimental flasks in triplicates containing 50 mL of F/2 medium. The cells were counted on every alternate day of post-inoculation using advanced Neubauer haemocytometer. Dry weight was estimated at an interval of 5 days following the method of Becker (1994). The specific growth rate (μ) was calculated following the equation (Guillard, 1973):

$$\mu = \frac{\ln (X_1 - X_0)}{T_1 - T_0} \quad \text{.....(3.1)}$$

where X_0 and X_1 are densities at the starting and end of the exponential phase at days T_0 and T_1 respectively.

Average biomass productivity P_B (mg L^{-1} day^{-1}) was calculated by

$$P_B = \frac{X_1 - X_0}{T_1 - T_0} \quad \text{.....(3.2)}$$

where X_1 and X_0 were the biomass dry weight concentrations (g L^{-1}) on days T_1 and T_0 (final and initial sampling days respectively) (Griffiths and Harrison, 2009).

The lipid productivity was calculated by the following equation:

$$P_{lipid} = L_c \times P_B \quad \text{..... (3.3)}$$

where, P_{lipid} is lipid productivity in mg L^{-1} day^{-1} , Lc is the total lipid content (g/g) and P_B is the biomass productivity in (mg L^{-1} day^{-1}).

3.2.3 Nile red staining

An aliquot each of 20th day old stationary phase culture was centrifuged at 3105 ×*g* for 5 min and the pellet was re-suspended in the same volume of phosphate buffered saline (pH 7.4). The cells were washed with phosphate buffer two times and Nile red (0.1 mg mL^{-1} in acetone) was added in the ratio of 1:100 v/v. The reaction mix was incubated for minimum 5 min in dark and observed under an inverted phase contrast fluorescent microscope (Leica DMIL connected with DFC 420C camera) and images were processed using Leica application suite (LAS) software (Greenspan et al., 1985).

3.2.4 Nile red fluorescence assay

Aliquots of 1 mL microalgal culture (Abs 0.2 at 750 nm, 5-40 ×10^5 cells mL^{-1}) were inoculated into 50 mL Erlenmeyer flasks containing F/2 medium for a period of 30 days. For the determination of neutral lipids, 1mL each sample was collected at an interval of 5 days and diluted to Abs 0.2 at 750 nm and 100 µL was mixed with 380 µL of 25% dimethyl sulphoxide (DMSO). Aliquots of 20 µL of nile red stain in acetone (1 mg mL^{-1}) was added and mixed well, incubated at 40 °C for 10 min. Fluorescence was recorded at excitation and emission wave lengths of 490 nm and 580 nm respectively in a spectrofluorometer (Thermo-scientific Varioskan flash,

Finland). Auto fluorescence from the algae were measured as above after mixing 100µL sample with 400 µL 25% DMSO alone. The difference in the reading of the two entities provided the relative fluorescence intensity (Chen et al., 2009). The data presented represent the mean ± standard error of the mean (SEM) or standard deviation (SD) as noted with n = 6.

3.2.5 Neutral lipid extraction by column chromatography

The crude lipid extracted (5-10 mg) from dry microalgal biomass cultured for period of 15 days (mid stationary phase of all of the strains) following Bligh and Dyer method (1959), was dissolved in 2 mL chloroform and loaded into a silica gel (230-400 mesh size, Merck) packed glass column (5 × 3.5 cm) (equipped with a glass wool plug) pre conditioned with n-hexane. The silica pack up to a height of 3 cm was prepared using slurry of 300 mg silica powder in 3 mL chloroform and the bed was leveled by adding clean dry sand on top of it. The neutral lipids (NLs) fractions were eluted (at 1 mL min^{-1}) with 4 mL of chloroform, glycolipids (GLs) were eluted with 4 mL of acetone/methanol (9:1 v/v), and phospholipids (PLs) were eluted with 4 mL of methanol. Each lipid fraction was collected into a glass vial and dried using nitrogen gas (Popovich et al., 2012, Liang et al., 2014).

3.2.6 Thin layer chromatography

The efficiency of the crude lipid fractionation was verified by thin-layer chromatography (TLC). In this method, small strips of pre-coated silica gel aluminium sheets (Silica gel G 60, Merck) were used. A fine spotting line was drawn with a pencil 2-3 cm from the bottom of the activated TLC sheet. Twenty µL of lipid sample in chloroform was applied on the line using a glass

micropipette and left at room temperature for 1-2 min to allow the residual solvent to evaporate. The strip was then transferred to a pre-conditioned TLC glass chamber containing the solvent system and the whole set up was left undisturbed for ascending chromatography until the solvent reached about 1cm from the top of the plate. For the separation of neutral lipids, the major solvent system used was petroleum ether/diethyl ether/acetic acid in the ratio 70:30:2 per volume. This system separated neutral lipids into monoacylglycerols, diacylglycerols, sterols, triacylglycerides and steryl esters. The polar components such as glycerophospholipids remain at the origin. The separated fractions can be visualized using specific staining methods. For detection of neutral lipids, manganese chloride ($MnCl_2$) charring method was adopted. The dry plates containing the separated fraction were sprayed with methanolic $MnCl_2$ solution (containing 0.63 g $MnCl_2.4H_2O$, 60 mL water, 60 mL methanol, 4 mL concentrated sulfuric acid) and heated in oven at 100°C for 30 min. The neutral lipid fractions will turn into brown or black spots after drying (Schneiter and Daum, 2006).

3.2.7 Neutral lipid fatty acid profiling

The neutral lipid fractions were trans-esterified to FAME by AOAC 996.06 method (AOAC, 1996). The extracted fraction was dissolved in 2 mL each chloroform and diethyl ether and evaporated to dryness under nitrogen. Subsequently, 2 mL 7% boron trifluoride and 1mL of toluene were added to each vial and heated at 100°C for 45min. After cooling them down, 5mL distilled water, 1mL hexane and 1g sodium sulphate were added and shaken well to allow phase separation. The top most hexane layer containing FAMEs were analysed using GCMS system (Perkin Elmer Clarus 680GC) equipped with a mass detector (Clarus 600T mass

spectrometer) and a fused silica capillary column (Elite -5MS column: ID-0.25 mm, length-30 m; film thickness-0.25 µm, Perkin Elmer with temperature limits: 60 to 325/350 °C). The injection volume was limited to 1µL. The carrier gas used was helium at a flow rate of 0.6 mL min^{-1}. The injector temperature was set at 240 °C with a split ratio of 20:1. The initial temperature of the oven was set at 60 °C with a hold for 1 min and then ramped to 2 °C per min to 200 °C with 4 min hold, then 5 °C per min to 280 °C and finally hold for 10 min. Turbo mass software was used for instrument control, data acquisition and data analysis chromatography. The detection of different classes of fatty acid methyl esters was made by comparing the retention time of the standards (C4 –C24) (Sigma Aldrich, India).

3.2.8 Biodiesel properties from FAME profile

The important biodiesel properties such as saponification value (SV), iodine value (IV), cetane number (CN), density (ρ) , long chain saturated factor (LCSF), cold filter plugging point (CFPP) and higher heating value (HHV) were calculated from the FAME composition. Saponification value (SV) in mg $KOHg^{-1}$, iodine value (IV) in g $I_2 100g^{-1}$ of fat, cetane number (CN), density (ρ) (g cm^{-3}), long chain saturated factor (LCSF) and cold filter plugging point (CFPP) (°C) was predicted by the following equations (Islam et al., 2013):

$$SV = \frac{\sum [(560 \times Ni)]}{MWi} \quad \text{.....} \quad (3.4)$$

$$IV = \frac{\sum [(254 \times Di \times Ni)]}{MWi} \quad \text{.....} \quad (3.5)$$

$$CN = 46.3 + (5458/SV) - (0.225 \times IV) \quad \text{.....} \quad (3.6)$$

$$\rho = \sum \mathrm{Ni}\left(0.8463 + 4.9/\mathrm{MW}i + 0.0118 \times \mathrm{D}i\right) \quad \text{.....(3.7)}$$

$$\mathrm{LCSF}=(0.1\times \mathrm{C16})+(0.5\times \mathrm{C18})+(1\times \mathrm{C20})+(2\times \mathrm{C24}) \quad \text{..... (3.8)}$$

$$\mathrm{CFPP}=(3.147\times \mathrm{LCSF})-16.477 \quad \text{..... (3.9)}$$

where, N*i* is the percentage of each FAME, MW*i* is the molecular weight of the *i*th FAME and D*i* is the number of double bonds in the *i*th FAME.

Higher heating values (MJ kg^{-1}) of biodiesels were calculated according to Ayhan Demirbas model (Demirbas, 1998):

$$\mathrm{HHV} = 49.43 - (0.015 \text{ x IV}) - (0.041 \text{ X SV}) \quad \text{.....(3.10)}$$

3.2.9 Biodiesel synthesis

Total lipid from 25 g lyophilized biomass of diatom *Navicula phyllepta* were extracted using the modified method of Bligh and Dyer (1959) as previously described. For conversion into biodiesel, transesterification of the oils was performed by refluxing microalgal lipid, methanol, and sodium hydroxide (NaOH) at a ratio of 10:2.3:0.123 (w/w/w) at 70 °C for 1 h. After the reaction, biodiesel was separated by centrifugation at 8000 × g for 5 min at room temperature. The separated biodiesel was washed with 100 mL distilled water and re-extracted using 100 mL n-hexane followed by rotavaporation to remove the solvent (modified from Nurachman et al., 2012).

3.2.10 Fourier transform infrared (FT-IR) analysis

The synthesised biodiesel was compared with commercially available petro diesel with a Fourier transform infrared spectrophotometer (Thermo Nicolet, Avatar 370). All spectra were acquired at 25 ± 1 °C and 32 scans with a spectral resolution of 4 cm^{-1} per analyzed sample were performed.

The analysis region ranged from 400 cm^{-1} to 4000 cm^{-1}. The spectral bands were identified using characteristic infrared band of various functional groups (Stuart, 2005).

3.2.11 Flame test

A simple flame test was performed on the biodiesel and petro-diesel by burning cotton wicks dipped in the samples.

3.2.12 Statistical analyses

Statistical analyses were carried out using one way and two-way analysis of variance (ANOVA). The differences in the values were considered significant at $p< 0.05$. Fisher's Least Significant Difference (LSD) test was calculated after ANOVA for post hoc comparisons.

3.3 Results and Discussion

3.3.1 Biochemical composition analyses

Lipids, carbohydrates and proteins are the major biochemical components in algal biomass, the composition of which varies with species, culture conditions and age. Dividing cells are characterized by a high protein and low carbohydrate content and when the cells reach stationary phase, more carbon is incorporated into carbohydrate and / lipid (Zhu et al., 1997). The analyses of the three major biochemical constituents such as total lipids, carbohydrates and proteins (expressed as percentage of dry weight of biomass) of the microalgal isolates in their late log phase is represented in Table 3.1. The results showed that the diatoms had a higher lipid content compared to those of others of different classes. The species with comparably higher lipid content were in the order *Amphora* sp.

MACC9 (30.12%), *N. phyllepta* (26.54%), *Amphora* sp. MACC4 (23.49%), *Nitzschia* sp. (22.26%) and *Biddulphia* sp. (19.23%). The green alga *Picochlorum* sp. (24%) and haptophyte *Prymnesium* sp. (21.67%) also had high lipid contents. However, it is to be noted that the total lipid quantities in the present study integrates chlorophyll as in all other studies following Bligh and Dyer total lipid extraction protocol (1959). Protein content was in the order *Picochlorum* sp. (69.43%), *Nannochloris* sp. (50.43%) and *Prymnesium* sp. (40.28%). A deeper understanding is required in understanding the carbon accumulation and allocation in microalgae during their different growth stages. In the present study, the total carbohydrate concentration was not competitive enough to that of total lipid content. Only *Picochlorum* sp., *Nannochloris* sp. and *Prymnesium* sp. showed carbohydrate content of greater than 10% with respect to dry weight. This might be due to the fact that during stationary phase when there is nutrient starvation, the total carbohydrate starts to partially degrade and accumulation inside the biomass ceases. The immediate response to stress is intensive production of carbohydrate, later getting converted to fatty acids and potential link between fatty acid and carbohydrate synthesis is rather complex (Recht et al., 2012, Bellou and Aggelis, 2013). This fact must be considered while using microalgae for large scale production of biodiesel and bioethanol. Comparison of biochemical composition using ANOVA showed that the lipid, protein and carbohydrate content varied significantly ($p < 0.05$) between species. The lipid content of *Amphora* sp. MACC9 and *Navicula phyllepta* MACC8 were significantly higher amongst the microalgal species investigated, whereas, *Picochlorum* sp. MACC13 had the highest concentration of carbohydrate and protein.

3.3.2 Comparison of growth rates, biomass and lipid productivities

The growth rate and oil content (% dry weight) are the mostly studied parameters in the high scale production of algal biomass for biofuel (Griffiths and Harrison, 2009). But it is seen that fast growth only rarely correlates, mostly negatively correlates, with high lipid productivity. Small cell size contributes to lower the biomass productivity, even when the lipid content is high (Rodolfi et al., 2009). Generally, high specific growth rate depends on cell multiplication and it need not reflect the microalga's specific capacity for producing and storing lipids (Hu et al., 2008). The longest exponential phase was observed in *Picochlorum* sp. and *Prymnesium* sp. till 12-14 days with a maximum growth between 4^{th} and 10th day with an exception of *N. phyllepta* showing maximum doubling during 4^{th} to 6^{th} day entering stationary phase after 10^{th} day. Due to small size and clumping nature, the cell counting of *Nitzschia* sp. MACC11 was not performed. Table 3.2 represents the specific growth rates, biomass and lipid productivities.

The highest growth rate was found for *N. phyllepta* (0.58 day^{-1}) followed by *Amphora* sp. MACC4 (0.45 day^{-1}) despite the lower cell numbers. In a survey of *in situ* growth rates of marine phytoplankton carried out by Furnas (1990), maximum doubling rates were measured for both pennate and centric diatoms, which were higher than those for other algae with corresponding sizes. Figure 3.2 shows the growth curve of the selected microalgal strains under uniform culturing conditions. The volumetric lipid productivity and the qualitative lipid composition (fatty acid composition) should be considered as important parameters to facilitate decision making on species selection for biodiesel (Nascimento et al., 2013). The highest lipid productivity was observed for members of Bacillariophyceae *Navicula*

phyllepta MACC8 (114 mg L^{-1} day^{-1}) followed by *Amphora sp.* MACC4 (105 mg L^{-1} day^{-1}). In terms of lipid productivity, diatoms having larger vacuoles take longer time to get subjected to nutrient limited compared to other phyla due to enhanced nutrient storage capability (Hildebrand et al., 2012).

The biomass production was high in diatoms such as *Amphora* sp. MACC4 with 450 mg L^{-1} day^{-1} followed by *Navicula phyllepta* MACC8 (431 mg L^{-1} day^{-1}) and *Amphora* sp. MACC9 (310 mg L^{-1} day^{-1}), which were also the top lipid producers in our study. This observation is in support with the studies showing that biomass productivity of diatoms are in equal range with that of well established high lipid producing species such as *Nannochloropsis salina* (Boussiba et al., 1987) and *Chlorella* sp. (Doucha and Livansky, 2006). The marine diatoms, which contribute to a larger part of marine primary productivity, are reported to hold a great promise in biofuel production (Ramachandra et al., 2009). Biomass yields may be considered as an adequate criterion for biodiesel production only when associated with lipid productivity (Griffith and Harrison, 2009). Though it is reported that high biomass does not correlate with lipid production (Nascimento et al., 2013), the biomass and lipid production have been found positively correlated in the present study. The one way ANOVA of data for growth rate, lipid productivity and biomass productivity showed a significant difference ($p< 0.05$) between the isolates. Considering the three parameters i.e. growth rate, lipid and biomass productivities together, *N. phyllepta* MACC8 showed a significant higher value than rest of the isolates.

3.3.3 Nile red staining and fluorescence assay

The nile red staining demonstrated neutral lipids including TAGs in yellow golden, while the others in red (Fig. 3.3). The isolates *Amphora* sp. MACC4, *Biddulphia* sp. MACC6, *Navicula phyllepta* MACC8 and *Nitzschia* sp. MACC11 were stained relatively intense. Nile red fluorescence techniques have been used previously as the rapid screening tool to detect neutral lipids as well as their relative content (Elsey et al., 2007; Damiani et al., 2010). Altogether, 7 microalgae isolates could be selected based on the lipid content and Nile red fluorescence assay (Fig. 3.4). The isolates belonging to class Bacillariophyceae showed significantly ($p= 0.0006$) high fluorescence, in which, *Navicula phyllepta* showed the maximum, reaching at 35 RFU mL^{-1} towards the late stationary phase (30^{th} day). All cultures showed a steady and significant ($p< 0.05$) increase in fluorescence after 10^{th} day of culturing.

3.3.4 Neutral lipid fatty acid profiling

The lipid or fatty acid composition has a crucial role on the technology of biodiesel production and product quality, and hence must be taken into consideration for strain screening for biodiesel production (Li and Du, 2013; Liang et al., 2014). The two most important properties of fatty acids that affect the fuel properties are the length of the carbon chain and the number of double bonds (Stansell et al., 2012). Both saturated fatty acid (SFA) and monounsaturated fatty acid (MUFA) contents should optimally balance for good biodiesel quality as the amount of each can influence the important biofuel properties such as cetane number, CFPP and density (Islam et al., 2013), whereas polyunsaturated fatty acids (PUFA) decrease its stability as biofuel (Demirbas, 2009). The proportion of polyunsaturated fatty acid methyl esters has been limited to less than 1% by the EN 14214 standards in

Europe. Furthermore, since triacylglycerides (TAG) are the main component of microalgal oil and have been considered as the major source for biodiesel, the exact profile of the intact TAG would provide important information regarding the potential of the microalgal oil for biodiesel applications. All the parameters required for meeting biodiesel quality can be improved by using the neutral lipid fraction rather than from the total biomass/crude lipid (Liang et al., 2014). Hence in the present study, the neutral lipid fractions of the isolates were investigated with the purpose of screening the microalgal oil with ideal biodiesel properties.

Table 3.3 shows the fatty acid profile of the neutral lipid fractions of the microalgal lipids subjected for the study. All the isolates predominantly had increased proportions of SFA and MUFA, while lesser amount of polyunsaturated fatty acids (PUFA). This is because the neutral lipid fractions or storage lipids are predominated by SFA and MUFA, while PUFA comprise the structural lipid fractions mainly glycolipids and phospholipids (Hu et al., 2008; Hamilton et al., 2015). In the present study, the diatoms such as *Biddulphia* sp. MACC6, *N. phyllpeta* MACC8 and *Amphora* sp. MACC9 showed somewhat equal proportions of SFA and MUFA in the neutral lipid. The most predominant fatty acids were C14:0, C16:0, C16:1, C18:0 and C18:1. The fatty acids showing considerable amount of C14, C16, C18 and low amount of polyunsaturated fatty acids are considered to be suitable for biodiesel production (Schenk et al., 2008). *Navicula phyllepta* MACC8 was found to have the highest amount of C16:1and C16:0 followed by *Amphora* sp. MACC4 and MACC9. In the present study, there were strain specific variations in fatty acids in *Amphora* sp. MACC4 and MACC9. The same finding was reported by Lang et al. (2011) that fatty acid contents may be

variable between species of the same genus and even among multiple isolates of the same species. The differences in fatty acid contents could be explained by genetic differences among the strains of the same species (Volkman et al., 1991; Alonso et al., 1992). Stearic acid (C18:0) was found to be the highest in the case of diatom *Nitzschia* sp. and the chlorophyte *Picochlorum* sp. *Prymnesium* sp. contained relatively higher concentration of C18:1 fatty acids compared to others, which supported the findings of Makri et al. (2011). Most of the microalgae consisted of small amount of eicosapentaenoic acid (EPA) C20:5(n-3), whereas in *Picochlorum* sp. and *Prymnesium* sp., it was absent in the present analysis.

Marine algae are known to be excellent producers of EPA (Patil et al., 2007). But this long chain PUFA is not generally found accumulated in the neutral lipid fractions except in some obligate phototrophic microalgae (Apt et al., 1999). Culture conditions, strains used and growth phases are important determinants of LC-PUFA production and partition into TAGs. Tonon et al. (2002) described the influence of growth stages on LC-PUFA such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) deposition in TAG extracts in several species. *Navicula phyllepta* gave the highest quantity of EPA in this present study. It is already reported that diatoms make appreciable amounts of EPA (Volkman et al., 1989; Brown, 2002), whereas the presence of significant amount of C20:5(n-3) is not a characteristic of chlorophytes (green algae) (Dunstan et al., 1992; Zhukova and Aizdaicher, 1995; Lang et al., 2011). Generally, the fatty acid profile of diatoms is distinguishable from other algal groups as it mainly comprises of C14:0, C16:0, C16:1, C20:5(n-3) and minor amount of C18 fatty acid precursors. Arachidonic acid C20:4(n-6) was found in detectable levels in

only three species namely *Amphora* sp. MACC4, *Biddulphia* sp.MACC6 and *Nitzschia* sp. MACC11, out of which *Biddulphia* sp. had the highest concentration which supported the results of Bromke et al. (2015) in which *Biddulphia biddulphiana* was rich in arachidonic acid. But another study by Volkman et al. (1980) on *Biddulphia sinensis* reported the presence of general fatty acids such C14:0, C16:0, C22:5, but the absence of C20:4(n-6). Dunstan et al. (1993), Renaud et al. (1999) and Scholz and Liebezeit (2013) showed that diatoms are good sources of arachidonic acid and cellular arachidonic acid can be found inside TAG reserves of some species of microalgae (Bigogno et al., 2002b). Growth phase/culture age has profound influence on the fatty acid composition of microalgae (Liang and Mai, 2005, Zhang and Hong, 2014). The increase in culture age (mid stationary phase) lead to the increase in concentrations of C16:0 and C16:1 fatty acids, which supported that those fatty acids are readily mobilizable reserves. It could be as result of complex mechanism of cell division, nitrogen metabolism and carbon fixation (Fidalgo et al., 1998; Liang et al., 2013). Behenic acid (C22:0) was only detected in green alga *Picochlorum* sp., which is agreement with the reports on lipid composition of green algae showing the presence of this long chain saturated fatty acid (Chaundhary et al., 2014; Akgul et al., 2015; Cicci et al., 2016). Generally, microbial association can be found in culture strains of almost any algal culture collection. Therefore, FA contents of the associated bacteria may also have contributed to the obtained FA profile. C15:0 and C 17:0 are considered as markers to detect the presence of bacterial contamination (Viso and Marty, 1993). Most of the analyzed strains contained small amounts methyl-15:0, methyl C17:0 and methyl C17; contributing minor percentages to the observed microalgal FA

profiles. Fatty acids contributing less than 1% were not taken into consideration in the present study.

There exists great diversity of fatty acid profiles of oils among different classes of microalgae. The fatty acid profiles can widely vary both quantitatively and qualitatively depending upon the strain, physiological status of microalgae and the changes in environmental conditions (Hu et al., 2008; Rodolfi et al., 2009), making it difficult to have any comparison of the microalgal species across experimental conditions (Grima et al., 1994). Culture conditions including medium composition, light intensity, aeration, culture age and growth systems have significant effect on fatty acid content and partitioning (Tonon et al., 2002; Chaung et al., 2012, Ryckebosch et al., 2014). Chen (2012) cultivated 12 species of marine diatoms in three different environments and concluded that these conditions altered the fatty acid composition of the diatom species, emphasizing that production and storage of lipids is species-specific. The culture of any particular species under artificial conditions does not warrant the conclusion that naturally growing pelagic cells would have the same fatty acid composition as during culture. There are changes in the relative proportions of fatty acids present, including shifts in the biosynthetic pathways of fatty acids.To conclude, the fatty acid profile of the selected microalgae, especially diatoms, showcases its suitability to be used for biodiesel production. The extraction and characterisation of TAG fraction of the lipid portion is specifically important because this process can beneficially improve the biodiesel properties, and such compositional information is necessary for selecting a suitable fuel type of algae. Moreover, the utilization of PUFA rich fractions

in nutraceutical or pharmaceutical applications will add to the cost effectiveness of oil production.

3.3.5 Fuel properties

Energy-related properties of biodiesel should be considered when compared to conventional diesel. In meeting the standards of biodiesel, quantification of individual compounds in biodiesel is not necessary, whereas the concentration of individual classes of the compounds is very much important (Knothe, 2006). The parameters attesting for the quality of the biodiesel were estimated in relation to the carbon chain length and the amount and/or position of double bonds (Islam et al., 2013). The standardized experimental procedures for estimation of biodiesel properties are generally very much time consuming, laborious, expensive and require large volume of samples. Hence, the empirical estimation of the important parameters of biodiesel based on the information on fatty acid profile of the microalgal oil allowed a comprehensive assessment of fuel quality (Talebi et al., 2014). The most important properties of biofuel, such as saponification value (SV), iodine value (IV), cetane number (CN), density(ρ), higher heating value (HHV) and cold filter plugging point (CFPP) have been evaluated in this study. The iodine value and saponification value determine the ignition quality of fuel, stability and degree of unsaturation (Mutanda et al., 2011). Saponification value has a negative correlation with the fatty acid chain length, while IV is positive to the extent of unsaturation in fatty acid (Lei et al., 2012). Cetane number (CN) is the measure of fuel's ignition delay and indicator for determining combustion behaviour of diesel. According to the ASTM D6751-02 and EN 14214 standards for biodiesel, the minimum CN should be 47.0, whereas, the IV is set to a maximum of 120 g $I_2 100g^{-1}$

fat (Islam et al., 2013). The heat of combustion is an important parameter for estimating fuel consumption. Greater the heat of combustion, the lower is the fuel consumption (Knothe, 2008). The density of the oil determines the performance of the engine. The more closely the density of biodiesel to that of petroleum diesel, the easier it is to blend with it in different ratios (Islam et al., 2013). Cold filter plugging point is the temperature at which the saturated fatty acids of the fuel crystallises and precipitates clogging the filters, pumps and injectors. Higher the cetane number, poorer the cold flow properties. The Long Chain Saturated Factor (LCSF) FAME profile of a feedstock is a critical parameter for CN, IV and CFPF of the biodiesel obtained. The LCSF of biodiesel and their cold flow properties (CPFF) are inversely related to each other (Joseph et al., 2016).

The saponification value (SV), iodine value (IV), higher heating value (HHV), density (ρ), cetane number (CN), long chain saturation factor (LCSF) and cold filter plugging point (CFPP) for all seven microalgal biodiesels are summarized in Table 3.4. All the microalgal isolates were within the range of standard value of CN and IV. The estimated CN value varied between the seven microalgal isolates from 58 to 83. Density (ρ), for which a standard value has been set at 0.86–0.90 g cm^{-3} according to EN 14214, is another important parameter for biodiesel quality. FAME profile-derived ρ-value of *Navicula phyllepta* MACC8 was within this range, whereas, *Amphora* sp. MACC4 and MACC9 were slightly below the range and rest of them barely met the specification value (0.86 g cm^{-3}). Higher heating values of all microalgal species investigated were found to comply with the standard range of 39.69 - 43.65 MJ kg^{-1} of normal biodiesel, which is normally slightly lower than that of gasoline (46 MJ kg^{-1}), but higher than

coal (32–37 MJ kg^{-1}) (Demirbas, 2007). *Prymnesium* sp. MACC15 had the highest HHV value due to lower degree of unsaturation in the fatty acid profile. Biodiesel rich in stearic and palmitic acid methyl esters have a tendency to present a poor CFPP (Mittelbach and Remschmidt, 2004). In the present study, *Nitzschia* sp. and *Picochlorum* sp. gave the poorest CFPP value due to the presence of high amount of stearic acid compared to other isolates. *Biddulphia* sp. MACC6, *Amphora* sp. MACC9 and *N. phyllepta* MACC8 gave good CFPP values indicating their feasibility for application in low temperature regions. In the present study, the LCSF of FAMEs of all the strains ranged from 4.44 to 15.64. *Biddulphia* sp. showed lower LCSF, whereas *Picochlorum* sp. showed higher values. The succinct comparison of the biodiesel properties of all the seven isolates showed that all the diatoms, especially *Navicula phyllpeta*, were promising for biodiesel production. The results are in compliance with the findings of Joseph et al. (2016) that oil from *Navicula* sp. could satisfy the various specifications set for biodiesel such as density, CFPP, CN, and IV.

Based on the FAME derived biodiesel property evaluation alone, it is very difficult to identify the best microlalgal isolate meeting the specified standards of a good biodiesel. The same conclusion was reported by Nascimento et al. (2013) in a study on screening of microalgae for biodiesel production based on FAME profile and suggested that best quality biodiesel can be obtained by mixing oils of different microalgal cultures. The fuel related properties of biodiesel can be improved by changing the fatty acid composition (Knothe, 2009; Dunn, 2011). In the present work, property estimation based on FAMEs derived from the neutral lipid fractions met the specific standards of biodiesel, as the removal of the glycolipid and

phospholipid fractions normally dominated by polyunsaturated FAMEs, contributed to this improvement (Mohammady, 2011; Liang et al., 2014). The study on diatom *Fistulifera* sp. by Liang et al. (2014) showed that nutrition stress did not affect the diesel quality derived from neutral lipids despite the variation of FAME profiles. From the present results, *Navicula phyllepta* was found to be most suitable candidate microalgae for the production of biodiesel.

