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CELL CULTURE OPTIMIZATION AND REACTOR STUDIES OF GREEN AND BROWN MICROALGAE FOR ENHANCED LIPID PRODUCTION

By

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CELL CULTURE OPTIMIZATION AND REACTOR STUDIES OF GREEN AND BROWN MICROALGAE FOR ENHANCED LIPID PRODUCTION

by

SYED MUHAMMAD USMAN SHAH

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DECLARATION OF THESIS

Title of thesis | CELL CULTURE OPTIMIZATION AND REACTOR STUDIES OF GREEN AND BROWN MICROALGAE FOR ENHANCED LIPID PRODUCTION

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DEDICATION

Dedicated to my beloved father

Syed Muhammad Hussain Shah

and

loving mother Imtiaz Bibi

for bringing me up to who I am today

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I owe my thanks to Almighty Allah, the Ultimate Creator, who has granted me to drink a drop of an ocean of His Creativity.

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ABSTRACT

Microalgae are a promising source of biofuels and other valuable chemicals. The low cell density and slow growth rate that have traditionally characterized microalgal cultures, however, have reduced their economical feasibility. To develop a sustainable microalgal process, it is necessary to increase culture productivity, maximize production yield, and reduce production costs. To achieve these complete understanding of the dynamic behaviour of microalgal cultures is of paramount importance. This study described the optimization of growth parameters that affected locally-isolated green *Nannochloropsis oculata* and *Tetraselmis suecica* and brown *Isochrysis galbana* and *Pavlova lutheri* microalgae using Response Surface Methodology (RSM). The cell densities, dry weight and lipid content increased with the increase of pH, salinity, photoperiod and light intensity for all the four tested species. The optimum pH and salinity were pH 8-9 and salinity of 35-40 ppt to achieve cell dry weight of 0.58-0.82 g L^{-1}; and lipid content of 27.4-37.3%. The maximum cell dry weight of 0.66-0.84 g L^{-1}; and lipid content of 23.9-36.6% were achieved at optimal 19.3-24 h illumination and light intensity of 162-198 µmol photons $m^{-2}s^{-1}$. Under deficiency conditions of 10-65 g L^{-1} KNO_3, 3-7.5 g L^{-1} Na_2HPO_4 and 2.5 g L^{-1} $FeCl_3$ the highest lipid accumulation of 37.3, 23.6, 28.3 and 37.2% with slightly reduced cell growth of 0.64, 0.49, 0.54 and 0.38 g L^{-1} were achieved for *N. oculata*, *T. suecica*, *I. galbana* and *P. lutheri*, respectively.

The interactions of pH, salinity, photoperiod and light intensity and macronutrients significantly influenced the biomass and lipid content positively. However, the interaction of phosphate-phospate for *N. oculata*, and nitrate-nitrate for *I. galbana* may affect cell growth negatively. The interaction of nitrate, phosphate, phosphate-phosphate and ferum-ferum interactions may all affect lipid content negatively.

The r^2 of 78.7-95.1% for biomass and 80.1-97.5% for lipid and mean absolute error percentage between experimental and predicted values of 0.02-0.042% and 0.61-1.66% for biomass and lipid content suggest good agreements between experimental and predicted values. Palm oil mill effluent (POME) was a promising potential economical medium for the algal growth and lipid enhancement whilst achieving POME remediation. Lipid contents of 39.1 ± 0.73, 27.0 ± 0.61, 26.3 ± 0.31 and 34.5 ± 0.82% were attained for *N. oculata*, *T. suecica*, *I. galbana* and *P. lutheri* respectively, under POME composition of 10-15% in sea water. The removal of COD (76.1-95%), TOC (43.1-74.8%), BOD (68.3- 97%), TN (48.7-90.8%) and oil and grease (59.1-94.9%) were achieved for 1–20% POME with co-cultivation of microalgae.

The optimized conditions from RSM were applied for mass production of microalgae in 5 L photobioreactor and 300 L open tank. Cultivation in 250 mL-30 L batch cultures showed that *N. oculata* and *P. lutheri* in 250 mL, *T. suecica* in 30 L and *I. galbana* in 1 L attained the maximum specific growth rate and lipid content. The highest biomass of 0.62-0.96 gL^{-1} and lipid content of 31.6-42.2% in photobioreactor, and 0.45-0.72 gL^{-1} and lipid content of 24.4-38.5% in open tank, were achieved for all four tested species. Composition analysis of the algal lipid produced in the optimized cultures shows that the lipid has a high quality as biodiesel precursor, in terms of the expected cetane number. The total saturated, monounsaturated and polyunsaturated fatty acids (PUFA) were obtained, with pentadecanoic acid (C15:0), palmitic acid (C16:0), palmitoleic acid (C16:1), heptadecanoic acid (C17:0), oleic acid (C18:1), eicosanoic acid (C20:0), eicosapentaenoic acid EPA (C20:5) and docosahexaenoic acid DHA (C22:6) as predominant fatty acids. Species belonging to genus *Nannochloropsis* was confirmed based on 18S ribosomal RNA gene, ITS region of ribosomal RNA transcription and *rbcL* gene. BLAST search of GenBank showed that the complete sequences of the species shared 97-99% similarity with *N. oculata* and was confirmed by the sequence alignment and phylogenetic tree analysis. The higher productivity and excellent lipid profile of the optimized microalgal culture make *N. oculata* and *P. lutheri* as exceptional sources for biodiesel production.

ABSTRAK

Sektor kejuruteraan alga kian mendapat perhatian untuk memenuhi keperluan penghasilan biotenaga yang lebih ekonomik, mesra alam sekitar serta penghasilan kompaun bernilai tinggi dalam sektor biokimia. Lipid dan asid lemak merupakan sasaran bahan kandungan utama dalam biotenaga. Parameter seperti pH yang optima, kemasinan, foto-kala, keamatan cahaya dan makronutrien dapat meningkatkan prestasi biojisim serta produktiviti lipid. Kajian yang telah dijalankan menunjukkan pertumbuhan sesuatu parameter yang boleh memberi kesan secara lokal terhadap isolasi spesis hijau mikroalga *Nannochloropsis oculata* dan *Tetraselmis suecica* serta spesis perang *Isochrysis galbana* dan *Pavlova lutheri*. Pengoptimuman telah dijalankan melalui kaedah Response Surface Methodology (RSM) untuk menjelaskan interaksi dan kaitan antara faktor-faktor. Ketumpatan sel, berat bersih dan kandungan lipid meningkat secara langsung dengan peningkatan pH, kemasinan, foto-kala dan kamatan cahaya untuk kesemua empat spesis. Tahap yang optima untuk pH dan kemasinan adalah 8-9 dan 35-40 ppt bagi setiap parameter untuk mencapai berat bersih 0.59-0.82 g L^{-1}; dan kandungan lipid dengan bacaan 27.4-37.3%. Seterusnya, berat bersih maksima dengan bacaan 66-0.84 g L^{-1}; dan kandungan lipid 23.9-36.6% tercapai melalui pencahayaan yang optima dari julat 19.3-24 jam serta keamatan cahaya dari julat 162-198 µmol photons $m^{-2}s^{-1}$. Kandungan terkumpul lipid yang tertinggi pada 37.3, 23.6, 28.3 dan 37.2% dengan sedikit pengurangan dalam pertumbuhan menerusi mikroalga *N. oculata*, *T. suecica*, *I. galbana* and *P. lutheri* apabila alga dikultur dalam kondisi medium yang kekurangan 10-65 g L^{-1} KNO_3, 3-7.5 g L^{-1} Na_2HPO_4 and 2.5 g L^{-1} $FeCl_3$.

Interaksi kaitan antara pH, kemasinan, foto-kala dan keamatan cahaya serta makronutrien secara langsung mempengaruhi kandungan biojisim dan lipid secara positif. Kaitan antara fosfat-fosfat untuk *N. oculata* dan nitrat-nitrat alga *I. galbana* menunjukkan impak negatif kepada pertumbuhan sel. Manakala, interaksi antara nitrat, fosfat, fosfat-fosfat dan ferum-ferum memberi kesan terhadap kandungan lipid

secara negatif. Ketepatan r^2 dari julat 78.7-95.1% untuk biojisim dan 80.1-97.5% untuk kandungan lipid dan peratusan min ralat mutlak di antara nilai bacaan eksperimen dan ramalan pada 0.02-0.042% dan 0.61-1.66% untuk kandungan biojisim dan lipid, menunjukkan persamaan yang baik antara kedua-dua bacaan. Sisa buangan kilang minyak kelapa sawit (POME) mempunyai potensi sebagai bahan media ekonomikal untuk pertumbuhan alga untuk penambahbaikan kandungan lipid, serta pemulihan sisa buangan. Kandungan lipid dari julat 39.1 ± 0.73, 27.0 ± 0.61, 26.3 ± 0.31 and 34.5 ± 0.82% untuk spesis *N. oculata*, *T. suecica*, *I. galbana* and *P. lutheri* telah dicapai bagi komposisi POME didalam air laut secara purata dari 10-15%. Pembuangan COD (76.1-95%), TOC (43.1-74.8%), BOD (68.3- 97%), TN (48.7-90.8%) dan minyak serta gris (59.1-94.9%) telah dicapai pada 1-20% komposisi POME dengan ko-pengkulturan mikroalga.

Kondisi yang optima telah diaplikasikan untuk jisim mikroalga dalam 5 L foto-bioreaktor dan 300 L tanki terbuka. Perbandingan antara 250 mL-30 L kultur kelompok menunjukkan spesis *N. oculata* and *P. lutheri* dalam 250 mL, *T. suecica* dalam 30 L dan *I. galbana* dalam 1 L mencecah kadar pertumbuhan spesifik yang maksima dengan bacaan 0.15-0.17 hari^{-1} dan kandungan lipid 27.2-37.1%. Tahap biojisim tertinggi 0.62-0.96 gL^{-1} dan kandungan lipid 31.6-42.2% dalam foto-bioreaktor, dan 0.45-0.72 gL^{-1} serta kandungan lipid dari 24.4-38.5 dalam tanki terbuka telah discapai untuk kesemua empat spesis. Jumlah asid lemak tak tepu (44.3-63.8% dan 30.4-55.03%); mono tak tepu (6.1-37.0% and 4.2-13.1%); dan poli tak tepu (PUFA) (8.3-22.3% dan 1.02-15.2%) telah diperolehi, dengan asid pentadekonik (C15:0), asid palmitik (C16:0), asid palmitolik (C16:1), asid heptadekanoik (C17:0), asid oleik (C18:1), asid eikosanoik (C20:0), EPA (C20:5) dan DHA (C22:6) sebagai asid lemak predominan. Spesis seperti genus *Nannochloropsis* telah dispastikan berdasarkan gen ribosomal RNA, transkripsi ITS region dan gen *rbcL*. BLAST search GenBank menunjukkan urutan-urutan spesis yang lengkap mempunyai persamaan hampir 97-99% dengan alga *N. oculata* dan ini telah dipastikan melalui urutan yang sejajar dan analisis pokok filogenetika.

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LIST OF ABBREVIATIONS

DW	Dry Weight
RSM	Response Surface Methodology
POME	Palm Oil Mill Effluent
FAME	Fatty acids Methyl Esters
ETE	Eicosatrienoic Acid
ETA	Eicosatetraenoic Acid
EPA	Eicosapentaenoic Acid
DHA	Docosahexaenoic Acid
MUFA	Monounsaturated Fatty Acid
PUFA	Polyunsaturated Fatty Acid
COD	Chemical Oxygen Demand
BOD	Biological Oxygen Demand
TOC	Total Organic Carbon
TN	Total Nitrogen
ppt	Parts Per Thousand
ANOVA	Analysis of Variance
DW_A	Average Dry Weight retained on Algal Filter
DW_c	Average Dry Weight retained on control Filter
W_d	Dry Weight of algae
V	Volume used
PBR	Photobioreactor

LIST OF SYMBOLS

N_{max}	Final cell concentration (10^6 cells mL^{-1})
X_{max}	Final cell dry weight (gL^{-1})
$X`_{max}$	Maximum biomass formation rate (gL^{-1} d^{-1})
μ_{max}	Maximum specific growth rate (d^{-1})
t_d	Doubling time (days)
y	Response variable
x_i, x_j	Independent coded variables
ε	Random error
β_0	Constant coefficient
β_i	Coefficient of linear
β_{ii}	Interaction regression
β_{ij}	Quadratic term
k	Number of independent values
Ai	Initial parameter
Af	Final parameter
X_o	Initial cell concentrations
X_t	Final cell concentrations
$Y\ x/s$	Yield of biomass ($g\ g^{-1}$)
$Y\ p/x$	Yield of lipid ($g\ g^{-1}$)

CHAPTER 1

INTRODUCTION

1.1 Alternative Sources of Energy

In 1970, during the first oil crisis, the US starts to develop biofuel technologies based on corn for ethanol and from soybeans for biodiesel. The Aquatic Species Program is an effort to produce biodiesel from high lipid content algae [1]. However the biofuel program shifts its focus to corn and soybean technologies in 1996 and the Aquatic Species Program is discontinued due to funding shortages, and the fact that corn and soybean fuels are considered proven technologies. In 2005, 14.3% of corn harvested in the US has been channelled towards ethanol production, and 1.5% of the soybeans harvested used to make biodiesel. Nevertheless, the production of these fuels offset a mere 1.72% and 0.09% of the US gasoline and diesel demand, respectively. Even if 100% of corn and soybeans harvested are to be used for biofuels, only 12% of the gasoline demand and 6% of the diesel demand will be met [2]. This however does not take into consideration the fossil fuels needed for farming and in the production of these fuels. Although one of the advantages of biofuels is the utilization of carbon dioxide for growth, corn and soybean farming require the use of fertilizers which only gives bioethanol and biodiesel, a 12% and 41% reduction in GHGs, respectively [2].

