Production optimization of the marine microalga *Picochlorum maculatum* MACC 3 as source of polyunsaturated fatty acids

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By

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Certificate

This is to certify that the research work presented in this thesis entitled ***"Production optimization of the marine microalga Picochlorum maculatum MACC3 as source of polyunsaturated fatty acids"*** *is based on the original work done by Mr. Arun Augustine under my guidance, at the National Centre for Aquatic Animal Health, School of Environmental Studies, Cochin University of Science and Technology, Cochin-682 016, 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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Declaration

I hereby do declare that the work presented in this thesis entitled **"Production optimization of the marine microalga *Picochlorum maculatum* MACC 3 as source of polyunsaturated fatty acids"** 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-682 016, 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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Arun Augustine

Contents

Chapter 1

GENERAL INTRODUCTION

•Contents•

1. Introduction

Microalgae constitute a highly diverse group of prokaryotic and eukaryotic organisms with a capital and ecological importance accounting for about 50% of the global organic carbon fixation and having enormous biotechnological potential (Field et al., 1998). Many species have been used for the production of high value-added compounds of application in feeds, dietetics, cosmetics, pharmaceutical, biofuel and fine chemical industries; and in various processes such as wastewater treatment or biofertilization (Apt & Behrens, 1999; Grossman, 2005; Richmond, 2008). Microalgae are key biological resources that have a wide range of biotechnological potential. Due to their high nutritional value, microalgae such as *Spirulina* and *Chlorella* are being mass culture for health food. A variety of high-value products including polyunsaturated fatty acids (PUFA), pigments such as carotenoids and phycobiliproteins, and bioactive compounds are useful as nutraceuticals and pharmaceuticals, as well as for industrial applications (Chu, 2012).

Many microalgal species have been produced at an industrial level including *Chlorella* sp., *Dunaliella salina*, *Botryococcus braunii* and *Nannochloropsis* sp. for

various biotechnological applications. *Spirulina* is being cultured on an industrial scale using open ponds for the biomass as a dietary supplement in countries such as Thailand, China, United States and India with an annual worldwide production of ranging from 3000 to 4000 metric tons. *Spirulina* is regarded as a nutritious food due to its high content of proteins, γ-linolenic acid, vitamins, and minerals (Chu, 2012; Gershwin & Belay, 2007; Habib et al., 2008; Koru, 2012; Patil et al., 2005; Sijtsma & Swaaf, 2004). The advent of modern biotechnological tools has led to a better understanding of the biosynthesis and physiological functions of the bioactive molecules in microalgae. Genomic projects have been embarked on many species of microalgae. The endless efforts have been undertaken to develop transgenic microalgae as 'green cell factories' to produce new pharmaceuticals using metabolic engineering (Fletcher et al., 2007; Johanningmeier & Fischer, 2011; Leon et al., 2008; León-Bañares et al., 2004; Purton, 2007; Surzycki et al., 2009; Yu et al., 2011).

In recent years, microalgae came into biotechnical focus for their great potential as bioreactors for the large-scale production of recombinant proteins (Cao et al., 2012; Fletcher et al., 2007; Johanningmeier & Fischer, 2011; Purton, 2007). Microalgae combine high growth rates like prokaryotic cells with all advantages of eukaryotic expression systems, i.e. post-transcriptional and post-translational modifications, the relatively short generation time of initial transformants and the assessment of protein expression, assembly of heterogeneous protein complexes (Franklin & Mayfield, 2004; Surzycki et al., 2007). Moreover, algae have a phototropic system; henceforth their cultivation is CO_2-neutral and involves moderately low costs only, these extent great advantage to so far used expression systems like bacteria, yeast, mammalian and insect cells (Harun et al., 2010; Matsunaga et al., 2005; Pereira et al., 2012; Pulz & Gross, 2004; Rosales-Mendoza et al., 2011; Warude et al., 2006). The microalgae in general and marine microalgae, in particular, are increasingly being recognized for potential application in human and animal nutrition, especially as a source of polyunsaturated fatty acids

1.1 The chemistry of fatty acids and polyunsaturated fatty acids

Fatty acids are the basic building blocks fats and lipids are made of. Fatty acids found in foods and fats stored in the body are mainly in the form of

triacylglycerols (TAGs), a glycerol molecule backbone to which three (termed sn-1; sn-2 and sn-3), often different, fatty acids are attached Figure 1-1 (A). Fatty acids are made up of a backbone of carbon atoms, with a carboxyl group (COOH) at one end [the delta (Δ) end] and a methyl group (CH_3) at another end [the omega (ω) or n-end] (Figure 1-1 (B). Hydrogen atoms are joined to the sequence of carbon atoms, forming a hydrocarbon chain. Carbon chain length and presence and absence of a double bond between the carbon atoms influence the characteristics of a fatty acid such as melting point and digestibility. Based on the bonding nature, they are divided into saturated fatty acid (SFA)- carbons in the fatty acid chain are linked by single bonds; monounsaturated fatty acid (MUFA)- only one double bond present in fatty acid chain and polyunsaturated fatty acid (PUFA) more than one double bond present in the fatty acid chain (Akoh & Min, 2008; Carvalho & Malcata, 2005; Gunstone, 1996; Tocher et al., 2003; Ying et al., 2000).

The polyunsaturated fatty acids have a fatty acid chain with more than one unsaturation and can be arranged in either *cis*- or *trans* Figure 1-1 (C). *cis*- form of fatty acids are predominantly found foods, where both hydrogen atoms are found on the same side of the fatty acid (Akoh & Min, 2008; Gunstone, 1996).

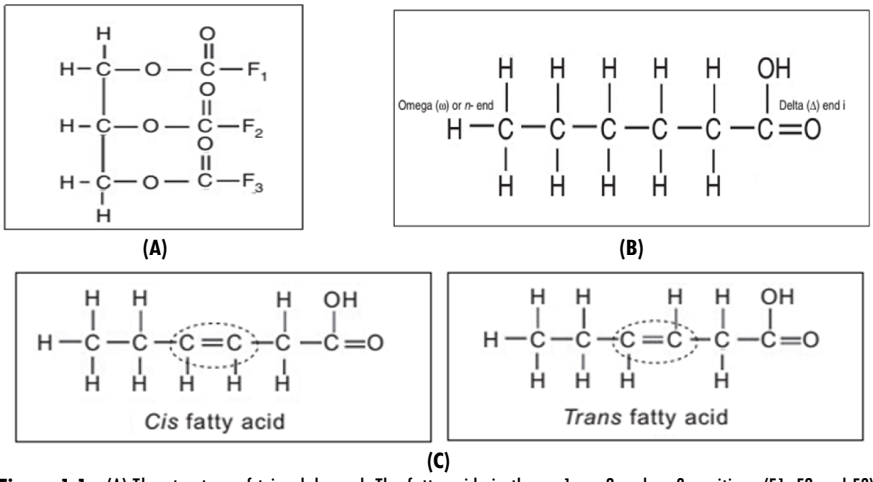


Figure 1-1. (A) The structure of triacylglycerol. The fatty acids in the sn-1, sn-2 and sn-3 positions (F1, F2 and F3). (B) The basic structure of a fatty acid with delta (Δ) and omega (ω) positon. (C) *cis*- and *trans*- configurations.
Picture adapted from (Lunn & Theobald, 2006)

The presence of a *cis*- bond in a fatty acid lowers the melting point of the fatty acid, making it more likely to be liquid at room temperature. *Trans*-fatty acids, where the hydrogen atoms are situated on opposite sides of the fatty acid, are less common in nature, but are typically found in small amounts in the fat of ruminant meats and in the milk being formed in the rumen (part of the gastrointestinal tract) during digestion and subsequently absorbed as an energy source for the animal. The major unsaturated fatty acids present in the diet are shown in Table 1-1.

Table 1-1. Main unsaturated fatty acids present in food (Lunn & Theobald, 2006)

Trivial name	Systematic name	Rich dietary sources
Monounsaturated fatty acids		
Palmitoleic (16:1n-7)	*cis*-9-hexadecenoic	Fish oil
Oleic (18:1n-9)	*cis*-9-octadecenoic	Olive oil, rapeseed oil, palm oil
Elaidic (18:1n-9 trans)	*trans*-9-octadecenoic	Partially hydrogenated fat
Trans-vaccenic (18:1n-7 trans)	*trans*-11-octadecenoic	Ruminant fats
Cis-vaccenic (18:1n-7)	*cis*-11-octadecenoic	Ruminant fats
Erucic (22:1n-9)	*cis*-13-docosenoic	Mustard seed oil
Polyunsaturated fatty acids		
Omega 6 (ω/ n-6)		
Linoleic (18:2n-6)	9,12-octadecadienoic	Sunflower oil, corn oil
Gamma (γ)-linolenic (18:3n-6)	6,9,12-octadecatrienoic	Evening primrose oil
Arachidonic (20:4n-6)	5,8,11,14-eicosatrienoic	Ruminant meats (low levels)
Omega 3 (ω/n-3)		
Alpha (α)-linolenic (18:3n-3)	9,12,15-octadecatrienoic	Flaxseed oil, linseed oil,
Eicosapentaenoic (20:5n-3)	5, 8,11,14,17-eicosapentaenoic	Fish oil, oil-rich fish
Docosahexaenoic (22:6n-3)	4,7,10,13,16,19-docosahexaenoic	Fish oil, oil-rich fish

PUFAs can be further classified as either n-3 (omega 3) or n-6 (omega 6) PUFAs; based on the position of first double bond in the fatty acid chain. In the n-6 family, first double bond between the sixth and seventh carbon atoms from the terminal methyl group, while in an n-3 family of fatty acids have their first double bond between the third and fourth carbon atoms (Sanders & Emery, 2003). Important n-3 and n-6 PUFA and their structure and the binding position shown in the picture (see Figure. 1.2).

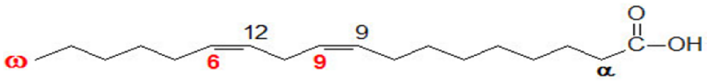
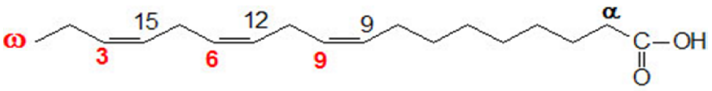
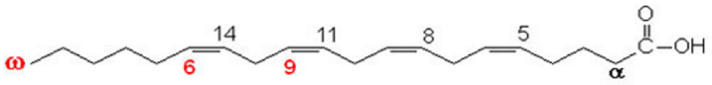
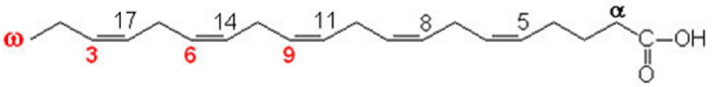
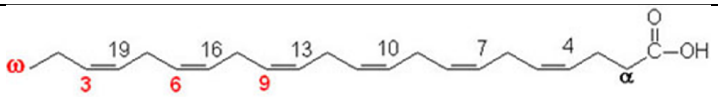
Common Name and Numerical Symbol	Structure of PUFA
Linoleic acid (LA) ω6 $18:2^{\Delta 9,12}$	
α-Linolenic acid (ALA) ω3 $18:3^{\Delta 9,12,15}$	
Arachidonic acid (AA)ω6 $20:4^{\Delta 5,8,11,14}$	
Eicosapentaenoic acid (EPA) ω3 $20:5^{\Delta 5,8,11,14,17}$	
Docosahexaenoic acid (DHA) ω3 $22:6^{\Delta 4,7,10,13,16,19}$	

Figure 1-2 n-3 and n-6 polyunsaturated fatty acids and structure with double bonding positions (Akoh & Min, 2008; Christie, 1998).

1.2 Polyunsaturated fatty acids (PUFA) in human nutrition and health

The interest in microbial lipids started over 130 years back and then onward the efforts to exploit them as alternative sources of oils and fat for human consumption (Nägeli & Loew, 1978). The problem with the microbial oil production was that the microbes had to be grown in culture medium containing glucose or sucrose as carbon sources derived from the agricultural crops at the cost of turning one agriculture commodity into another (Wynn & Ratledge, 2002). The investigation of Danish scientists on the reason why cardiovascular problems seemed nonexistent or affected significantly fewer people, in Greenland Eskimos in spite of the very high intake of fatty fish, helped to focus on the importance of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) as the two major PUFAs of fish oils. By the 1980s the importance of PUFA in human nutrition was established and by 1990s the particular health benefit of PUFA

during pregnancy, and the presence of DHA and arachidonic acid (ARA; 20:4n-6) in the mother's milk and their roles in the infant brain and retinal membrane development were established (Hunter, 2006; Lunn & Theobald, 2006).

Many of the fatty acids can be produced by humans, but our bodies do not synthesize sufficient essential omega 3 fatty acids and we must obtain them in our diet because humans are devoid of delta 12 and delta 15 desaturase enzymes those provide essential linoleic acid (LA; C18:2n−6) and alpha-linolenic acid (ALA; C18:3n−3). Omega-6 fatty acids such as arachidonic acid (AA; C20:4n−6) can be synthesized from LA and omega-3 fatty acids such as eicosapentaenoic acid (C20:5n−3; EPA) and docosahexaenoic acid (DHA; C22:6n−3) from ALA. However, the conversions of ALA to EPA and DHA are low. Therefore, both n−3 and n−6 PUFA are entirely derived from the diet and necessary for human health. The n−6:n−3 fatty acid ratio of 5:1 or less is desired, as suggested by nutrition experts (FAO/WHO, 1994).

PUFAs, with their unique structural and functional characteristics, are distinguished by two main functions. The first function relates to their roles in regulating the design, dynamics, phase transition and permeability of membranes, and regulating the behavior of membrane-bound proteins such as transport proteins, ATPases, receptors and ion channels. In addition, PUFAs control the expression of certain genes (Sessler & Ntambi, 1998) and thus affects processes including fatty acid biosynthesis and cholesterol transport in the body. Much more interest is focused on the second role of PUFAs as precursors of a wide variety of metabolites such as prostaglandins, leukotriene and hydroxy-fatty acids regulating critical biological functions (Chen et al., 2007c; Chow, 2007; Dyerberg, 1986). The various roles played by PUFAs make it apparent that they are required by every organ in the body to function normally (Adiv et al., 2004; Fekete et al., 2009). Ruxton et al. (2005) reported strong evidence on the clinical benefits of long-chain n−3 PUFA on cardiovascular diseases or rheumatoid arthritis. Reisman et al. (2006) reviewed the effect of LC n−3 PUFA on treating asthma. The potential use of n−3 PUFA in treating psoriasis has been reviewed well (Henneicke-von-Zepelin et al., 1993;

Pereira et al., 2012; Ziboh, 1998; Zulfakar et al., 2007) O'Sullivan & O'Morain (2006), Diamond et al. (2009) and Calder (2008) have reviewed the influence of omega-3 PUFA on bowel diseases. The potential of PUFA in maintaining the mental health (Bodnar & Wisner, 2005; Silvers & Scott, 2002) and on the prevention of several types of cancer (Bartsch et al., 1999; Chen et al., 2007c; Jiang et al., 1998; Lee et al., 1991; Nasrollahzadeh et al., 2008) have also been widely reviewed. Omega 3 fatty acids are effective for the prevention of rheumatoid arthritis (Calder & Zurier, 2001; Nielsen et al., 1992) and several reviews are published on the cardiovascular effects too (Kinsella et al., 1990; Mozaffarian et al., 2003; Tavazzi et al., 2008). Kinsella et al. (1990) and Jenner et al. (1992) studied the effect of PUFA on Parkinson's disease. The British Nutritional Foundation has well reviewed the health effects of dietary unsaturated fatty acids (Lunn & Theobald, 2006).

1.2.1 Polyunsaturated fatty acids (PUFA) in aquatic animal nutrition and health

Microalgae play a crucial nutritional role in the aquatic ecosystems as it considered as the primary producers. Most of the marine invertebrates exclusively depend on microalgae for their nutrition. Microalgae are utilized as a live feed for all the growth stages of bivalve molluscs such as oyster, scallops, clams, and mussels. Microalgae are used as the feedstock for crustaceans and some fish species and for zooplankton used in the aquaculture industries. So, the commercial success and productivity of any hatchery are directly related to the quality of the food sources used (Brett & Müller-Navarra, 1997; Guedes & Malcata, 2012; Spolaore et al., 2006).

In the exploration over last decades, several microalgae were screened for their potential for aquaculture industries, but only less than twenty species have been used commercially. The main microalgae used for the aquaculture industry include *Chaetoceros, Thalassiosira, Tetraselmis, Isochrysis, Nannochloropsis, Pavlova* and *Skeletonema.* The nutritional quality of microalgae varies significantly depending upon the species and culture conditions. Therefore, the microalgal strain selection and their nutritional package for growth are very important for the animal nutrition (Patil et al., 2007).

PUFA rich diets are essential for a wide range of marine and freshwater fishes, mollusks and crustaceans. There is a strong impact of PUFA on cladocerans (Ferrão-Filho et al., 2003), copepods (Klein Breteler et al., 2004), brine shrimps (Dobbeleir et al., 1980; Sargent et al., 1997), crabs (Chen et al., 2007a) and lobster (D'Abramo et al., 1980). Studies on prawns *Penaeus* and *Macrobrachium* showed the importance of PUFA rich nutrition on survival, growth, feed conversion, egg hatchability, molting and osmotic stress tolerance (D'Abramo & Sheen, 1993; Millamena & Pascual, 1990; Read, 1981)

1.3 Alternative sources of unsaturated fatty acids

The conventional commercial source of omega-3 fatty acids are cold water fatty fishes, and at present are not enough to satisfy the growing market demand. Moreover, fishes are devoid of enzymes for natural production of these essential fatty acids *de novo* and they accumulate these PUFA through food chain by the intake of marine microalgae- the primary PUFA producers in marine ecosystems (Bell & Tocher, 2009; Bergé & Barnathan, 2005; De Swaaf, 2003). The microalgal lipid content is critical for the growth and dietary makeup of secondary and tertiary consumers such as zooplankton, crustacean larvae, mollusks, some fishes, and finally higher organisms and humans. In the marine food web, EPA, and DHA, as well as certain levels of ARA, are required for optimal nutrition and stress tolerance of marine fish, especially at the larval and juvenile stages (Bell & Sargent, 2003; Bell & Tocher, 2009; Harel et al., 2002). It is predicted that fish production may diminish in the future. In addition to this, some fishes, especially marine fishes like salmon, sardine, tuna, anchovy, mackerel, are sometimes contaminated with heavy metals as copper or mercury, and organic pollutants as polychlorinated biphenyls or dioxins, which have a toxic effect on human health (Domingo et al., 2007). The PUFAs produced from fish oils also have many drawbacks such as poor taste, high purification cost and susceptibility to oxidation due to the high amount of omega-3 PUFA leading to the formation of toxic products such as peroxides, volatile compounds related to non-desirable off-flavors. This oxidation can be accelerated by factors such as temperature, exposure to light and oxygen or presence of trace metals.

Therefore, optimum processing, storage and packaging of fish oils are essential to preserve omega-3 PUFA from oxidation. Again, the quality oil depends on fish species, the season and the geographical location of the catching site. Moreover, marine fish oil is a complex mixture of fatty acids of varying chain lengths and unsaturation degrees, requiring that EPA is refined for pharmaceuticals (Gill & Valivety, 1997). The concerns over the quality and availability of the fish oil as a source of omega-3 fatty acids have generated interest in manufacturing PUFA from the alternative route (Domingo, 2007). Thus, there is an urgent need to find alternative sources of PUFA. Marine microalgae and transgenic plants are considered as the sustainable source of these edible fatty acids (Kamal-Eldin & Yanishlieva, 2002; Ratledge & Wilkinson, 1988).

1.4 Microalgae for polyunsaturated fatty acid production

1.4.1 Viability of microalgae for PUFA production

Microalgae are microscopic, prokaryotic or eukaryotic photosynthetic microorganisms inhabiting the world's oceans and other aquatic environments. They are accountable for at least half of global primary productivity, altering solar energy to organic energy and fixing carbon dioxide in the process and are the world's fastest-growing plants. They provide essential nutrition for aquatic animals, including omega-3 oils and other lipids, proteins and carbohydrates. Microalgae are rich in bioactive compounds and a source of genes for unique biosynthetic pathways, yet are a largely untapped resource, with only 10 percent of some 40 000 species isolated and cultured (Blackburn, 2014; Brown et al., 1997; Renaud et al., 1999). They are a renewable resource for human and animal nutrition, medical, biofuel and other industrial applications, and can be mass-produced in photobioreactors, ponds or fermenters. Microalgae can survive almost all earth ecosystems, representing a wide variety of species (Chisti, 2007; Li et al., 2008) ranging from unicellular to multicellular structures.

Unicellular eukaryotic microalgae are the product of over 3 billion years of evolution through primary, secondary and tertiary endosymbiotic events and are

highly diverse in nature comprising eight major phyla. These endosymbiotic events had significant effects on the metabolic pathways and regulation of fatty acid precursor synthesis (Falkowski et al., 2004). For example, fatty acid synthesis occurs in the chloroplast but is least regulated by nuclear-encoded gene products, and there are fundamental differences in the interaction between the nucleus and chloroplast in algae with different extents of endosymbiosis affected the distribution of DNA between the plastid and nucleus. Continued exploration of the evolutionary diversity of algae is important to identify species with high productivity under various environmental conditions (Wilhelm et al., 2006).

The extensive collections of microalgae during the last few decades have been created by researchers in different countries (Table 1-2) and are available to be selected for use in diverse applications, such as value added products for pharmaceutical purposes, food crops for human consumption, fine chemical for industrial applications and as an energy source. However, many strains in this collection have been cultivated for several years; some may lose original properties of nutrient requirement and mating ability. The new native strain directly from the unique environmental conditions will be providing new flexible and vigorous strain to meet the requirement of algal biotechnology industry (DOE, 2010).

Table 1-2. Major microalgal culture collections in the world

Major microalgal culture collection, country, year of establishment, type of organisms maintained and specialized and list of species and stain included in the table (Aizdaicher, 2008; Andersen, 2005)

Sl No.	Institution	Country	Year	Type	Species	Strain
1	University of Coimbra (ACOI)	Portugal		Freshwater microalgae	1000	4000
2	Goettingen University, Sammlung von Algenkulturen (SAG)	Germany	1920	Green algae (77%) cyanobacteria (8%)	1273	2213
3	University of Texas Algal Culture Collection (UTEX)	USA	1953	Green algae and cyanobacteria (200 genera)		3000
5	National Institute for Environmental Studies Collection (NIES)	Japan	1983	cyanobacteria, microalgae	700	2150
6	Australian National Algae Culture Collection (ANACC)	Australia		Majority of classes of marine and some freshwater microalgae	300	1000
7	National Center for Marine Algae and Microbiota (NCMA)	USA	1985	all major photosynthetic classes	-	2,800

1.4.2 Nutritional importance of microalgae

Around 221 species of macro and microalgae are harvested worldwide for different purposes, resulting in approximately 66% for use as foodstuffs. Spolaore et al. (2006) have reviewed some of the actual commercial applications of microalgae in human and animal nutrition and in the cosmetic industry. Cardozo et al. (2007) have also reviewed the most recent researches on the microalgal production of high-value compounds having economic relevance such as PUFA, sterols or carotenoids for application in food science, pharmacology or human health.

Microalgae are considered as the potent alternative source for PUFA and are primary producers of essential PUFAs in the marine food chain (Das et al., 2011; Khozin-Goldberg et al., 2011). The microbial oil sources carry several advantages over the conventional sources which include:- (i) the dynamic lipid synthesizing machine make them as a perspective oil source. (ii) microalgae contributes higher value PUFAs rather than resembling the bulk of low-priced commodities such as soybean oil, palm oil and sunflower oil; (iii) autotrophic microalgae cultivation of marine species in phototrophic systems can utilize non-arable lands and water resources considered unsuitable for agriculture, (iv) they possess simpler fatty acid profiles and high growth rates on wide varieties of substrates allowing utilization of cheap or zero-cost materials; (v) microalgal based oil production can be carried out throughout the year, which, is independent of seasonal or climatic variations,(vi) microbial sources can supply pharmaceutical grade with high controlled quality and concentration; (vii) microalgae have the proficiency to carry out numerous transformation reactions (e.g. oxidation, desaturation and hydrogenation) which allows the upgrading of PUFA structures, lipid and other products; (viii) availability of numerous mutants defective in specific enzymes (e.g. desaturases) improves production of tailor-made oils; (ix) microalgae provide model flat forms for studying lipid biochemistry, metabolic control and function because the shorter generation time and fewer organelle compartments in their cells providing answer to the key metabolic and biochemical questions more simply and faster than in complex multicellular systems of other organisms; (x) due to simplicity of microbial metabolic

regulation, they can be readily grown under controlled conditions with nutritional regimes that may stimulate or repress the key steps of fatty acid formation and allow the manipulation of the lipid yield and profile; (xi) in addition to the lipids they are rich in protein, trace elements, vitamins and antioxidants and therefore, they could be employed as source of macro- and micronutrients (Certik & Shimizu, 1999; Elrazak & Ahmed, 2012).

1.4.3 Distribution of PUFA in microalgae

The fatty acids are constituents of most algal lipids and rarely occur in the free form. They are mainly esterified to glycerolipids whose main classes in algae are the phosphoglycerides, glucosyl glycerides, and triacylglycerols (Adlerstein et al., 1997). The diversity of microalgal classes with respect to long-chain PUFA (LCPUFA) production is shown Table 1-3. The ω-3 LC-PUFA such as EPA is present in Cryptophyceae, Prasinophyceae, Rhodophyceae, Xanthophyceae, Glaucophyceae and Eustigmatophyceae and DHA is found in significant amounts mostly in Dinophyceae, Prymnesiophyceae, and Euglenophyceae (Imke et al., 2011). But, ω-6 LC-PUFA is relatively rare in algae, appearing mainly as precursors in the EPA biosynthesis. Most of the marine algal species have been a relatively low percentage of this ω-6 LC-PUFA accumulation (Thompson Jr & others, 1996). ARA is the major PUFA in fresh water chlorophyte, *Parietochloris incise*, but rare in other freshwater algae (Bigogno et al., 2002a; Iskandarov et al., 2009, 2010). Major PUFA producing group such as red algae, diatoms and eustigmatophytes are rich in LC- PUFA and low in C18 fatty acid precursors. But in other species, especially in green algae, LC-PUFAs occur along with the C18 PUFA like typical higher plants (Bigogno et al., 2002b; Khozin-Goldberg et al., 2011). Apart from the C18 PUFA, 18:3ω-3 and 18:3ω-6, representatives of certain microalgal classes contain highly unsaturated ω-3 C18 PUFA octadecatrienoic acid (18:4ω-3, alternatively steriadonic acid, SDA) and octadecapentaenoic acid (OPA, 18:5ω-3). Apart from the C18 PUFA, some algae from the group dinoflagellates of the genus *Pyrocystis* (Leblond et al., 2010) and coccolithophore *Emiliania huxleyi* (Sayanova et al., 2011) contain highly unsaturated

ω-3 C18 PUFA octadecatrienoic acid (18:4ω-3) and octadecapentaenoic acid (OPA, 18:5ω3).

PUFA have been found in a wide variety of photosynthetic and heterotrophic marine planktonic species belonging to different microalgal classes (Table 1-3), but some of them have less industrial production potential, mainly due to low specific growth rates and low cell densities when grown under conventional photoautotrophic conditions (Khozin-Goldberg et al., 2011; Wen & Chen, 2003). Abbadi et al. (2001) concluded that there is a vibrant technological need for the development and utilization of a safe, sustainable and cheap alternative source of ω-3 PUFA for human health and nourishment. Both the microalgae and fish have a similar type of omega-3 PUFA, but microalgal oil is devoid of unpleasant odour which makes them attractive for vegetarians. Furthermore, algal PUFA comprises cholesterol which contains squalene and phytosterol which offer additional health benefits to humans (Lewis et al., 2001). Conchillo et al. (2006) compared the advantage of microalgae oil over other PUFA sources and concluded that microalgae are advantageous from an industrial point of view as they can be easily cultivated without the effects of seasonal and climatic fluctuations with increased productivity of PUFA.

Table 1-3 Examples of LC-PUFA occurrence in various microalgae classes

Table contain the details of major microalgal division and classes which contain LC-PUFA and distribution of LC PUFA (Modified from (Khozin-Goldberg et al., 2011; Sijtsma & Swaaf, 2004; Wen & Chen, 2003))

Class; Genus; species	Type	Major PUFA	References
Chrysophyceae			
Monochrysis lutheri		EPA, AA	(Volkman et al., 1989)
Pseudopedinella sp.		EPA, AA	(Yongmanitchai & Ward, 1991b)
Coccolithus huxleyi		EPA, AA	(Yongmanitchai & Ward, 1991b)
Eustigmatophyta			
Eustigmatophyceae			
Nannochloropsis salina	P	EPA	(Yongmanitchai & Ward, 1991b)
Nannochloropsis sp.	P	EPA	(Bae & Hur, 2011)
Monodus subterraneus		EPA, AA	(Khozin-Goldberg & Cohen, 2006; Mühlroth et al., 2013)
N. salina, N. oculata	P	EPA	(Sukenik et al., 2009)

Chlorophyta			
Chlorophyceae			
Chlorella minutissima		EPA, AA	(Seto et al., 1992)
Mamiellophyceae			
Micromonas pusilla		EPA	(Zhukova & Aizdaicher, 1995)
Prasinophyceae			
Hetermastrix rotundra		EPA, DHA	(Yongmanitchai & Ward, 1991b)
Ostreococcus tauri		EPA, DHA	(Durako, 2009; Wagner et al., 2010)
Pyramimonas cordata			(Petrie et al., 2010)
Trebouxiophyceae			
Parietochloris incisa		ARA	(Bigogno et al., 2002a; Iskandarov et al., 2010; Tababa et al., 2012)
Picochlorum oklahomensis	P	EPA	(Zhu & Dunford, 2013)
Cryptophyta			
Cryptophyceae			
Chromonas salina		DHA	(Volkman et al., 1989)
Cryptomonas maculata		EPA, AA	(Chen & Chou, 2002)
Bacillariophyta (diatoms)			
Bacillariophyceae			
Asterionella japonica		EPA	(Cardozo et al., 2007; Sakshaug, 2004)
Navicula incerta		EPA	(Courtois de Viçose et al., 2012; Kang et al., 2011; Tan & Johns, 1996)
Navicula saprophila		EPA	(Kitano et al., 1997, 1998; Teoh et al., 2004)
Chaetoceros sp.		EPA	(Renaud et al., 1999, 2002)
Phaeodactylum tricornutum		EPA	(Arao et al., 1994; Cerón Garcia et al., 2006; Molina Grima et al., 1999; Yongmanitchai & Ward, 1991a)
Nitzschia laevis		EPA	(Cao et al., 2008; Chen et al., 2007b; Tan & Johns, 1996; Wen & Chen, 2003)
Coscinodiscophyceae			
Skeletonema costatum	P	DHA	(Berge et al., 1995)
Mediophyceae			
Odontella aurita		EPA	(Martins et al., 2013)
Dinoflagellata			
Dinophyceae			
Pyrocystis fusiformis, P. lunula, P.		EPA	(Imke et al., 2011)

noctiluca			
Crypthecodinium cohnii	H	DHA	(Mendes et al., 2009)
Amphidinium carterae	H	DHA	(Vazhappilly & Chen, 1998)
Haptophyta			
Prymnesiophyceae			
Isochrysis galbana	P	EPA, DHA	(Grima et al., 1993)
Emiliania huxleyi		DHA,	(Hansen et al., 1996; Sayanova et al., 2011)
Pavlovophyceae			
Pavlova lutheri	P	EPA, DHA,	(Carvalho et al., 2006; Dunstan et al., 1993; Guihéneuf & Stengel, 2013; Mühlroth et al., 2013; Tonon et al., 2003)
Rhodophyta			
Porphyridiophyceae			
Porphyridium cruentum	P	ARA, EPA	(Medina et al., 1998)
Xanthophyta			
Xanthophyceae			
Trachydiscus minutus		EPA	(Pilátová, 2013)
P- Phototrophic; H- Heterotrophic, ARA- Arachidonic acid, EPA- Eicosapentaenoic acid, DHA- Docosahexaenoic acid			
Classification according to (Guiry & Guiry, 2012)			

1.5 Biosynthesis of lipids and fatty acids in microalgae

Most of the fundamental biological processes, including lipid metabolism (Moellering et al., 2010) in microalgae are studied in model unicellular green alga *Chlamydomonas reinhardtii* (Harris, 2001), As in higher plants, the biosynthesis of fatty acids, the building blocks for TAG and membrane lipids, occurs in the chloroplast in *C. reinhardtii* (Sirevåg & Levine, 1972), and is catalyzed by two large, evolutionarily conserved enzymes: type-2 fatty acid synthase and acetyl-CoA carboxylase (ACCase). The resulting fatty acids can be used directly in the chloroplast to sequentially acylate glycerol-3-phosphate (G-3-P) by chloroplast-resident acyltransferases to produce lysophosphatidic acid (LysoPA) and phosphatidic acid (PA).

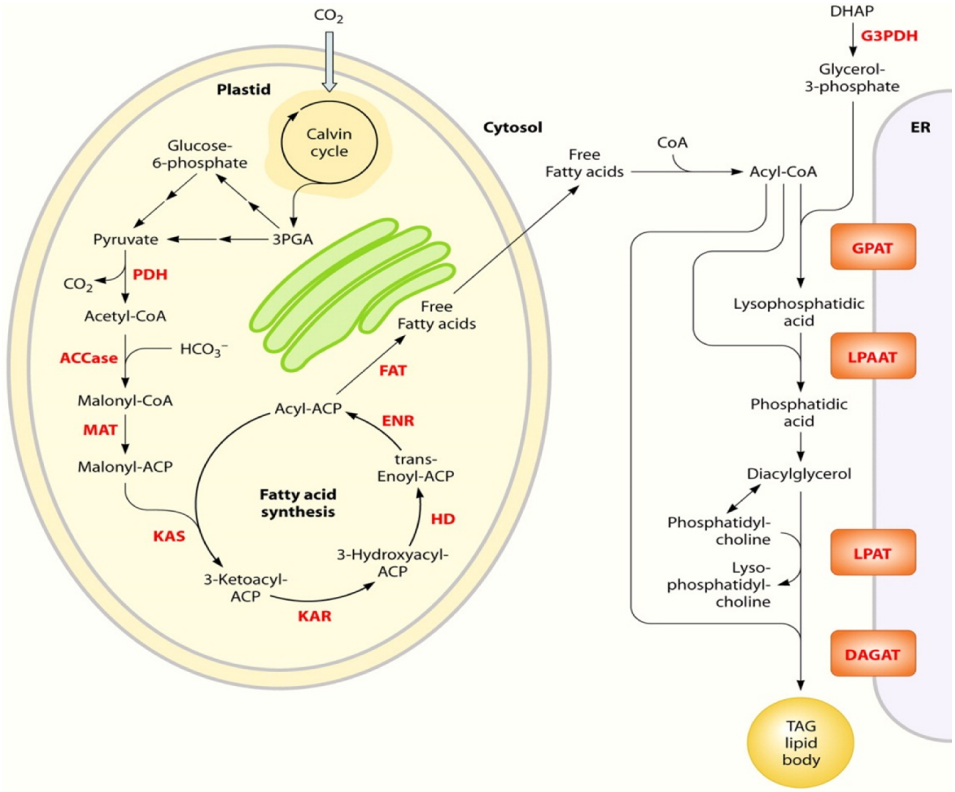


Figure 1-3 Simplified overview of the metabolites and representative pathways in microalgal lipid biosynthesis

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) adapted from Radakovits et al. (2010)

The PA and its dephosphorylated product diacylglycerol (DAG) generated in the chloroplast serve primarily as precursors for structural lipids of the photosynthetic membrane system (Browse & Somerville, 1991; Ohlrogge & Browse, 1995). Alternatively, fatty acids can be exported into the cytosol and used to sequentially acylate G-3-P in the ER by ER-resident acyltransferase isoforms. The resultant PA can be dephosphorylated to produce DAG that, in contrast to chloroplasts, can be used to synthesize both membrane lipids and storage TAG (Browse & Somerville, 1991; Ohlrogge & Browse, 1995). Due to the stringent substrate specificity of Lyso-

PA acyltransferases present either in the chloroplast or ER, lipids made by the chloroplast or ER pathway are characterized by the presence of a 16- or 18-carbon fatty acid at the sn-2 position of glycerol backbone, respectively (Fan et al., 2011, 2012; Frentzen, 1998; Giroud et al., 1988; Gouveia, 2011; Liu & Benning, 2013).

A large number of lower eukaryotes such as microalgae, fungi and protozoa are known to produce large amounts of PUFAs, so they are predicted to contain the complete array of enzymes necessary for the biosynthesis of EPA and DHA from stearic acid. The prospect for efficient microalgal PUFAs production requires an understanding of the process by which their biosynthesis is occurring within the cells. The distinct mechanism of microalgal PUFAs accumulation and the information about potential regulatory factors involved in the biosynthesis are yet poorly defined (Khozin-Goldberg & Cohen, 2011). There are a number of distinct pathways that could be involved in this biosynthetic process: (i) biosynthesis of fatty acids from glucose through *de novo*; (ii) the integration of exogenous fatty acids directly into lipid structures; and (iii) following desaturation and elongation of fatty acids. In addition, fatty acid bio-hydrogenation (saturation) and partial or total degradation (P-oxidation) also contribute to this process (Certik & Shimizu, 1999).

Usually, there are two major mechanisms by which living organisms synthesize unsaturated fatty acids (UFAs). Most of them use an oxygen-dependent fatty acid desaturation pathway, whereas, many prokaryotes, including *Escherichia coli*, *Shewanella* sp. and *Vibrio* sp. synthesize EPA/DHA anaerobically by polyketide synthase (PKS) pathway (Mansilla et al., 2004). In microalgae, *de novo* PUFAs biosynthesis occur through the aerobic mechanism, which is associated with membrane-bound enzymes and the desaturation system is composed of three proteins: NAD(P)H-cytochrome b5 reductase, cytochrome b5 and the terminal cyanide-sensitive desaturase. Desaturases involve the progressive addition of *cis* double bonds to specific positions in saturated C18 fatty acids via Δ9, Δ12 and Δ15 (or ω3) front-

end desaturases (inserting double bonds between the Δ9 bond and the carboxyl terminus) to give ALA and may follow different routes (Figure 1-4). Stearoyl-ACP desaturase (Δ9 desaturase) add the first double bond at stearic acid at the Δ9 position to form oleic acid. Subsequently, the LC-PUFA synthesis pathways in the endoplasmic reticulum (ER) are initiated by Δ12 desaturation of the chloroplast-derived oleic acid (OA; 18: 1 $^{\Delta 9}$, ω-9), producing LA (18:2 $^{\Delta 9;12}$,ω-6). Successively, LA may be further desaturated by a Δ15 (ω-3) desaturase, generating ALA (18:3$^{\Delta 9;12;15}$,ω-3). Therefore, these three fatty acids are the basic precursors of the ω - 9, ω -6 and ω -3 fatty acid cascades (Aguilar & De Mendoza, 2006). Conventionally, this sequence is Δ6 desaturase - elongase - Δ5 desaturase - elongase - Δ4 desaturase. In vertebrates, the conversion to EPA and DHA occurs through the Sprecher pathway (Sprecher et al., 1999) which involves 24:5n-3 and 24:6n-3 intermediates and a β-oxidation step (Figure 1-4) (Bell & Tocher, 2009; Meyer et al., 2004; Tonon et al., 2005). These fatty acids are further converted via the common ω-6 and ω-3 pathways, which are initiated with the Δ6 desaturation of LA or ALA, respectively. Alternative pathways that initiate with a Δ9-specific elongation of LA or ALA to eicosadienoic acid (20: 2$^{\Delta 11,14}$,ω-6) or eicosatrienoic acid (20:3$^{\Delta 11,14,17}$,ω-3), respectively, followed by sequential Δ8 and Δ5 desaturations, exist in some microalgae, such as the haptophytes *Isochrysis galbana* (Li et al., 2011; Qi et al., 2002), *Pavlova salina* (Zhou et al., 2007), and *E. huxleyi* (Sayanova et al., 2011), and the freshwater euglenophyte *Euglena gracilis* (Pereira et al., 2003) (Figure 1-4).

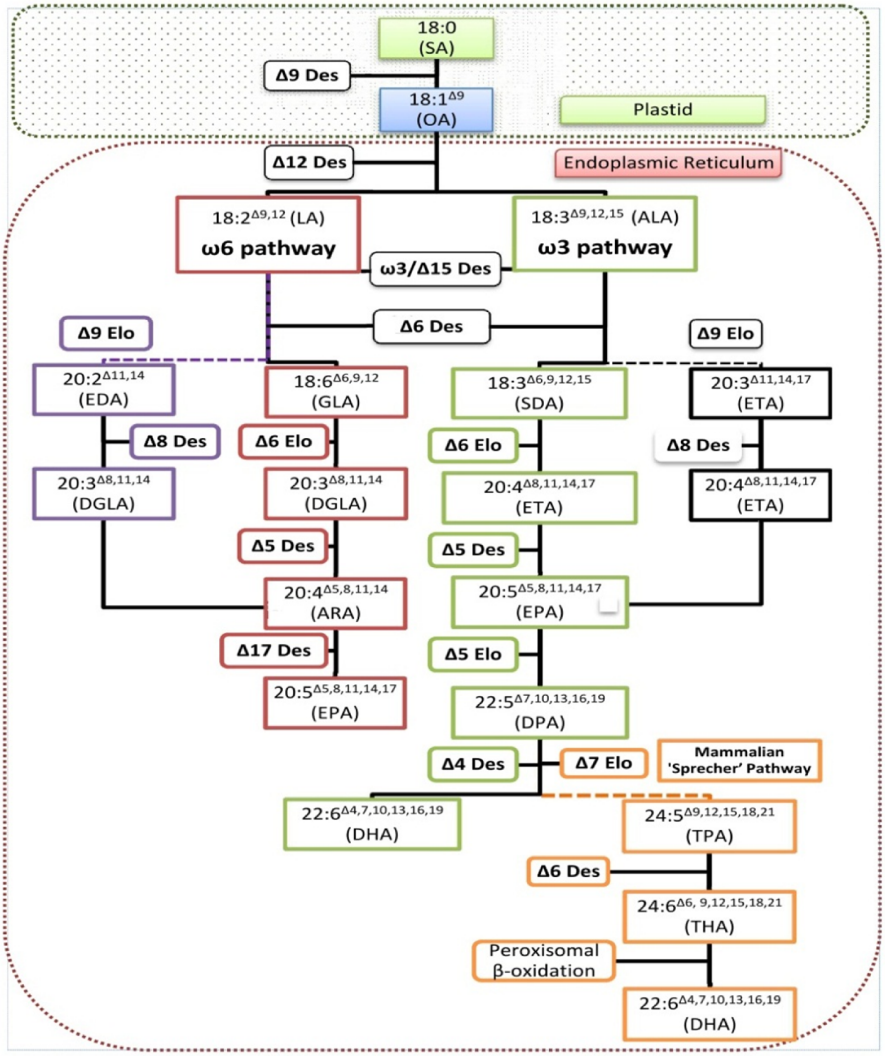


Figure 1-4. Pathway for the biosynthesis of long chain polyunsaturated fatty acids in microalgae

Biosynthesis of LC-PUFA starts from oleic acid (OA) which acts as the precursor for both ω3 and ω6 pathway. Red colour denotes for the ω6 pathway and green color denotes for the ω3 pathway. Blue and Black colour bocks denote for the alternative pathway for the PUFA biosynthesis and Orange colour bocks denote for the Sprecher pathway (modified from the Napier & Sayanova (2005) and Khozin-Goldberg et al. (2011)

1.6 Molecular biology of fatty acid desaturases and elongases

Numerous microalgal species are used for the production of high-value lipid compounds, such as long-chain polyunsaturated fatty acids (LC-PUFA) and carotenoid pigments, mainly as a feed source in aquaculture and for nutraceutical applications. Moreover, there is an increasing interest in the microalgal lipid biosynthetic pathway as the neutral lipids from oleaginous microalgae are recognized as a renewable feedstock for biodiesel production. Large numbers of genes encoding enzymes that mediate the key steps in fatty acid and TAG biosynthesis, as well as in lipid catabolism, are isolated and characterized over the last decades.

1.6.1 Desaturases

Fatty acid desaturase (FAD) are a class of enzymes that catalyze the addition of a double bond (unsaturation) in a fatty acyl chain. They are found in most all living cells and help to regulate the fluidity of membrane lipids and play a major role in the biosynthesis of PUFAs. FAD mediated desaturation is aerobic chemical reaction utilizing the molecular oxygen and reducing equivalent (electron) from electron transport chain (ETC). Based on the fatty acid ester attached to the fatty acid, they are categorized into three types: acyl-CoA, acyl-lipid, and acyl-ACP desaturase (Cook, 1996; Lim et al., 2014; Shanklin & Cahoon, 1998; Tocher et al., 1998).

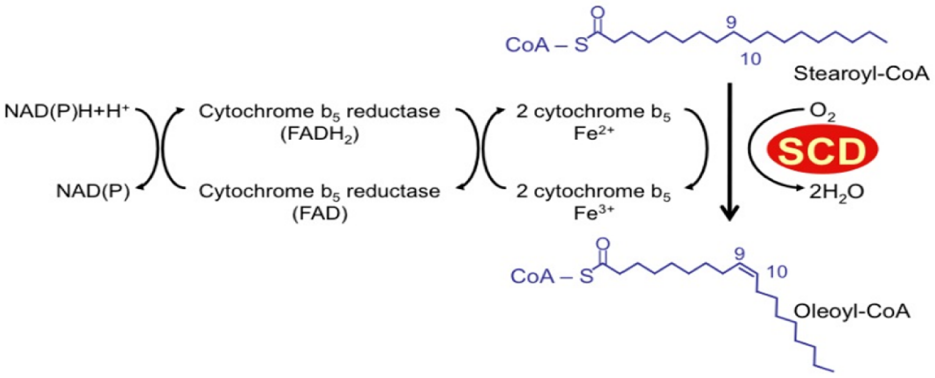


Figure 1-5 Mechanism of desaturation by stearoyl- CoA to Oleoyl-CoA by stearoyl-Co A desaturase

Adapted from Ntambi (1999; 2009)

1.6.1.1 Acyl-CoA desaturases (ACD)

The acyl-CoA desaturases introduce double bond to the fatty acids esterified to coenzyme A (CoA) and are found mainly in membrane-bound form and present in animals, yeasts, and fungal cells. They utilize cytochrome b5 as the electron donor and NADH-dependent cytochrome b5 reductase (Figure 1-5). Many of the acyl –CoA is hydrophobic proteins consisting of 300-350 amino acids residues. They span the lipid bilayer four times. The activities of Δ5, Δ6, Δ9 acyl-CoA desaturases have been described, and the only characterized protein are the gene of Δ9 acyl-CoA (stearoyl-CoA desaturase). These group of enzymes has three conserved motifs containing histidine residue and are considered to provide ligands to ferric ion at catalytic center (Table 1-4) (Los & Murata, 1998; Martin et al., 2007; Okayasu et al., 1981).

1.6.1.2 Acyl-Lipid Desaturases (ALD)

The acyl-lipid desaturases introduce unsaturated bonds in glycerolipid bound fatty acids. These are membrane-bound and found in plants, fungi, and cyanobacteria. Most of them are hydrophobic proteins consisting of 300-350 amino acids residues and span the lipid bilayer membrane four times. In cyanobacterial chloroplast, they use ferredoxin as the electron donor, whereas, in the cytoplasm of a plant cell, utilize cytochrome b5 and NADH-dependent cytochrome b5 oxidoreductase. Four ALD specific genes (Δ6, Δ9, Δ12, Δ15) were isolated and characterized from cyanobacteria *Synechocystis* sp. (Los et al., 1997; Reddy et al., 1993). The desaturases operate very strictly in the order in which they desaturate. First, the double bond is introduced by Δ9 and Δ6 and Δ12 add double bonds only on a fatty acid with Δ9. Likewise, Δ15 act only on the fatty acid containing a double bond in Δ12 position (Figure 1-6) (Andersson & Dörmann, 2009; Ohlrogge & Browse, 1995). These desaturases are characterized by the presence of three histidine clusters, which are located at strongly conserved positions in the amino acid sequence of each protein, involved in the formation of the active site of each desaturase, as has been demonstrated in other di-iron enzymes (Table 1-4) (Murata & Wada, 1995). Fatty acid desaturases are nonheme di-iron oxidases that transfer 4 e--, 2 from the fatty acid substrate and 2

from a reductant, to O_2 forming $2H_2O$. The ER desaturases require NADH, cytochrome b5, and cytochrome b5 reductase. The plastid desaturases apparently utilize reduced ferredoxin (Bates et al., 2012).

Table 1-4 Conservative histidine clusters in acyl-CoA and acyl-lipid desaturases

Desaturase Organism	Histidine cluster		
	1st	2nd	3rd
Δ9 Acyl-CoA Animal, yeast	HxxxxH	HxxHH	ExxHxxHH
Δ9 Acyl-lipid Cyanobacteria Higher plants	HxxxxH HxxxxH	ExxxxHRxHH ExxxxHRxHH	EGWHNNHH EGWHNNHH
Δ12 Acyl-lipid Cyanobacteria Higher plants	HDCGH HxCGH	HxxxxxHxxHH ExxxxxHxxHH	HxxHH HxxHH
ω3 Acyl-lipid Cyanobacteria Higher plants	HDCGH HDCGH	HxxxxxHRTHH HxxxxxHRTHH	HHxxxxHVAHH HHxxxxHVIHH
Δ6 Acyl-lipid Cyanobacteria Higher plants	HDxNH HDxGH	HxxxHH NxxxHH	QxxxHH QxxxHH

[a]Amino acids are shown in the single-letter code. The symbol x refers to any amino acid except histidine. Adapted from Los & Murata (1998)

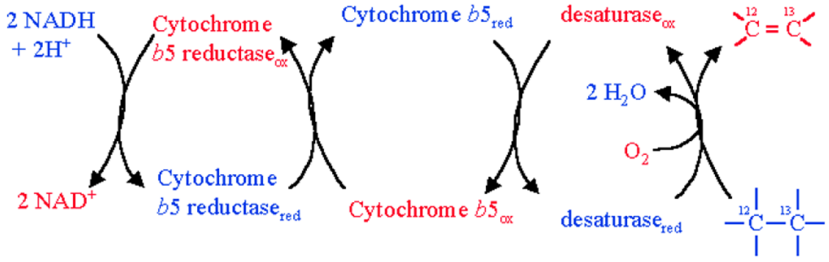


Figure 1-6 The ER Δ12 desaturase reaction and electron flow.

Figures adapted from (Bates et al., 2012)

1.6.1.3 Acyl-ACP Desaturases

The acyl-ACP desaturases are found in plant plastids in a soluble form and desaturated fatty acids linked to an acyl carrier protein (ACP). The main enzyme

under this category include stearoyl-ACP desaturase (Δ9 acyl-ACP desaturase) in the stroma of the chloroplast (plastids) that convert stearic acid to oleic acid The oleic acid produced is transported to the thylakoid membrane or into the cytoplasm for further desaturation in the lipid-bound form (Meesapyodsuk & Qiu, 2012; Roughan, 1987; Tocher et al., 1998).