3.3.6 FT-IR analysis of biodiesel

From the FT-IR spectra (Fig 3.6), it is clearly seen that the biodiesel from the candidate strain *N. phyllepta* have multiple absorption bands not present in the petrodiesel. There was a strong band at 771 cm^{-1} in biodiesel, which is assigned to C- H bend of aromatic hydrocarbons. The bands near 1200 cm^{-1} stretching vibrations of the bonds pertaining to the CC(=O)-O functional group characteristic to ester group. The region between 900 cm^{-1} and 1300 cm^{-1} known as the fingerprint region, corresponded to multiple vibrational movements attributed to different types of bonds and functional groups of biodiesel. The region between 1737 cm^{-1} presents a strong absorption band corresponding to stretching vibrations of the carbonyl (C=O) functional group. The region at 1460 cm^{-1} and interval from 2800 cm^{-1} to 2970 cm^{-1}, which overlapped with each other in both samples. The peaks with high absorption intensities between 2800 cm^{-1}- 3000 cm^{-1} are assigned to symmetric and asymmetric stretching vibrations and bands between 1450- 1470 cm^{-1} are assigned to C-H bend of the alkane and alkyl groups (Pena et al., 2014).

3.3.7 Flame test

The biodiesel from diatom *N. phyllepta* was observed to have shades of green colour due to presence of pigments. Upon combustion, the flame displayed yellowish in colour with little bits of black smoke which is similar to commercial petrodiesel (Fig 3.7).

Overall, these results further support the screening effort, which confirms *Navicula phyllepta* biomass as potential feedstock for biodiesel production.

3.4 Conclusions

Out of 14 monocultures isolated, seven species selected based on total and neutral lipid contents were screened for estimating the biodiesel production potential. The diatoms outranked the other tested species in terms of productivity of lipid, biomass, lipid content, their fatty acid profile and biodiesel properties. The diatom *Navicula phyllepta* was proven to be the best apparent feedstock for biodiesel production with a lipid content greater than 25%, the highest growth rate (0.58 day^{-1}), lipid productivity and biomass productivity of 114 and 431 mg L^{-1} day^{-1} respectively. The microalgal oil quality could comply with the international standards (ASTM D6751 and EN 14214) set for regular biodiesel. The FTIR spectra and the flame test also confirmed the compositional and combustion similarity of microalgal biodiesel with commercial diesel. Further studies for improving lipid and biomass productivities using various biochemical, genetic and metabolic engineering strategies, and development of integrated mass production and downstream process technologies for co-production of valuable byproducts are required to enable building up sustainable bio-refinery for biofuel production.

Table 3.1 Biochemical composition of the marine microalgal isolates used in the study

Strain	Percentage of dry biomass		
	Lipid%	**Carbohydrate%**	**Protein%**
Dixoniella sp. MACC1	9.63 ± 1.48	5.58 ± 0.20	23.07 ± 1.76
Biddulphia sp. MACC2	16.52 ± 3.18	4.29 ± 0.21	28.89 ± 2.75
Amphora sp. MACC4	23.49 ± 3.49	4.09 ± 0.63	36.08 ± 3.57
Biddulphia sp. MACC6	19.23 ± 0.27	9.95 ± 2.45	39.12 ± 3.19
Pleurocapsa sp. MACC7	1.34 ± 0.06	5.91 ± 0.47	26.03± 0.55
Navicula phyllepta MACC8	26.54 ± 4.77	4.28 ± 0.11	35.85 ± 3.16
Amphora sp. MACC9	30.12 ± 2.73	3.77 ± 0.60	43.79 ± 0.70
Durinskia baltica MACC10	7.81 ± 0.71	5.02 ± 0.20	34.45 ± 2.46
Nitzschia sp. MACC11	22.26 ± 0.97	9.92 ± 0.06	39.60 ± 4.68
Picochlorum sp. MACC13	24 ± 1.32	11.21 ± 1.33	69.43 ± 4.57
Nannochloris sp. MACC14	12.02 ± 2.29	10.00 ± 0.28	50.77 ± 4.01
Prymnesium sp. MACC15	21.67 ± 1.55	6.78 ± 0.52	40.28 ± 1.6
Prymnesium sp. MACC16	11.12 ± 2.21	11.69 ± 0.43	52.21± 3.95
Amphidinium sp. MACC17	7 ±1.02	6.11 ± 0.43	33.07 ± 0.36

Table 3.2 Lipid and biomass productivities and specific growth rate of the selected isolates

Strain	**Lipid productivity ($mgL^{-1}day^{-1}$)**	**Biomass productivity ($mgL^{-1}day^{-1}$)**	**Specific growth rate (day^{-1})**
Amphora sp. MACC4	105 ± 0.12	450 ± 0.35	0.45 ± 0.09
Biddulphia sp. MACC6	60.82 ± 0.003	316 ± 0.24	0.35 ± 0.12
N. phyllepta MACC8	114.38± 0.04	431 ± 0.39	0.58 ± 0.001
Amphora sp. MACC9	93.37± 0.03	310 ± 0.18	0.24 ± 0.01
Nitzschia sp. MACC11	49.28 ± 0.03	224 ± 0.09	-
Picochlorum sp. MACC13	36.4 ± 0.01	151 ± 0.06	0.31 ± 0.02
Prymnesium sp. MACC15	40.82 ± 0.02	194 ± 0.20	0.38 ± 0.02

Table 3.3 Neutral lipid fatty acid profiles (% of total FAME) of the marine microalgal strains investigated

Fatty acids	*Amphora* sp. MACC4	*Biddulphia* sp. MACC6	*Navicula phyllepta* MACC8	*Amphora* sp. MACC9	*Nitzschia* sp. MACC11	*Picochlorum* sp. MACC13	*Prymnesium* sp. MACC15
C14:0	7.85	-	4.43	7.28	-	20.5	19.3
C15:0	3.82	-	-	2.9	-	-	-
C16:1	28.5	30.76	42.97	41.24	7.27	1.98	3.02
C16:0	44.4	34.96	40.9	39.6	33.8	34.66	19.75
C17:1	1.21		-	-	1.42	1.73	1.07
C17:0	-	7.86	2.89	2.75	2.53	1.61	2.78
C18:1(n-9c)	1.47	-	-	1.53	1.33	-	7.5
C18:2(n-6)	1.71	-		-	-	3.8	3.74
C18:1(n-9t)	-	-	1.19	-	2.21	1.32	-
C18:0	4.16	1.9	2.41	1.18	19.05	19.74	7.62
C20:4(n-6)	1.21	4.3	-	-	1.39	-	-
C20:5(n-3)	2.3	-	4.07	1.84	1.85		-
C22:0	-	-	-	-	-	1.54	-
SFA	60.23	44.72	50.63	53.71	55.38	76.51	46.67
MUFA	31.18	30.76	47.73	42.77	12.23	28.79	11.59
PUFA	5.22	4.3	4.07	1.84	3.24	5.34	3.74

Table 3.4 Estimated properties of biodiesel from microalgal oils based on neutral lipid fatty acid profiles

Strains	SV (mg $KOHg^{-1}$)	IV (gI_2 $100g^{-1}$oil)	CN	LCSF	CFPP (°C)	HHV ($MJkg^{-1}$)	Density (g cm^{-3})
Amphora sp. MACC4	210.77	47.27	61.5	6.52	4	40.08	0.84
Biddulphia sp. MACC6	171.63	44.42	68.1	4.44	-2.52	41.72	0.69
N. phyllepta MACC8	216.67	61.01	58	5.29	0.14	39.69	0.86
Amphora sp. MACC9	214.67	50.15	60.4	4.55	-2.18	39.88	0.85
Nitzschia sp. MACC11	147.96	23.78	77.8	12.9	24	43	0.61
Picochlorum sp. MACC13	174.59	17.76	73.6	15.64	32.7	42	0.77
Prymnesium sp. MACC15	134.97	16.61	83	5.78	1.68	43.65	0.54
ASTM/EN biodiesel standard	-	< 120	> 47	-	(<5)	>35	0.86- 0.90

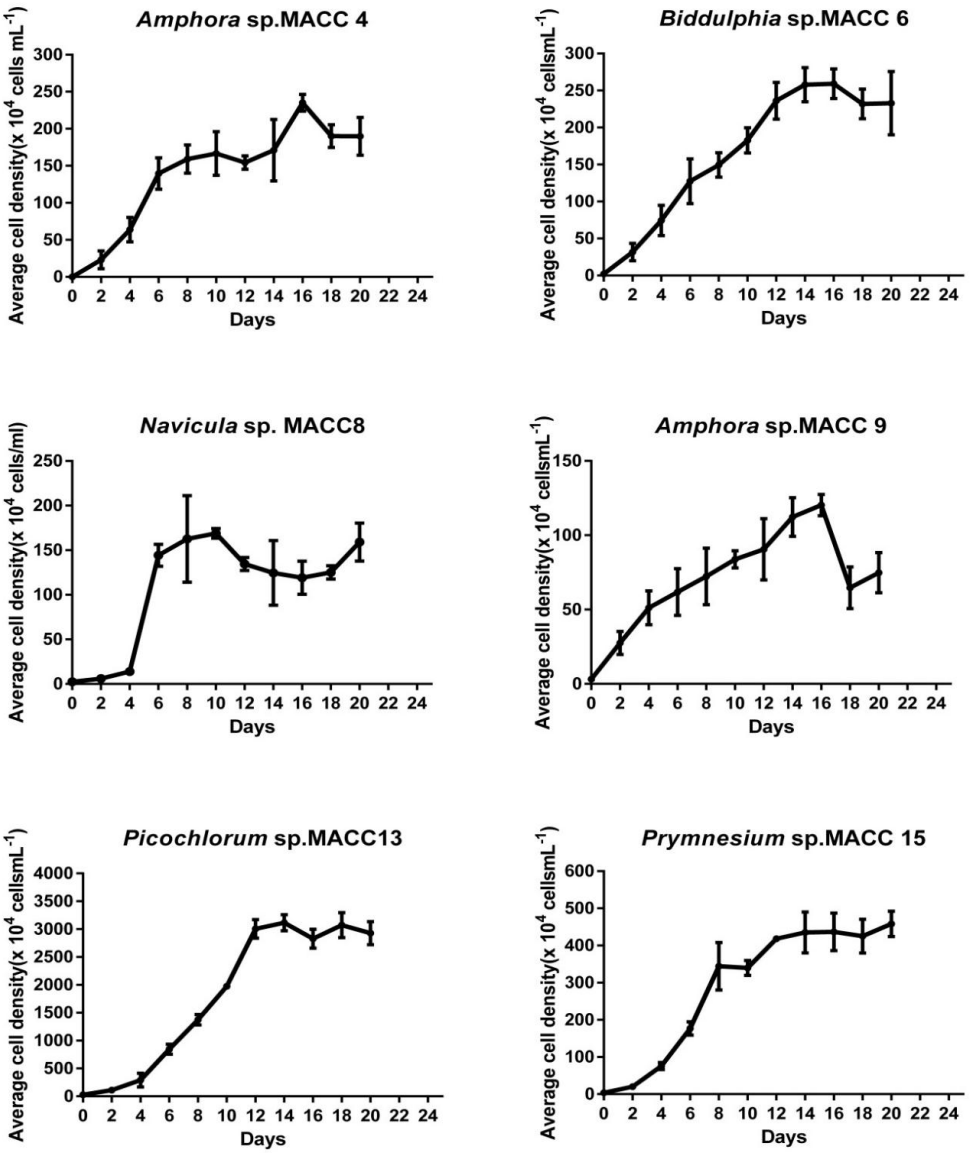


Fig. 3.2 Growth curve of *Amphora* sp. MACC4, *Biddulphia* sp. MACC6, *Navicula phyllepta* MACC8, *Amphora* sp. MACC9, *Picochlorum* sp. MACC13 and *Prymnesium* sp.MACC15. The data presented represent the mean ± standard error of the mean, n=3

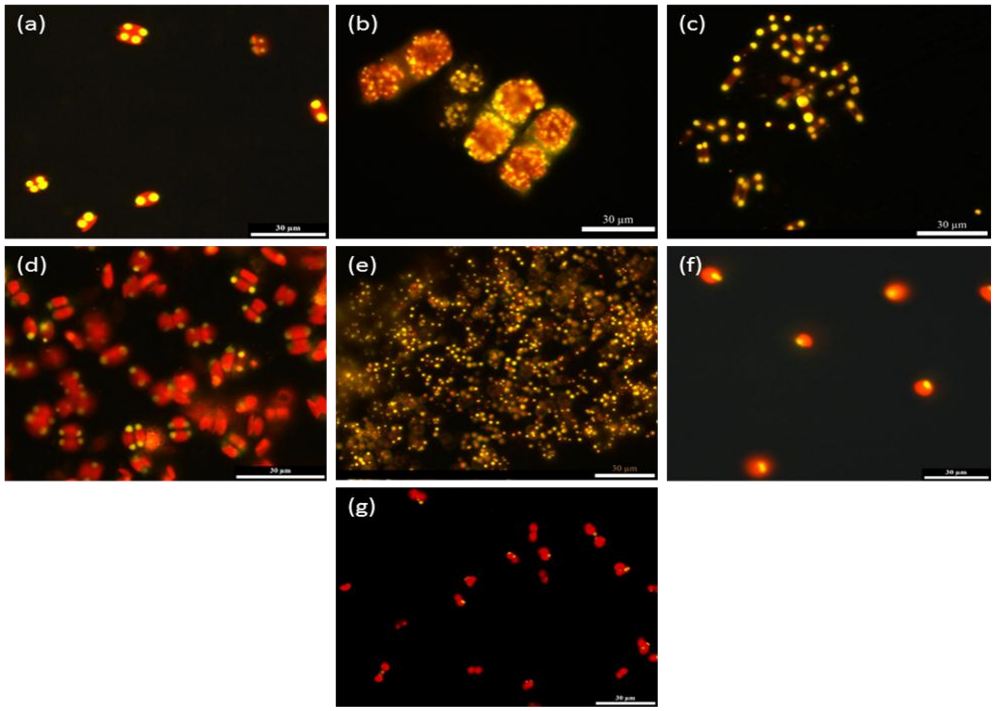


Fig. 3.3 Nile Red stained cells of (a) *Amphora* sp. MACC4 (b) *Biddulphia* sp. MACC6 (c) *Navicula phyllepta* MACC8 (d) *Amphora* sp. MACC9 (e) *Nitzschia* sp.MACC11(f) *Picochlorum* sp. MACC13 (g) *Prymnesium* sp. MACC15.Yellow stained portions shows the presence of neutrals in red stained cells. Scale bar = 30µm

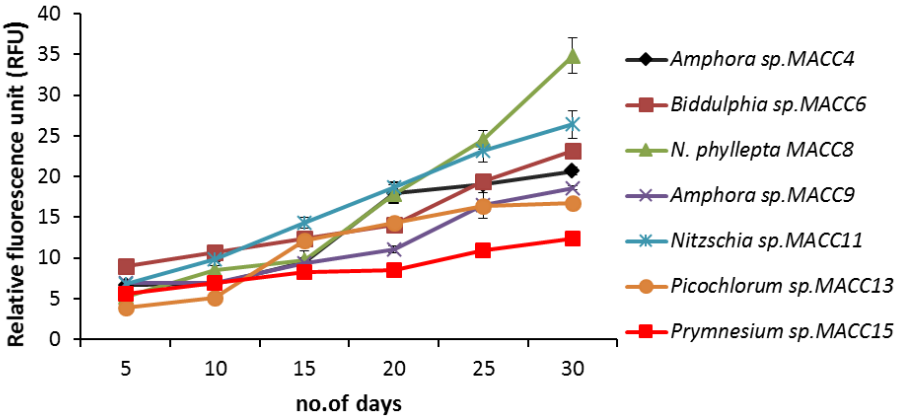


Fig. 3.4 Nile red fluorescence assay of the seven selected microalgal isolates for a period of 30 days. The data presented represent the mean ± standard error of the mean (SEM) n=6.

(a) (b)

Fig. 3.5 (a) Column chromatography of crude lipid for neutral lipid separation (b) Thin layer chromatography of the neutral lipid fraction

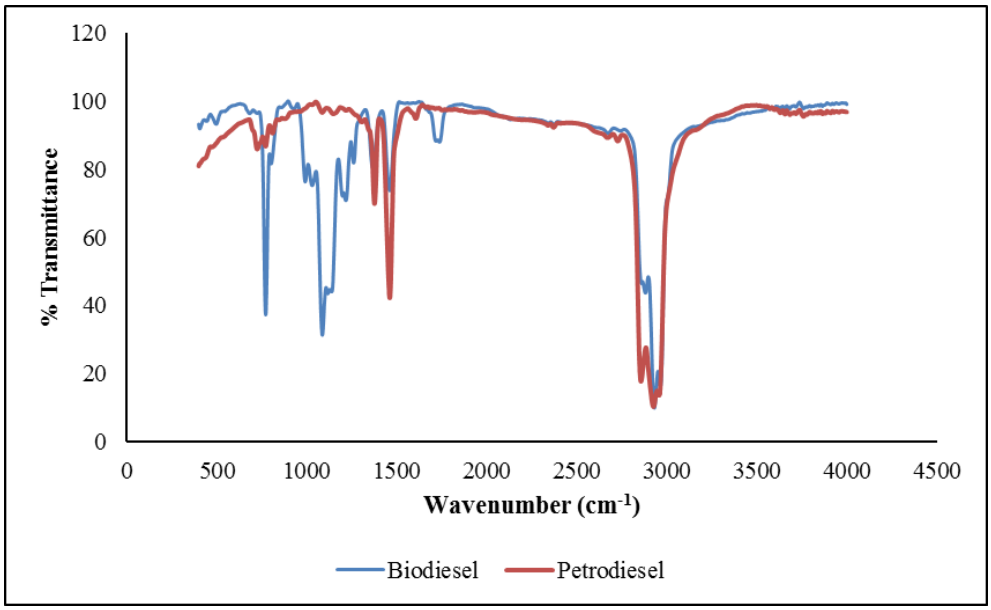


Fig. 3.6 FTIR spectra of biodiesel from *Navicula phyllepta* and petrodiesel

Fig. 3.7 Flame of biodiesel and petro-diesel

Chapter 4

OPTIMIZATION OF CULTURE CONDITIONS AND MEDIA COMPOSITIONS FOR HIGH BIOMASS AND LIPID PRODUCTION IN *NAVICULA PHYLLEPTA* MACC8 USING RESPONSE SURFACE METHODOLOGY

Contents

4.1 Introduction

Marine microalgae have been proved to be one of the most promising candidates for biofuel production. Reports on biofuel production from microalgae have been focussing more on green algae as they are easily being mass produced and more related to terrestrial plants. Recently, research on diatoms has started gaining momentum in the biofuel arena as they are found to be rich source of neutral lipids (Hildebrand et al., 2012; Levitan et al., 2014; D'Ippolito et al., 2015). The effective commercialisation of microalgae-based bio-fuel is limited due to the lack of technical and economic feasibility studies in mass production, biomass harvesting and downstream processing. Research on marine diatoms for bio-fuel applications is advantageous for use in large-scale raceway ponds due to its ability to

tolerate a wide range of salinity fluctuations; the actual use may be limited until conditions are optimized for diatom cell growth and lipid accumulation (Fields et al., 2014).

Navicula phyllepta MACC8 isolated from Cochin estuary in the west coast of India is identified as a potential biofuel feedstock based on its biomass and lipid productivities and fatty acid methyl ester composition. It is a most commonly reported benthic diatom from brackish and marine sediments (Clavero et al., 2000; Sabbe et al., 2003). *N. phyllepta* is ubiquitous in nature, suggesting its adaptation capability across a wide range of environmental conditions such as salinity, emersion time, and temperature (Witkowski et al., 2000; Sabbe et al., 2003). The lipids and fatty acids of some *Navicula* species have already been investigated (Mansour et al., 2005; Duong et al., 2015; Joseph et al., 2016) and the lipid quality indicated the potential of this microalga to be used as a feedstock for biodiesel production (Matusmuto et al., 2010; Sanjay et al., 2013). In addition, the characteristics of the cells of *Navicula* sp. to settle to the bottom of the vessel or to adhere to the surface of photobioreactors (non-suspended, membrane based cultivation) (Liu et al., 2013; Bilad et al., 2014; Katarzyna et al., 2015) can be helpful in easy harvesting the biomass, reducing positively the cost of biofuel production.

The quantity, quality and productivity of microalgal lipids depend not only on the strains but also on culture conditions. A number of factors such as optimum light, nutrient availability and temperature are involved in the economically feasible culturing of microalgae to yield high lipid content and growth rate (Araujo et al., 2011; Huang et al., 2013). Optimisation of

micronutrients in the growth medium is an important requirement in establishing a sustainable production system of microalgae. The process of optimization involves controlling a range of parameters affecting productivity. However, the conditions for neutral lipid accumulation and high growth rate are generally antagonistic. Identifying a trade-off between neutral lipid accumulation and growth is therefore a key issue for optimizing biodiesel productivity (Mairet et al., 2011). An active research is essential to understand as to why and how, under certain environmental conditions, some species of algae up-regulate neutral lipids (NL), which can be readily converted to biodiesel and other biofuels (Hu et al., 2008).

4.1.1 Statistical optimisation of growth media and conditions

Formulation of the accurate growth medium is an important criterion to be considered for the mass culture of microalgae for extracting any valuable product from them, towards which the optimization of medium is very much essential. The conventional 'one-factor-at-a-time' approach for optimizing media components has certain drawbacks as this method is time consuming and often leads to confusion in understanding the process parameters (Bezerra et al., 2008; Eckert and Trinh, 2016). For the production of a particular compound by any cell, there are various combinatorial interactions of the media components with the cell metabolism. Single variable optimisation method neglects the interaction among different variables, and thus is incapable of reaching the true optimum. An experimental design based on statistical modelling is beneficial in evaluating the relationship between a set of controllable experimental factors and observed responses (Iyer and Singhal, 2008; Myers et al., 2016).

4.1.1.1 Plackett - Burman Design

Plackett – Burman (PB) experimental design is used to identify the most important factors in the experiments when complete knowledge about the system is unavailable. It is an efficient screening method to identify the key and active factors using a few experimental runs (Box et al., 2005; Tyssedal, 2008; Torbeck, 2012). In PB designs, the main effects have a complicated confounding relationship with two-factor interactions. Therefore, these designs should be used to study main effects by assuming that two-way interactions are negligible. The most important feature of PB designs is that they all involve 4n experiments, where n = 1, 2, 3…n. In each case the maximum number of factors that can be studied is 4n-1, which means an 8-experiment design can study not more than 7 factors, a 12-experiment design will handle up to 11 factors, and so on. This may seem to be inconvenient, but it turns out to be a valuable feature of the method. PB designs utilise two levels for each factor, the higher level being denoted "+" and the lower "-" (Analytical Methods Committee, 2013).

4.1.1.2 Response surface methodology

Response surface methodology is a collection of mathematical and statistical techniques widely used for designing experiments, building models, determining optimum conditions of several factors influencing a mechanism or a system (Ghadge and Raheman, 2006; Acikel et al., 2010; Said and Amin 2016). It helps in screening key factors rapidly from multiple factors, which can avoid the defects brought by single-factor optimization (Zhang et al., 2012; Qin et al., 2013). Most of the reports related to the statistical optimization of microalgal media for biodiesel production are mainly on green algae (Azma et al., 2011; Chen et al., 2014; Jia et al., 2014;

Yang et al., 2014a; Fawzy, 2017). The research using directly RSM for improving the value of biomass and lipid production towards biofuel production by one-stage culture in diatoms has been scarcely reported. As differences among species and strains of the same genus exist, it is imperative to optimize the medium for each organism in order to obtain the maximum response. The main advantage of RSM is the reduced number of experimental trials needed to evaluate multiple factors and their interactions. Also, study of the individual and interactive effects of these factors will be helpful to find the target value. Hence, RSM provides an effective tool for investigating the aspects affecting the desired response if there are many factors and interactions in the experiment (Yin et al., 2009).

The application of RSM as an optimization technique consisting of the following stages: (1) the selection of independent variables of major effects on the system through screening studies and the delimitation of the experimental region, according to the objective of the study and the experience of the researcher; (2) the choice of the experimental design and carrying out the experiments according to the selected experimental matrix; (3) the mathematic–statistical treatment of the obtained experimental data through the fit of a polynomial function; (4) the evaluation of fitness of the model; (5) the verification of the necessity and possibility of performing a displacement in direction to the optimal region; and (6) obtaining the optimum values for each studied variable (7) checking the adequacy of the model (Maddox and Richert, 1977; Bezerra et al., 2008).

The most extensive applications of RSM are in situations, where several input variables potentially influence some performance measure or

qualitative characteristic of the process. This performance measure or quality characteristic is called the response and the input variables are known as independent variables (Sivaramakrishnan and Ravikumar, 2014; Myers et al., 2016). The field of response surface methodology consists of the experimental strategy for exploring the space of the process or independent variables, empirical statistical modelling to develop an appropriate relationship between the response/yield and the influencing variables, and optimization methods for finding the values of the process variables that produce desirable values of the responses. In most of the cases, either a first order or a second order model is used, out of which, the second-order model is widely used for being very flexible, easy to estimate parameters and work well in solving real response surface problems. For the case of two variables, the second-order model is

$$\eta = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 \quad (4.1)$$

where, 'η' is the estimated response, 'β_0' is a constant, 'β_1, β_2, β_3, β_{11}, β_{22}, β_{12}, are the coefficients for each term and 'x_1, x_2 and x_3 are the independent variables.

or

$$Y = \beta 0 + \Sigma \beta i X i + \Sigma \beta i i X i^2 + \Sigma \beta i j X i X j \quad (4.2)$$

where, Y represents the response variable, β_0 represents the interception coefficients, βi is the coefficient of linear effect, βii is the coefficient of quadratic effect and βij is the coefficient of the interaction effect (Bas and Boyaci, 2007; Jian and Nian-fa, 2007; Venil et al., 2009).

4.1.1.3 Central composite design

The most popular class of second-order designs is the central composite design (CCD) (Myers et al., 2016). This design can be easily constructed by supplementing the fractional factorial design that is used for estimating the first-order model. The use of CCD allowed determination of levels of various parameters to be carried out with the interrelation between each parameter evolving simultaneously (Shiow-Ling and Wen-Chang, 1997) This method is a well-established and widely used statistical method applied in the optimization of medium composition by determining the key factors from a large number of medium components by a small number of experiments (Khuri and Cornell, 1987; Soni et al., 2007). It has three groups of design points: a) Two-level factorial or fractional design points - all possible combinations of the +1 and -1 levels of the factors; b) Axial points or star points - axial points (outside the core), often represented by stars, emanate from the centre point, with all but one of the factors set to 0. The coded distance of the axial points is represented as a plus or minus alpha ("– α" or "+ α"); c) Center points - points with all levels set to coded leve l 0 (midpoint). Center points are usually repeated to get an estimate of experimental error (Anderson and Whitcomb, 2006; Stat-Ease, 2008).

The visualization of the predicted model equation in RSM can be obtained by the response surface plot (3D) or contour plot (2D). The response surface plot is the theoretical three dimensional plots showing the relationship between the response and the independent variables, whereas in the contour plot, lines of constant responses are drawn in the plane of the independent variables. The contour plot helps to visualize the shape of a response surface (Myers et al., 2016).

4.1.2 Factors effecting lipid and biomass production in diatoms

4.1.2.1 Temperature

Growth temperature is an important parameter that affects all biological reactions (Kleinschmidi and McMahon, 1970). Temperature stress has a major effect on lipids and fatty acids profile as these are the energy source for stress adaptation in algae (Teoh et al., 2013). The relationship between nutritional profile and temperature is of great importance in getting the desirable lipids from the cultured diatom. In general, the high growth temperature has been associated to a significant increase in lipid content in several species (Han et al., 2013; Teoh et al., 2013); however, other studies have found that the response of microalgae chemical composition to growth temperature varies from species to species (Renaud et al., 2002). Temperature has been found to have a major effect on the fatty acid composition of algae. A general trend towards increasing fatty acid unsaturation with decreasing temperature and increasing saturated fatty acids with increasing temperature has been observed in many algae. Insightful information about the effect of temperature on lipid synthesis and accumulation in microalgae is yet to be obtained. It is also reported that the rate of silicate dissolution in diatoms depends upon temperature (Lewin, 1961).

4.1.2.2 Culture age

The growth phase has been reported as an intrinsic factor influencing the growth rate and biochemical composition of microalgae (Renaud et al., 2002). Culture aging or senescence also affects lipid and fatty acid content and composition. It was reported in the diatom *Phaeodactylum tricornutum*,

that culture age had almost no influence on the total fatty acid content, although triacylglycerides accumulated and the polar lipids reduced (Alonso et al., 2000). The proportion of TAGs in the total lipid of healthy and actively dividing cells is usually low; however, during stationary phase certain species can have elevated proportions (Tonon et al., 2002; Mansour et al., 2003). The polyunsaturated fatty acids (PUFA) and TAG content can be optimized in desirable strains by manipulating the growth phase.

4.1.2.3 Salinity

The composition of intracellular lipid and growth of microalgae was reported to change in response to environmental salinity (Fava and Martini, 1988). Salinity changes normally affect phytoplankton in three ways: (1) osmotic stress (2) ion (salt) stress; and (3) changes of the cellular ionic ratios due to the membrane selective ion permeability (Moheimani, 2005). The increase in lipid content may correlate with the adaptive response to high NaCl concentrations, such as cell volume change and glycerol production. However, the mechanism of lipid content increase by high NaCl concentration is not clear. The amount of cell mass harvested from culture may decrease somewhat by NaCl addition during culture (Takagi et al., 2006). Marine diatoms are known to have a broad tolerance to salinity (Williams, 1964; Sonnekus, 2010)

4.1.2.4 pH

The uptake of inorganic carbon during photosynthesis can rapidly increase pH, and thus influence the growing conditions and physiology of the algal cells. The growth rate and structural lipids reduced and neutral lipid accumulated when pH increased to higher levels in diatoms due to

internal pH regulation in active algal cells (Smith and Raven, 1979; Taraldsvik and Myklestad, 2000; Spilling et al., 2013)

4.1.2.5 Agitation

A well-mixed microalgal culture is in a much more stable equilibrated state characterized stable pH (Persoone et al., 1980), a more efficient exchange of nutrients and metabolites (Grobbelaar, 1994) and reduced dissolved oxygen levels (Zittelli et al., 2006). Increasing the agitation rate in continuous cultures of microalgae enhanced biomass productivity by reducing the length of the continuous dark period (or light-limited period) experienced by the cells (Grobbelaar et al., 1996). Diatoms like *Phaeodactylum tricornutum* and *Phaeodactylum cruentum* have experienced various levels of cell damage at high agitation rates (Sobczuk et al., 2006).