The biodiesel industry would need to produce 530 billion liters annually to satisfy the US demand for transportation fuel [3]. At present, corn and sugarcane are the two major crops extensively utilized for bioethanol. In 2008, USA produces 34 billion liters of fuel grade bioethanol from corn, while Brazil comes second at 27.5 billion

liters of fuel grade bioethanol from sugarcane. Together, these two countries account for 89% of global production [4]. Motor vehicles in Brazil are currently using petroleum fuel blended with at least 25% bioethanol [5]. Table 1.1 compares the different sources of biodiesel and the oil yield. The major disadvantage of fuel crops is that they take up a significant amount of land, leading to a significant ecological impact. There are also issues about “food” versus “fuel” as corn, soybean crops or oil palm are major crops for edible oil. It seems that the only feasible source of biodiesel is microalgae as algae do not compromise a food stock or deplete nutrients in the soil though it still requires a nutrient source. In fact microalgae as oil-producing organisms for biodiesel production are well recognized. Lipids from microalgae can be turned into biodiesel through transesterification and used for energy development including simple combustion in boiler or in diesel engine [6, 7]. The entire process ranging from the cultivation of high-lipid microalgae to the production of biodiesel from microalgal oil has been explored. A typical algal culture system can generate 150 to 400 barrels of oil per acre per year, which is 30 times more than those produced via typical oilseed crops. The production cost of algal oil depends on many factors, such as yield of biomass from the culture system, oil content, scale of production systems, and the cost of recovering oil. Currently, algal-oil production is still far more expensive than petroleum diesel fuels. The petroleum-diesel price is at $3.80-$4.50 per gallon while the production cost of algae oil from a photobioreactor with an annual production capacity of 10000 tons per year, assuming the oil content of approximately 30 percent, is estimated to be $2.80 per liter ($10.50 per gallon) [3]. This does not include cost of converting algal oil to biodiesel, distribution and marketing costs for biodiesel, and taxes.

Table 1.1 Comparison of biofuel source [3].

Crop	Oil Yield (L/ha)	Land Area[a] (M ha)	Existing US cropping area[a] (%)
Corn	172	3081	1692
Soybean	446	1188	652
Canola	1190	445	244
Jatropha	1892	280	154
Coconut	2689	197	108
Oil palm	5950	89	48
Microalgae[b]	136900	3.9	2.2
Microalgae[c]	58700	9.0	5

[a]For meeting 100 % of all transport fuel needs of the United States.

[b]70 % oil (by wt) in biomass

[c]30 % oil (by wt) in biomass

1.2 Algae – A New Biology

Algae are one of the most abundant and primitive life forms on earth. They are less complex than the plants, and consist of one or more of the eukaryotic chlorophyll-containing cells. It may be made up of a single cell, colonies, filament of cells, or as simple as in the kelp tissues. The upper limit of algal species in nature is estimated at about 10 million, and only a small portion of these have been identified taxonomically. Fig 1.1 shows the placement of algae and higher plants in a phylogenetic tree. For many years, algae are categorized as plants because of their photosynthetic ability. Algae are now placed within the diverse kingdom Protista of eukaryotic, predominantly single-celled microscopic organisms [8].

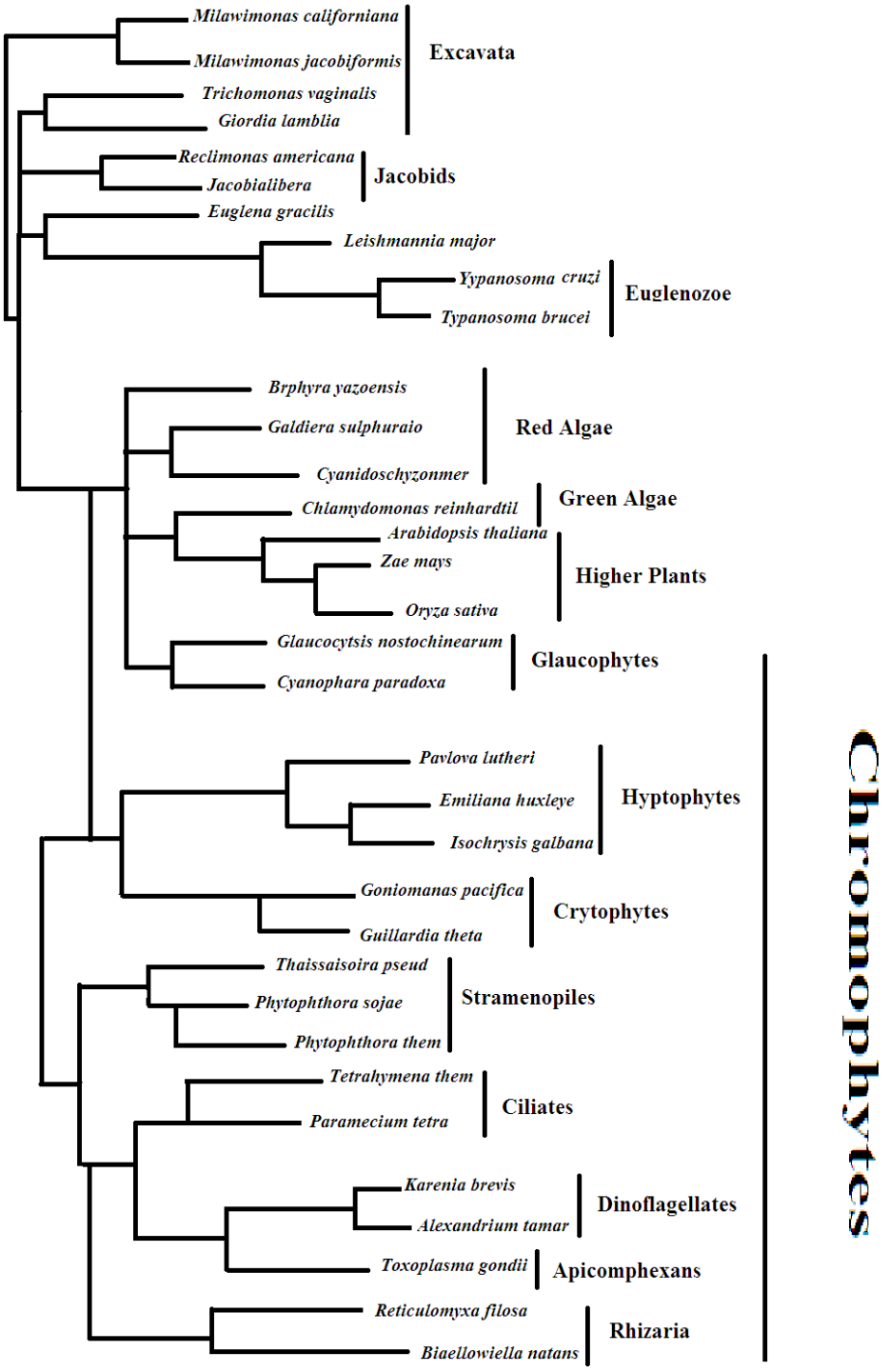


Fig 1.1 Phylogenetic tree showing the placement of algae and higher plants among some of their common protist neighbours [9].

Algae can be classified into two main groups:- 1) microalgae, which includes diatoms (Bacillariophyceae), green algae (Chlorophyceae), red algae (Rhodophyceae), yellow-green algae (Xanthophyceae), golden algae (Chrysophyceae), brown algae (Phaeophyceae), and Euglenoids; 2) macroalgae (seaweeds or multicellular algae). Microalgae are commonly categorized into three main sub-groups:- green (Chlorophyta), brown (Phaeophyta) and red (Rhodophyta) algae. Most of the red and brown algae are marine species, though there are some rare freshwater species in each group. Blue-green algae make up another group of single-celled organisms that use photosynthesis to create food but because they do not have eukaryotic cells, they are not classified as algae but as a type of chlorophyll-containing bacteria called cyanobacteria [8].

Algal cell doubling time is typically 1-2 days as compared to bacteria (20 mins), animal cells (20 hrs) and plant cells (1-4 days). Some species such as *Chlamydomanos* have been reported with doubling time as short as 6 hrs [10]. Algae can survive in dry conditions for a long time and can flourish in different extreme temperatures such as at 80 °C, in and around hot springs, in the snow and ice of the Arctic and Antarctic regions, and extreme pHs. Algae inhabit diverse ecological habitats ranging from seawater, freshwater or brackish water and can be found particularly in the oceans, rivers, lakes, streams, ponds, swamps and the soils [11]. In the tropics and subtropics, algae may be found on leaves, on woods and stones and some live within or on plants and animals.

1.3 Biochemicals from Algae

The three areas of research in aquatic natural products emerging in the last 3 decades are toxins, bioproducts and chemical ecology where more than 15000 novel compounds have been chemically determined. Algae therefore offer diverse spectrum of valuable products and environmental solutions such as food, nutritional compounds, omega-3 fatty acids, animal feed, energy sources (including jet fuel, aviation gas, biodiesel, gasoline, and bioethanol), organic fertilizers, biodegradable plastics, recombinant proteins, pigments, medicines, pharmaceuticals and vaccines.

Red algae are the most important source of many biologically active metabolites in comparison to other algal classes [12].

Recent trends in the field of drug research from natural sources indicate that algae is a promising group to provide novel biochemically active substances [13]. Algae have developed defence strategies that result in significant level of structural–chemical diversity from different metabolic pathways to survive in a competitive environment, freshwater and marine [14]. These compounds include pigments, antioxidants, β-carotenes, polysaccharides, triglycerides, fatty acids, vitamins, and biomass. Species such as *Cyanobacteria*, *Phormidium cebennse*, *Oscillatoria raciborskii*, *Scytonema burmanicum*, *Calothrix elenkinii*, and *Anabaena variabilis*, show anti HIV-1 activity, and give positive tests for the presence of sulfolipids. Hydrocolloids, alginate, agar, and carrageenan, produced from seaweeds are largely used as viscosity-modifying agents in foods and pharmaceuticals. As they are largely used as bulk commodities in industrial sectors as varied as pharmaceuticals, cosmetics, nutraceuticals, functional foods, and biofuels, readily available supply of extracts, fractions or pure compounds are of prime importance [15].

1.4 Algae as Feedstock for Biodiesel

The oil or lipid from algae is extracted using solvents and turned into biodiesel through transesterification. Oil levels of 20–50% are common in microalgae [3], though the ratios of lipid, carbohydrates and proteins are species-dependant. In some species, lipids can be up to 60% of the algal dry weight [16]. The average lipid content of algae can vary from 1–70% dry mass, depending on strain, and up to 90% in certain environments [17]. Algae have a trigger which when put into stressful environments such as nutrient deprivation, the algae change the use of carbon uptake from reproduction to energy storage in the form of oils. Athough algae with 70% oil content exist, a growth system to maintain the culture at this level of oil content has not been developed that would make oil from algae an economical option [3].

There are two main challenges in maintaining algae at theoretical high oil content. Firstly, although an outdoor system is more economical, algal biomass productivity is significantly reduced when compared to closed bioreactors. It is nearly impossible to maintain optimum laboratory culture conditions due to changing temperatures and invasive species. If controlled at optimized conditions in a closed system, algae cultures could be maintained in the exponential growth phase producing optimized biomass yields but controlled bioreactor system may be costly. Another hurdle is that algal reproduction decreases significantly when put in stressed environmental conditions. Even with higher lipid content per unit cell, the net oil gained may actually decrease in a nutrient deficient environment due to significantly reduced cell numbers [1]. A better understanding of separation between growth and production kinetics, may lead to a net increase in total oil productivity by carefully controlling the timing of nutrient depletion and cell harvesting.

1.5 Nutritional, Physiological and Environmental Factors

The quantity and quality of lipids within the cell may vary as a result of changes in growth conditions (temperature, light intensity, salinity and pH) or nutrient media concentrations (nitrogen, phosphates and iron) [18] and these are crucial to microalgal metabolism, and lipid productivity [19]. There is a relationship between substrate inhibition and maintenance energy. A variety of protective mechanisms developed in microorganisms against unfavorable stress conditions emphasizes the role of Na+/H+ antiporters, water and ionic channels and the synthesis of compatible solutes and salt stress-induced proteins [20]. Several microalgal strains have been screened such as Chlorella vulgaris, Chlorella protothecoides, Spirulina maxima, Chlamydomonas reinhardtii, Nannochloropsis sp., Neochloris oleoabundans, Scenedesmus obliquus, Nitzchia sp., Schizochytrium sp., and Dunaliella tertiolecta, for the highest lipid production in terms of quantity (combination of biomass productivity and lipid content) and quality (fatty acid composition) for biodiesel production [3, 19, 21]. Studies on Ochromonas danica and Nannochloropsis salina indicate that the cell growth and lipid content increase with increasing photoperiod [22]. Light is the driving force of photosynthesis, as well as for cell photo-acclimatization.

Physiological properties of phytoplankton and photosynthetic organisms can be changed upon exposure to photoperiod and light intensity.

The effects of light and photoperiod may also actually influence cultivation temperature where increased lipid content to 26-36% at 25-30^{o}C and reduced lipid level at 15-20% in *Isochrysis galbana* and *Nanochloropsis* at both extremes of low and high temperatures, have been reported [23]. Low temperatures reduce enzyme activity in glycolysis and the Krebs cycle and consequently the metabolism of carbon sources.