1.6.2 Elongases

Fatty acid elongation is a multi-step process including four sequential enzymatic reactions: rate limiting condensation (of malonyl-CoA and acyl-CoA), reduction, dehydration and enoyl reduction (Lassner et al., 1996). Several microsomal elongation systems with different specificities to the acyl chain length exist in various organisms. Recently PUFA-specific elongases, responsible for the elongation of PUFA were identified and characterized in the nematode *Caenorhabditis elegans* (Watts & others, 2002), mammals (Leonard et al., 2000), fish (Agaba et al., 2005), algae (Domergue et al., 2002), lower plants (Kaewsuwan et al., 2006) and fungi (Beaudoin et al., 2000; Parker-Barnes et al., 2000). In the PUFA elongases, the predicted protein is highly hydrophobic and has two strongly hydrophobic *trans*-membrane regions; the first located about 50 amino acids downstream of the N-terminus and the second in the vicinity of the C-terminus. A C-terminal lysine-rich motif, important for the endoplasmic reticulum targeting (Jackson et al., 1990), and four conserved motifs FYxSKxxEFxDT, QxxxLHVYHHxxI, NSxxHVxMYxYY, and TxxQxxQF, including a conserved histidine-rich box, are suggested to be functionally important for PUFA elongation (Meyer et al., 2004). These conserved motifs were not found in other classes of plant microsomal elongases, β-ketoacyl CoA synthases, and fatty acid elongases (FAE) involved in extra-plastidial elongation of saturated and monounsaturated fatty acids (Iskandarov et al., 2009). A variant histidine box containing three amino acid replacements in C18-D9-PUFA-specific elongase from *I. galbana* (IgASE1), was shown to be essential for optimal enzymatic activity rather than for substrate specificity (Li et al., 2011; Shi et al., 2012).

1.7 Triacylglycerol Biosynthesis

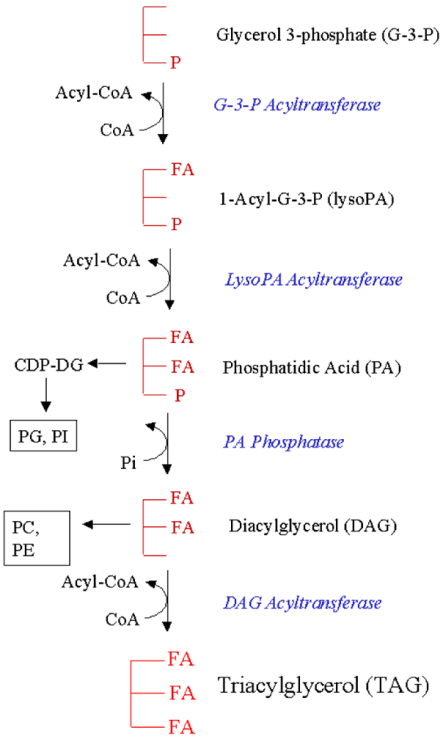


Figure 1-7 The Kennedy pathway of TAG biosynthesis
(http://www.uky.edu/~dhild/biochem/20/lect20.html)

After the desaturation reaction, the newly synthesized PUFA is distributed to the other cellular lipids. The main storage lipids in plants and many microorganisms are the triacylglycerols (TAG). TAG is synthesized via the so-called Kennedy pathway. This pathway apparently operates in the ER and the TAG accumulates in structures know as oil bodies which are surrounded by a phospholipid membrane monolayer rather than the usual bilayer, which are synthesized from sn-glycerol-3-phosphate and acyl-CoA through a sequential process. The oil bodies contain one major type of proteins known as oleosins. The Kennedy pathway involves 4 enzymatic steps involving glycerol 3 phosphate acyltransferases and a phosphatidate phosphatase. The 1st and 2nd acyltransferase reactions, which transfer fatty acids from fatty acid-CoAs to the *sn*-1 and *sn*-2 positions of glycerol, are common to membrane

lipid synthesis (as is the phosphatase). The 3rd acyltransferase, diacylglycerol acyltransferase which esterifies a fatty acid at the *sn*-3 position, is unique to TAG biosynthesis (Chapman & Ohlrogge, 2012; Lager et al., 2013, 2013; McMahon & Gallop, 2005) (Figure 1-7).

But, the gathering of three fatty acids onto the glycerol is not as straightforward as in the Kennedy pathway and most fatty acids are not instantly available for TAG biosynthesis (Certik & Shimizu, 1999). The acyl chains are bound to phospholipids, become desaturated or otherwise modified and then are available for TAG biosynthesis. This is one of the mechanisms for regulation of TAG unsaturation (Cases et al., 1998). In the first, acyl exchange occurs between the acyl-CoA pool and acyl-phospholipid (mainly phosphatidylcholine) by the combined reverse and forward reactions of acyl-CoA: phospholipid acyltransferase. The resulting newly formed PUFA-CoAs enrich the acyl-CoA pool, where they serve as acyl donors in TAG synthesis via the Kennedy pathway (Lager et al., 2013). The second mechanism involves the donation of the entire diacylglycerol portion from phosphatidylcholine catalyzed by reversible diacylglycerol choline phosphotransferase and these diacylglycerol moieties with newly modified PUFAs may be directly available for TAG synthesis. The acyltransferases and desaturases are tightly bound, which contribute to the specificity and selectivity of diverse acyl-CoA species and is responsible for PUFA distribution among individual lipid structures (Certik & Shimizu, 1999; Thompson Jr & others, 1996).

1.8 Induction of PUFA production in microalgae

1.8.1 Biochemical modulation and PUFA accumulation in microalgae

Microalgal lipid accumulation can be increased by nutrient starvation or stress in the favorable growth conditions and the accumulation of lipid is considered as the survival strategy in connection with the growth limiting factors. Many factors including temperature, UV radiation, pH, O_2 concentration, light and nutrient deprivation comprising nitrogen, phosphate, trace metals; vitamin and salinity were identified to have an impact on lipid accumulation in microalgae. Combination of

these approaches was also found to be favoring the lipid accumulation in algae, for example, by nitrogen starvation under controlled light (Harrison et al., 1990; Pal et al., 2011)

Omega-3 fatty acid biosynthesis also can be induced by environmental stress, mainly low temperature, and change in salinity and UV radiation. Tatsuzawa & Takizawa (1995) ad Guihéneuf & Stengel (2013) have reported increased EPA content in microalgae *Pavlova lutheri* from 20.3 to 30.3% when the culture temperature was reduced to 15°C. Similarly, *Phaeodactylum tricornutum* had a higher EPA content when the temperature was shifted from 25°C to 10°C for 12 h (Jiang & Gao, 2004). Increasing cell membrane fluidity through the induction of PUFA content is considered as the low-temperature survival strategy in microalgae. Salinity did not regulate the PUFA biosynthesis in *Crypthecodinium cohnii* ATCC 30556 in a consistent manner and the total DHA content increased up to 57 % of total fatty acids when cultured in 9 g/L NaCl (de Swaaf et al., 1999). *Phaeodactylum tricornutum* increased its EPA content up to 20 % when stressed with UV light (Liang et al., 2006). Some of the increased PUFAs are used to repair membrane damage, and also as antioxidant by scavenging free radicals as PUFAs contain many double bonds (Adarme-Vega et al., 2012).

1.8.2 Metabolic engineering of microalgae for enhanced PUFA production

The metabolic engineering of PUFA biosynthetic pathway has emerged as a promising approach to increase the production of fatty acids in microalgae. Metabolic engineering, a genetically based strain improvement, is the direct enhancement of product formation or cellular properties through modification of specific biochemical reactions using recombinant DNA techniques (Bailey, 1991). There are multiple strategies adopted for the genetic modification, including the production of new products by new pathways, removing or decreasing the activity of an enzyme, and amplifying genes to increase existing products (Yang et al., 1998). Metabolic engineering approaches in microalgae are recently reviewed by several authors (Cao

et al., 2012; Radakovits et al., 2010; Schuhmann et al., 2012). Similarly, metabolic engineering of plants for PUFA production is also a hot topic of research (Graham et al., 2007). The recent interest over microalgae for food and fuel triggered complete genome sequencing of several economically important microalgae. Genes encoding key enzymes involved in the fatty acid biosynthesis have been identified in *Ostreococcus tauri* (Wagner et al., 2010), *Thalassiosira pseudonana* (Tonon et al., 2004, 2005), *Phaeodactylum tricornutum* (Domergue et al., 2002), coldwater diatom *Fragilariopsis cylindrus* (Vaezi et al., 2013) and in particular, the model organism *Chlamydomonas reinhardtii* (Chi et al., 2008). At present, the mechanisms involved in the fatty acid biosynthetic pathways in microalgae have not been extensively studied and most information has been gathered from studies on plant metabolism.

Enhanced production of valuable metabolites in microalgae can be rendered possible by high-density cultivation through genetic manipulation (Matsunaga et al., 2005). Most diatoms are photoautotrophic and they can't grow in exogenous glucose in the absence of light (Hamilton et al., 2014; Radakovits et al., 2011). The transformable marine diatom *Phaeodactylum tricornutum* exhibited heterotrophic growth after the introduction of a single gene for glucose transporters glut1 or hup1. For this purpose, the plasmid (pPha-T1; glut1-gfp) was introduced into *Phaeodactylum tricornutum* using a biolistic procedure, and transformants were selected for zeocin resistance in the light. The trophic conversion of microalgae, such as diatoms, is a critical first step in engineering algae for successful large-scale cultivation (Daboussi et al., 2014; Zaslavskaia et al., 2001). Several works have been performed to create recombinant sources of ω-3 fatty acids in a variety of plants and microalgal systems with some success (Amiri-Jami & Griffiths, 2010; Damude & Kinney, 2007a, 2007b; Graham et al., 2007; Li et al., 2009; Radakovits et al., 2011).

In the future, ω-3 fatty acids may be produced by microalgae in larger quantities by regulating the expression of enzymes in the unsaturated fatty acid synthesis and lipid biosynthesis pathway (Haralampidis et al., 1998; Los & Murata, 1998; Mühlroth et al., 2013; Sakamoto & Murata, 2002; Yu et al., 2011). A promising cisgenic approach for microalgae may be to increase EPA or DHA production by

overexpressing at least some of their native elongases and desaturases. It may be necessary to use promoters inducible by external stimuli rather than constitutive promoters that may interfere with normal cell function and growth. Another, yet unexplored option may the inhibition of PUFA degradation at peroxisomes. So far, the mechanism behind the selection and storage and distribution of fatty acids for triacylglycerol production somewhat remains unclear (Adarme-Vega et al., 2012).

The understanding of the basic algal biology is still in infancy. In particular, gene regulatory mechanisms, presumably involving networks of transcription factors and noncoding RNAs, remain largely unexplored (Cerutti et al., 2011). Progress in this area will benefit greatly from the functional characterization of novel genes and from system level approaches aimed at understanding and modeling the biochemical and regulatory networks that control algal physiological and metabolic features. In this context, greater knowledge of RNA-mediated silencing pathways in algae may provide insights into both a mechanism(s) of gene regulation and a tool for the characterization of genes with unknown functions. Limited evidence suggests that small RNAs may function, in different algae, in defense mechanisms against transposon mobilization, in responses to nutrient deprivation and, possibly, in the regulation of recently evolved developmental processes. From a practical perspective, RNA interference (RNAi) is becoming a promising tool for assessing gene function by the sequence-specific knockdown. The development of RNAi technology in conjunction with system level "omics" approaches may provide the tools needed to advance our understanding of algal physiological and metabolic processes (Cerutti et al., 2011; Chi et al., 2008; Eamens et al., 2008; Li & Rana, 2012; Nevoigt, 2008; Perrine et al., 2012)

As most of the genes involved in the biosynthetic pathways have been cloned and characterized from different microalgae, it is now possible to recognize their roles in PUFA biosynthesis. Remarkable progress in engineering PUFAs producing strains has been made by many researchers in the last few years (Cao et al., 2012). Moreover, the characterization of enzymes in PUFA biosynthesis from microalgae can provide ample candidate genes for the production of the nutritionally important fatty acids in

transgenic plants (Chi et al., 2008). Rational metabolic engineering has been fairly successful in the construction of high-level PUFAs producing microalgae and should play a central role in the development of a cost-effective and clean alternative to fish oils (Nevoigt, 2008). Along with the development of systems biology and the growing information generated by global analysis methods, metabolic engineering techniques can be expected to further advance the performance of existing strains (Cao et al., 2012). We can easily combine it with other strategies such as fermentation engineering, enzyme engineering, and cell engineering to finally make the microbial EPA-producing system economically feasible.

1.9 Production Process of PUFA from Microalgae

1.9.1 Algal Cultivation

Algae can be cultivated via photoautotrophic or heterotrophic or mixotrophic methods, with varying challenges and advantages.

1.9.1.1 Photoautotrophic Cultivation

The innate capacity of the phototrophic algae is used for the cultivation. The efficiency of photosynthesis is a crucial determinate in their productivity, affecting growth rate, biomass production, and the accumulation of biomolecule precursors. Though theoretical biomass productivity values in the range of 100-200 g/ m^2 /day have been predicted (Chisti, 2007, 2008; Sirisansaneeyakul et al., 2011), there is no current consensus on the true maximum productivity of algae. Theoretical productivity is an important concept because it can be used to set achievable goals for both cultivation process design and strain improvement projects (Zhu et al., 2008). Multiple phototrophic approaches are employed depending upon the quality, quantity and value of the product required (open, closed, hybrid, and coastal or off-shore systems). Christine Rösch & Posten (2012) compared between the algal cultivation in an open pond and photobioreactor systems (Chen et al., 2011; Javanmardian & Palsson, 1991; Molina et al., 2001).

1.9.1.2 Heterotrophic and mixotrophic cultivation

In heterotrophic cultivation, algae are grown without light and are fed a carbon source, such as sugars, to generate new biomass. These carbon supplementations accelerate the lipid accumulation. The heterotrophic approach takes advantage of mature industrial fermentation technology and is already widely used to produce a variety of products at large scale. The advantages of heterotrophic production are the easiness to maintain optimal conditions for production and contamination prevention and the potential to utilize inexpensive agricultural and other industrial wastes such as lignocellulose sugar for algal growth. Heterotrophic cultivation also achieves high biomass concentrations in a short period of time that reduces the extent and cost of the algal culture and byproduct (Xu et al., 2006). A possible disadvantage of the system is the availability of suitable feedstock such as lignocellulose sugars which rely on primary productivity from other sources; they could compete for feedstock with other biofuel technologies. The addition of external carbon source increases the risk of contamination by undesired microbes living off the carbon source (Chen & Johns, 1995; Heredia-Arroyo et al., 2010; Jiang et al., 1999; Mendes et al., 2007; Wen & Chen, 2003)

The mixotrophic cultivation harnesses both the photoautotrophic and heterotrophic ability of algae. A mixotrophic culture can utilize both carbon and light sources for cell growth both in light and dark period yielding high cell densities in a short period of time. However, the mixotrophic cultures need a photobioreactor setup (Heredia-Arroyo et al., 2011).

1.9.2 Downstream Processing of Microalgae

The conversion of algal culture to bio-product require a long stretch and is money intensive processes collectively called downstream process, which, involve different steps such as harvesting, dewatering, and extraction of bioproduct (lipids, carbohydrates, protein, pigments) (Medina et al., 1998). The harvesting of small algal species in dilute suspensions at concentrations between less than 1 g/L (ponds) and 3-15 g/L (photobioreactors) are difficult as well as energy intensive. Dewatering to

about 20-30 % water content is important to reduce volume and weight, to minimize transportation and downstream costs and to extend the shelf-life of the microalgae concentrate. Dewatering can be achieved through different physical, chemical, and biological methods depending on the type of algae, the requirements of the downstream processes, and the desired product quality. The usually applied techniques involve flocculation, gravity sedimentation, centrifugation, filtration, and drying (Medina et al., 1998; Richmond, 2008).

1.9.2.1 Microalgal flocculation

Flocculation involves the addition of some chemical additives leading to sedimentation and harvesting. Chemical additives that bind algae or otherwise affect the physiochemical interaction between algae are known to promote flocculation (Knuckey et al., 2006; Lee et al., 1998). Alum, lime, cellulose, salts, polyacrylamide polymers, surfactants, chitosan, and other man-made fibers are some chemical additives that have been studied. Manipulating suspension pH with and without additives is also effective, and autoflocculation in the form of photosynthetically driven CO_2 depletion for pH control has been studied (Knuckey et al., 2006; Sirin et al., 2012; Sukenik & Shelef, 1984). Bio-flocculation, where algae are co-cultured with another organism that promotes sedimentation has also been considered. Finally, electro-flocculation and electrocoagulation offer the advantages of no added chemicals (Xu et al., 2010). Auto-flocculation and flocculation with alum, ferric chloride, chitosan, or hydrophobic absorbents and collection by means of dissolved air flotation, which thickens the material to 10 % dry weight content (100 g/L), are used as an initial step in dewatering to aggregate the microalgal cells, and they enhance the easiness for further processing, such as sedimentation or centrifugal recovery.

1.9.2.2 Gravity sedimentation

Gravity sedimentation, possibly enhanced by flocculation, is a separation technique with low energy demand and suitable for the harvesting of large microalgae at a reasonable cost, but it requires substantial area and the downstream processes and

product targets are tolerant to contamination by coagulants. The sediment sludge is more diluted than centrifugally recovered biomass, which substantially influences the economics of product recovery further downstream (Christine Rösch & Posten, 2012; Harith et al., 2009; Molina Grima et al., 2003).

1.9.2.3 Centrifugation

Centrifugation is widely used in algal harvesting. The efficiency is dependent on the selected species as related to size. Centrifugation technologies must consider large initial capital equipment investments, operating costs, and high throughput processing of large quantities of water and algae. The current level of centrifugation technology makes this approach cost-prohibitive for most of the envisioned large-scale algae biorefineries. Significant cost and energy savings must be realized before any widespread implementation of this approach can be carried out (DOE, 2010).

1.9.3 Extraction and Purification of PUFA from microalgae

Currently, limited volumes of byproducts are produced from the algal feedstock. Hence, there is a lack of well-defined industrial scale method for extracting and separating oils and lipids from algae. Most of the extraction techniques are suitable for analytical and laboratory scale procedure. To produce algal PUFAs as a competitive commodity, extraction techniques employed must be refined (Lee et al., 2010).

1.9.3.1 Mechanical cell breaking

Mechanical cell breaking include cell homogenizer, bead mills, ultrasounds and autoclaving (Andersen, 2005; Cravotto et al., 2008). Non-mechanical methods include freezing application, organic solvents, osmotic shock, acid, base and enzyme reactions (Rubio-Rodriguez et al., 2010), followed by the organic co- solvent mixture extraction by conventional chloroform and methanol (Bligh & Dyer, 1959). After the extraction, water is added to the co-solvent mixture until a two-phase system develops in which water and chloroform separate into two layers. Chloroform layer contains the lipid that can be recovered for further analysis and contain saponifiable lipids such

as pigments, lipoprotein and other lipids and non-lipid contaminants (Fajardo et al., 2007). Consequently, another co-solvent mixture has been proposed for the extraction of lipids (Medina et al., 1998).

1.9.3.2 Supercritical fluid extraction

Supercritical fluid extraction utilizes the enhanced solvating power of fluids above their critical point. It can be processed using solid and liquid feeds (Akoh & Min, 2008; Reverchon & De Marco, 2006). Supercritical fluid extraction has been applied for the extraction of functional ingredients and lipids from microalgae. Lipids have been selectively extracted from microalgae at temperatures of 40-50^{0}C and pressures of 241-379 bar (Gomez-Prieto et al., 2002; Mendes et al., 1994).

1.9.3.3 Quantification of total lipids and PUFAs

The common fatty acids of animal and plant origin have even-numbered chains of 16 to 22 carbon atoms, with zero to six double bonds of the *cis* configuration; methylene-interrupted double-bond systems predominate. The environment offers innumerable exceptions and odd- and even-numbered fatty acids with up to nearly 100 carbon atoms exist. In addition, double bonds can be of the *trans*-configuration, acetylenic and allenic bonds occur, and there can be innumerable other structural features, including branch points, rings, oxygenated functions, and much more. More than a thousand different fatty acids of natural origin must exist (Badami & Patil, 1980; Christie, 1998; Gunstone et al., 2012). There is a wide variety of analytical methodologies associated with the reported values, resulting in the potential for greater variability. On the basis of the analytical point of view the original method described by Bligh & Dyer (1959), the lipid is a total quantity of compound soluble in chloroform/ methanol solvent mixture, and then improved by Folch et al. (1957). Conventionally followed gravimetric extraction yields are highly dependent on the polarity of the solvents used and the composition of the algal lipids in the algal biomass, considering the complex mixture of polar and non-polar lipids (Laurens et al., 2012).

1.9.3.3.1 Gas Chromatography Analysis

Usually, gas chromatography (GC) with flame ionization detector (FID) analysis is used for accurate composition analysis of lipids (Han et al., 2011; Laurens et al., 2012). The lipids are esterified into corresponding fatty acid methyl esters (FAME) and analysed. This step is needed before GC analysis in order to improve volatility of the fatty acids and attain better resolutions in the chromatographic procedure (Brondz, 2002). The FAME conversion can either by lipid extraction and transesterification or direct transesterification without lipid extraction. For the analytical purpose later method is easy and most preferable (Carrapiso & Garcia, 2000).

1.10 PUFA production through biorefinery concept

The biorefinery approach involves the integrated production of biofuel, biochemical, antibiotics and additives to food and feedstock from a selected biomass. This type of multipurpose production of different industrially important products from the same substrate will open new opportunity that effectively extract compounds from the biomass material for direct use as food and feed additives (Jørgensen, 2012) and help the development of cost-effective microalgae-based biotechnologies.

The innate capacity of microalgae to produce multiple products such as oils, proteins and carbohydrates have encouraged the development of a biorefinery concept for processing. Diverse industries are able to use different algal products. For example, the pharmaceutical and nutraceutical industries use high-value bioactive products such as ω-3 fatty acids, carotenoids, and other pigments; the transport industry can use fatty acids from TAG for biodiesel, the chemical industry can use products such as glycerin, biomass can be used in agriculture and aquaculture as animal feed. Further practices include nutrient recycling, carbon sequestration, anaerobic digestion of wet biomass and pyrolysis for the production of biochar. Certainly, the biggest interest in microalgal use is for biodiesel production. It has the potential for a sustainable alternative to fossil fuels, as microalgal production

facilities do not need to compete for arable land or freshwater (Adarme-Vega et al., 2012; Subhadra & others, 2011; Subhadra, 2010).

1.11 Biotechnological and commercial prospects of microalgal lipid production

The photosynthetic microalgae belong to diverse and potent untapped resource for the commercial PUFA production. Currently, these essential nutraceuticals are produced from heterotrophic single cell organisms through fermentation. The marine microalgae *Crypthecodinium cohnii* (Jiang et al., 1999; Mendes et al., 2009; De Swaaf, 2003) and marine protist *Schizochytrium* sp.(Lippmeier, 2007; Martins et al., 2013) are sources of commercial microbial ω-3 LC-PUFA. The estimated market demand of microalgal PUFA through heterotrophic cultivation was about $195 million in 2004, and increasing the growth rate of 8% from 2004-2010 (Khozin-Goldberg et al., 2011). Numerous phototrophic microalgae PUFA producers are available and they are mainly used for aquaculture industry because of the relatively low cell densities. There are numerous challenges to be met the field of photo-biotechnology for the large scale production of PUFA for human and animal nutrition. Physiological and biochemical studies, advances in genetic and metabolic engineering approaches and availability of whole genome sequences of commercially important microalgae are all directed to enhance the growth, lipid productivity, light capturing capacity and resistance to contamination of microalgal species (screening for algae for growth in extreme conditions, which prevent the contamination with other organisms) (Becker, 1994). The increasing global attention over microalgae for food and fuel and large numbers of international events devoted to the microalgal cultivation and commercial product developments all will accelerate the commercialization of algae-based oil resource within 10 or 15 years (Wallis et al., 2002). Biorefinery approaches maximize the exploitation of valuable algal components for diverse industries. Transgenic plants have been proposed as an alternative source of essential fatty acids. Several authors reviewed the production of PUFA enriched vegetable species (Graham et al., 2007; Napier et al., 2007) and

transgenic oilseeds (Venegas-Calerón et al., 2010). However, the genetically modified plants in agriculture are not well accepted in many countries, especially in Europe.

From the biotechnological point of view, microalgae have several benefits for the commercial prospects, such as high growth rate, easiness in cultivation, ability to perform post-transcriptional and translational modifications and superior photosynthetic efficiency (three times efficient in using light than higher plants). Another feature of microalga is well-established chloroplast and nuclear genome transformation methods and relative short generation time to initial transformants screening and assessment of protein expression. Apart from that, most green algae are classified as ‘‘Generally Regarded As Safe’’ (GRAS), they can be cultivated in full control reducing any concern about environmental contamination (Franklin & Mayfield, 2004; Manuell et al., 2007; Mayfield et al., 2003; Rosales-Mendoza et al., 2011).

1.12 Significance of the study

The conventional sources of PUFA include oils of plant seeds and oils from certain marine fish. Fishes such as salmon, sardine, mackerel, tuna, are perfectly used for fish oil production, but PUFA**s** from fish oil have some disadvantages like fleshy odor, contamination with pesticides, heavy metals, and other marine pollutants, besides affecting the biodiversity of the source organism. To meet the future demand of PUFA, marine algae have been identified as the most important alternative source. The success of the microalgae-based PUFA production primarily depends on the characteristics of microalgal strain used, such as high growth rate and accumulation of desired product in easily extractable or separable form, versatile nutrient usage and adoption to grow in changing environmental and nutrient regimes. The growth of algae in extreme environmental conditions such as high salinity and high pH help to eliminate the growth of undesired organisms. The second important factor is the high cost of production related to nutrient supply and infrastructure. The nutrient related cost can be minimized by using medium optimization process, which, enable the supply minimum amount of nutrient for the maximum product accumulation. The

third aspect is the biotechnological approaches in the improvement of strain for biosynthetic pathway elucidation and characterization of the important enzymes involved in PUFA synthesis and its regulation in connection with various external stimuli and manipulation of the pathway for improved production through metabolic engineering approaches.

The vast biodiversity of marine microalgae in the Indian Ocean is yet to be explored fully for various biotechnological applications. In the present study, the marine microalga *Picochlorum maculatum* MACC3, earlier isolated from the west coast of India, was analyzed in depth for PUFA production potential to develop optimized processes for PUFA production and algal biomass downstream processing. The present study is undertaken with the following objectives, as an attempt to develop *P. maculatum* MACC3 biomass as a product for application in human and animal nutrition as an alternative source of PUFA.

1. Biochemical and molecular characterization of *Picochlorum maculatum* MACC3 and screening for PUFA production under phototrophic, mixotrophic and heterotrophic conditions
2. Optimization of medium and growth conditions for the production of biomass and gamma linolenic acid by *Picochlorum maculatum* MACC3 using response surface methodology
3. Scale up production, and optimization of harvesting technique of marine microalga *Picochlorum maculatum* MACC3 by flocculation with chitosan and aluminium sulphate using Response Surface Methodology
4. In-vitro cytotoxicity study of *Picochlorum maculatum* MACC3 harvested through centrifugation and flocculation with chitosan and aluminium sulphate in selected representative cell lines.

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Chapter 2

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF *PICOCHLORUM MACULATUM* MACC3 AND SCREENING FOR POLYUNSATURATED FATTY ACID PRODUCTION UNDER PHOTOTROPHIC, MIXOTROPHIC AND HETEROTROPHIC CONDITIONS

Contents

2.1 Introduction

The algal taxonomy has reached a critical turning point with the advances in molecular identification techniques, and morphological or biochemical identification alone can no more delineate an isolate to species level. Integrated and polyphasic approaches are required for resolving the conflicts arising out of morphological, ultra-structural and phylogenetic analyses in species identification. The green lineage or Viridiplantae comprises the land plants and green algae and these are the foremost groups of oxygenic photosynthetic eukaryotes. Green algae are diverse and ubiquitous in aquatic and some terrestrial habitats and they have played a vital role in the global ecosystem for hundreds of millions of years (Leliaert et al., 2012). The green lineage originated by the primary endosymbiotic event by which a heterotrophic eukaryotic host cell captured a cyanobacterium that became stably integrated into the eukaryotic cell and ultimately turned into a plastid. This primary endosymbiosis happened approximately between 1 and 1.5 billion years ago leading to the origin of the earliest oxygenic photosynthetic eukaryotes (Archibald, 2009; Cocquyt, 2009; Hedges et al., 2004) including green algae, red algae, and glaucophytes.

The chlorophytes are morphologically diverse, ranging from unicellular, multicellular, colonial, filamentous, siphonous to thallus forms. The classifications based on morphological, ultrastructural and phylogenetic characterizations differ significantly. The Chlorophyta, in general, can be classified into five classes, the Chlorophyceae, the Prasinophyceae, the Trebouxiophyceae, the Charophyceae and the Ulvophyceae (Matsunaga et al., 2005). The Trebouxiophyceae recently were separated from the Chlorophyceae. The Chlorophyta are primarily freshwater algae with approximately 500 genera comprising 16 000 species, with about 10% of which are marine species. The Ulvophyceae are primarily multicellular marine green algae. In addition, some species from the Prasinophyceae, Chlorophyceae, and Trebouxiophyceae families are found in the marine environment (Matsunaga et al., 2005).

Picochlorum a newly established genus, regroups species previously included in the genus *Nannochloris*, is a member of division Chlorophyta and class Trebouxiophyceae; most of them are small coccoid (non-motile), asexual, unicellular, green algae, and can withstand hypersaline conditions (Dahmen et al., 2014; Foflonker et al., 2014; Henley et al., 2004). Some species of *Picochlorum* are well studied. *Picochlorum oklahomensis* is slightly oval shaped green alga with a cell size of 1.5- 2.5 μm in diameter, and the cell contained chlorophyll a and b as the major pigments, in addition to the carotenoids such as lutein, β-carotene, violaxanthin, neoxanthin and vaucheriaxanthin ester (Henley et al., 2002; Hironaka, 2000). *Picochlorum oklahomensis* is reported to have a higher growth rate of 0.5 μ/day, 0.7 divisions per day, and a shorter generation time of 1.4 days (Zhu & Dunford, 2013).

Recently, the full genome sequence of the natural isolate, *Picochlorum* SE3 was analyzed and found to contain 13.5 Mbp nuclear genome encoding 7367 protein-coding genes, with 5795 introns, a G + C content of 46.1% and a gene density of 1.8 Kbp/gene. A maximum likelihood alignment of 4,80102 amino acids placed *Picochlorum* SE3 as a sister to *Chlorella variabilis* within Chlorophyta (100% bootstrap support) and revealed that its average protein evolutionary divergence rate (i.e. branch length) is elevated since its split from *C. variabilis* (Foflonker et al., 2014).

Biochemical analysis of *Picochlorum oklahomensis* showed that it contained 20% lipids and 35% protein of the total dry weight, and fatty acid methyl ester (FAME) analyses showed 27% saturated fatty acids, 22% monounsaturated fatty acids and 46 % polyunsaturated fatty acids of the total lipid. Because of high protein and oil, the alga is suitable for the food and feed industries. Although the oxidative stability of biodiesel produced from *P. oklahomensis* may be low due to its high unsaturated fatty acid content, high PUFA, specifically, linoleic and linolenic acid contents, enhances the high nutritional value of algal biomass produced (Zhu & Dunford, 2013).

Dahmen et al. (2014) reported that the lipid content of *Picochlorum* sp. increased from 163 mg/g to 524 mg/g of dry weight through medium optimization and contents of saturated and unsaturated fatty acids was 28% and 72%, respectively. The fatty acids profile was characterized by the predominance of short-chain unsaturated fatty acids, with 16:1 (17%) 16:2 (6%), 16:3 (13%), 18:2 (29.5%) and 18: 3 (23%) as the major fatty acids.

Microalgae are the primary producers of PUFA and a wide range of valuable products, but the commercial scale-up has been hindered by difficulties in obtaining high cell densities. The two main strategies adopted for high-density biomass production are the use of closed photobioreactors such as tubular photobioreactors, vertical alveolar panel (VAP) and fibre optical photobioreactors, and heterotrophic culture systems. The main purpose of the photobioreactors is to maximize the illumination area (surface to volume ratio) and to reduce the light limitation area (Chaumont, 1993; Chen & Johns, 1995; Ogbonna et al., 1997). However, light penetration in the culture medium is inversely proportional to the cell concentration (Oswald, 1988); closed photobioreactors tackle this problem in some extent. Javanmardian & Palsson (1991) have reported that light can penetrate 2 mm distance in 30 g/L biomass culture.

The phototrophic growth of microalgae is limited in productivity by light deficiency and mutual shading, and the requirements for more efficient, economical and controllable production systems make the development of heterotrophic growth process deeply desirable (Wen & Chen, 2003). The purpose of heterotrophic culture

is to utilize organic carbon as a source of energy requirement of microalgae and thereby eliminating the requirement of light. The main problem associated with heterotrophic microalgal production is that a limited number of microalgal species can use organic substrates, and they lack the ability to growth and produce light-induced products by organic substrate above a certain level (Chen & Johns, 1995; Pelletreau & Targett, 2008; Perez-Garcia et al., 2011).

In the present study, a marine microalga earlier isolated from the west coast of India and identified as *Nanochloris* sp. was confirmed as *Picochlorum maculatum* using polyphasic approaches and screened for polyunsaturated fatty acid production under different nutritional modes.

2.2 Materials and methods

2.2.1 Microalgal strain

The microalgal strain was originally isolated in Walne's medium from a seawater sample collected from Kannur region in the Arabian Sea in the west coast of India (11.87^0 N, 75.35^0 E) onboard FORV Sagar Sampada (Fisheries and Oceanographic Research Vessel, Government of India) cruise number 270. The monocultures obtained by serial dilution was initially identified as *Nannochloris* sp. MACC3 and maintained in the culture collection of National Centre for Aquatic Animal Health, Cochin University of Science and Technology, India, and confirmed in the present study as *Picochlorum maculatum* MACC3.

2.2.2 Microalgal culture conditions

The samples were plated on petri dishes with the f/2 medium in filtered sea water with 1% agar. Individual colonies, obtained after sequential sub-culturing, were picked up and transferred to fresh f/2 medium. The isolated colonies were treated with the antibiotics penicillin G (100 mg/L), streptomycin (25 mg/L) and gentamicin (25 mg/L) to inhibit the growth of possible contaminant bacteria. Petri plates were maintained at temperature and pH of 25^0C and 7.5 ± 0.2, respectively, under a photon irradiance of 50±10 µmol photon/m^2/s in 16:8 light and dark periods. Liquid cultures

were grown in Erlenmeyer flasks with f/2 liquid medium and agitated at 150 rpm in a rotary shaker (Orbitek, Scigenics Biotech. Pvt. Ltd., India), under the same light, temperature and pH as above. The algal cells were grown in two different culture media for comparing growth and identified using morphological and molecular methods.

2.2.3 Culture media

2.2.3.1 f/2 Medium

This is a common and widely used generally enriched seawater medium designed for growing coastal marine algae, especially diatoms. The concentration of the original formulation, termed "f Medium" (Guillard & Ryther, 1962) has been reduced by half.

To 950 mL of filtered natural seawater following components and trace element and vitamin solutions were added and the final volume was brought to 1 liter with filtered natural seawater. Since the green algae do not require silica, the addition of silicates was omitted in the present study.

Component	Stock solution (g/L)	Quantity (mL/L)	Molar concentration in the final medium	mg/L concentration In final medium
$NaNO_3$	75	1	8.82×10^{-4}	75
$NaH_2PO_4H_2O$	5	1	3.62×10^{-5}	5
Na_2SiO_3 $9H_2O$	30	1	1.06×10^{-4}	30
Trace metal solution	(see recipe below)	1	---	
Vitamin solution	(see recipe below)	0.5	---	

2.2.3.1.1 f/2 Trace Metal Solution

To 950 mL of dH_2O, the following components were added and the final volume was brought to 1 liter with dH_2O.

Component	Primary stock solution (g/L)	Quantity	Molar conc. In final medium	mg/L conc. In final medium (1mL/L of trace metal to the medium)
$FeCl_3$ $6H_2O$	-	3.15 g	1.17×10^{-5}	3.15
Na_2EDTA $2H_2O$	-	4.36 g	1.17×10^{-5}	4.36
$CuSO_4$ $5H_2O$	9.8	1 mL	3.93×10^{-8}	0.098
$Na_2MoO_42H_2O$	6.3	1 mL	2.60×10^{-8}	0.063
$ZnSO4$ $7H_2O$	22.0	1 mL	$7.65 \times 10^{-}$	0.022
$CoCl_2$ $6H_2O$	10.0	1 mL	4.20×10^{-8}	0.01
$MnCl_2$ $4H_2O$	180.0	1 mL	9.10×10^{-7}	0.18

2.2.3.1.2 f/2 Vitamin Solution

Dissolved the thiamine in 950 mL of dH_2O, and, added the amounts of the primary stocks as indicated in the quantity column below, and brought the final volume to 1 liter with dH_2O. The solution was filter sterilized and stored in a refrigerator or freezer.

Component	Primary stock solution (g/L)	Quantity	Molar conc. in final medium	mg/L conc. In final medium(0.5 mL/L of vitamin to the medium)
Thiamine HCl (vit. B_1)	-	200 mg	2.96×10^{-7}	0.1
Biotin (vit. H)	0.1	10 mL	2.05×10^{-9}	0.0005
Cyanocobalamin (vit. B_{12})	1.0	1 mL	3.69×10^{-10}	0.0005

2.2.3.2 Walne's Medium

To prepare, pasteurized 1 liter of filtered natural seawater; after cooling, aseptically added 1 mL of the nutrient solution and 100mL of the vitamins solution (Walne, 1970).

2.2.3.2.1 Nutrient Solution

Into 900 mL of high-quality dH_2O, dissolved the components and brought the final volume to 1 liter with high-quality dH_2O, filter sterilized, and stored at 4°C.

Component	Stock solution	Quantity used	Concentration in final medium (M)
$NaNO_3$	-	100 g	1.18×10^{-3}
H_3BO_3	-	33.6 g	5.43×10^{-4}
Na_2EDTA (anhydrous)	-	45.0g	1.54×10^{-4}
$NaH_2PO_4 \cdot H_2O$	-	20.0g	1.28×10^{-4}
$FeCl_3 \cdot 6H_2O$	-	1.3 g	4.81×10^{-6}
$MnCl_2 \cdot 4H_2O$	-	0.36 g	1.82×10^{-6}
Trace metals solution	(See following recipe)	1 mL	

2.2.3.2.2 Trace Metals Solution

Into 900 mL of high-quality dH_2O, dissolved the components. This solution is normally cloudy and acidified with a few drops of concentrated HCl to give a clear solution. The final volume was brought to 1 liter with high-quality dH_2O, filter sterilized and stored at 4°C.

Component	Quantity used	Concentration in final (M)
$ZnCl_2$	21.0 g	1.54×10^{-7}
$CoCl_2 \cdot 6H_2O$	20.0 g	8.41×10^{-8}
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	9.0 g	7.28×10^{-9}
$CuSO_4 \cdot 5H_2O$	20.0 g	8.01×10^{-8}

2.2.3.2.3 Vitamins Solution

The thiamine HCl and cyanocobalamin were dissolved in 950 mL distilled water and the final volume was brought to 1 liter, filter sterilized, and stored in a refrigerator or freezer.

Component	Quantity used	Concentration in final medium (M)
Thiamine HCl (vitamin B1)	1.0 g	2.9×10^{-10}
Cyanocobalamin (vitamin B12)	50.0 mg	3.69×10^{-12}

2.2.4 Morphological identification

2.2.4.1 Light microscopy

The microalgal cells were observed under a light microscope (Olympus CX41) and observed for morphological characters. The cultures were microscopically monitored during all experiments. The digital images were taken with micro publishers 3.3 RTV camera and images analysed by Q-imaging software. A measuring ocular calibrated to the different magnifications (10x, 40x and 100x magnitude oculars) was used to calculate cell sizes. All microscopic observations were performed frequently to monitor the growth of the alga, and to check that cultures were free of any contaminants.

2.2.4.2 Fluorescent microscopy

The viability of cells during different nutritional regimes was examined by fluorescent microscopy (Olympus CX41, USA) using fluorescein diacetate (FDA, Sigma, India). The stock solution of fluorochrome (FDA) was dissolved in dimethyl sulfoxide (DMSO, Merck) to a concentration of 5 mg/mL and stored at 4^0C. 1 µL of FDA stock was added to 1 mL microalgal culture and incubated for 20 min at an agitation of 120 rpm and under a photon irradiance of 50±10 µmol photon/m^2/s and

visualized under fluorescent microscope (Onji et al., 2000). Fluorescence excitation and emission of FDA were at 485 nm and at 540 nm respectively (Figure 2-1).

2.2.4.3 Electron microscopic analyses

2.2.4.3.1 Scanning and transmission electron microscopy

For SEM and TEM analyses, 2 mL of microalgal cultures were harvested by centrifugation at 8000 rpm for 5 min in separate eppendorf tubes and washed twice with PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.2 4g KH_2PO_4 in 1litre of water and pH adjusted to 7.4) and centrifuged at 8000 rpm at room temperature for 5 min. The cells were fixed overnight at 4^0C in 1 mL of 2.5 % glutaraldehyde EM grade (Electron Microscopy Sciences, PA, USA) in 0.1M sodium cacodylate buffer (Himedia, India), prepared in distilled water and filter sterilized. After the incubation, cells were washed twice with 0.1M sodium cacodylate buffer and centrifuged at 8000 rpm for 15 min. The supernatant was discarded and again incubated with 1% osmium tetroxide, (OSO_4) (Electron Microscopy Sciences, PA, USA) prepared in 0.1M sodium cacodylate buffer stored at 4^0C for 3 hrs, and then washed with sodium cacodylate buffer. The cells were dehydrated with increasing concentrations of ethanol 50-100% and stored at 4^0C until use in 200 µL of new CB buffer (Durako, 2009; Moheimani, 2005; Sarokin & Carpenter, 1982). The cells were spread on SEM stubs dried in critical point drying apparatus, platinum coated and observed under JEOL Analytical Scanning Electron Microscope (JSM 6390 LV, Tokyo, Japan) at the SAIF, CUSAT, Kochi, India. Transmission electron microscopic analysis done using Hitachi H-7650 systems at Sree Chitra Tirunal Institute for Medical Sciences, Trivandrum, India.

2.2.5 Molecular identification by genomic DNA based methods

2.2.5.1 Genomic DNA isolation

The protocol for DNA extraction from marine microalgae by Cheng & Jiang (2006) was modified and used to isolate DNA from 20 ml of a liquid culture of *P.maculatum* grown in the f/2 medium at late log phase under the conditions mentioned earlier. 18 days old culture of approximate cell count of 3.4 x10^7 cells/mL were pelletized by centrifugation at 7000 g for 5 min, washed twice with Tris-EDTA

buffer (pH- 7.5) and re-suspended in 450 µL of TEG (25mM Tris-HCl; 10mM EDTA; 50 mM glucose) buffer (pH- 8) with 100 µL lysozyme (5mg/mL) and vortexed with glass beads, and 50 µL 10% sodium dodecyl sulfate (SDS) was added. The tubes were then incubated on ice for 10 min and 8 µL proteinase K (20 mg/mL) was added and incubated at 60^0C for 1 hr in a water bath. Once the cells were completely lysed, the DNA was purified following standard phenol/chloroform extraction (Sambrook et al., 1989). After that 1 mL of phenol: chloroform: iso-amyl alcohol (25: 24: 1 v/v) mixture was added and gently inverted for 5 or 6 times, followed by centrifugation at 13000 g for 10 min at 4^0C. The aqueous phase was separated to a new microcentrifuge tube using cut tips to avoid DNA damage, washed twice with chloroform:isoamyl alcohol (24:1) by centrifuging at 13000 g for 10 min at 4^0C. The supernatant was collected in 1.5 mL of the microcentrifuge tube to which double the volume of ice cold absolute ethanol was added and incubated overnight at -20^0C followed by centrifugation at 13000g for 15 min at 4^0C. The supernatant was discarded and the pellet washed with 75% ethanol twice just by inverting the tube 4-5 times. The pellet was air dried for 1hr and re-dissolved in 50 µL of TE buffer (10 mM Tris; 1 mM EDTA of pH 8) and stored at 4^0C for further use.

The isolated DNA was quantified by spectrophotometry (A_{260}). 10 µL of isolated DNA was mixed with 990 µL of autoclaved Milli-Q water, and the absorbance at 260 nm and 280 nm each was measured using UV-visible spectrophotometer (Hitachi, Japan). A_{260}/A_{280} was calculated to determine the purity of DNA. The DNA was analysed by electrophoresis using 1% agarose gel along with 1 Kb DNA marker (New England Biolabs, USA). The isolated DNA was quantified using the following equation (2-1):

$$\text{Concentration of DNA (µg/mL)} = A_{260nm} \text{ x dilution factor x } 50 \qquad (2\text{-}1)$$

2.2.5.1 PCR-based amplification of molecular marker genes

PCR primers for 18S rRNA gene were designed using *Nannochloris* sp. 18S rRNA gene sequences from the National Centre for Biotechnology Information

(NCBI) database with accession numbers. AY220081, AB080300, AB080306 and AY195983 (Tsagkogeorga et al., 2009). The ITS (Internal Transcribed Spacer) region was amplified using ITS universal primer spanning a 700 bp containing ITS1, 5.8S, and ITS2 regions (White et al., 1990). 23S rRNA gene was amplified using Chlorophyceae specific primer (Del Campo et al., 2010). The ribulose-1, 5-bisphosphate carboxylase large subunit (rbcL) plastid gene was amplified using a primer designed using *Nannochloris* sp. rbcL sequences retrieved from NCBI database with accession no. AF446090.

Table 2-1: Details of primers used for PCR amplification, annealing temperature, and product size

Primer Name	Primer sequence	Anneal. Tm (°C)	Product size(bp)
18S F	5'-CCTGGTTGATCCTGCCAG-3'	57	1800
18S R	5'-TTGATCCTTCTGCAGGTTCA-3'		
ITS F	5'-TCCGTAGGTGAACCTGCGG-3'	52	690
ITS R	5'-TCCTCCGCTTATTGATATGC-3'		
23S F	5'- AGGGGTAAAGCACTGTTTCG-3'	59	800
23S R	5'- CCTTCTCCCGAAGTTACG-3'		
rbcL F	5'-AAGAATGTGGTGCAGCGGTAG-3'	55	800
rbcL R	5'-GGAAGTGGATACCGTGGTTAC- 3'		

PCR was conducted in a 25 µL reaction volume containing 2.5 µL of 10X buffer, 2.5 mM dNTP (2.5 µL), 1 µL of 10 pmol/µL each of forward and reverse primers, 1.0 µL of 0.5U/µL of Taq DNA polymerase and 1 µL of 50 µg/mL genomic DNA template, in a PCR thermal cycler (Eppendorf Mastercycler). The thermal cycling conditions were, an initial denaturation of 95^0C for 5 min followed by 35 cycles each consisting of a denaturation at 94^0C for 1 min, annealing at 57^0C for 1 min (18S rRNA gene), 52^0C for 45 seconds (ITS), 59^0C for 45 seconds (23S rRNA gene), and 55^0C for 45 sec for (rbcL gene), and extension at 72^0C for 1 min followed by the final extension at 72^0C for 7 min. An aliquot of 10 µL PCR product each was analyzed by 1% agarose gel electrophoresis stained with ethidium bromide and visualized under ultraviolet light, the product size was confirmed in comparison to 100 bp or 1 Kb ladder and documented using Gel Doc™ XR+ imaging system (Bio-Rad Inc., USA).

2.2.5.3 Cloning into pGEM-T easy vector

pGEM®-T Easy vector (Promega, USA; Figure 2-1) is a linearized vector with a single 3′-terminal thymidine at both ends. The T-overhangs at the insertion site greatly improve the efficiency of ligation of PCR products by preventing recircularization of the vector and provide a compatible overhang for PCR products generated by Taq DNA polymerase. This vector is a high-copy-number vector, containing T7 and SP6 RNA polymerase promoters flanking multiple cloning regions within the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide allows identification of recombinants by blue/white screening on indicator plate. The vector contains numerous restriction sites within the multiple cloning regions with recognition sites EcoRI, BstZI, and NotI, providing three single-enzyme digestions for the release of the insert. Alternatively, a double-digestion may be used to release the insert vector construction of PCR product in the pGEM-T easy vector.

The A tailed PCR product of 18S rDNA, ITS1, 23S rDNA and rbcL gene were ligated with pGEM-T Easy vector (Promega, USA) by following the manufacturer's instructions. Briefly, 10 µL ligation mixture containing 0.5 µL pGEM- T vector (50 ng/µL), 3.5 µL PCR product, 1 µL ligation buffer (10X), 1 µL T4 DNA ligase (3 Weiss units/µL) and 4 µL nuclease-free MilliQ water were incubated at 4°C overnight. This allowed the ligation of PCR products with pGEM- T easy vector.

2.2.5.4 Transformation into *E. coli* DH5α

The *E. coli* DH5α competent cells were thawed by placing on ice for 5-10 min. The ligation reaction (10 µL) was added to a sterile 15 mL culture tube already on ice, 100 µL of competent cells were transferred into the 15 mL tubes (containing ligation mix) on ice. The tubes were gently flicked to mix and placed on ice for 20 min. The cells were given heat shock at 42°C for 90 sec in a water bath and immediately returned to ice for 2 min. Super optimal broth with catabolite repression (SOC; composition for 10 mL: 0.2 g tryptone; 0.05 g yeast extract; 0.005 g NaCl, 100 µL 1M KCl; 50 µL 2 M MgCl2; 200 µL 1 M glucose) was added (600 µL). $MgCl_2$

and glucose were added just before transformation to the tubes containing cells transformed with ligation mixture, incubated for 2 hr at 37^0C with shaking at 220-230 rpm, plated 100 µL of each transformation culture onto duplicate plates containing ampicillin (100 µg/mL), Xgal (80 µg/mL) and IPTG (100mM) and the plates were incubated overnight (12-16 hr) at 37^0C.

2.2.5.5 Colony PCR for confirmation of gene insert in the selected clones

The positive clones were selected and patched on LB plate containing ampicillin (100 µg/mL), X-gal (80 µg/mL) and IPTG (100 mM) to reconfirm the transformation. All individually streaked colonies were subjected to colony PCR using vector specific T7 forward and SP6 reverse primers designed from either side of the multiple cloning site (MCS) of the vector so that whatever be the product inserted in the MCS, primer could amplify it from either side. The 25 µL PCR reaction mixture contained 2.5 µL 10X buffer, 2.5 µL dNTP (2.5 mM), 1µL Taq DNA polymerase (0.5 U/µL), pinch of colony, 1 µL of T7 forward and SP6 reverse primers each (10 pmol/µL) and the mixture was made up to 25 µL with MilliQ water. The hot start PCR programme used was initial denaturation at 95^0C for 5 min followed by holding at 80^0C for Taq DNA polymerase addition, 35 cycles of denaturation at 94^0C for 15 sec, annealing at 57^0C for 45 sec, extension at 72^0C for 1 min, followed by final extension at 72^0C for 10 min. 5 µL of PCR product was analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide, visualized and documented using gel documentation system (Gel Doc™ XR+ imaging system, Bio-Rad, USA).