4.1.2.6 Nutrients

Nutrient availability has a significant impact on growth and multiplication of microalgae and broad effects on their lipid and fatty acid composition. Environmental stress conditions when nutrients are limited, invariably causing a steady decline in cell division rate (Sharma et al., 2012). In diatoms, silicon is an equally important nutrient that affects cellular lipid metabolism. Silicon deprivation resulted in increased neutral lipids content and increased quantity of saturated and mono-unsaturated fatty acids than cells grown on silicon rich ambience (Roessler, 1988). Nitrogen deficiency caused metabolic imbalance reduced protein synthesis and photosynthesis rates leading to increase in intracellular levels of TAGs (Xin et al., 2010, Moll et al., 2014). The combination of silicate and nitrate limitation has the potential to increase TAG accumulation in lipid

producing diatoms (Moll et al., 2014). Phosphorus is an essential nutrient for nitrate absorption, photosynthetic respiration, energy transfer and signal transduction (Singh et al., 2015). Phosphorus starvation to induce lipid accumulation in diatoms has been studied as a sole stress or in combination with nitrogen limitation (Valenzuela et al., 2012; Burrows et al., 2012).

4.1.2.7 Iron

Iron is the most important trace metal for biochemical catalysis. Iron limitation can result in detrimental physiological effects in diatom cells resulting in increased cell density due to silicification and lipid accumulation (Allen et al., 2008; Liu et al., 2008). Rao (1981) had shown that varied levels of trace metals had varied effects on microalgae during the division of cells, and that addition of chelating agents like ethylene diamine tetraacetic acid (EDTA) would increase the availability of trace metals to the algae.

The present study deals with identification and evaluation of the effects of different growth factors on biomass and lipid production in the oleaginous microalga *Navicula phyllepta* MACC8 identified as a biofuel feedstock and to develop an economic growth medium by optimizing the significant factors using statistical modelling.

4.2 Materials and methods

4.2.1 Microalgal culture

The pennate diatom, *Navicula phyllepta* MACC8 (KC178569), was isolated from brackish waters of Cochin Estuary (9°55'35''N, 96°17'53''E). The strain was grown in F/2 medium (Guillard, 1975) at 26-28 °C under 27 μmol m^{-2} s^{-1} with 16:8 h light and dark photoperiods.

4.2.2 Light and transmission electron microscopy studies on *N. phyllepta* MACC8

Algal cells were collected by centrifuging at 4000*g* for 5 min. A small drop of the cell pellet was mounted on a clean glass slide with a cover and viewed under oil immersion under a light microscope (Olympus CH20iBIMF, India).

The cultures were observed under transmission electron microscope for studying the ultrastructure of the cell during lipid accumulating stationary stage. A sample of 5 mL microalgal cells was harvested during stationary phase (18-20 days) and washed in 1X phosphate buffer solution (PBS) for 2-3 times. The cells were centrifuged at 8000 ×*g*, supernatant was discarded and 500 µL of 2.5% gluteraldehyde added and incubated at 4 °C. The cells were harvested after 12 h, washed in 1X PBS 2-3 times and 200 µL of 2% osmium tetroxide was added and incubated at 4°C for 4 h or more till the culture got stained black/dark brown. The cells were harvested and washed with 1X PBS 2-3 times. The cells were dehydrated with a graded series of acetone and embedded in epoxy resin. The embedded specimen was cut into ultrathin sections and stained with uranyl acetate and lead citrate (Lewis and Knight, 1977). Transmission electron micrograph was recorded using TECNAI 200 TEM (FEI, Electron Optics, USA) at All India Institute of Medical Sciences (AIIMS), Delhi.

4.2.3 Selection of nitrogen source and salinity for high growth

Equimolar concentrations (2 mM) of ammonium chloride, sodium nitrate and urea were used as nitrogen sources in the basal medium F/2. An aliquot of 1 mL of 1×10^6 cells was inoculated in to 50 mL sterilised medium

keeping the other culture conditions constant. The cell count was determined using Neubeaur haemocytometer by withdrawing 1 mL of sample from the each culture flask every alternate day upto 14 days. For measuring salt tolerance, the diatom was cultured in F/2 medium with salinities 0, 10, 20, 30, 35 and 40 g kg^{-1}. Different salinities were prepared by diluting sea water (30 g kg^{-1}) with distilled water, and the salinity was measured using a refractometer. Tap water was used for zero salinity. An aliquot of 1 mL of 1×10^6 cells was inoculated into 100 mL Erlenmeyer flasks containing 50 mL sterilised F/2 medium at 26-28 °C with a photoperiod of 16:8 h light/ dark cycles under fluorescent white light (27 μmol m^{-2} s^{-1}). The cell counts and growth rates were determined every alternate day upto 14 days. The experiments were carried out in triplicate under different nitrogen sources, salinities and cultivation time and the mean values measured at different experimental conditions (different nitrogen sources/ salinities) and cultivation time were analysed by two way analysis of variance (ANOVA).

4.2.4 Selection of growth medium

One mL culture of *N. phyllepta* at a cell density 1×10^6 cells was inoculated into 100 mL Erlenmeyer flasks containing different sterilised enriched sea water media of 30 gkg^{-1} such as F/2 (Guillard, 1975), modified F/2 for diatoms (modified from Anderson, 2005), L1 medium (Guillard and Hargraves, 1993), diatom artificial medium (DAM) (Gagneux-Moreaux et al., 2007) and modified sea water medium (MSWM) (Nurachman et al., 2012) at 26-28 °C with a photoperiod of 16:8 h light/ dark cycles under fluorescent white light (27 μmol m^{-2} s^{-1}) and cultured for 14 days, in triplicates. Nutritional composition of each medium is given in Table 4.1.

Cell count was measured every alternate day and the growth rates of the isolate in different media were determined for a period of 14 days following Guillard (1973). The significances of the differences in mean growth rate of the diatom in different culture media and cultivation time were tested using two way ANOVA.

4.2.5 Enumeration of cell count using Neubeaur haemocytometer

The concentration of cells was calculated following the method given by Guillard and Sieracki (2005) as:

$$\text{cell density} = \frac{\text{number of cells x } 10000}{\text{number of squares}} \quad \text{.....(4.3)}$$

4.2.6 Determination of specific growth rate

The specific growth rate (μ) based on cell density was calculated following the equation (Guillard, 1973):

$$\mu = \frac{\ln (X_1 - X_0)}{T_1 - T_0} \quad \text{.....(4.4)}$$

where X_0 and X_1 are densities at the starting and end of the exponential phase at days T_0 and T_1 respectively.

4.2.7 Plackett - Burman experimental design based screening for significant variables

Plackett- Burman design was employed for screening eight variables such as urea, sodium metasilicate, sodium dihydrogen phosphate, ferric chloride, salinity, temperature, pH and agitation influencing lipid and biomass production. Each variable was set at a higher (+) and lower (-)

value to identify which factor had significant influence on the production (Table 4.2). An experimental design of 12 experiments or runs was formulated for the eight factors predicted by Design Expert software version 9.0 (Stat-Ease Inc, Minneapolis, MN, USA) based on the range of the variables provided. The experiments were carried out in 100 mL Erlenmeyer flasks containing 50 mL MSWM medium, and in an incubator shaker (Orbitek® LEIL, Scigenics Biotech, India) under illumination of 27 μmol m^{-2} s^{-1}, 16:8 h light and dark photoperiods. Five percent inoculum containing 1.5 × 10^6 mL^{-1} of cells in the exponential growth phase was added to the culture medium. The responses were measured in terms of dry weight of biomass (g L^{-1}) and total lipid content (g L^{-1}) towards the end of exponential phase (12^{th} day). The dry weight was estimated by harvesting 10 mL of 12 day culture at 4000 × *g* for 3-5 min, washed with sterilised distilled water and lyophilised at -72 °C for 12 h and the weight was determined (Becker, 1994). The total lipids were extracted from microalgal biomass following the modified method of Bligh and Dyer (1959) using lyophilized microalgal biomass from 30 mL microalgal culture to which 0.6 mL water, 1 mL methanol and 1mL chloroform were added. The solution was mixed for 30 s and an additional 1 mL chloroform and 1 mL water were added and the contents of the culture tube were mixed for 30 s. The tubes were centrifuged at 3105 × g for 10 min. The upper layer was withdrawn using a pipette and the lower chloroform phase containing the extracted lipids was transferred into another culture tube, the residue was extracted twice as above and the chloroform phases were pooled together and dried under nitrogen. Thereafter, the total lipids were measured gravimetrically, and the lipid content was calculated. The responses obtained were subjected to ANOVA and the

significant ($p< 0.05$) variables optimized for their concentration for biomass and total lipid content.

4.2.8 Response surface methodology

A 2^4 Factorial Central Composite Design (FCCD) was used to optimize the concentrations of the factors selected, keeping rest of the factors constant. An experimental design of 30 experiments or runs was formulated using the Design Expert software. The experiments were conducted in 100 mL Erlenmeyer flasks containing 50 mL medium (pH 7, 30 gkg^{-1}) prepared according to the design. Five percent inoculum containing 1.5×10^6 cells mL^{-1} in exponential phase was added to the culture medium. The cultures were incubated in an incubator shaker at 120 ×g under 27 μmol m^{-2} s^{-1}, 16:8 h light and dark photoperiods and the biomass (g L^{-1}) and total lipid content (g L^{-1}) were determined from 30 mL culture volume at the end of 12 days. Response surface methodology 3D plots were generated to understand the interaction between different factors and to find the optimum concentration of the medium components favouring the responses. The optimised values obtained were confirmed using point prediction.

4.2.9 Validation of the optimised media

The predicted responses (biomass and lipid) were experimentally validated in the optimised medium under optimised culture conditions predicted by the software at the end of the analyses by shake flask experiments and it was compared with the un-optimized medium for understanding the effectiveness of the whole optimisation process. The experiments were carried out in triplicates.

4.2.10 Statistical analyses of data

The data was processed and analysed by the statistical software, Design-Expert 6.0.9 (Stat-Ease, Inc., Minneapolis, USA) to estimate the coefficient of regression of experimental data and to plot response surface. ANOVA was used to determine the significance of each term in the fitted equations and to estimate the goodness of fit in each case.

4.2.11 Scale up production in outdoor conditions

4.2.11.1 Preparation of inoculum in 2L culture medium

The inoculum for outdoor cultivation was obtained from the indoor cultures, which were scaled up stepwise, starting from 100 mL to 500 mL volumes and then to final volume of 2 L. 10% inoculum with a cell density of 1.5×10^6 cells mL^{-1} at their exponential phase prepared in 500 mL flask was added to the sterilized 2 L media in 3 L Haffkine's flask. The culture was continuously aerated using air pumps fitted with air stones. The inoculum culture was cultivated at 25-28 °C under an illumination of 27 µmol photon m^{-2} s^{-1} in 16:8 h photoperiod. The cell dry weight of culture in 2 L culture medium was measured at regular intervals till 18^{th} day.

4.2.11.2 Sterilization

Twenty litres polyethylene tetraphthalate (PET) water jars of 490 mm and 270 mm diameter were used for mass production of the microalgae under study. Chlorination was selected as the means of sterilization. The jars were sterilized by filling it with plain tap water and chlorinating it with 10 ppm 4% sodium hypochlorite. The water was aerated overnight using 0.2 µm air filters along with air stones. After 12 h, the tap water was discarded and the jars were filled with 30 g kg^{-1} sea water and 10 ppm active

chlorine was added and aerated overnight. The residual chlorine was neutralized using 12 ppm sodium thiosulphate and strongly aerated for one hour before medium preparation (Moretti et al., 2005).

4.2.11.3 Media preparation and inoculation

Growth media was prepared aseptically by adding the optimised concentration of minimal sea water media (MSWM) components in natural sea water of salinity 30 g kg^{-1}. Twelve days old culture cultivated in 3L Haffkine's flask of cell density 1.5x 10^6 cells mL^{-1} was inoculated at 10% of the culture volume. The outdoor cultivation was carried out in a shady environment with an air temperature of 28-30°C and water temperature was around 25-26 °C. Sunlight along with artificial illumination of 20µmol photon/m^2/s was provided for a photoperiod of 16:8 h. Continuous mixing was provided by sparging sterile air (0.2 µm filter) using silicon tubes fitted with air stones. The experiment was carried out in triplicates.

4.2.11.4 Harvesting

The biomass was harvested from the 20 L water jars by stopping the aeration and allowing the cells to settle for 2-3 h. After settling period, the clear supernatant was siphoned out without disturbing the cell biomass settled at the bottom of the jars. The wet biomass was centrifuged after 10 days of culturing in heavy duty centrifuge at 3500 × g for 10 min. The wet biomass and dry biomass after lyophilisation were determined by gravimetric method.

4.3 Results and discussions

4.3.1 Microalgal culture

Tropical marine diatoms are generally difficult to adapt to laboratory conditions (Nurachman et al., 2012), but we were successful in culturing *Navicula* sp. under our laboratory conditions. This benthic diatom was isolated from the water samples collected from Cochin Estuary, India. The diatom *Navicula phyllepta* is a unicellular and uninucleate appearing brown to slightly green in colour (Fig. 4.1 a). The primary photosynthetic pigments in *Navicula* sp. are chlorophyll a and c and ß-carotene masked by fucoxanthin and xantophylls (Kuczynska et al., 2015). The ultrastructure of the cell (Fig. 4.1 b) showed the presence of chloroplasts at the two ends of the cell. The large spherical nucleus was attached to the chloroplasts. Tubular shaped mitochondrion was localised in the peripheral cytoplasm layer which is a characteristic of diatoms. The lipid bodies were found at the centre or periphery (Dawes, 1998).

4.3.2 Effect of different nitrogen sources

On assessing the effect of different sources of nitrogen on the growth of the benthic diatom, urea gave higher cell densities compared to sodium nitrate and ammonium chloride as nitrogen source (Fig. 4.2 a). There were significant differences in cell densities of *N. phyllepta* MACC8 in different media ($p = 0.0003$) and between the culturing periods ($p = 0.007$). The growth rates of the diatom in sodium nitrate ($NaNO_3$), urea and ammonium chloride (NH_4Cl) were 0.44, 0.40 and 0.21day^{-1} respectively. Similar results were reported in a study using diatom *Cylindrotheca fusiformis*, where $NaNO_3$ and urea were equally good in promoting growth (Suman et al.,

2012). The possible explanation for this improved growth mechanism could be that the various genes involved in nitrate assimilation/acquisition in microalgae were actively expressed in the presence of nitrate and urea medium, but repressed in the presence of ammonium medium (Hildebrand and Dahlin, 2000; Imamura et al., 2010; McDonald et al., 2010). Nitrate and urea were better nitrogen sources than ammonium salts in *Phaeodactylum tricornutum* (Yongmanitchai and Ward, 1991), *Chlorella vulgaris* and *Scenedesmus* sp. (Crofcheck et al., 2009, Wijanarko et al., 2011; Muthu et al., 2013). Most marine and fresh water microalgae can effectively use NO^{3-}, NO^{2-}, N_2 or NH^{4+} as nitrogen sources, but through absolutely different pathways (Glass et al., 2009). Urea with double nitrogen groups (NH_2) as the nutrient source leads to availability of nitrogen sources, ammonium and nitrate in the medium along with higher nitrogen uptake efficiency, thereby increasing the amount of nitrogen in vivo and also resulting in higher biomass concentration. It is also known to boost the algal growth as it acts as a complementary source of organic carbon (Saumya et al., 2016). Moreover, the use of urea in growth media stabilises pH due to lack of ionic charge (Eustance et al., 2013). From the study, both urea and sodium nitrate were proved to be good nitrogen sources but since urea is more cost effective (Wijanarko et al., 2011; Kim et al., 2016), urea based media was selected as a more feasible one for mass production of *N. phyllepta* MACC8, a potential biodiesel producer.

4.3.4 Effect of different salinities on diatom growth

Navicula phyllepta, an estuarine isolate, exhibited wide salinity tolerance by growing in all tested salinities 0, 10, 20, 30, 35 and 40 g kg^{-1}. *Navicula phyllepta* is a dominant member of communities along estuarine

gradient (5-30 g kg^{-1}) (Vanelslander et al., 2009; Smol et al., 2010; Bellinger and Sigee, 2015). *Navicula phyllepta* has been reported from a broad range of salinities, from electrolyte- rich freshwaters (Krammer and Lange-Bertalot, 1986) to hypersaline environments with salinities at 75 g kg^{-1} (Clavero et al., 2000). In the present study, the highest cell densities were obtained in higher salinities ranging from 20-40 g kg^{-1}. There was significant difference in growth between the extreme salinities 0 and 40 g kg^{-1} ($p< 0.05$). However, there was no significant growth difference within a salinity range of 10-40 g kg^{-1} and cultivation time (Fig. 4.2b). The proportional increase in lag phase with increasing salinity is clearly evident form the graph. Growth in zero salinity showed a long lag phase of 8 days, whereas in salinities higher than 30 g kg^{-1}, the exponential phase started immediately after 4^{th} day. Similar studies reported that estuarine diatom *Thalassiosira weissflogii* had higher growth rate and better biochemical composition at 25 and 30 g kg^{-1} (Garcia et al., 2012). The diatom *Chaetoceros calcitrans* had significant growth when cultured at salinity of 30 g kg^{-1} (Adenan et al., 2013). Similarly, *Nannochloropsis salina* belonging to Eustigmatophyceae, a potential biofuel feedstock showed significant increase in growth rate, biomass and lipid content at higher salinities in the range of 22-34 g kg^{-1} and higher salinities helped in controlling the invading non targeted algae and grazers (Bartley et al., 2013). The results of the present study showed that after a long lag phase of 8 days, the diatom showed a quiet steady growth in zero salinity i.e. fresh water. This indicates the reason for the abundance of the species at low salinities and also imparts competitive advantage in oligohaline and mesohaline parts of the estuary (Vanelslander et al., 2009). The tolerance to low salinities is important during heavy rainfall, when the salinity of sediment

top layer almost reduces to fresh water conditions (Coull, 1999). The growth rate was found to be the highest in the salinity range of 10- 40 g kg^{-1} with an average rate of 0.46 day^{-1} with the least in zero salinity (0.36 day^{-1}). Hence, the isolate can be cultured in sea water (30 g kg^{-1}), encouraging the utilisation of non-potable waters for mass production and minimising the invasion of contaminating invasive organisms.

4.3.5 Selection of growth medium

Of the five types of enriched sea water media tested such as F/2 , modified F/2 for diatom (change in quantities, not final concentration), L1, DAM and MSWM, the modified sea water medium was found to be a cheaper and better medium for the stable growth of *Navicula phyllepta*. Growth studies showed that *N. phyllepta* MACC8 had a significant growth difference in different media tested and the cultivation time ($p<0.05$) (Fig. 4.2 c). The highest growth rate was in MSWM (0.48 day^{-1}), followed by F/2 (0.41 day^{-1}) and L1 (0.32 day^{-1}). There was no significant growth difference in modified F/2 medium with a growth rate of 0.26 day^{-1} and DAM with 0.25 day^{-1}. The major obstacle in the large scale production of biodiesel from microalgae is the high cost in production and one of the factors contributing to the cost is the medium used for culturing the organism. The conventional medium used for culturing this strain contains many nutrients and trace metals which increase the overall cost of the culturing medium. An optimal media is the one which should contain the minimum quantity of nutrients to support maximum growth of the microalgae (Crofcheck et al., 2012). Different media have varying nutrient quantities that can significantly change the quantity of cell biomass and its biochemical composition during cultivation (Mandalam and Palsson, 1998). In addition to that, it was proved

in this study that the media composition can cause change in cell characteristics. *Navicula* sp. is a major biofilm producer as the cells are capable for producing transparent exopolymer particles (TEP), which are colonized by bacteria. These bacteria produce extra cellular polysaccharides in response to the presence of phytoplankton, thereby initiating cell adhesion and cell to cell attachment (Buhmann et al., 2011; Amin, 2012). Such a property is very disadvantageous in terms of the mass cultivation of this diatom in any form of cultivation systems. In this present experiment, the culturing of the diatom *Navicula phyllepta* grown in the minimal sea water medium helped to reduce the cell adhesion and cell aggregation compared to the conventional F/2 medium. The cells were almost homogenously suspended in the media upon agitation. Figure 4.3 (a) and (b) shows the change in cell aggregation in MSWM and F/2 media respectively. It could be due to reduced number of bacterial population in the media devoid of external addition of trace metals and vitamins as it was reported by Windler (2015) in his study that xenic cultures of benthic diatom *Achnanthidium minutissimum* showed visible aggregates, whereas the axenic cultures were almost suspended or less aggregated. It may be also due to that some diatom secretome inhibited the biofilm formation under the changed environmental conditions (Doghri et al., 2016). Another possible argument is that urea, a major protein denaturant, must have denatured/ solubilised the cell wall proteins involved in diatom cell adhesion (Nguyen and Harvey, 2001; Lee and van der Vegt, 2006; Wills et al., 2013). Finally, taking all the factors discussed above into consideration, the minimal sea water medium was selected for further statistical optimization.

4.3.6 Screening of variables using Plackett- Burman design

Plackett- Burman design helps us to screen the important factors affecting the desired response with limited number of experiments. Table 4.3 shows the distribution of different variables and the responses in the study. Of the seven factors tested, urea, sodium silicate, sodium phosphate and temperature were considered as significant variables influencing the responses. The factors silicate and temperature had a positive coefficient on biomass production in *N. phyllepta*. In the case of lipid content, temperature had a significant positive coefficient, while urea and phosphate showed a negative coefficient. The rest of the variables had no significant effects on the responses and were kept constant in further experiments. Statistical analysis based on t-test statistic was carried out, at 95% confidence interval (CI) throughout. Table 4.4 represents the ANOVA results of the selected variables having significant effect on biomass and lipid productions. Increasing the silica concentration in the culture media enhanced the cell division in diatoms, which is vital for improving algal biodiesel productivity in terms of increased biomass (Moll et al., 2014; Yang et al., 2014). Most of the published studies on phytoplankton are carried out between 20 °C and 30 °C and this is suitable for mass cultivation (Karthikeyan et al., 2010; Adenan et al., 2013). The results indicated that the concentration of silicon and temperature could be increased in their levels for further study as there is scope for improving the biomass production.

4.3.7 Identification of the best culture media and conditions using response surface methodology

Following screening, response surface methodology using face centred composite design was employed to understand the interactions

between various nutritional and physical factors affecting biomass and lipid production. Table 4.5 summarizes the results of FCCD experiment of each run with results of the response. The results were analysed by standard analysis of variance (ANOVA) which gave the quadratic equation:

$$\text{Biomass} = +0.74+0.33\times A+0.075\times B-0.018\times C+0.073\times D+0.030\times AB-0.029\times AC+0.024\times AD-0.013\times BC-0.026\times BD+0.011\times CD+0.025\times A^2-0.014\times B^2-0.049\times C^2-0.068\times D^2 \quad (4.5)$$

$$\text{Lipid} = +0.15+6.66E\text{-}004\times A+6.22E\text{-}003\times B-2.167E\text{-}003\times C-7.44E\text{-}003\times D+3.125E\text{-}004\times AB-3.938E\text{-}003\times AC-7.312E\text{-}003\times AD+7.312E\text{-}003\times BC-9.938E\text{-}003\times BD-3.812E\text{-}003\times CD+4.035E\text{-}004\times A^2-0.015\times B^2-4.096E\text{-}003\times C^2-8.596E\text{-}003\times D^2 \quad (4.6)$$

where, A is sodium silicate, B is urea, C is sodium phosphate and D is temperature. The ANOVA results for biomass and total lipid production (Table 4.6) showed that the model was significant ($p< 0.05$). In the case of biomass, the model F-value of 57.49 implied that the model was significant. There was only a 0.01% chance that an F-value this large could occur due to noise. In this case A, B, D, AB and AC were significant model terms. The predicted R square value 0.9006 was reasonably in agreement with the adjusted R square value 0.9646. The "lack of fit f-value" of 2.09 implied that the lack of fit was not significant relative to the error. There was a 21.56% chance that a "lack of fit f value" of this large could occur. Non-significant lack of fit was good. The results showed that silicate, urea, temperature and interactive effect between silicate and urea, silicate and phosphate were significant in biomass production. The predicted R-squared value of 0.8181 for model to predict lipid production was in fairly reasonable agreement with the adjusted R-squared of 0.9235. The model

F- value of 25.99 implied the model was significant. There was only a 0.01% chance that an F-value of this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicated model terms were significant. In this case, B, D, AC, AD, BC, BD, CD, B^2 and D^2 are significant model terms. The "lack of fit f-value" of 1.44 implied that the lack of fit was not significant relative to the pure error. There was a 36.07% chance that a "lack of fit f-value" of this large could occur due to the noise. The model showed that the lipid content in the diatom was controlled by a number of individual factors such as urea, temperature and interactive factors such as silicate-phosphate, silicate-temperature, urea-phosphate, urea-temperature, and phosphate-temperature. Perturbation graphs were plotted to compare the effect of all the individual factors at a particular point in the design space. The response is plotted by changing only one factor over its range, while keeping the other factors constant. The lines showing curves or steep slope are the variables showing significant effect on the responses. The silicate (A) had great influence on the biomass whereas urea (B) and temperature (D) showed major effect on the lipid production (Fig. 4.4 (a) and (b)).

Three-dimensional response surfaces were plotted on the basis of the model equation, to investigate the interactions among the variables and to determine the optimum concentration of each factor for maximum response (Fig. 4.5 and Fig. 4.6). Each figure presents the effect of two factors, while keeping the other factor at zero level. It was clearly seen that silicate had important role in the biomass production, whereas urea, phosphate and temperature showed an interactive effect towards total lipid production. The predicted values obtained from this model were as follows: 4.89 mM

sodium metasilicate, 0.90 mM urea, 0.1 mM sodium dihydrogen phosphate and 30.8°C temperature with a resultant biomass of 1.18 gL^{-1}, whereas, 4.69 mM sodium metasilicate, 0.76 mM urea, 0.13 mM sodium dihydrogen phosphate and 25 °C temperature for lipid production of 0.16 g L^{-1} on 12^{th} day of culturing.

Amorphorus silica is vital for cell growth as it is an essential component for frustule formation (Martin-Jezequel et al., 2000) and silicon availability is thus a key factor in the regulation of diatom growth in nature. But the concentration of silicate is critical as the higher concentrations can be inhibitory as reported by Alverson (2007). There are various studies focussing on the significance of mutual effects of nutrients and environmental stress on growth and lipid production rather than their individual effects (Juneja et al., 2013; Spilling et al., 2015; Singh et al., 2015). Nitrogen has been already identified as limiting agent for increased lipid production in marine microalgae. This could be due to alteration in metabolic pathways (gene regulation) under stress conditions leading to lipid accumulation (Yang et al., 2013). The combined limitation of both nitrogen and phosphorus resulted in the highest lipid concentrations in *Phaeodactylum tricornutum* (Valenzuela et al., 2012; 2013), *Chlamydomonas reinhardtii* (Kamalanathan et al., 2015) and *Chlorella minutissima* (Arora et al., 2016). Increase in lipid during deprivation or limitation of nitrogen, phosphorus source or both could be due to decrease in protein synthesis, causing the excess carbon to get channelized into storage molecules such as neutral lipids (triacylglycerides) and starch (Arora et al., 2016). Temperature affects the physiological processes by changing the rate of chemical reactions and the stability of cellular components (Sandnes et al., 2005; Wagenen et al., 2012). The

response of microalgal lipid content to high and low growth temperatures varies from species to species (Renaud et al., 2002; Wu et al., 2013). The results in the present study are in accordance with the results of study by Wu et al. (2013) on *Monoraphidium* sp., in which lipid content was the highest at 25 °C, while high biomass and lipid productivity was achieved at 30 °C. Similarly, Wah et al. (2015) stated in his study on the effect of different temperatures on the lipid profile of a benthic diatom *Amphora subacutiuscula* that the total lipid content was the highest at 23 °C when tested in a range of 5-35 °C and saturated fatty acids content was more at lower temperatures. This could be the result of the adaptive mechanism of the organism at low temperatures. Fakhry and El Maghraby (2015) also reported that the degree of nitrogen availability in coupled effect with temperature had been identified as a critical factor for the maximal production of lipid in microalgae. In this work, the high diurnal temperature range of 25–35 °C makes *Navicula phyllepta* suitable for the outdoor culture in tropical regions, but more outdoor experiments are needed to evaluate the feasibility.