Different geographical strains have different preferences towards salinity. Optimal salinity may be a function of immediate conditions from which the strain is initially isolated. Species isolated at higher salinities will grow better at higher salinities and not as good at lower salinities [24]. The effects of low salinity level on growth retardation has been observed in *N. gregari* [25], and on lower lipid contents (18-19% DW) in nutrient replete conditions in *Chlamydomonas applanata* and *C. reinhardtii* [16]. Higher lipid content (60.6% DW) has been reported for *Dunaliella* in salt concentration as high as 0.5 M NaCl (equivalent to seawater, or 35 psu) [26] and lipid increases as NaCl increases. The highest lipid content of 50% for *S. obliquus* has been reported after 10 day stress period when NaCl at 1 M is used [27]. Culture pH influences influx and efflux of anions and cations into the cellular system. Optimal pH for *Chlorella vulgaris*, is pH 7-8 [28], and for cyanobacteria, pH 8.2-8.7 [29].

A strain of algae put into a nitrogen deficient environment can enhance the oil content per dry mass from 22% to 58% [1]. A study on lipid accumulation in nitrogen deficient and silica deficient media suggests that rapid cell division occurs with sufficient nitrogen present. When nitrogen is depleted, cell division ceases, although the biomass may continue to accumulate mainly a result of an increase in intracellular components or lipid content. Silica depletion as studied in diatoms – a type of algae that utilizes silica in its cell walls, similarly show a decreased cell division in silica depleted media. Some strains show increase in lipid content, particularly neutral lipids while other strains have the lipid content remain unchanged with decreased growth rate and much lower total biomass [1]. Five strains of green alga *Chlorella* in Wantanabe and low-nitrogen media in a 2-L stirred bioreactor show varied growth in

low-nitrogen media but with increased lipid content, as compared to the growth in Wantanabe medium. In *C. vulgaris*, lipid increases from 18-40% dry weight, and the lipid content in *C. emersonii* increases from 29-63% [30].

1.6 Engineering Microalgae for Enhanced Productivity

The productivity can be enhanced by efficient cultivation method and by genetic and metabolic engineering, which is likely to have the greatest impact on improving the economics of biodiesel production from microalgae [31]. There are now intensive global research efforts aimed at increasing and modifying the accumulation of lipids, alcohols, hydrocarbons, polysaccharides, and other bio-based compounds in algae through genetic engineering. During the Aquatic Species Program, acetyl-CoA carboxylase (AACase) activity is observed to increase two fold in the diatoms during silica deficiency. Later studies conclude that the AACase enzyme plays a key role in lipid accumulation. The enzyme is then isolated and cloned for a study on gene expression. The studies conclude that algae alter enzyme activity during silica deprivation for increased synthesis of lipids. Genetic engineering of an algal cell with increased activity of AACase during optimized cell growth produces a strain of algae efficient for bio-oil production. Although the Aquatic Species Program does make strides in genetic manipulations and mutations, the efforts are unsuccessful at manipulation of algal strain for biofuel production [1].

To cope with unfavorable conditions such as lack of nitrogen, some species may have developed a number of adaptations, including alteration of lipid metabolism and synthesis of non-membranous lipids such as triacylglycerols (TAG) and carotenoids [32]. These mechanisms have been described in chlorophytes such as *Dunaliella salina*, *Dunaliella bardawil*, *Haematococcus pluvialis,* and *Parietochloris incise* [33-36]. Although nitrogen deficiency appears to inhibit the cell cycle and the production of almost all cellular components, the rate of lipid synthesis remains high, which leads to the accumulation of neutral lipids in starved cells [11].

The TAG accumulation is often deposited in cytoplasmic lipid globules referred to as oil bodies (OB), which increase vastly in sizes and numbers under mineral nutrition deficiency, high salinity and high irradiances [36].

Lipids, particularly TAG, are thought to be a storehouse for the excessive photosynthates, which could not be utilized under unfavorable conditions. These adaptive responses help to ensure the survival of cells during times of stress, while lipids serve as energy stores [37].

Algal biotechnology breakthroughs directly enable new approaches to generate algae with desirable properties for the production of biofuels and bioproducts.

The strategies to introduce genes, to delete or disrupt genes, and to modify genes or gene expression in particular species of algae is carried out to enhance physiological properties of algal strains and optimization of algal production systems. Though the potential for genetic manipulation exists, not much attention has been made for these efforts [3]. To date, no commercial application of genetically manipulated algae has been reported. Limitation in this field is mainly due to the inability to silence foreign genes causing instability in the gene expression. If the regulation of the gene expression for algae is better understood, controlling the gene expression may be possible [38]. Also, secrecy in this field may exist due to competitiveness in finding an optimal source for biofuels which causes additional difficulty in advancing the field through shared knowledge.

1.7 Problem Statement

The main challenge in microalgal lipid is to make it more economical through effective and efficient reactor design for maximum growth, basic culture conditions and product harvesting. Important factors to be optimized are pH, salinity, photoperiod, light intensity and nutrient deficient media. Among important nutrient components for algal productivity are nitrate, phosphate and iron. An alternative and cost effective source of media must be developed to reduce dependency on commercial media.

An understanding on the the interaction between factors is important to provide the basis to formulate cultivation strategies and for the prediction of optimal conditions for growth and lipid accumulation. As direct translation of lab-scale optimal conditions may not always be feasible, mass cultivation of microalgae in photobioreactors and open tanks need to identify scale-up kinetics factors and to asses reproducibility issues including mode of mixing, aeration, representative sampling and harvesting.

Nannochloropsis is a genus of marine eukaryotic unicellular algae and species in this genus are morphologically similar and it is difficult to identify species in this genus through traditional morphological observations. The gene sequences need to be used to identify species in this genus on molecular level.

1.8 Objectives

The objectives of this study were:

i) To determine optimal conditions and elucidate interactions between different factors such as pH, salinity, photoperiod, light intensity and macronutrients using Response Surface Methodology, that could affect productivity of locally-isolated *Nannochloropsis oculata*, *Tetraselmis suecica*, *Isochrysis galbana* and *Pavlova lutheri*.

ii) To develop an alternative and economical medium and to asses palm oil mill effluent (POME) remediation by algal cultivation.

iii) To establish the kinetics of cell growth and lipid production in 5 L photobioreactor and 300 L open tanks

iv) To carry out molecular identification of *Nannochloropsis oculata* strain by the sequences of 18S rRNA, *rbcL* gene and internal transcribed spacer (ITS) region of rRNA transcription unit

1.9 Scope of Study

The scope involved investigation of the contribution of different growth factors (pH, salinity, photoperiod, light intensity, phosphate, nitrate and iron) for growth and lipid enhancement of microalgae. Selections of locally isolated marine microalgae (*Nannochloropsis oculata* and *Tetraselmis suecica* for green microalgae and *Isochrysis galbana* and *Pavlova lutheri* for brown microalgae) were evaluated based on high growth rates and lipid content. Comparison between commercial media (TMRL Enrichment) with palm oil mill effluent (POME) was made based on optimized conditions as established by Response Surface Methodology (RSM). Analysis of cell density, specific growth rate and comparison of kinetics between 250 mL, 1 L, 5 L, 30 L, 300 L batch cultures and 5 L photobioreactor were established to identify optimization parameters for scale-up. Molecular identification of *Nannochloropsis oculata* was made by using different gene sequences.

1.10 Thesis Organization

Chapter 1 covers the introduction on microalgae and describes the motivation behind algae as the feedstock for biodiesel. Nutritional, physiological and environmental factors, lipid productivity enhancement, problem statement, objectives and scope of research are also discussed. Chapter 2 describes the biology of algae, bioenergy and biochemicals, engineering considerations for microalgal productivity, Abiotic and nutritional requirements, microalgal reactor and metabolic engineering. Chapter 3 presents the methodology and equipments used for microalgal growth, media conditions and preparations, RSM model and POME characterization. Chapter 4 describes basic culture conditions, optimization and interaction between factors, effects of pH, salinity, photoperiod and light intensity on biomass and lipid content. Chapter 5 covers the effects of macronutrients and iron and the economical palm oil mill effluent media on microalgal productivity. Chapter 6 explores the establishment of kinetics, cell growth, lipid production and fatty acid profiles between 5 L photobioreactor and 300 L open tank cultivation at optimal conditions. Chapter 7 describes the DNA purity, PCR products, DNA sequencing and phylogenetic analysis

for molecular identification of a species in genus *Nannochloropsis*, and finally Chapter 8 covers the conclusion and recommendations for future work.

CHAPTER 2

LITRATURE REVIEW

2.1 The Biology of Algae

2.1.1 Characteristics and Classes of Algae

Algae which exist as either macroalgae or microalgae are thallophytes, lacking the stems, mesophyll cells, bundle sheaths, roots or leaves [39]. Microalgae are unicellular or multicellular photosynthetic organisms of usually 50 μm maximum diameter that use light energy and carbon dioxide with higher photosynthetic efficiency than plants for biomass production [40]. Microalgal cell structures are either prokaryotes or eukaryotes. Prokaryotic cell algae, categorized as cyanobacteria (cyanophyceae), lack membrane-bound organelles such as plastids, nuclei or mitochondria and photosynthesis takes place in the cytoplasm instead of the organelles. Eukaryotic cell algae have organelles in which photosynthesis occur. Majority of the microalgae possess a nucleus which aid in cell function allowing the cells to metabolize, survive and reproduce. As they thrive in extreme environmental conditions, such as saline or alkaline habitats, cold arctic regions, hot springs and arid soils, significant numbers live parasitically or in symbiotic relationship with other organisms [41].

Eukaryotic microalgae are classified into variety of classes based on their life cycle, basic cellular structure, and pigmentation. The different division or phyla may also be based on specific food reserves and cell wall constituents in each species. Pigmentation determines habitat each genera or species are found, apart from the varying adaptability potential [42].

1) Chlorophyta (green algae): This group is a diverse aquatic or marine, unicellular or multicellular organisms and posses variability in morphology. They contain chlorophyll *a* and *b* and several cartenoids. Cell walls are mainly cellulosic with some species having hemicelluloses and calcium carbonate. Starch and oil are the food reserve of green algae, that they are mostly used for biofuel because of the stored products which could be used for either ethanol or biodiesel [42, 43].

2) Bacillariophyta (diatoms): Unicellular, with high silica embedded in the cell wall which aid in resistance to decay. Exist in freshwater, moist soil and moist surfaces but diatoms deposit most of the limestones as well as fossil fuel. Chrysolaminarin (polysaccharide) and oil are the stored products which can be used for biodiesel with significant amount of polyunsaturated fatty acids (PUFAs) especially eicosapentaenoic acid for use as food additives [43].

3) Chrysophyta (Golden-brown algae): Occur mainly in freshwater especially oligotrophic water column with low calcium concentration [42, 44]. Cell wall is cellulosic with large deposition of silica and consists of photosynthetic pigments chlorophyll *a* and *c*. The stored products are chrysolaminarin and oil [44].

4) Rodophyta (Red algae): Multicellular and filamentous forms, with chlorophyll *a* and *d*, phycobiliproteins and starch as stored products [42], which accumulate in the cytoplasm outside the chloroplast.

5) Cyanobacteria (Blue-green algae): Prokaryotic aquatic bacteria that that are not related to any of the other eukaryotic algal groups, but the different species can be red, brown, or yellow. There are two types of pigmentation which enable photosynthesis: - chlorophyll *a* together with various proteins called phycobilins, which give the cells a typical blue-green to greyish-brown colour; and chlorophyll *b* for a few genera, which lack phycobilins as well as having bright green colour [45]. Cyanobacteria may be single-celled or colonial. Colonies may form filaments, sheets or even hollow balls and blooms i.e. dense masses on the surface of a water body, depending on the species and environmental conditions.

6) Phaeophyta (Brown algae): Mainly marine algae and grow in colder region. The brown color comes from the dominance of fucoxanthin over chlorophyll *a* and *c*.

Cell wall consists of an inner cellulose microfibrillar and outer amorphous layer of alginates, and the stored products are carbohydrates and oil [41].

7) Xanthophyta (Yellow-green algae): Mostly unicellular or colonial species and live in freshwater and terrestrial habitats. The colour is attributable to both chlorophyll *a* and cartenoids, while chlorophyll *c* is minute and fucoxanthin is absent. The cell wall is cellulosic composed of two overlapping halves. The storage products are chrysolaminarin and oil (arachidonic and eicosapentaenoic acids) [42].

Among green algal species are:

a) *Nannochloropsis* belonging to the class of Eustigmatophyceae. The genus is widely used in aquaculture due to its relatively high growth rate, resistance to shear and contamination, high nutritional values and high lipid content. These characteristics fit the needs of the biofuel industry [46]. The oil productivity of microalgae is higher than the best producing oil crops [47].

b) *Tetraselmis suecica* is a marine green flagellate, belonging to Chlorophyceae. It is widely used in aquaculture as feed for bivalve molluscs, penaeid shrimp larvae and rotifers. It has good nutritional properties and contains C16:0 and C18:1 as predominant fatty acids [48] which are suitable for biodiesel.

Among brown algal species are:

a) *Isochrysis galbana* is a haptophyceae which is of substantial interest in aquaculture, principally to feed mollusk larvae, as well as fish and crustaceans in the early stages of growth [49].

b) *Pavlova lutheri*, a phytoflagellate, is a good example of high lipid producing marine microalgae. It is a common member of the Pavlovophyceae (Haptophyta), often used as food source for aquatic filter-feeders and cultured in laboratories to produce high levels of PUFAs. The size of 4-6 µm, cell density of 0.4-1.5 g L^{-1} and 15-35 % lipid content has been reported, with doubling time of 3-4 days [50, 51].