2.2.5.6 Broth culture of transformed DH5 α *E. coli* cells

The positive clones *(E. coli* DH5α with recombinant pGEM-T vectors) were transferred to 10 mL LB broth with ampicillin (100 µg/µL) and incubated at 37 °C with shaking at 220 rpm (12 hr).

2.2.5.7 Plasmid extraction

Plasmid was extracted using GenElute HP Plasmid Miniprep kit (Sigma Life Sciences, USA) following manufacturer's instructions. Briefly, an aliquot of 6 mL

culture after overnight incubation was pelletized at 12,000 g for 1 min. The pellet was resuspended in 200 µL resuspension solution containing RNase A and lysed by adding 200 µL lysis buffer. An aliquot of 350 µL neutralization solution was added and centrifuged at 12,000 g for 10 min to remove the cell debris. Clear lysate was loaded into GenElute HP Miniprep binding column inserted into a microcentrifuge tube and centrifuged at 12,000 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 10 mM Tris-Cl was added and centrifuged at 12,000 g for 1 min. The plasmid DNA was stored at -20^{0}C. The purity of plasmid DNA was analysed by agarose gel electrophoresis and by determining the ratio of the absorbance at 260/280 nm in a UV-VIS spectrophotometer (U-2800, Hitachi, Japan).

2.2.5.8 Restriction digestion of plasmid DNA

The insert in the plasmid was confirmed by restriction digestion with Sal 1 or Sac 1 to linearize the plasmid (single cut) and EoR1 or Not 1 to release the insert (double cut). The PCR reaction mix contained 15.3 µL milliQ, 2 µL 10X buffer, 0.2 µL BSA, 2 µL of plasmid DNA and 0.5 µL of either of the restriction enzyme. The plasmid DNA was digested at 35^{0}C for 1 hr followed by 65^{0}C for 20 min in water bath. The insert release was checked on 1% agarose gel electrophoresis.

2.2.5.9 Sequencing and sequence analyses

The plasmid from selected positive clone each was sequenced using ABI PRISM 3700 BigDye^{TM} Sequencer (Switzerland) using T7 and SP6 primers. DNA sequences obtained from the positive clones of were assembled using the Gene Tool software (Gene Tool Lite Launcher) and checked for vector contamination using Vecscreen application from NCBI site (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The nucleotide Basic Local Alignment Search Tool (BLASTn) algorithm (Altschul et al., 1990) was used to search the GenBank database for homologous sequences.

2.2.5.10 Phylogenetic Analyses

The phylogenetic analyses of the 18S rRNA gene, ITS1 region, 23S rRNA gene and rbcL gene sequences were done by Molecular Evolutionary Genetics Analysis (MEGA 6) software (Tamura et al., 2013). The sequences were multiple aligned using the programme ClustalW2. The phylogenetic tree was constructed using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) and Maximum Parsimony tree constructed using MEGA6 software. Finally, the constructed trees were analyzed for elucidating the phylogenetic relationship of the alga (Kumar et al., 1994; Tamura et al., 2013). The sequences were submitted to GenBank database.

2.2.6 Microalgal growth under phototrophic, mixotrophic and heterotrophic conditions

The growth of *Picochlorum maculatum* MACC3 under phototrophic, mixotrophic and heterotrophic conditions were assessed in terms of biomass production and proximate composition analyses of the biomass. All the analyses were carried out using cultures in stationary phase, 18 days old for phototrophic and mixotrophic cultures, and 6 days old for heterotrophic culture.

2.2.6.1 Growth under Phototrophic Conditions

The *Picochlorum maculatum* MACC3 cells under phototrophic conditions were grown in f/2 and Walne's media (compositions mentioned in session 2.2.3.1 and 2.2.3.2). Five hundred mL Erlenmeyer flasks containing 300 mL growth media each was inoculated with *P. maculatum* MACC3 cells at an initial cell density of $3X10^4$ cells for 18 days and incubated under conditions as mentioned in session 2.2.2 5 mL samples were taken on every 3rd day for monitoring the growth in terms of cell count and optical density. All the analyses were done in triplicate

2.2.6.2 Growth under Mixotrophic Conditions

The growth of *P. maculatum*MACC3 under mixotrophic conditions were assessed using f/2 as the basal medium supplemented with seven carbon sources such as fructose (10 g/L), glucose (10 g/L), sucrose (10 g/L), sodium acetate (3 g/L)

methanol (10 mL/L), ethanol (10 mL/L) and glycerol (5 g/L) and inoculated with a 10% of stationary phase mixotrophic culture of 3.4 X 10^7 cells/mL. The microalgal cultures were maintained at a temperature and pH of 25^0C and 7.5 ± 0.2, respectively, under a photon irradiance of 50±10 µmol photon/m^2/s in 16:8 light and dark periods and agitated with 150 rpm in rotary shaker (Orbitek, Scigenics Biotech Pvt. Ltd., India). The ranges of carbon sources were selected based on literature (Cerón Garcia et al., 2000, 2006; Chen & Zhang, 1997; Heredia-Arroyo et al., 2010; Wan et al., 2011; Zhang et al., 2011)

2.2.6.3 Growth under Heterotrophic Conditions

The growth of alga under heterotrophic conditions were assessed using f/2 medium with carbon sources such as fructose (10 g/L), glucose (10g/L), sucrose (10 g/L), sodium acetate (3 g/L) methanol (10 mL/L), ethanol (10 mL/L) and glycerol (5 g/L). The media with the respective carbon sources were inoculated with 10% of heterotrophic cultures of approximate cell number of 4x10^6 cells/mL. The cultures were maintained in a shaker at 120 rpm and incubated under darkness by covering with black cloth. Stationary phase algal culture was taken on the 6th day of culture for further analyses. The algal cells did not grow in sodium acetate at a concentration above 3 g/L. The concentration of carbon sources were selected based on literature (Chen et al., 1997; Heredia-Arroyo et al., 2010; Mitra et al., 2012; Tsavalos & Day, 1994; Wen & Chen, 2003).

2.2.7 Proximate composition analyses of *P. maculatum* MACC3 biomass

The proximate composition of *P. maculatum* MACC3 was calculated as percentage of total algal dry weight (DW) from the absolute concentration for each biochemical component. The raw percentage data for moisture, ash content, biomolecules (protein, carbohydrate, lipids) and pigments (chlorophyll a, chlorophyll b, carotenoids), microalgal yield in terms of biomass per liter of culture and each biomolecule yield were calculated under various culture conditions such as phototrophic (Walne's and f/2 media), mixotrophic (glucose, fructose, sucrose,

glycerol, sodium acetate) and heterotrophic. The heterotrophic growth in glucose alone was analyzed, as the algal biomass yield was negligible in all other carbon sources.

2.2.7.1 Dry weight determination and cell counting

A 20 mL sample of culture from the each of the three replicates was filtered through pre-weighed Whatman grade GF/C glass microfiber filter paper and washed with 0.5M ammonium formate (NH_4HCO_2) solution. The filters were then oven dried overnight at 100^0C, cooled in a desiccator and weighted. The dry weight was obtained from the difference between initial and final weights. The number of cells was counted in a Neubauer chamber using an Olympus CX 41 optical microscope (Andersen, 2005; Zhu & Lee, 1997).

2.2.7.2 Moisture content

The moisture content of wet algal biomass was measured by drying a representative 2 g sample at 100 ± 5^0C for 20 hr or till constant weight was attained and the difference in weight was calculated (Tokucsoglu & Üunal, 2003)

2.2.7.3 Ash content

The ash content was determined by incineration of a representative 0.5 g of algal sample at 450^0C for 5 hr in a pre-weighed silica crucible. The residue in the crucible was weighed and the difference in weights was calculated as the ash content (Fidalgo Paredes et al., 1998; Zhu & Lee, 1997)

2.2.7.4 Biochemical analyses

The biochemical composition of *P. maculatum* MACC3 biomass was estimated from 20 mL of culture pellets. The pellet was first treated with 0.2N perchloric acid ($HClO_4$) and extracted with chloroform methanol 2:1 (v/v) to obtain lipid component. Lipid free pellets were treated with 2 mL, 2N sodium hydroxide (NaOH) at 95^0C for 10 min, and from this aliquots were drawn for protein and carbohydrate estimations (Lee et al., 1985).

2.2.7.4.1 Protein estimation by Bradford Method

The assay relies on the binding of the dye Coomassie Brilliant Blue G250 to the protein molecule. The cationic form of the dye, which predominates in the acidic assay reagent solution, has a λmax of 470 nm. The dye binds to protein as the anionic form, which has a measured λmax of 595 nm.

100 µL of alkali digested microalgal samples were treated with 5 mL of Bradford reagent (For preparing 5X concentration of Bradford reagent: 50 mg of Coomassie Brilliant Blue G250 was added to 25 mL methanol and 50 mL 85 % phosphoric acid and final volume was made up to 100 mL with distilled water), vortexed well and incubated at room temperature for 10 min and the optical density was measured at 595 nm. The bovine serum albumin (BSA) at different concentrations of 10, 20, 40, 60, 80, 100 µg/mL was used as standard for the estimation of proteins. The amount of protein was expressed in terms of protein yield in liter of culture and also in percentage of the dried biomass (Barbarino & Lourenço, 2005; Bradford, 1976; Kruger, 1994; Pandey & Budhathoki, 2010)

2.2.7.4.2 Estimation of carbohydrate by phenol-sulphuric acid method

Microalgal samples digested with NaOH were taken for carbohydrate analyses. 100 µL sample each was made up to 2 mL with distilled water, treated with 50 µL of phenol reagent (90 % phenol solution in water), mixed thoroughly and rapidly and 5 mL of sulphuric acid (H_2SO_4) was added and mixed it well, which promote heat development in the assay. The reaction mixture was allowed to stand for 30 min at room temperature and the absorbance was measured at 485 nm. The glucose at different concentrations 0, 10, 20, 40, 60, 100, 120 150, 200 µg/mL was used as standard for the estimation of carbohydrate. The weight of carbohydrate was plotted against the corresponding absorbance to generate a standard graph, concentration of carbohydrates were predicted from the graph and expressed in carbohydrate yield per liter of microalgal culture and also as percentage of the dried biomass (Fidalgo Paredes et al., 1998)

2.2.7.4.3 Quantification of total lipids by gravimetric method

The total lipids were extracted from approximately 500 mg of dry algal biomass (from 500 mL of algal culture) with 50 mL chloroform–methanol (2:1 v/v). The mixtures were transferred into a separatory funnel and shaken for 5 min. The lipid fraction was then separated from the separatory funnel and solvent allowed to evaporate. The crude lipid obtained was weighed in an electronic balance (Bligh & Dyer, 1959).

2.2.7.4.4 Estimation of lipids by sulfo phospho-vanillin (SPV) method

The lipid reacts with vanillin in presence of concentrated H_2SO_4 and phosphoric acid to form pink coloured complex which can be measured spectrophotometrically at 535 nm.

The lipid samples (50 µL) were taken in a 10 mL acid washed test tube with light cap and evaporate to dryness by keeping in 80^0C oven. The dried samples were incubated with 150 µL of 96 % sulphuric acid at 85^0C for 20 min in hot air bath in a lightly capped test tube to prevent the acid leakage. The samples were then cooled rapidly by placing in ice-water for 5 min, vortexed and transferred 100 µL of the aliquot into 96 well plates and the pre- vanillin absorbance was read at 535 nm. The micro-well plate was incubated with 50 µL vanillin solution (10 mg of vanillin in 10 mL 85% phosphoric acid and made up to 50 mL using H_2O) for 10 min in dark and recorded the post vanillin chromophore absorbance at the same wavelength (A535). The final SPV response of samples was defined as the difference between the final post-vanillin and pre-vanillin 535 nm absorbance readings. The concentrations of samples were calculated from the plot of corn oil (Sigma, India) lipid standards at concentrations of 10, 20, 50, 100, 200, 300, 400, 500 µg/mL. The amount of lipid were expressed in terms of lipid yield and percentage of lipid of the total dried algal biomass (Anderson, 1973; Frings et al., 1972; Izard & Limberger, 2003; Knight et al., 1972; McMahon et al., 2013).

2.2.7.5 Estimation of pigments

5 mL each of *Picochlorum* culture suspensions from 18 days old phototrophic and mixotrophic cultures was centrifuged at 5000 g for 5 min and the supernatant was discarded. The cells were re-suspended in 5 mL of methanol, incubated overnight in the dark and the extracts were centrifuged to remove cellular debris and made up to 5 mL depending on the actual chlorophyll concentration. The absorption spectra were measured at A_{470}, A_{653} and A_{666} in a spectrophotometer (Hitachi japan). The concentrations of chlorophylls a, b and carotenoids were determined using the equations (2-2), (2-3) and (2-4) by Hartmut (1983)

For methanol (mg/L of algal extracts)

$$Chlorophyll\ a = (15.65\ X\ \ A_{666}) - (7.3\ X\ A_{653})\ () \quad (2\text{-}2)$$

$$Chlorophyll\ b = (27.05\ X\ \ A_{653}) - (11.21\ X\ A_{666}) \quad (2\text{-}3)$$

$$Carotenoids = \left(\frac{(1000\ X\ \ A_{470}) - (2.86\ X\ Chl_a) - (129.2\ X\ Chl_b)}{245}\right) \quad (2\text{-}4)$$

2.2.7.6 Analyses of fatty acids

100 mL of algal cells were harvested and lyophilized for fatty acid analysis (stationary phase culture of phototrophic culture (Walne's and f/2); mixotrophic and heterotrophic cultures (using glucose as carbon sources). In mixotrophic and heterotrophic cultures, the algal biomass yield from a liter of culture was only half of the biomass obtained from phototrophic culture. The fatty acid methyl esters (FAME) were prepared from dried algal biomass according to (Indarti et al., 2005). The method involved the addition of a 4 mL mixture of methanol, concentrated sulfuric acid, and chloroform (1.7:0.3:2.0, v/v/v) into a tube containing 20 mg of dried algal biomass and 1 mg heptadecanoic acid (C17:0) as an internal standard. The screw-capped tubes were tightly closed with Teflon cap to avoid leakage. The tubes were heated in a water bath at 90^0C for 40 min, and then later cooled down to room temperature, and 1 mL of distilled water was added. Mixed thoroughly for 1 min and allowed to settle for the phase separation. The lower phase containing the FAME was transferred to a clean vial and passed through anhydrous Na_2SO_4 for removing the

water content completely. The solvent was removed using a rotary vacuum evaporator and residual chloroform removed by evaporation under a stream of nitrogen. The dried fatty acid was re-dissolved in HPLC grade hexane and transferred into a vial and analyzed using gas chromatography with mass spectrometry (GC-MS)

2.2.7.6.1 Instrument conditions (GC-MS)

Gas chromatographic analysis of FAME was performed on a Perkin Elmer Clarus 680 GC equipped with a mass detector (Clarus 600 T mass spectrometer) and a fused silica capillary column (Elite-5 MS column: ID-0.25 mm; length-30 m; film thickness-0.25 µm, Perkin Elmer with temperature limits: 60 to 325/350^{0}C). Samples were injected in split mode (split ratio 20:1). Helium was used as a carrier gas. The injector and detector temperatures were 60^{0}C and 200^{0}C, respectively. The temperature was programmed as follows, initially at 60^{0}C for 1 min; then 2^{0}C/ min to 200^{0}C, hold for 4 min, then 5^{0}C/min to 280^{0}C and finally hold for 10 min. Turbo mass software was used for instrument control, data acquisition and data analysis (integration, retention times and peak areas). The FAME was identified by co-chromatography with authentic commercially available FAME standards (Supelco™ 37 Component FAME Mix, Catalog No.: 47885-U, Supelco, Bellefonte, PA, USA). The fatty acids concentrations in the algal samples were quantified by comparing their peak area with that of the internal standard (C17:0).

2.2.7.7 Analysis of pigments

P. maculatum MACC3 cell pigments were analyzed using HPLC method by De la Vega et al. (2011). Briefly, 10 mL of cells were collected by centrifugation at 6000 g, and algal biomass was extracted in 10 mL methanol. The mixture was centrifuged and supernatant filtered through cellulose acetate filter (0.45µm). Chromatographic analysis of pigments was accomplished on Dionex ultimate 3000 HPLC equipped with Phenomenex Luna 5U C18 (2) column (5µm; 250 x 4.6 mm) and a flow rate of 1 mL/min. Instrument control is by Chromeleon™ Chromatography Data System (CDS) software package. The mobile phase consisted of solvent A, ethyl acetate (Merck HPLC Grade); solvent B, acetonitrile/ water (9:1

v/v). Gradient program applied was 0-16 min 0-60% A; 16-30 min 100% A; 30-35 min 100% B. Injection volume was 50 µL and pigment detection was carried out at 450 nm. Pigment peaks were identified by comparing retention times with pure carotenoids standard (Sigma).

2.2.7.8 Analysis of minerals (Metals)

Trace metal analyses of *Picochlorum maculatum* biomass at the stationary phase of culture under three nutritional modes (phototrophic (f/2 medium), mixotrophic and heterotrophic in glucose medium)) were carried out using inductively coupled plasma atomic emission spectrometry (ICP-AES). 1 g of dried microalgal biomass was completely digested with 10 mL of HNO_3 at 80^0C for 2 hrs and after digestion was made up to 50 mL with distilled water and filtered through Whatman No 1 filter paper. Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb and Zn were determined by ICP-AES. Metal analyses were carried out using Thermo Scientific icap 7000 series ICP-OES. A Multi-element standard mixture supplied by thermo was used to standardize the instrument. Metal data were obtained from 3 different analyses and each was analyzed in triplicate using ICP-AES (n = 3) (Tokucsoglu & Üunal, 2003).

2.3 Results and discussions

2.3.1 Microscopic Observations

2.3.1.1 Identification of the microalga

The microalga *Picochlorum maculatum* MACC 3 isolated from the west coast of India was successfully purified to an axenic culture. The optical and fluorescent microscopic, SEM and TEM images of the *P. maculatum* MACC3 are shown in Figure 2-3, Figure 2-4, Figure 2-5 A & B respectively. A thick cell wall and chloroplast could be visualized in the TEM image. The microalga was small green unicellular and slightly oval in shape. The algal cells approximately ranged from 1 -2 µm in width and 1.2 -2.5 µm in length depending on different growth stage. To further determine the taxonomic position, molecular phylogenetic analyses were used.

2.3.1.2 Fluorescent microscopic observation

Actively dividing heterotrophic cells of *P. maculatum* MACC3 were visualized in homogeneously bright green colour by FDA staining (Figure 2-4 B&C). The application of FDA staining is used for assessment of the viability of algae in heterotrophic culture. In the esterified state, FDA is a lipophilic, non-fluorescent molecule that diffuses freely across the plasma membrane. Nonspecific esterase hydrolyzes FDA in the cytoplasm to produce fluorescein, which is retained by the viable cells. Fluorescein fluorescence reflects the esterase activity and cell membrane integrity, both of which indicate cell viability (Dorsey et al., 1989)

The viability of the *P. maculatum* MACC3 cells was assessed under different culture conditions. The bright chlorophyll fluorescence masks the fluorescein fluorescence in the cytoplasm and prevents the proper recording of the viability of cells grown under phototrophic and mixotrophic conditions (Figure 2-4 A&D). In algal cells of poor physiological conditions or in aged algal cultures, autofluorescence of chlorophyll is lower and in such conditions the FDA staining could be visualized as a bright yellow fluorescence (Markelova et al., 2000; Onji et al., 2000; Pouneva, 1997).

FDA staining was used for viability test in green marine alga like *Enteromorpha intestinalis* which had a small lens like chloroplast, not only for distinguishing the living and dead cells but also studying the cell intermediate state and metabolic activity of the cells (Saga et al., 1987). Klut et al. (1988) studied the morphological changes of algal cells and metabolic state of cell components in 4 different taxonomical divisions using FDA based staining. FDA is extensively used in the rapid assessment of metabolic activity, ecotoxicological studies (Blanchette, 2006; Clarke et al., 2001; Correa et al., 1986; Dorsey et al., 1989; Franklin et al., 2001; Gilbert et al., 1992; Machado & Soares, 2013; Sánchez-Monedero et al., 2008), in flow cytometry (Combrier et al., 1989, 1989; Coury et al., 1995; Peperzak & Brussaard, 2011) and for assessing the microbial activity of soil ecosystems and forest soils (Sánchez-Monedero et al., 2008)

The FDA based fluorochrome studies can be effectively used to test the viability of algal cells under heterotrophic culture conditions, where, the chlorophyll autofluorescence was not masking the FDA based staining.

2.3.2 Phylogenetic Analyses

2.3.2.1 18 S ribosomal RNA gene analysis

The 18S rRNA gene was amplified as a 1791 bp sequence (Figure 2-6). The BLAST analysis of the sequence showed 98% query coverage with 99% similarity to 18S rRNA gene sequence of *Picochlorum maculatum* with accession no. AB080302. The phylogenetic analysis of the sequence (Figure 2-11) showed that it is closely related *P. maculatum* MACC3. The 18S rRNA sequence is submitted to the Genbank with accession no. KM055115.

2.3.2.2 23S ribosomal RNA gene analysis

The 23S ribosomal DNA could be amplified from *P. maculatum* MACC3 DNA as an 850 bp sequence (Figure 2-7). On the BLAST analysis, the sequence showed 100 % query coverage with 90% similarity with published 23S rRNA gene sequence of *Picochlorum eukaryotum* with accession no. X76084, class Trebouxiophyceae and the same was supported by phylogenetic analysis (Figure 2-13). The 23S rRNA gene sequences are submitted to the Genbank with accession no.KP213855

2.3.2.3 ITS region of ribosomal gene analysis

The ITS region of 500 bp could be amplified (Figure 2-8), and sequenced. The BLAST analysis of the sequence showed 97 % query coverage with 96 % similarity with published ITS sequence of *Nannochloris* sp. AICB 424 with accession No. JQ922411 of the class Trebouxiophyceae The phylogenetic tree is shown in (Figure 2-14). The ITS sequences are submitted to the GenBank with accession no.KP213856.

2.3.2.4 Ribulose bisphosphate carboxylase large chain (rbcL) gene analysis

The rbcL gene of 800 bp was amplified (Figure 2-9) and sequenced. The BLAST analysis of the sequences showed 100 % query coverage with 94% similarity with published rbcL sequence of *Nannochloris* sp. with accession no. AF446090 and 100 % query coverage with 91% similarity with a rbcL sequence of *Picochlorum oculatum* strain UTEX LB, with accession no EF113455, class Trebouxiophyceae. The phylogenetic tree is shown in (Figure 2-12). The rbcL gene sequence is submitted to the Genbank with accession no. KP190042.

A detailed phylogenetic analysis based on 18S ribosomal DNA, actin gene and ITS region of a great number of *Nannochloris*-like algae carried out by (Henley et al., 2004; Yamamoto et al., 2001) have provided a new taxonomic classification of these related taxa. Based on this phylogenetic analysis, cell division pattern and habitat, most marine strains previously assigned as *Nannochloris* or *Nannochlorum* should really be included in a new genus designated *Picochlorum* (De la Vega et al., 2011).

The sequence and phylogenetic analyses of 23S rDNA, ITS region of rDNA and rbcL gene placed the new strain isolated from marine habitat in the *Picochlorum* sub-clade and class Trebouxiophyceae. Further, phylogenetic analysis based on 18S rDNA showed maximum similarity to *Nannochloris maculata,* which is recently named as *Picochlorum maculatum* based on GenBank data. Moreover, based on the results, the microalga previously identified as *Nannochloris* was confirmed as *Picochlorum maculatum* MACC3. The sequences used for these phylogenetic analyses are widely applied for the identification of many species on different algal taxonomical levels. For higher plants and algae, chloroplast DNA (cpDNA) variation is commonly used for molecular and phylogenetic analysis (Despres et al., 2003). But the low evolutionary rate of cpDNA limits its use in the identification of organisms to genus and species level. The rbcL gene was found to be able to distinguish the algal species in this study. In addition, the ITS region is more variable than 18S rDNA (Hall et al., 2010; Mai & Coleman, 1997; Saunders & Kucera, 2010; Schlotterer et al.,

1994; Wolf et al., 2005). The combination of these two regions is widely used to identify organisms in the taxa below the species level.

2.3.3 Proximate composition analyses

Under phototrophic culture conditions, the maximum algal biomass and cell density of *P. maculatum* MACC3 at the stationary phase on the18th days were 1.4 g DW/L and 3.4±2 x 10^7 cells/mL of culture, respectively. In mixotrophic and heterotrophic cultures, the cell counting was difficult as the cells were aggregated due to the addition of carbons sources. In heterotrophic cultures, the pigments were not at detectable levels. The proximate composition analyses of the biomass under all culture conditions were done in triplicate, and data is presented in the Table 2-2 as a mean value with standard deviation.

Table 2-2: Proximate composition of *P. maculatum* MACC3 biomass grown under different nutritional modes Phototrophic (Walne's and f/2 media), Mixotrophic (glucose, fructose, sucrose, glycerol, sodium acetate) and Heterotrophic (glucose)

	P-Walne's	P-F/2	M-Glucose	M-Fructose	M-Sucrose	M-Glycerol	M-Sodi Ace	H-Glucose
Biomass	1.40 ± 0.04	1.31 ± 0.02	1.10 ± 0.00	1.04 ± 0.04	0.95 ± 0.01	0.51 ± 0.3	0.53 ± 0.01	0.51 ± 0.01
Moisture content	7.1 ± 0.19	5.0 ± 0.28	6.6 ± 0.29	9.2 ± 0.16	6.4 ± 0.20	8.2 ± 0.73	8.4 ± 0.29	8.7 ± 0.94
Ash Content	9.2 ± 0.32	8.2 ± 0.49	10.1 ± 0.62	9.2 ± 0.10	10.2 ± 0.19	11.2 ± 0.89	8.8 ± 0.69	9.9 ± 1.42
Carbohydrates	13.1 ± 0.44	14.6 ± 0.54	21.6 ± 1.16	20.1 ± 1.04	21.8 ± 1.14	16.5 ± 2.06	16.4 ± 0.30	22.4 ± 0.28
Proteins	30.4 ± 1.41	35.7 ± 0.41	29.2 ± 0.50	27.2 ± 2.47	26.1 ± 0.98	25.3 ± 0.85	30.2 ± 3.13	27.0 ± 1.33
Lipids	27.7 ± 0.36	30.1 ± 0.31	22.4 ± 0.89	20.4 ± 0.88	22.2 ± 2.47	28.1 ± 1.90	28.9 ± 1.20	30.5 ± 2.35
Carotenoids	1.84 ± 0.18	2.10 ± 0.08	0.86 ± 0.01	0.21 ± 0.01	0.85 ± 0.12	0.84 ± 0.01	0.67 ± 0.04	-
Chlorophyll a	1.78 ± 0.05	1.95 ± 0.08	0.64 ± 0.14	0.18 ± 0.01	0.83 ± 0.06	0.88 ± 0.05	0.62 ± 0.04	-
Chlorophyll b	0.98 ± 0.05	0.98 ± 0.07	0.17 ± 0.02	0.06 ± 0.00	0.41 ± 0.07	0.21 ± 0.07	0.12 ± 0.04	-

P- Phototrophic, M- Mixotrophic, H- Heterotrophic

2.3.3.1 Biomass yield

The phototrophic growth of *P.maculatum* MACC3 in Conway and f/2 media gave 1.40, 1.31 g/L of dried algal biomass respectively. In mixotrophic growth, the biomass yield for each carbon source was in the order of glucose (1.10 g/L), fructose (1.02 g/L), sucrose (0.95 g/L), with very low biomass yield in sodium acetate (0.53 g/L), glycerol (0.51 g/L) methanol (0.51 g/L) and ethanol (0.53 g/L) respectively. In heterotrophic conditions the biomass yields for carbons sources were in the order of glucose (0.50 g/L), fructose (0.47 g/L), sucrose (0.48 g/L), methanol (0.34 g/L), ethanol

(0.40 g/L), glycerol (0.47 g/L) and sodium acetate (0.42 g/L) respectively ($p< 0.5$). The proximate composition analyses of heterotrophic with glucose alone were analyzed (Figure 2-15. and Table 2-2) and pigments were below detectable levels moisture and ash content is shown in figure 2-16 and 2-17.

2.3.3.2 Carbohydrates

In phototrophic conditions, the biomass obtained in Walne's medium was 185 g/L and in the f/2 medium was 190 g/L ($p> 0.05$). In mixotrophic conditions using glucose, fructose, sucrose, glycerol and sodium acetate the biomass were 224 mg/L, 225 mg/L, 298 mg/L, 95 mg/L, 87 mg/L respectively ($p<0.05$). In a heterotrophic medium, the carbohydrate yield was 113 g/L (Figure 2-18 and Table 2-22). In *P. atomus* 250 mg/g of carbohydrate was reported in phototrophic culture (Von Alvensleben et al., 2013).

2.3.3.3 Protein

In phototrophic growth in Conway and f/2 media, the maximum protein yields were 428 and 466 respectively, which was 30 and 35 % of the total biomass ($p<0.05$). In mixotrophic growth the protein yields were 319 mg/L, 283 mg/L, 247 mg/L, 128 mg/L, 160 mg/L for glucose, fructose, sucrose, glycerol and sodium acetate with 29%, 27%, 26%, 25 % and 30 % of the total biomass dry weight respectively ($p< 0.05$). In heterotrophic growth with glucose, the protein yield was 137 mg/L, which was 25 % of the cell dry weight (Figure 2-19 and Table 2-2). In *P. atomus* 300 mg/g of protein was reported in phototrophic culture (Von Alvensleben et al., 2013).

2.3.3.4 Lipids

The lipid yields under phototrophic growth were 390 mg/L and 391 mg/L in Conway and f/2 media, which was 28 and 30 % of the total algal biomass ($p>0.05$). Under mixotrophic conditions, the lipid yields were 244 mg/L (glucose), 213 mg/L (fructose), 210 mg/L (sucrose), 142 mg/L (glycerol) and 153 mg/L (sodium acetate) ($p<0.05$). In the heterotrophic culture, lipid yield was 155 mg/L (Figure 2-20 and Table 2-2). Based on high lipid content and lipid productivity of *P. maculatum* MACC3 in f/2 medium, it was used for further experiments.

2.3.3.5 Pigments estimation

In phototrophic cultures in Walne's and f/2 media, the carotenoid contents were 26 mg/L and 28 mg/L, chlorophyll *a* 25 mg/L and 26 mg/L, and chlorophyll *b* 14 mg/L and 13 mg/L respectively. In mixotrophic growth, carotenoid contents were 9.3 mg/L, 2.18 mg/L, 8.3 mg/L, 8.3 mg/L, 4.3 mg/L and 3.6 mg/L in media supplemented with glucose, fructose, sucrose, glycerol and sodium acetate respectively ($p< 0.05$), whereas the chlorophyll *a* concentrations were 7 mg/L, 1.8mg/L, 7.85 mg/L, 4.4 mg/L, 3.3 mg/L respectively ($p<0.05$). Chlorophyll *b* concentrations of 1.87 mg/L, 0.61mg/L, 3.89 mg/L, 1.05 mg/L, 0.063 mg/L were obtained in media with glucose, fructose, sucrose, glycerol and sodium acetate ($p<0.05$). The carotenoids were found higher in the present analysis, might be because the analyses were done towards the end of exponential phase of the culture, when the proportional biochemical composition of the algal cells changed as well as the medium composition. In heterotrophic growth, the pigments were in below detectable levels as the cultures were grown in darkness. The details of pigments are shown in Figure 2-21, Figure 2-22, Figure 2-23 and Table 2-2.

2.3.3.6 Fatty acid analyses

The fatty acid methyl ester analyses of *P. maculatum* MACC3 cultures grown under phototrophic, mixotrophic and heterotrophic conditions are given in Table 2-3. For mixotrophic and heterotrophic conditions, the FAME analyses were carried out only for those cultures supplemented with glucose. In all culture conditions, the same fatty acid composition was observed with a variation in concentration with the nutritional mode. The fatty acids identified were lauric Acid (LA: C12:0) myristic acid (MA: C14:0), palmitic acid (PA: C16:0), palmitoleic acid, (C:16:1), stearic acid (SA: C:18), oleic acid (OA: C18:1n9), linoleic acid (LA: C18:2n6), γ-linolenic acid (GLA: C18:3n3), α-linolenic acid (ALA: C18:3n6), eicosatetraenoic acid (ETA: C20:4n3) and eicosapentaenoic acid (EPA: C20:5n3). The PUFA yield under phototrophic, mixotrophic and heterotrophic conditions was 200 mg/L, 111 mg/L and 99 mg/L respectively comprising 51%, 50% and 56% of the total lipid and 15%, 11% and 15%

of the total biomass. In all the culture conditions, gamma linolenic acid (GLA) was the major PUFA contributing to 26% (105 mg/L), 31% (78 mg/L) and 29% (56 mg/L) of the total lipids in phototrophic, mixotrophic and heterotrophic conditions respectively (Table 2-3; Figure 2-24). The ANOVA results showed a significant difference in fatty acid content between cultures grown under different nutritional modes ($p< 0.05$).

Thus, *P. maculatum* MACC3 oil could be a good source of essential fatty acids and can be recommended for food and feed industry because of the high GLA and PUFA contents. The fatty acid yield of *P. maculatum* MACC3 has reflected its nutritional mode with decreasing the concentration of PUFA from autotrophic via mixotrophic to heterotrophic. Interestingly, the same tendency was found in another chlorophyte *Chlamydomonas* sp., which showed autotrophy as the main nutritional mode in situ (Poerschmann et al., 2004; Viña, 2002) Regardless of the Chlorophytes, the inclination of declining PUFA concentrations with increasing phagotrophy is possibly due to a biochemical response to the metabolic adaptations imposed on organisms that change their growth pathway (Viña, 2002).

In *P. maculatum* MACC3, the total PUFA contents were 51, 50, 56% of the total lipid yield under phototrophic, mixotrophic and heterotrophic conditions, in contrast to 39% in *Chlorella*, 24% in *Isochrysis* and 23% in *Spirulina* under phototrophic conditions as reported by Tokucsoglu & Üunal (2003). Zhu & Dunford (2013) reported 46% of PUFA of total lipids in the phototrophic culture of *Picochlorum oklahomensis* less than the value of 51% in the present study in the same nutritional mode. *P. maculatum* MACC3 had 28% lipid under the phototrophic conditions, 21% in mixotrophic and 26% in heterotrophic conditions with glucose as carbon source. The comparative nutrition study in *Chlorella vulgaris* reported that cells accumulated 38% of lipids under phototrophic conditions, whereas, 21% in mixotrophic and 23% in heterotrophic culture with glucose as the sole carbons source (Liang et al., 2009). Obligate photo-trophic nutrition is common in the algal world. But some microalgae can grow faster on organic carbon sources in laboratories in the dark conditions (Cerón Garcia et al., 2000, 2006; Chen et al., 1997; Heredia-Arroyo et al., 2010, 2011; Kitano et al., 1997; Mitra et al., 2012; Ngangkham et al., 2012; Tsavalos & Day, 1994; Zhang et al., 2011).

In some mixotrophic microalgae like *Chlorella vulgaris*, the concentration of glucose is the medium is a critical factor for its growth. 1-2% glucose improves the cell growth significantly as compared to the 5-10 % as higher concentrations exert an inhibitory effect on the cell growth. The same trend was observed in *Chlorella protothecoides* when grown in the glucose concentration higher than 6% (Xiong et al., 2008). Docosahexaenoic acid (DHA) producing marine microalga *Schizochytrium limacinum* grew well on glycerol (Pyle et al., 2008).

Table 2.3: Fatty acids content of *P. maculatum* MACC3 during stationary growth phase
Phototrophic (P), mixotrophic (M) and heterotrophic (H)

	Fatty Acids	Fatty acids yield (mg/L)			Fatty acid content of biomass (%)			Fatty acid content of total lipids (%)		
		P	M	H	P	M	H	P	M	H
C12:0	Lauric Acid (LA)	5.2± 0.2	4.90± 0.4	1.38± 0.1	0.71	0.84	0.29	2.2	3.8	3.23
C14:0	Myristic Acid (MA)	10.8± 0.2	9.31± 0.3	8.13± 0.2	0.83	0.98	1.17	2.75	4.42	4.31
C16:0	Palmitic Acid (PA)	62.3± 0.4	23.87± 0.3	13.8± 0.2	4.78	2.17	1.99	15.86	9.75	7.36
C:16:1	Palmitoleic acid	38.3± 0.2	23.84± 0.2	25.3± 0.2	2.94	2.17	3.63	9.74	9.73	13.42
C:18	Stearic acid (SA)	20.8± 0.3	15.38± 0.4	20.8± 0.4	1.60	1.40	2.99	5.30	6.28	11.04
C18:1n9	Oleic Acid (OA)	36.1± 0.2	35.3± 0.2	12.8± 0.4	2.77	3.22	1.84	9.19	14.45	6.81
C18:2n6	Linoleic (LA)	70.8± 0.2	12.35± 0.4	33.8± 0.2	5.43	1.12	4.85	18.01	5.04	17.94
C18:3n3	α-Linolenic Acid (ALA)	8.99± 0.3	20.99 ± 0.4	8.99± 0.4	0.69	1.91	1.29	2.29	8.57	4.77
C18:3n6	γ-Linolenic Acid (GLA)	105 ± 0.7	78.13± 0.8	56.1± 0.8	8.05	7.12	8.06	26.70	31.91	29.78
C20:4n3	Eicosatetraenoic acid (ETA)	7.83± 0.3	6.83± 0.3	2.38± 0.2	0.75	0.83	0.40	1.88	2.58	1.3
C20:5n3	Eicosapentaenoic acid (EPA)	9.33± 0.2	6.18± 0.4	5.34± 0.3	0.71	0.56	0.77	2.37	2.52	2.83
	Saturated FA (SAFA)	94.4± 3.3	50.07± 3.4	42.8± 3.2	7.20	4.56	6.14	23.90	20.45	22.71
	Monounsaturated FA (MUFA)	74.2± 3.4	59.2± 3.6	38.1± 4.2	5.70	5.39	5.47	18.93	24.19	20.23
	Polyunsaturated FA (PUFA)	200± 2.2	111.5± 7.1	99.0± 2.2	15.32	11.25	15.16	50.85	50.43	56.05
	Total	368 ± 8.1	220± 9.2	180 ± 9.8	28.23	21.20	26.78	93.68	95.06	99.00

2.3.3.7 Pigment profile of *P. maculatum* MACC3

The chromatogram of pigment profile of *Picochlorum maculatum* MACC3 under phototrophic (Figure 2-25) and mixotrophic conditions supplemented with glucose (Figure 2-26) showed that the major carotenoid found under both the conditions was lutein followed by α- carotene, β- carotene, neoxanthin, zeaxanthin, and violaxanthin.

Chlorophyll-*a* is the primary photosynthetic pigment in all algae and is the only chlorophyll of the cyanobacteria and the Rhodophyta. Analogous to all higher plants, Chlorophyta and Euglenophyta contain chlorophyll-*b* as well (Raven, 1987). The total amount of chlorophyll in algae is in the range of 0.5–1.5% of dry weight.

The average concentration of carotenoids in algae amounts to about 0.1–0.2% of the dry weight (Del Campo et al., 2007). Under appropriate culture conditions, a much higher concentration of β-carotene (up to 14% per dry weight) could be found in the unicellular, wall-less Chlorophyceae *Dunaliella* sp. (Hosseini Tafreshi & Shariati, 2009; Neidhardt et al., 1998).

De la Vega et al. (2011) reported that high light intensity induced a strong decrease in the violaxanthin content while zeaxanthin was extraordinarily increased to reach a content of 1.8 mg/g. Baroli & Niyogi (2000) reported that zeaxanthin production was also stimulated, by a minor extent, by other stress conditions such as high salinity or nitrogen starvation. The fact that the content of zeaxanthin in algae is increased under different stress conditions is due to the well-known xanthophyll cycle, catalyzing the conversion of violaxanthin into zeaxanthin under stress conditions (Demmig-Adams et al., 1996). Under normal conditions, the presence of zeaxanthin was not detected in most of the chlorophytes such as *Chlamydomonas reinhardtii* (Niyogi et al., 1997) or *Chlorella zofingiensis* (Del Campo et al., 2004; Chen & Wang, 2013), but the content of pigment increased when the cells were under stress.

Many studies have shown that dietary intakes of zeaxanthin and lutein reduced the risk of chronic eye diseases, including age-related macular degeneration (AMD) and cataracts (Carpentier et al., 2009). While lutein is a common carotenoid found in most fruits and vegetables, zeaxanthin is present only in very small quantities in most of them (Ribaya-Mercado & Blumberg, 2004). A microalga with high content in both lutein and zeaxanthin should be desirable as a source for natural eye vitamin supplements. Some chlorophyte microalgae, such as *Muriellopsis* sp. and *Picochlorum* sp. HM1 with high content in this pigment have been proposed as a source of lutein (Del Campo et al., 2007; De la Vega et al., 2011). *Picochlorum maculatum* MACC3 with a high content of both lutein and zeaxanthin can be used as a potential source for natural eye vitamin supplements. Moreover, lutein is an antioxidant with potential applications in aquaculture as a carotenoid source for fish larvae.

2.3.3.8 Mineral analyses

The minerals in *Picochlorum maculatum* under different nutritional conditions are given in Table 2-4 & Figure 2-27. The phototrophic culture was in f/2 medium and mixotrophic and heterotrophic cultures were in f/2 medium supplemented with glucose.

The major mineral constituents of *P. maculatum* MACC3 were Mg and Fe, and Ni, Cd, and Pb were the least. The mineral content was the maximum of 366 µ/g under phototrophic growth conditions, followed by mixotrophic 359 µ/g and heterotrophic 190 µ/g (p<0.05).

Table 2-4: Mineral composition of *P. maculatum* MACC3

Mineral composition of alga presented in (µ/g) of algal biomass

	Phototrophic	Mixotrophic	Heterotrophic
Cadmium (Cd)	0.029 ± 0.001	0.030 ± 0.001	0.034 ± 0.004
Cobalt (Co)	0.101 ± 0.007	0.025 ± 0.001	0.025 ± 0.002
Chromium (Cr)	0.773 ± 0.028	1.230 ± 0.087	1.224 ± 0.049
Copper (Cu)	3.051 ± 0.018	1.020 ± 0.007	1.063 ± 0.028
Nickel (Ni)	-	-	-
Lead (Pb)	0.084 ± 0.007	0.221 ± 0.014	0.275 ± 0.014
Zinc (Zn)	9.080 ± 0.036	3.370 ± 0.086	13.249 ± 1.414
Iron (Fe)	133.691 ± 0.100	81.618 ± 2.121	47.508 ± 2.828
Magnesium (Mg)	211.897 ± 2.121	267.634 ± 7.071	125.795 ± 1.414
Manganese (Mn)	7.422 ± 0.028	4.458 ± 0.425	1.151 ± 0.175
Total	**366.128**	**359.606**	**190.325**

The wide-ranging algal products supplement the daily intake of some trace elements for adults: Fe, 10-18 mg; Zn, 15 mg; Mn, 2.5-5 mg and Cu, 2-3 mg. Seaweeds contained high proportions of ash (21.1- 39.3%) and sulphate (1.3 -5.9%). In brown algae, ash content (30.1-39.3%) was higher than in red algae (20.6- 21.1%). (Akoh & Min, 2008; Mabeau & Fleurence, 1993; Rupérez, 2002)

Most of the microalgae which are living in an aquatic biotope are capable of accumulating heavy metals at concentrations in different orders and magnitude higher

than those present in the surrounding. In algal cells, saturation with heavy metals usually will be reached within 24 hr (Kratochvil & Volesky, 1998; Richmond, 2008). A higher amount of various heavy metals was a major problem encountered in the large-scale production of algae of the algal biomass. As per the WHO/ FAO guidelines, an adult person of 60 kg body weight should not include more than 3 mg of lead, 0.5 mg of cadmium, 20 mg of arsenic, and 0.3 mg of mercury per week through food and beverages (Becker, 2004; Committee, 2004; Habib et al., 2008).

There are no distinctive levels exist, for toxic metals in microalgae; the concentrations are variable, with culture condition, culture media, and contaminations during processing, or even improper analytical techniques. At present, no official standards exist for the heavy metal content of microalgal products. On a voluntary basis, some algae manufacturers have established internal guidelines for metal levels in their products. As per IUPAC guidelines (1974), the permissible limits are Pb- 5.0 ppm, Cd- 1.0 ppm and Hg- 0.1 ppm and As- 2.0 ppm in single cell proteins (SCP) (Committee, 2004). The maximum permissible weekly intake is Pb- 3 mg, Cd- 0.5 mg, Hg- 0.3 mg and As- 2.0 mg as per the WHO 1972 guidelines (FAO/WHO, 1994).

Most of the green algae are photoautotrophic primary producers and they use light energy to synthesize energy-rich organic molecules during photosynthesis. Some microalgae are heterotrophic organisms depending on the organic carbon from their nutritional media. But some organisms fit more than one trophic level combining the abilities for photosynthesis as well as heterotrophic nutrition. They act like primary producers and consumers. These particular nutritional modes have been developed by organisms to overcome the adverse situations (Boëchat et al., 2007; Jones, 1994). Mixotrophs can utilize both photosynthetic ability and nutrition uptake capacity, even though it is high energy costing, but it possesses the nutritional advantage to survive in adverse conditions (Rothhaupt, 1996). These types of changes in the nutritional regime have a tremendous impact on the metabolic pathways and it may alter the biochemical composition, as reported for a fatty acid profile of green alga *Chlamydomonas* sp. growing in acidic conditions. The nutritional quality of planktonic dependent organisms has been associated with high unsaturated fatty acids

and sterols (Ahlgren et al., 2009; Allard et al., 2011; Brett & Müller-Navarra, 1997; Poerschmann et al., 2004; Sleigh, 2000)

2.4 Conclusions

In most of the studied strains of algae, the lipid production is tightly coupled to cell stress. Consequently, an ideal candidate should display high biomass coupled with stable lipid production and require strain specific investigations on culturing conditions and genetics. The present study identified *Picochlorum maculatum* MACC3 as a new candidate with potential applications in aquaculture and neutraceutical industries. The higher biomass productivity, specific growth rate, protein content, favorable PUFA and carotenoid profiles, and a lower generation time of *P. maculatum* MACC3, point to the commercial prospects of this marine microalga. Preliminary data on this newly identified *Picochlorum* shows the need for further research and investigations to obtain optimal biomass, specific growth rate and high amounts of fatty acids, amino acids, minerals, pigments and carbohydrates and an optimized biomass harvesting technique for large-scale culture for industrial applications. Moreover, genetic studies including engineering of lipids and polyunsaturated fatty acid biosynthesis will help explain some of the mechanisms underlying the biological activities of this newly isolated *Picochlorum* species.

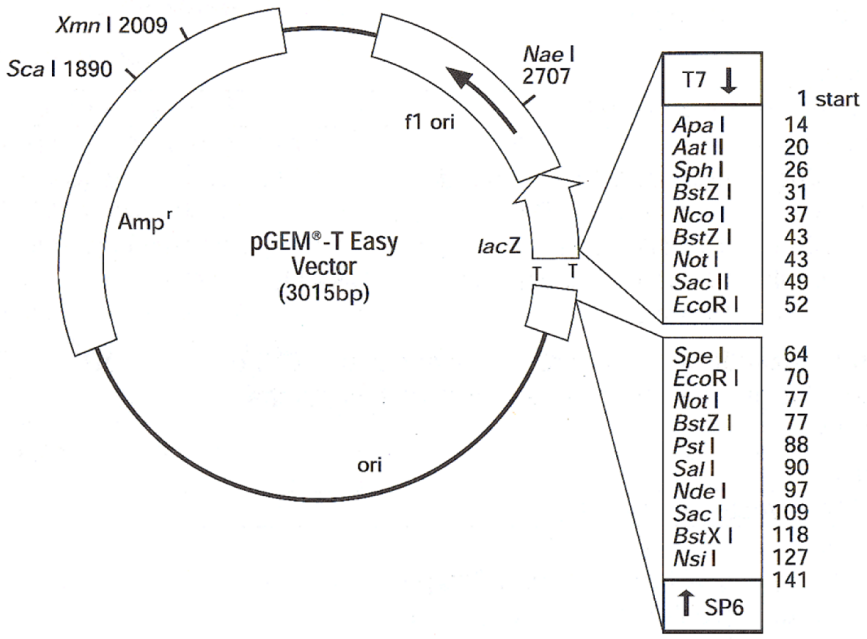


Figure 2-1: pGEM T- Easy vector map

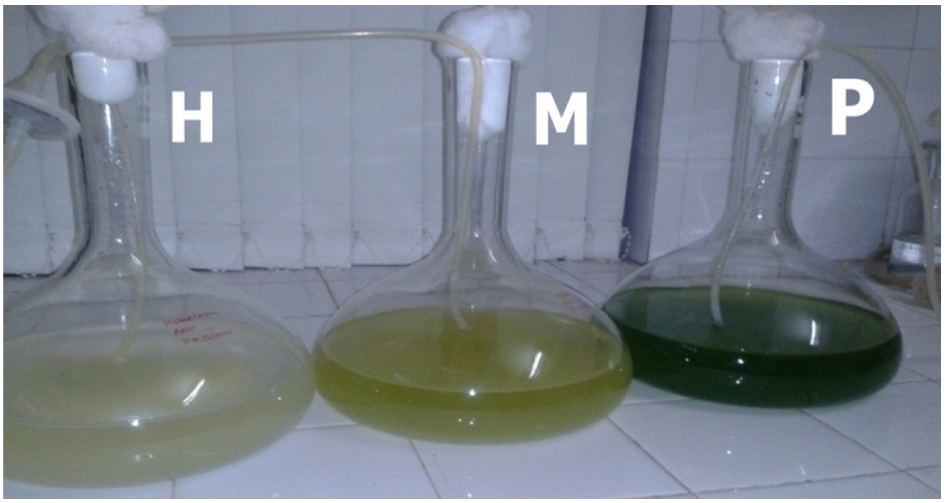


Figure 2-2: Growth of *P. maculatum* MACC3 under Phototrophic (P), Mixotrophic (M) and Heterotrophic (H) culture conditions during stationary phase of growth

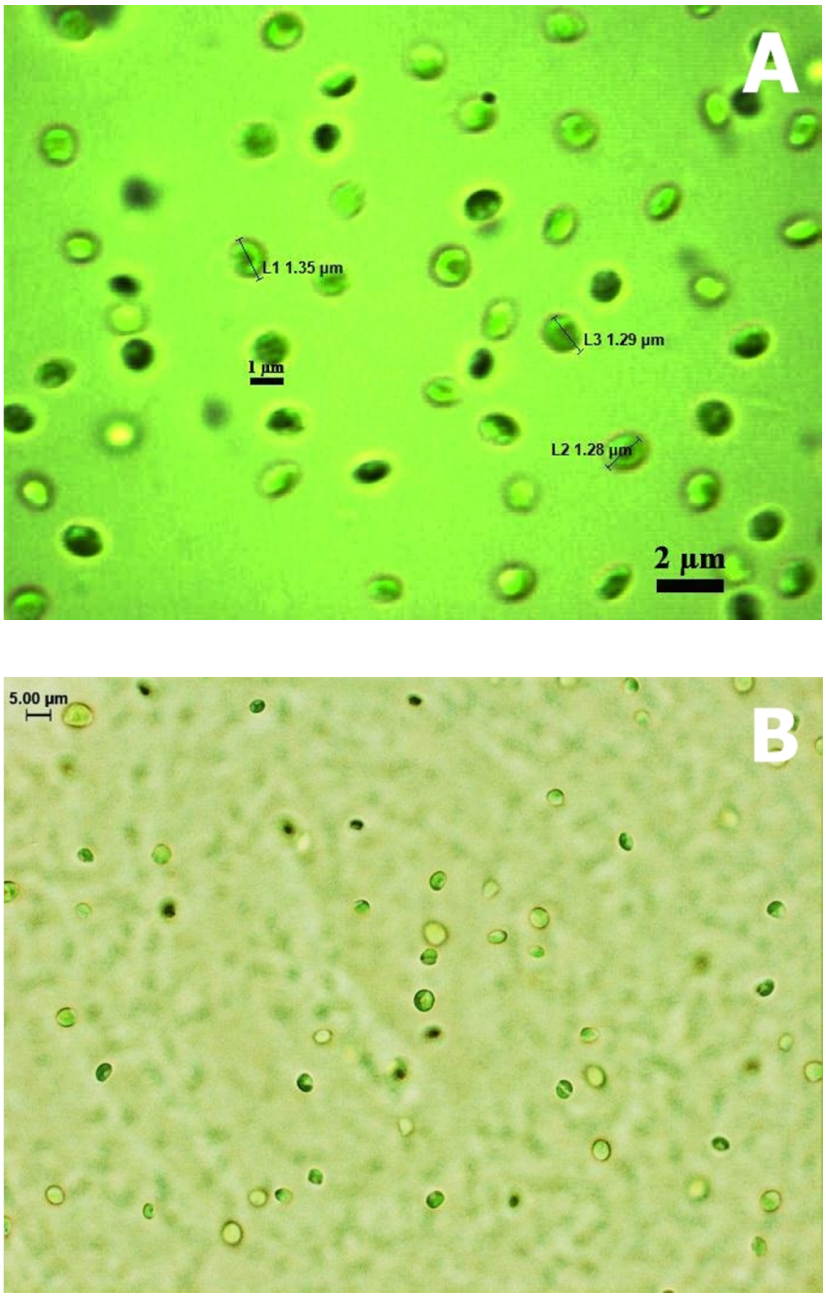


Figure 2-3: *Picochlorum maculatum* MACC3 under a light microscope.