4.3.8 Validation of the model

Statistical optimisation of growth medium and conditions of *Navicula phyllepta* towards high biomass and lipid production using RSM design provided the most simplest and accurate means for obtaining the most efficient medium with the best combination of interacting factors. The validation experiments for confirming the adequacy of the model designed in this study yielded a maximum biomass of 1.2 ± 0.08 g L^{-1}, which was 1.62 fold higher (64% increase) than under un-optimized conditions (0.74 ± 0.08 g L^{-1}). The total lipid was measured to be 0.11 ± 0.003 g L^{-1}

which was 1.2 fold higher (22% increase) than in un-optimized conditions (0.09 ± 0.009 gL^{-1}). A study on improved culturing conditions for green microalga *Scenedesmus* sp. by Yang et al. (2014) showed an increase of 13.41% in biomass and 36.32% in lipid content compared to the original conditions. A report on application of factorial design of experiment for biofuel production by the haptophyte *Isochrysis galbana* (Chen et al., 2012) demonstrated a 3.93% increase in lipid content upon modifying the main interacting variables. The maximum predicted value of biomass (2.95 g L^{-1}) obtained was increased by 1.3 times when compared with the original medium (2.27 g L^{-1}) in the case of microalgae *Chlorella pyrenoidosa* (Yadavalli and Rao, 2013). The maximum biomass attained after RSM based optimisation in microalga *Desmodesmus* sp. was only 0.758 g L^{-1} (1.3 fold higher than initial medium) at the end of 14^{th} day of culturing (Ji et al. 2013). Cheng et al. (2013a) reported that statistical optimization of culture media in two stage cultivation method of *Chlorella protothecoides* gave a biomass concentration of 1.19 g L^{-1} in optimized biomass production medium after 11 days of cultivation which was 1.8 times higher than that in the original medium, whereas 12.9% lipid content was obtained from the biomass in the lipid production medium, which was three times higher than that from the original medium. In this study, the amount of lipid obtained for 1.2 g L^{-1} of biomass in biomass production medium was 0.132 g L^{-1} (lipid content 11% dcw) and 0.56 g L^{-1} of biomass yielded 0.11 g L^{-1} of lipid in lipid production medium was (lipid content 19.6% dcw) at the end of 12 days. On comparison with these reports, it could be concluded that the optimisation of media components and culture conditions enhanced the biomass and lipid production in the diatom from that of the original

conditions without subjecting to any stress conditions. The results indicated that the media conditions optimised for high biomass production can be adopted for culturing the diatom, and the lipid productivity can be further augmented by subjecting the cells to stress conditions by altering the growth conditions. The present study on RSM based optimisation of biomass and lipid production in the newly isolated *Navicula phyllepta* identified the important parameters favouring augmented production and, thus is the first step towards designing a two stage cultivation method for increased biomass and lipid production in this microalga.

4.3.9 Scale up production in outdoor conditions

The scaling up of unialgal cultures in outdoor open culture systems is often a challenging process, which relies on the type of algal strain, weather conditions, water chemistry (salinity, turbidity, pH, dissolved oxygen, chemicals), biological contamination and cultivation system operational factors (pond design, hydrodynamics and mixing, dilution/harvesting frequency) (Lebeau and Robert, 2003; Fon Sing, 2010). The first step towards mass cultivation involves the supply of good sea water free of any contaminant, either chemical or biological. Sterilization of the seawater can be done by many ways like filtration, autoclaving, pasteurization, UV irradiation, chlorination, acidification or ozonisation (Biswajit et al., 2016). Sterilisation by chlorination has been proved to be and effective and cheaper means of water sterilisation, though good care has to be taken for the complete removal of residual chlorine before culture inoculation.

One of the main challenges of large-scale cultivation of microalgae in outdoor conditions is that biomass productivities obtained over extended

periods of time are generally found to be low. Though the cultivation conditions are easy to maintain in the lab, maintaining them at large scale is challenging and cost intensive. In the present study, the inoculum culture of the diatom in 3L flasks showed a steady growth till the 18th day of culturing under controlled laboratory conditions (Fig. 4.7). The dry weight of the dry biomass weighed 0.9 g L^{-1} by 9th day and 1.6 g L^{-1} at the end of 18th day (Fig. 4.8). The mass culturing of the diatom in 20 L PET water jars (Fig. 4.9) under outdoor conditions gave a wet biomass of 4.7-5-5 g L^{-1} and dry weight was 0.6 g L^{-1} by 10^{th} day of culturing. In this present study, it was clearly evident that the amount of biomass produced in outdoor environment was very low compared to indoor conditions, where the governing factors such as temperature and light intensity were regulated. The growth of microalgae in outdoor culture systems is largely dependent on the intensity of incident light and on air temperature, which under daily and seasonal weather and climatological conditions can fluctuate appreciably from one extreme to another. Matsumoto et al. (2017) reported that diatom *Fistulifera solaris*, which is phylogenetically related to *Navicula* sp. showed steady growth in raceway and column-type bioreactors without temperature control from spring to autumn seasons (upto 42 °C), but showed a declined growth during winter season (below 10 °C). Though several studies have shown outdoor cultivation with temperature control units (Del Campo et al., 2001; Acien et al., 2003; Zittelli et al., 2006), controlling temperature and light is not practical in the open cultivation system and is cost intensive in the closed cultivation system (Converti et al., 2009; Christenson and Sims, 2011). In the present work, the cells showed auto settling behaviour after 10^{th} day of culturing period which indicated that the cells were collapsing.

Control of light intensity is very much essential as less light is required during the stationary and declining phases. Too much of light will cause the culture for the early declining. Nurachman et al. (2012) reported that high light intensity from sunlight resulted in decrease of *Navicula* sp. growth. This feature was consistent with the fact that origin habitat of the *Navicula* sp. was in deep waters results in the requirement of low sunlight exposure for growth. Similarly, aeration is also important for developing and maintaining mass outdoor culture. Aeration is very much essential to enhance the exponential phase of growth of micro-algae for required temperature, the maintenance of oxygen sufficiency, helps in mixing the nutrient in the culture media uniformly in the medium and preventing the algae from settling at the bottom of the culture tanks. Large-scale batch culture of benthic diatoms may be performed in clear plastic bags or, more commonly, in solid plastic containers. Diatoms are well adapted to turbulence, and in fact are more productive in high turbulence conditions than other classes of marine microalgae (Margalef, 1997). Some benthic diatoms grow in suspension when agitated, while others do not (Lebeau and Robert, 2003). However, *Navicula phyllpeta* showed a suspended growth upon aeration up to 10 days of culturing. From the indoor strain comparison study and from the glasshouse cultivation trials by Mercz (1994), it was hypothesised that benthic diatom like *Amphora coffeaeformis* could be suitable candidates for cultivation in outdoor open raceway ponds for biodiesel production. The harvesting of highly dense diatoms cells by gravity settling/ sedimentation without the use of any flocculants proved to be cost efficient (Fig. 4.10) (Al Hattab, 2015). However, more series of experiments has to be carried out in outdoor cultivation of the diatom

Navicula phyllepta to investigate strain tolerance to the outdoor growth conditions and higher biomass productivity

4.4 Conclusions

In this study, an optimized process was developed for biomass and lipid production in the lipid rich marine diatom *Navicula phyllepta* MACC8. The high growth rate of the algal cells in modified sea water medium with minimum components and urea as nitrogen source offers a great potential for the mass production of the diatom in a cost effective process. The Plackett - Burman design and response surface methodology based optimization of biomass and lipid production in *Navicula phyllepta* MACC8 resulted in 1.62 fold increase (64%) in biomass and 1.2 fold increases (22%) in lipid production. The optimized process parameters and growth medium obtained in this experiment can be further utilized for large scale biomass production from *N. phyllepta* and augmented lipid production by limiting the factors such as urea, silicate, phosphate and temperature as identified in the present study. The mass culturing of the diatom in 20L PET water jars under outdoor conditions gave a wet biomass of 4.7-5-5 g L^{-1} and dry weight was 0.6 g L^{-1} by 10^{th} day of culturing. However, an intensive research is required for the outdoor large scale cultivation of *N. phyllepta* for sustainable production of high biomass for biodiesel production

Table 4.1: Nutrient composition of different media used in the study

Constituents	DAM (gL^{-1})	F/2 (gL^{-1})	Modified F/2 (for diatoms) (gL^{-1})	L1 (gL^{-1})	MSWM (gL^{-1})
Urea	-	-	-	-	0.060
$NaNO_3$	25.5	75	150	75	-
NaH_2PO_4	1.38	5	10	5	0.030
$Na_2SiO_3.9H_2O$	28.4	60	30	30	0.150
NaCl	20.57	-	-	-	-
Na_2SO_4	3.067	-	-	-	-
$CaCl_2.2H_2O$	1.15	-	-	-	-
$MgCl_2.6H_2O$	11.1	-	-	-	-
H_3BO_3	1.50	-	-	-	-
KBr	5	-	-	-	-
KCl	35	-	-	-	-
NaF	0.15	-	-	-	-
$NaHCO_3$	10	-	-	-	-
$SrCl_2.6H_2O$	0.85	-	-	-	-
Trace metals					
$CoCl_2.6H_2O$	10	10	9.9	10	-
$CuSO_4.5H_2O$	9.8	9.8	9.8	2.45	-
$MnCl_2.4H_2O$	0.8	180	179	180	-
$NaMoO_4.2H_2O$	6.30	6.3	6.2	19.9	-
$NiCl_2.6H_2O$	0.74	-	-	-	-
$Na_2SeO_3.5H_2O$	0.85	-	-	-	-
$ZnSO_4.7H_2O$	22	22	21.9	22	-
$FeCl_3.6H_2O$	3.15	3.15	6.3	3.15	0.005
$Na_2EDTA.2H_2O$	4.36	4.36	8.8	4.36	0.050
$NiSO_4.6H_2O$	-	-	-	2.7	-
Na_3VO_4	-	-	-	1.84	-
K_2CrO_4	-	-	-	1.94	-
$ZnCl_2$	-	-	-	-	-
Vitamin solutions					
Thiamin HCl	0.2	0.2	0.4	0.20	-
Cyanocobalamin	1	1	1	1	-
Biotin	0.1	0.1	0.2	0.1	-

DAM- Diatom artificial medium ; MSWM-Modified sea water medium

Table 4.2: Higher and lower limits of the variables selected for the Plackett Burman experimental screening

Variable	Higher limit(+)	Lower limit (-)
Urea (mM)	10	0.5
Sodium silicate (mM)	25	0.25
Ferric chloride (mM)	0.1	0.01
Salinity (gkg^{-1})	40	25
Temperature (°C)	30	20
Sodium phosphate (mM)	1.0	0.1
pH	9	7
Agitation (rpm)	125	115

Table 4.3: Plackett- Burman experimental design and range of factors

Run	Urea mM	$NaSiO_3$ mM	$FeCl_3$ mM	Salinity gkg^{-1}	Temp °C	NaH_2PO_4 mM	pH	Agitation rpm	Biomass gL^{-1}	Lipid gL^{-1}
1	0.5	2.5	0.01	25	20	1.0	7	115	0.241±0.02	0.063±0.003
2	10	0.25	0.1	25	20	0.1	7	125	0.1±0.014	0.068±0.006
3	10	2.5	0.01	40	20	0.1	9	115	0.214±0.03	0.06±0.003
4	0.5	0.25	0.1	40	30	0.1	7	125	0.209±0.05	0.082±0.002
5	10	2.5	0.01	40	30	0.1	7	115	0.65±0.02	0.085±0.003
6	0.5	0.25	0.01	25	20	0.1	7	125	0.135±0.04	0.072±0.001
7	10	0.25	0.1	40	20	1.0	9	115	0.11±0.03	0.056±0.002
8	0.5	0.25	0.01	40	30	1.0	9	115	0.24±0.06	0.074±0.002
9	10	0.25	0.01	25	30	1.0	9	125	0.241±0.09	0.063±0.006
10	0.5	2.5	0.1	40	20	1.0	9	125	0.238±0.03	0.064±0.004
11	0.5	2.5	0.1	25	30	0.1	9	125	0.605±0.01	0.102±0.002
12	10	2.5	0.1	25	30	1.0	7	115	0.32±0.07	0.060±0.007

Values represent mean of three biological replicates

Table 4.4: Statistical analyses for biomass and total lipid production of selected factorial model under Plackett-Burman design

Source	Biomass (gL^{-1})			Total lipid (gL^{-1})		
	Sum of Squares	F-Value	p- Value	Sum of Squares	F-Value	p- Value
Model	0.25	12.63	0.0024	1.586E-003	11.95	0.0025
Urea	-	-	-	3.521E-004	7.96	0.0225
$NaSiO_3$	0.13	12.69	0.0061	-	-	-
Temperature	0.13	12.57	0.0063	5.741E-004	12.97	0.0070
NaH_2PO_4	-	-	-	6.601E-004	14.92	0.0048

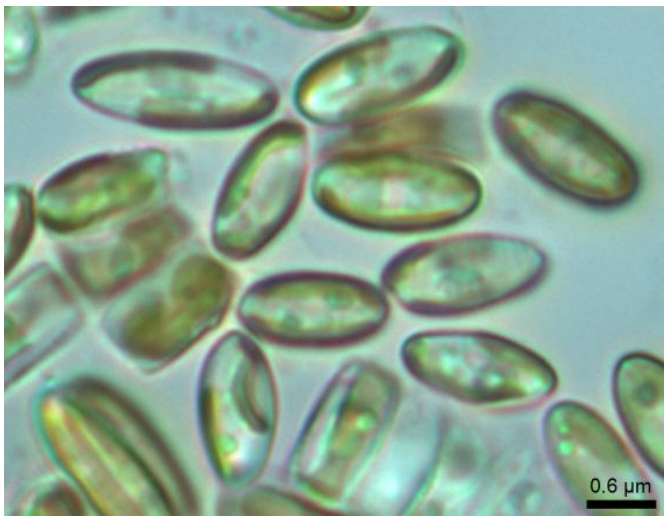
Table 4.5: Central composite design matrix with experimental values of biomass and total lipid production

Run	A	B	C	D	Biomass (gL^{-1})		Total lipid (gL^{-1})	
					Actual value	Predicted value	Actual value	Predicted value
1	5.00	0.90	0.15	25	0.895±0.04	0.93	0.164±0.01	0.17
2	1.00	0.10	0.15	25	0.22±0.028	0.20	0.097±0.01	0.096
3	5.00	0.10	0.15	25	0.722±0.05	0.70	0.119±0.003	0.12
4	5.00	0.10	0.05	35	1.05±0.062	1.01	0.121±0.002	0.12
5	1.00	0.10	0.15	35	0.339±0.01	0.37	0.105±0.001	0.11
6	3.00	0.50	0.10	30	0.78±0.03	0.75	0.154±0.001	0.15
7	1.00	0.90	0.15	35	0.391±0.01	0.38	0.111±0.003	0.11
8	1.00	0.90	0.15	25	0.313±0.01	0.31	0.144±0.004	0.14
9	1.00	0.90	0.05	25	0.282±0.17	0.34	0.128±0.006	0.13
10	1.00	0.10	0.05	25	0.24±0.033	0.17	0.114±0.001	0.12
11	3.00	0.90	0.10	30	0.863±0.02	0.86	0.144±0.008	0.14
12	5.00	0.10	0.05	25	0.749±0.07	0.79	0.126±0.005	0.12
13	1.00	0.90	0.05	35	0.377±0.006	0.36	0.122±0.002	0.12
14	3.00	0.50	0.05	30	0.692±0.01	0.71	0.144±0.01	0.15
15	3.00	0.50	0.10	30	0.852±0.02	0.75	0.149±0.002	0.15
16	1.00	0.10	0.05	35	0.31±0.01	0.30	0.145±0.003	0.14
17	3.00	0.50	0.10	25	0.585±0.02	0.61	0.149±0.013	0.15
18	3.00	0.50	0.10	35	0.737±0.08	0.75	0.132±0.019	0.13
19	3.00	0.50	0.10	30	0.74±0.04	0.75	0.156±0.004	0.15
20	3.00	0.50	0.10	30	0.76±0.02	0.75	0.147±0.004	0.15
21	5.00	0.50	0.10	30	1.07±0.07	1.10	0.142±0.006	0.15
22	3.00	0.50	0.15	30	0.667±0.03	0.68	0.146±0.1	0.14
23	3.00	0.50	0.10	30	0.748±0.06	0.75	0.144±0.005	0.15
24	3.00	0.10	0.10	30	0.567±0.01	0.66	0.125±0.003	0.13
25	5.00	0.10	0.15	35	0.995±0.02	0.97	0.106±0.004	0.10
26	5.00	0.90	0.05	25	1.146±0.01	1.08	0.146±0.009	0.14
27	3.00	0.50	0.10	30	0.747±0.03	0.75	0.147±0.003	0.15
28	1.00	0.50	0.10	30	0.437±0.01	0.44	0.157±0.003	0.15
29	5.00	0.90	0.15	35	1.103±0.02	1.10	0.113±0.002	0.11
30	5.00	0.90	0.05	35	1.143±0.07	1.20	0.098±0.001	0.1

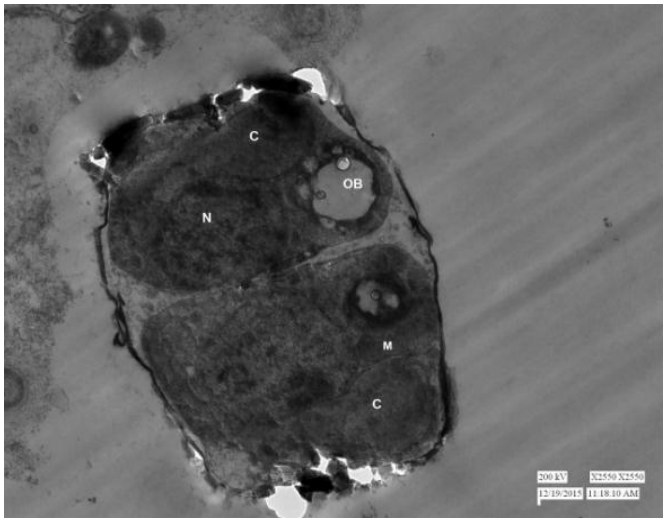
A- $NaSiO_3$ mM, B- Urea mM, C- NaH_2PO_4 mM, D- Temperature °C. Values represent mean of three biological replicates

Table 4.6. ANOVA results for biomass and total lipid production under response surface quadratic model

Source	Biomass(gL^{-1})			Total lipid (gL^{-1})		
	Sum of Squares	**F-Value**	**p- Value**	**Sum of Squares**	**F-Value**	**p- Value**
Model	2.32	57.49	< 0.0001	9.927E-003	25.99	< 0.0001
A-$NaSiO_3$	1.99	69.36	< 0.0001	8.000E-006	0.29	0.5961
B-Urea	0.10	34.96	< 0.0001	6.969E-004	25.55	0.0001
C-NaH_2PO_4	5.576E-003	1.93	0.1850	8.450E-005	3.10	0.0988
D-Temp	0.097	33.52	< 0.0001	9.976E-004	36.57	< 0.0001
AB	0.015	5.06	0.0399	1.562E-006	0.057	0.8141
AC	0.013	4.58	0.0491	2.481E-004	9.09	0.0087
AD	9.206E-003	3.19	0.0944	8.556E-004	31.36	< 0.0001
BC	2.657E-003	0.92	0.3527	8.556E-004	31.36	< 0.0001
BD	0.011	3.78	0.0707	1.580E-003	57.92	< 0.0001
CD	2.066E-003	0.72	0.4110	2.326E-004	8.53	0.0106
A^2	1.570E-003	0.54	0.4723	4.219E-007	0.015	0.9027
B^2	4.993E-004	0.17	0.6835	5.520E-004	20.24	0.0004
C^2	6.318E-003	2.19	0.1598	4.348E-005	1.59	0.2261
D^2	0.012	4.13	0.0601	1.915E-004	7.02	0.0182



(a)



(b)

Fig. 4.1 (a) Light microscopic image of *Navicula phyllepta* MACC8 under oil immersion (100X magnification), scale bar=0.6µm. (b) electron microscopic image of cross section of a dividing cell showing chloroplast (C), nucleus(N), mitochondrion(M), oil bodies(OB).

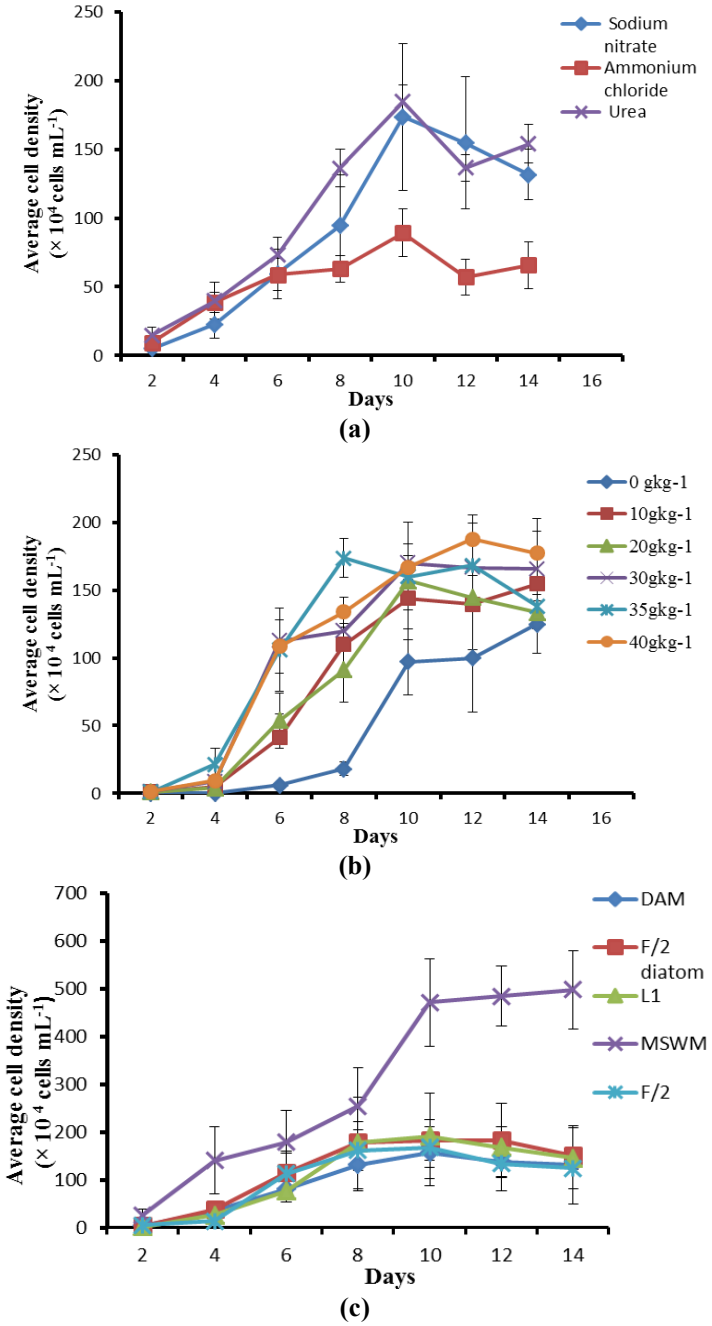
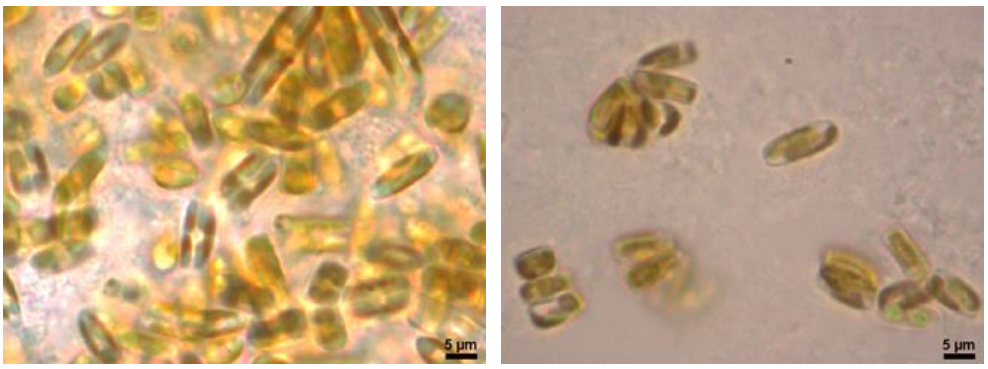
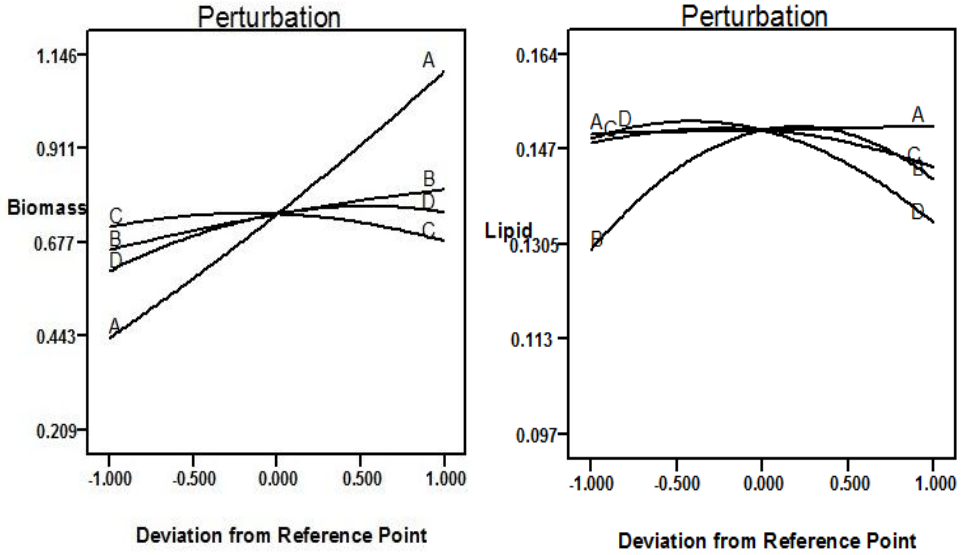


Fig. 4.2 The growth of *Navicula phyllepta* MACC8 in (a) F/2 medium with different nitrogen sources, (b) different salinities, and (c) different media



(a) (b)

Fig. 4.3 Light microscopic image of (a) Aggregation of cells grown in F/2 (conventional medium) medium (b) non- aggregated diatoms grown in MSW medium, viewed under 100X magnification. Scale bar=5µm



(a) (b)

Fig. 4.4 Perturbation graph of independent variable affecting (a) biomass and (b) total lipid. A-sodium silicate, B- urea, C- sodium phosphate D- temperature

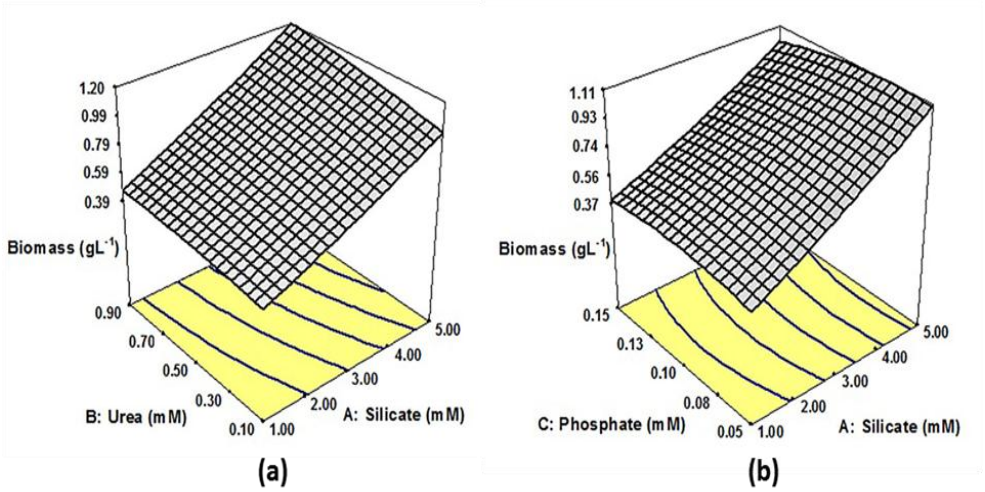


Fig. 4.5 RSM plots of biomass as a function of (a) urea and silicate (b) silicate and phosphate in MSW medium

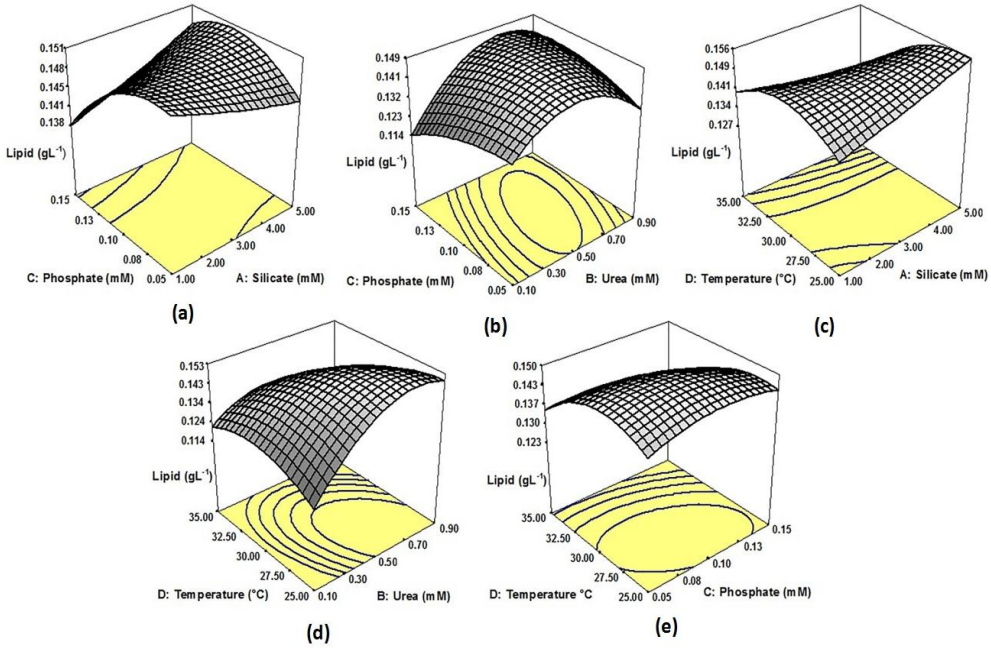


Fig. 4.6 RSM plots of lipid as a function of (a) phosphate and silicate (b) phosphate and urea (c) temperature and silicate (d) temperature and urea (e) temperature and phosphate in MSW medium

Fig. 4.7 Inoculum preparation for upscale cultivation of *Navicula phyllepta* in 3L Haffkine flask under laboratory conditions

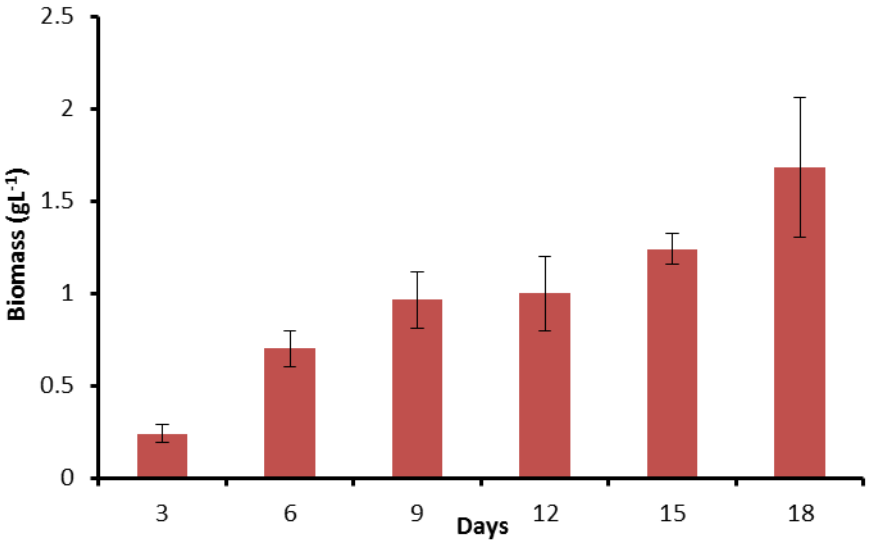


Fig. 4.8 Dry weight of biomass of the diatom cultured in controlled laboratory conditions in 3L flasks at an interval of 3 days

Fig. 4.9 Mass cultivation of *N. phyllepta* in 20L PET water jars under outdoor conditions

Fig. 4.10 Auto settling cells of diatom *N. phyllepta* by stopping aeration

Chapter 5

ENHANCEMENT OF LIPID PRODUCTION IN *NAVICULA PHYLLEPTA* MACC8 UNDER SELECTED STRESS CONDITIONS USING TWO STAGE CULTIVATION METHOD

Contents

5.1 Introduction

Storage lipid bodies remain among the least characterized organelles till date, as their biological importance had been recognized just a decade ago (Farese and Walthe, 2009). Micro- algal lipid droplets have garnered increasing attention as storage organelles for biofuel molecules, though the understanding of the real dynamics behind their biosynthesis is lacking (Merchant et al., 2011). It is a very well investigated fact that the formation of lipid droplets in microalgae is triggered by cellular stresses such as nutrient deprivation, high light exposure and temperature fluctuation (Hu et al., 2008; Pal et al., 2011). Among those stresses, nutrient deprivation is easily accomplished through a change in growth medium composition and is widely used to experimentally induce lipid accumulation inside the

microalgal cells and can be easily reversed by replenishing the nutrients in the growth medium. Thus, by simply changing the growth medium, manipulation of the lipid production can be easily achieved making microalgae excellent models to study fundamental mechanisms of lipid droplet formation or mobilization (Liu and Benning, 2013).