2.1.2 Respiration and Photosynthesis

Microalgae assimilate inorganic carbon in the photosynthesis with the present of light either sunlight or from other sources. The solar energy will then be converted to chemical energy with O_2 as a by-product, and the chemical energy is subsequently used to assimilate CO_2 and convert it to sugars [52]. The metabolic process by which an organism obtains energy by reacting oxygen with glucose to give water, CO_2 and ATP (energy) is called respiration. Reduction in temperature activates enzymes as an adaptive mechanism to maintain respiration and photosynthesis. The microscopic algae uses photosynthetic process similar to that of higher-developed plants. Photosynthesis involves two steps: - light reaction that only occurs when the cells are illuminated (Eq 1); and carbon-fixation reactions, also known as dark reactions (Fig. 2.1) that occur both in the presence and absence of light (Eq 2) [53].

$$2H_2O + 2NADP^+ + 3\,ADP + 3P_i + light \rightarrow 2NADPH + 2H^+ + 3ATP + O_2 \quad (1)$$

$$3CO_2 + 9ATP + 6NADPH + 6H^+ \rightarrow C_3H_6O_3\text{-phosphate} + 9ADP + 8P_i + 6NADP^+ + 3H_2O \quad (2)$$

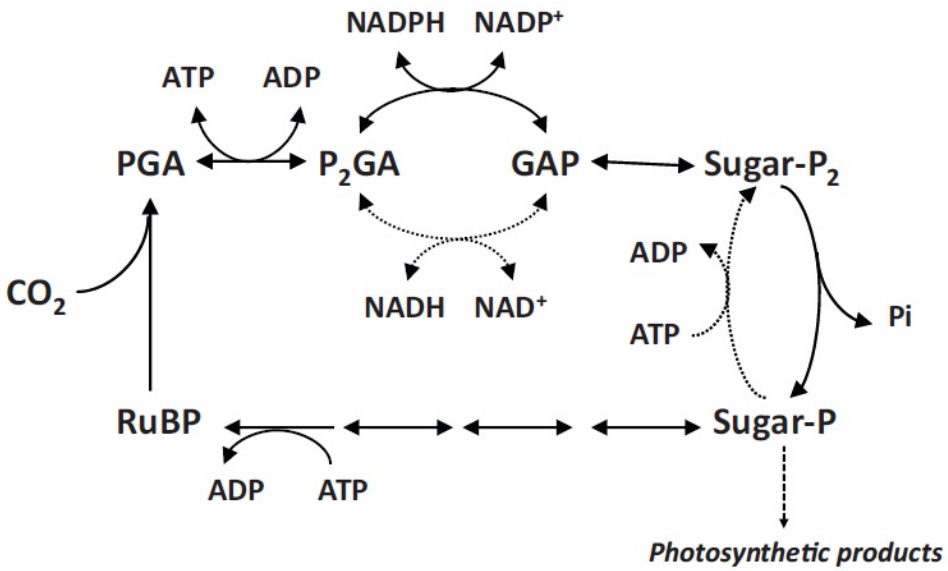


Fig 2.1 A generalized structure of the Calvin cycle [54]

Biofixation of CO_2 using microalgae has emerged as a potential option as microalgae have the advantages of efficient photosynthesis. Light is first absorbed by the antenna pigments of photosystem (PS) I and II. The absorbed energy is transferred to the reaction center chlorophylls, P_{680} in PSII, P_{700} in PSI. Absorption of 1 photon of light by PSII removes 1 electron from P_{680}. With its resulting positive charge, P_{680} is sufficiently electronegative that it can remove 1 electron from a molecule of water. When these steps have occurred 4 times, requiring 2 molecules of water, a total of 1 molecule of oxygen and 4 protons (H^+) are released. The electrons are transferred to the cytochrome b_6/f complex where they provide the energy for chemiosmosis. Activation of P_{700} in PSI enables it to pick up electrons from the cytochrome b_6/f complex (by way of plastocyanin). It raises them to a sufficiently high redox potential that, after passing through ferredoxin (Fd), they can reduce $NADP^+$ to NADPH [55].

2.2 Bioenergy and Biochemicals from Microalgae

The first generation biofuel, bioethanol, is produced from corn and sugar cane, while biodiesel from palm oil, soybean oil, and oilseed rape. However, these may not be eco-friendly as the debate rages on “food” over “fuel” [56, 57]. Second generation biofuel from cellulosic biomass is a better option as wood residues, sawdust and other agricultural cellulosic sources are abundant and do not impede the production of food. Lignocellulosic energy crops can be grown on insignificant lands that would not otherwise be used as farmland and can be sustainably harvested for conversion into diesel, ethanol jet fuel and gasoline [57]. The third generation biofuel is produced from algae and cyanobacteria. Algae appear to represent the only means of renewable current generation of biofuels [3, 6]. Due to their naturally high-lipid content, semi-steady state production and suitability in variety of climates [58], microalgal biofuels have much lower impact on the environment and on the world’s food supply than conventional biofuel-producing crops. Having low viscosity, high calorific value and low density, these properties are more appropriate for biofuel than lignocellulosic materials. Fig. 2.2 shows resources needed for microalgal conversion to intermediates and fuels.

Fig. 2.3 shows typical algal lipid production process. If algal production could be scaled up to industrial capacity to meet the current fuel demand, less than 6 million hectares would be needed worldwide. This consists of less than 0.4% of arable land which is an achievable goal from global agriculture perspective. There are some desirable characteristics of algal strains to be considered as candidates for biofuel production [59] :- 1) robust and able to survive the shear stresses common in photobioreactors; 2) able to dominate wild strains in open pond production systems; 3) high CO_2 sinking capacity; 4) limited nutrient requirements; 5) tolerant to a wide range of temperatures resulting from the diurnal cycle and seasonal variations; 6) potential to provide valuable co-products; 7) fast productivity cycle; 8) high photosynthetic efficiency; and 9) display self-flocculation characteristics.

Most of the efficient algal species are marine, thus requiring no freshwater in culturing phase [56]. The major challenge in achieving the dual benefits of microalgae is determining a way that allows for downstream processing that is suitable for producing biofuel and other bioproducts [60]. The choice of specific microalgae depends on biofuel products which could be biodiesel, ethanol or propanol, while the residue obtained can be used for animal feed, cosmetics, pharmaceutical products, and biofertilizer [61].

2.2.1 Biodiesel

Biodiesel production from photosynthetic algae which grow on CO_2 has great potential as biofuel as it provides maximum net energy. Oil conversion into biodiesel is much less energy-intensive than methods for conversion to other fuels. The feature has made biodiesel the desired end-product from algae, but this requires selecting high-oil content strains, and contriving cost effective methods of harvesting, oil extraction and conversion of oil to biodiesel. Biodiesel typically produced from the oil of oleaginous crops such as soybean, sunflower, rapeseed and palm, involve mono-alcoholic transesterification process, in which triglycerides react with mono-alcohol (most commonly ethanol or methanol) with catalysts [31, 62]. Fig. 2.4 shows the general chemical reaction of the methyl ester production.

Triacylglycerols are typically non-volatile that transesterification with short alkyl moieties, methyl or ethyl residues are required to manufacture biodiesel. There are several chemical reaction steps: - reversible hydrolysis where triglycerides are converted into diglycerides; diglycerides converted into monoglycerides; and monoglycerides into free fatty acids and glycerol (as byproduct); followed by re-esterification with short chain alcohol (ethanol or methanol), in the presence of a catalyst.

Lipases are excellent for various vegetable oil conversions to methyl ester. With the molar ratio of oil to alcohol 3:1–6:1 and the conversion temperature of 35–50°C at atmospheric pressure, more than 90% crude oil yield can be achieved [63, 64]. If lipase is used, hydrolysis and esterification may take place concurrently, but the thermal liability of enzyme becomes a major stumbling block for industrial scale production. *In situ* transesterification is a promising alternative to reduce processing costs, as this may lead to conversion of fatty acids to alkyl esters right inside the biomass, thereby eliminating the solvent extraction step and facilitating the harvesting biomass and drying. The method which includes alcoholysis leads up to 20% higher biodiesel yield than the conventional process and reduces wastes [65].

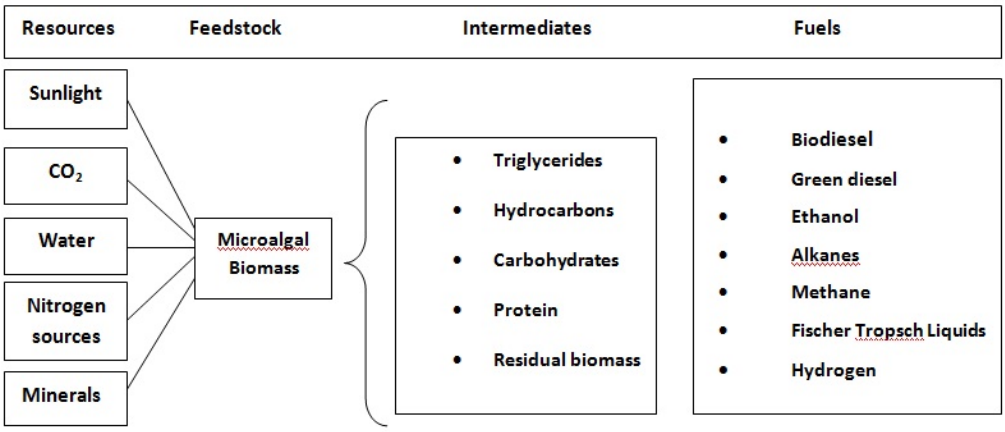


Fig. 2.2 Fuel Production options from Microalgal Cell Components [66].

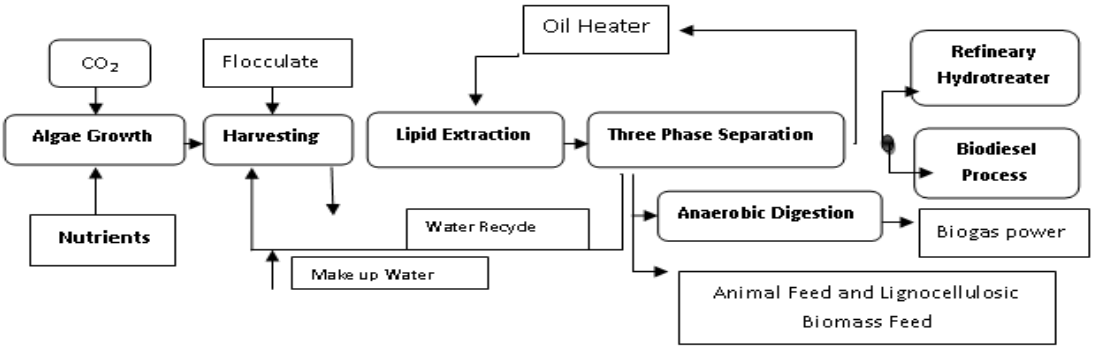


Fig. 2.3 Algal Lipid Production Process [66].

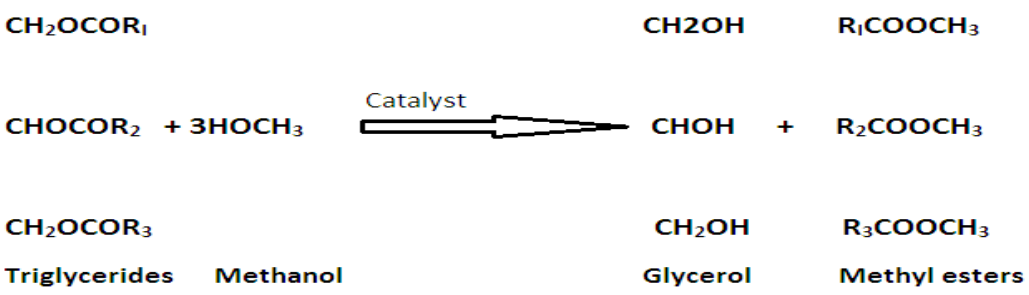


Fig. 2.4 The chemical reaction of the transesterification process

2.2.2 Bioethanol

There has been significant interest on microalgal utilization as an advanced energy feedstock for bioethanol production [11]. This is based on the fact that 75% of algal complex carbohydrates could be hydrolyzed into a fermentable hexose monomer with 80% theoretical fermentated yield of ethanol [67]. Microalgae can also assimilate cellulose which can be fermented to bioethanol and it is easy to supply optimal levels and minimal nutrients for microalgae culturing from the well-mixed aqueous environment as compared to the soil. Absence of non-photosynthetic supporting structures (roots, stems or leaves) also favors microalgal cultivation as there will be less energy spent towards distribution and transportation of storage molecules like starch between tissues [10].

Generally two methods are used, namely fermentation (biochemical process) and gasification (thermo-chemical process) [68]. After oil extraction from the microalgal biomass, fermentation process ensues utilizing gluco–amylase, α–amylase and yeast, bacteria or fungi for fermenting the sugars to ethanol and carbon dioxide with used water that can be recycled [69].

$$C_6H_{12}O_6 + 2ADP + 2Pi \longrightarrow 2C_2H_5OH + 2CO_2 + 2ATP + 2H_2O$$

The biomass preparation can be carried out with mechanical presss or enzymatic cell wall break down to make carbohydrates more available, as well as breaking down large molecules of carbohydrates. When cells are disrupted, the yeast *Saccharomyces cerevisiae* is added to the biomass and fermentation begins. In this way, sugar is converted into ethanol and ethanol is purified by distillation [70]. CO_2 can be recycled during fermentation as a nutrient or residual biomass in the process of anaerobic digestion for methane production, such that in essence all the organic matter is accounted for [68, 71].

2.2.3 Biomethane

Biogas is mainly composed of a mixture of methane (55–75%) and carbon dioxide (25–45%) produced by anaerobic microorganisms during anaerobic digestion. It involves the breakdown of organic matter to produce biogas with an energy content of about 20–40% lower heating value of the feedstock. Anaerobic digestion occurs in three sequential stages: hydrolysis, fermentation and methanogenesis. During hydrolysis, complex organic biopolymers (e.g carbohydrates, lipids and proteins) are hydrolyzed and broken down into soluble sugars. The fermentation carried out by bacteria converts into alcohols, acetic acid, volatile fatty acids and gas containing H_2 and CO_2, which is primarily metabolized by methanogenesis into CH_4 (60–70%) and CO_2 (30–40%). Anaerobic digestion is suitable for high moisture content (80–90%) organic wastes which can be useful for wet algal biomass. Microalgal biomass has the advantage over the conventionally used plant biomass for the production of biogas, in that they grow in a liquid medium and the space available for cultivation is two-dimensional. Microalgae contain lower cellulose and no lignin, leading to good process stability and high conversion efficiencies [72]. Methane can be used as fuel gas and also be converted to produce electricity [73], CO_2 can be removed, should pure methane to be used [31]. The conversion of microalgal biomass into biogas even recovers energy through the extraction of lipids that can be used for biodiesel production [61].