(A) 100X and (B) 40X

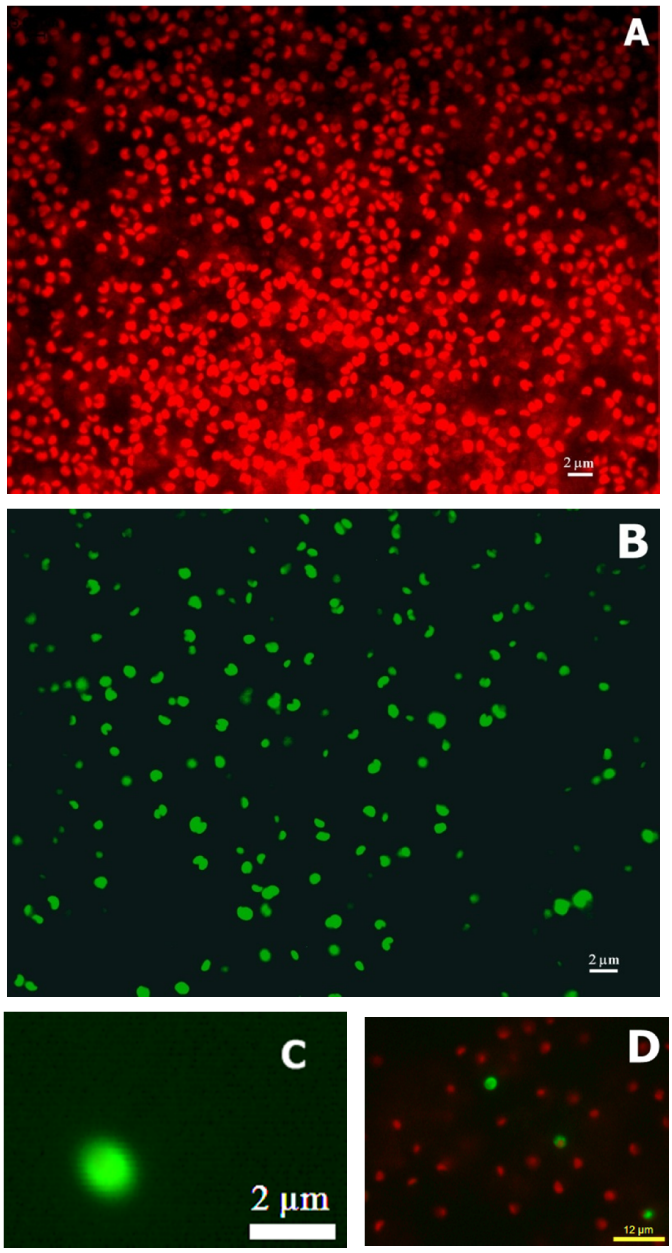


Figure 2-4: Fluorescent microscopic image of *Picochlorum maculatum* MACC3

(A) Autofluorescence of chlorophyll in phototrophic cell culture concentrates (40X)
(B) Green fluorescence of fluorescein diacetate (FDA) in heterotrophic cell culture concentrates (40X)
(C) *P. maculatum* MACC3 cell stained with FDA (100 X) **(D)** Phototrophic *P. maculatum* MACC3 cells stained with FDA Green colour (live cells) Orange colour (dead cells) (40 X)

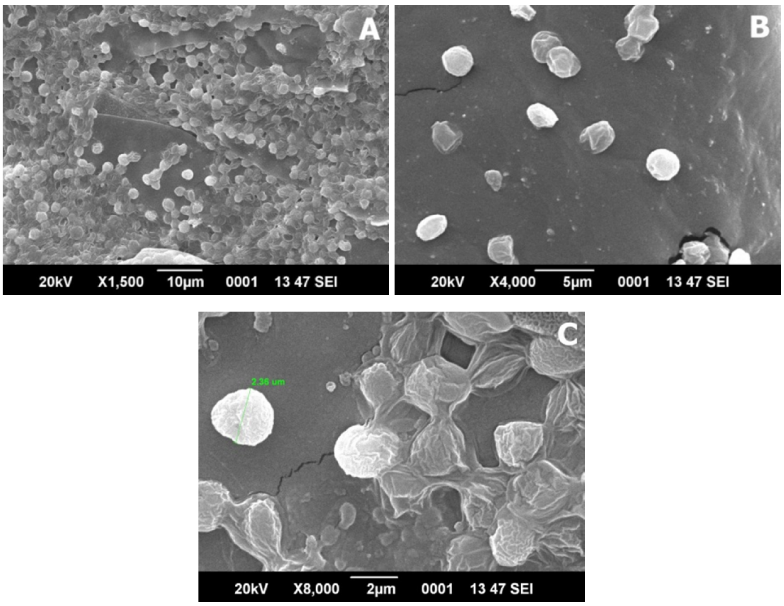


Figure 2-5A: Scanning Electron Micrographs of *Picochlorum maculatum* MACC 3
(A) 1500X, (B) 4000X & (C) 8000X magnification

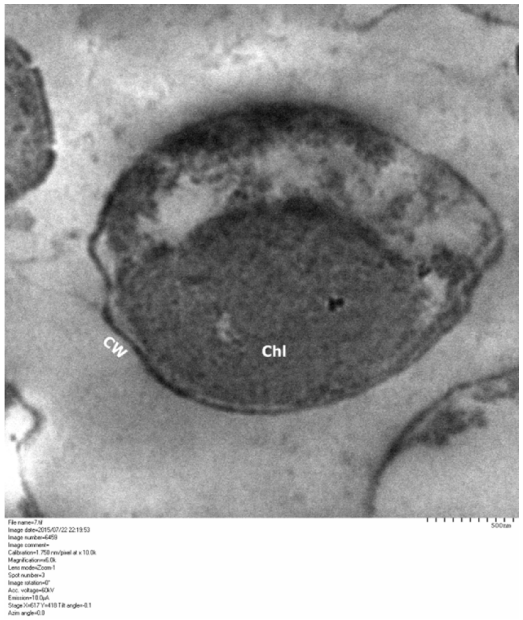
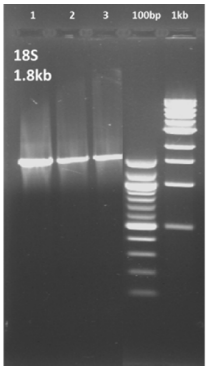


Figure 2-5B: Transmission Electron Micrographs of *Picochlorum maculatum* MACC 3
6000X magnification (Chl- Chloroplast; CW- Cell wall)



Picochlorum maculatum 18S ribosomal RNA gene, partial sequence, strain MACC3

cctggttgatcctgccagtagtcgtatgcttgtctcaaagattaagccatgcatgtctaagtataaaactgctttata
ctgtgaaactgcgaatggctcattaaatcagttatagtttatttgatggtacctacttactcggatacccgtagtaa
ttctagagctaatacgtgcgtacatcccgacttctggaagggacgtatttattagataaaaggccgaccgggct
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gcgtatatttaagttgctgcagttaaaaagctcgtagttggatttcgggtggggcctgccggtccgccgtttcg
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ggcgaggttactttgagtaaattagagtgttcaaagcagcctacgctctgaatacattagcatggaaataacac
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tatttcattgtcagaggtgaaattcttggatttatgaaagacgaactactgcgaaagcatttgccaaggatgtttt
cattaatcaagaacgaaagttgggggctcgaagacgattagacaccgtcctagtctcaaccataaacgatgc
cgactagggatcggcgggtgttttttttgatgaccccgccggcaccttatgagaaatcaaagtttttgggttccg
gggggagtatggtcgcaaggctgaatattaaaggaattgacggaacggcaccaccccgcgtggagcctgc
ggcttaatttgactctacccgggaaaacttaccagctcctcacatattgttgattcacacatttagagctctttctt
gcttctatgggtggtggtgcatggccttttattatttggtgggttgccttgtcaggttgattccggtaacgaacgag
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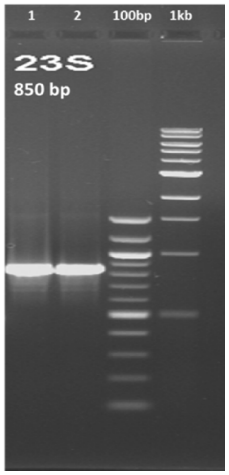
attgcaattattaatcttcaacgaggaatgcctagtaggcgcaagtcatcagcttgcgtcgattacgtccctgcc
ctttgtacacaccgcccgtcgctcctaccgattgggtgtgctggtgaaatgttcggattggcggcgtcgtgcg
gtttccgcgcgtcgctgtcgagaagttcattaaaccctcccacctagaggaaggagaagtcgtaacaaggttt
ccgtaggtgaacctgcggaaggatcat

Figure 2-6: PCR products of the 18 S rRNA gene amplified

Yellow and green colour area are forward and reverse primer binding sites



Picochlorum maculatum 23S ribosomal RNA gene, partial sequence, strain MACC3.

aggggtaaagcactgtttcggtgcgggcggcgaaagctgtaccaaatcgtggcaaactctgaatactagatatgctattt
tctggccagtgagacagtgggggataagcttcattgtcaagagggaaacagcccagatcactagctaaggccccaaa
atgatcgttaagtggcaaaggcggtgagaatgcttagacagccagaaggtttgcttagaagcagccatcctttaaagag
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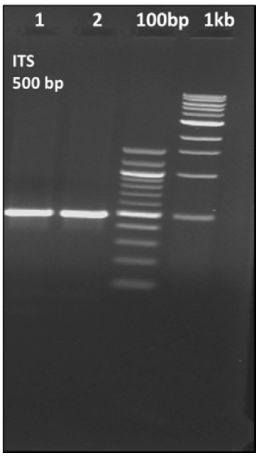
ggtaccgagggacggagaaggtagagatttgctgttattggatttcagtgcaagtcttcgaggtgttgagaggtagaag
aaaaactatccttgagctaagaaacgatgcgcacttacttttaagtaagtgtttattatctatcatgcttccaagaaaagctc
gaactactgttaaatataaaatacctgtaccgtaaaccgacacaggtaggttagtagagtatactaaggggcgcgaga
taactctctctaaggaactcggcaaaatgacccgtaacttcgggagaagg

Figure 2-7: PCR products of the 23S ribosomal RNA gene amplified



Picochlorum maculatum internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2,partial sequence, strain MACC3
tccgtaggtgaacctgcggaaggatcattgaatcgatcaaacacacaccgcgaacacg
cgcgacgcgacctcgtttgttgtccgggatcgcgtcgtcgtcgtcgttgttgtttccgttcc
gtcgacagctatcactgctgtggtcggcaacgcactccacacgtgtcaaacgccactctg
aagccgacgtctcgtgtcgtgtcttgggggggttggtcgtgggcttcgcgctccgcgtcc
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cgtgaaccatcgaagttttgaacgcaccttgcgcccgaggcttcggccgagggcatgtc
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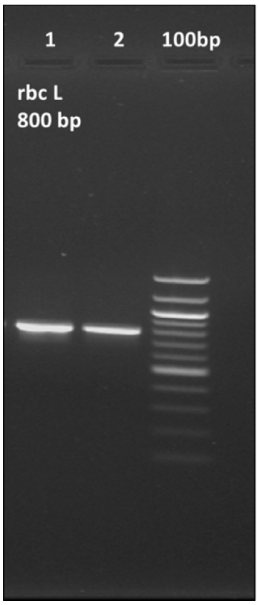
cgcagcggcttgagcgacgaccccgttcgagggcgacgacttggtaggtactgtctca
gtgcacgtcctttcgtcgtggcccgaggcgtctcgcttggagctcctttgtatgtacattcat
catcacatgattcgacctgagctcaggcaagagcacccgctgaacttaagcatatcaata
agcggagga

Figure 2-8: PCR products of the ITS region amplified



Picochlorum maculatum ribulose 1, 5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast, strain MACC3.
aagaatgtggtgcagcggtagcagctgaatcatcaactggtacttggactacagtatgga
ctgatggtttaactagtttagatcgttacaaaggtcgttgttacgacatcgaacctgtaccag
gtgaagacaaccaatacatcgcatatgttgcttacccattagatttatttgaagaaggttctg
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gacttcactaaagatgatgaaaacgttaactcacaaccattcatgcgttggagagatcgttt
cttattcgtagctgaagcaatctacaaagctcaagctgaaactggtgaaattaaaggtcac
tacttaaacgcaacagctgctacttgtgaagaaatgcttaagcgtgctgcttgtgctaaag
atttaggtgtacctattatcatgcacgattacttaacaggtggtttcacagctaacactagctt
atctcactactgtcgtgacaacggtttattattacacattcaccgtgcgatgcacgctgtaat
tgaccgtcaacgtaaccacggtatccacttcc

Figure 2-9: PCR products of the rbcL gene amplified

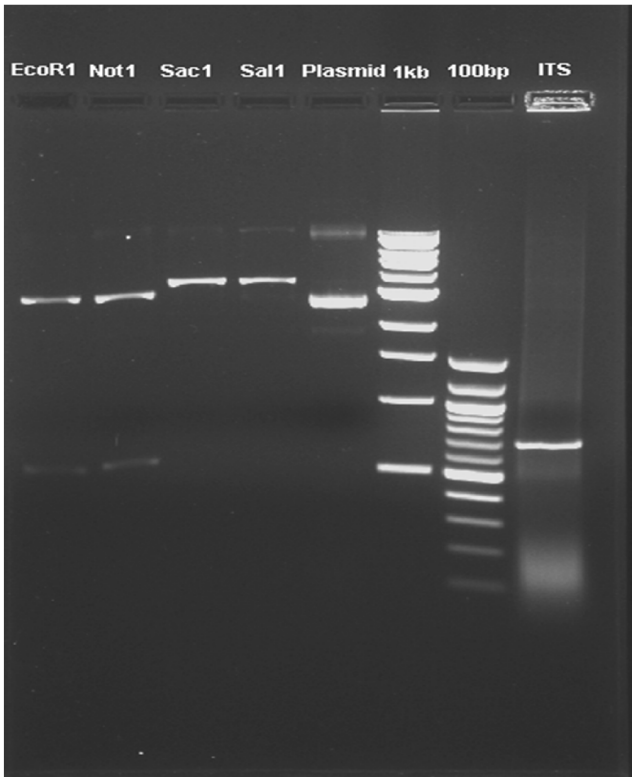


Figure 2-10: Plasmid DNA restriction digestion

Line (1) & (2) Plasmid DNA digested with EcoR1 and Not1 which make double cut in the pGMET vector release the inserted DNA sequence, (3) & (4) Sac1 and Sal1 make single cut in the pGEMT vector which linearize the plasmid and slightly see the band slightly higher than the plasmid (5) pGEMT vector with insert (6) & (7) are 100bp and 1 kb makers (8) Originally inserted DNA of ITS region

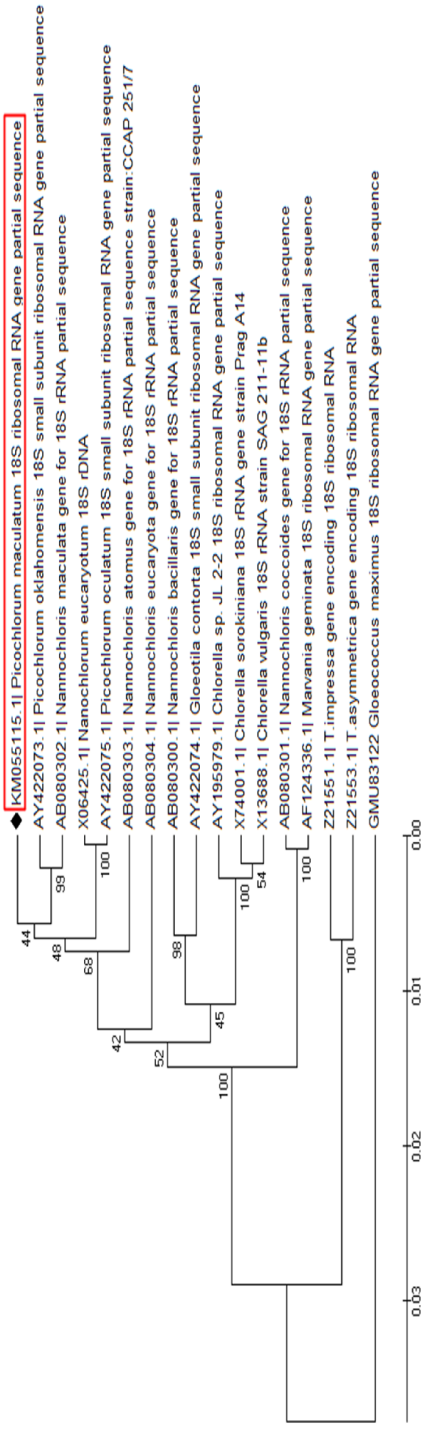


Figure 2-11: Phylogenetic tree of *Picochlorum maculatum* MACC3 based on 18S ribosomal RNA gene sequence

The evolutionary history was inferred using the UPGMA method (Sneath et al., 1973). The optimal tree with the sum of branch length = 0.19710634 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The analysis involved 17 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1666 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

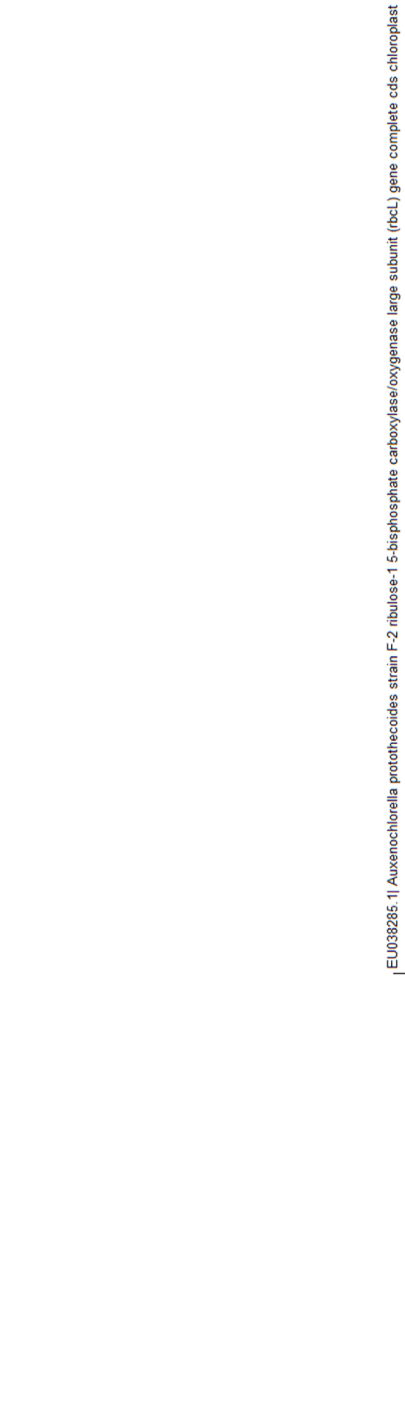


Figure 2-12: Phylogenetic tree of *Picochlorum maculatum* MACC3 based on rbcL gene sequence

The evolutionary history was inferred using the UPGMA method (Sneath et al., 1973). The optimal tree with the sum of branch length = 0.37339708 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The analysis involved 14 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 441 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013)

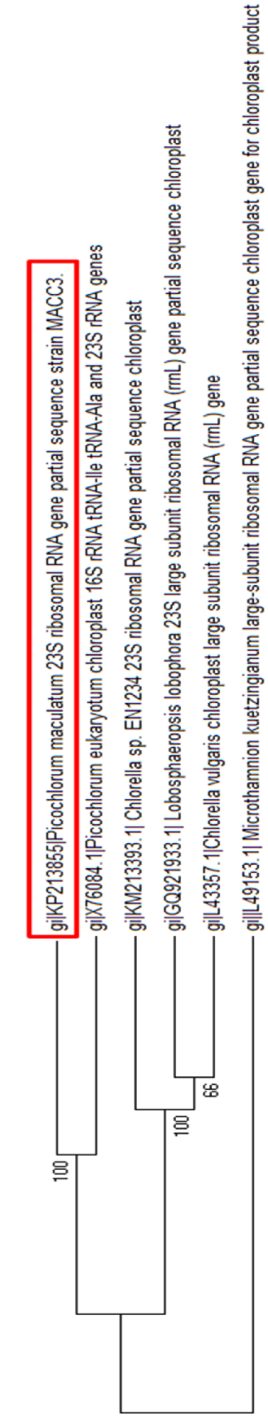


Figure 2-13: Phylogenetic tree of *Picochlorum maculatum* MACC3 based on 23S ribosomal RNA gene sequence

The evolutionary history was inferred using the UPGMA method (Sneath et al., 1973). The optimal tree with the sum of branch length = 0.42501603 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The analysis involved 6 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 663 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013)

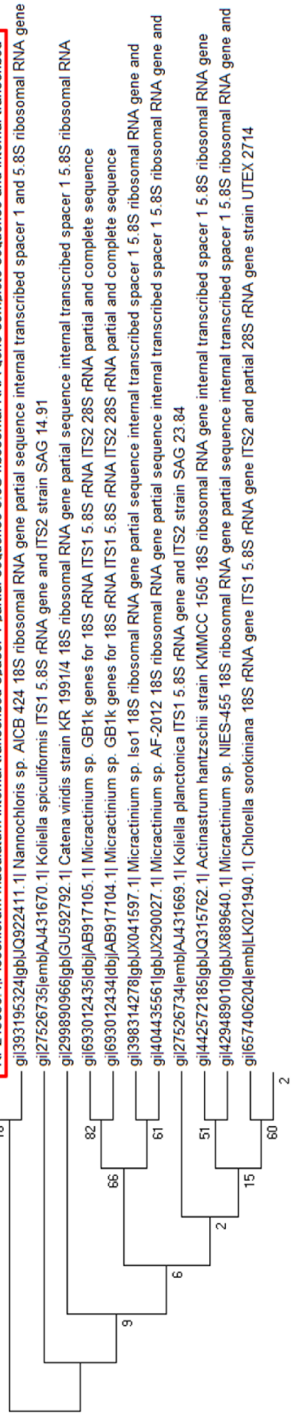


Figure 2-14: Phylogenetic tree of *Picochlorum maculatum* MACC3 based on the ITS region sequence

The evolutionary history was inferred using the Maximum Parsimony method. Tree #6 out of 7 most parsimonious trees (length = 16) is shown. The consistency index is (0.700000), the retention index is (0.896552), and the composite index is 0.728448 (0.627586) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (Felsenstein, 1985). The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (pg. 126 in ref. (Nei & Kumar, 2000)) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The analysis involved 29 nudeotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 178 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

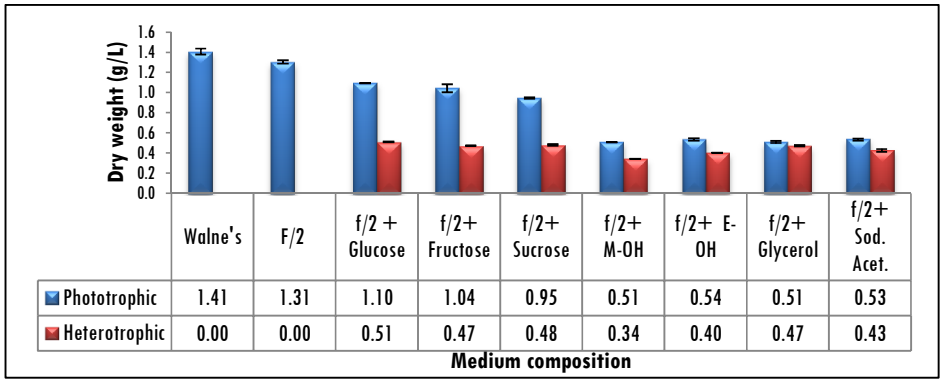


Figure 2-15: Biomass production of *P. maculatum MACC3* under phototrophic, mixotrophic and heterotrophic conditions during stationary phase of growth

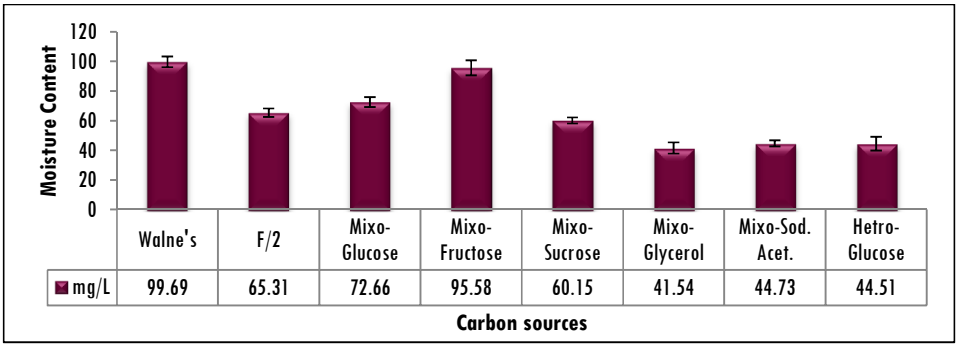


Figure 2-16: Moisture content of *P. maculatum* MACC3 in phototrophic, mixotrophic and heterotrophic cultures during stationary phase of growth

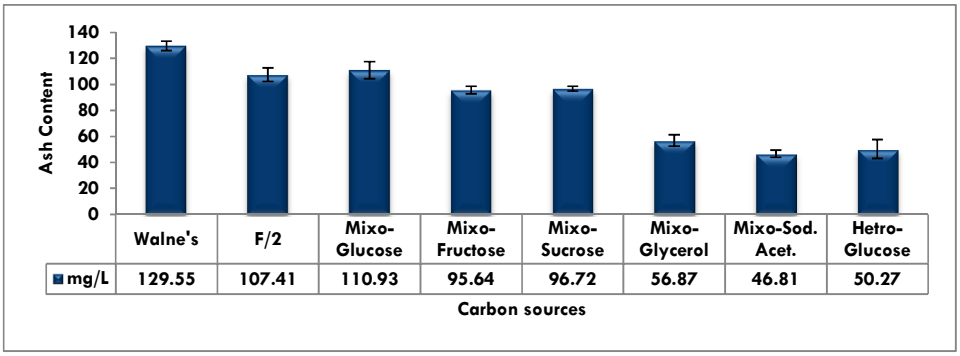


Figure 2-17: Ash content of *P. maculatum* MACC3 in phototrophic and mixotrophic and heterotrophic cultures during stationary phase of growth

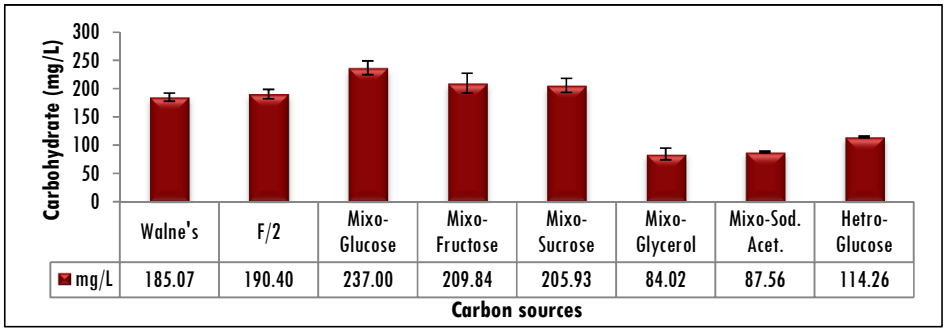


Figure 2-18: Carbohydrate yield of *P. maculatum* MACC3 in phototrophic and mixotrophic and heterotrophic cultures during stationary phase of growth

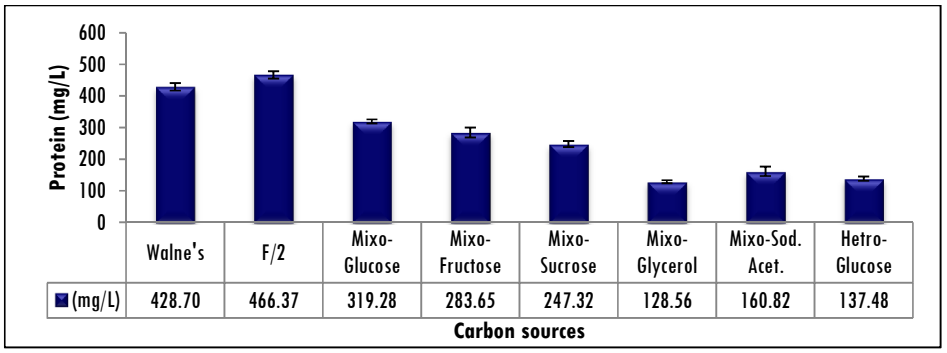


Figure 2-19: Protein yield of *P. maculatum* MACC3 in phototrophic, mixotrophic and heterotrophic cultures during stationary phase of growth

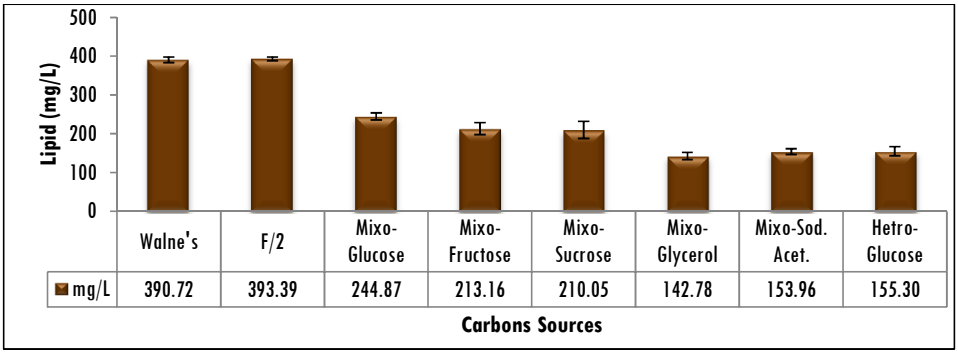


Figure 2-20: Lipid yield of *P. maculatum* MACC3 in phototrophic, mixotrophic and heterotrophic cultures during stationary phase of growth

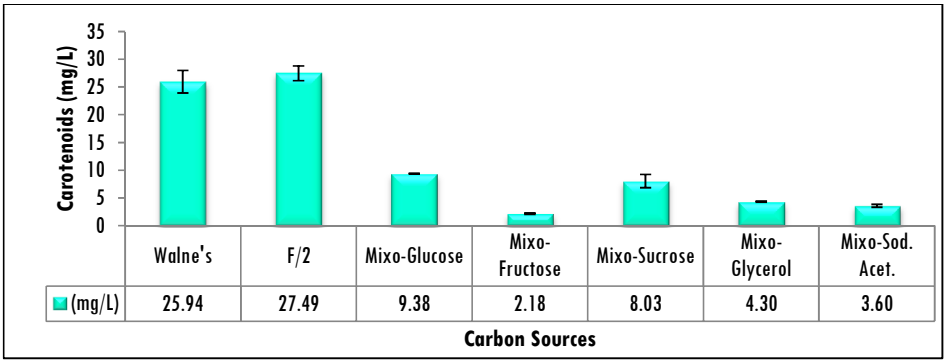


Figure 2-21: Carotenoids yield of *P. maculatum* MACC3 in phototrophic and mixotrophic cultures during stationary phase of growth

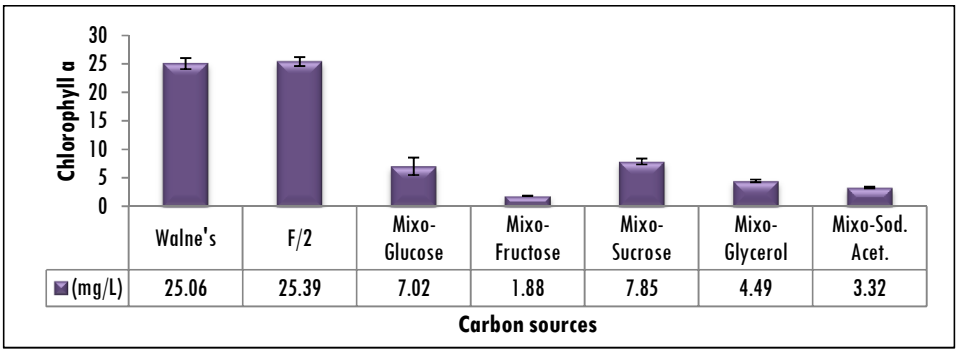


Figure2-22: Chlorophyll *a* yield of *P. maculatum* MACC3 in phototrophic and mixotrophic cultures during stationary phase of growth

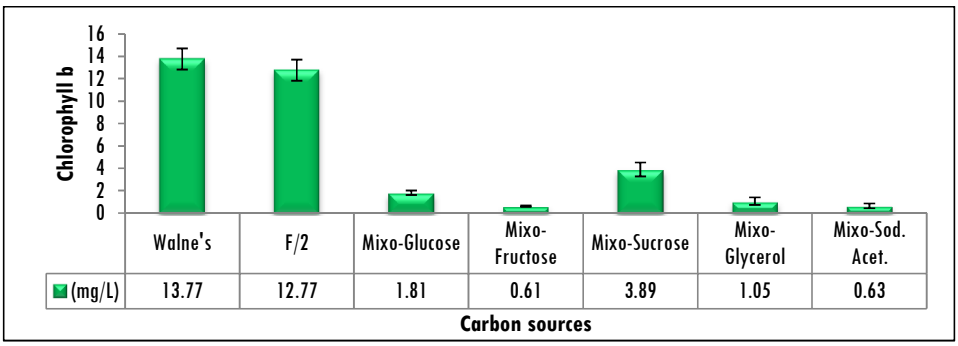


Figure 2-23: Chlorophyll *b* yield of *P. maculatum* MACC3 in phototrophic and mixotrophic cultures during stationary phase of growth

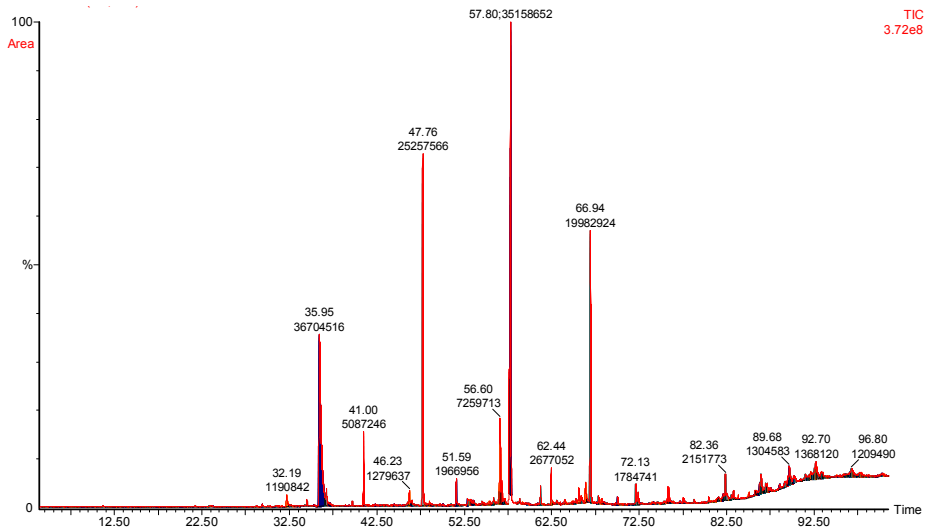


Figure 2-24: GC-MS chromatogram of fatty acid methyl esters in *P. maculatum* MACC3 in the f/2 medium during the stationary phase of growth.

Retention Time (RT)	Fatty acid
35.95	Lauric Acid (LA)
41.00	Myristic Acid (MA)
46.00	Palmitoleic acid
47.20	Palmitic Acid (PA)
56.6	Heptadecanoic acid
57.80	γ-Linolenic Acid (GLA)
62.40	Linoleic (LA)
66.94	α-Linolenic Acid (ALA)
67.10	Oleic Acid (OA)
67.84	Stearic acid (SA)
72.13	Eicosapentaenoic acid (EPA)
73.21	Eicosatetraenoic acid (ETA)

Fatty acid compositions analyzed by comparing the retention time with the standards fatty acid retention time. The concentration of fatty acids determined by the peak area of internal standard (Heptadecanoic acid) used.

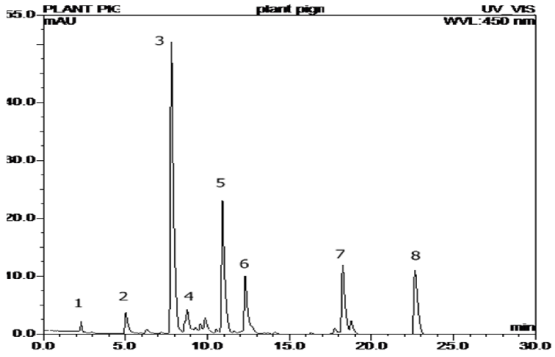


Figure 2-25: HPLC chromatogram showing the pigment profile of *P. maculatum* MACC3 in phototrophic culture grown in f/2 medium during stationary phase of growth

1. Neoxanthin, 2. Violaxanthin, 3. Lutein, 4. Zeaxanthin, 5. Chlorophyll b, 6. Chlorophyll a, 7. α Carotene, 8. β- Carotene. (Peaks identified by comparing with the retention time of standards)

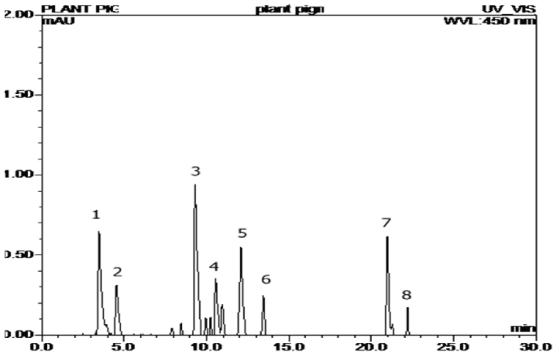
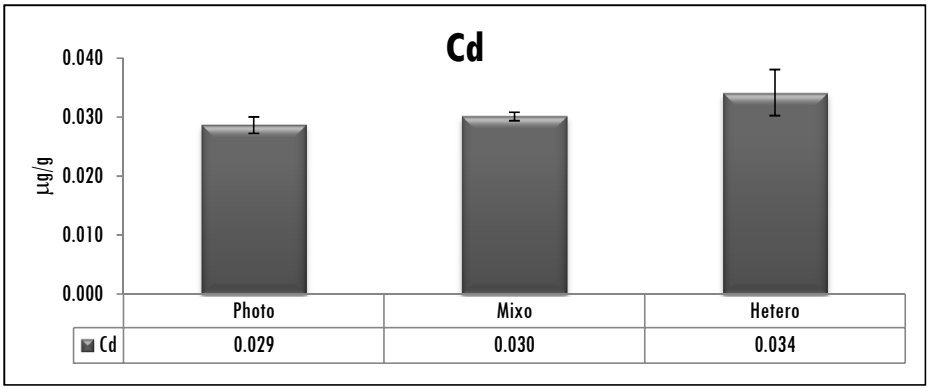
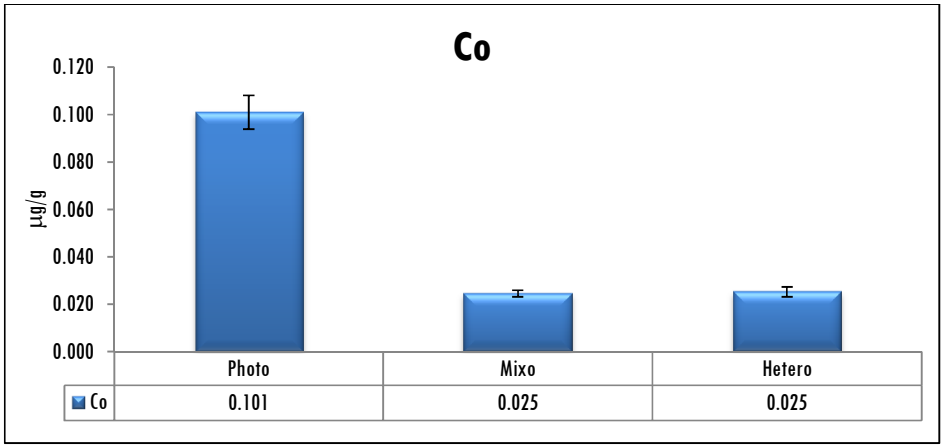


Figure 2-26: HPLC chromatogram showing the pigment profile of *P. maculatum* MACC3 in mixotrophic culture grown in medium with glucose as the carbon source during stationary phase of growth

1. Neoxanthin, 2. Violaxanthin, 3. Lutein, 4. Zeaxanthin, 5. Chlorophyll b, 6. Chlorophyll a, 7. α Carotene, 8. β- Carotene.

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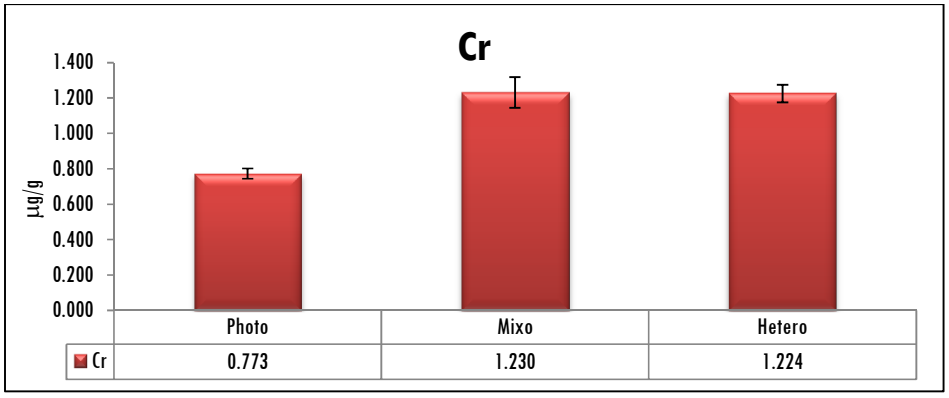
µg/g
0.120
0.100
0.080
0.060
0.040
0.020
0.000
Photo
Mixo
Hetero
Co
0.101
0.025
0.025


Cr
µg/g
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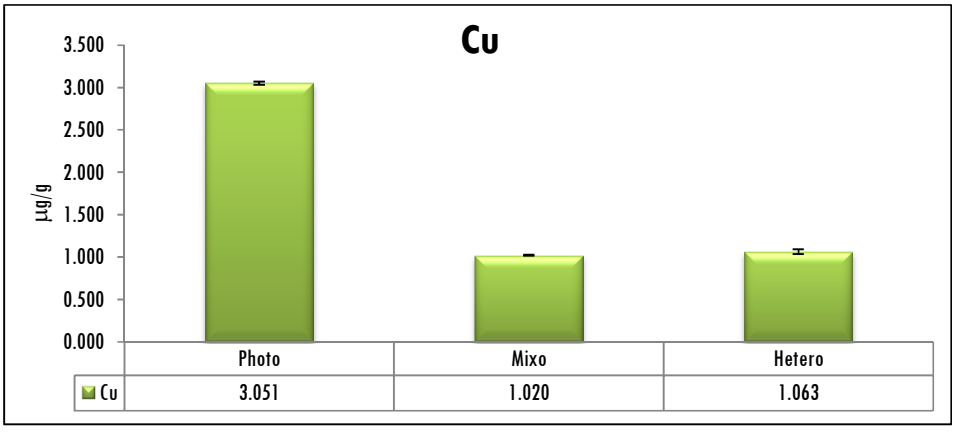
1.200
1.000
0.800
0.600
0.400
0.200
0.000
Photo
Mixo
Hetero
Cr
0.773
1.230
1.224


Cu
µg/g
3.500
3.000
2.500
2.000
1.500
1.000
0.500
0.000
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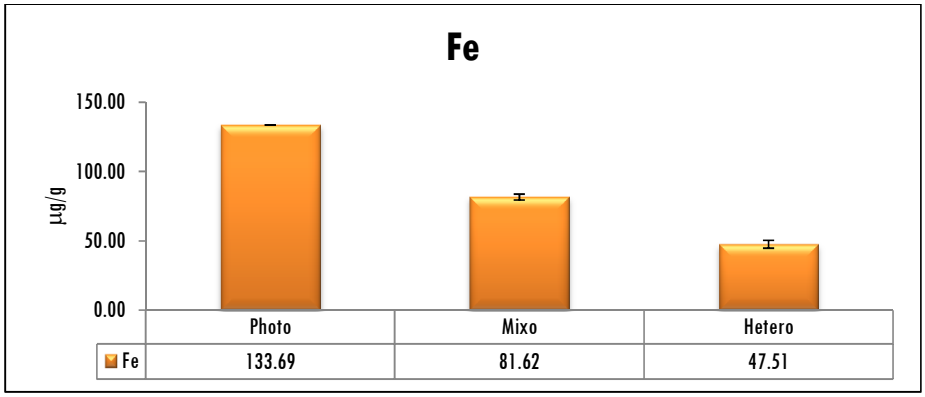
Mixo
Hetero
Cu
3.051
1.020
1.063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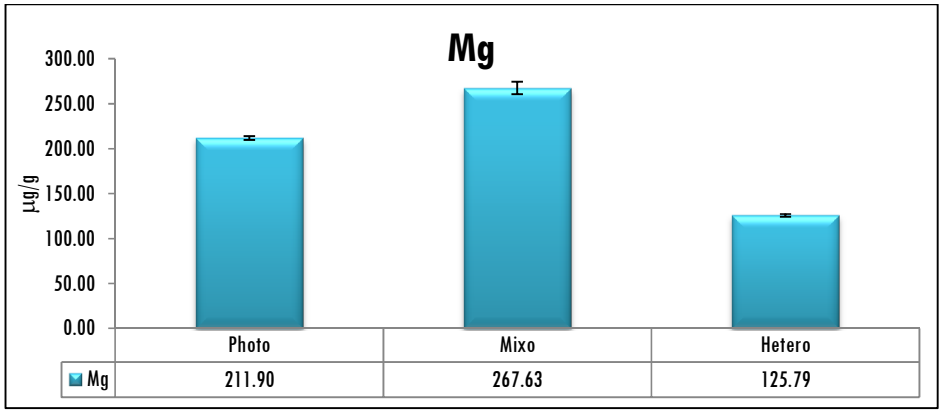
100.00
50.00
0.00
µg/g
Photo
Mixo
Hetero
Fe
133.69
81.62
47.51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250.00
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50.00
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µg/g
Photo
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Hetero
Mg
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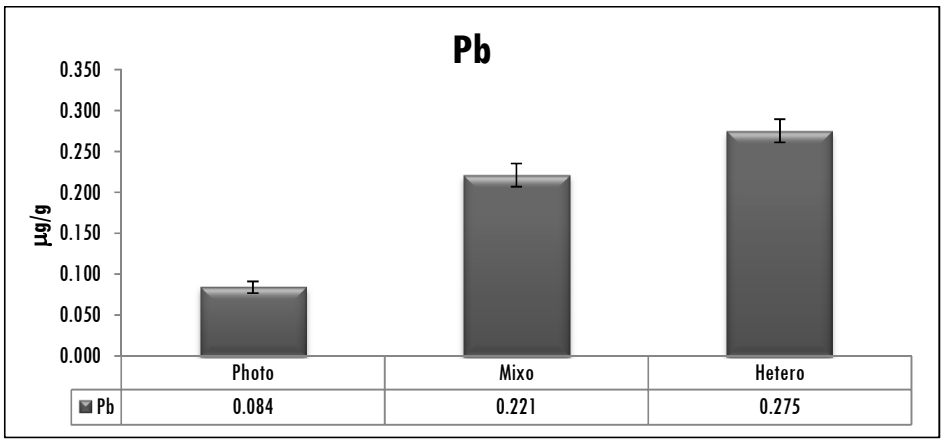
125.79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0.250
0.200
0.150
0.100
0.050
0.000
µg/g
Photo
Mixo
Hetero
Pb
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0.221
0.275

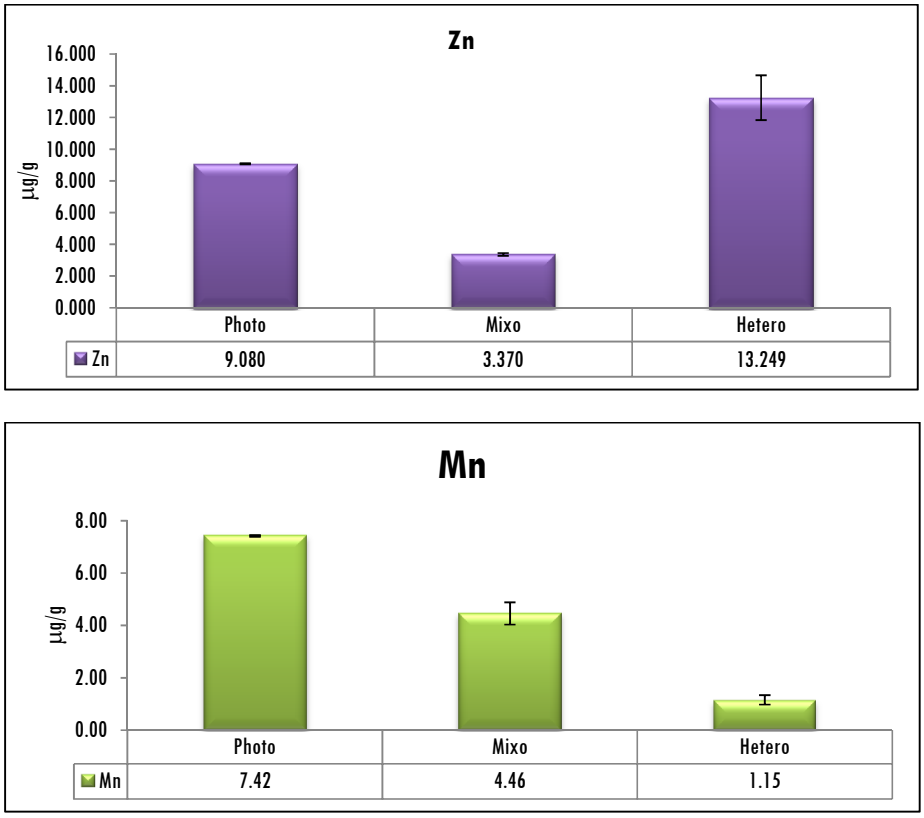

Figure 2-27: Minerals composition (Cd, Co, Cr, Cu, Fe, Mg, Pb, Zn and Mn) of *P. maculatum* MACC3 in phototrophic mixotrophic and heterotrophic cultures during stationary phases of growth

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Chapter 3

OPTIMIZATION OF MEDIUM AND GROWTH CONDITIONS FOR THE PRODUCTION OF BIOMASS AND GAMMA LINOLENIC ACID BY *PICOCHLORUM MACULATUM* MACC3 USING RESPONSE SURFACE METHODOLOGY

•Contents•

3.1 Introduction

Polyunsaturated fatty acids (PUFAs) are essential requirements for human and animal nutrition. The conventional sources for this nutraceutical are marine fishes and certain mammals. Owing to the increasing demand on PUFA over the last decades and also due to the disadvantage and limitation of the conventional PUFA sources, large numbers of alternative potential sources were screened. The microalgae are considered as the best potential alternative for this nutraceutical (Bell & Tocher, 2009; Elrazak & Ahmed, 2012; Hossain, 2011; Sanders, 2000; Turon, 2013; Warude et al., 2006). Most of the industrial production of PUFA is achieved through the microalgal fermentation process. The success of any microalgal industry is based on the optimization of the production process for which integrated approaches are required for the delineating the interactive effects of critical factors affecting biomass and bioproduct formation and biomass harvesting.