There are several theories or hypotheses regarding why stress conditions induce lipid accumulation in the algal cells. One popular hypothesis is that the microalgae normally accumulate lipid during the cell cycle prior to cell division and unfavourable/stress conditions often block cell cycle progression, as a result of which, there is a shift in the lipid biosynthetic pathways towards synthesis of more neutral lipids than synthesizing membrane lipids required for the cell wall formation (Converti et al., 2009; Singh et al., 2011; Ji et al., 2013). Reduced photosynthetic rate leads to accumulation of NADH, which inhibits enzyme citrate synthase and prevents acetyl CoA from entering into the TCA cycle. High concentrations of acetyl CoA activate acetyl CoA carboxylase, which converts acetyl CoA to malonyl CoA, which is the rate limiting step in fatty acid biosynthesis (Praveenkumar et al., 2013). Another hypothesis involves rearranging the entire cellular components inside the cells under stress conditions. A decrease in protein content per cell and rearrangement of membrane lipids (in particular chloroplast lipids) is observed, which usually results in little actual increase in lipid content of the culture as a whole, however lipid content as a function of dry weight is markedly increased. The third hypothesis involves maintaining cellular redox homeostasis. It is generally believed that under stress conditions lipid biosynthesis acts as a sink for electrons. Normally, the cells cannot shunt all solar energy absorbed into

non-photochemical quenching (NPQ) and therefore, accumulate excess amounts of reducing energy in the form of NADPH (reducing power). In order to avoid over reduction for the cells, lipid production is increased as they are highly reduced molecules (Klok et al., 2013, Lucker and Kramer, 2013).

Microalgae are able to survive in extreme environments by altering their metabolism according to changing environmental conditions (Adams et al., 2013; Zhang et al., 2013). Lipid enhancement strategies involving alteration of the nutrient regime and cultivation conditions such as light, temperature, CO_2 and salinity are widely explored and applied in microalgal cultivation practices (Mata et al., 2010; Merchant et al., 2011; Pittman et al., 2011; Sharma et al., 2012). Nutrient limitation and induction of stress by controlled cultivation have been the norm to improve lipid content in microalgae (Jiang et al., 2012; Klok et al., 2013; Singh et al., 2014). Nutrients such as nitrogen, iron, phosphorus, magnesium and silicon are very important for cellular mechanisms such as photosynthesis, respiration, cell division, intracellular transportation, protein synthesis in microalgae (Zhang et al., 2014). In contrast to large number of studies on nutrient limitation in phytoplankton growth (Ornolfsdottir et al., 2004; Yin et al., 2004; Gobler et al., 2006), there are less reports on the effects of nutrient limitation on marine diatoms (De Castro Araujo and Garcia, 2005; Gobler et al., 2006). Nutrient composition alterations are the preferred choice for the enhancement of lipid accumulation, because of easy applicability at both laboratory and large scale cultivation. However, the main challenge for this strategy is the trade-off between biomass and lipid yields. The application of any of the stress conditions to boost lipid accumulation decreases biomass productivity

(Guschina and Harwood, 2006). Nutrients like nitrogen have significant influence on both the biomass generation as well as the lipid accumulation in the cell. Increase in the nitrogen concentration in the medium directly enhances the biomass and inversely affects the lipid productivity (Adams et al., 2013).

To overcome the challenges in nutrient deprivation studies, two-stage cultivation was adopted to improve the lipid yield without affecting the biomass (Farooq et al., 2013, Xia et al., 2013). In two-stage cultivation, microalgae are initially grown under nutrient-sufficient conditions to obtain maximum cell density and thereafter in the second stage, the cultivation conditions are altered (mostly limited) to trigger the accumulation of lipid droplets inside the cell (Doan and Obbard, 2014) (Fig. 5.1). In a two-stage marine microalgae cultivation, transferring from nitrogen replete medium to nitrogen free medium caused lipid content to increase to 20-26% due to metabolic stress (Jiang et al., 2012). Another aspect of the two-stage cultivation is switching from photoautotrophic algal growth to heterotrophic growth for enhanced production of desired product (Miao and Wu, 2006; Zheng et al., 2012). Oyler (2008) described a process for production of oil from algae via a reverse sequential phototrophic and heterotrophic growth to overcome the disadvantages of single phototrophic or heterotrophic culture. Despite of several economic benefits, the main limitation of the two stage cultivation is that most of these results are strain specific and hence its efficiency may vary. None of the studies have proved universal application of two-stage cultivation for all microalgal strains (Singh et al., 2016).

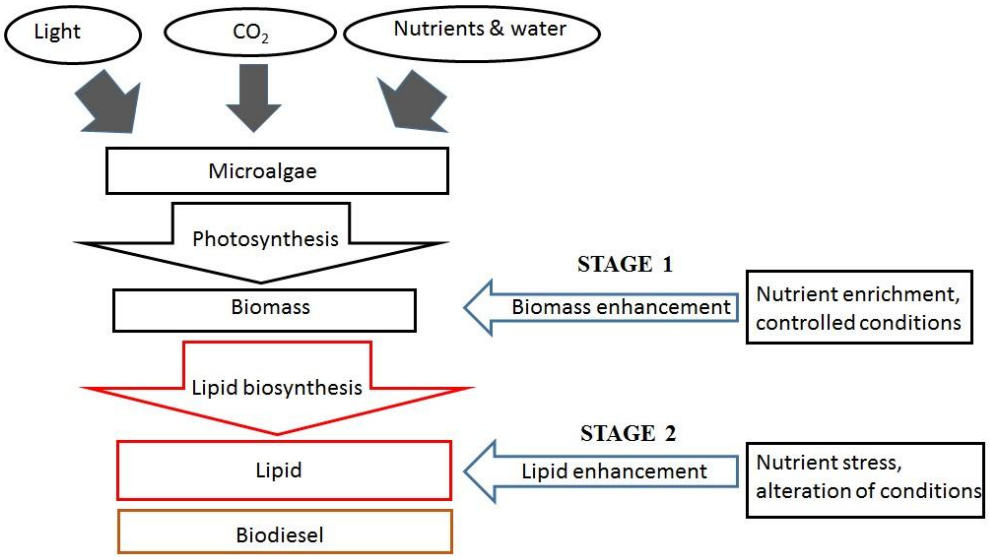


Fig. 5.1 Two stage cultivation strategy for enhanced biomass and lipid production

The aim of the present study was to identify the main stress factors those will enhance the lipid production in the diatom *Navicula phyllepta* without compromising growth in a two stage cultivation system and also to understand the subsequent biochemical changes during the stress conditions.

5.2 Materials and methods

5.2.1 Two stage cultivation approach- Design of experiments

In order to determine the effect of two stage cultivation approach towards enhanced lipid production without comprising biomass, three sets of experiments were conducted.

5.2.1.1 Set I

A batch culture of 150 mL of modified sea water medium with composition and growth conditions optimised for high biomass production

was used in the first stage of culturing. The medium contained 4.89 mM sodium metasilicate, 0.90 mM urea, 0.1 mM sodium dihydrogen phosphate, 0.05 mM ferric chloride and 0.2 mM disodium EDTA. The diatoms cells at the exponential phase of growth with a cell density of 1.5×10^6 cells mL^{-1} was inoculated (10% of total volume) into the medium and cultured at 30.8 °C temperature, 30 g kg^{-1} salinity and agitation at 125 rpm. After culturing for 12 days, the biomass was harvested by centrifugation at 1400 × g, washed with sterilised sea water and transferred to another batch culture of 150 mL of modified sea water medium with composition and conditions optimised for high lipid production (second stage). The medium consisted of 4.69 mM sodium metasilicate, 0.76 mM urea, 0.13 mM sodium dihydrogen phosphate, 0.05 mM ferric chloride and 0.2 mM disodium EDTA and incubated at 25 °C temperature, with 30 g kg^{-1} salinity and agitation at 125 rpm. The control cultures were maintained in the first stage itself at 30 °C throughout the experiment period. Total dry weight and lipid content were estimated by gravimetric method on every 3^{rd}, 6^{th}, 9^{th}, 12^{th}, 15^{th} and 18^{th} day of cultivation. All the experiments were carried out in triplicates.

5.2.1.2 Set II

Ten percent culture inoculum with a cell density of 1.5×10^6 cells mL^{-1} was cultured in a batch culture system of 150 mL of modified sea water medium with composition and conditions optimised for high biomass production as the first stage of culturing. The medium was prepared by using 4.89 mM sodium metasilicate, 0.90 mM urea, 0.1 mM sodium dihydrogen phosphate, 0.05 mM ferric chloride and 0.2 mM disodium EDTA and the cultures were incubated at 30.8 °C, with 30 g kg^{-1} salinity and at 125 rpm agitation. After culturing for 12 days, the biomass from the

cultures were harvested by centrifugation at 1400 ×g, washed with sterilised sea water and transferred to another batch culture set (stage 2) of 150 mL of modified sea water medium with one nutrient deprived (i.e. silicon or nitrogen or phosphorus) at a time. The temperature was reduced to 25 °C and other factors were kept constant. Total dry weight and lipid content were estimated by gravimetric method on every 3^{rd}, 6^{th}, 9^{th}, 12^{th}, 15^{th} and 18^{th} day of cultivation. A control experiment was set by transferring biomass into a medium with composition for high biomass production (nutrient replete) at 25 °C. All the experiments were carried out in triplicates.

5.2.1.3 Set III

Ten percent culture inoculum with a cell density of 1.5×10^6 cells mL^{-1} was cultured in a batch culture system of 150 mL of modified sea water medium with composition and conditions optimised for high biomass production as the first stage of culturing. The medium was prepared by using 4.89 mM sodium metasilicate, 0.90 mM urea, 0.1 mM sodium dihydrogen phosphate, 0.05 mM ferric chloride and 0.2 mM disodium EDTA and incubated at 30.8°C temperature, 30 g kg^{-1} salinity and agitation at 125 rpm. After culturing for 12 days, the biomass from cultures were harvested by centrifugation at 1400 × g, washed with sterilised sea water and transferred to another batch culture system (second stage) of 150 mL of modified sea water media subjected to three sets of stress conditions as given below.

Stress 1 phosphorus (sodium phosphate) deprived medium at temperature 25 °C

Stress 2 phosphorus deprived, silicon limited (20% of the original concentration of sodium silicate i.e. 0.978 mM) medium at 25 °C

Stress 3 phosphorus deprived, nitrogen limited (20% of the original concentration of urea i.e. 0.18 mM) medium at 25 °C.

A control experiment was set using medium with all the nutrients available in their original concentration optimized for high biomass production (nutrient replete) at a temperature of 25 °C. All other factors were kept constant. Total dry weight and lipid content were estimated by gravimetric method on every 3rd, 6th, 9th, 12th, 15th and 18th day of cultivation. All the experiments were carried out in triplicates. The biomass from stress 3 experiments were analysed in detail, as this design with multiple stress conditions was expected to induce maximum lipid production.

5.2.2 Nile red staining

An aliquot of culture from each batch of Set III experiments was centrifuged at 3105 ×*g* for 5 min and the pellet was re-suspended in the same volume of phosphate buffered saline (pH 7.4). The cells were washed with phosphate buffer two times and Nile red (0.1 mg mL^{-1} in acetone) was added in the ratio of 1:100 v/v. The reaction mix was incubated for minimum 5 min in dark and observed under an inverted phase contrast fluorescent microscope (Leica DMIL connected with DFC 420C camera) and images were processed using Leica application suite (LAS) software (Greenspan et al., 1985).

5.2.3 Fatty acid profiling

Twenty milligrams of lyophilised microalgal biomass from each test flask of set III after a period of 12 days was taken in 20 mL vial. The samples were incubated at 90 °C for 120 min with methanol-HCl-

chloroform (10:1:1 v/v/v). One mL of milli-Q water was added and the fatty acid methyl esters were extracted by adding 2.0 mL hexane- chloroform (4:1 v/v), vortexing and recovering the top layer. The process was repeated twice (Lewis et al., 2000). The collected fatty acid methyl esters were analysed in GCMS system (Perkin Elmer Clarus 680GC) equipped with a mass detector (Clarus 600T mass spectrometer) and a fused silica capillary column (Elite -5MS column, ID-0.25 mm, length -30 m; film thickness-0.25 µm, temperature limits: 60 to 325/350 ° C). The injection volume was limited to 1 µL. The carrier gas used was helium at a flow rate of 0.6 mL min^{-1}. The injector temperature was set at 240 °C with a split ratio of 20:1. The initial temperature of the oven was set at 60 °C with a hold for 1 min and then ramped to 2 °C per min to 200 °C with a 4 min hold, then 5 °C per min to 280 °C and finally hold for 10 min. Turbo mass software was used for instrument control, data acquisition and data analysis chromatography. The detection of different classes of fatty acid methyl esters was made by comparing the retention time of the standards (C4 –C24) (Sigma Aldrich, India).

5.2.4 Whole cell analysis by FTIR

Approximately 1mg of freeze dried microalgal biomass from second stage (12^{th} day) of set III was used to estimate the biochemical composition of the test samples using FTIR spectrometer (Thermo Nicolet, Avatar 370). Thirty two scans of absorbance spectra were collected with a spectral resolution of 4 cm^{-1} between 4000 and 400 cm^{-1} for each sample. Scans were co-added and averaged. Band assignments to molecular groups of algae were based on those previously published (Mudrock and Wetzel, 2009). The peak areas of bands were determined for each sample and relative absorption

area ratio of carbohydrate to amide I, lipid to amide I and amide I to amide A was calculated.

5.2.5 Oxidative stress indices

5.2.5.1 Lipid peroxidation (Heath and Packer, 1968)

The level of lipid peroxidation (LPX) was measured in terms of malondialdehyde (MDA), a product of LPX estimated by thiobarbituric acid (TBA) reaction. Fresh wet algal sample (0.1 g) was homogenized in 1 mL of 10% (w/v) trichloroacetic acid (TCA), and the homogenate was centrifuged at 7000 ×g for 10 min. One mL of the supernatant was mixed with 2 mL of 0.5% TBA solution (in 10% TCA). Then the mixture was heated at 95 °C for 45 min and then cooled under room temperature. The supernatant was read at 532 nm after the removal of any interfering substances by centrifuging at 4000 ×g for 10 min. The change in absorbance was recorded every 30 s upto 3 min in thermostated UV-Vis spectrophotometer (UV-1601, Shimadzu, Japan), with 10% TCA solution as blank. The amount of thiobarbituric acid reactive substances (TBARS) formed was calculated by using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

$$\text{Concentration of MDA (nmol g}^{-1}) = \frac{A_{532}}{1.56 \times 10^5} \times \frac{\text{reaction volume (mL)}}{\text{fresh weight (g)}} \quad .....(5.1)$$

5.2.5.2 Superoxide dismutase (SOD) (Marklund and Marklund, 1974)

Fresh microalgal homogenate (0.1 g) was prepared in 1mL of 50 mM Tris -EDTA (pH 8.5). Blank was adjusted to zero with Tris- EDTA. A volume of 33 µL of each sample was mixed with 933 µL of buffer solution and placed in spectrometer. A volume of 33 µL of 0.2 mM pyrogallol prepared in 0.01 N

HCl was added, mixed and absorbance measured at 420 nm for 3 min. The control tube was prepared by replacing sample with distilled water. The change in absorbance was recorded at every 30 s upto 3 min.

$$\text{Rate} = \frac{\text{OD final-OD initial}}{3} \quad \text{.....(5.2)}$$

$$\% \text{ of inhibition} = \frac{\Delta\,\text{OD}_{\text{control}} - \Delta\text{OD}_{\text{test}}}{\Delta\text{OD}_{\text{control}}} \times 100 \quad \text{.....(5.3)}$$

$$\text{SOD activity (U g}^{-1}) = \frac{\%\text{ of inhibition}}{50} \times \frac{V_T\ (\text{mL})}{V_S\ (\text{mL})} \times \frac{\text{n}}{\text{fresh weight (g)}} \quad \text{.....(5.4)}$$

where V_T= total reaction volume; V_S= volume of enzyme used;
n = dilution factor

5.2.5.3 Catalase (Aebi, 1974)

Wet microalgal biomass (0.1 g) was homogenized in 1 mL phosphate buffer (0.5 M, pH 7.5), centrifuged at 12400 × g at 4 °C for 30 min and the supernatant was taken for catalase (CAT) activity. A reaction mixture containing 1.6 ml phosphate buffer (pH 7.3), 100 µL EDTA (3 mM), 200 µL H_2O_2 (0.3%) and 100 µL supernatant was taken in a cuvette. Catalase activity in the supernatant was determined by monitoring the disappearance of H_2O_2, by measuring a decrease in absorbance at 240 nm against a blank of same reaction mixture without 0.3% H_2O_2 upto 3 min.

$$\text{Catalase activity (U g}^{-1}) = \frac{2.303}{3} \times \frac{\log \text{OD}_{\text{zero}}}{\log \text{OD}_{\text{3min}}} \times \frac{1}{\text{fresh weight (g)}} \quad \text{.....(5.7)}$$

5.2.5.4 Peroxidase (POD) (Reddy et al., 1995)

Fresh wet biomass (0.1 g) was homogenized in 1mL 0.1M phosphate buffer (pH 6.5) and 0.1 mL of the enzyme extract was added to 3 mL pyrogallol solution and mixed well. The absorbance was adjusted to zero at 430 nm. To the test cuvette, 0.5 mL of 1% H_2O_2 (in 0.1M phosphate buffer at pH 6.5) was added and mixed. The increase in absorbance was recorded at every 30 s upto 3 min in a spectrophotometer. One unit of peroxidase is defined as the change in absorbance/min at 430 nm.

$$\text{Rate} = \frac{\text{OD final-OD initial}}{3} \quad \text{.....(5.5)}$$

$$\text{Peroxidase activity (U g}^{-1}\text{)} = \frac{\Delta OD_{test}-\Delta OD_{blank}}{12} \times \frac{V_T\ (mL)}{V_S\ (mL)} \times \frac{n}{\text{fresh weight (g)}} \quad \text{.....(5.6)}$$

where, V_T = Total reaction volume; V_S = volume of enzyme used; n = dilution factor; 12 = extinction coefficient of 1 mg mL^{-1} of purpurogallin at 420 nm

5.2.6 Statistical analyses

Statistical analyses were carried using one way and two-way analysis of variance (ANOVA). The differences in the values were considered significant at $p< 0.05$. Fisher's Least Significant Difference (LSD) test was calculated after ANOVA for post hoc comparisons.

5.3 Results and discussion

5.3.1 Two stage cultivation approach

The set 1 experiment (Fig. 5.2) showed the advantage of two stage cultivation method over single stage method using the media and culture conditions optimised using response surface methodology in chapter 4. In the two stage cultivation (test) experiment, there was proportional increase in biomass as well as lipid concentrations in the optimised medium for higher lipid production till 18th day from the start of second stage. However in the single stage cultivation (control) maintained in the optimised media for high biomass production, there was a decline in the total biomass and subsequent lipid concentration after 18th day from the start of first stage. In the test samples, the total biomass reached 1.9 g L^{-1}, lipid concentration reached 0.4 g L^{-1} and the lipid percentage was 24% by the second stage of culturing. Whereas in the control, it was 20 % of total lipid with a concentration of 0.27 g L^{-1} and a maximum biomass of 1.24 g L^{-1} by the end of first stage of culturing. Two way ANOVA showed that the two parameters i.e. biomass and total lipid concentration had significant difference ($p< 0.05$) between the test and control. However, significant change in lipid percentage was not observed in this case. Finally, from the experiment, it can be concluded that though the two stage cultivation approach had an effect on the increased lipid production along with high biomass production compared to single stage cultivation approach, the desired lipid production can be achieved by subjecting the organism to single or multiple stress conditions.

The set II experiment (Fig. 5.3) was carried out to understand the effect of nutrient deprivation on the biomass and lipid production in the second stage of two stage cultivation approach, as nutrients can be readily manipulated compared to other influencing conditions. The results showed that phosphorus deprivation resulted in reduction in the growth and lipid production, but not a significant effect compared with the control culture with no modification in the original concentrations. The silicon and urea removal showed a significant ($p< 0.05$) difference in the biomass and lipid concentration compared to control cultures. The biomass concentration was the lowest in silicon deprived media giving 0.84 g L^{-1} by the end of the experiment, which proves the role of silicate in biomass production. The urea deprived cultures initially showed a steady growth reaching 1.54 g L^{-1}, but crashed after 9^{th} day. The silicon deprivation resulted in the highest lipid percentage of 28.78 % of weight at the end of 18^{th} day. The phosphorus deprived medium produced only 25% of lipid during 18^{th} day, which was attained by urea deprived cultures by the end of 9^{th} day of second stage culturing. ANOVA analyses showed that silicon and phosphorus deprived and nutrient replete cultures had significant difference in lipid percentages with urea deprived cultures. In all the tests, there was steady increase in parameters between the stress days, but not significant ($p< 0.05$). It can also inferred from the analysis that more than one stress factor contribute to the increased production of lipid as well as biomass in a two stage cultivation strategy.

In set III experiment (Fig. 5.4), the multiple nutrient stress factors were studied for increasing the production of biomass along with lipid quantity. In this study, the test cultures under stress 2 and stress 3 were

subjected to more than two nutrient stress conditions at a time along with temperature stress for a period of 12 days in second stage (Stage 2) . In the light of crashing of cells in urea deprived medium in set II, in the present design of experiments, the concentration of the selected nutrient was reduced or limited in the culture medium, rather than completely removing the nutrient that is important for the survival of the diatom. The experimental results showed that multiple nutrient stress conditions had profound influence on the increased rate of lipid production. The cultures subjected to stress 3 experiment i.e. phosphate deprivation, urea limitation and temperature reduction in the second stage could give a higher concentration of lipid 0.39 gL^{-1} proportional to its biomass of 1.44 g L^{-1}. There was no significant difference in biomass in stress experiments 1 and 2, which clearly showed that urea even in low concentration in stress 3 experiment, had a significant role in the growth of the diatom. Stress 2 experiment with phosphate deprivation, silicate limitation and temperature reduction gave a significant increase in total lipid percentage of 32.13 % at the end of stage 2 of culturing at the cost of reduced biomass (1.1 g L^{-1}). The lipid percentages reached 27.58% and 23.54% for stress 3 and stress 1 (phosphate deprivation, temperature reduction) respectively. There was significant difference in the responses between the initial and final culturing days. Post hoc analysis showed that there was significant difference in the lipid percentage in stress 2 and stress 3 experiments compared to stress 1 and control cultures.

Two-stage cultivation is considered as one of the most promising strategy in microalgal culturing for enhanced lipid production. Several studies reported two-stage cultivation strategies to explore the potential of microalgae

as a feedstock to produce biofuels and other high-value products. Ra et al. (2015) employed a two-stage strategy to culture four species of microalgae to examine biomass production in the first stage, and oil production with salt stress in the second stage. Ratha et al. (2013) successfully employed a two-stage cultivation approach to improve the lipid productivity in *Chlorella* and *Scenedesmus* sp. Alvarez-Diaz et al. (2014) obtained an increase of 36.5-45.5% in lipid accumulation using two-stage cultivation of *Ankistrodesmus falcatus*. The present results also supported the earlier studies that the two-stage cultivation is indeed a promising approach.

Generally, nitrogen and phosphorous are considered to be the two major macronutrients controlling the cellular metabolism in microalgae (Juneja et al., 2013). It is proved that oleaginous microalgae, especially diatoms, produce small amounts of neutral lipids, mainly TAG, under favourable growth conditions, and they start to accumulate lipid droplets upon stresses, especially nutrient starvation (Eizadora et al., 2009; Norici et al., 2011; Sharma et al., 2012; Yin-Hu et al., 2012; Valenzuela et al., 2012). Nitrogen deprivation in the two phase cultivation was the most preferred methodology to obtain high biomass with high lipid content according to some earlier reports (Widjaja et al., 2009; Yeesang and Cheirsilp, 2010; Chen et al., 2010; Praveenkumar et al., 2011). Microalgal biomass contains approximately 1% phosphorus of total dry biomass, but the consumption is more due to the luxurious uptake of phosphorus by microalgal cells. During phosphorus starvation, cells uptake phosphorus from the culture medium and store it in the form of polyphosphate granules (poly-P) for further utilisation (Yin-Hu et al., 2012). Khozin-Goldberg and Cohen (2006) reported that phosphate starvation resulted in reduced cell dry weight of fresh water

eustigmatophyte *Monodus subterraneus* compared to nutrient sufficient medium, but cell doubling was still observed, which indicated that intracellular phosphate was sufficient for at least one cell division. Previous studies have frequently deployed nitrogen or phosphorus starvation as the major stress factor for increasing lipid yield, but there is paucity in the studies concerning synergistic utilization to achieve high lipid productivity (Belotti et al., 2013; Singh et al., 2015; Kamalanathan et al., 2016). During nutrient starvation, microalgae release nitrogen from the photosynthetic pigments and utilize the same for the metabolic processes, while deficiency of phosphorus is fulfilled by utilization of the poly-P granules accumulated (Qu et al., 2008; Praveenkumar et al., 2011). Arora et al. (2016) studied on synergistically optimized nitrogen and phosphorous concentrations for attainment of maximum lipid productivity in *Chlorella minutissima* and showed that nitrogen stress was mainly responsible for increasing the lipid accumulation, which was higher in comparison to phosphate deficiency/limitation. Previous studies also reported that nitrogen and phosphorus co-starvation made it possible for an increase in total lipid productivity with a visible shift in lipid composition from polar to non-polar lipids (Solovchenko, 2012; Fakhry and Maghraby, 2015). Kamalanathan et al. (2016) reported that combination of nitrogen plus phosphorus starvation had a more profound effect on the physiology and macromolecular pools of *Chlamydomonas reinhardtii* than phosphorus starvation alone resulting in higher oil production. Dhup et al. (2017) stated that phosphorus limitation may be the cause for a decrease in nitrogen uptake, suggesting that phosphorus induces nitrogen removal resulting in increased lipid content.

According to various findings, cell division and silicon metabolism are closely related (Martin-Jezequel et al., 2000). In some species, including *Chaetoceros gracilis* (Lombardi and Wangersky, 1995), the production of storage lipid (triacylglycerol or TAG) was stimulated when Si availability was limiting for cell division (Araujo et al., 2011; Hildebrand et al., 2012). Very little information is known about the physiological mechanism by which silicon deficiency signals towards accumulation of lipids (Merchant et al., 2012). Further studies identified that silicon depleted cells directed newly assimilated carbon more towards lipid production and less towards carbohydrate production or else silicon depleted cells slowly converted non-lipid cell components to lipids (Gupta et al., 2011). Several reports suggest that silicon limitation stimulates lipid formation more rapidly resulting in higher lipid content (Shifrin and Chisholm, 1981; Enright et al., 1986; Taguchi et al., 1987; Mortensen et al., 1988; Parrish and Wangersky, 1990; Lombardi and Wangersky, 1991; McGinnis et al., 1997; Adams and Bugbee, 2014). There are other reports also on diatoms showing that lipid content increased under nitrogen and silica starvation (Coombs et al., 1967; Parrish and Wangersky, 1990; Lombardi and Wangersky, 1995; Thajuddin et al., 2015).