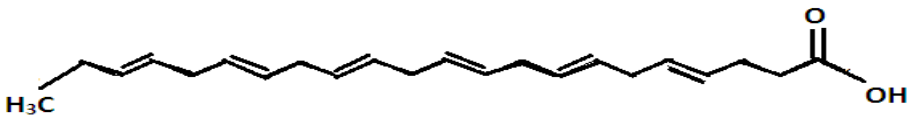
2.2.4 Biohydrogen

Hydrogen is an important fuel and is widely applied in fuel cells, liquefaction of coal and upgrading of heavy oils. Hydrogen can be produced by steam reformation of bio-oils, dark and photo fermentation of organic materials, and photolysis of water catalyzed by special microalgal species [74, 75]. Cyanobacteria are able to diverge the electrons emerging from the two primary reactions of oxygenic photosynthesis directly into the production of H_2, making them attractive for H_2 production from solar energy and water [76].

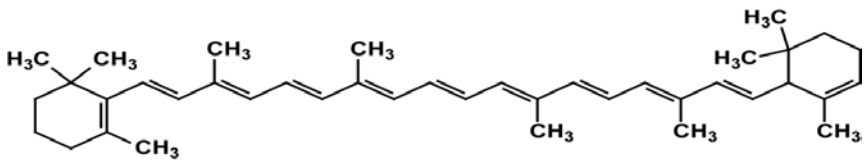
The two natural pathways for H_2 production in cyanobacteria are: - 1) H_2-production as a by-product during nitrogen fixation by nitrogenases; and 2) H_2-production directly by bidirectional hydrogenase [77]. Nitrogenases require ATP whereas bidirectional hydrogenases do not require ATP, making them more efficient and favorable for H_2-production with a much higher turnover.

2.2.5 High Value Biochemicals

An algal cell may be considered as a multi-functional nano-scale factory with the capacity to produce a range of products of interest [11]. To date, only a few microalgae have been chemically characterized for potentials as biofuel feedstocks or pharmaceutical products [78]. Species such as *Nannochloropsis oculata* is well known for its high biomass and lipid productivity for biodiesel production; *Arthospira* sp., and *Dunaliella salina* are known for face and skin care products [79]. Microalgae are rich in PUFAs such as docosahexanoic acid (DHA) and eicosapentaenoic acid (EPA) both found as lipids in microalgae. Although not suited for biodiesel production, both can be separated and purified as high value products. DHA (Fig 2.5 a) is vital for child brain and eye, and for adult cardiovascular health [80] and EPA helps to lower the risk of cardiac arrest [81]. Pigments such as carotenoids (Fig 2.5 b) can be used as food and cosmetic colourants [82]; astaxanthin as an anti-oxidant nutraceutical [67]; and phycobiliproteins as food dyes and cosmetics [83].



a)



b)

Figure 2.5 Structure of a) Docosahexaenoic acid (DHA), b) β-Carotene

2.3 Engineering Considerations for Microalgae

With more knowledge on algal biology and technological advances, the commercialization of algal biofuel production will be feasible in not too distant future provided the issues related to large-scale methods to cultivate and harvest algal cells, extraction of lipid and conversion into diesel are properly addressed. Manipulation of processing conditions such as temperature, salinity, light, pH, nutrients as well as culture duration allows modulation of cell growth, biochemical and lipid composition for consequent optimization of overall yield and productivity [51].

2.3.1 Growth Conditions

Microalgae are naturally acclimatized to a range of aquatic habitats, and it is sensible to use strains isolated from native environments. Microalgal growth processes can be categorized into three modes: - autotrophic, heterotrophic and mixotrophic. Autotrophic or photoautotrophic involves photosynthesis to produce complex organic compound from simple inorganic molecules like salts [59]. Heterotrophic microalgae do not carry out photosynthesis but use organic substrates like glucose or acetate as the carbon and energy source to stimulate growth, while mixotrophic microalgae derive energy from both photosynthesis and chemical oxidation and they combine both photoautotrophic and heterotrophic mechanism to sustain growth [84].

Heterotrophic culture utilizes organic carbon source for growth in the dark thus eliminating the requirement for light [85]. Some microalgae, when grown in the presence of a fixed carbon source (glucose), have higher biomass (> 20 gL^{-1}) and oil productivity (> 50% of the dry weight as lipid) than phototrophic production [86]. Other advantages of heterotrophic culture include: - i) microalgal cell densities of over 20 times higher than phototrophic culture, rendering cell harvesting more efficient; ii) reduced water demand; and iii) with no light requirement, there are associated benefits in energy and space availability for other uses [85]. Bioethanol production by yeast is mainly a two step process: i) production of bioethanol from sugar; and ii) separation of bioethanol from water.

Production of FAME from heterotrophic microalgae requires four distinct steps: i) using sugar as a substrate to grow the cells; ii) extraction of the oil from the harvested biomass; ii) producing biodiesel from the extracted lipid; and iv) separation of biodiesel from the solvent mixture [86]. This may render it in competition for starch and sugars, as the case for first generation biofuels, hence reducing its economic competitiveness.

2.3.2 Cell Productivity

Table 2.1 lists the growth rate, lipid content and optimum culture temperature of the most studied microalgae. Oil productivity of microalgae as high as 100000 $Lha^{-1}yr^{-1}$ or 27.5 ml $m^{-2}d^{-1}$ for a shallow, open pond system (raceway pond), has been reported [3, 87]. This would actually require a biomass productivity of 36.7 g $m^{-2}d^{-1}$ with 75% oil content, or 50 g $m^{-2}d^{-1}$ with 50% oil content, or 91.6 g $m^{-2}d^{-1}$ with 30% oil content and none of these has been reported. *Botryococcus brauni* accumulates 70-80% of cell weight as lipid, but has low biomass productivity of only 3 g $m^{-2}d^{-1}$ [3, 64]. Other fast growing strains may achieve areal productivity of 12-47.6 g $m^{-2}d^{-1}$, but the intracellular lipids levels are typically lower than 30%. When projecting oil productivity, both biomass productivity and oil content must be considered, and this is most accurately defined by multiplying the areal biomass productivity (or the annual average) and the intracellular oil content at the time of harvesting.

Table 2.1 Growth rate and lipid content of some of the microalgae

Strain name	Growth rate (d^{-1})	Lipid content (% of DW)	Optimum temperature	Reference
Tetraselmis suecica	0.86	11.5	25 ^{o}C	[88]
Isochrysis galbana	0.62	25.0	30 ^{o}C	[89]
Phaeodactylum tricornutum	0.60	18.0	23 ^{o}C	[90]
Nannochloropsis oculata	0.43	29.7	21 ^{o}C	[91]
Chlorella minitussima	0.43	31.0	25 ^{o}C	[30]
Haematococcus pluvialis	0.25	25.0	18 ^{o}C	[67]
Botryococcus Braunii	0.18	75.0	25 ^{o}C	[92]

2.3.3 Tolerance to Extreme Culture Conditions

Lipids in eukaryotic photoautotrophic cells function as a structural component of cell membranes or to modulate cellular activity, and serve as energy storage compounds. Under specific circumstances such as stressed conditions, microalgae accumulate high concentrations of carbon in the form of triacylglycerol (TAG) [47, 93]. These high-carbon lipids are the potential source of sustainable oil production. One of the main biological functions of neutral lipids (TAG) in microalgal cells is to provide energy for immediate and delayed metabolic requirements. Once the stationary phase of the growth cycle is reached and nutrients are depleted, microalgae switch cell metabolism and store energy in the form of lipids as an environmental stress response [94, 95]. The switch can be protracted with no net biomass productivity or lowering the productivity. Variations in cell biochemical composition are reported under favourable environmental conditions but different culture media. For single species of chlorophyta *Chlorella*, *Botryococcus braunii*, and *Dunaliella salina*, with estimated biochemical composition of 30-50% protein, 20-40% carbohydrate and 8-15% lipids. Under stressed conditions the species accumulate 80% fatty acid, 80% hydrocarbon and 40% glycerol on dry weight basis, respectively [42]. Under nutrient deficient condition, oil productivity may be lesser than that in control at 5 g $m^{-2}d^{-1}$ [94]. Metabolic stress agent such as SAN 9785 herbicide, enhances the EPA production by 28% in *Porphyridium cruentum* [96]. However, the use is limited to indoor, closed culture systems due to expenses and environment concerns.

Contaminations of pure culture from other invasive microalgae species are normally associated with outdoor open pond systems, or raceway ponds [69, 87]. Addition of organic carbon at the start of the culture cycle in open systems, must be conducted with care, preferably at a late stage in the biomass production cycle to minimize contamination of hetrotrophic bacteria. Invasive species may have superior growth rates allowing them to suppress the strain of interest and impair lipid yields and overall productivity. A superior microalgae strain must be selected that can grow and tolerate extreme conditions.

Three microalgae strains that have been mass cultured in outdoor open ponds, in extreme conditions with consistent productivity are *Dunaliella salina* in hypersaline water, *Spirulina platensis* grown at high pH and *Chlorella* sp. grown at high nutrient loading [85]. Altering pH of the entire culture is feasible but requires addition of acids or bases, and extreme eutrophy may require excessive use of fertilizers. An increased salinity represents a favourable option, as daily evaporation loss from the open pond would increase the salinity and a saline-tolerant strain should be able to survive.

2.3.4 Abiotic Requirements

2.3.4.1 Light

Light is important for cell growth and the quantity of photon energy absorbed by each cell is a combination of factors such as cell density, length of optical path, thickness of layers, photon flux density and the rate of agitation [97]. Apart from solar radiation, the fluorescent tubes are normally used especially those emitting either blue or red light spectrum as these are the most active light spectrum for photosynthesis. The photo-period (light: dark) varies between 18: 6, 12: 12 or 16: 8. Excess light intensity may result in photo-inhibition which affects cell generation time. Cells respond to high light intensity by reducing chlorophyll *a* and pigments involved in photosynthesis to boost photoprotective agents like secondary carotenoids (zeaxanthin, beta-carotene, astaxanthin), thus preventing the absorption of surplus light energy by the photosynthetic machinery [98, 99]. The carotenoids, found in significant quantity in unique structures like plastoglobuli of plastids or cytoplasmic lipid bodies, are normally elevated due to disruption in carbon and nitrogen flow inside the cells under stressed conditions.

2.3.4.2 Temperature

Temperature is very much associated with irradiation. At temperature close to optimum growth, algae could tolerate much higher irradiation before photo-inhibition takes place. Temperature in physiological tolerant range affects biochemical composition and the quantity of cellular lipid and fatty acids [97, 99]. Optimal temperature for most strains are 16-28ºC, although, some survive extremes of -5ºC and above 90ºC. At lower than 16^{o}C, there may be reduced growth, but any temperature higher may lead to photosynthetic deficiency [100]. Temperature below optimal range causes an increase in unsaturation of lipids in the membrane. This improves stability and fluidity of cell membranes especially thylakoid membrane via increased unsaturated fatty acids to prevent photoinhibition at low temperature [101, 102].

2.3.4.3 pH Range

Algae grow best at neutral pH (7-8.5) and when buffer is added to the medium for pH adjustment [103]. As culture age, pH increases due to the release of OH^- and accumulation of minerals and oxidation of nutrients. Tris (2-amino-2-[hydroxymethyl] 1-1-propanediol) and glycylglycine are commonly used to buffer pH in culturing marine algae to avoid or reduce precipitation of salts in stock solution [104]. However, Tris is quite toxic in some species while glyclyglycine is non-toxic but acidic. Both pH buffers are adjusted with HCl or 1N NaOH to obtain the desired pH of the medium [103]. Nutrient medium is pre-adjusted to pH 6.5 before algal cultivation. Cultures under pH 7.5 are usually maintained for optimum growth [105], but some species such as cyanobacterium *Anabaena variabilis* achieves optimal productivity at pH 8.2–8.4. The productivity is slightly lower at 7.4–7.8, decreasing significantly above pH 9, and the cells unable to thrive at pH 9.7–9.9 [106]. In algal cultures, pH usually increases due to photosynthetic CO_2 assimilation [107]. In high rate algal ponds, pH increase can be compensated by respiration deeper in the ponds, and the pH can then be regulated by letting in more organic material and thereby

enhancing the respiration [52]. Even if pH is high for other reasons than photosynthetic CO_2-exhaustion, pH may regulate the availability of inorganic carbon.

Assimilation of nitrate increases the pH due to the release of OH^-. With ammonia, the pH of the medium decreases due to H^+ release, which is too acidic to support growth [108].

High pH can also lead to precipitation of phosphate in the medium by formation of calcium phosphates, and may redissolve if pH drops at night. If the concentration of ammonia is high at usually high pH, photosynthesis will be inhibited. High pH induces flocculation of some algae, which can actually facilitate harvesting but this leads to reduced nutrient uptake and growth. In order to avoid extreme pHs, agitation and mixing can promote the gas exchange between water and air which in turn regulates pH [52].