DHA was found to be the predominant PUFA of heterotrophic marine dinoflagellate like *Crypthecodinium cohnii*, which accumulated 25-60% of DHA and palmitic acid of the total fatty acid and no other PUFA were present more than 1%,

which makes them attractive for industrial production and makes the separation and purification economical (Mendes et al., 2007, 2009; De Swaaf, 2003; Tuttle & Loeblich, 1975). Efforts have been made to produce DHA in photobioreactors, but it is challenging to achieve high biomass concentrations and high DHA productivities. This is due to two unsolved problems, namely light constraint and oxygen accumulation, in photoautotrophic cultures (Lee et al., 1995; Molina et al., 2001). Heterotrophic production of DHA from *C. cohnii* was commercialized by Martek Biosciences in Maryland. Vazhappilly & Chen, (1998) evaluated the potential of EPA and DHA production in 20 phototrophically growing microalgae algae and the highest EPA yield was reported in *Monodus subterraneus* UTEX 151 (96.3 mg/L), *Phaeodactylum tricornutum* UTEX 642 (43.4 mg/L), *Chlorella minutissima* UTEX 2341 (36.7 mg/L), and *Porphyridium cruentum* UTEX 161 (17.9 mg/L) owing to their relatively high biomass concentrations. The DHA yield was high in *C. cohnii* UTEX L1649 (19.5 mg/L) and *Amphidinium carterae* UTEX LB 1002 (8.6 mg/L).Cao et al. (2008) reported the heterotrophic EPA production in diatom *Nitzschia laevis* 110 mg/L. The gamma linolenic acid production was reported to be the highest in the cyanobacterium *Spirulina* (Tanticharoen et al., 1994). However, the PUFA production potential of microalgae have not been explored much and a comprehensive analysis of more than 2000 strains from the SAG culture collection showed that there is a hidden array of fatty acids in the microalgae, the diversity of which needs to be explored in detail (Lang et al., 2011).

3.1.1 Physical and nutritional parameters of microalgae for PUFA production

3.1.1.1 Environmental factors

3.1.1.1.1 Light

Light is an important factor affecting the growth, metabolism and fatty acid composition of photosynthetic microalgae because the chloroplast membrane is composed of highly saturated polar lipids (Andersson & Dörmann, 2009; Thompson Jr & others, 1996). The red alga *Porphyridium cruentum* produce high EPA and DHA

when grown at 25^0C and high light intensity (Brody & Emerson, 1959). A reduction in light intensity resulted in a decrease in the proportion of both EPA and DHA and increase in the saturated fatty acids (Guschina & Harwood, 2009; Richmond, 2008). However, in some green algae, such as *Monodus subterraneus* increased EPA production was observed at low light intensity (Khozin-Goldberg & Cohen, 2006; Mühlroth et al., 2013). In *Chlorella vulgaris, an* increase in light intensity from 37.5 to 62.5 µmol photons/m^{-2}/s resulted in increased growth and biomass production; with a further decrease in biomass with an increase in light intensity to 100 µmol photons/m^{-2}/s. This demonstrated that light regime is an important factor controlling biomass in *C. vulgaris* (Khoeyi et al., 2011).

3.1.1.1.2 Temperature

Temperature has a vital role in the PUFA formation in microorganisms. An increasing the synthesis of unsaturated fatty acids has been observed at low temperatures in algae, bacteria, blue-green algae, yeast, and fungi. This increased unsaturation help to adapt cold temperature by maintaining the cell membrane fluidity (Jiang & Chen, 2000).

3.1.1.1.3 pH

pH is another physicochemical factor affecting the PUFA production in microalgae. In the cultures of *P. tricornutum*, EPA yield reached a maximum when the initial pH was 7.6 (Yongmanitchai & Ward, 1989). Jiang & Chen (2000) reported that pH had a significant effect on the DHA-producing microalgae *Crypthecodinium cohnii* grown under heterotrophic conditions with glucose as the sole carbon source. pH 7.2 was found to be the best in terms of specific growth rate, cell density and degree of unsaturation in the fatty acid.

3.1.1.2 Nutritional factors

3.1.1.2.1 Nitrogen

The nitrogen concentration in the medium affects the relative proportion of saturated and unsaturated fatty acids in the green algae, bacteria, and fungi since the

nitrogen level in the medium control the switching of metabolisms between protein and lipid synthesis. Under the nitrogen stress in *Dunaliella* and *Botryococcus* species produced a higher percentage of EPA. In contrast, the PUFA content in the freshwater algae *Chlorella* and *Scenedesmus* increased with increased nitrogen concentrations (Yongmanitchai & Ward, 1991b).

3.1.1.2.2 Phosphorus

It is an important constituent of the membrane phospholipids and nucleic acids. In cells, a high proportion of PUFA exists in the form of polar lipids such as phospholipids, phosphate content of the cell significantly affect the cellular PUFA contents (Yongmanitchai & Ward, 1991a, 1991b).

3.1.1.2.3 Salinity

The salinity of the medium may influence the physiological properties of microalgae. In *Dunaliella* sp. the n-3 PUFA content of the cells decreased with increasing salinity (Xu & Beardall, 1997). In contrast, Seto et al. (1992) investigated the effect of salt on the growth rate and fatty acid composition of *Chlorella minutissima* and showed that the cells grown in the NaCl-enriched medium contained higher percentages of EPA. In the culture of the marine diatom *Nitzschia laevis*, EPA yield was the highest at half the salinity of the artificial seawater (Wen & Chen, 2000). Fatty acid composition of *Phaeodactylum tricornutum* was significantly affected by the salinity of the medium. EPA content of the cells increased when the medium salinity was increased (Yongmanitchai & Ward, 1991a). In *Dunaliella salina,* the saturated fatty acids decreased and unsaturated fatty acids increased with increasing the NaCl concentration (Peeler et al., 1989; Takagi et al., 2006). The salt concentration in the medium has a direct involvement in the electron transport and/or photophosphorylation and result in a decrease in the quantum efficiency of photosynthesis (Seemann & Critchley, 1985).

3.1.1.2.4 Minor nutrients

Several metal ions including Mn^{2+}, Co^{2+}, Zn^{2+}, Mg^{2+}, promote the synthesis of lipids and PUFA in microorganisms, as they take part in the process of fatty acid

biosynthesis as a cofactor of certain enzymes. Supplementation of Fe^{2+}, Cu^{2+}, Mn^{2}+, $Co^{2+}$and Zn^{2+} to the medium enhanced the DHA productivity in *Thraustochytrium* sp. High levels of trace elements were shown to inhibit the growth of the green alga *Haematococcus pluvialis* (Carvalho et al., 2006; Certik & Shimizu, 1999; Song et al., 2012).

3.1.1.2.5 Vitamins

The supplementation of vitamin B12 to the culture medium of *Phaeodactylum tricornutum* increased the EPA yield (Yongmanitchai & Ward, 1991a).

3.1.2 Statistical optimization of microalgal production

The product and process optimization are the backbones of any industrial production and even small improvements can make a giant commercial achievement. In modern industrial production processes, the optimization of nutritional and physical parameters significantly improves the productivity. To be economically feasible, it is necessary to engineer optimum culture conditions to minimize the cost and to maximize production. The conventional one variable at a time (OVAT) method in which one independent variable is studied while maintaining all the other factors at a fixed level is an operation frequently used in the fermentation industries for the production of high yield of the desired metabolite from the microbial systems (Tyssedal et al., 2006; Vanaja & Shobha Rani, 2007; Wen & Chen, 2003). However, it is time-consuming and can lead to misinterpretation of results, especially because it doesn't consider the interaction among the various physicochemical parameters. In order to overcome these problems and to determine the interaction between the factors, different optimizations processes are employed (Chen et al., 2009; De Coninck et al., 2000; Gorret et al., 2004; Tokcaer et al., 2006; Weuster-Botz, 2000). Response surface methodology is a statistical experimental design technique, involving building a model evaluating the combined effect of different factors for defining optimum growth media or conditions for maximizing the production of biomass or the desired response.

The two main applications of experimental design are screening, wherein the factors that influence the biomass or product formation are identified, and optimization, wherein the optimal settings or conditions for an experiment are found. Screening techniques such as factorial designs allow the experimenter to select the significant factors and the level at which they are significant (Bas & Boyaci, 2007; Proust, 2009). Design experiments reduce the number of experiments, which help to save money and time. In comparison to OVAT, we can predict and quantify the interactions between the factors by the design of experiments. The response surface methodology extensively used in various processes or products optimization is a proven technique for medium optimization and fermentation technology (Carvalho et al., 2006; Chen et al., 2009; Francis et al., 2003; Patil et al., 2011; Saelao et al., 2011; Sarada et al., 2002; Song et al., 2007).

According to the review by Hill & Hunter (1966), the RSM method was introduced by Box and Wilson in 1951. Box and Wilson suggested to use a first-degree polynomial model to approximate the response variable and though not accurate such a model is easy to estimate and apply even when little is known about the process. In RSM, an output (response) is optimized in response to input (explanatory) variables based on the multiple regression analysis using quantitative data obtained from appropriately designed experiments.

3.1.2.1 Plackett–Burman experimental design

The Plackett–Burman (PB) designs are used for the preliminary screening of main factors from a large number of variables influencing the product formation or optimization process, with a minimum number of experiments (n+1), where n is the number of variables. The number of experimental trials in PB is a multiple of 4 (12, 20, 24, 28 etc.) and PB design with 12 runs enable the screening of up to 11 variables (Elrazak & Ahmed, 2012). PB was used to screen the medium components for PUFA production from microorganisms (Chodok et al., 2010; Pote & Bhadekar, 2014), and also used in the EPA and AA production from microalgae (Tababa et al., 2012; Wen & Chen, 2001). PB was applied for screening the most significant factors affecting

PUFA production, by the fungus *Mortierella alpina* CBS 754.68 (Jang et al., 2005), GLA production from *Hansenula polymorpha* (Khongto et al., 2011) and DHA production from *Schizochytrium limacinum* (Song et al., 2007)

3.1.2.2 Central composite design (CCD)

Response surface methodology was used to optimize the concentrations of the significant parameters identified by Plackett-Burman design. In order to evaluate the interactive effect of the variables, experiments involving different combinations of variables were designed (Pote & Bhadekar, 2014). CCD contains fractional factorial and embedded factorial designs and has three groups of design values.

a) Fractional design points- all possible combinations of the +1 and -1 levels of the factors (2^k).

b) Axial points or star points - all of the factors set to 0, the midpoint, except one factor, which has the value +/- alpha (+α or -α). However, in the case of axial points of face-centered central composite design, all the factors are set to 0 (midpoint), except one factor, which is at the +1/-1 value

c) Center points - points with all levels set to coded level 0 (midpoint).

The "α" is set the default at 1.68179 in coded units, is the axial distance from the center point and makes the design rotatable and provides equally good predictions at points equally distant from the center. Center points are usually repeated to get an estimate of experimental error. Thus the central composite design requires five coded levels of each factor: "-1" or "+1" (factorial points), "-α" or "+ α" (axial points), and all zero level (center point). The variables are coded according to the following equation:(Khuri & Mukhopadhyay, 2010)

$$Coded\ value = \frac{\left(Actual\ value - {}^{1}/_{2}\,(high\ level + lower\ level)\right)}{\left({}^{1}/_{2}\ (high\ level - low\ level)\right)} \qquad (3\text{-}1)$$

RSM gives the graphical representation of the model equation and optimal operating conditions. The visualization of the predicted model equation can be obtained graphically by the 3D response surface plot or 2D contour, which, could be

used to describe the individual and collective effect of the test variables on the response and to determine the mutual interactions between the test variables and their subsequent effect on the response. Each contour curve in a 2D plot signifies a countless number of combinations of two test variables with all the others at fixed levels. The yield values for different concentrations of the variables can also be predicted from the respective contour plots, the shape of the contour plot, circular or elliptical, indicates whether the mutual interactions between the corresponding variables are significant or not (Manimekalai & Swaminathan, 1999; Xiong et al., 2004).

(Song et al., 2007) optimized the fermentation conditions of *Schizochytrium limacinum* OUC88, a heterotrophic marine fungus using RSM for biomass and DHA production. Based on the PB based screening, it was found that the temperature, aeration rate, agitation speed and inoculum age were the significant factors for the biomass and DHA production, and these factors were further optimized using the RSM. The optimum levels of significant factors were temperature 23.5^0C, aeration rate 1.48 L/min, agitation 250 rpm and yielded maximum biomass and DHA of 24.1 and 4.7 g/L respectively, which were 27.2 and 28.6 % higher than the production achieved before optimization. Saelao et al. (2011) optimized the biomass and arachidonic acid (ARA) production from *Aureispira maritime* using RSM design, through the optimization biomass and ARA production increased 4.02 fold (2.05 g/L) and 3.59 fold (21.50 mg/g) respectively. de Swaaf et al. (1999) studied the optimization of DHA production in *Crypthecodinium cohnii,* through the optimization in batch cultures and reported that the biomass concentration increased from 1.5 to 27.7 g/L, and total lipid and DHA content increased from 7.5-13.5 % and 36.5- 43.6 % respectively. Various other reports are available on the use of RSM for optimization PUFA production such as, DHA optimization in *Crypthecodinium cohnii* (De Swaaf, 2003), optimization of macro and micronutrients for EPA and DHA production in *Pavlova lutheri* (Carvalho et al., 2006), production optimization of ARA and EPA in fungus *Pythium ultimum* (Gandhi & Weete, 1991), and the effect of culture media and conditions on PUFA production by *Mortierella alpine* (Jang et al., 2005).

The significant increases observed in biomass and product of interest simply by optimizing species-specific significant variables using statistical design based bioprocess development, point to the importance of applying such strategies in the microalgal production process. The aim of this work was to screen the most significant variables affecting biomass and gamma linolenic acid production in *P. maculatum* by Plackett-Burman experimental design, and central composite design using response surface methodology. The significant variables were further optimized for maximum production of biomass and gamma linolenic acid.

3.2 Materials and methods

3.2.1 Initial screening of media components for biomass production

An initial screening was carried out by one-variable-at-a-time approach (Table 3-1 & Table 3-2) to find the suitable concentrations of medium components for maximum yield of *P. maculatum* MACC3 biomass. All experiments were done in triplicate in 250 mL flasks with 150 mL of the f/2 medium under a photon irradiance of 50±10 µmol photon/m^2/s in 16:8 light and dark period and agitated at 150 rpm. The initial cell density was 3.4 x 10^4 cells/mL. The cultures were incubated at temperature and pH of 25^0C and 7.5 ± 0.2, respectively on a rotary shaker (Orbitek, Scigenics Biotech Pvt. Ltd., India). The cells were harvested for final biomass analyses during stationary growth phase (18th day). The biomass concentration was determined as dry weight discussed in chapter 2 (session 2.2.7.1) and expressed as g/L in all the screening experiments.

Table 3-1: Nutrients and the concentrations selected for initial optimization

Component	Concentrations
$NaNO_3$	37, 75, 150, 225 and 300 mg/L
$NaH_2PO_4H_2O$	0, 2.5, 5, 10, 15, 20 mg/L
Salinity	0, 10, 20, 30, 35, 40, 50 ppt
Metal solution (4.16 gm/L Na_2EDTA, 3.15 gm/L $FeCl_3$. $6H_2O$, 10 mg/L $CuSO_4.5H_2O$, 22 mg/L $ZnSO_4.7H_2O$, 10 mg/L $CoCl_2.6H_2O$, 180 mg/L $MnCl_2.4H_2O$, 6 mg/L $Na_2MoO_4.2H_2O$)	0, 0.5, 1, 1.5, 2.0, 2.5 mL/L
Vitamin Solution (500 µg/L vitamin B_{12}, 0.1 mg/L vitamin B_1 and 500 µg/L biotin)	0, 0.5, 1, 2 mL/L

Table 3-2: Physical parameters selected for initial optimization

Parameter	Values
pH	5, 6, 7, 8, 9 and 10
Temperature (^{0}C)	10, 15, 20, 25, 30, 35, 40 and 50
Agitation (rpm)	0, 50, 100, 150, 200 and 250
Inoculum size (%)	1, 2, 4, 8, 16, 32 % of culture with optical density of 1OD used as the inoculum

3.2.1.1 Screening of nitrate sources

Seven different nitrate sources (ammonium chloride, urea, ammonium nitrate, sodium nitrate, peptone, yeast extract, and malt extract) were screened for their effect on growth and biomass accumulation in *P. maculatum* MAAC3 at a concentration of 75 mg/L in 250 mL flasks with 150 mL of f/2 medium. The concentrations of nitrate sources were fixed based on the nitrate concentration in the f/2 medium. The media was inoculated with 10% inoculum of *P. maculatum* with a cell density of 3.4 x 10^7 cells/mL. The cultures were incubated under the conditions mentioned in the previous section.

3.2.1.2 Optimization of nitrate

The optimum concentration of $NaNO_3$ (nitrate source used in the f/2 medium) was tested at different concentrations of 37, 75, 150, 225 and 300 mg/L in 250 mL flasks with 150 mL of f/2 medium. The cultures were incubated under the growth conditions mentioned earlier.

3.2.1.3 Optimization of phosphate

The optimum concentration of $NaH_2PO_4H_2O$ required for maximum biomass production in *P. maculatum* was determined by screening different concentrations of 2.5, 5, 10, 15 and 20 mg/L in 250 mL flasks with 150 mL of f/2 medium, along with phosphate free control. The cultures were incubated at temperature and pH of 25^0C and 7.5±0.2, respectively on a rotary shaker under conditions mentioned earlier. The biomass concentration on the 18^{th} days was determined as dry weight and expressed as g/L.

3.1.2.4 Optimization of salinity

The *P.maculatum* cultures were grown at salinities of 0, 10, 20, 30, 35, 40 and 50 ppt in 250 mL flasks with 150 mL of f/2 medium, to test the optimum salinity for maximum biomass production. The initial cell density was 3.4 X 10^7 cells/mL. The cultures were incubated at temperature and pH of 25^0C and 7.5±0.2, respectively on a rotary shaker under conditions mentioned earlier. The biomass concentration was determined on the 18^{th} day as dry weight and expressed as g/L.

3.1.2.5 Optimization of metal concentration

The concentration of metals in the growth medium was optimized in terms of metal solution volume added. The different volumes of metal solution (1 mL/L metal solution contain 3.15 mg/L $FeCl_3$ $6H_2O$; 4.36 mg/L Na_2EDTA $2H_2O$, 0.098 mg/L $CuSO_4$ $5H_2O$; 0.063 mg/L $Na_2MoO_4 2H_2O$; 0.022 mg/L ZnSO4 $7H_2O$; 0.01 mg/L $CoCl_2$ $6H_2O$; 0.18 mg/L $MnCl_2$ $4H_2O$) tested were 0.5, 1, 1.5, 2.0, 2.5 mL/L in 250 mL flasks with 150 mL of f/2 medium, along with metal solution free control. The cultures were incubated at temperature and pH of 25^0C and 7.5 ± 0.2, respectively on a rotary shaker under conditions mentioned earlier. The biomass concentration was determined on the 18^{th} day as dry weight and expressed as g/L.

3.1.2.6 Optimization of vitamin concentration

The concentration of vitamins in the growth medium was optimized in terms of volume of the vitamin solution (0.5 mL/L of vitamin solution contains 0.1 mg/L thiamine HCl (vitamin B_1); 0.0005 mg/L biotin (vitamin H) and 0.0005 mg/L cyanocobalamin (vitamin B_{12})). The vitamins were tested at volumes of 0.5, 1, 1.5, 2.0, mL/L in 250 mL flasks with 150 mL of the f/2 medium along with a control without vitamins. The cultures were incubated at temperature and pH of 25^0C and 7.5 ± 0.2, respectively on a rotary shaker under conditions mentioned earlier. The biomass concentration was determined on the 18^{th} day as dry weight and expressed as g/L.

3.1.2.7 Optimization of pH

P. maculatum MACC3 cells were incubated in 250 mL flasks with 150 mL of the f/2 medium at different pH of 5, 6, 7, 8, 9 and 10 at an initial cell density of 3.4 x 10^7 cells/mL and incubated on a rotary shaker under conditions mentioned earlier. The biomass concentration was determined on the 18th day as dry weight and expressed as g/L.

3.1.2.8 Optimization of temperature

P. maculatum MACC3 cells were incubated at different temperatures of 10, 15, 20, 25, 30, 35, 40 and 50^{0}C in 250 mL flasks with 150 mL of f/2 medium and initial pH of 7.5 ± 0.2 on a rotary shaker under conditions as mentioned earlier. The cells were harvested during stationary phase on the 18th day. The biomass concentration was determined as dry weight and expressed as g/L.

3.1.2.9 Optimization of Agitation

P. maculatum MACC3 cells were incubated in 250 mL flasks with 150 mL of the f/2 medium at different agitation speeds of 50, 100, 150, 200, 250 rpm on a rotary shaker and incubated under conditions mentioned earlier. The cells were harvested during stationary phase on the 18th day. The biomass concentration was determined as dry weight and expressed as g/L.

3.1.2.10 Optimization of inoculum size

The optimum inoculum size was selected based on screening different initial inoculum densities of 1, 2, 4, 8, 16, and 32% of 1.4 OD culture (approximate cell density of 3.4 X 10^7 cells/ mL) in 250 mL flasks with 150 mL of f/2 medium. The cultures were incubated under conditions mentioned earlier.

The stationary phase cultures (18th day) were taken for gravimetric analysis of dry weight. Fatty acid analyses were carried out by GC- MS based FAME analysis, the protocol is discussed in Chapter 2 (Session 2.2.7.1 and 2.2.7.6)

3.2.2 Screening of growth parameters using Plackett and Burman design

Based on the results of screening experiments by the one-variable-at-a-time approach, the significant growth conditions and medium components affecting biomass and gamma linolenic acid production in *P. maculatum* were identified using Plackett and Burman statistical experimental design using Design Expert software (Version 7, Stat-Ease Inc., USA). The variables tested and their upper (+) and lower limits (-) are given in Table 3-3. A set of 12 (N or k + 1) experiments (run) were carried out in triplicate and mean value was considered as the response (Lin & Draper, 1992; Plackett & Burman, 1946; Wang & Wu, 1995). The f/2 medium was used as the basal medium to which 10% of exponentially growing *P.maculatum* cells were inoculated at an initial cell density of $3.5x10^5$ cells/mL and incubated in a shaker incubator (Scigenics Biotech, India) at a light intensity of 50±10 µmol photon/ m^2/s and 16:8, light: dark period for 18 days. The responses, biomass, and gamma linolenic acid production were estimated by gravimetric estimation of dry weight and fatty acid methyl ester (FAME) analysis by gas chromatography-mass spectrometry using heptadecanoic acid as the internal standard (C:17), respectively.

Table 3-3: Levels of variables tested in Plackett-Burman design for screening of significant variables for biomass and GLA Production in *P. maculatum* MACC3

	Variables	Unit	Coded values	
			(-1) Lower	(+1) Higher
1	Temperature	^{0}C	15	35
2	Agitation	rpm	100	200
3	pH		6	9
4	$NaNO_3$	mg/L	37.5	150
5	NaH_2PO_4	mg/L	2.5	10
6	NaCl	g/L	15	40
7	Metal Solution	mL/L	0.2	2
8	Vitamin Solution	mL/L	0.25	1

Table 3-4: Plackett-Burman design matrix of media components for the screening of significant variables for biomass and GLA production in *P. maculatum* MACC3

Run	A	B	C	D	E	F	G	H
	^{0}C	rpm		mg/L	mg/L	g/L	mL/L	mL/L
1	-1(15)	-1(100)	-1(6)	1(150)	1(10)	1(40)	-1 (0.5)	1(1)
2	-1(15)	-1(100)	1(9)	1(150)	1(10)	-1(15)	1(2)	1(1)
3	-1(15)	1(200)	1(9)	-1(37.5)	1(10)	-1(15)	-1 (0.5)	-1(0.25)
4	1(35)	-1(100)	-1(6)	-1(37.5)	1(10)	1(40)	1(2)	-1(0.25)
5	-1(15)	1(200)	-1(6)	-1(37.5)	-1(2.5)	1(40)	1(2)	1(1)
6	1(35)	1(200)	-1(6)	1(150)	-1(2.5)	-1(15)	-1 (0.5)	1(1)
7	1(35)	-1(100)	1(9)	-1(37.5)	-1(2.5)	-1(15)	1(2)	1(1)
8	-1(15)	-1(100)	-1(6)	-1(37.5)	-1(2.5)	-1(15)	-1 (0.5)	-1(0.25)
9	-1(15)	1(200)	1(9)	1(150)	-1(2.5)	1(40)	1(2)	-1(0.25)
10	1(35)	-1(100)	1(9)	1(150)	-1(2.5)	1(40)	-1 (0.5)	-1(0.25)
11	1(35)	1(200)	1(9)	-1(37.5)	1(10)	1(40)	-1 (0.5)	1(1)
12	1(35)	1(200)	-1(6)	1(150)	1(10)	-1(15)	1(2)	-1(0.25)

A: Temperature, B: Agitation, C: pH, D: Nitrate, E: Phosphate, F: NaCl, G: Metal Solution, H:Vitamin solution (The actual values of factors are given in brackets)

The Plackett-Burman experimental design is based on the first-order polynomial model:

$$Y = \beta_0 + \sum \beta_i X_i \qquad (i= 1,\ldots\ldots, k) \tag{3-2}$$

where, Y is the estimated response (production of biomass, or GLA yield), $\beta 0$ is a model intercept, βi is the regression coefficient, Xi is the level of the independent variable, k is a number of variables. The effect of each variable was determined by following standard equation:

$$E_{Xi} = \frac{2[\sum R_I^+ - \sum R_i^-]}{N} \tag{3-3}$$

where, $E_{(Xi)}$ is the effect of the tested variable. R_i^+ and R_i^- are responses (production of biomass, GLA production) when variables were at high and low levels, respectively. N is total number of experiments or runs (N=12). Experimental error was estimated by calculating the variance among the dummy variables as follows:

$$V_{eff} = \frac{\sum (E_d)^2}{n} \quad (3\text{-}4)$$

where, V_{eff} *is* the variance of the effect of high/low levels of a variable, *Ed* is the effect of high/low levels of dummy variable and *n* is the number of dummy variables. The standard error (SE) of the high/low levels of variable is the square root of the variance of an effect, and the significance level (*p* value) of each effect of high/low levels of variable was determined using the Student's *t* test:

$$t_{X_i} = \frac{E_{(X_i)}}{SE} \quad (3\text{-}5)$$

where, $E_{(Xi)}$ is the effect of the variable *Xi*.

The variables at or above the 95% confidence level ($p<0.05$) were considered to have significant effects on responses (biomass or GLA production) (Chodok et al., 2010; Deshmukh & Puranik., 2010; Miller & Sitter, 2001; Rajendran et al., 2008; Vanaja & Shobha Rani, 2007).

3.2.3 Central composite design

Once the variable having the greatest influence on the response were identified by the Plackett- Burman design, response surface methodology was used to determine the optimum level of the significant factors for the biomass and GLA production. In this design, the total number of treatment combination (n) is calculated by the following equation

$$n = 2^k + 2K + n_0 \quad (3\text{-}6)$$

where, the k is the number of independent variables and n_o is the number of a repeat of the experiment at the central point (Bradley, 2007; Khuri & Mukhopadhyay, 2010; Myers et al., 2009; Pujari & Chandra, 2000)

A 2^4 factorial central composite design (CCD) with 8 axial points, 16 quadrant points, and 6 replicates at central point leading to a table of 30 sets of experiments, was used to optimize the temperature (x_1) and nitrate (x_2) for high

biomass, and pH (x_3) and vitamin concentration (x_4) for GLA production. The other parameters (phosphate, agitation, metal and NaCl concentrations), which did not significantly influence the production were kept at constant levels. The coding of variables was carried out according to the following equation:

$$x_i = \frac{(X_i - X_{cp})}{\Delta X_i} \qquad (i = 1, 2, 3 \dots \dots \dots k) \tag{3-7}$$

where, x_i is the dimensionless value of an independent variable, *Xi* is the real value of an independent variable, *Xcp* is the real value of an independent variable at the central point, and ΔXi is the step change of variable *i*

The relationship of the independent variables and the response is calculated from the second order polynomial equation response.

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i<j}^{k} \beta_{ij} x_i x_j + \sum_{i=1}^{k} \beta_{ii} x_i^2 \tag{3-8}$$

where, y represent the predicted response (biomass or GLA production), β_0 is the model constant, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, B_{ij} is the interaction coefficient and k is the number of factors. The analysis of variance for the experimental data and model coefficient were calculated by using Design Expert software. In addition, response surface and contour plots were constructed to visualize the observation and interaction effects of the significant variables on the response.

Table 3-5: Levels of variables tested in central composite design

Name	Unit	-α (-2)	-1	0	+1	+α (+2)
Temperature	^{0}C	9	15	21	27	33
Nitrate	mg/L	25	50	75	100	125
pH		3.5	5	6.5	8	9.5
Vitamin	mL/L	0	0.5	1	1.5	2
Each variable was tested at five levels, Axial value low (-α), low (-1), middle (0), high (+1) and Axial value high (+α)						

Table 3-6: Central Composite Design matrixes of the four variables

Run	A: Temperature	B : Nitrate	C: pH	Vitamin Solution
	^{0}C	mg/L		mL/L
1	21 (0)	75 (0)	6.5 (0)	1 (0)
2	21 (0)	75 (0)	6.5(0)	2 (+2)
3	21 (0)	75 (0)	6.5 (0)	1 (0)
4	21 (0)	75 (0)	3.5 (-2)	1 (0)
5	27 (+1)	100 (-1)	8 (+1)	1.5 (+1)
6	21 (0)	75 (0)	6.5 (0)	1 (0)
7	27 (+1)	100 (+1)	8 (+1)	0.5 (-1)
8	21 (0)	25 (-2)	6.5 (0)	1 (0)
9	21 (0)	125 (+2)	6.5 (0)	1 (0)
10	21 (0)	75 (0)	9.5 (+2)	1 (0)
11	27 (+1)	100 (+1)	8 (+1)	1.5 (+1)
12	27 (+1)	50 (-1)	8 (+1)	0.5 (-1)
13	33 (+2)	75 (0)	6.5 (0)	1 (0)
14	9 (-2)	75 (0)	6.5 (0)	1 (0)
15	15 (-1)	50 (-1)	8 (+1)	0.5 (-1)
16	27 (+1)	50 (-1)	5 (-1)	0.5 (-1)
17	15 (-1)	100 (+1)	8 (+1)	0.5 (-1)
18	15 (-1)	50 (-1)	8 (+1)	1.5 (+1)
19	27 (+1)	50 (-1)	5 (-1)	1.5 (+1)
20	15 (-1)	50 (-1)	5 (-1)	0.5 (-1)
21	15 (-1)	100 (+1)	8 (+1)	1.5 (+1)
22	15 (-1)	100 (+1)	5 (-1)	1.5 (+1)
23	15 (-1)	100 (+1)	5 (-1)	0.5 (-1)
24	21 (0)	75 (0)	6.5 (0)	0 (-2)
25	21 (0)	75 (0)	6.5 (0)	1 (0)
26	27 (+1)	100 (+1)	5 (-1)	1.5 (+1)
27	27 (+1)	100 (+1)	5 (-1)	0.5 (-1)
28	15 (-1)	50 (-1)	5 (-1)	1.5 (+1)
29	21 (0)	75 (0)	6.5 (0)	1 (0)
30	21 (0)	75 (0)	6.5 (0)	1 (0)

3.2.4 Validation of the model

The statistical model was validated with respect to biomass, PUFA and GLA production under the conditions predicted by the model. Samples were withdrawn at desired intervals and biomass, PUFA and GLA measurements were carried out as described earlier. The experiments were carried out in triplicate and results were expressed as a mean ± standard deviation. The experimental values were subsequently compared with the predicted values.

3.3 Results and discussions

3.3.1 Initial screening for growth conditions and media components for biomass production

3.3.1.1 Effect of different nitrogen source on the biomass production in *P. maculatum* MACC3

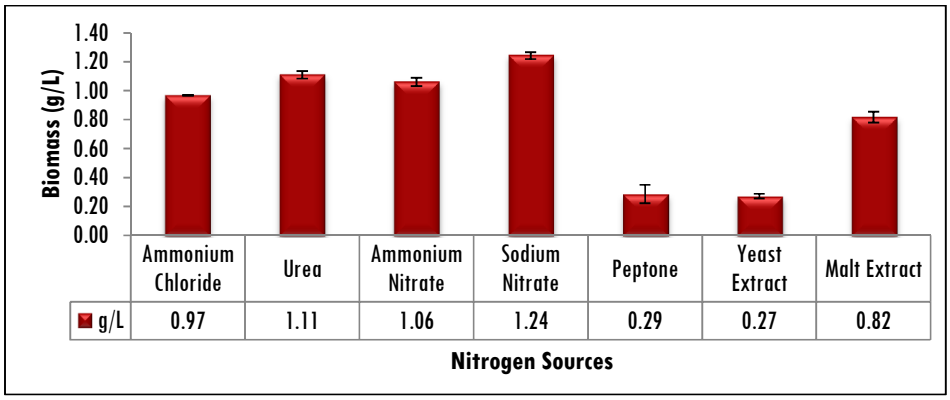


Figure 3-1: Effect of different nitrogen sources on the growth of *P. maculatum* MACC 3

The effect of different nitrogen sources on the biomass production of *P. maculatum* showed (Figure 3-1) that the maximum dry biomass was obtained from sodium nitrate at 1.24 g/L, which is a component of nitrogen source in f/2 medium, which is recommended by the algal culture collection at the University of Texas, Austin and also it is less costly (Li et al., 2008). This was followed by urea at 1.11 g/L, ammonium nitrate at 1.06 g/L, ammonium chloride at 0.97 mg/L, malt extract at

0.82 g/L concentrations. ANOVA test showed that there was a significant difference between the nitrate sources for biomass production ($p<0.05$). Sodium nitrate was selected as a nitrogen source for further studies.

A range of nitrogen compounds, both organic and inorganic, promote the growth of microalgae. Utilization of nitrite, ammonia or urea is common in microalgae. Carvalho et al. (2006) reported that different nitrogen sources do not affect the EPA and DHA yield, and the choice of nitrogen source was determined based on other criteria such as the cost of raw materials or susceptibility to bacterial degradation. (Yamaberi et al., 1998) reported that the highest lipid productivity in *Nannochloris* sp. UTEX LB1999 was 76.5 mg/L/ day. Li et al. (2008) compared the lipid productivity of different microalgae.

3.3.1.2 Effect of sodium nitrate on the biomass production in *P. maculatum* MACC3

The biomass concentrations increased with increasing the sodium nitrate concentrations (Figure 3-2). 75 mg/L is the concentration used in the f/2 medium, maximum biomass production obtained was 1.784 g/L at 300 mg/L sodium nitrate concentration. ANOVA test showed that there was a significant difference between the effect of different nitrogen concentrations on biomass production in *P. maculatum* MACC3 ($p<0.05$).

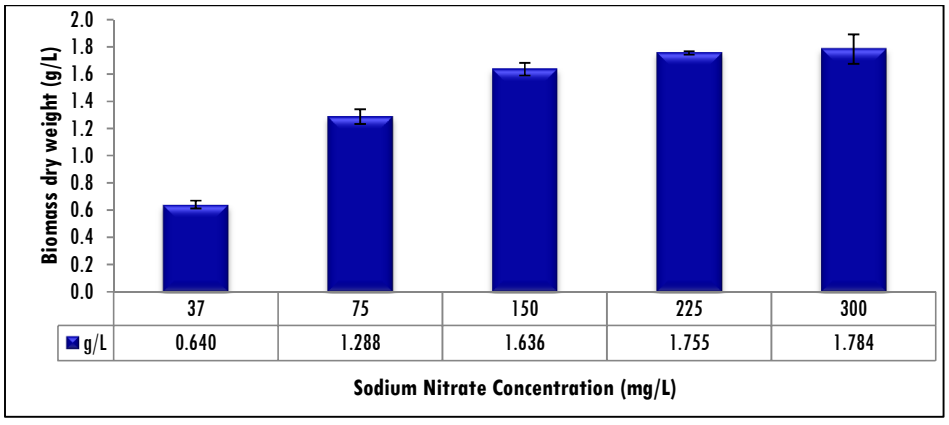


Figure 3-2: Effect of different nitrate concentrations on the growth of *P. maculatum* MACC 3

3.3.1.3 Effect of phosphate on the biomass production in *P. maculatum* MACC3

The phosphate concentration at 5 mg/L yielded maximum biomass ($p<0.05$) (Figure 3-3) and concentrations above 5 mg/L did not show any difference in the biomass production by *P.maculatum* MACC3. Phosphate is the major nutrient for microalgal growth; it plays an important role in the most of the cellular process especially involved in the energy transfer and nucleic acid synthesis (Richmond, 2008).

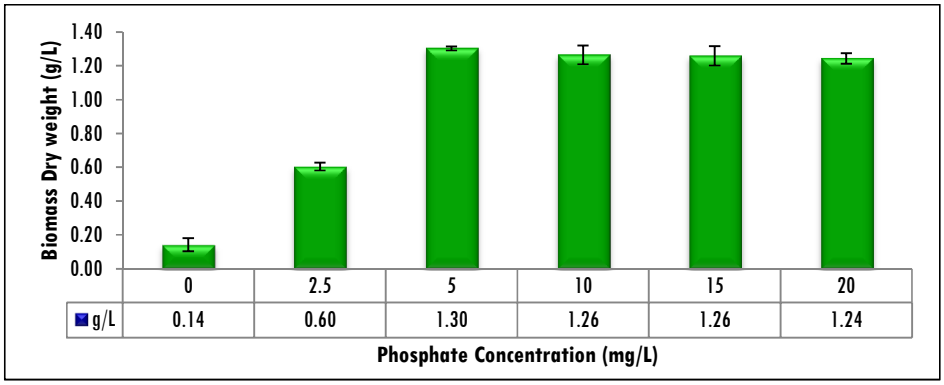


Figure 3-3: Effect of different phosphate concentrations on the growth of *P. maculatum* MACC 3

3.3.1.4 Effect of salinity on the biomass production in *P. maculatum* MACC3

P. maculatum MACC3 survived in a wide range of salinities from 0 to 50 ppt, from 35 to 50 ppt salinity the biomass yield was almost equal (Figure 3-4). The ANOVA showed that the salinity has a profound influence on the biomass production in *P.maculatum* MACC3 ($p< 0.05$).

The microalgal cell membrane has a hydrophobic nature due to the presence of fatty acids, which form an impermeable barrier to water and other polar molecules. The characteristic of membrane lipid determined is mainly by the fatty acid constituent in the lipid and their interaction between other biomolecules (Xu et al., 1998). The environmental interactions change the fatty acid profile of lipids. The effect of the interaction of medium salinity on growth and fatty acid profile of microalgae has been studied (Jiang & Chen, 1999; Xu & Beardall, 1997). The degree

of unsaturation directly depended on the fluidity and conformation of the cell membrane. Any environmental factor which affects the cell membrane fluidity will affect the degree of unsaturated fatty acids.

Hypersaline or increased salinity led to a decrease in EPA, but the levels of DHA are not affected in *Porphyridium cruentum* (Lee et al., 1989) and *Phaeodactylum tricornutum* (Yongmanitchai & Ward, 1991a). But the modification of medium salinity did not affect the EPA content or significantly affect the biomass yield in *Nannochloropsis* sp. (Zittelli et al., 1999).

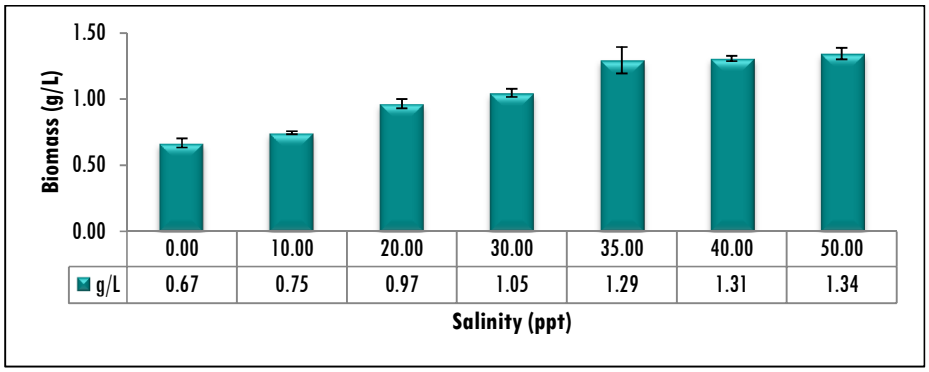


Figure 3-4: Effect of different salinities on the growth of *P. maculatum* MACC3

3.3.1.5 Effect of vitamin concentration on the biomass production *in P. maculatum* MACC3

P. maculatum MACC3 was not dependent on the vitamin for growth and with increasing concentrations of vitamin, the biomass yield decreased (Figure 3-5). The ANOVA showed that decrease in biomass yield with vitamin concentration was significant ($P < 0.05$).

Thiamine (B1) and cyanocobalamin (B12) are the most usually cited essential vitamin for microalgae. In *P. lutheri* B12 was essential for the growth, but B1 was considered to have a stimulant effect on the growth (Droop, 1958). The increased availability of these vitamins detrimental for algal growth (Droop, 1961). Over half of all microalgal species require an exogenous supply of vitamin B_{12} (cobalamin), 20% require vitamin B_1 (thiamine) and a smaller proportion (5%) require biotin (vitamin

B_7) (Croft et al., 2006) however, the effect of these organic metabolites on the growth and diversity of phytoplankton communities has been poorly studied.

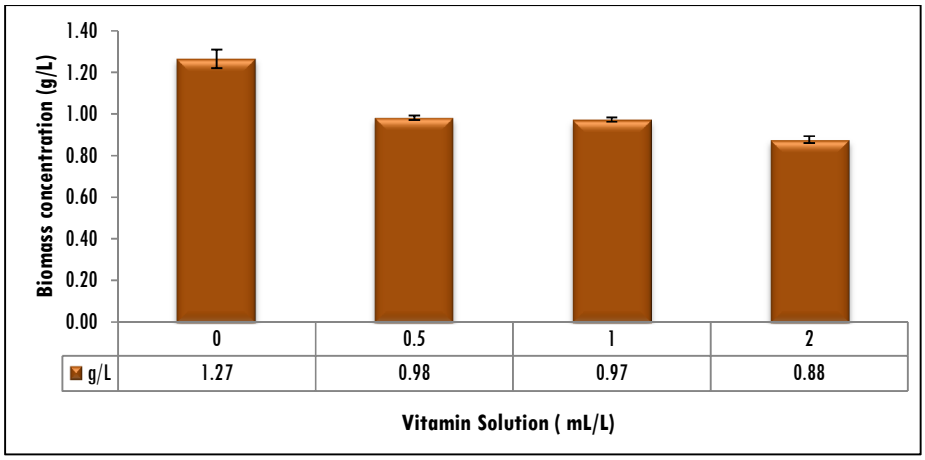


Figure 3-5: Effect of different vitamin concentrations on the growth of *P. maculatum* MACC 3

3.3.1.6 Effect of metal concentration on biomass production in *P.maculatum* MACC3

As shown in Figure 3-6, 1 mL/L concentration of the metal solution was the most effective and gave the maximum biomass production. Increasing the metal concentration beyond this had a negative effect on the algal growth ($p< 0.05$). Without micronutrients no medium can be adequate for microalgal growth; they play a direct physiological role in the algal growth. The major elements in the algal medium involve B, Mn, Zn, Co, Cu and Mo. In most of the medium formulation studies, the micronutrients together are considered as a single-variable (factor) and sometimes some of the nutrients may not be essential for algal growth. This type of unessential component may have some inhibitory effect on the production of the desired compound (Fábregas et al., 2000). Fe is essential for oxidation–reduction and chlorophyll production, and manganese is used for chlorophyll synthesis (Becker, 1994)

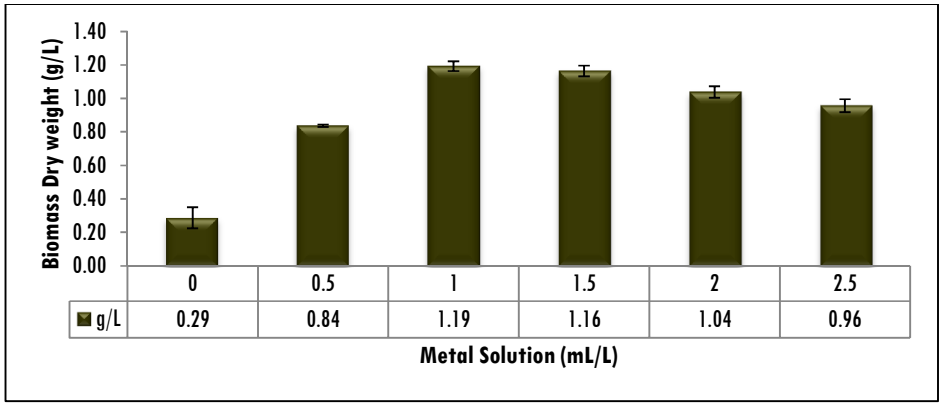


Figure 3-6: Effect of different metal concentrations on the growth of *P. maculatum* MACC 3

3.3.1.7 Effect of pH on the biomass production in *P. maculatum* MACC3

pH 8 was found to be optimal for the growth of *P. maculatum (*Figure 3-7), and increasing or decreeing pH led to a reduction in the biomass yield ($p< 0.05$).

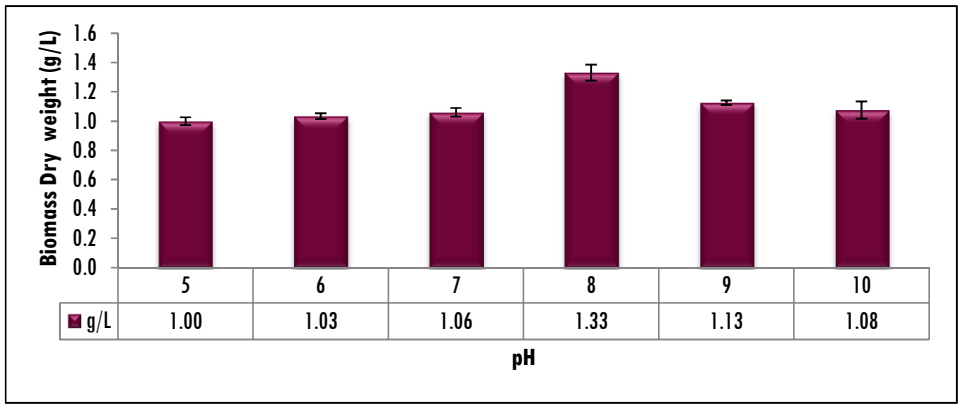


Figure 3-7: Effect of different pH on the growth of *P. maculatum* MACC 3

3.3.1.8 Effect of temperature on the biomass production in *P. maculatum* MACC3

P. maculatum MACC3 Figure 3-8 showed an increased biomass with increasing the temperature up to 35^0C, beyond which the biomass yield reduced (Figure 3-8) ($p< 0.05$).

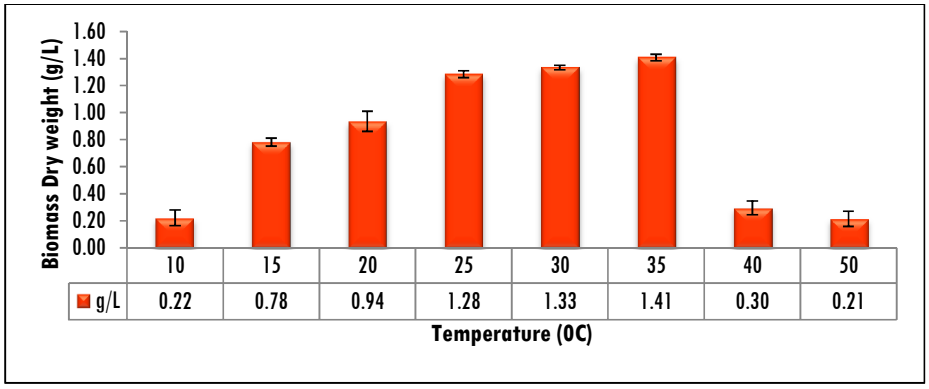


Figure 3-8: Effect of different temperatures on the growth of *P. maculatum* MACC 3

3.3.1.9 Effect of agitation on the biomass production in *P. maculatum* MACC3

Figure 3-9 shows that slow mixing had an influence on the biomass production, but more than 150 rpm did not influence the yield much. The mixing and mass transfer are critical in large-scale production than small volume cultures.