The present study demonstrated the potential of combining nutrients and abiotic stress factors to improve the lipid productivity in microalgae. To obtain maximum yields, it is imperative to have the knowledge of the synergistic effects of these factors as well as significance of each factor with regard to the lipid accumulation (Cao et al., 2014, Sun et al., 2014). The effects of multi-factor collaborative stresses on *Nannochloropsis oculata* was investigated by Wei and Hang (2017) and found that there was increase

in biomass along with improved oil yielding performance. Breuer et al. (2013) investigated temperature on TAG deposition under nitrogen deficient conditions and found temperature to be the major influencing factors for TAG accumulation under nutrient starvation. Variations of temperature (a decrease from 30 °C to 25 °C) and decrease in the concentration of nitrate in the medium resulted in a significant change in cell composition during batch cultures, favouring the accumulation of lipid bodies in microalgae *Nannochloropsis oculata* and *Chlorella vulgaris* (Converti et al., 2009). Higher lipid production was demonstrated by lowering temperature and manipulating nitrogen and phosphorus sources in green algae and cyanobacteria (Bohnenberger and Crossetti, 2014). The major advantage of combined strategy is that in this approach, one factor may compensate the negative effect of the other. Moreover, this approach can easily be employed for large scale production of microalgal biodiesel (Singh et al., 2016).

In this present study, the two-stage cultivation method proved to be effective in providing improved quantity of lipid without compromising biomass of the biofuel feedstock. Though silicon deficiency along with phosphorus deprivation and temperature variation resulted in the highest lipid percentage in the diatom cells, nitrogen (urea) limitation along with phosphorus deprivation along with temperature variation was found to be the most favourable post-harvest treatment in order to attain high biomass and high lipid content per cell with a minor loss in biomass.

5.3.2 Nile red staining

The lipid bodies were located by Nile red staining through fluorescence microscopy. The bright yellow fluorescence from intracellular

lipid droplets and red fluorescence from chlorophyll were observed (Fig. 5.5). After 9 days of stress period, the numbers of yellow spots or lipid bodies within the cells increased especially in stress 2 and stress 3 test samples compared to the control, but their sizes were similar. After 12 days in stage 2, the size of the oil bodies increased reaching upto 3-4 µm in size by 18^{th} day in silicate (stress 2) and urea limited (stress 3) samples, which visually proved the fact that lipid accumulation increased with the application of multiple stresses on diatoms cells compared to nutrient replete condition. Yang et al. (2013) demonstrated the increase in oil bodies in Nile red stained *P. tricornutum* under nitrogen starvation, whereas as same results were reported by Msanne et al. (2012) in *Chlamydomonas* sp. and *Coccomyxa* sp. Similarly Dhup et al. (2017) also reported that lower phosphorus concentrations induced substantially higher lipid accumulation in *Monoraphidium* sp resulting in progression of number and volume of oil bodies inside the cells with time. White et al. (2011) showed similar accumulation of neutral lipid bodies after Nile red staining in multiple stress conditions of *Chlorella* sp.

5.3.3 Fatty acid composition analyses

The important fatty acids determined in this study were C14:0, C16:0, C16:1, C18:0, C18:1 and C20:5(n-3), which are the characteristics of marine diatoms (Volkman et al., 1989; Brown, 2002; Sabu et al., 2017).The fatty acid composition of the diatom *Navicula phyllepta* cultured in stage 2 varied substantially among the different nutrient (single or multiple) stress conditions compared to the control, which was subjected to temperature stress alone (Fig. 5.6 a). The most predominant fatty acids were C16:0, C16:1 and C18:0, which are the essential fatty acids determining the

biodiesel quality. The relative percentage of total fatty acids of C16:0 and C16:1 was found to be highest in stress 3 (51.77%, 27.08%) followed by stress 2 (46.78%, 18.39%) compared to control (43.5%, 18.13%), whereas the stress 1 samples with phosphate deprivation and temperature reduction gave slightly lesser concentrations (32.04%, 11.07%) compared to the control. The percentage of stearic acid C18:0, which contributed to total saturation of microalgal oil was greatly enhanced in the multiple and double stress samples compared to the control. It was quantified as 21.27% in stress 2 and 21.13% in stress 3, whereas 15.97% in stress 1 and only 2.93% in the control. Enhancement of C18:0 fatty acids were reported by Saha et al. (2013) in his studies on various stress regulatory factors on lipid production in *Haematococcus pluvialis*. It has been proved that complete removal of phosphate caused an increase in proportion of C18:0 (Khozin-Goldberg and Cohen, 2006). The polyunsaturated fatty acid eicosapentanoic acid (EPA) was found in all samples except in stress 3. A comparatively high concentration of EPA C20:5(n-3) was reported in stress 1 samples compared to the rest. The total percentage of saturated (72.9%) and monounsaturated fatty acids (27.08%) was found to be the highest in stress 3 (Fig. 5.6 b). Similar findings were reported by Thajuddin et al. (2015), in which nitrogen deficiency profoundly affected the fatty acid profile of both the diatoms *Navicula* sp. and *Amphora* sp. causing an increase in SFA and MUFA with reduced PUFA content. In the same study, the percentage of EPA in *Navicula* sp. was found to be the highest in silicon deficient stress condition compared to the nitrogen deficient. Adams and Bugbee (2014) reported a shift in fatty acid chain length from C18 to C16 on reducing the silicon concentration.

One of the most commonly observed changes associated with the alteration in the level of unsaturation of fatty acids in the lipid membrane is the temperature shift (Thompson, 1996; Harwood, 2004; Guschina and Harwood, 2009). Studies showed that temperature stress can lead to an increase in unsaturation level even under nutrient deplete or replete conditions for retaining the membrane fluidity (Hodgson et al., 1991; Hu and Gao, 2006; Roleda et al., 2013). Rousch et al. (2003) also demonstrated the change in fatty acid composition and degree of saturation in marine diatoms upon temperature stress. Generally, the cultivation under suboptimal/stressful conditions resulted in decreased fatty acid chain length and lower levels of unsaturation, which was associated with reduced growth and increased oil levels (Hodgson et al., 1991; Roessler et al., 1994; Tonon et al., 2002). The presence of high proportion of saturated and monounsaturated fatty acids in the diatom *N. phyllepta* under multiple stresses are considered to be optimal from a fuel quality perspective.

5.3.4 Whole cell response to the stresses

FTIR spectroscopy is capable to visualize the macromolecular composition of algal cells and microorganisms, and the relative intensities correlate with chemically measured content of cellular molecules like protein, carbohydrate, lipids and silicate (Naumann et al., 1991; Giordano et al., 2001). In the diatom *Navicula phyllepta* (Fig. 5.7), the infrared spectra of biomass preparations were dominated by the protein amide I (mainly C-O stretching) and amide II (mainly N–H bending) vibrational bands around 1658 and 1545 cm^{-1} respectively. The shape of these bands depends on the secondary structure of the proteins present in the sample. The band at 3300-3400 cm^{-1} was attributed to presence of the amide A/B (N–H stretching

vibrations of the peptide groups) (Fabian and Mantele, 2002). The algal carbohydrate content determined by FT-IR was due to the C-OH and C-O-C stretching vibration peaks at 1000–1200 cm^{-1}. The bands at 2850-2970 cm^{-1} were attributed to asymmetric and symmetric C-H vibrations, mainly due to methyl and methylene groups in fatty acids, which were mainly considered for quantification of the total lipid content (Pistorius et al., 2009). The prominent bands present~ 1740–1640 cm^{-1} were due to the presence of C=O of esters or fatty acids. The bands at 800-1100 cm^{-1} attributed to silica frustules of diatom cells, which sometimes overlap with carbohydrate portion (Stehfest et al., 2005).

Generally, nitrogen, silicon and phosphorus limitation stresses induced a decrease in photosynthetic activity, but the impact on biochemical pools was dependent on the limiting nutrient. Nitrate depletion mainly affected the lipid content of diatoms (Shifrin and Chisholm, 1981), whereas depletion of both nitrate and phosphate reduced the protein pool, while increasing carbohydrate synthesis (Guerrini et al., 2000). Lippemeier et al., (1999) clearly showed that silicic acid metabolism had strong influences on the photosynthetic parameters of diatom cells. In the present study, on comparing the relative change in the carbohydrate/ amide I ratio (Fig. 5.8 a), the stress 3 showed the highest value (7.46) followed by stress 2 (7.1), stress 1(6.7) and control (6.32). The lipid/ amide I also followed a similar consistent pattern with the highest value in the case of stress 3 (Fig. 5.8 b). The ratio of control could not be estimated due to limited band showing the presence of lipid. The amide I and amide A ratio showed similar values for control, stress 1 and stress 3 samples with higher value for stress 2 test samples (Fig. 5.8 c). Protein pools are highly affected by nutrient starvations such as

silicate, phosphate and nitrate (Soler et al., 2010). Nitrogen limitation demonstrated a decrease in protein content and increase in starch content, which could be indicated as the rise in carbon/amide I area ratio (Yao et al., 2012). Nitrogen deprivation led to arrested protein synthesis, allowing fixed carbon from photosynthesis more prone to be diverted to carbohydrate and neutral lipid/ total lipid (Meng et al., 2014). Shifrin and Chisholm (1981) also suggested that diatoms under nitrogen stress stored carbohydrates initially and shifted to lipid storage only after extended periods of stress. Stress studies on *P. tricornutum* by Stehfest et al. (2005) showed that the carbohydrate/amide ratio was higher in nitrogen deplete compared to phosphate deplete conditions. According to Dean et al. (2008) FTIR absorption spectra from low phosphate cultures had more prominent carbohydrate and lipid bands compared to high phosphate algal cells. Nutrient limitation has been shown to induce production and accumulation of extracellular polysaccharides in a number of diatom species (Guerrini et al., 2000, Magaletti et al., 2004; Abdullahi et al., 2006). Generally, phosphorus limitation is a stronger inducer of exopolysaccharide production than nitrogen limitation, although studies of the opposite result also exist (Ai et al., 2015). Redalje and Laws (1983) also showed that variation in temperature can modify biochemical composition especially protein and polysaccharide content of a nitrogen-limited marine diatom *Thalassiosira alleni.* But it can be also seen that there is no consistent trend in proximate biochemical composition as a function of temperature. De Castro Araujo and Garcia (2005) found that higher lipid and carbohydrate contents were obtained at lower temperatures (20 and 25 °C) compared to high temperature (30 °C) in diatom *Chaetoceros wighamii*, while protein content remained unaffected.

Hence, the changes observed with FTIR spectroscopy of the test and control cultures in the present study were found to be consistent with the effects described previously in the literature.

5.3.5 Oxidative stress indices

When plants or microalgae are subjected to biotic or abiotic stresses, the major biochemical changes occur resulting in the production of reactive oxygen species (ROS) (Allen, 1995; Marshall and Newman, 2002). The organelles, mitochondria and the chloroplast are the important intracellular generators of reactive oxygen species. In chloroplasts, ROS can be generated by direct transfer of excitation energy from chlorophyll to produce single oxygen, or by univalent oxygen reduction of photosystem I, in the Mehler reaction (Asada, 1999). Despite the fact that molecular oxygen itself is not harmful for cells, in the absence of any protective mechanism, ROS can seriously disrupt normal metabolism through oxidative damage to lipids, protein and nucleic acids (Alscher et al., 1997; Mallick and Mohn, 2000; Meloni et al., 2003). Anti-oxidative enzymes are the most important components in the scavenging system of ROS. Superoxide dismutase (SOD) is a major scavenger of superoxide (O^{2-}), and its enzymatic action results in the formation of H_2O_2 and O_2. The hydrogen peroxide produced is then scavenged by catalase and a variety of peroxidases (POD). Catalase, which is apparently absent in the chloroplast dismutates H_2O_2 into water and molecular oxygen, whereas POD decomposes H_2O_2 by oxidation of co-substrates such as phenolic compounds and/or antioxidants. Stress induces a variety of cellular changes, such as damage to membrane integrity reduction in photosynthesis and

impairment of CO_2, which may produce ROS and the resulting lipid peroxidation (Pise et al., 2013).

In order to demonstrate the effect of various stress conditions on the intracellular oxidative stress status, four oxidative stress indicator antioxidant enzymes were analysed during the set III experimental set up in stage 2 culturing. Catalase activity was the highest in stress 2 followed by stress 3 indicating increased rate of hydrogen peroxide production (Fig. 5.9a). As the silicon limited cultures gave very less biomass, the catalase activity was the highest compared to others, demonstrating the extreme stress on the diatom upon sudden transfer from high silicate to low silicate concentration in two phase cultivation. There was significant difference in catalase activity between the silicon limited and urea limited cultures. Lipid peroxidation, measured in terms of malondialdehyde (MDA) content in the cells, was higher in urea limited cultures (stress 3) compared to others (Fig. 5.9b). There was significant difference $p< 0.05$ in activity between stress 3 and stress 2 stress days till 9^{th} day of culturing (starting of stationary phase) compared with the control and also between the culturing period till 9^{th} day. It signifies the high lipid degradation inside the cells due to the limitation of extracellular nitrogen uptake. This result was in support to the findings of Yilancioglu et al. (2014) and Al-Rashed et al. (2016). The values of SOD were in correspondence to catalase activity in all the stress conditions (Fig. 5.10a). This data suggests that superoxide may be elevated under silicate deficient and nitrogen deficient conditions, necessitating increased SOD activity. In this case, the activity showed significant difference between stress conditions. The values of POD (Fig. 5.10 b) showed the highest value in stress 2 cultures till 9^{th} day, after which it increased in stress 3 set of cultures,

showing the decomposition of hydrogen peroxide generated. An increased activity of antioxidants such as SOD, peroxidases and catalase were reported in microalgae under stress conditions, especially under nitrogen deprivation (Gigova and Ivanova, 2015; Al Rashed et al., 2016). Lauritano et al. (2015) showed that antioxidant enzymes are good indicators of highly stressed diatom *Skeletonema marinoi* in silicon starved/limited conditions. Choo et al. (2004) reported enhanced activity of SOD, peroxidase and catalase in filamentous green algae by altering temperature. The concentration of MDA and catalase activity increased significantly to reduce the stress of phosphorus limitation and compensate for its deficiency in diazotrophic cyanobacterium *Cylindrospermopsis raciborskii* (Wu et al., 2012b). A number of studies revealed that the induction of superoxide-detoxifying enzymes (SOD) in response to stress in cyanobacterial species (Campbell and Laudenbach, 1995; Li et al., 2002). Ruiz-Domínguez et al. (2015) stated that nutrient-limited cultures of *Coccomyxa* sp. showed a significant increase in the intracellular activity levels of the enzymes such as peroxidases and catalase indicating a connection between nutrient deprivation and oxidative stress.

The role of reactive oxygen species in lipid accumulation is not well explored. Recent research studies show that oxidative stress is a mediator for lipid accumulation in various microalgae like *Dunaliella salina* (Yilancioglu et al., 2014) and *Chlorella pyrenoidosa* (Fan et al., 2014). Association of increased ROS levels and lipid accumulation under different environmental stress conditions was shown in some previous studies in green microalgae (Li et al., 2011). A study on diatoms also showed that nitrogen depletion results in the co-occurrence of ROS species and lipid accumulation (Liu et al., 2012b). A

mechanistic understanding of the interrelationship between ROS rise and increased lipid accumulation in microalgae species requires further investigation (Hong et al., 2008). Microalgae can modify its photosynthetic machinery under stress, resulting in a decrease in the gene expression of various proteins forming up the photosystem complexes (Zhang et al., 2004). Such metabolic modifications are thought to occur for reducing oxidative stress via decreasing rate of photosynthesis (Nishiyama et al., 2001). It is found that nitrogen depleted conditions trigger reactive oxygen species accumulation, increased cellular lipid content and protein production impairment. Nitrogen deprivation is closely associated with the degradation of ribulose-1, 5-bisphosphate carboxylase oxygenase to recycle nitrogen (Garcia-Ferris and Moreno, 1993). Degradation of this protein may result in alterations in photosynthesis rate as a result of which, the overall anabolic reaction flux was severely constrained (Cakmak et al., 2012). In this context, microalgal cells may favour storage of energetic molecules, such as lipids, instead of consumption. It has been reported that oxidative stress tolerant microalgae are efficient for biofuel production than non-oxidative tolerant microalgae (Osundeko et al., 2013). Hence the higher activities of anti-oxidant enzymes in *Navicula phyllepta* show its high tolerance to stressful conditions. The present results also provide evidence supporting the scientific facts that oxidative stress on the diatom due to nutrient depletion/starvation can mediate lipid accumulation inside the cells.

5.4 Conclusion

In this study, two-stage cultivation strategy was found to be an effective method compared to single stage in stimulating increased

production of lipid in the diatom *Navicula phyllepta* without compromising the biomass. Multiple stress conditions involving alteration of nutrients along with abiotic stress such as temperature during the two stage cultivation was found to be more productive compared to single or double stress treatments. The study leads to the conclusion that the two-phase culturing system, with multifactor stress application, especially nitrogen limitation, along with phosphate starvation and temperature stress as post-harvest treatment, would be the suitable method for gaining maximum biomass productivity and lipid content. The highest lipid percentage of 32 % of cell dry weight was obtained upon silicon limitation, phosphate starvation and temperature stress condition at 25° C. The application of multiple stresses resulting in high amount of saturated and monounsaturated fatty acids with less/no polyunsaturated content, especially in nitrogen limited condition, favoured its suitability towards biodiesel production. The changes in biochemical composition and oxidative stress parameters within the various stress conditions demonstrated the profound influence of the selected stress factors on the biodiesel productivity of the diatom under study. Since the energy consumption in process systems is a significant parameter that affects the total production costs, the criteria of choosing low energy consuming techniques such as nutrient starvation would be the most economical in two stage cultivation approach. In addition, the present study included experiments in small volumes, and therefore, based on the results of the study, large scale culturing and biomass production need to be carried out as the next step.

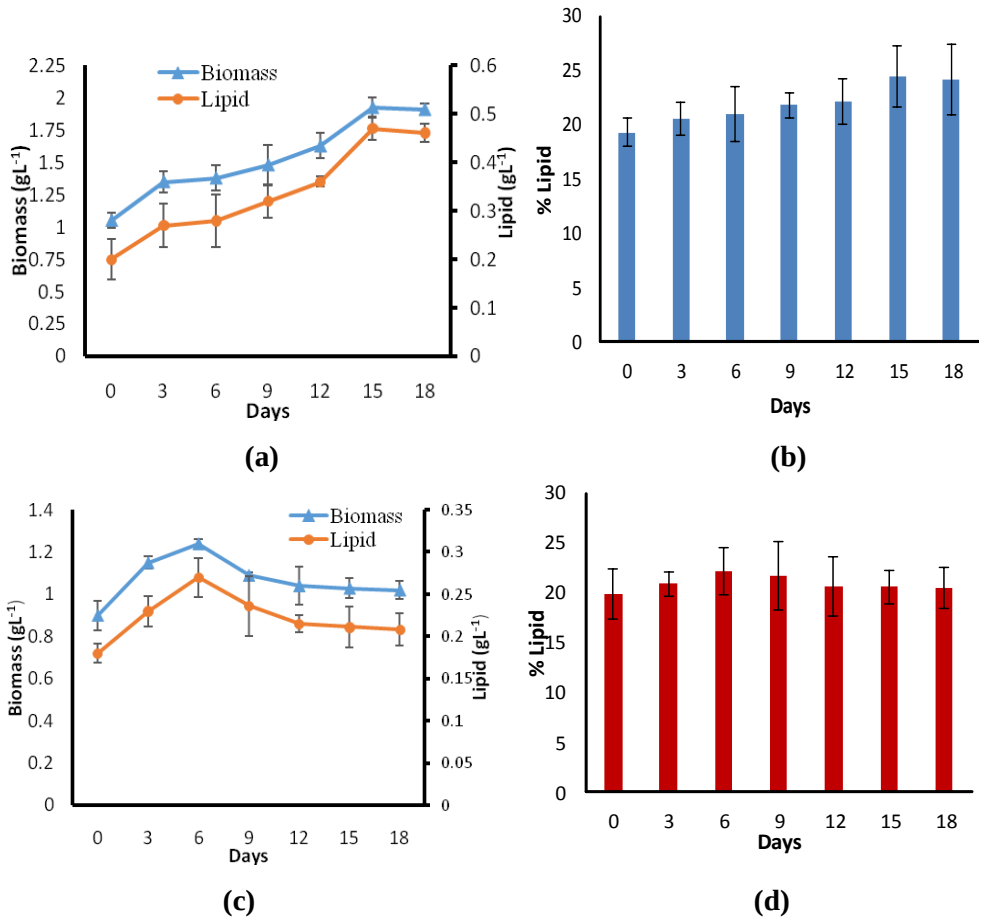


Fig. 5.2 Biomass, lipid concentration, lipid percentage of *Navicula phyllepta* in set I design of experiment, in which the cells were grown in medium and growth conditions optimized for biomass production for 12 days (stage 1), and subsequently in those for lipid production (stage 2) (a, b) for 18 days from the start of stage 2. Control set was maintained at stage 1 itself (c, d) till 18 days.

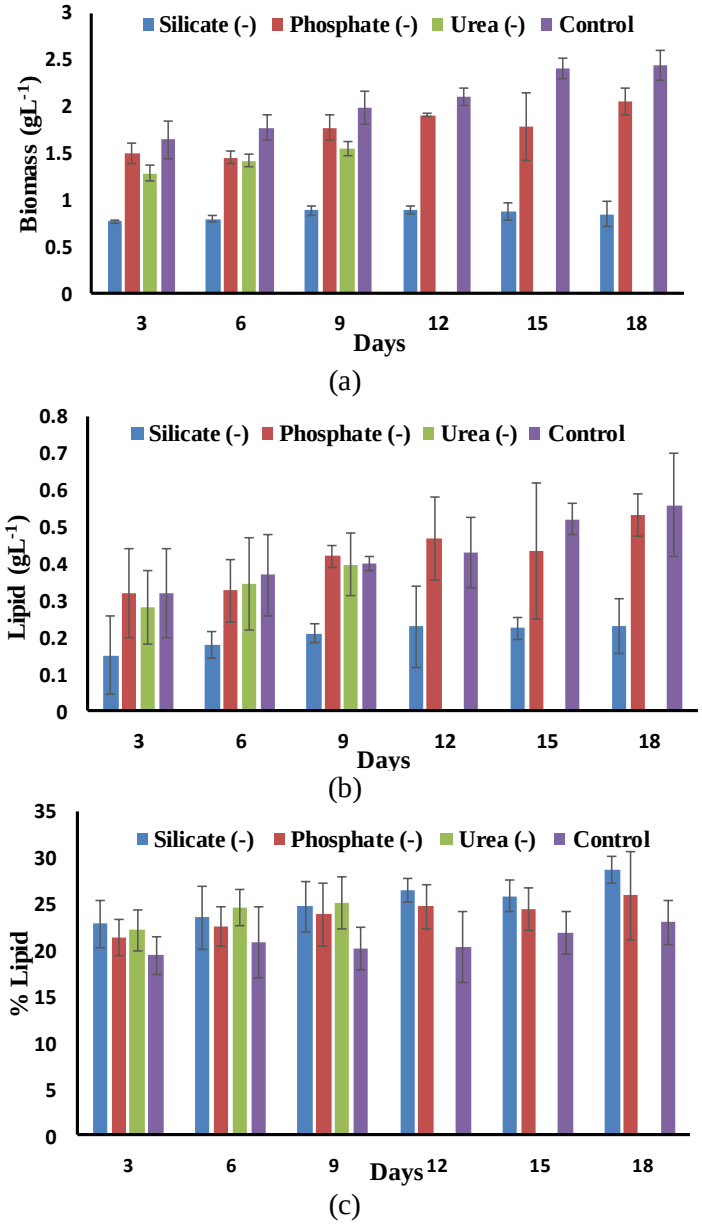
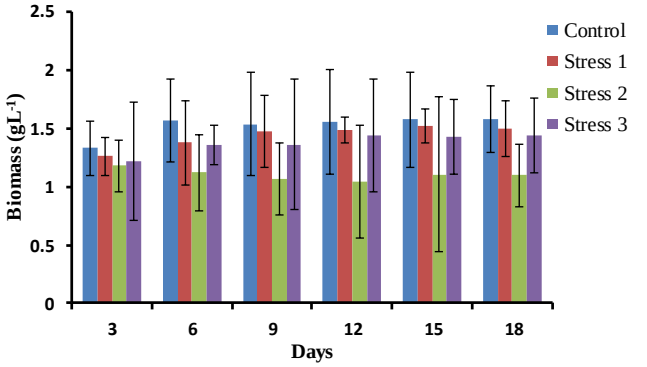
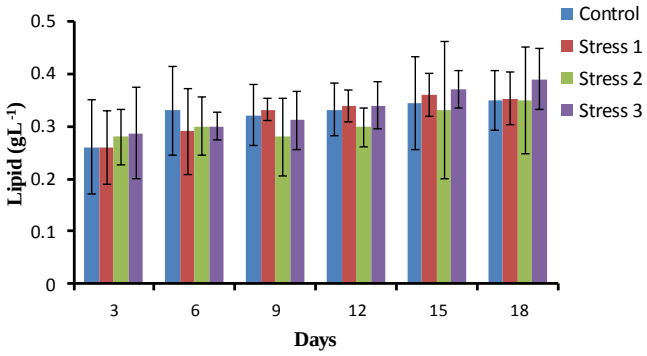


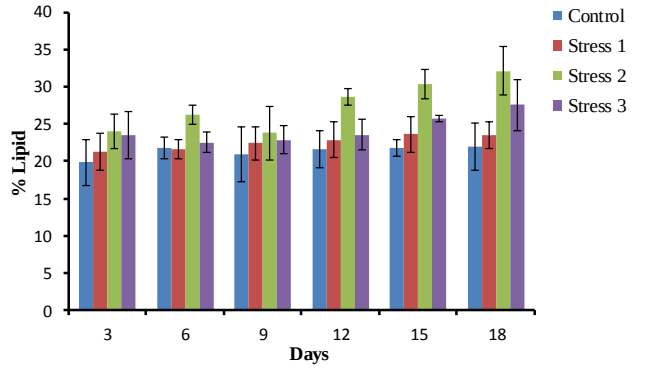
Fig. 5.3 Biomass (a), lipid concentration (b), lipid percentage (c) in *Navicula phyllepta* of set II design of experiment, in which the cells were grown in medium and growth conditions optimized for biomass production for 12 days, and subsequently in the medium deprived of urea, phosphate or silicate at 25°C . Control was nutrient replete medium at 25°C.



(a)



(b)



(c)

Fig. 5.4 Biomass (a), lipid concentration (b), lipid percentage (c) in *Navicula phyllepta* of set III design of experiment in which the cells were grown in medium and growth conditions optimized for biomass production for 12 days, and subsequently at a temperature 25°C in the medium 1) deprived of phosphate, 2) deprived of phosphate and silicate limited medium and 3) deprived of phosphate and urea limited medium. Control was nutrient replete medium at 25°C.