2.3.4.4 Water and Salinity

Due to heavy metal contamination of natural tap water and distilled water, culturing of freshwater algae is ideal with spring water [103]. In some species, oligotrophic water is ideal because of its low concentration of trace metal and nutrient which can be supplemented via enriched medium having less phytoplankton and sediments. Alternatively, aged natural water or nano-pure water (water purified using a Barnstead/Thermolyne Nanopure lab water system) can be used. Seawater is however vital for marine algae productivity. Natural seawater can be left to age for six months in the dark at 5°C, while spring and lake water are aged for two months in the dark at 20°C [103]. Salt concentration is the major contributor to the osmotic effects of ions. Algae typically tolerate moderate salt concentration. High salt or high sugar normally leads to loss of water from the cells, affects the rate of respiration, distribution of minerals, ion toxicity, photosynthetic rate and permeability of the cell membranes [109] and ultimately cell death. Early signs of salinity damage are brighter colour of algae than the normal colour of green [110].

Compatible solutes like proline and glycine are formed within cells during osmotic stress and acts as osmoprotectants to stabilize the enzymes. The growth of microalgae is retarded during salinity stress due to the accumulation of these compatible solutes to balance the external salt concentrations, although lipids may also be involved in the protection against salt stress [111]. The percentage of saturated fatty acid in microalgae decreases as the concentration of NaCl increases, while the percentage of highly unsaturated fatty acid increases [112].

For cultures growing in freshwater with low salinity and nearly neutral pH, inorganic carbon source may be required to boost photosynthesis [113]. The decrease in photosynthetic activity observed under salt stress may be due to limitations in photosynthetic electron transport and partial stomatal closure [114]. In *Synechocystis*, the combination of light and salt stress is reported to assert strong synergistic and damaging effect on PSII, due to the fact that salt stress inhibits the activity of PS II from light-induced inactivation [115].

2.3.5 Nutritional Requirements

2.3.5.1 Carbon and Nitrogen

The CO_2 concentration in natural air is low to aerate culture medium and sustain optimal growth and biomass productivity. For large scale CO_2 supply from waste gas of industrial combustion process, diesel engine, cement plants or fermentation may be an option. Good mixing or agitation could help to improve CO_2 distribution in a CO_2-enriched air supplied to the media. The assimilation of CO_2 and bicarbonate by rapidly growing algae could discharge of OH^- and elevate pH. In mass cultivation, pH should be maintained to keep it in an optimal range and to prevent carbon depletion.

Nitrogen is a vital component for composition, formation and functionality of protein and DNA and is normally supplied in the form of nitrate, nitrite, ammonia or urea. The most widely used nitrogen source for microalgae culture is ammonia and urea because they are readily assimilated and economical.

Nitrate and nitrite are quite expensive and require high metabolic energy for absorption, followed by reduction into organic molecules (ammonia) before incorporation by the cells. The average nitrogen requirement for most green algae is between 5-10% or 5-50 mM [113]. When nitrogen supply is sufficient, majority of microalgae are unable to produce nitrogen storage materials, while only few are found with nitrogen storage compounds like cyanophycin and phycocyanin. Microalgae grown in nitrogen limited conditions demonstrate a degradation of phycobilisomes during cell division when new phycobilisome synthesis ceases [116]. Nitrogen starvation can either improve the biosynthesis and accumulation of lipids or reduce the process in some species. When lipid production is enhanced in nitrogen starved conditions, neutral lipids in the form of TAGs are predominant in the depleted cells [117].

2.3.5.2 Phosphorus and Sulphur

Phosphorous occur as either orthophosphate or organic phosphate combination. Orthophosphate is incorporated into organic components via several phosphorylation process. Phosphates are essential for cellular metabolic process via formation of structural and functional constituent necessary for normal growth, biosynthesis of nucleic acid, and energy transfer [113, 118]. Phosphorous concentration is often growth limiting in natural aqueous environment. Inorganic phosphate could occur in cells as polyphosphate where distinct polyphosphate granules accumulate in cells when phosphorus is sufficient, but invisible when phosphorous is deficient. The optimal absorption rates of phosphorous for most strains are 50 μg L^{-1}- 20 mg L^{-1}. Deficient phosphorous is similar to nitrogen starved condition, as it leads to increase in lipid and carbohydrate content, a decrease in protein, nucleic acid and chlorophyll *a* content [113]. There is less phycobilisome degradation in phosphorous deficiency as compared to in nitrogen deficiency.

Sulphur is vital to cells because it is a constituent of essential amino acids (methionine, cysteine and cystine), vitamins and sulphur-lipids. It is normally provided as inorganic sulphate in the culture medium, although certain species may utilize organic sulphur sources like sulphur containing amino acids. Sulphur absorption is an active phosphorylation-driven requiring light energy and temperature-sensitive process. It also requires a reduction of nitrate before it is absorbed or incorporated into cells [42, 113].

2.3.5.3 Trace Elements and Metal ions

Trace elements are required in small amounts of micro-, nano- or even pictogram per liter. The major trace elements in algae media are manganese, nickel, zinc, boron, vanadium, cobalt, copper, molybdenum and these can not be replaced by other elements [42, 113]. Trace elements influence growth in a representative number of species, and show direct physiological and positive effects on algae growth. Cultures lacking these elements may show reversible signs of deficiency.

Important metal ions for cellular functions include Ca, K, Na, Mg and Fe. The physiological role of calcium is implicit, but when required it should be in minute ratio or supplied in composition with other nutrient. Calcium ions protect the cytoplasmic membrane, aid salt formation with colloids and precipitation of $CaCO_3$. It is responsible for calcite or aragonite formation in or on cell wall of some strains, which prevents decay in diatoms [42, 113]. Potassium is particularly required in marine and halophilic microalgae in trace concentration and toxic at high concentration especially to freshwater microalgae. It aids nitrogen fixation during conversion of molecular nitrogen to ammonia. Insufficient supply of potassium can lead to reduced photosynthesis, growth, development and high respiration. Deficient cells can be revived by addition of potassium or rubidium. Because of the tight correlation in chemical properties with K, Na can replace K in any culture media and involve in protein synthesis and osmotic regulation [42, 113].

Mg is the fundamental atom of chlorophyll molecules required for photosynthesis, ribosomes aggregation in cell functional units and catalyst formation. Mg deficiency interrupts cell division, resulting in abnormal and large etiolated cells (made pale due to lack of light) [42, 113]. Fe being a constituent of cytochromes is vital for metabolism, for redox properties and effective in nitrogen assimilation and fixation, photosynthesis, respiration, DNA synthesis and for cell structure and constituent. As a functional part of ferredoxin, Fe affects the synthesis of phycocyanin and chlorophyll. Bleaching and yellowing of algae culture are often an indication of iron deficiency in the medium. The mode of supply is not clear because the fractions of particulate, colloidal or soluble iron available to algae are not clear [113].

The effect of iron with iron source $FeCl_3$ or EDTA on lipid accumulation and biomass productivity has been investigated on marine *Chlorella* strain under laboratory conditions. The final cell density increases when chelated Fe^{3+} is added to the culture medium during late exponential growth phase. The total lipid content also increases when cells are re-inoculated into a new medium containing high level of iron [119].

2.4 Reactor Engineering and Mode of Operations

2.4.1 Open and Closed Culture Systems

Most of the commercial, large-scale outdoor algal cultivation are artificial open ponds because they are cheap to build and easy to operate and scale up [59]. The raceway ponds (Fig. 2.6), usually lined with plastic or cement, are only 20-35 cm deep to ensure adequate exposure to sunlight. The ponds equipped with paddlewheels to provide motive force and keep algae suspended are supplied with water and nutrients, and mature algae are continuously removed at one end. The raceway ponds hold relatively low capital and maintenance costs while circular ponds are less attractive because of expensive concrete construction, high energy consumption of stirring, the mechanical complexity of supplying CO_2 and inefficient land use [10].

Though open pond systems are economical, there are several disadvantages such as low productivity and biomass yield, high harvesting cost, water loss through evaporation, limited number of species which can be grown in ponds, vulnerability to contamination and lower efficiency of carbon dioxide use [3, 6]. Temperature fluctuations due to diurnal variations are difficult to control in open ponds [3].

In Australia, China, Japan, Taiwan, India, US and Israel, open culture techniques are currently used to produce food supplements and fish feed [85]. In actual fact large-scale microalgal cultivation has mainly been focused for the production of live feed (rotifers) in aquaculture and for food additive purposes [46, 120], until the last two decades due to emphasis on renewable fuel sources, and on microalgal lipid accumulation and composition [4, 19, 121].

Laboratory results have shown the potential of the scaling-up for microalgal feedstock to replace petroleum oil-based fuels [67]. However, reproducibility of laboratory results to larger outdoor systems is hampered by constraints related to seasonal climate variations, contamination, salinity changes due to evaporation, and CO_2 supply. There is limited understanding on seasonal lipid variation or accumulation as a function of light and temperature in outdoor large-scale production.

The limitations of open pond systems lead to the development of large-scale enclosed photobioreactors (PBRs). Closed systems provide excellent reproducibility due to operational control, superior light and CO_2 utilization, minimal water loss, and lower risk of contamination [87, 122]. The structure, narrow light path, large illuminating area and relatively controllable environment, facilitate higher cell density in photobioreactors than in open pond system [123]. There are two major types of enclosed PBRs – tubular and plate types but only the tubular reactor (Fig 2.7) is used commercially and mainly for high value pharmaceutical products and not for lower value biofuel feedstocks [79]. In Germany, the closed-system PBR, with a capacity of 700 m^3, has been in operation since 1999 [124]. Table 2.2 suggests that PBR is clearly superior to open pond with the capability of producing nearly thirty times more concentrated biomass but requiring significantly less surface area at 5681 m^2.

The hurdles to using PBR for the mass production of biofuels are high expenses costs, excessive energy demand for pumping, mixing of the culture medium and cell harvesting, gradients of pH, dissolved oxygen and CO_2 along the tubes and the problems of wall growth, fouling and hydrodynamic stress [123, 125]. Techno-economic studies on open ponds, PBRs and on hybrid systems combining both open ponds and PBR systems suggest that the financial feasibility of PBRs is substantially lower than for open ponds. In the base case, average total costs of production for lipids, including financial costs, are only $12.73/gal for open tanks as compared to $31.61/gal for PBRs [126].

Innovative PBR concept with reduced energy demand, higher biomass concentration, and lower production costs can be expected in the future [127]. The use of PBR to serve as inoculum for open pond system may overcome many of the limitations. The combined use of both PBRs and raceway ponds for biomass production involves the use of high quality inoculum into a raceway pond which can be of much larger capacity, for generation of large amount of biomass with a substantially lowered risk of productivity impairment due to culture contamination [67].

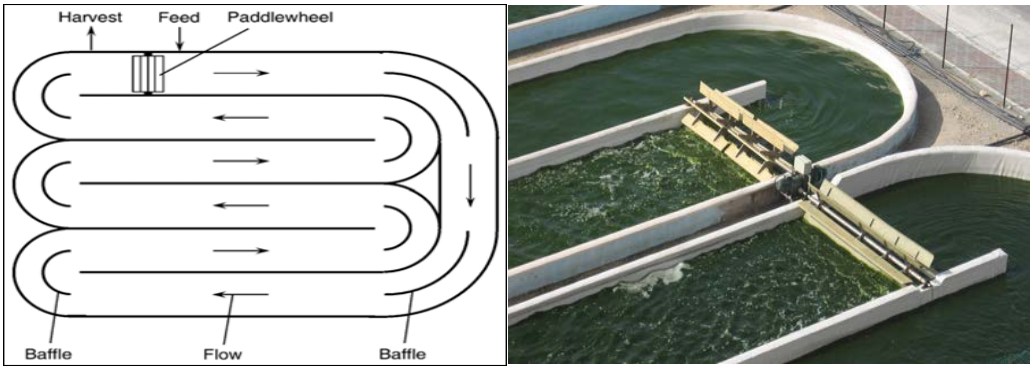


Fig. 2.6 Arial view of a raceway pond [3].

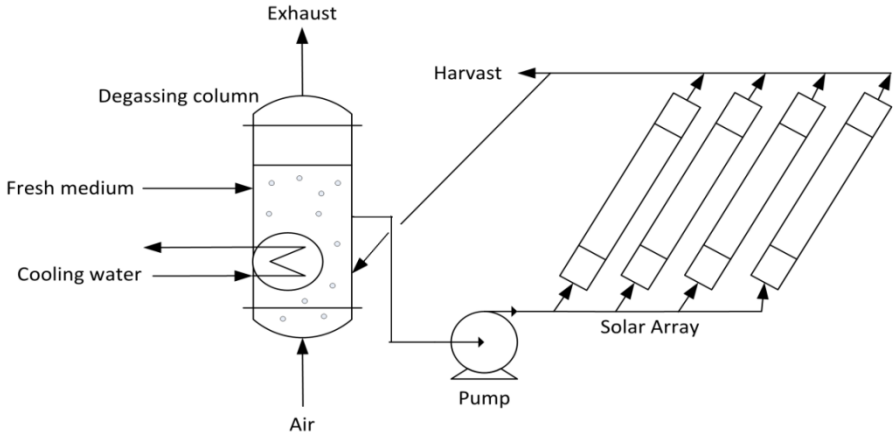


Fig. 2.7 Tubular Horizontal Photobioreactors [3]

Contamination of the raceway pond can also be minimized by adopting several strategies:

1) High ratio of inoculum to pond capacity to minimize culture time, and the associated maintenance cost. The inocula ratio can be adjusted according to strain growth rate, lipid accumulation and harvesting period.

2) Minimum growth period in the raceway pond (usually not more than 3-4 days) so as to minimize evaporation losses. Although maximum productivity is usually attained by harvesting a major fraction (50% or higher) of the pond each day, maintaining such cultures for longer time period renders the pond culture susceptible to contamination [67].

3) Resilient microalgal strain that can grow in extreme culture conditions. *Chlorella* invasion of *Spirulina* culture can be minimized by adding bicarbonate and raising pH; and amoeba grazers can be suppressed by using ammonia as the N-source [96]. *Dunaliella salina* can grow in higher salinities, *Chlorella* sp. can grow in nutrient-rich conditions and *Glaradaria sulphuria* can grow at pH 2 [128, 129].