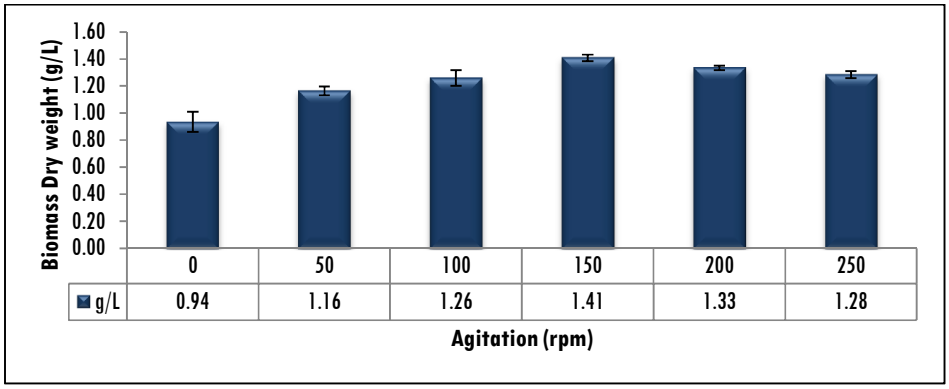
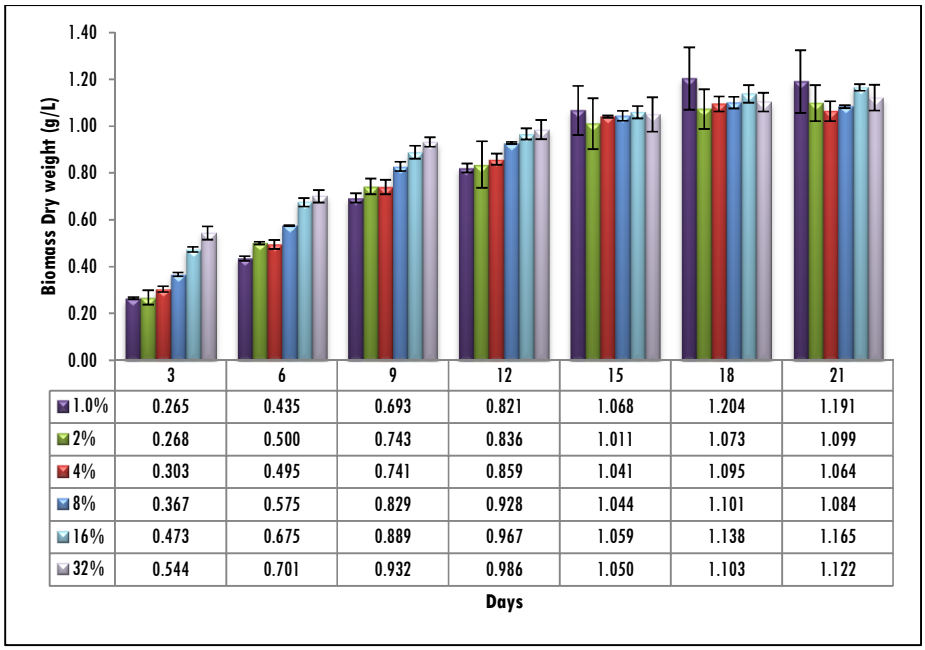


Figure 3-9: Effect of different agitation on the growth of *P. maculatum* MACC 3

3.3.1.10 Effect of inoculum size on the biomass production in *P. maculatum* MACC3

The Figure 3-10 shows that the inoculum size had an influence on the initial day of algal growth, but after 9 days the algal growth reaches nearly equal in cell

density. ANOVA showed that biomass yield during stationary phase culture had not much difference within respect to the inoculum size ($p > 0.05$).



	3	6	9	12	15	18	21
1.0%	0.265	0.435	0.693	0.821	1.068	1.204	1.191
2%	0.268	0.500	0.743	0.836	1.011	1.073	1.099
4%	0.303	0.495	0.741	0.859	1.041	1.095	1.064
8%	0.367	0.575	0.829	0.928	1.044	1.101	1.084
16%	0.473	0.675	0.889	0.967	1.059	1.138	1.165
32%	0.544	0.701	0.932	0.986	1.050	1.103	1.122

Figure 3-10: Effect of different inoculum size on the growth of *P. maculatum* MACC 3

3.3.2 Plackett and Burman Experimental Design based screening for media components

PBD was used for the screening of components by a limited number of experiments. The eleven selected variables along with their corresponding experimental and predicted values are given in Table 3-7. The statistical significance of the effect of different variables on biomass and GLA production are given in Table 3-8 and Table 3-9 respectively. The models were statistically significant for both biomass ($p<0.05$, $R^2= 0.985542$) and GLA ($p<0.05$, $R^2= 0.9689$) productions. Through the PB experimental screening maximum biomass of 1.56 g/L obtained from the run no. 12 with a temperature of 35^0C, nitrate 150 mg/L, pH of 6, phosphate 10 mg/L, salinity 15 ppt metal solution of 2 mL/L and vitamin 1 mL/L. The significant factors affecting biomass production were temperature (p=0.0246), and nitrate (p=0.0010), whereas, for GLA production the maximum yield of 113.4 mg/L was obtained from

the rum no. 6 with temperature 35^{0}C, agitation 200 rpm, pH 6, nitrate 37.5 mg/L, phosphate 2.5 mg/L, salinity 15 ppt, metal solution 0.5 mL/L and vitamin 1 mL/L. pH (p=0.0123) and vitamin concentration (p=0.0104) were significant for GLA production and these factors were further selected for the optimization using Central Composite Design.

Table 3-7: Plackett-Burman matrix for medium components with the corresponding observed values of Biomass and GLA production in *P. maculatum* MACC3

Run	A	B	C	D	E	F	G	H	D1	D2	D3	GLA	Biomass
	^{0}C	rpm		mg/L	mg/L	g/L	mL/L	mL/L				mg/L	g/L
1	-1	-1	1	-1	1	1	-1	1	1	1	-1	64.98	1.15
2	1	-1	1	1	1	-1	-1	-1	1	-1	1	30.00	1.31
3	-1	1	-1	1	1	-1	1	1	1	-1	-1	17.41	0.52
4	1	1	1	-1	-1	-1	1	-1	1	1	-1	29.93	0.86
5	1	-1	1	1	-1	1	1	1	-1	-1	-1	63.47	0.57
6	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	113.04	1.18
7	1	1	-1	1	1	1	-1	-1	-1	1	-1	72.96	0.84
8	-1	1	1	-1	1	1	1	-1	-1	-1	1	47.86	0.61
9	1	1	-1	-1	-1	1	-1	1	1	-1	1	13.24	1.03
10	1	-1	-1	-1	1	-1	1	1	-1	1	1	24.98	1.25
11	-1	1	1	1	-1	-1	-1	1	-1	1	1	40.20	0.63
12	-1	-1	-1	1	-1	1	1	-1	1	1	1	59.92	1.56

A: Temperature, B: Agitation, C: pH, D: Nitrate, E: Phosphate, F: NaCl, G: Meta Solution, H:Vitamin solution, D1- D3 dummy factors

Table 3-8: Statistical analyses of the the Plackett-Burman design for maximum biomass production

Source		Sum of Squares	*df*	Mean Square	Coefficient	*F*- value	*p*-value
Code	Variables						
Model		1.245	8	0.1557	-	25.5616	0.0111
A	Temp.	0.107	1	0.1075	0.09	17.6511	0.0246
B	Agitation	0.024	1	0.0242	-0.04	3.9756	0.1402
C	pH	0.010	1	0.0104	-0.03	1.7147	0.2816
D	Nitrate	0.996	1	0.9964	0.29	163.6269	0.0010
E	Phosphate	0.023	1	0.0232	0.04	3.8036	0.1462
F	NaCl	0.024	1	0.0240	-0.04	3.9490	0.1411
G	Metal Solution	0.058	1	0.0575	0.07	9.4506	0.0544
H	Vitamin	0.002	1	0.0020	-0.01	0.3213	0.6105

Coefficient determination (R^2) = 0.985542, Adjusted (R^2) = 0.946986, Predicted (R^2) = 0.7686.

Table 3-9: Statistical analysis of the Plackett-Burman design for maximum GLA production

Source		Sum of Squares	*df*	Mean Square	Coefficient	F- value	p-value
Code	Variables						
Model		8631.07	8	1078.88	-	11.70	0.0339
A	Temperature	902.40	1	902.40	0.87	9.792	0.0521
B	Agitation	111.37	1	111.37	0.30	1.208	0.3519
C	pH	2712.01	1	2712.01	-1.50	29.42	0.0123
D	Nitrate	98.25	1	98.25	0.29	1.066	0.3778
E	Phosphate	722.36	1	722.36	-0.78	7.838	0.0679
F	NaCl	908.10	1	908.10	-0.87	9.853	0.0517
G	Metal Solution	126.46	1	126.46	-0.32	1.372	0.3260
H	Vitamin	3050.08	1	3050.08	1.59	33.09	0.0104

Coefficient determination (R^2) = 0.9689, Adjusted (R^2) = 0.8861, Predicted (R^2) = 0.5033.

3.3.3 Optimization of media components by Central Composite Design (CCD) of RSM

Through the CCD experiment, the maximum biomass of 1.5 g/L, PUFA yield of 366 mg/L and GLA yield of 160 mg/L were obtained from the run no 2 with a temperature of 21^{0}C, nitrate 75 mg/L, pH 6.5 and vitamin 2 mL/L (Table 3-10).

Table 3-10: Central composite design matrixes of the four variables along with the experimental and predicted values

Run	A: Temperature	B : Nitrate	C: pH	D: Vitamin Solution	Biomass yield	PUFA yield	GLA yield
	OC	mg/L		mL/L	g/L	mg/L	mg/L
1	21 (0)	75 (0)	6.5 (0)	1 (0)	1.42	267.85	152.23
2	21 (0)	75 (0)	6.5(0)	2 (+2)	1.52	366.52	160.85
3	21 (0)	75 (0)	6.5 (0)	1 (0)	1.53	260.17	148.83
4	21 (0)	75 (0)	3.5 (-2)	1 (0)	1.35	7.36	4.14
5	27 (+1)	50 (-1)	8 (+1)	1.5 (+1)	0.97	55.03	18.41
6	21 (0)	75 (0)	6.5 (0)	1 (0)	1.44	229.94	131.71
7	27 (+1)	100 (+1)	8 (+1)	0.5 (-1)	1.25	138.18	66.35
8	21 (0)	62.5 (-2)	6.5 (0)	1 (0)	1.12	140.73	67.56
9	21 (0)	125 (+2)	6.5 (0)	1 (0)	1.48	185.43	80.16
10	21 (0)	25 (0)	9.5 (+2)	1 (0)	1.48	83.05	14.25

11	27 (+1)	100 (+1)	8 (+1)	1.5 (+1)	1.32	97.73	42.71
12	27 (+1)	50 (-1)	8 (+1)	0.5 (-1)	1.14	100.27	45.36
13	33 (+2)	75 (0)	6.5 (0)	1 (0)	0.2	8.23	2.87
14	9 (-2)	75 (0)	6.5 (0)	1 (0)	0.8	20.6	5.64
15	15 (-1)	50 (-1)	8 (+1)	0.5 (-1)	1.25	134.3	40
16	27 (+1)	50 (-1)	5 (-1)	0.5 (-1)	1.1	48.26	5.26
17	15 (-1)	100 (+1)	8 (+1)	0.5 (-1)	1.27	128.7	13.48
18	15 (-1)	50 (-1)	8 (+1)	1.5 (+1)	1.36	77.78	6.78
19	27 (+1)	50 (-1)	5 (-1)	1.5 (+1)	1.01	112	48.73
20	15 (-1)	50 (-1)	5 (-1)	0.5 (-1)	1.31	135.03	54.08
21	15 (-1)	100 (+1)	8 (+1)	1.5 (+1)	1.42	114.03	8.64
22	15 (-1)	100 (+1)	5 (-1)	1.5 (+1)	1.34	192.6	88.61
23	15 (-1)	100 (+1)	5 (-1)	0.5 (-1)	1.25	63.24	23
24	21 (0)	75 (0)	6.5 (0)	0 (-2)	1.51	237.5	99.31
25	21 (0)	75 (0)	6.5 (0)	1 (0)	1.51	283.01	159.19
26	27 (+1)	100 (+1)	5 (-1)	1.5 (+1)	1.14	45.5	19.57
27	27 (+1)	100 (+1)	5 (-1)	0.5 (-1)	1.21	41.2	10.19
28	15 (-1)	50 (-1)	5 (-1)	1.5 (+1)	1.21	235.75	113.53
29	21 (0)	75 (0)	6.5 (0)	1 (0)	1.49	258.38	147.74
30	21 (0)	75 (0)	6.5 (0)	1 (0)	1.49	238.4	138.39

3.3.3.1 Central composite design and response of biomass yield

The quadratic regression model for biomass production was highly significant ($p<0.0001$). The 'lack of fit' was insignificant for the model and R^2 had a value of 0.9692, indicating that the model could explain up to 96.92% of the variability in the response. The predicted R^2 value 0.8488 was in reasonable agreement with adjusted R^2 value 0.9405. For biomass production, A, B, AB, A^2, B^2 were the significant model terms, where the interactive effects were significant. Adequate precision measures such as signal to noise ratio greater than 4 are desirable. The signal to noise ratio (adequate precision) for the model was higher than 4 (26.117) for biomass production, indicating an adequate signal which could be used to navigate the design space (good fit).

Table 3-11: Analysis of variance (ANOVA) for the fitted quadratic polynomial model of central composite design based on the biomass yield

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	2.078	14.000	0.148	33.798	< 0.0001
A-Temperature	0.254	1.000	0.254	57.918	< 0.0001
B-Nitrate	0.102	1.000	0.102	23.256	0.0002
C-pH	0.019	1.000	0.019	4.238	0.0573
D-Vitamin	0.000	1.000	0.000	0.005	0.9472
AB	0.021	1.000	0.021	4.679	0.0471
AC	0.000	1.000	0.000	0.006	0.9399
AD	0.016	1.000	0.016	3.749	0.0719
BC	0.004	1.000	0.004	0.805	0.3839
BD	0.015	1.000	0.015	3.444	0.0832
CD	0.007	1.000	0.007	1.504	0.2389
A^2	1.556	1.000	1.556	354.366	< 0.0001
B^2	0.041	1.000	0.041	9.276	0.0082
C^2	0.002	1.000	0.002	0.512	0.4854
D^2	0.007	1.000	0.007	1.565	0.2300
Residual	0.066	15.000	0.004		
Lack of Fit	0.057	10.000	0.006	3.213	0.1048
Pure Error	0.009	5.000	0.002		
Cor Total	2.144	29.000			

The experimental results of the CCD for biomass yield fitted with the second order polynomial equation (3-9)

$$\begin{aligned} Biomass\ yield = {} & (-1.12478) + (0.252579x_1) + (0.001806x_2) \\ & + (0.011077x_3) + (-0.26084\,x_4) + (0.000239x_1x_2) \\ & + (0.000141x_1x_3) + (-0.01069x_1x_4) + (0.000396x_2x_3) \\ & + (0.002459x_2x_4) + (0.027092x_3x_4) + (-0.00662x_1^2) \\ & + (-6.2\mathrm{E}-05x_2^2) + (-0.00402x_3^2)(0.063322x_4^2) \end{aligned} \quad (3.9)$$

where, x_1, x_2, x_3, and x_4 were the temperature, nitrate, pH and vitamin respectively

The ANOVA results showed (Figure 3-11) that the temperature and nitrate were the significant factors for the biomass production ($p<0.05$) and the interaction between

temperature and nitrate was significant ($p<0.05$) for the biomass production in *P. maculatum* as reported generally for microalgae. Nitrogen is the most crucial nutrient for growth and lipid production in *P.maculatum*. The temperature is the most crucial environmental factor for biomass and lipid production in *P.maculatum*. Tornabene et al. (1983) reported that the high lipid content in green algae obtained at lower $NaNO_3$. Illman et al. (2000) reported that *Chlorella vulgaris* increased lipid production in low nitrate level. *Picochlorum* cells grown under optimal conditions transferred to phosphorus and nitrogen-depleted medium supplemented with sodium carbonate, carbon dioxide, glucose, and sodium chloride responded differently in the improvement in the lipid accumulation (Dahmen et al., 2014).

3.3.3.2 Central composite design and response of PUFA yield

The quadratic regression model for PUFA production was highly significant ($p<0.0001$). The 'lack of fit' was insignificant for the model and R^2 had a value of 0.9322, indicating that the model could explain up to 93.22% of the variability of the response. The predicted R^2 value 0.641 was in reasonable agreement with adjusted R^2 value 0.8689. For PUFA production A, B, D, AC, BC, CD, A^2, B^2, C^2 were the significant model terms, where the interactive effects were significant. Adequate precision measures such as signal to noise ratio greater than 4 are desirable. The signal to noise ratio (adequate precision) for the model was higher than 4 (14.87) for PUFA production, indicating an adequate signal which could be used to navigate the design space (good fit). The experimental results of the CCD for PUFA yield fitted with the second order polynomial (Equation 3-10 and Table 3-12)

$$\begin{aligned} PUFA\ yield = {} & (-1516.918) + (58.4621473x_1) + (2.435558552x_2) \\ & + (285.5290734x_3) + (291.2025478x_4) \\ & + (0.038057206x_1x_2) + (2.194882164x_1x_3) \\ & + (-3.677872013x_1x_4) + (0.499590969x_2x_3) \\ & + (0.079184467x_2x_4) + (-37.91779736x_3x_4 \\ & - 1.789411522x_1^2) + (-0.043606751x_2^2) \\ & + (-25.21023019x_3^2)(29.91780331x_4^2) \end{aligned} \quad (3\text{-}10)$$

where, x_1, x_2, x_3 , and x_4 were the temperature, nitrate, pH and vitamin respectively

Table 3-12: Analysis of variance (ANOVA) for the fitted quadratic polynomial model of central composite design based on the PUFA yield

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	241467.596	14.000	17247.685	14.738	< 0.0001
A-Temperature	9125.911	1.000	9125.911	7.798	0.0137
B-Nitrate	6.165	1.000	6.165	0.005	0.9431
C-pH	639.063	1.000	639.063	0.546	0.4713
D-Vitamin	6643.751	1.000	6643.751	5.677	0.0309
AB	521.406	1.000	521.406	0.446	0.5146
AC	6243.490	1.000	6243.490	5.335	0.0355
AD	1947.851	1.000	1947.851	1.664	0.2165
BC	5615.801	1.000	5615.801	4.799	0.0447
BD	15.675	1.000	15.675	0.013	0.9094
CD	12939.834	1.000	12939.834	11.057	0.0046
A^2	113822.639	1.000	113822.639	97.259	< 0.0001
B^2	20373.737	1.000	20373.737	17.409	0.0008
C^2	88251.449	1.000	88251.449	75.409	< 0.0001
D^2	1534.414	1.000	1534.414	1.311	0.2701
Residual	17554.612	15.000	1170.307		
Lack of Fit	15673.677	10.000	1567.368	4.166	0.0644
Pure Error	1880.935	5.000	376.187		
Cor Total	259022.2084	29			

df –Degree of freedom, R^2 =0.9322, Adjusted R^2 = 0.8689, Predicted R^2 = 0.641

The ANOVA results based on the PUFA yield showed (Figure 3-12 and 3-13) that temperature and vitamin were the significant factors for PUFA production ($p<0.05$). Singh & Ward (1997) reported that at low temperature the solubility of oxygen increases and this provide a large amount of intracellular oxygen molecules available for the oxygen-dependent desaturation reaction. Increased production of unsaturated fatty acid in microalgae at low temperature is a means of adaptation to the cold environment by increasing the unsaturated fatty acids can help to the organisms to maintain cell fluidity at low temperature (Yongmanitchai & Ward, 1989).

Psychrophilic microorganisms with an optimum temperature below 20^0C typically contain more highly unsaturated fatty acids than the mesophiles.

Even through higher PUFA content can be obtained from microalgae grown at lower temperatures, the biomass yield is reduced and overall productivity. High energy cost related to maintaining cooling is another disadvantage of industrial production of PUFA. To overcome this constraint temperature shifting strategy was employed for the heterotrophic culture of *Thraustochytrium* sp. and *Crypthecodinium cohnii* for better production of DHA by shifting from 25^0C to 15^0C at the later stage of cultivation (Jiang & Chen, 2000). However, in the cultivation of *Thraustochytrium roseum,* the optimum DHA production was at 25^0C (Jiang et al., 1999; Raghukumar, 2008). High level intracellular molecular oxygen was another critical factor for increased PUFA production at low temperature because the desaturation and elongation of fatty acids are an oxygen depended reaction (Los et al., 1997; Ma et al., 2011; Renaud et al., 2002). Low temperature induced PUFA productions were observed in *Phaeodactylum tricornutum* (Jiang & Gao, 2004) *Nannochloropsis* sp. (Rukminasari et al., 2013) and *Porphyridium purpureum* (Nuutila et al., 1997). These results suggest that the effect of temperature on cell growth and n-3 PUFA production should be carefully studied for individual microalgal species.

3.3.3.3 Central composite design and response of GLA yield

The quadratic regression model for GLA production was highly significant ($p<0.01$). The 'lack of fit' was insignificant for the model and R^2 had a value of 0.9538, indicating that the model could explain up to 95.38 % of the variability of the response. The predicted R^2 value 0.7567 was in reasonable agreement with adjusted R^2 value 0.9106. For GLA production D, AC, CD, A^2, B^2 and C^2 were the significant model terms, where the interactive effects were significant. Adequate precision measures such as signal to noise ratio greater than 4 are desirable. The signal to noise ratio (adequate precision) for the model was higher than 4 (14.87) for GLA production, indicating an adequate signal which could be used to navigate the design

space (good fit). (Table 3-13). The experimental results of the CCD for GLA yield fitted with the second order polynomial equation (3-11)

$$\begin{aligned} GLA\ yield = {} & (-962.048) + (29.06018x_1) + (2.95264x_2) + (177.1887x_3) \\ & + (252.3551x_4) + (0.042376x_1x_2) + (2.07926x_1x_3) \\ & + (-1.76538x_1x_4) + (0.16808x_2x_3) + (0.018833x_2x_4) \\ & + (22.2135x_3x_4) + (1.06339x_1^2) + (0.03341x_2^2) \\ & + (16.4655x_3^2)(27.3053x_4^2) \end{aligned} \quad (3\text{-}11)$$

x_1, x_2, x_3 and x_4 where the temperature, nitrate, pH and vitamin respectively

Table 3-13: Analysis of variance (ANOVA) for the fitted quadratic polynomial model of central composite design GLA yield

	Sum of		**Mean**	**F**	**p-value**
Source	Squares	df	Square	Value	Prob > F
Model	86808.790	14.000	6200.630	22.100	< 0.0001
A-Temperature	392.550	1.000	392.550	1.400	0.2553
B-Nitrate	49.360	1.000	49.360	0.180	0.6808
C-pH	425.240	1.000	425.240	1.520	0.2372
D-Vitamin	1878.880	1.000	1878.880	6.700	0.0206
AB	646.480	1.000	646.480	2.300	0.1498
AC	5603.030	1.000	5603.030	19.970	0.0005
AD	448.790	1.000	448.790	1.600	0.2252
BC	635.670	1.000	635.670	2.270	0.153
BD	0.890	1.000	0.890	0.003	0.9559
CD	4440.970	1.000	4440.970	15.830	0.0012
A^2	40196.780	1.000	40196.780	143.290	< 0.0001
B^2	11958.580	1.000	11958.580	42.630	< 0.0001
C^2	37646.130	1.000	37646.130	134.190	< 0.0001
D^2	1278.140	1.000	1278.140	4.560	0.0497
Residual	4208.020	15.000	280.530		
Lack of Fit	3722.640	10.000	372.260	3.830	0.0755
Pure Error	485.380	5.000	97.080		
Cor Total	91016.820	29.000			

df–Degree of freedom, R^2 =0.9538, Adjusted R^2 = 0.9106, Predicted R^2 = 0.7567

The ANOVA results based on the GLA yield showed that the model was significant ($p<0.05$) for the GLA production. The factor vitamin (D) concentration was significant for GLA production. Significant interactions were observed between temperature and pH (AC), and pH and vitamin concentration (CD). The GLA optimization based on the CCD model resulted in an increase in the GLA production in *P. maculatum* MACC3 from 113 mg/L to 143 mg/L (Figure 3-14 to 16).

The two-dimensional contour plots and their respective three-dimensional contour response surface plots demonstrated significant interaction effects between the variables. The optimal values obtained from the contour plots have been almost equal to the results obtained from the regression equation.

3.3.4 Modified composition of f/2 medium for GLA production in *P.maculatum* MACC3

Based on the optimization the GLA production medium for *P.maculatum* was reconstituted for maximum production. The modified growth medium contains the ingredients given below (Table 3-14).

Table 3-14: Modified f/2 medium and growth conditions for GLA production in *P.maculatum* MACC3

Parameters	Quantity
Medium components	
Nitrate	91 mg/L
Phosphate	5 mg/L
Salinity	35 ppt
Metal solution	1 mL/L
Vitamin solution	1.49 mL/L
Physical factors	
Light	60±10 µmol photon/m^2/s
Light : dark period	16:8
Agitation	120 rpm
pH	7.71
Temperature	19^{0}C
Inoculum size	10% of 3.4 X10^7 cells/mL

3.3.5 Validation of the Experimental Model

The aim of the optimization was to determine a set of variables (parameters) that would lead to the development of the best model, which would maximize GLA production. The solutions obtained from the model are given in Table 3-15. The maximum predicted value for the GLA yield was 143 mg/L with optimum medium components such as nitrate 90 mg/L, vitamin 1.51 mL/L and at temperature 19^0C and pH 6.32. The model was validated by repeating the experiment under optimum conditions in shake flasks which resulted in biomass yield of 1.53±0.07 g/L, PUFA yield of 280±20 mg/L and GLA yield of 150 ± 10 mg/L, indicating a good correlation between predicted and experimental values, proving the validity of the model.

Table 3-15: Solution predicted by the model for GLA production in *P. maculatum* MACC3

Solutions	Temperature*	Nitrate*	pH*	Vitamin*	Biomass	PUFA	GLA	Desirability	
Number	(0C)	mg/L		mL/L	(g/L)	mg/L	mg/L		
1	16.36	73.91	7.26	0.55	1.41	197	110	1	
2	22.13	87.4	5.5	0.55	1.45	171	112	1	
3	16.16	96.89	7.32	1.41	1.51	203	94	1	
4	26.25	50.31	6.42	0.56	1.13	121	77	1	
5	19.07	90.96	6.32	1.09	1.53	268	143	1	Selected

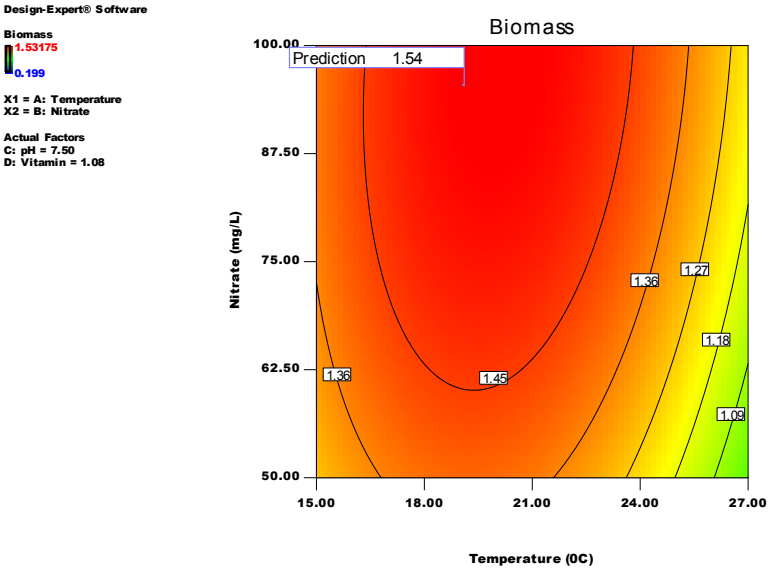
Only a few studies have been carried out on the PUFA and GLA production optimization in marine microalgae. But a number of studies were carried out in the optimization of microalgal culture medium for EPA and DHA production (Carvalho et al., 2006; Cho et al., 2007; Otero et al., 1997; Park et al., 2011; Song et al., 2007). They have particularly demonstrated the effect of low temperature on high production of unsaturated fatty acids.

Nitrogen starvation and related increased lipid content production were reported in *Picochlorum sp.* The lipid content of the Chlorophytes raised up to 45% of dry weight by stress or nutrient starvation (Converti et al., 2009; El-Kassas, 2013; Rukminasari & others, 2013). Such type of studies conducted in several microalgae showed that significant increase in lipid was reported in association with nitrogen stress.

Mourente et al. (1990) reported that *Nannochloris* sp. and *Chlorella* species (Trebouxiophyceae) showed a high proportion of short chain polyunsaturated fatty acids as the major fatty acids. Roncarati et al. (2004) reported that high level of concentrated CO_2 generally increased unsaturated long chain PUFA. The addition of CO_2 has a significant influence on the increase in unsaturated long chain fatty acids concentration. Ben-Amotz et al. (1985) reported that among marine microalgae, *Picochlorum* genus has high intracellular lipids. The fatty acid profile of *Picochlorum* species showed that high predominance (65%) of 18 carbons unsaturated fatty acids with 26 % omega- 3 and 24 % omega 6

3.4 Conclusions

In this chapter, different media components were investigated and screened to determine the significance of their effect on the biomass production and GLA yield of the newly isolated marine microalga *Picochlorum maculatum* MACC3. One factor at a time approach was used to fix the range of medium components for screening experiment using Plackett and Burman method. PB was used for the initial screening of the significant factors for biomass and GLA production. Based on the PB experiment results, the most statistically significant positive media components for biomass production were temperature and nitrate, and for GLA production, pH, and vitamin concentration. These four parameters were further optimized by using central composite design to determine the optimum concentration of these components and the interaction effect between the parameters for maximum growth and GLA production. The maximum amount of biomass, PUFA and GLA yields achieved by the design combination was 1.53±0.5 g/L, 280±20 mg/L and 150±10 mg/L respectively. The validations of experiments were also carried out to verify the adequacy and the accuracy of the model and the results showed that the predicted values were in agreement with the experimental values. The optimized culture medium obtained from the experiment will be used for the scale-up culture of *Picochlorum maculatum* MACC3.



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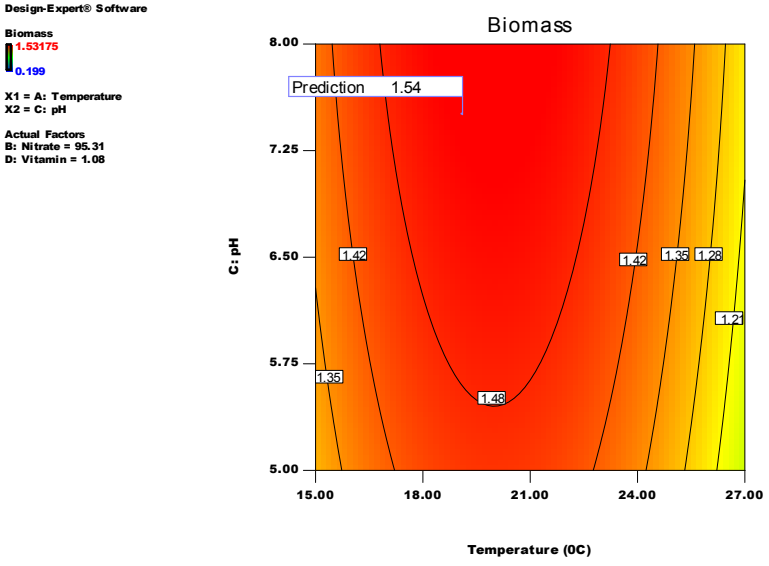
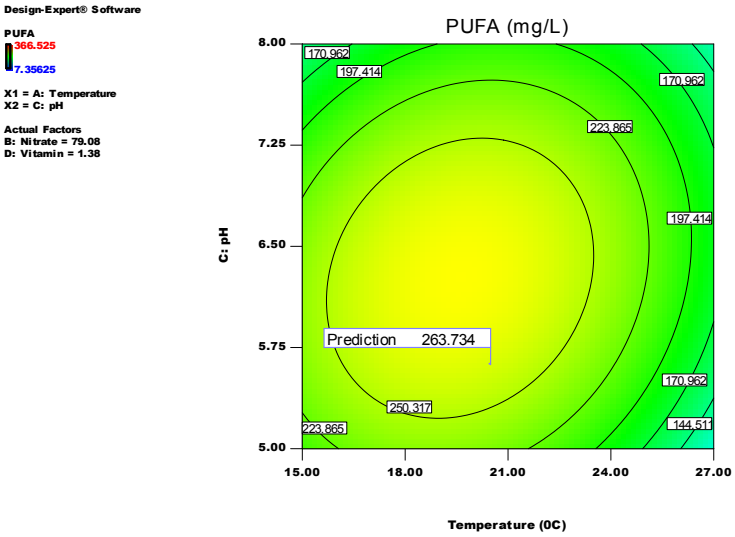


B

Figure 3-11: Contour plot of biomass yield of *P. maculatum* MACC3

(A) Interaction between temperature and nitrate (B) interaction between pH and temperature



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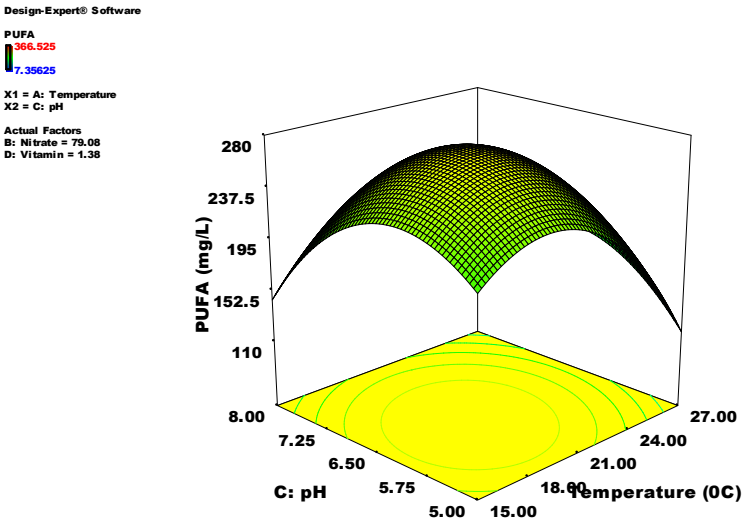
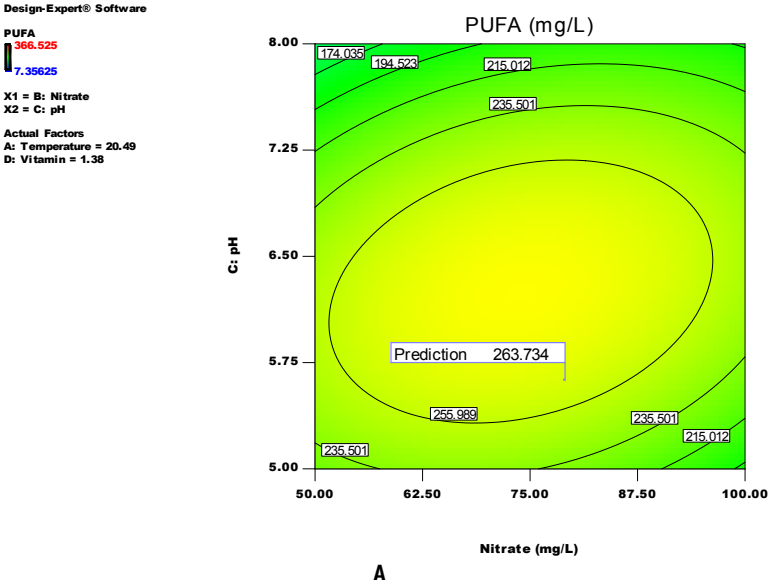


B

Figure 3-12: Contour plot and response surface plot of PUFA yield of *P. maculatum* MACC3

(A) Interaction between temperature and pH (B) interaction between temperature and pH (RSM 3D plots)



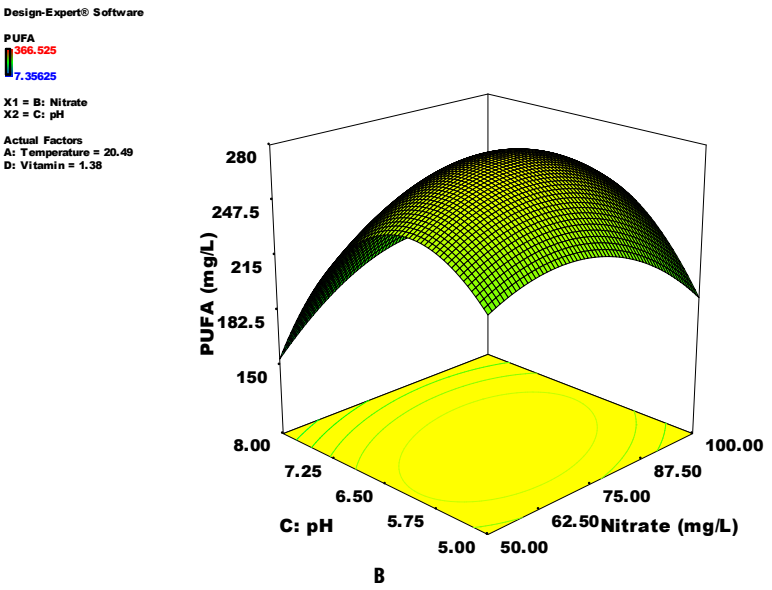
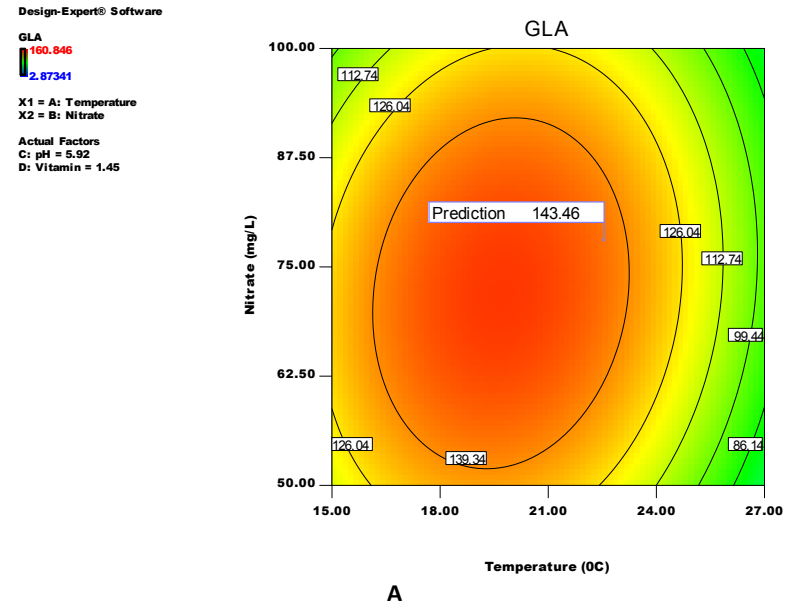


Figure 3-13: Contour plot and response surface plot of PUFA yield of *P. maculatum* MACC3

(A) Interaction between nitrate and pH (B) interaction between nitrate and pH (RSM 3D plot)



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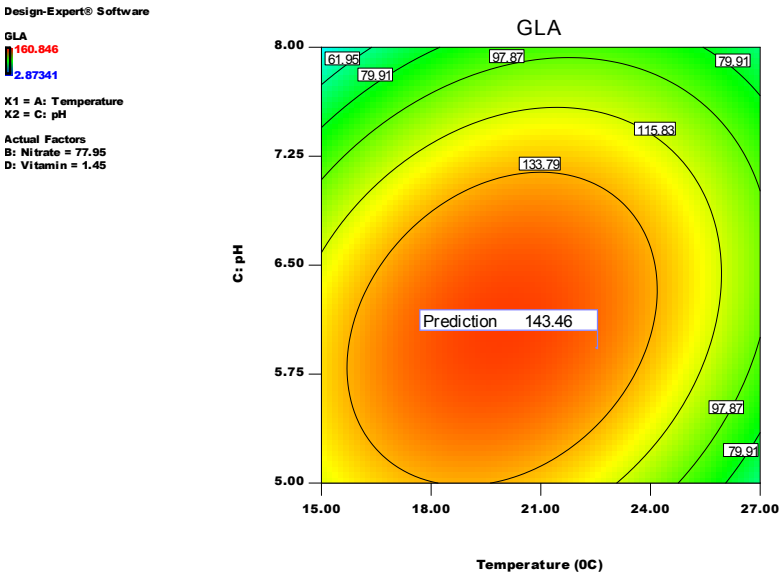
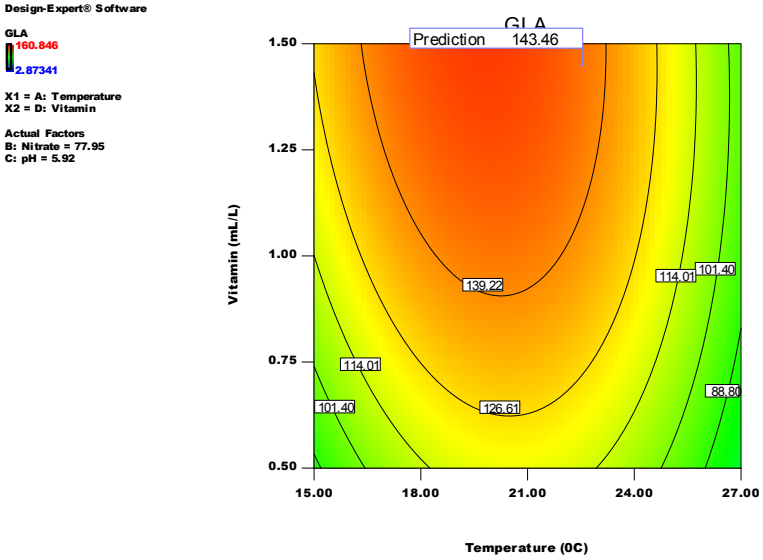


B

Figure 3-14: Contour plot of GLA yield of *P. maculatum* MACC3
(A) Interaction between nitrate and temperature (B) interaction between pH and temperature



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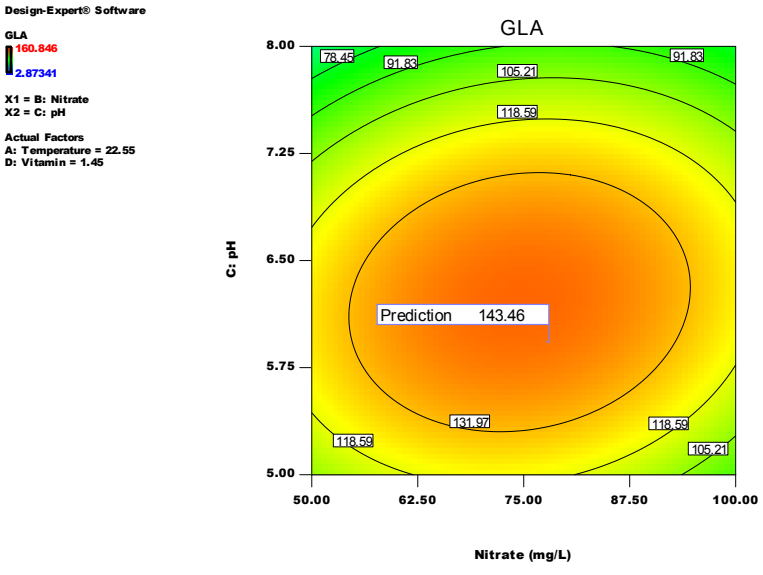
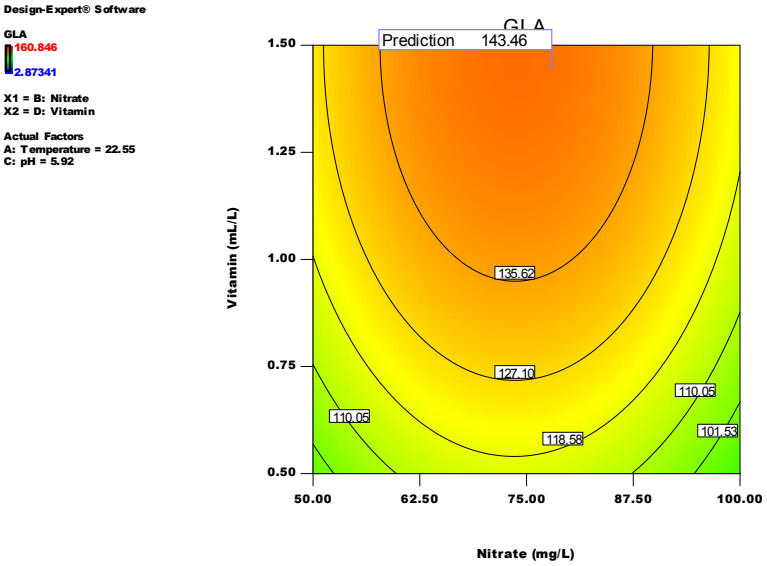


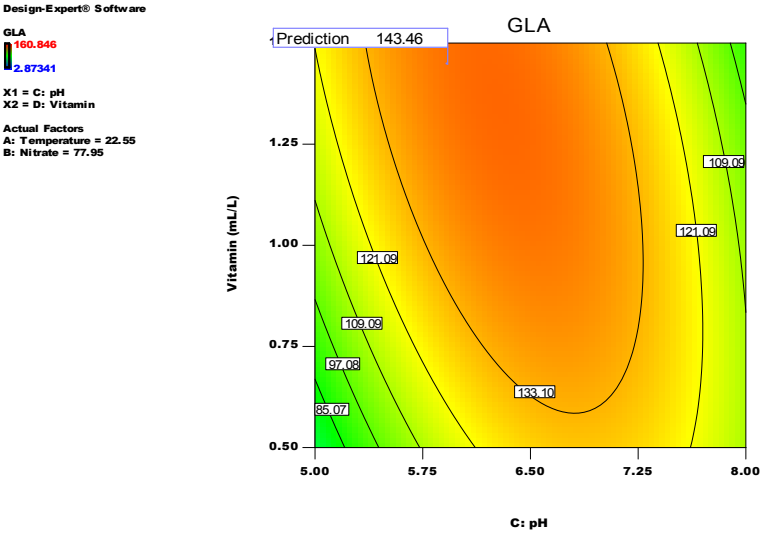
B

Figure 3-15: Contour plot of GLA yield of *P. maculatum* MACC3

(A) Interaction between temperature and vitamin (B) interaction between nitrate and pH



A



B

Figure 3-16: Contour plot of GLA yield of *P. maculatum* MACC3
(A) Interaction between nitrate and vitamin (B) interaction between vitamin and pH

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Chapter 4

SCALE UP PRODUCTION, AND OPTIMIZATION OF HARVESTING TECHNIQUE OF MARINE MICROALGA *PICOCHLORUM MACULATUM* MACC3 BY FLOCCULATION WITH CHITOSAN AND ALUMINIUM SULPHATE USING RESPONSE SURFACE METHODOLOGY

•Contents•

4.1 Introduction

Mass production and downstream processing of the microalgal biomass are the most critical components limiting the technical and economic feasibility of commercialization processes of many potent microalgal biotechnologies. Several attempts are made for the large-scale cultivation of microalgae in different cultivation systems for providing future food and fuel (Matsunaga et al., 2005). The mass production of microalgae as food and feed are limited to a few species such as *Nannochloropsis oculata*, *N. gaditana*, *Chlorella* sp., *Tetraselmis suecica, Pavlova lutheri, Isochrysis galbana* and *Dunaliella tertiolecta, Dunaliella salina* (Masoji dek & Prasil, 2010). Edible blue-green algae including *Nostoc* and *Arthrospira* (Spirulina) have been used for food for thousands of years (Borowitzka, 1999; Spolaore et al., 2006). These species are selected based on their nutritional value, culture easiness and absence of side effects, such as toxicity. Most of the microalgae show great versatility in their nutritional profile not only among the different species but also in a genetically different population of the same species (strains). In addition, the nutritional profile of microalgae varies depending on the culture conditions and the

chemical and physical properties of culture media (Guedes & Malcata, 2012; Harwood & Guschina, 2009; Lavens et al., 1996).

After isolation, identification, and screening of a microalgal strain for the product of interest, the next step is the development of mass production process to establish the link between discovery and commercialization (Barbosa, 2003). The large-scale culture systems vary depending upon the microalgal strain and the product of interest. Open pond/ culture systems can be used only for fast growing strains, which grow in extreme environmental conditions such as high salinity and pH that prevent the growth of other unwanted organisms. For high value-added products used in pharmaceutical and food industries mono algal or axenic cultures are needed to meet the standards of closed photobioreactor systems developed for this purpose (Pulz & Scheibenbogen, 1998; Volesky et al., 1999).

High-density cultures of microalgae in photobioreactors are achieved through proper reactor design and process optimization. Most important scale-up and operational parameters influencing the microalgal production is light, mass transfer, shear and mixing rates. These parameters are closely interrelated and they determine the efficiency and productivity of the systems and optimization of these critical factors maximize the production yield (Barbosa, 2003; Richmond, 2008). The main limiting factor for the development of high-density culture photobioreactors is the light gradient formed in the reactor, due to light absorption and mutual shading by the cells. Depending upon the mixing characteristics of the systems, the cell will circulate between the light and dark zones of the reactor.

Harvesting of microalgae suspensions

Algal biomass harvesting is the most difficult and expensive part in the execution of microalgal technology (Riaño et al., 2012), and it contributes nearly 20- 30 % of the total production cost and 10% of the total energy utilization (Barbosa, 2003). While considering the large quantity of algal biomass recovery, suitable harvesting technologies are needed to achieve several physical, chemical, and biological ways to reach the optimum solid- liquid separation. Most of the common harvesting techniques such as

centrifugation and filtration have drawbacks such as high energy cost and difficulty in recovering small algae like *Chlorella* and *Scenedesmus* (Mata et al., 2010). Membrane microfiltration and ultra- filtration are the other possible alternatives suitable for fragile cells and small-scale production processes, but these are expensive because of membrane replacement and pumping. There are no universal harvesting techniques and it is an active field of research to develop appropriate and economical harvesting systems for potent algal species. The combination of flocculation is used to aggregate the microalgal cells to increase the effective particle size, which, help to achieve the sedimentation, centrifugal recovery and filtration (Molina Grima et al., 2003). Most of the harvesting methods discriminate based on size and density to perform the biomass separation. Micro-strainers are attractive for harvesting because of their mechanical simplicity and large unit size (Weissman & Goebel, 1987).

Richmond (2008) suggested that the desired product quality is the criteria for the selection of a harvesting process- gravimetric sedimentation enhanced by flocculation for low-value products, and continuous centrifugation and for high value added products for food and aquaculture applications. Another important benchmark for selecting the harvesting procedure is the density or the acceptable level of moisture in the resulting concentrate. Gravity sedimentation of biomass is generally more watery than centrifugally recovered biomass, which significantly influence the recovery, downstream processes, and the product cost. The costs of thermal drying are much higher than those of mechanical dewatering. For reducing the overall production cost, a concentrated biomass with higher solids content is required. This has been achieved through a combination of pre-concentration methods with a mechanical dewatering step such as microstrainer, filtration, and centrifugation followed by post concentration by means of a screw centrifuge or a thermal drying (Molina Grima et al., 2003; Richmond, 2008).