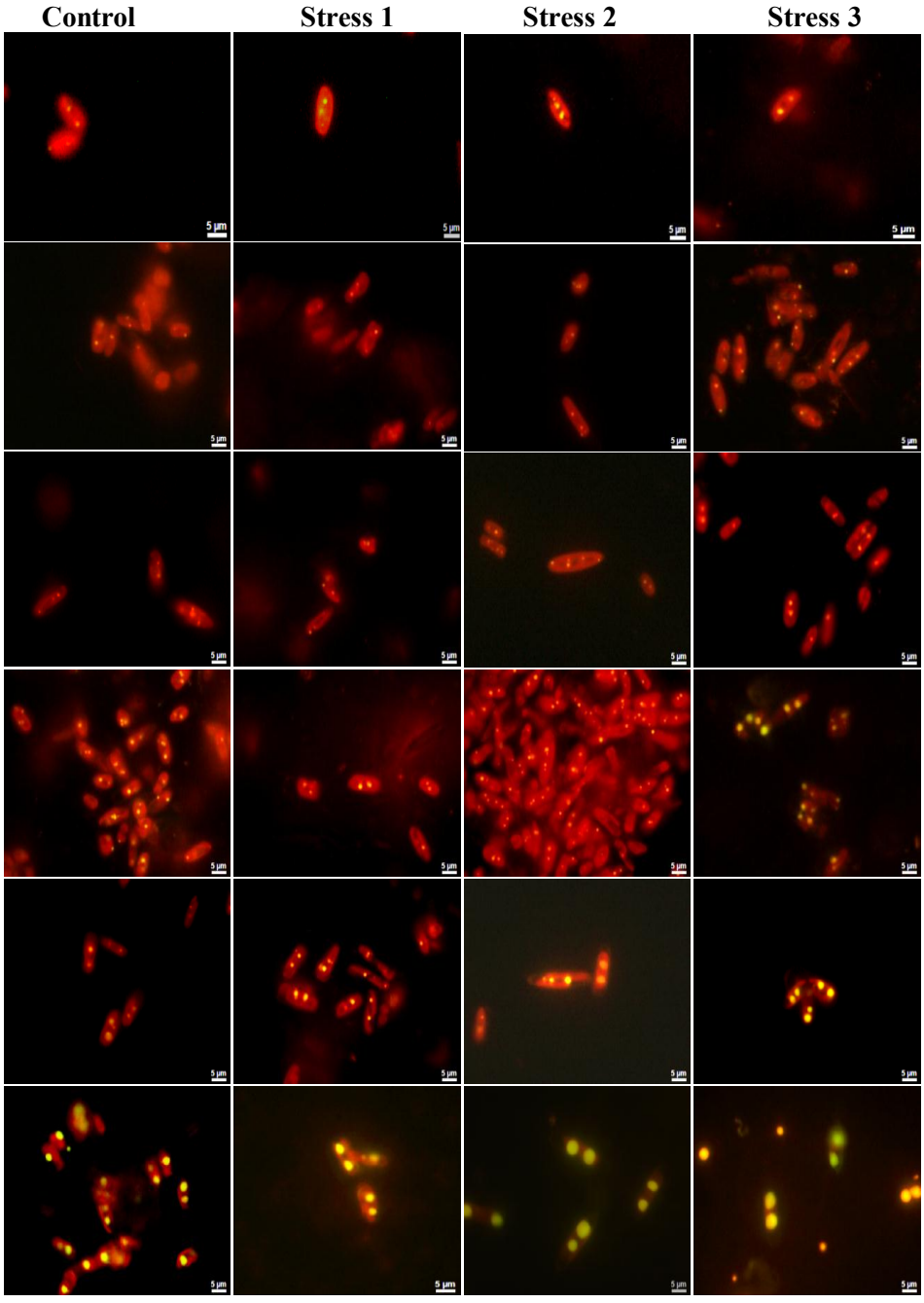
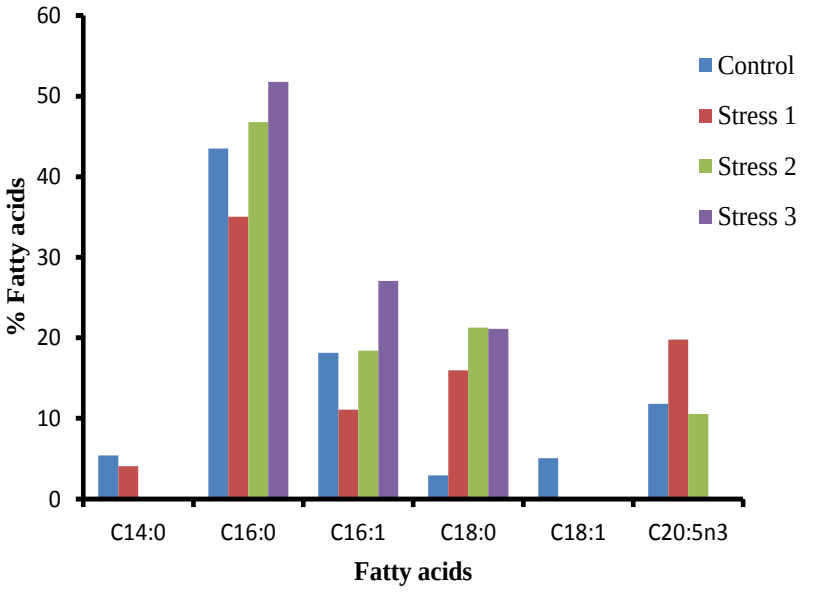
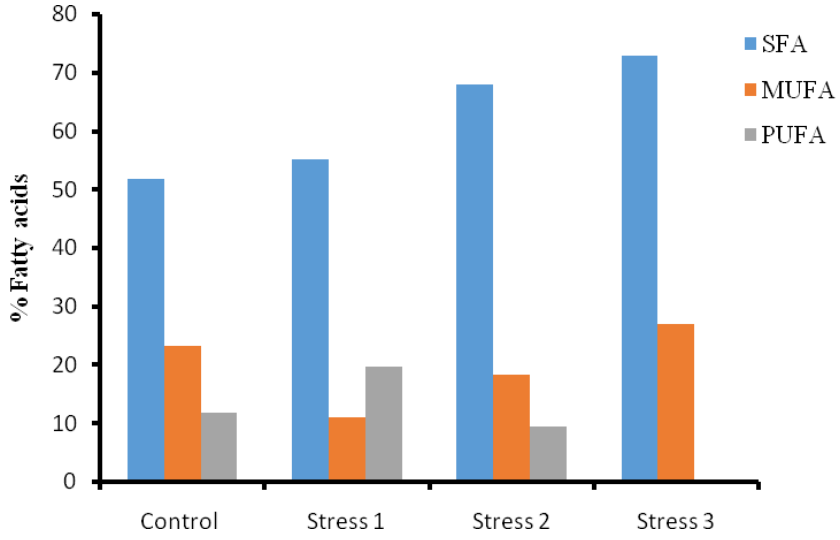


Fig. 5.5 Nile red stained images of *Navicula phyllepta* showing yellow oil bodies under stress and control conditions of set III during different days of cultivation



(a)



(b)

Fig. 5.6 Percentage of (a) individual fatty acids and (b) total fatty acids based upon degree of saturation present in *Navicula phyllepta* in control and stress conditions of set III after 12 days

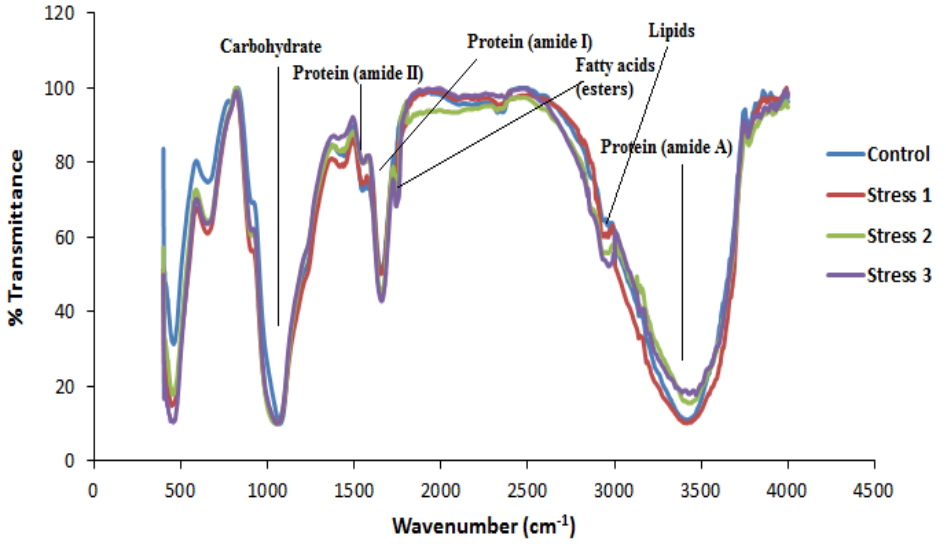
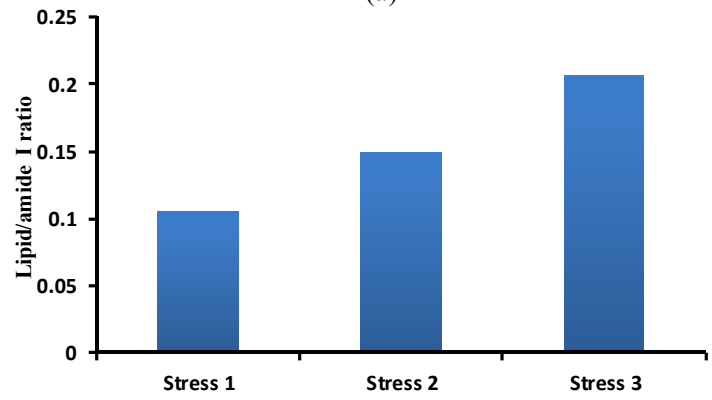
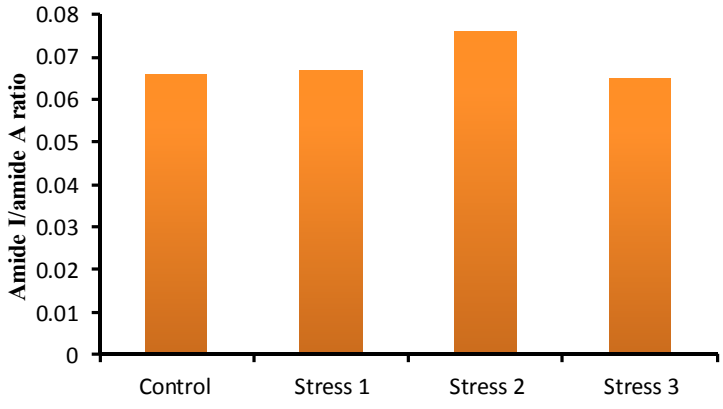


Fig. 5.7 FTIR spectrum of control and test samples on12th day of stress experiments of set III

(a)

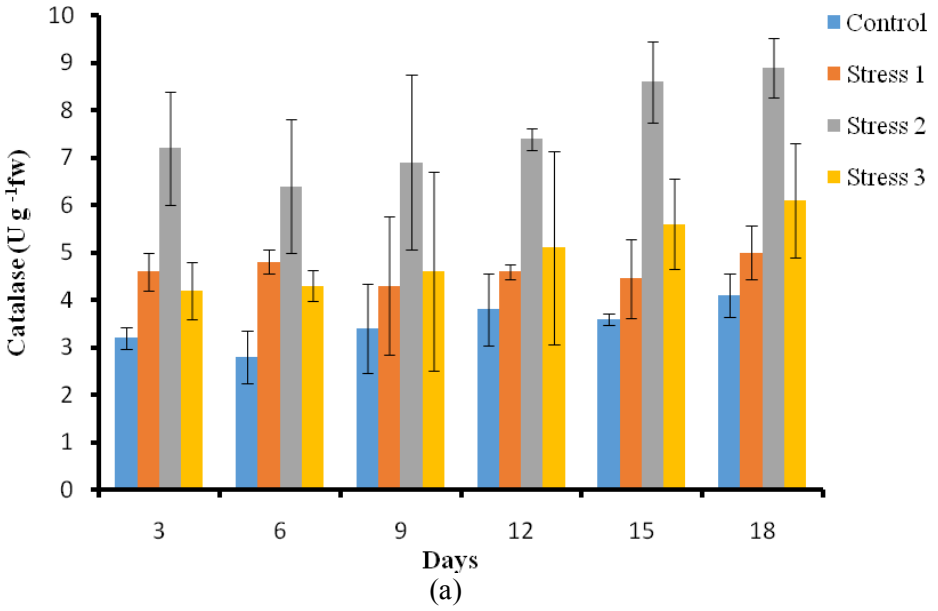


(b)

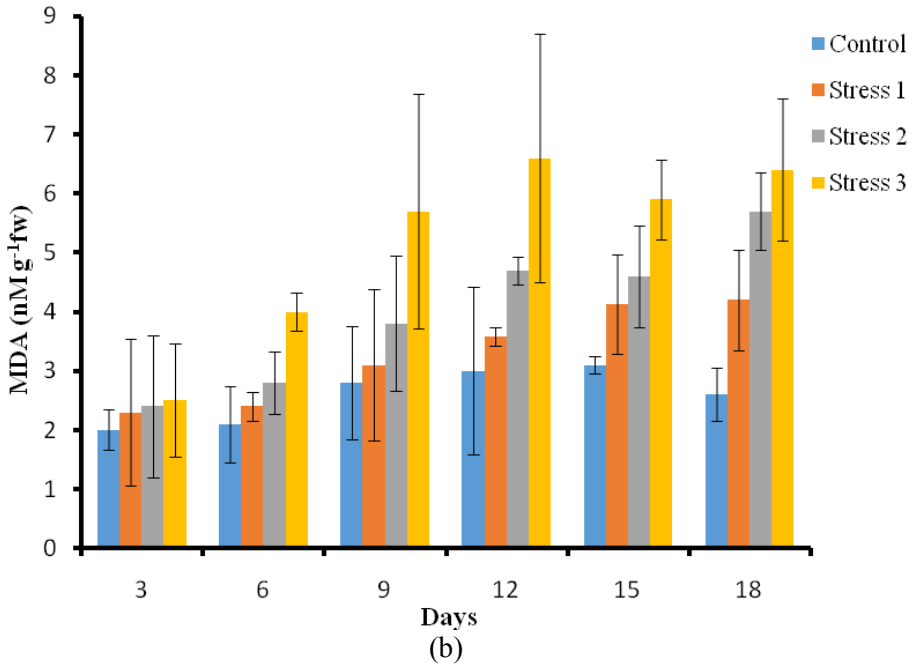


(c)

Fig. 5.8 Graphs showing the carbohydrate/ amide I (a), lipid/ amide I (b) and amide I and amide A (c) ratio between test and control samples on 12th day of stress experiments of set III

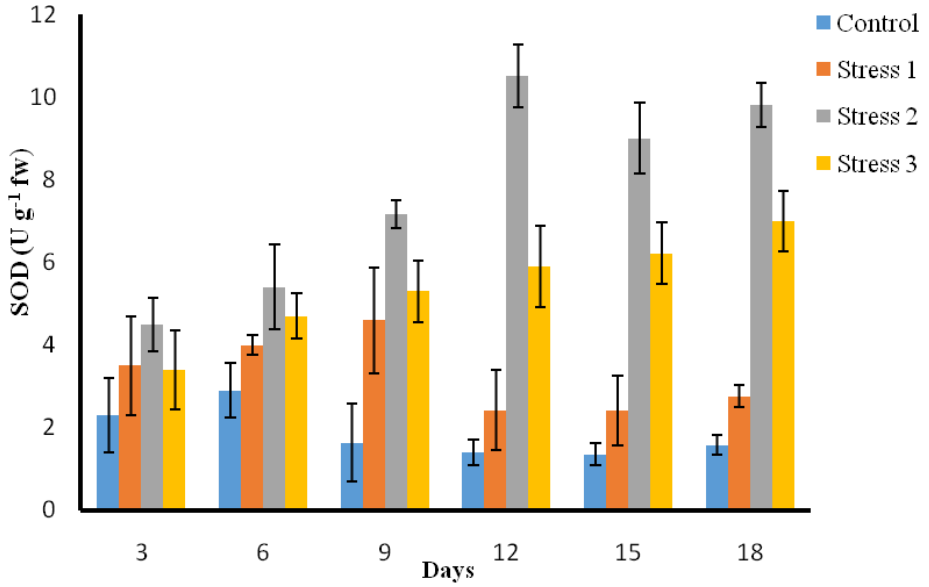


(a)

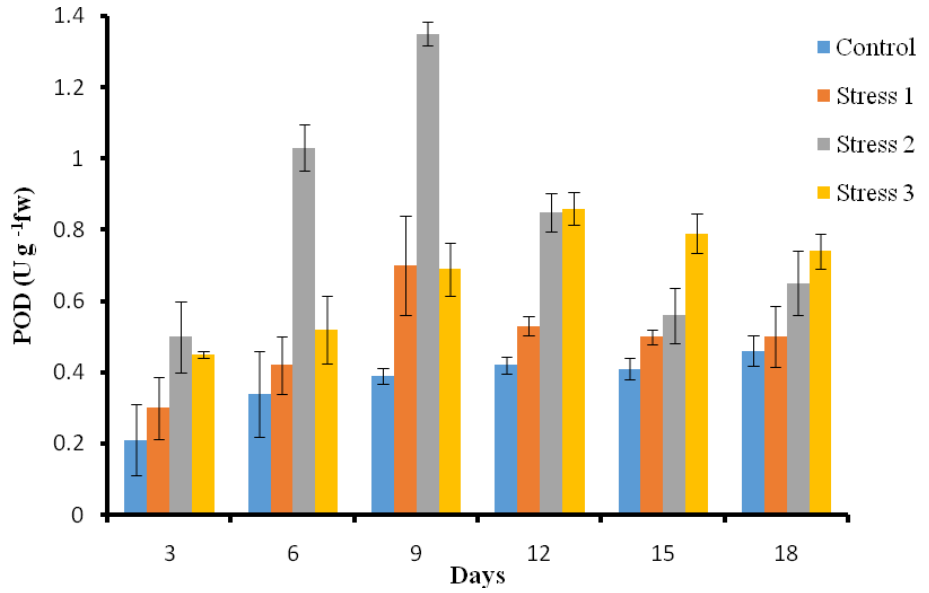


(b)

Fig. 5.9 Catalase (a) and lipid peroxidation (b) activities in *Navicula phyllepta* in control and stress (test) conditions of set III experiments



(a)



(b)

Fig. 5.10 Superoxide dismutase (SOD) (a) and peroxidase (POD) (b) activity in *Navicula phyllepta* in control and stress (test) conditions of set III experiments

Chapter 6

SUMMARY AND CONCLUSIONS

Contents

6.1 Introduction

The risk of climate change, inevitable fuel shortages, environmental unsustainability, and compromised national security has necessitated the search for other alternative liquid fuels from various biological renewable sources. The use of biofuels will ultimately lead to the reduction of harmful emissions of green-house gases, which can help in reducing the green house effects and global warming. The selection of biodiesel feedstock is based on higher yields, short duration, lower production cost and less land usage. Among the various biodiesel feed stocks, the microalgal oil has the potential to replace the conventional diesel fuel. There has been increasing interest in the study of microalgae in recent years due to their great biotechnological potential, especially as a source of biofuels. The potential of microalgae as renewable source for biodiesel production is very promising due to higher growth rates, its ability grow in any climatic zone and growth medium; and the capability to accumulate higher amounts of lipids (from 20% until 80%

of dry weight) than conventional oil crops (not more than 5% of dry weight). Moreover the choice of marine microalgae helps in reducing competition with fresh water for its mass cultivation Though this field has gathered momentum; still challenging tasks are ahead of the scientific community as algae are not nearly as well understood as other organisms due to their morphological and biochemical diversity, polyphyletic nature and the various bottlenecks associated with the cost in microalgal mass production and downstream processing. The identification of local microalgal strains which are lipid rich is still the major challenge in microalgae based biofuel production.

In this context, a study was undertaken to isolate, identify and characterize marine microalgae with biodiesel potential from the Indian waters, which have been very scarcely explored. The growth and lipid production of the selected isolate was optimized using statistical optimization tools and a cheap culture medium and an appropriate cultivation approach was standardized for enhancing its lipid productivity.

6.2 Objectives of the investigation

- Isolation, identification and phylogenetic analyses of the microalgal strains from west coast of India
- Screening for the most potent microalgal strain for biofuel production
- Optimization of the culture conditions and media compositions for high biomass and lipid production in *Navicula phyllepta* MACC8 using response surface methodology

- Enhancement of lipid production in *Navicula phyllepta* MACC8 under selected stress conditions using two stage cultivation method

6.3 Salient findings

The salient findings of the present study are:

I. Isolation, identification and phylogenetic analyses of the microalgal strains from west coast of India

- About fourteen monocultures of microalgae were isolated from the marine water samples collected from west coast of India and some parts of Cochin Estuary.
- Media recipes based on sea water such as F/2 medium, Walne's medium and Keller's K medium and BG11 medium helped in isolating native mixed microalgal strains under laboratory conditions.
- Standard plating and serial dilution methods were used to separate algae from mixed populations.
- Successful culturing of tropical benthic diatoms was achieved, which is generally considered to be difficult under laboratory conditions.
- The fourteen microalgal monocultures isolated belonged to various microalgal phyla such as Rhodophyta, Bacillariophyta, Chlorophyta, Dinoflagellata, Haptophyta and Cyanobacteria.
- An understanding of morphological features of the microalgal strains with the aid of light and scanning electron microscopy helped in their partial identification.

- Specific identification was successfully achieved using molecular based approaches especially using conserved gene markers such as 18S rRNA, ITS, 23S rRNA and LSU rRNA.
- 18SrRNA proved to be the universal marker for microalgal identification**.**
- Three DNA extraction methods were compared in which methods B (modified method of Wu et al. 2000) and C (Cheng and Jiang 2006) were identified to be the most effective ones in terms of quantity and quality.
- The marine microalgal isolates were identified as *Dixoniella* sp. MACC1(JF428838), *Biddulphia* sp. MACC2 (JX524545), *Amphora* sp. MACC4 (JX896689), *Biddulphia* sp. MACC6 (KR007589), *Pleurocapsa* sp. MACC7 (KJ845342), *Navicula phyllepta* MACC8 (KC178569), *Amphora* sp. MACC9 (KJ845340), *Durinskia baltica* MACC10 (KC161251), *Nitzschia* sp. MACC11 (KT270819), *Picochlorum* sp. MACC13 (KP098569), *Nannochloris* sp. MACC14 (KJ845339), *Prymnesium* sp. MACC15 (KJ845343), *Prymnesium* sp. MACC16 (KJ845344) and *Amphidinium* sp. MACC17 (KJ845341).
- Phylogenetic analyses of the monocultures with the related strains confirmed the taxonomic position of each isolate and also depicted the closeness to high lipid producing species, which were already reported in most of the cases, with no earlier information regarding a few.

II. Screening for the most potent microalgal strain for biofuel production

- The total lipid analyses of the 14 microalgal isolates in their late log phase showed that diatoms had a higher lipid content compared to other microalgae belonging to different classes.
- The species with comparably higher lipid content above 20 % were identified as *Amphora* sp. MACC9 (30.12%), *N. phyllepta* (26.54%), *Amphora* sp. MACC4 (23.49%), *Nitzschia* sp. (22.26%) and *Biddulphia* sp. (19.23%), *Picochlorum* sp. (24%) and *Prymnesium* sp. (21.67%).
- Protein content was in the order *Picochlorum* sp. (69.43%), *Nannochloris* sp. (50.43%) and *Prymnesium* sp. (40.28%).
- Only *Picochlorum* sp., *Nannochloris* sp. and *Prymnesium* sp. showed carbohydrate content greater than 10% of dry weight.
- Analysis of variance showed that the lipid, protein and carbohydrate contents varied significantly between species.
- Growth rate studies showed that the longest exponential phase was observed in *Picochlorum* sp. and *Prymnesium* sp. till 12-14 days, with a maximum growth between 4^{th} and 10th day with an exception in *Navicula phyllepta*, which showed the maximum doubling during 4^{th} to 6^{th} days, entering stationary phase after 10^{th} day.
- The highest growth rate was for *Navicula phyllepta* (0.58 day^{-1}) followed by *Amphora* sp. MACC4 (0.45 day^{-1}).

- The highest lipid productivity was observed for members of Bacillariophyceae *Navicula phyllepta* MACC8 (114 $mgL^{-1}day^{-1}$) followed by *Amphora sp.* MACC4 (105 $mgL^{-1}day^{-1}$).
- The biomass productivity was high in diatoms such as *Amphora* sp. MACC4 with 450 $mgL^{-1}day^{-1}$ followed by *Navicula phyllepta* MACC8 (431 mg L^{-1} day^{-1}) and *Amphora* sp. MACC9 (310 mg L^{-1} day^{-1}), which were also the top lipid producers in the present study.
- One way ANOVA of data for growth rate, lipid productivity and biomass productivity showed a significant difference between the isolates.
- Based on growth rate, lipid and biomass productivities together, *N. phyllepta* MACC8 showed a significantly higher value ($p < 0.05$) than rest of the isolates.
- The isolates *Amphora* sp. MACC4, *Biddulphia* sp. MACC6, *Navicula phyllepta* MACC8 and *Nitzschia* sp. MACC11 were stained relatively intense in Nile red staining method for qualitative determination of lipid bodies inside cells.
- The isolates belonging to class Bacillariophyceae showed significantly high fluorescence, in which, *Navicula phyllepta* showed the maximum, reaching at 35 RFU mL^{-1} towards the late stationary phase (at 30^{th} day).
- Fatty acid profile of the neutral lipid fractions of all the isolates predominantly had increased proportions of SFA and MUFA, with lesser amounts of polyunsaturated fatty acids (PUFA).

- The most predominant fatty acids were found to be C14:0, C16:0, C16:1, C18:0 and C18:1.
- *N. Phyllepta* MACC8 was found to have the highest amount of C16:1and C16:0 followed by *Amphora* sp. MACC4 and MACC9, whereas stearic acid (C18:0) was found to the highest in the case of diatom *Nitzschia* sp. and chlorophyte *Picochlorum* species.
- Most of the microalgae consisted of small amounts of eicosapentaenoic acid (EPA) C20:5(n-3), whereas it was absent in *Picochlorum* sp. and *Prymnesium* sp.
- The empirical estimation of the important parameters for biodiesel based on the information on fatty acid profile of the microalgal oil allowed a comprehensive assessment of fuel quality.
- All the microalgal isolates were within the range of standard value of cetane number (CN) and iodine value (IV). The estimated CN value varied between the seven microalgal isolates from 58 to 83.
- Oil density value of *Navicula phyllepta* MACC8 was within this range, whereas, *Amphora* sp. MACC4 and MACC9 were slightly below the range and rest of them barely met the specification value (0.86 g cm^{-3}).
- Higher heating values (HHV) of all microalgal species investigated were found to comply with the standard range of 39.69 - 43.65 MJ kg^{-1} of normal biodiesel.

- *Biddulphia* sp. MACC6, *Amphora* sp. MACC9 and *N. phyllepta* MACC8 gave good cold filter plugging points (CFPP) values indicating their feasibility for application in low temperature regions.
- Comparison of biodiesel properties of all the seven isolates showed that all the diatoms, especially *Navicula phyllepta*, were promising for biodiesel production.
- The FTIR spectra and the flame test confirmed the compositional and combustion similarity of microalgal biodiesel with commercial diesel.

III. Optimization of the culture conditions and media compositions for high biomass and lipid production in *Navicula phyllepta* MACC8 using response surface methodology

- Urea as nitrogen source in growth medium proved to be ideal in terms of high cell densities of diatom *Navicula phyllepta* compared to sodium nitrate and ammonium chloride.
- The growth rates of the diatom in sodium nitrate ($NaNO_3$), urea and ammonium chloride (NH_4Cl) were 0.44, 0.40 and 0.21 day^{-1} respectively. There were significant differences in cell densities of *N. phyllepta* MACC8 during growth in different media and between the culturing periods.
- The highest cell densities were obtained in higher salinities ranging from 20-40 g kg^{-1}. There was significant difference in growth between the extreme salinities 0 and 40 g kg^{-1} ($p< 0.05$). No significant growth difference was seen within a salinity range of 10-40 g kg^{-1} and cultivation time.

- Modified sea water medium (MSWM) was found to be a cheaper and better medium for the stable growth of *Navicula phyllepta*, with the highest growth rate in MSWM (0.48 day^{-1}), followed by F/2 (0.41 day^{-1}) and L1 (0.32 day^{-1}).
- Plackett- Burman design of experiments showed that urea, sodium silicate, sodium phosphate and temperature were the significant variables influencing the biomass and lipid content out of the seven variables (urea, sodium silicate, sodium phosphate, temperature, ferric chloride, pH, salinity, agitation) tested.
- The factors silicate and temperature had a positive coefficient on biomass production in *N. phyllepta*. In the case of lipid content, temperature had a significant positive coefficient, while urea and phosphate showed a negative coefficient.
- The central composite design model for both biomass and lipid was found to be significant.
- The results showed that silicate, urea, temperature and the interactive effects between silicate and urea, silicate and phosphate were significant in biomass production.
- The predicted R^2value 0.9006 was reasonably in agreement with the adjusted R^2 value 0.9646.
- The model showed that the lipid content in the diatom was controlled by a number of individual factors such as urea, temperature and interactive factors such as silicate-phosphate, silicate-temperature, urea-phosphate, urea-temperature, and phosphate-temperature.

- In case of lipid content, the predicted R^2- value of 0.8181 for the model to predict lipid production was in fairly reasonable agreement with the adjusted R^2 value of 0.9235.
- It was clearly seen that silicate had important role in the biomass production, whereas urea, phosphate and temperature showed an interactive effect in total lipid production.
- The validation experiments for confirming the adequacy of the model yielded a maximum biomass of 1.2 ± 0.08 g L^{-1}, which was 1.62 fold higher (64% increase) than under un-optimized conditions (0.74 ± 0.08 g L^{-1}).
- The total lipid was measured to be 0.11 ± 0.003 g L^{-1}, which was 1.2 fold higher (22% increase) than in un-optimized conditions (0.09 ± 0.009 g L^{-1}).
- The results were in agreement with the predicted values 1.18 g L^{-1} of dry biomass and 0.16 g L^{-1} of total lipid.
- Sterilisation by chlorination proved to be an effective and cheapest means of water sterilisation for mass cultivation of the diatom in outdoor conditions.
- The mass culturing of the diatom in 20L PET water jars under outdoor conditions gave a wet biomass of 4.7-5-5 g L^{-1} and dry weight of 0.6 g L^{-1} by 10^{th} day of culturing.
- Light intensity and temperature were found to influence the productivity in outdoor microalgal mass cultivation.

- Harvesting of highly dense diatoms cells by gravity settling/ sedimentation without the use of any flocculants was proved to be cost effective.

IV. Enhancement of lipid production in *Navicula phyllepta* MACC8 under selected stress conditions using two stage cultivation method

- Two-stage cultivation approach was found to be more productive in terms of biomass and lipid production over single stage approach in set I experiment in which the cells were growing in medium and growth conditions optimized for biomass production for 12 days (stage 1), and subsequently in those for lipid production (stage 2).
- There was proportional increase of biomass as well as lipid concentration in the second phase of two-stage cultivation till the end of experiment, whereas there was a decline in the biomass and subsequent lipid concentration in the single stage experiment.
- The total biomass reached 1.9 g L^{-1} and lipid concentration reached 0.4 g L^{-1} in two stage cultivation. The percentage of lipid reached 24%, whereas it was 20 % in the case of single stage at the end of experiment period, where the maximum biomass attained was 1.24 g L^{-1} and total lipid 0.27 g L^{-1}.
- Two way ANOVA showed that both biomass and total lipid concentration had significant difference between the single stage and two stage experimental setup.
- In set II experiment in which the cells were growing in medium and growth conditions optimized for biomass production for 12

days, and subsequently in the medium deprived of urea, phosphate or silicate; nutrient deprivation was found to have profound effect on biomass and lipid production in the diatom cultured in two-stage cultivation method.