Table 2.2 Comparison of Photobioreactor and Raceway production methods [3].

Variable	Photobioreactor	Raceway pond
Annual biomass production (kg)	100,000	100,000
Volumetric productivity (kg $m^{-3}d^{-1}$)	1.535	0.117
Areal productivity (kg $m^{-2}d^{-1}$)	0.072	0.035
Biomass concentration in broth (kg m^{-3})	4	0.14
Dilution rate (d^{-1})	0.384	0.25
Area needed (m^2)	5681	7828
Oil yield (m^3ha^{-1})	58.7	42.6
Annual CO_2 consumption (kg)	183,333	183,333
System geometry	132 parallel tubes/unit; 80 m tubes; 0.06 m tube diameter	978 m^2/pond; 12 m wide, 82 m long, 0.30 m deep
Number of units	6	8

2.4.2 Culture Harvesting and Dewatering

The economics of mass production by microalgae could be enhanced by improvements in cultivation technique and low energy, simple and yet effective downstream processing. Harvesting algae from either open pond or photobioreactor is not easy as the goal is to separate the media and algae in the quickest, most energy efficient, and cheapest possible way. Harvesting and dewatering small algal species in dilute suspensions at concentrations between less than 1 gL^{-1} (ponds) and 3–15 gL^{-1} (photobioreactors) may be energy intensive. Dewatering to about 20–30% water content is necessary to reduce volume and weight, to minimize transportation and downstream costs and to extend the shelf-life of the microalgae concentrate [130]. Depending on the type of algae, the requirements of the downstream processes, and the desired product quality, different physical, chemical, and biological methods can be applied to harvest algae: gravity sedimentation, floatation, flocculation, centrifugation, or filtration. Each has its pros and cons, as summarized in Table 2.3.

Gravity sedimentation is low energy method which allows cells to naturally settle to the bottom but may need substantial area and the downstream processes and product targets tolerant to coagulant contamination. Although some algal species do float naturally, floatation is usually induced by micro-air bubbles to lift cells to the surface [59, 131]. Flocculation starts by adding multivalent metal salts such as ferric chloride and aluminum sulfate to the media, to reduce natural negative charges between the cells making algae clumping together, thereby facilitating cell harvesting by other methods. pH changes can stimulate natural flocculation, thus avoiding the need to add metal salts [59]. Chitosan or hydrophobic absorbents with dissolved air flotation have been reported which thickens the material to 10% dry weight content (100 gL^{-1}) [130]. Sediment sludge is however more diluted than centrifugally recovered biomass that high-speed continuous centrifugation is attractive and used commercially to harvest high-value metabolites from hatcheries and nurseries in aquaculture. Centrifugation produces *g*-forces between 5000 and 10000 to quickly separate the pellet out [131]. The drawback is that strong gravitational and shear forces can damage cell structure [71], and the technology must consider large capital

investment, operating cost and high throughput processing of large quantities of water and algae [132].

Filtration by membrane, micro-pressure, or vacuum filters have proven to be the most competitive harvesting option for large-scale microalgae because of mechanical simplicity and availability in large unit sizes, but again hampered by extensive operation costs and hidden pre-concentration requirements [71]. Filtration making use of packed bed filters can be used with or without additional pressure and works best at low algal cell concentrations [131]. Cross-flow, micro, and ultra-filtrations are other methods explored with the same efficiency as centrifugation, but possibly at a much lower price [59, 64, 131].

Once the algal biomass is separated out, the next challenge is to reduce the cell water content as the biomass may have 5-15% dry solid content. The algae cells may require drying to extend the storage time and for use with the extraction process. For large-scale cultivation such as *Chlorella, Scenedesmus,* and *Spirulina*, drying is required to achieve high biomass concentrations, and the costs climb steeply with incremental temperature and duration. Common methods include sun, thermal, spray, and freeze-drying. Sun or air-drying is the cheapest and possible in low-humidity climates, but may be subjected to climatic variations, large area and considerable time. Thermal, spray and freeze drying are rapid, but are substantially more expensive. Spray drying can cause damage to the pigments and thermal drying may compromise the lipid content when heated above 60°C where the level of triglycerides may be reduced. Freeze drying on the other hand eases lipid extraction whilst retaining a high level of triglycerides [59, 133].

Table 2.3 Pros and Cons of various algal harvesting methods [59, 131].

Harvesting Method	Advantages	Disadvantages
Gravity Sedimentation	- Simple - Most common with wastewater treatment	- Only usable with large algae species (>70 μm) - Very slow
Floatation	- Can be used with flocculant - Some algae species naturally float	- Not proven in saline growth media
Flocculation	- Clump cells together to facilitate other methods easier/more efficient	- Requires addition of flocculants - Contamination of co-products by flocculant
Centrifugation	- Rapid cell separation - High harvesting efficiency - For high value products	- High cost (capital and energy)
Filtration	- Low concentrations of algae cells can be harvested from large amounts of media with low energy consumption - micro and ultra filtration to harvest small algae species (<30μm) at small scale	- Only usable with large algae species (>70 μm) - High cell concentrations require pressure filtration, increasing energy costs

2.4.3 Product Extraction and Purification

For industrial applications, the extraction solvents should be cheap, easy to remove, have low toxicity, insoluble in water, efficient in dissolving targeted components, and ideally recyclable. Organic solvent extraction is widely used for lipid extraction from traditional oilseed plants, and different extraction systems have also been tested with algae cultures [134]. The different solvents include hexane/isopropanol, hexane/ethanol [134], dimethyl sulfoxide/petroleum ether [135] and chloroform/methanol [136]. The concept of like dissolves like is the basis behind the earliest and well-known co-solvent extraction procedure [136]. To maximize extraction efficiency, the organic solvent used has to match the lipid polarity profile in the cells.

After the extraction reaction is complete, water (immiscible with chloroform) is added to the co-solvent mixture until a two-phase system develops in which water and chloroform separate into two immiscible layers. Microalgal chloroform extractables include hydrocarbons, carotenoids, chlorophylls, sterols, TAGs, wax esters, long-chain alcohols, aldehydes and free fatty acids. The methanol extractables include phospholipids and traces of glycolipids, many of which are non-lipid compounds [134]. The hexane system has been promoted because hexane and alcohol will readily separate into two separate phases when water is added, thereby improving downstream separations. There is also a need to develop a low-energy oil recovery method from the wet biomass. FAME could be produced directly from the wet biomass harvested by centrifugation but since centrifugation is a more energy intensive process, it is necessary to see its performance in the presence of residual coagulants [86, 67].

2.4.4 Wastewater Remediation

The cost of current algae production for biodiesel is approximately $3000/tonne. To remain competitive with petroleum-diesel, that cost needs to be reduced to less than $340/tonne [3] and this can be achieved by improving the medium to grow algae. While a variety of nutrient mixtures may be used as media constituents for growing algae at lab scale (250 ml to 5L), this may no longer be feasible at commercial large-scale because of economic consideration and negative environmental impact from upstream production of these nutrients [58, 128]. Wastewater is an ideal medium on which to grow mixotrophic algae, as it is abundant, renewable, low cost, and contain high levels of nutrients. Microalgae can moderate the effects of nutrient and organic rich wastewater [63], sewage effluent and industrial sources by utilizing inorganic nitrogen and phosphorus for growth, removing heavy metals, as well as some toxic organic compounds thus helping to reduce secondary pollution [137], and eutrophication in the aquatic environment. For this, microalgae must tolerate a wide variation in medium conditions (e.g. salinity, extreme pH and heat).

Some freshwater microalgae have the ability to uptake organic matter in mixotrophic metabolism in order to support photosynthesis in conditions where light penetration is limited. A locally collected algae polyculture grown on municipal wastewater removes over 99% of ammonium and phosphate levels under semicontinuous operation [138]. Microalgae have been used to metabolically sequester and remove macronutrients from eutrophic natural water bodies and industrial waste water. *Chlorella vulgaris* has been reportedly able to remove 1.5-3.5 mg L^{-1} of phosphate from waste water [139]. In wastewater treatment lagoons, microalgae are used to sequester excess nutrients and provide oxygen to aerobic microbes to lower biochemical oxygen demand (BOD) [140-142]. The different wastewater sources tested for algal remediation and lipids for biofuel including municipal [138, 141, 143, 144, 145], dairy [132, 138] and carpet mill [142] wastewaters (Table 2.4). Over 75% reduction in nutrient levels have been reported with the lipid content of 29% on dairy wastewater and 25.25% on municipal wastewater [138, 143].

Some studies report 50% N level reduction and nutrient removal of 96-99% after only three days [138, 142, 144]. As there is no standard growth and measurement procedure established, the studies have to be interpreted bearing in mind the different growth conditions and rate of removal.

Table 2.4 Nutrient removal and lipid production of algae growing on wastewater.

Algae Species	Wastewater media	Nutrient Removal (%)				Growth Rate	Lipid Production (%)	References
		NH_4-N	PO_4-P	Total N	Total P			
Polyculture	Municipal	> 99	> 99				11.3	[138]
	Dairy	96	> 99					
C. vulgaris	Municipal			50% [c]				[144]
Polyculture	85-90% CM[a] 10-15 % Municipal	99.7-99.8	98.8-99.1			9.2-17.8 ton/ha/yr	6.82	[142]
S. obliquus	Municipal			100%	98%	0.0438/hr		[146]
C. vulgaris	Municipal	60.1	80.3					[141]
S. obliquus		100	83.3					
C. vulgaris	Municipal	100 [b]	36 [b]					[145]
C. sorokiniana		100 [b]	36 [b]					
C. reinhardtii	Municipal			55.8 $mgL^{-1}d^{-1}$	17.4 $mgL^{-1}d^{-1}$	2.00 $gL^{-1}d^{-1}$	25.25	[143]

[a]CM – Carpet Mill wastewater.

[b]Levels achieved when cultured with growth-promoting bacterium *Azospirillum brasilense* Cd.

[c]50% reduction achieved after three days of growth.

2.4.5 CO_2 Enrichment

Among various methods for CO_2 reduction (absorption, adsorption, chemical fixation, etc.), biological CO_2 fixation by plants and photosynthetic microorganisms has attracted much attention because it leads to the production of biomass energy. For photosynthetic organisms, water, nutrients and carbon dioxide are vital to growth, but the low atmospheric CO_2 concentration may limit the growth of these organisms. Increased CO_2 capture by terrestrial plants is however expected to remove only 3–6% of fossil fuel emissions, largely due to the slow growth rates. Thus a cheap source of CO_2 to fuel the photosynthetic process is needed [147]. Biological CO_2 fixation by algal photosynthesis has been proposed as an economically feasible method [148]. Macroalgae have the ability to fix CO_2 and capture solar energy with efficiency 10 to 50 times greater than that of terrestrial plants. Microalgal growth rate is approximately 50 times higher than the terrestrial plants during exponential growth, having higher photosynthetic efficiencies with more proficient carbon capture [62, 149].

Flue gases from industrial plants are capable of being used as a feed to phototrophic microalgae and do not harmfully affect algal growth [150]. The use of microalgae as a carbon sequestrator is only considered feasible if they are used as biofuel feedstock rather than merely as a carbon sequester. The desired microalgae strains should have the following characteristics: 1) high growth rate and biomass productivity; 2) high tolerance to trace amount of acidic components from flue gases such as NOx and SOx; and 3) able to sustain growth even under extreme culture conditions (high temperature of water due to direct introduction of flue gases) [59]. Species that are suitable include *Chlorella, Emiliania huxley, Spirulina platensis, Nannochloropsis sp*, and *Phaeodactylum* [150].

There are specific parameters to be optimized such as how efficiently the microalgae could use CO_2 to avoid release of excess CO_2 into the atmosphere, and the design of effective closed and open system and irrigation, planting, fertilization and harvesting. Open system may contain various species of microalgae with different utilization efficiencies and may directly release excess CO_2 into the atmosphere, but closed systems allow easy control of the CO_2.

If the purpose of algae cultivation is to sequester industrial CO_2 outputs, it is important to consider operation during night time and cloudy days when algae slow down reproduction rate and take up less CO_2. This may require installation of gas storage facilities to cope with the influx of CO_2 accumulation at night. The challenge of limited availability of land for large scale microalgal CO_2-capturing from industrial or power plants have to be overcome by sophisticated area-efficient techniques to recycle CO_2 [151].

Solar energy-driven CO_2 fixation technologies using macroalgae have been utilized to convert CO_2 in the stack gas from thermal power station. Since flue gases from industries such as steel-making plants and thermal power stations contain about 500 times higher concentration of CO_2 [10-20 % (v/v)] than that in the air, selection and screening of suitable algal strains having tolerance to high CO_2 concentration must be carried out [3]. There may be algal growth inhibition by high CO_2 concentration, that there is a need for large amount of nutrients such as N and P due to low CO_2 conversion and short gas retention time. A pilot scale system is successfully developed to culture microalgae using industrial flue gases where *Scenedesmus obliquus* tolerates high CO_2 concentration up to 12% (v/v) with optimal removal efficiency of 67% [152]. CO_2 tolerance of *Chlorella vulgaris* is enhanced by gradual increase of CO_2 concentration, while *S. obliquus, Chlorella kessleri* and *Spirulina* sp. show good tolerance to high CO_2 contents (up to 18% CO_2). CO_2 consumption rate of 549.9 $mgL^{-1}day^{-1}$ is achieved for a maximum *S.obliquus* biomass productivity and lipid productivity of 292.50 $mgL^{-1}day^{-1}$ and 78.73 $mgL^{-1}day^{-1}$ (38.9% lipid content) respectively, in two-stage cultivation with 10% CO_2 [153].