4.1.1 Flocculation

Compared to the conventional algal harvesting methods, flocculation is reasonably effective with low capital investment and low operating costs. It also allows rapid treatment of large quantities of algae with scalability (Davis, 2011).

Flocculants trigger the aggregation of cells and help biomass recovery. Normally microalgal cell surfaces possess partial negative charge that prevents them from forming aggregation in suspension culture, and flocculants neutralize these charges and allow the cells to flocculate. The flocculation of algal biomass is dependent on several factors such as pH, the ionic strength of the culture medium, culture age, cell size, flocculants dose and concentration of algal biomass in the culture (Cui, 2013; Shen et al., 2013). Flocculation potential of different algae depends on the properties, such as the cell wall composition, physiological conditions, culture age and type of extracellular excretions (Avnimelech et al., 1982). Therefore, a suitable flocculation method for microalgae harvesting should be determined based on these characteristics.

4.1.1.1 Inorganic flocculants

Flocculants are used in fast solid-liquid separations involving the aggregation of particles, and are classified into inorganic and organic polymeric materials (Guibal & Roussy, 2007; Renault et al., 2009; Szygula et al., 2009). Inorganic flocculants including mineral additives (calcium salt, lime), hydrolyzing metal salt (aluminium sulphate [$Al_2(SO_4)_3$], ferric chloride [$FeCl_3$], ferric sulphate [$Fe_2(SO_4)_3$]) pre-hydrolyzed metals (poly aluminum chloride, poly-aluminosilicate) and polyelectrolytes (coagulant acids) have been studied extensively (Renault et al., 2009). Currently, the uses of inorganic flocculants have been limited due to a large amount of flocculants required to achieve the flocculation efficacy and it produces a large amount of slurry. Most of the inorganic flocculants are highly sensitive to pH and inefficient towards small algal cells. Most of the inorganic flocculants are temperature dependent. Polyferric chloride (PFC) is a newly introduced inorganic polymeric flocculant which, contain complex polynuclear ions formed by OH bridging having high molecular weight and high cationic charges. PFC is more effective at a comparatively lower dose and can be used in wide range of pH and temperature due to their high level of hydrolysis (Granados et al., 2012; Sirin et al., 2012).

4.1.1.1.1 Aluminium sulphate

The inorganic salt aluminium sulphate (alum) is one of the most widely used coagulants in conventional water purification systems. It is widely used for its low cost, ease of use and availability. However, it produces ample amount of sludge that is difficult to dehydrate, its efficiency entirely depended on the pH

4.1.1.2 Organic flocculants

Commercially available organic flocculants are two types, synthetic materials based on various monomers like acrylamide and acrylic acid and natural polymers like starch, cellulose, alginate and natural gums. The advantage of natural polymeric flocculants is their ability to produce large, dense compact flocs that are stronger and have good settling characteristics compared to those obtained by coagulation achieved using inorganic flocculants. Polymeric flocculants are easy to handle and immediately soluble in aqueous systems and also reduce sludge volume. High removal efficiencies can be achieved even with a small amount of flocculant, which generates a small volume of sludge. The performance of polymer is less dependent on pH. The flocculation performance primarily depends on the type of flocculant used and type of algae and medium composition. Polyacrylamide (PAM) is a commonly used organic polymer with various functions, which can be used to produce a good settling performance at relatively low cost (Uduman et al., 2010; Zeng et al., 2008).

As compared with the inorganic flocculants, polymers are required in low flocculant doses, efficient at low temperature, less pH dependent, and generate a small volume of sludge. The synthetic polymers are water soluble, but lack biodegradability and are toxic and costly.

4.1.1.3 Bioflocculants

Most of the commercial polymers are also derived from petroleum-based raw materials, that is not always safe or environmentally friendly and therefore, there is growing interested in developing natural low-cost alternatives to synthetic polyelectrolytes. Numerous biological products have recently been proposed and

studied as effective coagulants and flocculants for replacing conventional materials. These include biopolymers (starches, chitosan, alginates), and microbial materials produced by microorganisms including bacteria, fungi, and yeast, which are safe and biodegradable with no secondary pollution (Wang et al., 2006), with potential applications in food and fermentation processes, down steaming processing and water treatment systems.

4.1.1.3.1 Chitosan

Chitosan, a cationic natural polyelectrolyte, extensively used as an algal flocculant is a natural polymer obtained from the marine crustaceans, shrimps, and crabs (Kurita, 2006). Chitin is the second most abundant biopolymer in the world after cellulose and shows unique properties due to the presence of primary amino group and has a commercial importance due to the presence of high nitrogen content. It is a linear copolymer of D-glucosamine and N-acetyl –D-glucosamine produced by the deacetylation of chitin, insoluble in either water or organic solvents, soluble in dilute acids such as acetic acid, formic acid and inorganic acids with a remarkable exception of sulphuric acid, the free amino groups are protonated and the biopolymer becomes fully soluble (Kurita, 2006; Rinaudo, 2006). The pKa of the amino group of glucosamine residue is about 6.3 and at acidic pH, chitosan becomes a soluble cationic polymer with high charge density. The treatment of chitosan with acid produces protonated amine groups along the chain, facilitating electrostatic interaction between the polymer chain and the negatively charged algal cells (Roberts, 1992). The flocculation properties of chitosan depend on the molecular weight, degree of deacetylation and crystallinity (Renault et al., 2009; Rinaudo, 2006; Zeng et al., 2008). In particular, chitosan is a promising bioflocculant for down streaming and purification process, and is environmental friendly (Salehizadeh & Shojaosadati, 2001).

4.1.1.4 Electroflocculation

The electro flocculation has been applied for efficient microalgal biomass harvesting in recent years. Electroflocculation is a process that uses electric currents

to dissolve sacrificial metal to supply the ions required for the flocculation and is a physical/chemical process that has the advantages of being non-species specific, simpler to operate and results are more predictable (Lee et al., 2013). However, this is energy requiring process and therefore, will affect the economic feasibility of the process development.

In the present study, widely used inorganic flocculant aluminium sulphate and natural bioflocculant chitosan were evaluated for their potential in harvesting *P.maculatum* MACC 3 biomass cultivated in 10 L carboy jars. The selection of these two flocculants was based on the wide acceptance of these flocculants in microalgal industries (Heasman et al., 2000; Knuckey et al., 2006). The effects of pH, flocculant dose, algal biomass concentration and settlement time on flocculation efficiency were also tested. The chitosan based bio-flocculation was compared with the chemically induced flocculation by aluminium sulphate, in terms of recovery efficiency and time needed for sedimentation. Finally, optimized processes for biomass harvesting using the two flocculants were designed based on statistical optimization using response surface methodology.

4.2 Materials and methods

4.2.1 Scale up production of *Picochlorum maculatum* MACC 3

4.2.1.1 Sterilization of sea water

The sea water was sterilized by chlorination. Active chlorine is a strong oxidizing agent. The commercially available sodium hypochlorite (NaClO) with 10 mg/L active chlorine was used for sea water sterilization for 1 hr, and the residual chlorine was neutralized using 12 mg/L sodium thiosulphate ($Na_2S_2O_3$). The sterilized sea water was aerated strongly for an hour before using for medium preparation (Morretti, 1999).

4.2.1.2 Inoculation

P. maculatum MACC3 was mass produced in 10 L sterilized Nalgene carboys with 45 cm height 30 cm diameter and 0.3 mm thickness. Liquid cultures were grown

in with optimized Guillard f/2 liquid medium (recipe as in Chapter 2) in natural filtered seawater of salinity 35 ppt. 18th day old exponentially growing *P. maculatum* cells of 1.2- 1.5 optical density (3.4 x 10^7 cells/mL) was inoculated at 10 % of the culture volume of 8L. The cultures were maintained at temperature and pH of 25^0C and 7.5 ± 0.2, respectively under a LED (Light-Emitting Diode) light panel with photon irradiance of 100 ±20 µmol photon/m^2/s and 16:8 light and dark period. The medium optical density was measured at 600 nm on every two days and pH was monitored regularly. Mixing was provided by sparging sterile air (0.2µm filter) continuously, into the cultures using aquarium pumps and aeration tube fitted with a stone sparger.

The growth rate of algae was characterized based on cell counts every two days using Neubauer haemocytometer under light microscope (Olympus CX41, magnification 20-40X), and optical density was measured at 750 nm wavelength (OD750 nm) in a Shimadzu UV- 1601 spectrophotometer. The specific growth rate was calculated from the slope of the linear regression of time (days) and cell density (cells/mL) (Doan et al., 2011; Wood et al., 2005)

$$\mu = (lnN_t - lnN_0)/(t - t_0) \quad (4\text{-}1)$$

where, N_t is cell density at time (t), and N_0 is cell density at the start of the exponential phase (to). All the flocculation experiments were carried out in triplicate using cultures with an initial OD_{750} ~ 1.5 corresponding to the cell density of 3.4 X 10^7 cell/mL.

4.2.2 Flocculants

The stock solution of 5 g/L chitosan in 1% acetic acid was prepared by continuously stirring until a clear solution was obtained. Aluminium sulphate stock solution of 5 g/L was prepared in distilled water.

4.2.3 Calculation of flocculating efficiency

50 mL of *P. maculatum* culture in f/2 medium with 1 g/L concentration was poured into 100 mL beaker and placed on a magnetic stirrer (n=3). pH of the medium

was adjusted with 0.1N HCl or 1N NaOH solutions. The culture pH was measured using Eutech pH meter (Thermo Fisher Scientific). Different concentrations of flocculants were added to 50 mL algal cultures in a continuous shaking mode, followed by vigorous stirring at a maximum speed for the 30s followed by stirring at 100 rpm for 15 min and settling for 15 min. After flocculation of the algal cells, an aliquot of the culture was withdrawn from a height of two third from the bottom and optical density was measured at 750 nm (Sirin et al., 2012). Flocculating activity was evaluated by the flocculating efficiency (FE) according to the Equation

$$Flocculation\ Efficiency\ (\%) = \left(1 - \frac{A - C}{B - C}\right) X\ 100 \qquad (4\text{-}2)$$

where, A is the OD_{750} of sample and B is the OD_{750} of the initial culture and C is the OD_{750} of the reference blanks with seawater and flocculants at the respective (Lee et al., 2009).

4.2.4 Single factor flocculation experiments

The appropriate range of significant factors affecting flocculation was selected based on one-factor flocculation experiments. Using the one-factor flocculation experiments, the approximate range of flocculant concentrations (chitosan and aluminium sulphate) and pH were fixed for experimental design using the central composite design of response surface methodology.

4.2.4.1 pH induced flocculation

The effect of pH on the flocculation was tested in the pH range of 5-11. The experiments were conducted by changing the pH of algal culture using 0.1N HCl or 1N NaOH solutions. The experiments were conducted in 100 mL cylindrical beakers with 50 mL of microalgal cultures. The continuous mixing and homogenous pH of algal cultures were achieved using magnetic stirring, followed by slow shaking at 100 rpm for 15 min and settling for 15 min. All flocculation experiments were done in triplicate.

4.2.4.2 Effects of flocculant dosage on harvesting efficiency

Chitosan (sigma Aldrich, India) and aluminium sulphate were added to the cultures (n=3) at increasing concentrations of 10, 20, 40, 80, 100, 150, 200, 300, 400 mg/L. pH of the culture was maintained at 8.5.

4.2.4.3 Effect of pH on the flocculation efficiency

The effects of pH of the medium on the flocculation efficiency of chitosan and aluminium sulphate at 60, 120 mg/L respectively, were carried out by adjusting the pH of the culture using 0.1N HCl or 1N NaOH solutions. The cultures were shaken vigorously for homogeneity of pH in the medium. The cultures were shaken at 300 rpm for 1 min and then 100 rpm for 15 min and allowed to stand still for 15 min for settling. An aliquot of the supernatant was taken for measuring the flocculation efficiency.

4.2.5 RSM experimental design for optimization of biomass flocculation

A central composite design (CCD) constructed using the Design-expert 7 software (Stat-Ease, Minneapolis, MN, USA) was used for the optimization of algal biomass flocculation. The three factors used for experimental design to study the effect on the flocculating activity were, the algal biomass concentration (ABC) (x_1), pH (x_2) and flocculant dosage (x_3). All factors were tested at five levels and the quality of analysis model was evaluated based on an analysis of variance (ANOVA). The response variable (y) that represents the flocculating activity was fitted using a second-order model in the form of a quadratic polynomial equation (4-3):

$$y = \beta_0 + \sum_{i=1}^{m} \beta_i x_i + \sum_{i<j}^{m} \beta_{ij} x_i x_j + \sum_{i=1}^{m} \beta_{ii} x_i^2 \qquad (4\text{-}3)$$

where, y is the response variable to be modeled x_i and x_j are the independent variables that determine y. β_0, β_i, β_{ii} are the offset terms, the i linear coefficient, and the quadratic coefficient, respectively. B_{ij} reflects the interaction between x_i and x_j (Yang et al., 2009).

A total 18 experiments each for chitosan and aluminium sulphate (Table 4-2; Table 4-5) were designed including 8 fractional factorial designs (2^3), 6 star points (2x3), and 6 replicates at central points. Based on the results of single factor experiments, the algal biomass concentration ranged from 1 to 2 g/L with a central point of 1.5 g/L, pH from 7 to 11 with a central point of 9 for chitosan and pH 8-11 with central point 9.5 for aluminium sulphate and flocculant dosage from 10 to 100 mg/L with central point of 55 mg/L for chitosan and 50 to 150 mg/L with central point of 100 mg/L for aluminium sulphate.

Algal biomass concentration (ABC) was adjusted by adding fresh culture medium to the pre-concentrated cells. pH of each sample was adjusted by adding 0.1N HCl or 1N NaOH solutions. The flocculants were added to the samples at the concentration ranges, mixed well; the pH of the final medium was adjusted as per the design and transferred to the settling cylinder. After settlement for 15min, the upper clear supernatant was removed using fixed volume pipette without disturbing the bottom concentrated algal biomass for optical density reading at 750 nm.

4.2.6 Cell viability

The viability of cells was calculated by staining using Evan's blue. Aliquots of 10 mL *P. maculatum* cultures were treated with 1 mL of 1% (w/v) solution of stain which then allowed standing for 30 min at room temperature, and observed under light microscope (Olympus CX41). The stain penetrates through the dead algal cell wall, imparting a blue colour to the cells, whereas viable cells retain their natural colour. The total cell count was taken using Neubauer haemocytometer (Harith et al., 2009) and the percentage cell viability *(CV)* was calculated as follows:

$$CV\ (\%) = \text{Viable cells/Total cells}\ X\ 100 \qquad (4\text{-}4)$$

4.2.7 Scanning electron microscopic (SEM) analysis

For the SEM analysis, 3 mL of *P.maculatum* culture was harvested by centrifugation at 8000*g* for 5 min (kept as the control) and flocculation using chitosan and aluminium sulfate. Methodology discussed in chapter 2 (session 2.2.4.3.1)

4.3 Results and discussion

4.3.1 Growth rate of algae

A linear regression equation (4-4) describing the relation between dry biomass concentration and optical density was calculated for *Picochlorum maculatum* MACC3

$$y = 1.0046x - 0.1019\ (R^2 = 0.9768) \quad (4\text{-}4)$$

where, y=dry biomass concentration (g/L), x= optical density at 600 nm.

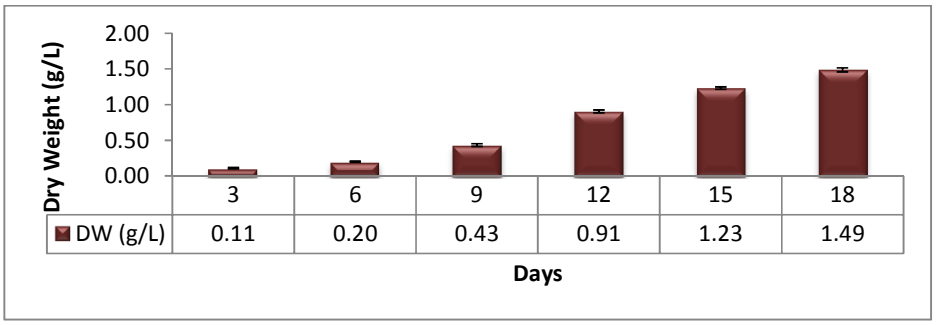


Figure 4-1: Growth of *P.maculatum* MACC3 in f/2 medium in 10 L Nalgene carboys

Table 4-1: Biomass and growth characteristics of *P. maculatum MACC3* on 18th day of culture

Growth properties	
Maximum biomass concentration (g/L)	1.49 ± 0.21
Growth rate (per day)	0.482 ± 0.13

The growth of algae in mass culture conditions is an important selection criterion for industrial applications. *P. maculatum* MACC3 reached maximum growth on the 18th day (Figure 4-9) under the experimental conditions (Figure 4-1) with a biomass production of 1.5 g/L. *P. maculatum* had a higher growth rate of 0.48 per day (Table 4-1). Zhu & Dunford (2013) reported that the *Picochlorum oklahomensis* which has a biomass production of 2.1 g/L on (stationary phase) 18th-day culture and a growth rate of 0.5 per day and division rate of 0.7 division/ day with a generation time of 1.4. In comparison with the *Nannochloropsis oculata* which had a biomass yield of 1.2 g/L during stationary phase (11 days) with a growth rate of 0.3 and division rate of 0.5 and a generation time of 2.2 per day.

4.3.2 Effect of pH induced flocculation

The change in pH change without using flocculant induced a few cells to flocculate, when the pH was 9 (Figure 4-2). The pH induced flocculation test in *P. maculatum* MACC3 resulted in a flocculation efficiency of nearly 3% in neutral pH, with an increase up to 16 % by increasing alkalinity and of 5.5 % in acidic pH ($p<0.05$).

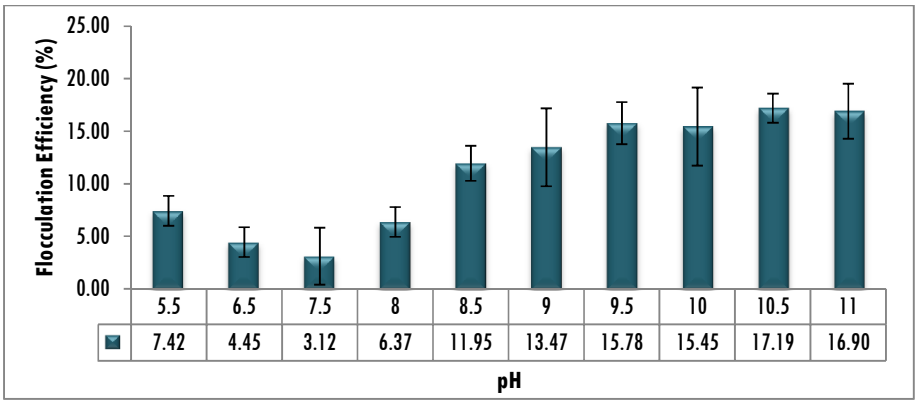


Figure 4-2: Effect of pH on induced flocculation on *P.maculatum* MACC3 after 15 min of settlement

The cell surface charge strongly depends on the pH of the culture medium. When the pH of the medium was more acidic or basic- below 7 or above 9, the negative charge of the cell surface was reduced. The microalgal cell membrane is composed of extra polysaccharides, lipids, and proteins and at low pH, the amine groups dissociate and carboxyl groups protect the dissociation, as a result the negative surface charge of the algal cell is weakened (Cheng et al., 2011; Sukenik & Shelef, 1984). When the pH is above 9, the microalgal cells release extracellular polysaccharides changing the net surface charge of the algal cells (Davis, 2011; Zhang et al., 2011).

4.3.3 Effect of flocculant dosage on flocculation activity

The single factor analysis of different flocculant concentrations at an algal biomass concentration (ABC) of 1 g/L and pH of 8.5 showed that the flocculation efficiency reached 85-95% at chitosan concentration of 50-100 mg/L and aluminium sulphate concentration of 100-200 mg/L (Figure 4-3). The results of two-way ANOVA

showed a significant difference in the flocculation efficiency between different flocculant concentrations ($p< 0.05$), but not between the flocculants ($p>0.05$). *P. maculatum* cells exhibited nearly 90% cell viability at increasing concentrations of chitosan (10-500 mg L^{-1}) and aluminium sulfate concentration below 100 mg L^{-1}. Cell viability declined gradually with increasing aluminium sulfate concentrations above 100 mg L^{-1} (Figure 4-11). Evan's Blue is widely used as a specialist stain for assessing cell viability during the trial to optimum method of microalgal cell harvesting, preservation and storing of concentrated microalgal paste and slurries. The stain finds application in bioassay experiments as well as live feed in hatcheries (Harith et al., 2009).

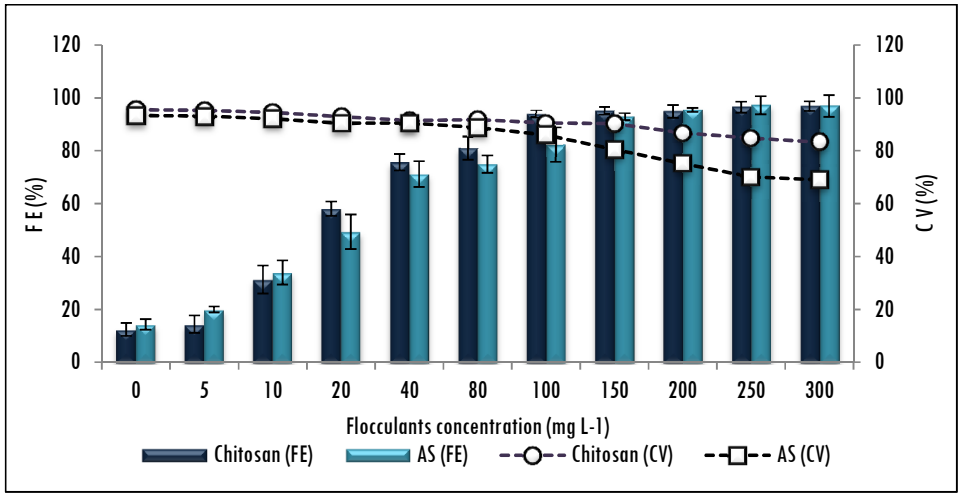


Figure 4-3: Effect of chitosan and aluminium sulphate concentration on the flocculation efficiency (FE) and cell viability (CV) of *P. maculatum*

The optimum chitosan concentration reported for flocculation of *Tetraselmis chuii*, *Isochrysis* sp. and *Thalassiosira pseudonana* were 40 mg/L, whereas for *Chaetoceros muelleri* 150 mg/L, with no apparent consistency in concentrations for efficient flocculation within different algal taxonomical groups (Heasman et al., 2000). Heasman et al. (2000) tested the effect of six different organic solvents such as citric acid, acetic acid, tartaric acid, glycolic acid, lactic acid and dichloroacetic acid to prepare the chitosan solution on flocculation and observed no significant difference in flocculation between chitosan prepare in different solvents.

4.3.4 Effect of pH on flocculation

The effect of varying pH on the flocculation at constant biomass (1 g/L) and flocculant concentration (75 and 150 mg/L for chitosan and AS respectively) showed that flocculation efficiency reached 90- 95 % at pH 8.5-9 for chitosan and 9-9.5 for aluminium sulphate, respectively (Figure 4-4).

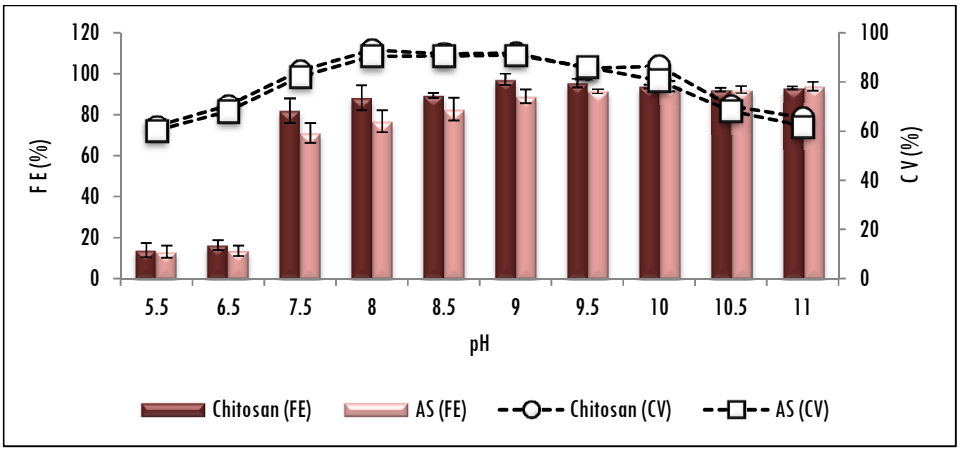


Figure 4-4: Effect of pH on chitosan and aluminium sulphate based flocculation efficiency (FE) and cell viability (CV) of *P. maculatum*

The flocculant efficacy of chitosan and aluminium sulphate in flocculating *P. maculatum* MACC3 biomass was tested at different pH and constant flocculant concentration. Chitosan gave above 90 % flocculation efficiency at pH 8- 8.5 and aluminium sulphate 90% flocculation efficiency at pH 8.5- 9. Two-way ANOVA result showed that change in pH had a significant effect on flocculation efficiency ($p< 0.05$), which varied with the flocculant used ($P<0.05$). Evan's blue based cell viability analysis showed that the algal cells exhibited nearly 90% cell viability at pH ranging from 7 to 9 and gradually reduced to 70- 85% with increasing (9-10.5) or decreasing (4-7) pH values. Cell integrity and viability were significantly reduced at a pH below 4 and above 10.5. After comparing Evan's blue based cell viability analysis results of microalgae such as *Chlorococcum ellipsoideum, C. nivale* and *Scenedesmus* sp. Liu et al. (2013) concluded that the decrease in pH value (up to 3.5) did not lead to cell lysis.

4.3.5 Optimization of flocculation using Response Surface Methodology

The algal biomass concentration, pH, and flocculant dosage had a significant effect ($p<0.001$) on the flocculation of *P. maculatum* MACC3 with both the chitosan and aluminium sulphate. The data from the optimization experiments were fitted with the general polynomial equation for the three factors (4-5).

$$FE = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 \quad (4\text{-}5)$$

4.3.5.1 RSM based flocculation study in P. maculatum MACC3 with chitosan

A central composite design (CCD) was performed to estimate the optimum level of each variable. Three variables were selected for the optimization of flocculation in *P. maculatum* MACC3. The components were algal biomass concentration (ABC), pH and flocculant concentration (chitosan). A CCD experiment with 20 trials was performed (Table 4-2) to optimize the flocculation efficiency and evaluate the effect of each factor in addition to the interaction between them.

Table 4-2: Central composite design matrixes of the three variables of flocculation using chitosan

	Factor 1	Factor 2	Factor 3	Response 1
	A: Algal Biomass Concentration (ABC)	B: pH	C: Chitosan concentration	Flocculation Efficiency (FE)
Std.	g/L		mg/L	%
1	1.00	7.00	10.00	26.15
2	2.00	7.00	10.00	3.41
3	1.00	11.00	10.00	60.39
4	2.00	11.00	10.00	52.76
5	1.00	7.00	100.00	82.80
6	2.00	7.00	100.00	57.52
7	1.00	11.00	100.00	94.80
8	2.00	11.00	100.00	96.92
9	0.66	9.00	55.00	88.56
10	2.34	9.00	55.00	74.02
11	1.50	5.64	55.00	24.19
12	1.50	12.36	55.00	90.82

13	1.50	9.00	-20.68	2.04
14	1.50	9.00	130.68	83.41
15	1.50	9.00	55.00	81.85
16	1.50	9.00	55.00	84.96
17	1.50	9.00	55.00	86.50
18	1.50	9.00	55.00	91.54
19	1.50	9.00	55.00	80.84
20	1.50	9.00	55.00	92.46

Table 4-3: ANOVA for response surface quadratic model for flocculation with chitosan

Source	Sum of Squares	DF	Mean Square	F- value	*p*-value
Model	17538.03	9.00	1948.67	105.74	< 0.0001
A-ABC	445.30	1.00	445.30	24.16	0.0006
B-pH	4469.01	1.00	4469.01	242.49	< 0.0001
C-Chitosan Conc.	7790.36	1.00	7790.36	422.71	< 0.0001
AB	225.89	1.00	225.89	12.26	0.0049
AC	6.50	1.00	6.50	0.35	0.5658
BC	129.52	1.00	129.52	7.03	0.0243
A^2	41.96	1.00	41.96	2.28	0.1622
B^2	1474.66	1.00	1474.66	80.02	< 0.0001
C^2	3391.73	1.00	3391.73	184.04	< 0.0001
Residual	184.29	10.00	18.43		
Lack of Fit	67.46	5.00	13.49	0.58	0.7193
Pure Error	116.83	5.00	23.37		
Cor Total	17722.33	19.00			
The coefficient of determination (R^2) of the model was 0.9896					

The experimental results of the CCD for Chitosan flocculation fitted with the second order polynomial equation (4-6)

$$FE_{Chitosan} = (-197.32) + (-40.970x_1) + (49.053x_2) + (1.706x_3) + (5.313x_1x_2) + (0.040x_1x_3) + (-0.044x_2x_3) + (-6.82x_1^2) + (-2.528x_2^2) + (0.00075x_3^2) \quad (4\text{-}6)$$

where, X_1, X_2 and X_3 are ABC, pH and flocculant concentration respectively.

The model was significant with the coefficient of determination R^2 of 0.9896 showing the goodness of fit of the model. ANOVA of the quadratic regression model (Table 4-3) indicated that the model was highly significant ($p<0.0001$) in predicting the flocculation efficiency. The 'lack of fit' was insignificant for the model. The closer the R^2 value to 1, stronger the model and better it predicts the response. R^2 had a value of 0. 9896, which indicated that the model could explain up to 98.96% of the variability in the response, and the model could not explain only 1.04 % of the total variation. The value of R^2 indicated a good agreement between the experimental and predicted values for flocculation with chitosan. An adequate precision measure such as signal to noise ratio greater than 4 is desirable. The signal to noise ratio (adequate precision) for the model was higher than 4 (31.74) for chitosan based flocculation model, indicating an adequate signal which could be used to navigate the design space (good fit). The predicted R^2 value 0.9599 was in reasonable agreement with adjusted R^2 value 0.9802. These results indicate that model was suitable for describing the relationship between flocculation efficiency and the significant factors.

For chitosan based flocculation algal biomass (A), pH (B) and chitosan concentration (C) were significant and significant interaction occurring between algal biomass and pH (AB) ($p<0.01$), pH and chitosan concentration (BC) ($p<0.05$). The regression model developed was represented in 2D contour plots (Figure 4-5, Figure 4-6, Figure 4-7 and Figure 4-8) to gain a better understanding of the interaction between the variables and to determine the optimum level of each variable for maximum flocculation efficiency.

4.3.5.1.1 Validation of the Experimental Model

RSM based optimization of flocculation of *P. maculatum* biomass with chitosan yielded a high flocculation efficiency of 96 %. The model predicted optimum parameters were 1.5 g/L of algal biomass concentration at pH 9 with a chitosan concentration of 86.76 mg/L (Figure 4-10).

4.3.5.2 RSM based optimization of flocculation of P. maculatum MACC3 biomass with aluminium sulphate

The experimental result of the CCD for aluminium sulphate flocculation (FE_{AS}) (Table 4-4) was fitted with the second order polynomial Eq.(4-7).

Table 4-4: Central composite design matrixes of the three variables for flocculation using aluminium sulphate

	Factor 1	Factor 2	Factor 3	Response 1
	A: Algal Biomass Concentration (ABC)	B: pH	C: Aluminium Sulphate (AS)	Flocculation Efficiency (FE)
Std	g/L		mg/L	%
1	1.00	8.00	50.00	50.05
2	2.00	8.00	50.00	50.88
3	1.00	11.00	50.00	87.17
4	2.00	11.00	50.00	71.37
5	1.00	8.00	150.00	90.69
6	2.00	8.00	150.00	80.59
7	1.00	11.00	150.00	96.30
8	2.00	11.00	150.00	85.95
9	0.66	9.50	100.00	92.78
10	2.34	9.50	100.00	74.82
11	1.50	6.98	100.00	45.83
12	1.50	12.02	100.00	94.05
13	1.50	9.50	15.91	51.51
14	1.50	9.50	184.09	97.12
15	1.50	9.50	100.00	80.54
16	1.50	9.50	100.00	86.54
17	1.50	9.50	100.00	87.54
18	1.50	9.50	100.00	85.04
19	1.50	9.50	100.00	87.25
20	1.50	9.50	100.00	86.78

Table 4-5: ANOVA for the response surface quadratic model of flocculation with aluminium sulphate

Source	Sum of Squares	DF	Mean Square	F-value	p-value
Model	4958.56	9	550.95	28.067	< 0.0001
A-ABC	315.34	1	315.34	16.065	0.0025

B-pH	1640.09	1	1640.09	83.551	< 0.0001
C-Aluminium sulphate	2134.94	1	2134.94	108.760	< 0.0001
AB	35.64	1	35.64	1.816	0.2075
AC	3.75	1	3.75	0.191	0.6715
BC	271.89	1	271.89	13.851	0.0040
A^2	2.20	1	2.20	0.112	0.7446
B^2	403.48	1	403.48	20.554	0.0011
C^2	202.00	1	202.00	10.291	0.0094
Residual	196.30	10	19.63		
Lack of Fit	161.61	5	32.32	4.658	0.0583
Pure Error	34.69	5	6.94		
Cor Total	5154.86	19			

The coefficient of determination (R^2) of the model was 0.961

$$FE_{AS} = (-343.206) + (24.5539x_1) + (63.9822x_2) + (1.3290x_3) + (-2.8144x_1x_2) + (-0.0273x_1x_3) + (-0.0777x_2x_3) + (-1.5632x_1^2) + (-2.3516x_2^2) + (-0.0015x_3^2) \quad (4\text{-}7)$$

The models were significant with the coefficient of determination R^2 of 0.9619 ANOVA of the quadratic regression model (Table 4-5) indicated that the models were highly significant ($p<0.0001$). The 'lack of fit' was insignificant for the model. R^2 had a value of 0.9619, which indicated that the model could explain up to 96.19% of the variability in the response. The value of R^2 indicated a good agreement between the experimental and predicted values of flocculation. The signal to noise ratio (adequate precision) for the model was higher than 4 (18.04) for aluminium sulphate based flocculation model, indicating an adequate signal, which could be used to navigate the design space (good fit). The predicted R^2 value 0.7420 was in reasonable agreement with adjusted R^2 value 0.9276. The results indicated that model was suitable to describe the relationship between flocculation efficiency and the significant factors. The experimental results of the CCD for Aluminium sulphate flocculation fitted with the second order polynomial equation (4-7)

For aluminium sulphate based flocculation, the algal biomass concentration (A) ($p<0.01$), pH (B) ($p<0.0001$) and aluminium sulphate concentration (C) ($p<0.0001$) were significant factors, with significant interaction occurring between pH

and aluminium sulphate (BC) concentration ($p<0.01$). The regression model is represented in 2D contour plots (Figure 4-5 to 8) to gain a better understanding of the interaction between the variables and to determine the optimum level of each variable for maximum flocculation efficiency. RSM based optimization of *P. maculatum* biomass with aluminium sulphate yielded a high flocculation efficiency of 92.81 %. The model predicted parameters for optimum flocculation were algal biomass concentration of 1.5 g/L at pH 9.99 and aluminium sulphate concentration of 124 mg/L (Figure 4-9b).

The experimental results obtained were in good agreement with the predicted values and the both flocculation models gave flocculation efficiencies greater than 90 % demonstrating the feasibility of chitosan and aluminium sulphate for *P. maculatum* MACC3 biomass flocculation. The efficiency of flocculation was influenced by the pH and flocculant concentration. Shen et al. (2013) reported that for the flocculation of *Nannochloropsis oculata* with aluminium sulphate, the optimum factors were 383 μM aluminium sulphate, pH 8.3 and *N. oculata* biomass of 1.7 g/L. Both the flocculants were effective in flocculating *P. maculatum* MACC3 biomass even though the cells were unicellular and small in size. The flocculation efficiency of chitosan was 96 % (pH 9 and 86.76 mg/L of chitosan) and aluminium sulphate 92 % (pH- 9.9, 124 mg/L of AS) at an algal biomass concentration of 1.5 g/L.

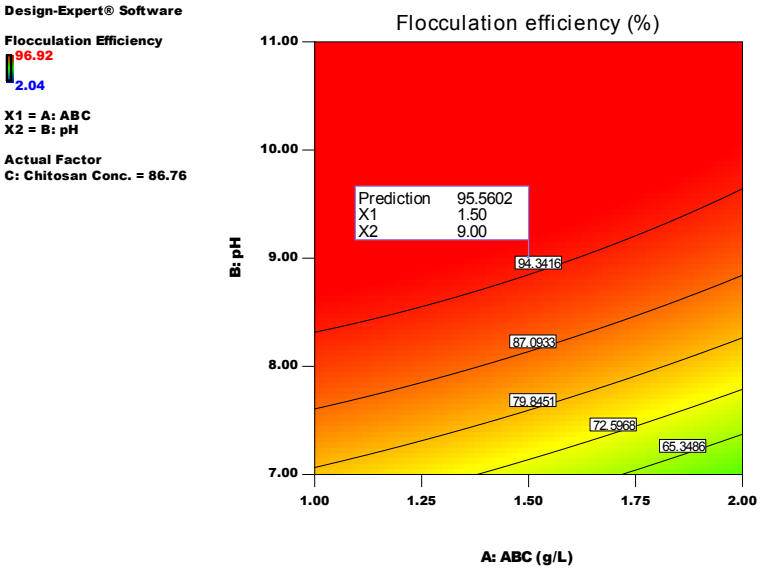
4.3.6 Scanning electron microscopy

The examination of SEM images of *P. maculatum* indicated that the algal cells do not change morphologically, due to different harvesting techniques such as centrifugation (Fig. 4-12 A), and flocculation (Fig. 4-12 B for chitosan and Fig. 4-12 C for aluminium sulphate). Apart from this, chitosan based flocculation resulted in highly concentrated and thick biomass as compared to that of aluminium sulphate. Somogyi et al. (2013) reported that the cell wall thickness of *Picochlorum* sp. range from 50 to 90 nm with an electron dense and electron transparent layer. This thick cell wall may help to prevent the cell disruption through the harvesting process. Rashid et al. (2013) reported no microalgal cell lysis for *Chlorella vulgaris* after examining the SEM images of flocculation utilizing chitosan.

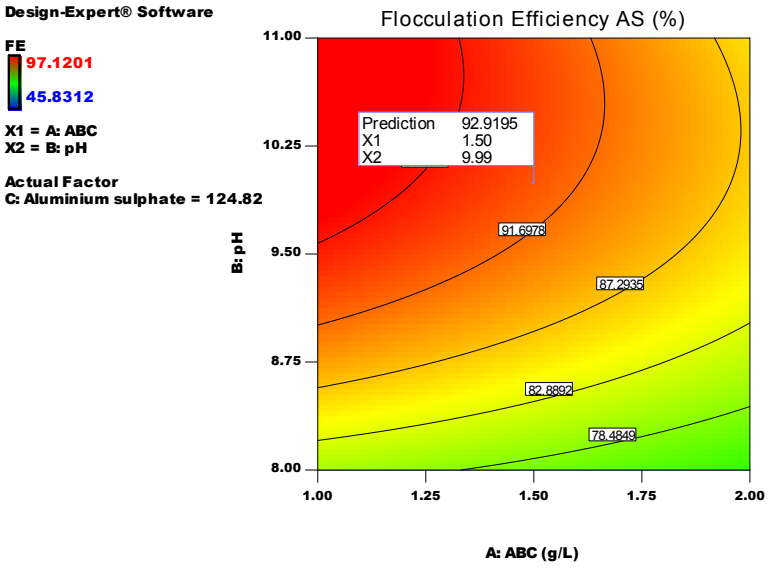
Flocculation aggregates the microalgal cells and enhances further processing, such as sedimentation or centrifugal recovery. Gravity sedimentation is possibly enhanced by flocculation, which is a highly economical harvesting technique, but it needs a large area. The sediment sludge is more diluted than centrifugally recovered biomass, which substantially influences the economics of product recovery further downstream (Christine Rösch & Posten, 2012; Grima et al., 2003). The harvesting of microalgal cells by flocculation is seen to be a superior method to conventional harvesting methods such as centrifugation and flotation because it allows the treatment of large quantities of microalgal culture (Lee et al., 1998). Of the two flocculants tested, chitosan is the most suitable one for algal biomass targeted for the live feed and food applications as it is a bio-flocculant. Chitosan possesses several intrinsic properties such as non-toxicity, biodegradability, and outstanding chelation behavior; those make it an effective flocculant for the algal biomass recovery. The high cationic charge density and long polymer chains, lead to bridge aggregates (Renault et al., 2009). More studies are required to refine the optimization of the properties of chitosan such as the degree of deacetylation and molecular weight which can influence the flocculation.

4.4 Conclusions

The work focused on the response surface methodology based optimization of flocculation process for biomass harvesting of the marine microalga *Picochlorum maculatum* MACC3 using two different flocculants- chitosan and aluminium sulphate. A maximum flocculation efficiency of 96 % was obtained chitosan, whereas 92 % with aluminium sulphate. Based on the cell viability, cell integrity, flocculation efficiency and flocculating parameters such as pH and flocculant dosage, chitosan is recommended as a preferred flocculant for the biomass harvesting of *P. maculatum* MACC3 for feed and nutraceutical applications. As a good source of unsaturated fatty acid for nutraceutical purpose, flocculation with chitosan is preferable as chitosan is a 100 % natural product.

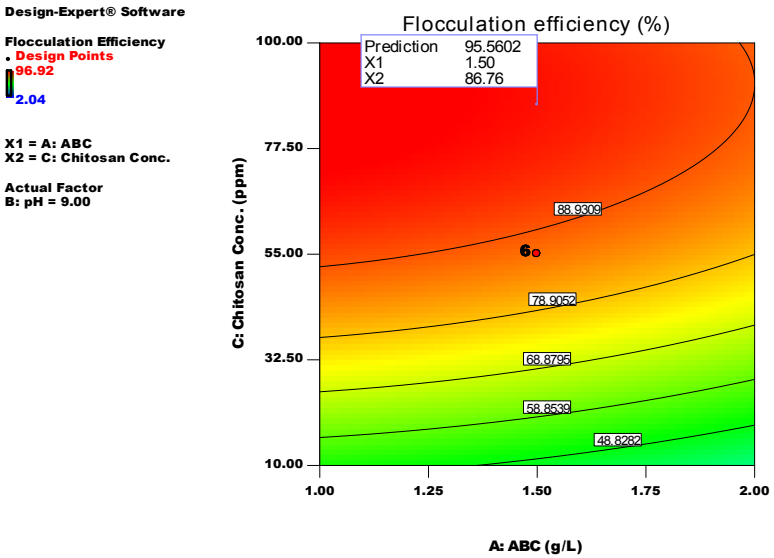


A

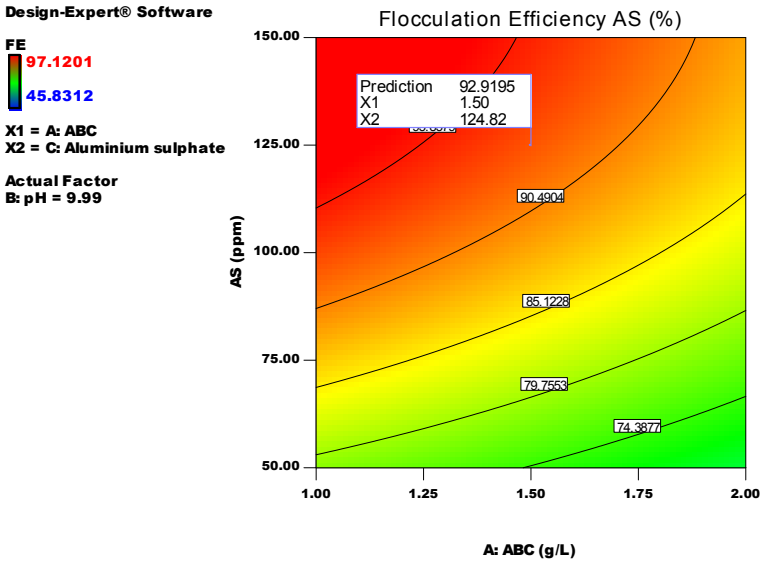


B

Figure 4-5: Contour plots of the flocculation efficiency vs algal biomass concentration and pH. (A) chitosan (p=0.01) (B) aluminium sulphate (p= 0.2075)

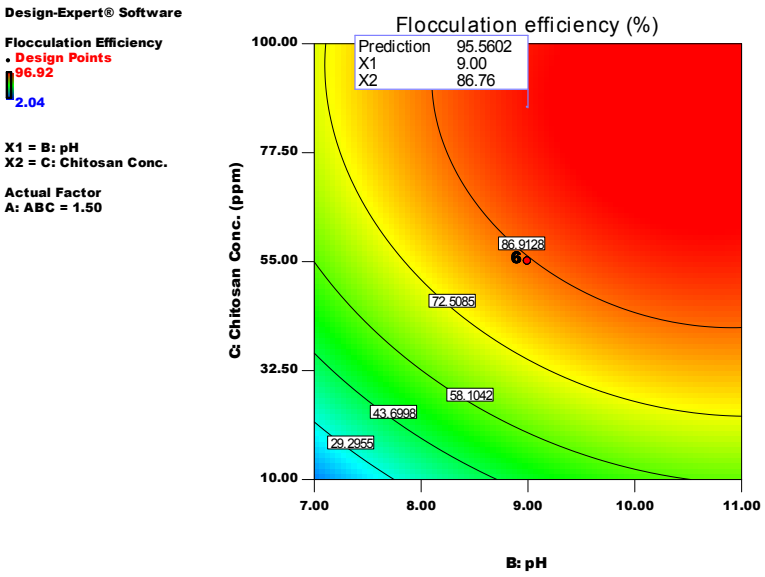


A

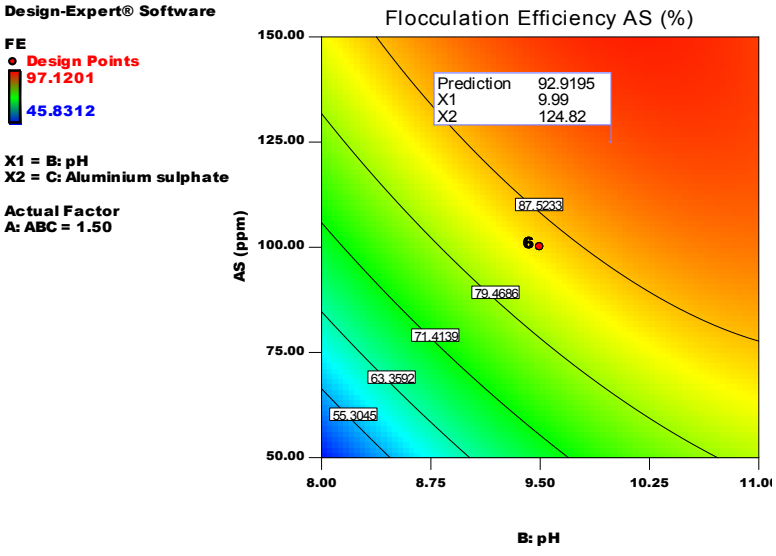


B

Figure 4-6: Contour plot of the flocculation efficiency vs algal biomass concentration and flocculant dose (A) Chitosan (p=0.5658) (B) Aluminium sulphate (p=0.6715)

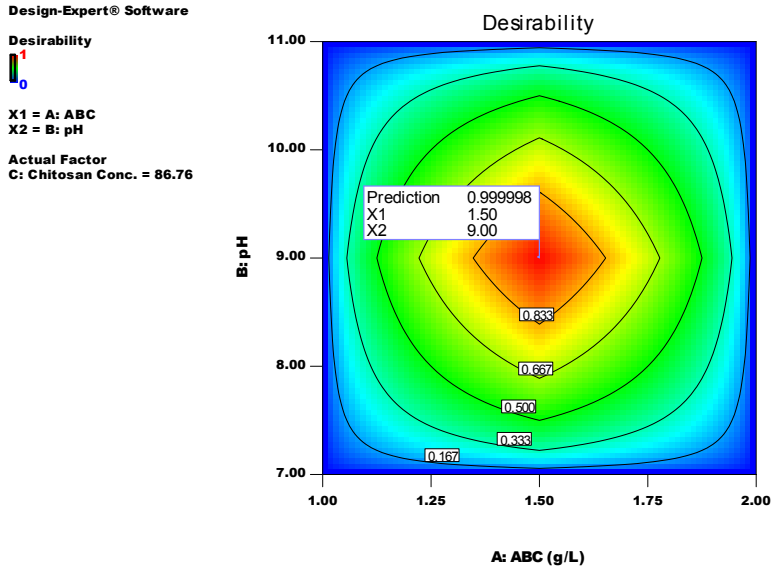


A

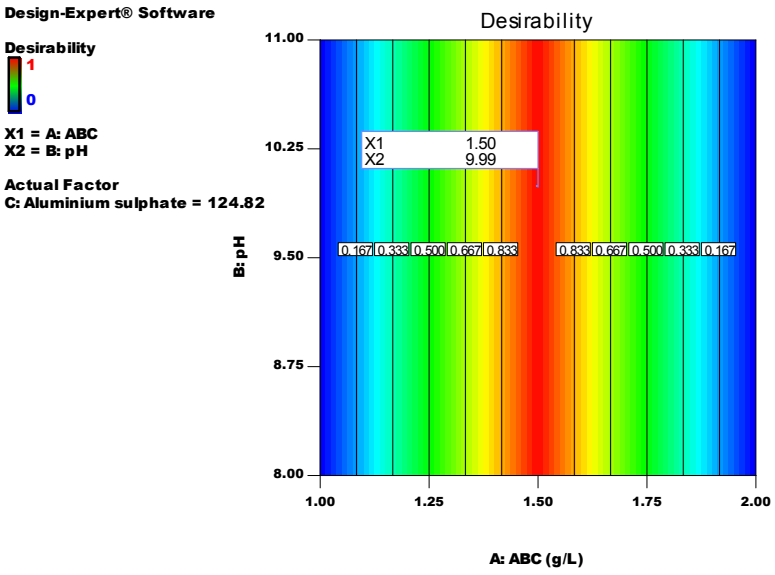


B

Figure 4-7: Contour plots of the flocculation efficiency vs pH and flocculant dose (A) Chitosan ($p<0.05$) and (B) Aluminium sulphate ($p<0.01$)



A



B

Figure 4-8: Contour plots of the desirability of ABS, pH and flocculant concentration (A) chitosan (B) aluminium sulphate

Figure 4-9: Scale up production of *P. maculatum* MACC3 in 10 L carboy jar (6-day old culture)

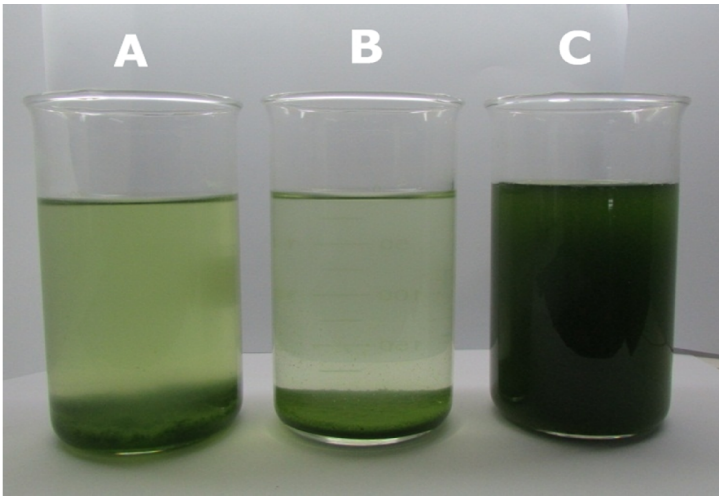


Figure 4-10: Flocculation experiments and validation of the design

(A) Aluminium sulphate based flocculation (B) Chitosan based flocculation (C) *P. maculatum* culture without flocculation (1.5 g/L biomass, 18th-day culture)

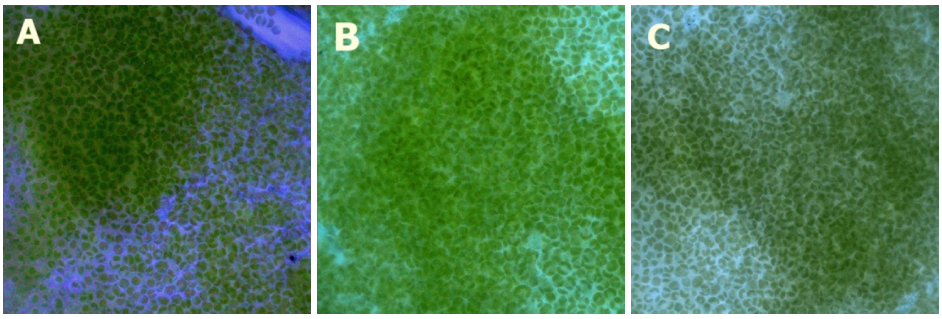


Figure 4-11: Evan's Blue based cell viability test of *Picochlorum maculatum* under optimized harvesting conditions by Centrifugation **(A)** flocculation by Chitosan **(B)** and Aluminium sulphate **(C)**

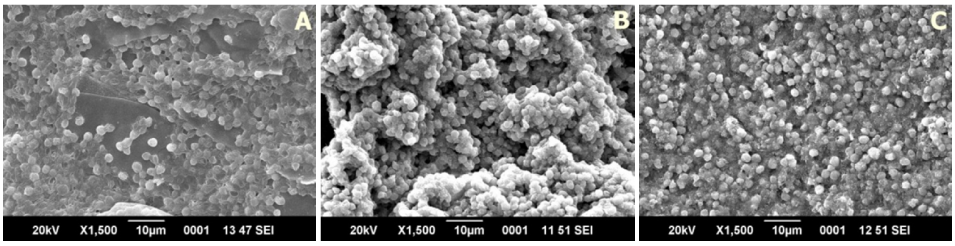


Figure 4-12: Scanning electron microscopic analysis of *Picochlorum maculatum* harvested by centrifugation **(A)**, flocculation by chitosan **(B)** and aluminium sulphate **(C)**

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Chapter 5

IN-VITRO CYTOTOXICITY STUDY OF *PICOCHLORUM MACULATUM* MACC3 HARVESTED THROUGH CENTRIFUGATION AND FLOCCULATION WITH CHITOSAN AND ALUMINIUM SULPHATE IN SELECTED REPRESENTATIVE CELL LINES

Contents

5.1 Introduction

Marine microalgae are chief primary producers in the marine ecosystems (Manilal et al., 2009). Algae are promising source of novel bioactive compounds such as fatty acids, polysaccharides, steroids, phycoproteins, amino acids, dietary minerals carotenoids, halogen compounds and diverse antioxidants such as tocopherols and polyphenols which serve as antioxidant activity which suppress the activity of free radicles (Plaza et al., 2008; Shalaby, 2011). Microalgae are rich sources of protein, carbohydrate, enzyme and essential fibers. Apart from that, they are a promising source of essential fatty acids for human nutrition. They are a rich source of essential minerals, it is used as a food especially in Asian countries, and some of the most widely commercialized green algae include *Chlorella vulgaris, Dunaliella salina Haematococcus pluvialis,* and the Cyanobacteria *Spirulina maxima,* which are widely used as nutritional supplements for human and animal feed additives. *Spirulina platensis* blue-green alga considered as the most nutritious food to man, which is a good source of protein (Colla et al., 2007; Habib et al., 2008; Priyadarshani & Rath, 2012; Spolaore et al., 2006).