- ANOVA results showed that silicate and urea removal showed a significant difference in the biomass and lipid concentration compared to control cultures, where only temperature was utilised as a stress factor.
- The biomass concentration was the lowest in silicate deprived medium resulting in 0.84 g L^{-1} by the end of the 18^{th} day, which proves the role of silicon in biomass production. But the urea deprived cultures though initially showed a steady growth reaching 1.54 g L^{-1}, which crashed after 9^{th} day.
- In terms of lipid percentage, silicon deprivation resulted in the highest percentage of 28.78 % with respect to dry weight at the end of 18^{th} day, while phosphate deprived media could produce only 25% of lipid. The similar percentage was attained by urea deprived cultures by the end of 9^{th} day of culturing.
- Results of set III experiment showed that multiple nutrient stress conditions had profound influence on the increased rate of lipid production.
- The cultures subjected to stress 3 conditions, where there was phosphate deprivation, urea limitation and temperature reduction could give a higher concentration of lipid 0.39 g L^{-1} in proportional to its biomass of 1.44 g L^{-1}.

- Experiments with stress 2 (phosphate deprivation, silicate limitation and temperature reduction) gave a significant increase in total lipid percentage 32.13 % at the cost of reduced biomass (1.1 gL^{-1}), whereas the lipid percentages reached 27.58% and 23.54% for stress 3 (phosphate deprivation, urea limitation and temperature reduction) and stress 1 (phosphate deprivation, temperature reduction) respectively.
- Two-stage cultivation method proved to be effective in providing improved quantity of lipid without comprising biomass of the biofuel feedstock. Nutrient modifications such as nitrogen limitation and phosphorus deprivation along with temperature change was found to be the most favourable post-harvest treatment in order to attain high biomass and high lipid content per cell with a minor loss in biomass.
- Increase in size and number of oil bodies showed that lipid accumulation increased with the application of multiple stresses on diatom cells compared to nutrient replete condition.
- The fatty acid composition of the diatom *Navicula phyllepta* varied substantially among the different nutrient (single or multiple) stress conditions compared to control, which was subjected to single stress alone.
- The most predominant fatty acids were C16:0, C16:1 and C18:0 which were the essential fatty acids determining the biodiesel quality.

- The relative percentage of total fatty acids of C16:0 and C16:1 was the highest in stress 3 (51.77%, 27.08%) followed by stress 2 (46.78%, 18.39%) compared to control (43.5%, 18.13%).
- The percentage of stearic acid C18:0, which contributed to total saturation of microalgal oil was greatly enhanced in the multiple and double stress samples compared to the control.
- The polyunsaturated fatty acid eicosapentanoic acid (EPA) was found in all samples except in stress 3.
- The total percentage of saturated (72.9%) and monounsaturated fatty acids (27.08%) was found to be highest in stress 3.
- On comparing the relative change in the carbohydrate/ amide I ratio, the stress 3 showed the highest value (7.46) followed by stress 2 (7.1), stress 1 (6.7) and control (6.32). The lipid/ amide I also followed a similar consistent pattern with the highest value in the case of stress 3. The amide I and amide A ratio showed similar values for control, stress 1 and stress 3 samples with higher value for stress 2 test sample. The results indicated that the phosphorus, silicon and nitrogen limitation caused changes in the proximate biochemical composition of the microalgae and the impact is dependent on the nutrient limited.
- Catalase activity was seen highest in stress 2 followed by stress 3 indicating increased rate of hydrogen peroxide production. Lipid peroxidation was higher in urea limited cultures (stress 3) compared to others. The superoxide dismutase (SOD) values

were in correspondence to catalase activity in all the stress conditions. Peroxidase (POD) values showed highest value in stress 2 cultures till 9th day after which it increased in stress 3 set of cultures which shows the decomposition of hydrogen peroxide generated.

- A higher activity of anti-oxidant enzymes in *Navicula phyllepta* showed its high tolerance to stressful conditions.

By combining all the results, it could be concluded that there is rich source of microalgal biodiversity in the Indian marine waters with biofuel production potential. Of the 14 microalgal monocultures isolated, 7 were rich in lipid content, especially the diatoms. Based on biochemical composition, biomass and lipid productivities, growth rate and FAME composition, the less explored diatom *Navicula phyllepta* isolated from Cochin estuary was identified as a potential feedstock for biodiesel production. A simple and cheap modified sea water medium with minimum composition was found to enhance the growth of the diatom *Navicula phyllepta* along with reduced cell adhesion. Auto settling behaviour and less cell adhesion property of the diatom contributed towards economic benefit in harvesting of the cells. Two stage cultivation was found to be a promising approach towards enhanced lipid production without comprising much on biomass by altering/ limiting important growth factors such silicon, nitrogen, phosphorus and temperature.

6.4 The way forward

- Mass production strategies for large scale biomass production under outdoor cultivation conditions have to be standardized.
- Transcriptome analyses for understanding the quantitative gene expression of different regulatory genes in neutral lipid accumulation in diatom *Navicula phyllepta* have to be accomplished.
- Development of tools for genetic, genomic and metabolic engineering in unsequenced marine diatom *Navicula phyllepta* for enhanced lipid production without compromising biomass production need to be achieved.

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Zhu L, Hiltunen E, Shu Q, Zhou W, Li Z, Wang Z (2014a) Biodiesel production from algae cultivated in winter with artificial wastewater through pH regulation by acetic acid. Appl Energ 128:103–110

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Appendix

Gen Bank Submission

Genbank details of the marine microalgal isolates

Biddulphia sp. MACC2 18S ribosomal RNA gene, partial sequence

LOCUS : JX524545 438 bp DNA linear PLN 01-DEC-2014
DEFINITION: *Biddulphia* sp. MACC2 18S ribosomal RNA gene, partial sequence
ACCESSION: JX524545
SOURCE : *Biddulphia* sp. MACC2
ORGANISM : *Biddulphia* sp. MACC2
Eukaryota; Stramenopiles; Bacillariophyta; Mediophyceae; Biddulphiophycidae; Biddulphiales; Biddulphiaceae; Biddulphia
REFERENCE : 1 (bases 1 to 438)
AUTHORS : Sreelakshmi,P.R., Sanyo,S., Ramya,R.N., Vrinda,S., Valsamma,J. and Bright Singh,I.S.
TITLE : Marine microalgae from the west coast of India: Isolation and screening for neutral lipid
JOURNAL : Unpublished
REFERENCE : 2 (bases 1 to 438)
AUTHORS : Sreelakshmi,P.R., Sanyo,S., Ramya, R.N., Vrinda,S., Valsamma,J. and Bright Singh,I.S.
TITLE : Direct Submission
JOURNAL : Submitted (21-AUG-2012) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India
FEATURES : Location/Qualifiers
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rRNA <1..438

/product="18S ribosomal RNA"

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Biddulphia sp. MACC 6 18S ribosomal RNA gene, partial sequence

LOCUS : KR007589 1804 bp DNA linear PLN 21-SEP-2015
DEFINITION : *Biddulphia* sp. MACC 6 18S ribosomal RNA gene, partial sequence.
ACCESSION: KR007589
SOURCE : *Biddulphia* sp. MACC 6
ORGANISM : *Biddulphia* sp. MACC 6
: Eukaryota; Stramenopiles; Bacillariophyta; Mediophyceae;
: Biddulphiophycidae; Biddulphiales; Biddulphiaceae; Biddulphia.
REFERENCE : 1(bases 1 to 1804)
AUTHORS : Sanyo, S., Bright, S. I. S. and Valsamma, J.
TITLE : Isolation and evaluation of high neutral lipid potent marine microalgae from west coast of India
JOURNAL : Unpublished
REFERENCE : 2 (bases 1 to 1804)
AUTHORS : Sanyo,S., Bright,S.I.S. and Valsamma,J.
TITLE : Direct Submission
JOURNAL : Submitted (22-MAR-2015) National Centre for Aquatic Animal Health,Cochin University of Science and Technology, Fine Arts Avenue,Cochin, Kerala 682016, India
FEATURES : Location/Qualifiers
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Pleurocapsa sp. MACC 7 23S ribosomal RNA gene, partial sequence

LOCUS : KJ845342 387 bp DNA linear BCT 01-DEC-2014
DEFINITION: *Pleurocapsa* sp. MACC 7 23S ribosomal RNA gene, partial sequence.
ACCESSION : KJ845342
SOURCE : *Pleurocapsa* sp. MACC 7
ORGANISM : *Pleurocapsa* sp. MACC 7
: Bacteria; Cyanobacteria; Pleurocapsales; Hyellaceae; Pleurocapsa
REFERENCE : 1 (bases 1 to 387)
AUTHORS : Sanyo,S., Bright Singh,I. and Valsamma,J.
TITLE : Isolation and evaluation of high neutral lipid potent marine microalgae from west coast of India
JOURNAL : Unpublished
REFERENCE : 2 (bases 1 to 387)
AUTHORS : Sanyo,S., Bright Singh,I. and Valsamma,J.
TITLE : Direct Submission
JOURNAL : Submitted (16-MAY-2014) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue,Cochin, Kerala 682016, India
FEATURES : Location/Qualifiers
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Navicula phyllepta isolate MACC 8 18S ribosomal RNA gene, partial sequence

LOCUS : KC178569 1677 bp DNA linear PLN 01-DEC 2014
DEFINITION: *Navicula phyllepta* isolate MACC 8 18S ribosomal RNA gene, partial sequence.
ACCESSION: KC178569
SOURCE : *Navicula phyllepta*
ORGANISM : *Navicula phyllepta*
Eukaryota; Stramenopiles; Bacillariophyta; Bacillariophyceae; Bacillariophycidae; Naviculales; Naviculaceae; Navicula.
REFERENCE : 1 (bases 1 to 1677)
AUTHORS : Sanyo, S., Valsamma, J. and Bright Singh, I.S.
TITLE : Molecular identification and screening for neutral lipid potent microalgal species from marine and brackish waters
JOURNAL : Unpublished
REFERENCE : 2 (bases 1 to 1677)
AUTHORS : Sanyo,S., Valsamma,J. and Bright Singh,I.S.
TITLE : Direct Submission
JOURNAL : Submitted (16-NOV-2012) National Centre for Aquatic Animal Health,Cochin University of Science and Technology, Fine Arts Avenue,Cochin, Kerala 682016, India
REFERENCE : 3 (bases 1 to 1677)
AUTHORS : Sanyo,S., Valsamma,J. and Bright Singh,I.S.
TITLE : Direct Submission
JOURNAL : Submitted (01-DEC-2014) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India
FEATURES : Location/Qualifiers

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1621 attgtgacca gttcacttca ttgtgagttg gttgtgagaa cttgtctaaa ccttatc

***Amphora* sp. MACC 9 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence**

LOCUS : KJ845340 894 bp DNA linear PLN 18-DEC-2014

DEFINITION : *Amphora* sp. MACC 9 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

ACCESSION : KJ845340

VERSION : KJ845340.2

SOURCE : *Amphora* sp. MACC 9

ORGANISM : *Amphora* sp. MACC 9

Eukaryota; Stramenopiles; Bacillariophyta; Bacillariophyceae; Bacillariophycidae; Thalassiophysales; Catenulaceae; Amphora; unclassified Amphora.

REFERENCE : 1(bases 1 to 894)

AUTHORS : Sanyo,S., Bright Singh,I. and Valsamma,J.

TITLE : Isolation and evaluation of high neutral lipid potent marine microalgae from west coast of India

JOURNAL : Unpublished

REFERENCE : 2 (bases 1 to 894)

AUTHORS : Sanyo,S., Bright Singh,I. and Valsamma,J.

TITLE : Direct Submission

JOURNAL : Submitted (16-MAY-2014) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India

REFERENCE : 3 (bases 1 to 894)

AUTHORS : Sanyo,S., Bright Singh,I. and Valsamma,J.

TITLE : Direct Submission

JOURNAL : Submitted (18-DEC-2014) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India

REMARK : Sequence update by submitter

COMMENT : On Dec 18, 2014 this sequence version replaced gi: 726968592.

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Durinskia baltica isolate MACC 10 23S ribosomal RNA gene, partial sequence; chloroplast

LOCUS : KC161251 834 bp DNA linear PLN 13-FEB-2013

DEFINITION: *Durinskia baltica* isolate MACC 10 23S ribosomal RNA gene,partial sequence; chloroplast.

ACCESSION: KC161251

SOURCE : *Durinskia baltica*

ORGANISM : *Durinskia baltica*
Eukaryota; Alveolata; Dinophyceae; Peridiniales; Peridiniaceae; Durinskia.

REFERENCE: 1 (bases 1 to 834)

AUTHORS : Sanyo,S., Valsamma,J. and Bright Singh,I.S.

TITLE : Molecular identification and screening for neutral lipid potent microalgal species from marine and brackish waters

JOURNAL : Unpublished

REFERENCE: 2 (bases 1 to 834)

AUTHORS : Sanyo,S., Ramya,R.N., Valsamma,J. and Bright Singh,I.S.

TITLE : Direct Submission

JOURNAL : Submitted (13-NOV-2012) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue,Cochin, Kerala 682016, India

FEATURES : Location/Qualifiers

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ORIGIN

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Nitzschia sp. MACC11 18S ribosomal RNA gene, partial sequence

LOCUS : KT279819 400 bp DNA linear PLN 19-DEC-2015
DEFINITION: *Nitzschia* sp. MACC11 18S ribosomal RNA gene, partial sequence.
ACCESSION: KT279819
SOURCE : *Nitzschia* sp. MACC11
ORGANISM : *Nitzschia* sp. MACC11
Eukaryota; Stramenopiles; Bacillariophyta; Bacillariophyceae; Bacillariophycidae; Bacillariales; Bacillariaceae; Nitzschia; unclassified Nitzschia.
REFERENCE: 1 (bases 1 to 400)
AUTHORS : Sanyo,S., Bright,S.I.S. and Valsamma,J.
TITLE : Isolation and evaluation of high neutral lipid potent marine microalgae from west coast of India
JOURNAL : Unpublished
REFERENCE: 2 (bases 1 to 400)
AUTHORS : Sanyo,S., Bright,S.I.S. and Valsamma,J.
TITLE : Direct Submission
JOURNAL : Submitted (12-JUL-2015) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India
FEATURES : Location/Qualifiers
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/isolation_source="marine"
/db_xref="taxon:1766654"
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rRNA <1..>400
/product="18S ribosomal RNA"

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     61 cgtcgccatc cttgggtgga acctgtgtgg cattaggttg tcgtgcaggg gatgcccatc
    121 gtttactgtg aaaaaattag agtgttcaaa gcaggcttat gccgttgaat atattagcat
    181 ggaataataa gataggacct tggtactatt ttgttggttt gcgcaccaag gtaatgatta
    241 atagggacag ttgggggtat tcgtattcca ttgtcagagg tgaaattctt ggatttttgg
    301 aagacgaact actgcgaaag catttaccaa ggatgttttc attaatcaag aacgaaagtt
    361 aggggatcga agatgattag ataccatcgt agtcgtcata
//
```

Picochlorum sp. MACC13 18S ribosomal RNA gene, partial sequence; chloroplast

LOCUS : KP098569 429 bp DNA linear PLN 07-MAR-2015

DEFINITION: *Picochlorum* sp. MACC13 18S ribosomal RNA gene, partial sequence;chloroplast.

ACCESSION: KP098569

SOURCE : *Picochlorum* sp. MACC13

ORGANISM : *Picochlorum* sp. MACC13
Eukaryota; Viridiplantae; Chlorophyta; Trebouxiophyceae; Trebouxiophyceae incertae sedis; Picochlorum.

REFERENCE: 1(bases 1 to 429)

AUTHORS : Sanyo,S., Bright,S.I. and Valsamma,J.

TITLE : Isolation and evaluation of high neutral lipid potent marine microalgae from west coast of India

JOURNAL : Unpublished

REFERENCE: 2 (bases 1 to 429)

AUTHORS : Sanyo,S., Bright,S.I. and Valsamma,J.

TITLE : Direct Submission

JOURNAL : Submitted (05-NOV-2014) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India

FEATURES : Location/Qualifiers

source 1..429
/organism="Picochlorum sp. MACC13"
/organelle="plastid:chloroplast"
/mol_type="genomic DNA"
/strain="MACC13"
/isolation_source="marine environment"
/db_xref="taxon:1620275"
/country="India: west coast"

rRNA <1..>429

/product="18S ribosomal RNA"

ORIGIN

1 attcgtattt cattgtcaga ggtgaaattc ttggatttat gaaagacgaa ctactgcgaa

61 agcatttgcc aaggatgttt tcattaatca agaacgaaag ttgggggctc gaagacgatt

121 agataccgtc ctagtctcaa ccataaacga tgccgactag ggatcggcgg gtgttttttt

181 gatgaccccg ccggcacctt atgagaaatc aaagtttttg ggttccgggg ggagtatggt

241 cgcaaggctg aaacttaaag gaattgacgg aagggcacca ccaggcgtgg agcctgcggc

301 ttaatttgac tcaacacggg aaaacttacc aggtccagac atagtgagga ttgacagatt

361 gagagctctt tcttgattct atgggtggtg gtgcatggcc gttcttagtt ggtgggttgc

421 cttgtcagg

Nannochloris sp. MACC 14 small subunit ribosomal RNA gene, partial sequence

LOCUS : KJ845339 626 bp DNA linear PLN 01-DEC-2014

DEFINITION: *Nannochloris* sp. MACC 14 small subunit ribosomal RNA gene, partial sequence.

ACCESSION: KJ845339

SOURCE : *Nannochloris* sp. MACC 14

ORGANISM : *Nannochloris* sp. MACC 14
Eukaryota; Viridiplantae; Chlorophyta; Trebouxiophyceae; Chlorellales; Chlorellaceae; Nannochloris.

REFERENCE: 1 (bases 1 to 626)

AUTHORS : Sanyo,S., Bright Singh,I. and Valsamma,J.

TITLE : Isolation and evaluation of high neutral lipid potent marine microalgae from west coast of India

JOURNAL : Unpublished

REFERENCE : 2 (bases 1 to 626)

AUTHORS : Sanyo,S., Bright Singh,I. and Valsamma,J.

TITLE : Direct Submission

JOURNAL : Submitted (16-MAY-2014) National Centre for Aquatic Animal Health,Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India

FEATURES : Location/Qualifiers

source 1..626
/organism="Nannochloris sp. MACC 14"
/mol_type="genomic DNA"
/strain="MACC 14"
/isolation_source="West coast of India-open sea"
/db_xref="taxon:1537147"
rRNA <1..>626
/product="small subunit ribosomal RNA"

ORIGIN

1 cgaatggctc attaaatcag ttatagttta tttgatggta cttacttact cggatacccg
61 tagtaattct agagctaata cgtgcgtaca tcccgacttc tggaagggac gtatttatta
121 gataaaaggc cgaccgggct tgcccgactc gcggtgactc atgataactt cacgaatcgc
181 atggccttgc gccggcgatg tttcattcaa atttctgccc tatcaacttt tgatggtagg
241 atagaggcct accatggtgg taacgggtga cggagaatta gggttcgatt ccggagaggg
301 agcctgagaa acggctacca catccaagga aggcagcagg cgcgcaaatt acccaatcct
361 gatacaggga ggtagtgaca ataaataaca ataccgggcc tttggtctgg taattggaat
421 gagtacaacc taaacacctt aacgaggatc aattggaggg caagtctggt gccagcagcc
481 gcggtaattc cagctccaat agcgtatatt taagttgctg cagttaaaaa actcgtagtt
541 ggatttcggg tgggaatggc cggtccgccg tttcggtgtg cacttgctac gcccaccttg
601 ctgccgggga cgtgctcttg agttcc

Prymnesium sp. MACC 15 large subunit ribosomal RNA gene, partial sequence

LOCUS : KJ845343 576 bp DNA linear PLN 01-DEC-2014
DEFINITION: *Prymnesium* sp. MACC 15 large subunit ribosomal RNA gene, partial sequence
ACCESSION: KJ845343
SOURCE : *Prymnesium* sp. MACC 15
ORGANISM : *Prymnesium* sp. MACC 15
Eukaryota; Haptophyceae; Prymnesiales; Prymnesiaceae; Prymnesium.
REFERENCE: 1 (bases 1 to 576)
AUTHORS : Sanyo,S., Bright Singh,I. and Valsamma, J
TITLE : Isolation and evaluation of high neutral lipid potent marine microalgae from west coast of India
JOURNAL : Unpublished
REFERENCE: 2(bases 1 to 576)
AUTHORS : Sanyo, S., Bright Singh, I. and Valsamma, J.
TITLE : Direct Submission
JOURNAL : Submitted (16-MAY-2014) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India
FEATURES : Location/Qualifiers
source 1..576
/organism="Prymnesium sp. MACC 15"
/mol_type="genomic DNA"
/strain="MACC 15"
/isolation_source="Cochin Estuary"
/db_xref="taxon:1537485"
rRNA <1..>576
/product="large subunit ribosomal RNA"

ORIGIN

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      1 gggcctgggg taggagagta agagtgcttg aaatcggcaa gaggaaaccc gtttgcgccc
     61 ggtgtcgtct cttcggctca gcaatgcctc ttggtgtggt gcacttcgga gagacgggtc
    121 agctcgcgct ggggggcgtc acacacatcc aggtagggct ccggtgagcc tggttggatg
    181 gcgcgcgctc ggggccgggc tgacggaaac gggcgcaacc gacccgtcta gaaacacgga
    241 ccaaggagtc tgacacgtgt gcgagtaccc gggtggcaag cccgcgtgcg caatgaaagt
    301 gaagggcgtt cgggcgccaa ccgaccttga tcttctgtga ggggttttga gtggagcaca
    361 cttgtcggga cccgaaagat ggtgaactat gcctgaggag gtgaagccag aggaaactct
    421 gggggagctc gtagcgatac tgacgtgcaa atcgttcgtc gaacttgggt atagggcgaa
    481 agactaatcg aaccatctag tagctggtcc ctccgagttt ccctcaggat agcttgaacg
    541 ccgacggttt tgtcaggtaa gcgaatgatt agagca
//
```

Prymnesium sp. MACC 16 large subunit ribosomal RNA gene, partial sequence

LOCUS : KJ845344 578 bp DNA linear PLN 01-DEC-2014
DEFINITION: *Prymnesium* sp. MACC 16 large subunit ribosomal RNA gene, partial sequence.
ACCESSION: KJ845344
SOURCE : *Prymnesium* sp. MACC 16
ORGANISM : *Prymnesium* sp. MACC 16
Eukaryota; Haptophyceae; Prymnesiales; Prymnesiaceae; Prymnesium.
REFERENCE: 1(bases 1 to 578)
AUTHORS : Sanyo, S., Bright Singh, I. and Valsamma, J.
TITLE : Isolation and evaluation of high neutral lipid potent marine
: microalgae from west coast of India
JOURNAL : Unpublished
REFERENCE: 2 (bases 1 to 578)
AUTHORS : Sanyo, S., Bright Singh, I. and Valsamma, J.
TITLE : Direct Submission
JOURNAL : Submitted (16-MAY-2014) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India
FEATURES : Location/Qualifiers
source 1..578
/organism="Prymnesium sp. MACC 16"
/mol_type="genomic DNA"
/strain="MACC 16"
/isolation_source="West coast of India-open sea"
/db_xref="taxon:1537486"
/lat_lon="21.00 N 68.00 E"
rRNA <1..>578
/product="large subunit ribosomal RNA"

ORIGIN

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      1 tcagccgcgc ggaatgatag taagagtgct tgaatcggca gaggaaaccc gtttgcgccc
     61 ggtgtcgtct cttcggctca gcaatgcctc ttggtgtggt gcacttcgga gagacgggtc
    121 agctcgcgct gggggggcgt cacacacatc caggtagggc tccggtgagc ctggttggat
    181 ggcgcgcgct cggggccggc tgacggaacg ggcgcaaacc gacccgtcta gaaacacgga
    241 ccaaggagtc tgacacgtgt gcgagtaccc gggtggcaag cccgcgtgcg caatgaaagt
    301 gaagggcgtt cggcgccaac cgaccttgat cttctgtgaa gggtttgagt ggagcacact
    361 tgtcgggacc cgaaagatgg tgaactatgc ctgaggaggg tgaaccagag gaaactctgg
    421 tggaggctcg tagcgatact gacgtgcaaa tcgttcgtcg aacttgggta taggggcgaa
    481 agactaatcg aaccatctag tagctggttc cctccgaagt ttccctcagg atagcttgac
    541 gccgacggtt ttgtcaggta aagcgaatga ttagagca
//
```

Amphidinium sp. MACC 17 large subunit ribosomal RNA gene, partial sequence; chloroplast

LOCUS : KJ845341 551 bp DNA linear PLN 01-DEC-2014

DEFINITION: *Amphidinium* sp. MACC 17 large subunit ribosomal RNA gene,partial sequence; chloroplast.

ACCESSION: KJ845341

SOURCE : *Amphidinium* sp. MACC 17

ORGANISM : *Amphidinium* sp. MACC 17
Eukaryota; Alveolata; Dinophyceae; Gymnodiniales; Gymnodiniaceae; Amphidinium.

REFERENCE: 1 (bases 1 to 551)

AUTHORS : Sanyo,S., Bright Singh,I. and Valsamma,J.

TITLE : Isolation and evaluation of high neutral lipid potent marine microalgae from west coast of India

JOURNAL : Unpublished

REFERENCE : 2 (bases 1 to 551)

AUTHORS : Sanyo,S., Bright Singh,I. and Valsamma,J.

TITLE : Direct Submission

JOURNAL : Submitted (16-MAY-2014) National Centre for Aquatic Animal Health,Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India

FEATURES : Location/Qualifiers

source 1..551
/organism="Amphidinium sp. MACC 17"
/organelle="plastid:chloroplast"
/mol_type="genomic DNA"
/strain="MACC 17"
/isolation_source="West coast of India-open sea"
/db_xref="taxon:1537483"
/lat_lon="7.11 N 77.30 E"
rRNA <1..>551
/product="large subunit ribosomal RNA"

ORIGIN

```
      1 tttgttgcta gtaagattgc aggctgcagt tctcacttgt gggatgctgt gtactgtgtc
     61 tcttactact cggttgggct ttgtgtagtg ctcagaatct tagcgtggac tggctgaagg
    121 gttgcgacct ggaccatgtt cttgcctact cttggcgcaa cataactgct tgaccaaata
    181 gttcaatttt acccgtcttg aaacacggac caaggagtct aatgcgtgtg caagtgtaag
    241 ggtttgacac ctgacagctc aacaaacgtg actgctggga tccttgcacc agcaaccaac
    301 tgatcgatta ggagatgttt gtgtatgagc acacttacta ggacccgaaa ggtggtgaac
    361 tatgcctgag aagggtgacg tcaggggaaa ctctgatgga agctcgtagc gctactgacg
    421 tgcaaatcgt tcgtctgact tgggtatagg ggcgaaagac taatcgaacc atctagtagc
    481 tggttccctc cgaagtttcc ctcaggatag ctggagcaga tcagttttat caggtaaagc
    541 gaatgattag a
//
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List of Publications

From thesis

[1] **Sanyo Sabu**, Bright Singh I.S. and Valsamma Joseph (2017) Molecular identification and comparative evaluation of tropical marine microalgae for biodiesel production. Marine Biotechnology 19(4): 328-344

[2] **Sanyo Sabu**, Bright Singh I.S. and Valsamma Joseph (2017) Optimisation of critical media components and culture conditions for enhanced biomass and lipid production in the oleaginous diatom *Navicula phyllepta*: a statistical approach. Environmental Science and Pollution Research 24: 26763-26777

Outside thesis

[1] Arun Augustine, Jisha Kumaran, Jayesh Puthumana, **Sanyo Sabu**; Bright Singh I.S. and Valsamma Joseph (2017) Multifactorial interactions and optimization in biomass harvesting of marine picoalga *Picochlorum maculatum* MACC3 with different flocculants. Aquaculture 474:18-25

[2] Sreelakshmi P.R., **Sanyo Sabu**, Bright Singh I.S. and Valsamma Joseph (2014) Isolation and evaluation of marine microalgae from west coast of India for neutral lipids, Proceedings of 26th Kerala Science Congress conducted by KSCSTE (08-38), p 211

Book of abstracts (conferences)

[1] **Sanyo Sabu**, I.S.Bright Singh, Valsamma Joseph (2015) Statistical optimization of biomass and lipid production in the marine diatom *Navicula phyllepta*, a potential biodiesel feedstock isolated from Cochin estuary. International conference on New Horizons in Biotechnology, Trivandrum pp 357

[2] **Sanyo Sabu**, I.S.Bright Singh, Valsamma Joseph (2015)Evaluation of marine diatoms isolated from the west coast of India for potential biodiesel production.Proceedings of 5th International Conference on Algal Biomass, Biofuels and Bioproducts, SanDiego, USA. P1.16

[3] Arun Augustine, Jisha Kumaran, **Sanyo Sabu**, T.R. Gireesh Kumar, I.S. Bright Singh, Valsamma Joseph (2015) Biomass and gamma linolenic acid production in marine microalga *Picochlorum maculatum* MACC3: Screening for significant variables using two level Plackett–Burman experimental design. Proceedings of 5th International Conference on Algal Biomass, Biofuels and Bioproducts, SanDiego, USA. P1.41

[4] **Sanyo Sabu**, I.S. Bright Singh, Valsamma Joseph (2015) A comparative study on the biofuel potential of three classes of marine microalgae isolated from the west coast of India. Proceedings of the 103rd Indian Science Congress, ISCA, Mumbai, pp 108.

[5] **Sanyo Sabu**, I.S.Bright Singh, Valsamma Joseph (2014) Biodiesel production potential of the haptophyte microalga *Prymnesium parvum* isolated from Cochin estuary.Proceedings of the international symposium MECOS2- marine ecosystems oppotunities and challenges.MBAI, Kochi, pp113.

[6] Sreelakshmi P.R.,**Sanyo Sabu**, Bright Singh I.S., Valsamma Joseph (2014) Isolation and evaluation of marine microalgae from west coast of India for neutral lipids, Proceedings of the 26th Kerala Science Congress, Wayanad, Kerala, pp 21