The importance of microalgae in animal and human nutrition as a functional food is increasingly being realized in recent years. The most important ingredient of algal biomass that is of health implication is the polyunsaturated fatty acids (Chu, 2012; Das et al., 2011, 2011; Ibañez & Cifuentes, 2013; Kovavc, 2013; Pereira et al., 2012; Wargovich et al., 2010; Whelan & Rust, 2006). *P. maculatum* is a rich source of gamma-linolenic acid and carotenoids, especially lutein and zeaxanthin as established in the present study. However, the biomass produced in the optimized medium and harvested by flocculation need to be tested for any cytotoxicity. Considering the potential for wide applications of *P. maculatum* MACC3 as live feed and a source of nutraceuticals, in the present study toxicity of *P. maculatum* MACC3 extracts prepared in DMSO (dimethyl sulphoxide) after centrifugation and flocculation with chitosan and aluminium sulphate were tested on human, fish and insect cell lines to facilitate its safe usage. Hep-2 (human laryngeal epithelial cells), RTG-2 (a fish cell line derived from Rainbow trout gonad; *Oncorhynchus mykiss*) and Sf9 (Insect cell line derived from a clonal isolate of *Spodoptera frugiperda*) were used for the cytotoxicity studies.

Hep-2 cell line was used in the study to test the toxicity in humans so that microalga could be used in supplementary food in different food industries and also to assess its toxicity while handling. RTG-2 was used to evaluate the application of microalgae as a live feed or processed food in aquaculture industries. Sf9 cell line was used as a representative for invertebrates to assess its toxicity level. For toxicity evaluations, cellular morphology and mitochondrial function (MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay), were assessed under control and exposed conditions (24 h of exposure).

5.2 Materials and methods

5.2.1 Preparation of *Picochlorum maculatum* MACC3 algal samples

Microalgal samples were harvested by centrifugation and also by using flocculation with two different flocculants such as chitosan and aluminium sulphate. The sample was lyophilized and homogenized in DMSO.

5.2.1.1 Centrifugation

18 days old *P.maculatum* cells were harvested by centrifugation at 6000 g for 10 min. after the cells were washed with sterile seawater followed by 2 times with distilled water to remove the medium components, salt content, and other debris. Samples were lyophilized using (Lyolab, Lyophilization Systems India Pvt Limited, Hyderabad, India). Lyophilized cells were used for further analyses.

5.2.1.2 Flocculation

18 days old algal culture of *P. maculatum* cells were harvested using flocculation with 87 mg/L chitosan at a pH 9 and 125 mg/L aluminium sulphate at pH 9.9. The flocculated cells were washed with sterile seawater followed by sterile distilled water and the cells were lyophilized for further analysis.

5.2.1.3 Preparation for cytotoxicity studies

One gram of lyophilized algal biomass was homogenized in 10 ml of dimethyl sulfoxide (DMSO) and adjusted the pH of the extract using 0.1N HCl or 0.1 N NaOH. The extract was clarified by filtration using Whatman No.1 filter paper followed by filter sterilization using 0.22 μm membrane filters.

Filtrates were concentrated using rotary evaporator at 50˚C. The resulting concentrated extract was further concentrated by the use of a vacuum oven to remove residual solvent and sterilized extracts were stored at –80°C until use. The extracts were dissolved in the cell culture medium in appropriate concentrations for further cytotoxicity studies.

5.2.2 Cytotoxicity analysis of *P. maculatum* MACC3 extract

Cytotoxicity assays are *in vitro* bioassay methods used to predict the toxicity of substances to various tissues or animals. The potential cytotoxic activity of *P. maculatum* was assessed in three different cell lines for evaluating the safety of the algal biomass for use in animal and fish feed.

5.2.3 Cell lines used for the study and their growth conditions

Insect cell line, Sf9, originated from *Spodoptera frugiperda* pupal ovarian tissue, fish cell line, RTG-2, originated from rainbow trout (*Oncorhynchus mykiss*) gonadal tissue and human laryngeal epithelial cell line, Hep-2, were used for the study. These cell lines were purchased from National Centre for Cell Science, Pune, India. The insect cell line, Sf9 was maintained at 28°C in TNM-FH (Trichoplusia ni Medium-Formulation Hink) medium, fish cell line, and human cell line maintained in Eagle's MEM (Minimum essential medium) supplemented with 2 mM L-glutamine and 1.5g/ L sodium bicarbonate. Hep-2 was maintained at 37 ^{0}C while RTG-2 at 25 ^{0}C. All the media were supplemented with 10% fetal bovine serum and antibiotic mixture containing 100 µg/mL streptomycin and 100 IU/ml penicillin (Freshney, 2000, 2005)

5.2.4 Exposure of cell lines to algal extracts

The cell cultures in 96 well plates were developed from each cell line by adding 0.2 ml cell suspension in the growth medium containing approximately 5 x10^5 cells/ ml and incubating for 12 h at 37 ºC, 28 ºC and 25 ºC for human, insects and fish cells respectively. Different concentrations of algal extracts prepared in growth media were added to the wells to attain final at a concentration of 10, 25, 50, 75, 100, 125, 250, 500, 1000 µg/mL for MTT assay, to demonstrate the mitochondrial dehydrogenase activity. Cells without algal extracts were kept as negative control and a solvent control using DMSO.

After 24 h incubation, the wells were observed under Inverted phase contrast microscope (Leica, Germany) and cytotoxicity assays were performed.

5.2.5 MTT Assay

In spite of the visual observation, mitochondrial dehydrogenase activity was measured as the cell viability and metabolic activity which depended on an intact mitochondrial membrane and the respiratory chain. MTT assay measures the mitochondrial dehydrogenase which reflects the metabolic activity of the cells. Succinate dehydrogenase belonging to the mitochondrial respiratory chain reduces

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, Sigma-Aldrich Co.) to insoluble formazan crystals, which when solubilized in dimethyl sulphoxide (DMSO) yield a purple-coloured solution (Mosmann, 1983). The assay is a colorimetric method based on the determination of cell viability utilizing the reaction of a tetrazolium salt (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) with the mitochondria of metabolically active cells. After incubation, the medium was replaced with 50 µl MTT (Sigma-Aldrich Co.) having the strength of 5 mg/ml in phosphate buffered saline (PBS) and incubated for 4 hrs in dark at 37 ºC, 28 ºC and 25 ºC for human, insects and fish cells, respectively. The medium was removed and MTT-formazan crystals were dissolved in 200µl DMSO (HiMedia Laboratories, Mumbai). Absorbance was recorded immediately at 570 nm in a microplate reader (TECAN Infinite Tm, Austria) with a reference wavelength at 690 nm. The percentage of cell viability (growth) against control cells were calculated as follows:

$$Cell\ viability\ \% = [(OD_{570}\ of\ treated\ cells / OD_{570} of\ control\ cells)]\ X\ 100 \qquad (1)$$

5.2.6 Statistical Analysis

The results in the graph are average values of 3 replicates ± standard deviation. The effects of treatments were statistically analyzed by a single factor and two-factor analysis of variance (ANOVA). Differences were considered significant at $P < 0.05$.

5.3 Results and discussions

5.3.1 Cytotoxicity of *P. maculatum*

The cytotoxicity of *P. maculatum MACC3* extracts prepared through different harvesting methods at a concentration of 10, 25, 50, 75, 100, 125, 250, 500, 1000 µg/mL were tested on Hep-2 cells, RTG-2 cell line and Sf9 cell line for 24 hrs. At concentrations from 10 – 1000 µg/mL, the algal extracts did not cause any significant change in the cell morphology, despite the particles from the extracts attached on the surface of the cells (Figure 5-1, Figure 5-2, and Figure 5-3). Moreover, the inhibition

of mitochondrial dehydrogenase activity was undetectable (Figure 5-4, Figure 5-5 and Figure 5-6). The accumulation of dead cells in the negative control wells (without extract) was found almost equal to the wells which were treated with algal extracts, suggesting the harvesting methods tested did not cause any significant effect or toxicity towards cell lines.

The measurement of cellular metabolic activity through mitochondrial dehydrogenase system showed that it required a higher concentration of algal extracts for its inhibition. Similar observation was reported by Abdo et al. (2012) that up to 2 mg/ml of algal extract were nontoxic to Hep2 cell line. However, Abdo et al. (2012) used extracts from five algal species such as *Anabaena sphaerica*, *Chroococcus turgidus*, *Oscillatoria limnetica*, *Spirulina platensis* and *Cosmarium leave* and studied the toxicity using trypan blue dye exclusion method and by morphology evaluation. This is the first report of *in vitro* toxicity studies using *P. maculatum* extracts.

Though the biomass harvested using both chitosan and aluminium sulphate did not differ significantly, and both were safe based on cell line based assays, chitosan is preferable because it is from a natural source and with many benefits. Chitin is a natural polymer found in the shell of crustaceans, insects, and fungi. The deacetylated product chitosan has a commercial value it was used extensively in flocculating algae and also as a supplemented food for various fishes such as Rainbow trout (*Oncorhynchus mykiss*) boost up the innate immune response (Vahedi & Ghodratizadeh, 2011). The role of immunostimulants in the control of fish diseases is very well established and studies were carried out on the immunostimulant activity of chitosan against various fish pathogens such as disease protection in *Tilapia mossambicus* against opportunistic pathogen *Aeromonas hydrophila* in freshwater culture systems (Balcázar et al., 2006).

The biodegradability, nontoxicity, and biocompatibility of chitosan make it preferable for potential applications in different areas such as biotechnology, food processing, environmental protection and medicine (Chen, 2013; Zhao et al., 2010). It can be easily processed in diverse form and allow the design of various medical and pharmaceutical devices (Synowiecki & Al-Khateeb, 2003). It is also used as effective

drug delivery systems because of the unique physicochemical properties and biological characteristics. Because of the low molecular weight, chitin and chitosan are useful carriers for molecular drug delivery systems (Kato et al., 2003). Chitosan was combined with drugs such as doxorubicin, paclitaxel, docetaxel, and norcantharidin, as drug carriers (Patel et al., 2010). Their biodegradability makes them dissolve with time when it used in wound healing (Dai et al., 2011; Vinsova & Vavrikova, 2011).

While evaluating the safety of *P. maculatum* MACC3 harvesting technique for its use as animal and fish feed additive, it could be demonstrated that it was safe to be applied as a feed additive to fishes as it did not cause any toxicity to fish cell line. Moreover, there was no toxicity to either human or insect cell lines too. Being a bio-flocculant, the microalgal biomass harvested using chitosan is safe and will have added health benefits to the aquatic animals as feed, which need to be further explored based on *in vivo* studies.

5.4 Conclusions

The study demonstrated that the algal extracts were not toxic to all the three cell lines tested. The overall cytotoxicity study with algal extracts revealed that the IC_{50} values were multifold higher and safe for various applications. Additionally, all harvesting methods tested did not cause any negative impact on the safety of its use as food or feed supplement. Moreover, it also gives primary evidence that the alga *P.maculatum* MACC 3 will be a potential candidate for live feed and nutraceuticals applications.

Based on the results we could recommend the *P. maculatum* for application as fish feed additive after accomplishing *in vivo* studies under laboratory and field conditions

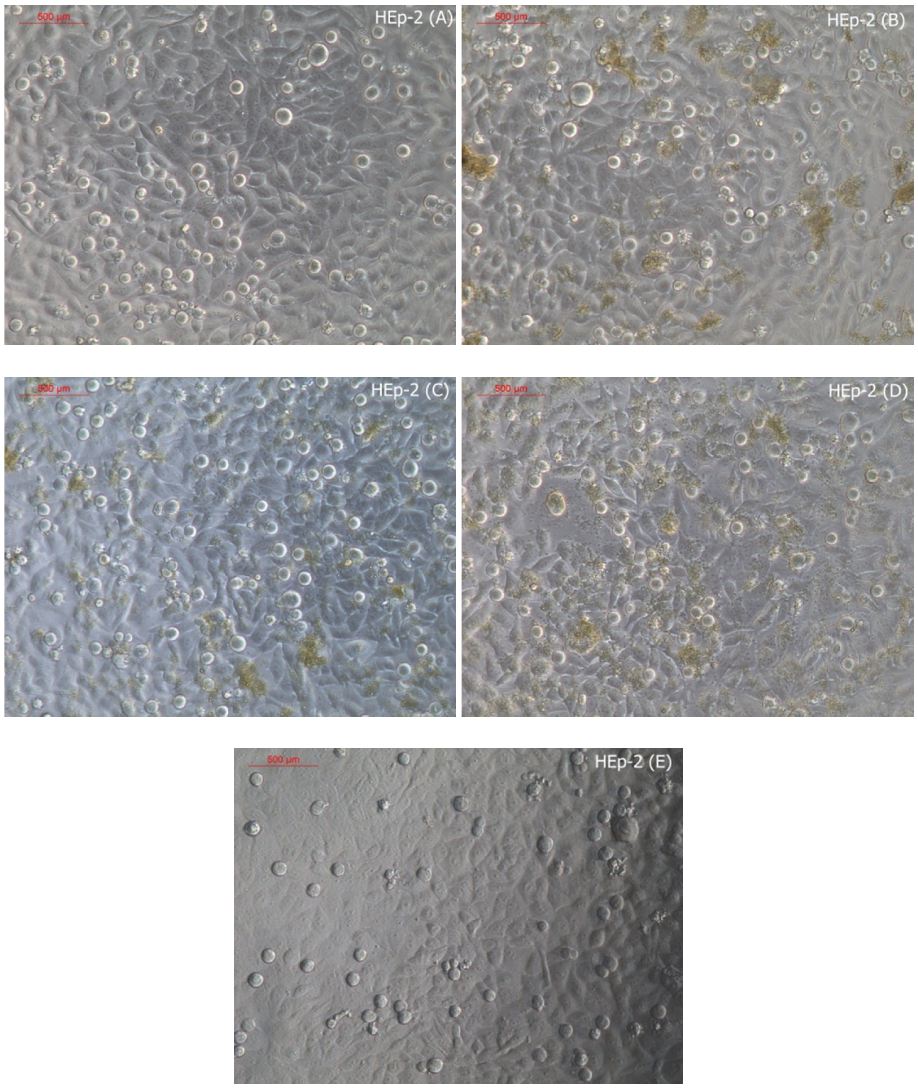


Figure 5-1: Cytotoxic effects of algal extract on HEp-2 cell lines

(A) DMSO (Solvent control) (B) Centrifuged algal cells (C) Chitosan flocculated algal cells (D) Aluminium sulphate flocculated algal cells (E) Normal HEp-2 cells

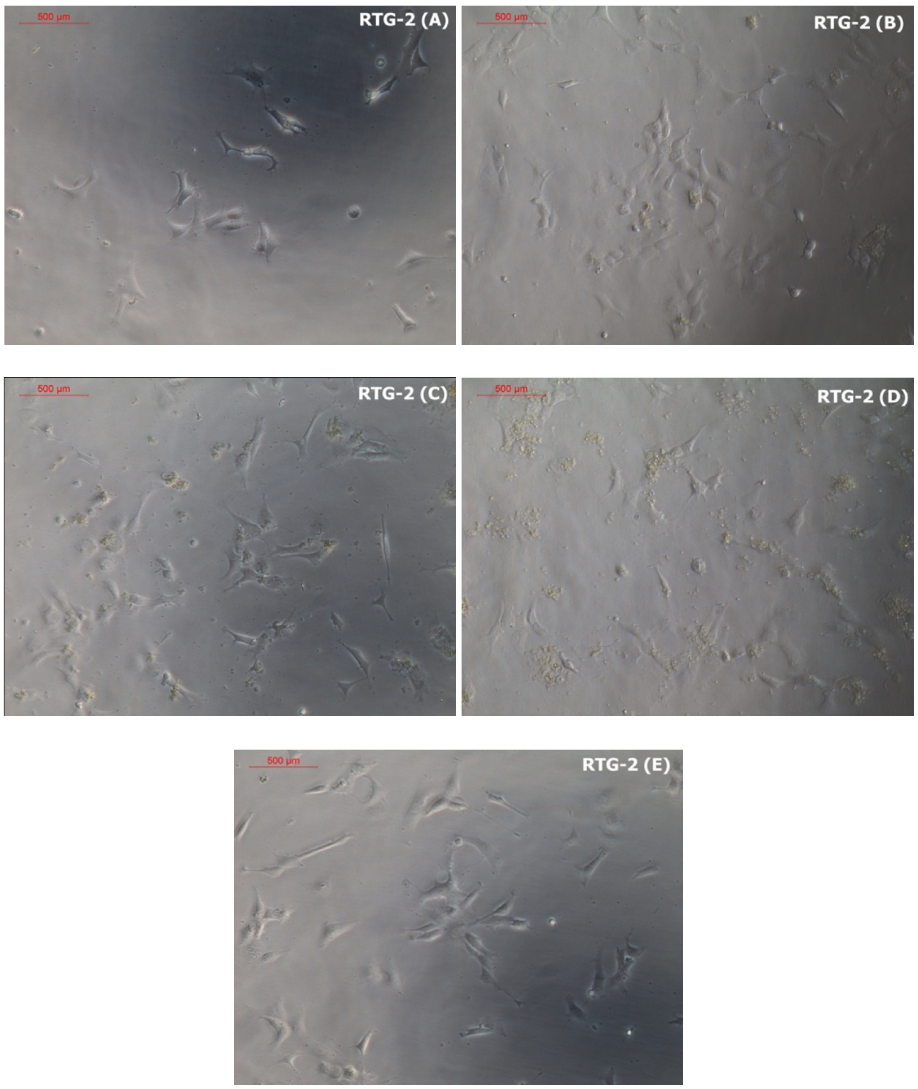


Figure 5-2: Cytotoxic effects of algal extract on RTG-2 cell lines

(A) DMSO (Solvent control) (B) Centrifuged algal cells (C) Chitosan flocculated algal cells (D) Aluminium sulphate flocculated algal cells (E) Normal RTG-2 cells

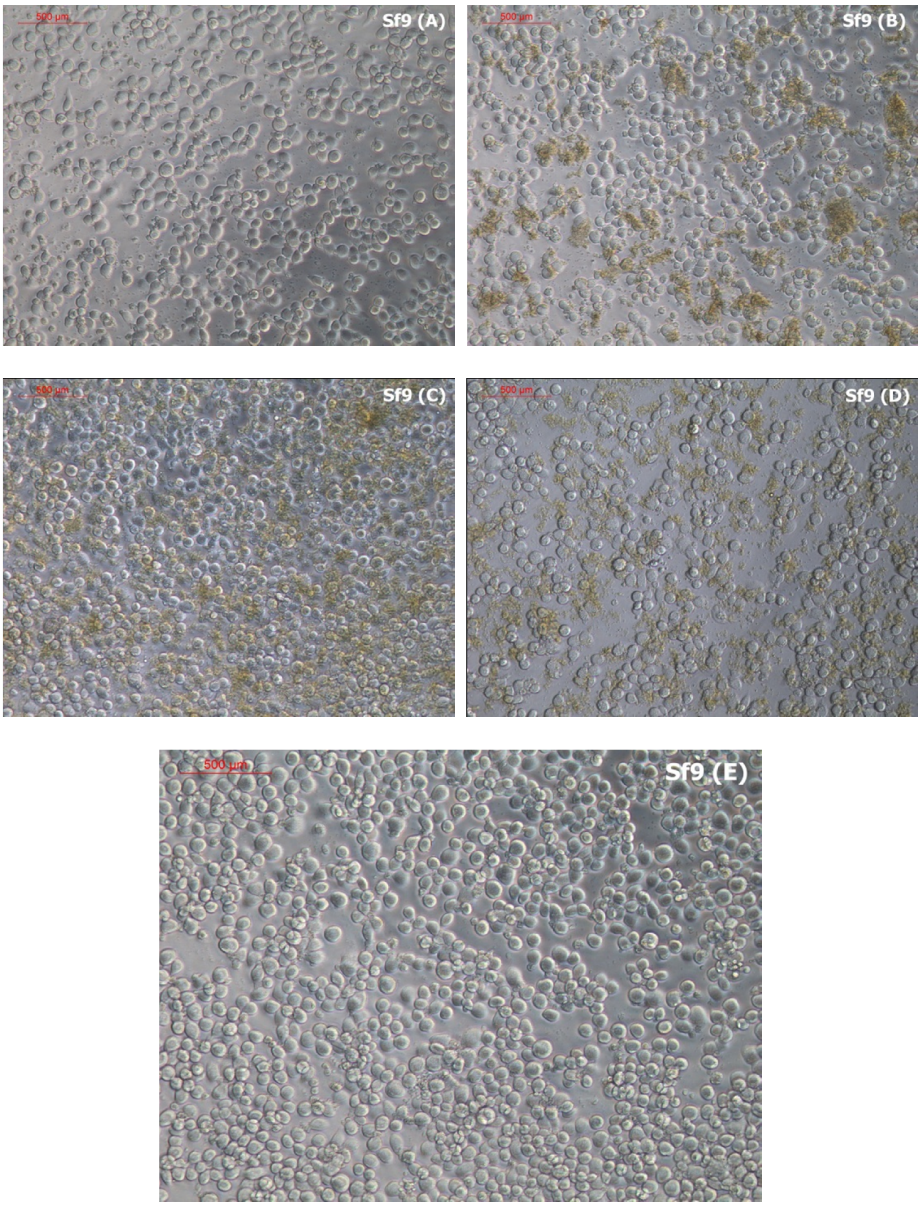
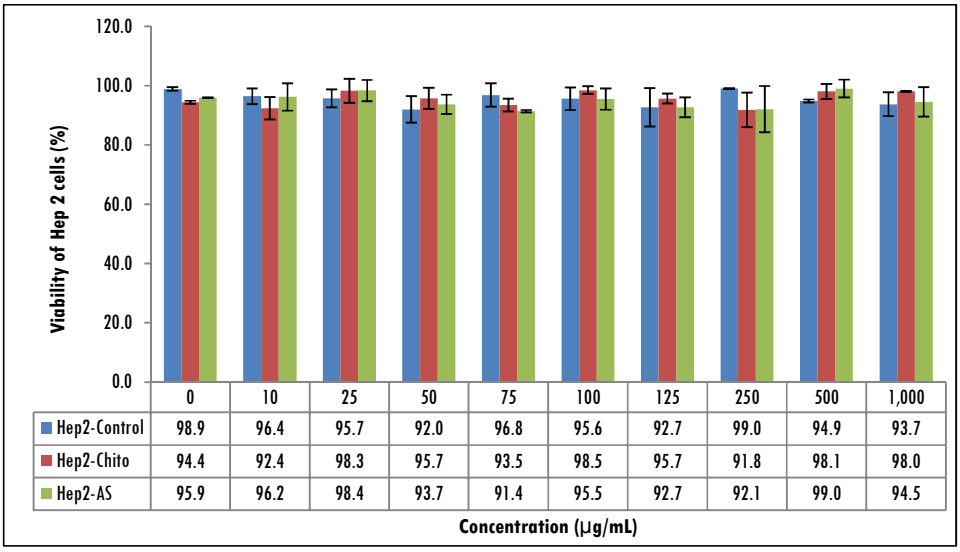


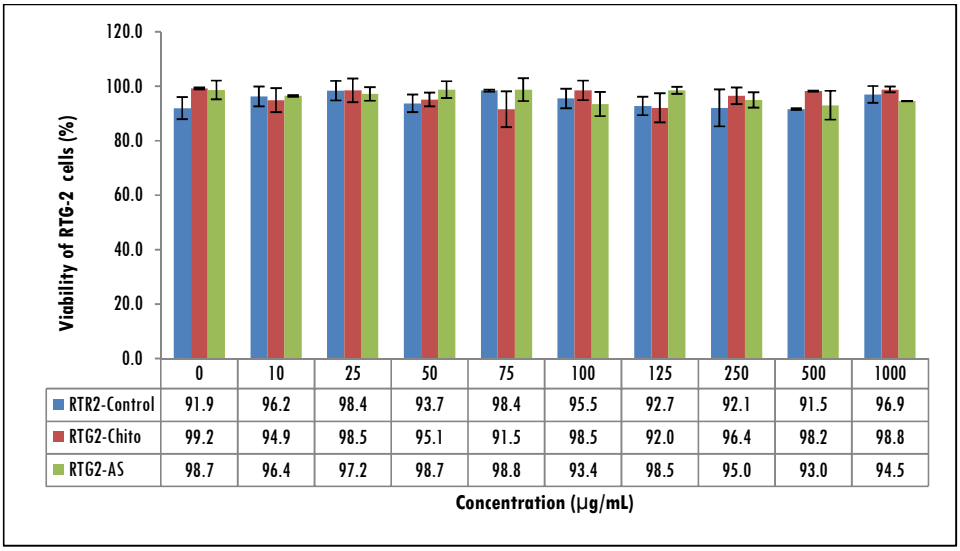
Figure 5-3: Cytotoxic effects of algal extract on Sf9 insect cell lines

(A) DMSO (Solvent control) (B) Centrifuged algal cells (C) Chitosan flocculated algal cells (D) Aluminium sulphate flocculated algal cells (E) Normal Sf9 cells



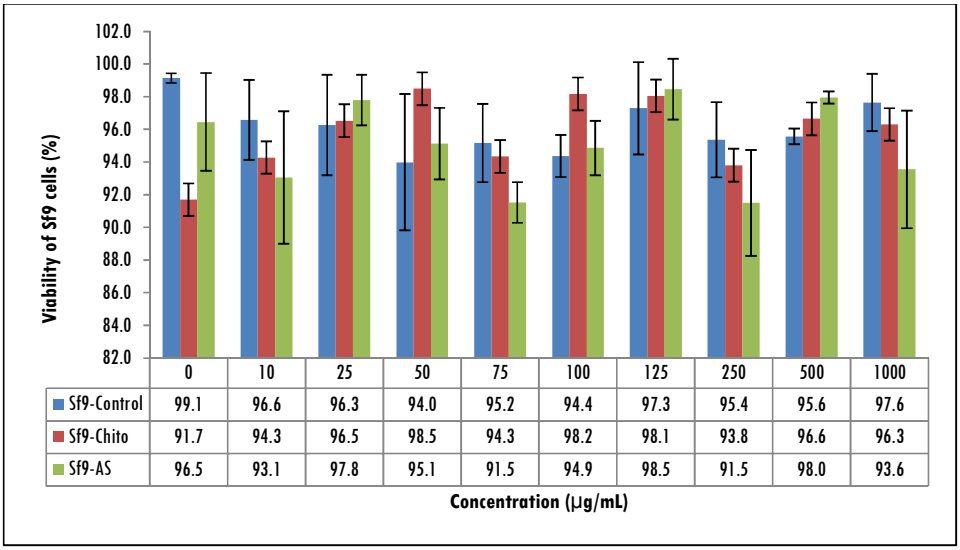
	0	10	25	50	75	100	125	250	500	1,000
Hep2-Control	98.9	96.4	95.7	92.0	96.8	95.6	92.7	99.0	94.9	93.7
Hep2-Chito	94.4	92.4	98.3	95.7	93.5	98.5	95.7	91.8	98.1	98.0
Hep2-AS	95.9	96.2	98.4	93.7	91.4	95.5	92.7	92.1	99.0	94.5

Figure 5-4: Bar graph of MTT assays for Hep2 cells incubated with different concentrations of algal extracts from different harvesting methods [Centrifugation (Control), Chitosan (Chito) and Aluminium sulphate (AS)]. Values are given as percentage cell viability ± standard error (n=3) (P>0.05).



	0	10	25	50	75	100	125	250	500	1000
RTR2-Control	91.9	96.2	98.4	93.7	98.4	95.5	92.7	92.1	91.5	96.9
RTG2-Chito	99.2	94.9	98.5	95.1	91.5	98.5	92.0	96.4	98.2	98.8
RTG2-AS	98.7	96.4	97.2	98.7	98.8	93.4	98.5	95.0	93.0	94.5

Figure 5-5: Bar graph of MTT assays for RTG 2 cells incubated with different concentrations of algal extracts from different harvesting methods [Centrifugation (Control), Chitosan (Chito) and Aluminium sulphate (AS)]. Values are given as percentage cell viability ± standard error (n=3) (P>0.05).



	0	10	25	50	75	100	125	250	500	1000
Sf9-Control	99.1	96.6	96.3	94.0	95.2	94.4	97.3	95.4	95.6	97.6
Sf9-Chito	91.7	94.3	96.5	98.5	94.3	98.2	98.1	93.8	96.6	96.3
Sf9-AS	96.5	93.1	97.8	95.1	91.5	94.9	98.5	91.5	98.0	93.6

Figure 5-6: Bar graph of MTT assays for Sf9 cells incubated with different concentrations of algal extracts from different harvesting methods [Centrifugation (Control), Chitosan (Chito) and Aluminium sulphate (AS)]. Values are given as percentage cell viability ± standard error (n=3) (P>0.05).

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Chapter 6

SUMMARY AND CONCLUSIONS

The demand for polyunsaturated fatty acids (PUFA) as additives in animal and human diet is ever increasing. The animals are deprived of desaturase and elongase genes for PUFA synthesis; hence PUFA is supplemented in the diet. Recent research indicates a strong correlation between intake of PUFA and health beneficial effects, such as the prevention of cardiovascular diseases, Alzheimer's disease, abatement of depression, and inhibition of cancer.

At present omega, fatty acids are being produced from oily fish, but the concerns regarding the accumulation of pollutants as well as the continued exploitation of fish stocks in order to meet the demands of an expanding market are drawing the attention to other resources. Since omega PUFA in fish oils are actually derived via the marine food chain from zooplankton consuming omega PUFA-synthesizing microalgae, marine microalgae are the natural omega 3 factories of the sea. Fish are devoid of genetic makeup to synthesize these beneficial omega 3 oils, but microalgae do have; the beneficial oils are then passed up the food chain to fish and then human. Marine microalgae are considered as the potent and alternative source for the production of industrially important nutritional fatty acids by either large-scale cultivation or transformation of PUFA biosynthetic genes to oilseed crops (Meyer et al., 2004), as the conventional sources are not sufficient to meet the market demand.

The chapter 1 of the thesis gives an overall review on the recent advancements in the production and applications of PUFA, specifically on microalgae as a source of PUFA. Microalgae-based PUFA is superior to any other sources as it is free from contamination and seasonal independence. They offer a renewable source of omega oil for use in human and animal diets, depending on how well they adapt to

live in large scale culture facilities. But so far, only relatively few wild-type microalgae are known to produce ω-3 PUFA such as ALA (alpha-linolenic acid C18:3, n3), EPA (eicosapentaenoic acid, C20:5, n3) and DHA (docosahexaenoic acid, C22:6, n3) and ω-6 PUFA such as LA (linoleic acid, 18:2, n6), GLA (gamma linolenic acid, 18:3, n6), AA (*arachidonic acid,* 20:4, n6) in high concentrations, such as *Schizochytrium* (DHA), *Crypthecodinium* (DHA) and *Phaeodactylum* (EPA). In a recent study screening on the screening of around 2000 microalgae for fatty acids, it was found that microalgae are a rich source of fatty acids, with varying abundance in different species and groups (Imke et al., 2011). The mega-diversity of marine microalgae is not explored to the fullest, and therefore, holds a great storehouse of potential PUFA producers.

In this context, a study was undertaken to delineate the roles of different growth factors and conditions on the biomass and PUFA production in a marine microalga. The microalgal strain used in the present study was originally isolated in Walne's medium from a seawater sample collected from Kannur region in the Arabian Sea off the west coast of India. The monocultures obtained by serial dilution was initially identified as *Nannochloris* sp. MACC3 and maintained in the culture collection of National Centre for Aquatic Animal Health, Cochin University of Science and Technology, India. In the present study, the strain was identified at the species level based on morphology and molecular phylogeny, analyzed in in-depth for PUFA production potential, and optimized the bioprocesses for biomass and PUFA production and biomass harvesting based on statistical experimental design tools.

The present study is undertaken with the following objectives, with an ultimate goal to develop *P. maculatum* MACC3 biomass as a product for application in human and animal nutrition as an alternative source of PUFA.

1. Biochemical and molecular characterization of *Picochlorum maculatum* MACC3 and screening for PUFA production under phototrophic, mixotrophic and heterotrophic conditions

2. Optimization of medium and growth conditions for the production of biomass and gamma linolenic acid by *Picochlorum maculatum* MACC3 using response surface methodology
3. Scale up production, and optimization of harvesting technique of marine microalga *Picochlorum maculatum* MACC3 by flocculation with chitosan and aluminium sulphate using Response Surface Methodology
4. In-vitro cytotoxicity study of *Picochlorum maculatum* MACC3 harvested through centrifugation and flocculation with chitosan and aluminium sulphate in selected representative cell lines.

The salient findings of the present study are summarized as given below:

- ✓ Based on morphological identification by light, fluorescent and electron microscopy and molecular identification using 23S rDNA, ITS and rbcL genes as phylogenetic markers, the microalgal strain was identified as *Picochlorum sp.* MACC3. Based on phylogenetic analysis using 18 S rDNA sequences (1800bp), it was confirmed as *Picochlorum maculatum* MACC3 and the sequences were deposited in the GeneBank database with accession numbers KP190042, KP213855, KP213856 and KM055115, respectively.
- ✓ The growth of *P.maculatum* MACC3 was assessed in f/2 and Walne's media in terms of dry weight, and biomolecule profile under phototrophic conditions.
- ✓ Proximate compositions of the alga under three different nutritional regimes such as phototrophic, heterotrophic and mixotrophic were analysed in terms of biomolecules such as protein, carbohydrates, lipids, pigments (carotenoids, chlorophyll a , chlorophyll b), minerals, ash and moisture contents.
- ✓ The phototrophic growth of *P. maculatum* MACC3 in Walne's medium gave better biomass yield and f//2 medium give maximum protein, lipid, and carbohydrate yield, the f/2 medium was selected for further experiments.

- ✓ The fatty acid, pigment and mineral profile of *P. maculatum* at different nutritional regimes such as phototrophic, mixotrophic and heterotrophic were carried out.
- ✓ GC-MS analyses of fatty acids of *P. maculatum* MACC3 were done in three nutritional regimes- phototrophic condition in f/2 medium, mixotrophic and heterotrophic condition in f/2 medium supplemented with glucose and analyzed during the stationary phase of the culture. Seven major fatty acids were identified in all culture conditions, which include Lauric Acid (LA: C: 12:0), myristic acid (MA: C14:0), palmitic acid (PA: C16:0), palmitoleic acid, (C:16:1), stearic acid (SA: C:18), oleic acid (OA: C18:1n9), linoleic acid (LA: C18:2n6), γ-linolenic acid (GLA: C18:3n3), α-linolenic acid (ALA: C18:3n6), eicosatetraenoic acid (ETA: C20:4n3) and eicosapentaenoic acid (EPA: C20:5n3). The PUFA yields under phototrophic, mixotrophic and heterotrophic conditions were 200 mg/L, 111 mg/L and 99 mg/L respectively comprising 15.32%, 11.25% and 15.16 % of the total biomass.
- ✓ *P.maculatum* MACC3 was capable of producing GLA as the major fatty acid under all nutritional regimes, and the maximum production of GLA was obtained at the end of logarithmic growth phase, from 18th day culture in phototrophic culture in f/2 medium (105 ± 0.7 mg/L), followed by mixotrophic (78.13± 0.8 mg/L), and heterotrophic cultures (56.1± 0.8 mg/L).
- ✓ The pigments from *P. maculatum* grown under the phototrophic condition, and mixotrophic condition supplemented with glucose as the carbon source, were analyzed using HPLC. Under both the conditions, the pigment composition was the same such as chlorophyll a and b and lutein followed by β- carotene, neoxanthin, zeaxanthin, and violaxanthin, with the relatively higher percentage of pigment in phototrophic culture.
- ✓ ICP- AES analyses of minerals were carried out using cultures grown in phototrophic (f/2 medium), mixotrophic and heterotrophic conditions (f/2 medium supplemented with glucose). The maximum mineral yields were obtained in phototrophic condition (366±5 µg/g), followed by mixotrophic

(359±6 µg/g) and heterotrophic conditions (190± 8 µg/g). Out of 10 metals (Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb and Zn) checked, Mn is the major mineral in all culture conditions and the concentrations of Cd, Cr, Ni, and Pb were below the permissible limits.

- ✓ Optimization of biomass and GLA production could be initially done by one variable at a time approach. Based on this result, the ranges for variables were fixed and the significant factors were screened by Plackett-Burman experimental design. The optimum level of significant factors was further optimized by using Central Composite Design of Response Surface Methodology
- ✓ Based on the Plackett-Burman design, temperature and nitrate were found to be significant for biomass production, and pH and vitamin concentration were significant for the GLA production.
- ✓ Altogether, 30 different medium compositions were checked in terms of biomass and GLA production as suggested by the Design expert software (2^4 full factorial points, 8 axial points, and 6 center points).
- ✓ Analysis of variance (ANOVA) of the quadratic model of medium optimization for biomass, PUFA, and GLA yield $p<0.05$ showed that the model was adequate.
- ✓ The observed R^2 values of 0.9692, 0. 93227 and 0.9538 for biomass, PUFA and GLA explained that the fitted model could explain 96.92%, 93.22% and 95.38 % of the total variation in the response.
- ✓ An adequate precision value of 26.17, 14.87 and 13.62 for biomass PUFA and GLA suggested that the polynomial quadratic model was an adequate signal, and could be used to navigate the design space.
- ✓ The temperature and nitrate were the significant factors for biomass yield and significant interactions occurred between temperature and nitrate (AB). The RSM based optimization study in *P. maculatum* MACC3 yielded a maximum biomass of 1.53 g/L.

- ✓ The temperature and vitamin were the significant factors affecting PUFA yield. The significant interactions occurred between temperature and pH (AC), nitrate and pH (BC), pH and vitamin (CD). The maximum PUFA obtained through optimization was 268 mg/L.
- ✓ The vitamin concentration was the significant factor affecting GLA yield. The interactions between temperature and pH (AC), and pH and vitamin concentration were significant. The maximum GLA yield obtained from the model was 143 mg/L.
- ✓ The optimized concentration of nitrate was 90 mg/L, vitamin solution 1.53 mL, pH 6.3 and incubation temperature 19^0C. The validation experiments yielded 1.55 g/L of biomass, 280 mg/L of PUFA and 160 mg/L of GLA.
- ✓ The flocculation efficiencies of chitosan and aluminium sulphate were optimized for harvesting the biomass of *P. maculatum* MACC3
- ✓ Central composite design of response surface methodology was used for optimizing the flocculation variables such as algal biomass concentration, pH, and flocculant dosage and the flocculation efficiency was taken as the response.
- ✓ Altogether, 20 different combinations of variables were checked in terms of flocculation efficiency of both chitosan and aluminium sulphate as suggested by the Design expert software (2^3 full factorial points, 6 axial points, and 6 center points).
- ✓ The algal biomass (A), pH (B), chitosan concentrations (C) were significant for chitosan based flocculation, and significant interactions occurred between algal biomass and pH (AB) and pH and chitosan concentration (BC). RSM based optimization study in *P. maculatum* with chitosan yielded a high flocculation efficiency of 96%. The model predicted parameters for maximum flocculation efficiency of chitosan were algal biomass concentration of 1.5 g/L, pH 9 and chitosan concentration of 86.76 mg/L.
- ✓ For aluminium sulphate based flocculation, the algal biomass concentration (A), pH (B) and aluminium sulphate concentration (C) were significant, and

significant interactions occurred between pH and aluminium sulphate (BC) concentrations.

- ✓ RSM based optimization study in *P. maculatum* MACC3 with aluminium sulphate yielded high flocculation efficiency of 92.81 %. The model predicted parameters for maximum flocculation efficiency of aluminium sulphate were algal biomass concentration of 1.53 g/L, pH 9.99 and aluminium sulphate concentration of 124 mg/L
- ✓ Analysis of variance (ANOVA) of the quadratic model of chitosan and aluminium sulphate (Prob> F= 105) and (Prob> F= 28.07) showed that the model was adequate with no significant lack of fit
- ✓ The observed R^2 values of 0.9896 and 0. 9691 for chitosan and aluminium sulphate respectively, explained that the fitted model could explain 98.66% and 96.96 % of the total variation in the data.
- ✓ An adequate precision value of 31.749 and 18.045 suggested that the polynomial quadratic model was an adequate signal, and could be used to navigate the design space.
- ✓ Both chitosan and aluminium sulphate based flocculation models were validated by repeating the experiments in 1-liter volume indicating a good correlation between predicted and experimental values proving the validity of the model.
- ✓ The assessment of cytotoxicity of extracts of *P. maculatum* MACC3 harvested using chitosan and aluminium sulphate on HEp-2, RTG-2, and sf9 cells did not cause any significant change in cell morphology, on testing at concentrations of 1- 1000 µg/mL of extracts, The results of MTT assay showed that none of the extracts were toxic to HEp-2, RTG-2 or sf9 cells. Based on the results of cytotoxicity test, it was concluded that *P. maculatum* MACC3 could be used for supplementing fish feed.

Precisely, this work screened and characterized the biomass and GLA production potential of *P. maculatum* MACC3 and optimized the medium for the maximum production of biomass, PUFA, and GLA. Further, the protocols for biomass harvesting by flocculation were optimized with both chitosan and aluminium sulphate and the

flocculation efficiencies of chitosan and aluminium sulphate were compared. The safety of the microalgal biomass harvested through flocculation was evaluated by testing the cell cytotoxicity in human, fish and insect cell lines and was assessed safe.

Based on the results of the present study, the marine microalga *Picochlorum maculatum* MACC3 is proposed as a potential source of polyunsaturated fatty acids, specifically gamma-linolenic acid, for application as fish feed additive after accomplishing *in vivo* studies under laboratory and field conditions.

Suggestions for Further Research

- ✓ Optimization of large-scale production in photobioreactors and standardization of the downstream process for GLA production from *P. maculatum* MACC3
- ✓ Screening for nutritional effects and toxicity if any, of *P. maculatum* MACC3 biomass in *in-vivo* models to evaluate the potential for food and feed for humans and animals. The *P. maculatum* biomass is a storehouse of bio-molecules and the functional food prospects of the alga need to be further explored.
- ✓ Study the nutritional quality and adaptability of the *P. maculatum* MACC3 as the live feed for finfish and shellfishes under laboratory and field conditions.
- ✓ Study on expression of the key genes in PUFA biosynthesis pathway of *P. maculatum* MACC3 and the relative expression of genes in response to varying physicochemical parameters.
- ✓ Recombinant production of PUFA from *P. maculatum* MACC3 using genetic and metabolic engineering approaches.

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Picochlorum maculatum 18S ribosomal RNA gene, partial sequence

GenBank: KM055115.1

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  AUTHORS   Arun,A., Sreelakshmi,P.R., Vrinda,S., Bright Singh,I.S. and
            Joseph,V.
  TITLE     Polyunsaturated fatty acid production in marine microalga
            Nannochloris maculata MACC3
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1791)
  AUTHORS   Arun,A., Sreelakshmi,P.R., Vrinda,S., Bright Singh,I.S. and
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  TITLE     Direct Submission
  JOURNAL   Submitted (14-JUN-2014) National Centre for Aquatic Animal Health,
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  TITLE     Production of polyunsaturated fatty acids in marine microalga
            Picochlorum maculatum MACC3
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 851)
  AUTHORS   Arun,A., Vrinda,S., Jisha,K., Sanyo,S., Sreelakshmi,P.R., Bright
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  TITLE     Direct Submission
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GenBank: KP213856.1

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  TITLE     Production of polyunsaturated fatty acids in marine microalga
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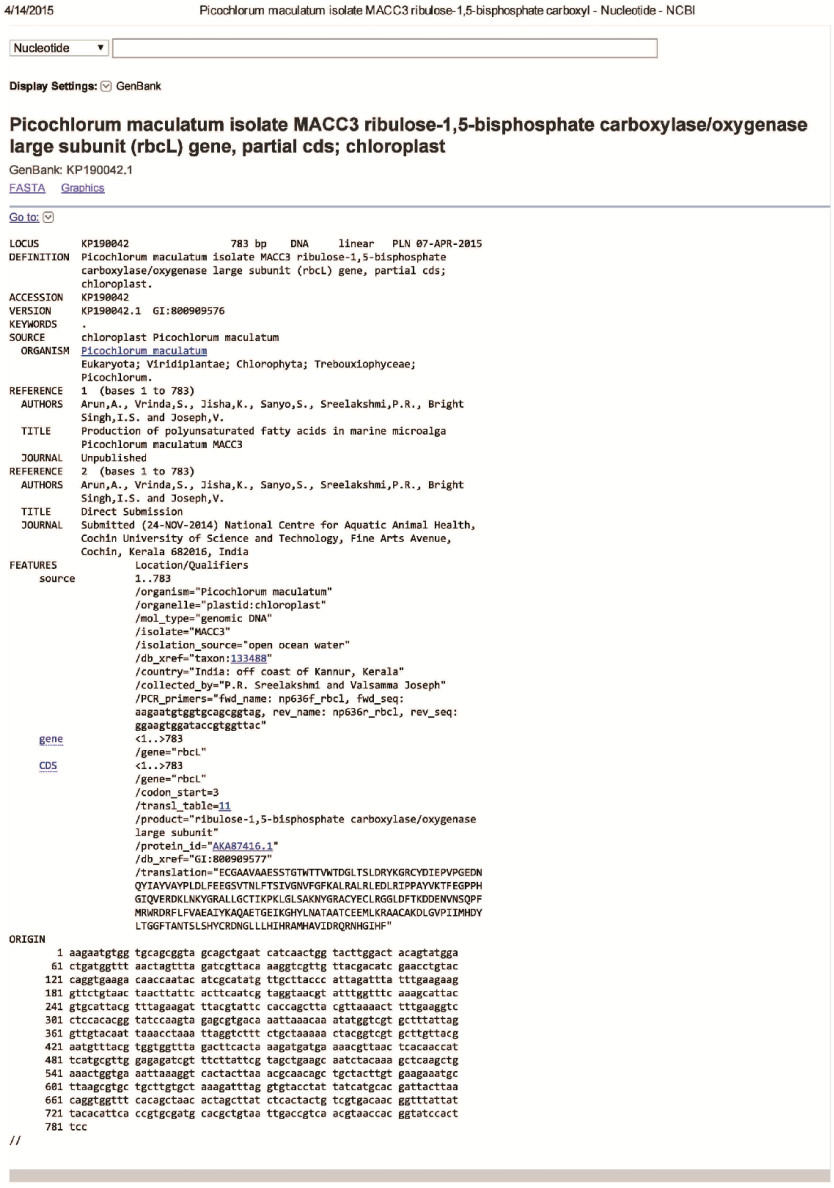

4/14/2015 Picochlorum maculatum isolate MACC3 ribulose-1,5-bisphosphate carboxyl - Nucleotide - NCBI

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GenBank: KP190042.1

FASTA Graphics

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  TITLE     Production of polyunsaturated fatty acids in marine microalga
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      661 caggtggttt cacagctaac actagcttat ctcactactg tcgtgacaac ggtttattat
      721 tacacattca ccgtgcgatg cacgctgtaa ttgaccgtca acgtaaccac ggtatccact
      781 tcc
//
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