2.5 Algal Metabolic and Catabolic Pathways

2.5.1 Carbohydrate

The most common source of metabolic energy is sugar among living organisms. Sugar catabolic pathways are active mainly during the dark phase of the light–dark cycle. These pathways are responsible for producing NAD(P)H and other biosynthetic metabolites involved in the normal cellular functions. The main enzymes involved in the sucrose synthesis are sucrose-phosphate synthase (sps) and sucrose-phosphate phosphatase (spp) [154]. The major route of glucose degradation is the oxidative pentose phosphate pathway (OPP) cycle and is considered as the main CO_2 fixation mechanism in cyanobacteria.

The key enzymes in the oxidation of glucose-6-phosphate (G6P) through the OPP cycle are G6P dehydrogenase and 6-phosphogluconate dehydrogenase. G6P dehydrogenase controlled at the level of gene expression is important in regulation. In addition, low RuBP levels significantly reduce G6P dehydrogenase activity [155]. The enzyme ADP-glucose diphosphorylase (AGPase encoded by the agp gene, also known as glgC) controls glycogen synthesis in bacteria in an ATP-dependent reaction and seems to be regulated by 3PG (activator) and Pi (inhibitor) [156].

$$G1P + ATP \leftrightarrow ADP - Glc + PPi$$

2.5.2 Lipid Metabolism

Photosynthetic reactions not only provide a carbon source but also generate reducing power (NADH and NADPH) and energy (ATP) for fatty acid synthesis. Fatty acids are the building blocks of many cellular lipids including triacylglycerols. Fig 2.8 shows fatty acid biosynthesis in plants. Fatty acid and protein biosynthetic pathways possess phosphoenolpyruvate (PEP) as common substrate. Thus, when PEP is converted to oxaloacetate (OAA) by PEP carboxylase (PEPC), it enters into protein

synthesis and is directed to fatty acid synthesis when transformed to malonyl-CoA. PEP is converted to pyruvate by pyruvate kinase and then by pyruvate dehydrogenase in a second reaction to form acetyl-coenzyme A (acetyl-CoA). Pyruvic acid can be converted to alanine and thus participates in protein metabolism [157]. Acetyl-CoA can be converted to malonyl-CoA in a rate-limiting reaction catalysed by acetyl-CoA carboxylase, which is the first step towards fatty acid synthesis.

High concentrations of acetyl-CoA or free fatty acids stimulate PEPC activity in *E. coli*. In certain cyanobacterial strains, the increased levels of acetyl-CoA do not influence PEPC activity. PEPC from *Synechococcus vulcanus* is strongly activated by fructose-1, 6-diphosphate, while aspartate acts as a strong suppressor and has been reported to reduce the PEPC activity from *Coccochloris peniocystis.* In *Synechococcus sp.* PEPC seems to divert the carbon flux away from fatty acid biosynthesis that the antisense expression of PEPC-coding gene (ppc) leads to increase lipid content [158].

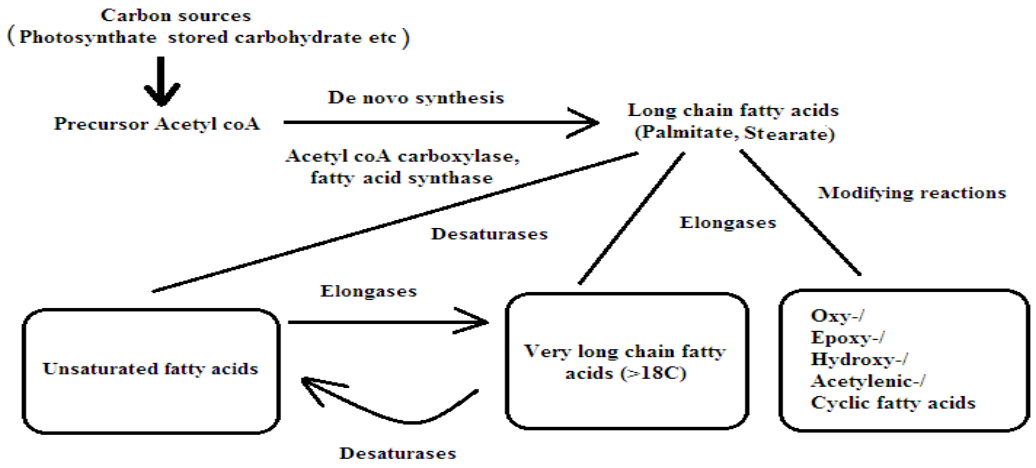


Figure 2.8 Fatty acid biosynthesis in plants [159].

Microalgae can fix CO_2 into sugars using energy from the sun, and fixed sugars are further processed to produce acetyl-CoA, with more than one pathway contributing to maintain the acetyl-CoA pool. Acetyl-CoA from photosynthesis serves as the precursor for fatty acid synthesis in the chloroplast. The first committed step catalyzed by a multifunctional enzyme complex, acetyl CoA carboxylase (ACCase)

which produces malonyl-CoA from acetyl CoA and bicarbonate. Before being used by the fatty acid synthase machinery, the malonyl group is transferred from CoA to ACP (acetyl carrier protein) catalyzed by a malonyl-CoA: acyl carrier protein malonyltransferase. For most algae species, the final acyl chains emerging from the chloroplast are 16- or 18- carbons in length. These 16- or 18-carbon fatty acids are formed by a series of two-carbon chain elongating reactions catalyzed by a multi-subunit fatty acid synthase (FAS) assisted by stoichiometric amount of ATP, acetyl CoA and NADPH for each two carbons added to the growing acyl chain. The chain-elongating reaction is terminated by acyl-ACP thioesterase (FAT) whose specificity determines the final chain length. The released free fatty acids can move across the plastid envelop membrane where they are esterified to CoA via another enzymatic reaction catalyzed by long chain acyl-CoA synthases (LACS) [160].

Studies on algal lipid biochemistry have shifted from a few model organisms to a much larger number of unusual algae. This has led to the discovery of new compounds, including major membrane components, elucidation of lipid signalling pathways, and genes that code for proteins involved in the production of long-chain PUFA such as arachidonic, eicosapentaenoic and docosahexaenoic acids. Early work on fatty acid biosynthesis has been made on *C. vulgaris* and this has moved towards elucidating the metabolism and functions of acyl lipids. Biosynthesis of glycerolipids has been investigated in *Chlorella kessleri* with special emphasis on the fatty acid distribution at the *sn*-1 and *sn*-2 positions in membrane lipids [161]. Lipids, in the form of triacylglycerides, typically provide storage function in the cell that enables microalgae to endure adverse environmental conditions. Essentially algal biomass and triacylglycerides compete for photosynthetic assimilates and a reprogramming of physiological pathways is required to stimulate lipid biosynthesis [161].

2.5.3 Carotenoids

Carotenogenesis pathways and their enzymes are mainly investigated in cyanobacteria and terrestrial plants among oxygenic phototrophs. Algae have common pathways with terrestrial plants, but also additional algae-specific pathways which are solely

proposed based on the chemical structures of carotenoids. Some common carotenogenesis genes in algae are suggested from homology of the known genes [162], but most genes and enzymes for algae-specific pathways are still unknown.

All carotenoids in oxygenic phototrophs are dicyclic carotenoids: β-carotene, α-carotene and their derivatives, and are all derived from lycopene except for myxol glycosides and oscillol diglycosides in cyanobacteria which are monocyclic and acyclic carotenoids, respectively. Lycopene is cyclized into either β-carotene through γ-carotene, or α-carotene through γ-carotene or δ-carotene. Three distinct families of lycopene cyclases have been identified in carotenogenetic organisms. One large family contains CrtY in some bacteria except cyanobacteria, and CrtL (CrtL-b, Lcy-b) in some cyanobacteria and plants. Lycopene ε-cyclases (CrtL-e, Lcy-e) from plants and lycopene β-monocyclases (CrtYm, CrtLm) from bacteria are also included. Their amino acid sequences exhibit significant five conserved regions and have an NAD (P)/ FAD-binding motif [162].

Isopentenyl pyrophosphate (IPP), a C5-compound, is the source of isoprenoids, terpenes, quinones, sterols, phytol of chlorophylls, and carotenoids. There are two known independent pathways of IPP synthesis: the classical mevalonate (MVA) pathway and the alternative, non-mevalonate, 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway. In the MVA pathway, acetyl-Coenzyme A is converted to IPP through MVA, and the enzymes and genes are well studied. The pathway is found in plant cytoplasm, animals and some bacteria. The DOXP pathway found in the 1990s, proposes that pyruvate and glyceraldehyde are converted to IPP [163]. Three enzymes from *Rhodophyta*, *Cyanidioschyzon merolae*, and *Dunaliella salina* and *Haematococcus pluvialis* of Chlorophyceae are functionally confirmed. Under stressful environment such as high light intensity, UV irradiation and nutrition stress, some Chlorophyceae such as *Haematococcus*, *Chlorella* and *Scenedesmus*, accumulate ketocarotenoids, canthaxanthin and astaxanthin, which are synthesized by combining CrtR-b and β-carotene ketolase (CrtW, BKT) [164].

2.5.4 Metabolic Flux Analysis

A deeper understanding of the interactions between biochemical energy, carbon fixation and assimilation pathways through mathematical modeling could be a valuable tool to evaluate maximum theoretical product yield and to help developing improved strategies. Metabolic flux analysis (MFA) is a powerful methodology for the determination and quantification of metabolic pathway and network fluxes. Network analysis can direct microbial development efforts towards successful strategies and enable quantitative fine-tuning of the network for optimal product yields while maintaining the robustness of the production. The intracellular fluxes are calculated by using a stoichiometric model (metabolic pathway map) for the major intracellular metabolic reactions, the extracellular fluxes and the known internal fluxes as input and the metabolic fluxes for the rest of intracellular reactions under pseudo-steady state condition are calculated by means of flux balance analysis (FBA) [165]. Genome-scale model-driven algal metabolic design promises to facilitate optimization by directing the utilization of metabolites in the complex, interconnected metabolic networks. The application of such analytical approaches to algal systems is limited to date, although metabolic network analysis can improve understanding of algal metabolic systems and play an important role in expediting the adoption of new biofuel technologies [166].

Microbial pathway engineering has traditionally been applied mainly to industrial processes for biosynthesis of products of high economical value. The aim of accurate quantification of pathway fluxes is to convert as much substrate to a desired metabolic product via strain improvement. There have been several initiatives towards development of metabolic flux models for different organisms in order to gain quantitative information about metabolic physiology of the cultures [167].

2.6 Summary

A general description of microalgae and microalgal characteristics and classes with the bioenergy and biochemicals from microalgae are presented. Engineering

considerations for microalgae like growth conditions, cell productivity, tolerance to extreme culture conditions, abiotic requirements and nutritional requirements and comprehensive discussion of reactor engineering and mode of operation are reviewed. The current trends in process analytical technologies from perspectives of culture harvesting and dewatering, product extraction and purification, wastewater remediation and CO_2 enrichment are covered. Finally a brief review of the algal metabolic and catabolic pathways concludes the chapter.

CHAPTER 3

METHODOLOGY

3.1 Materials

Four species of microalgae (*Nannochloropsis oculata*, *Pavlova lutheri*, *Isochrysis galbana* and *Tetraselmis suecica*) used in the present study, were collected from Fisheries Research Institute, Pulau Sayak, Sungai Petani, Kedah, Malaysia. Raw POME was collected from Oil, Palm Mill, FELCRA Nasaruddin Bota, Perak, Malaysia and stored at 4°C. All chemicals and solvents were obtained from Merck (Darmstadt, Germany). The instruments used were Stirring Hot Plate (Fisher Scientific, 1110250SH), Lux Meter (LX1330B), Ultrasonic Homogenizer (150/VT), Centrifuge, Rotary Evaporator, Microscope (10 x 40 MAG), Gas Chromatography (7890A GC System), COD Thermoreactore (DRB 200), Spectrophotometer (HACH DR 500), BOD track, Photobioreactor (Biosys 7-L x 6 Plus), Autoclave (75 X), the Deionized Water System (Thermo Scientific, TK Japan), Nano Photometer (IMPLEN) and PCR (MJ Mini Thermal Cycler BIORAD) (Appendix A).

3.2 Cultivation of Microalgae

3.2.1 Glassware and Tubing

Borosilicate glassware and tissue culture-grade polycarbonate or polystyrene plasticware were used exclusively for all glassware, including stock bottles, beakers, and cultural tubes and flasks. Teflon and plastics were used to reduce breakage. All containers and tubing used for cultures and media stocks were carefully selected to avoid toxic compounds. Teflon-lined caps were autoclaved, because new caps may

release toxic phenolics when heated [104]. Likewise, rubber stoppers were autoclaved separately from media because they may release volatile compounds when heated.

3.2.2 Preparation of Sea Water and Storage

Filtered seawater was sterilized by autoclaving for 20 minutes at 121°C and 15 psi. After autoclaving, the media was left for 2-4 hrs to allow gases such as CO_2 to diffuse into the medium. To avoid precipitation during autoclaving, 1.44 mL of 1N HCl and 0.12 g of $NaHCO_3$ per liter were added. These indirectly lower the pH, which help to reduce precipitation during autoclaving [104]. CO_2 may be added directly by bubbling the medium before autoclaving. The seawater was cooled quickly after autoclaving to room temperature, which also helped to prevent precipitation. Filtered seawater was stored in either glass or plastic bottles, often 5 liters for ease of handling. New containers were bleached for 24 hrs with diluted (i.e., 10%) HCl and then rinsed thoroughly before use. The seawater was kept cool (cold room) and in the dark.

3.2.3 Culture Media Preparation

The chemical composition of Conway (AQUACOP) and TMRL [102, 168] solutions are given in Appendix B1 and B2. For cultivation of microalgae, 10% (v/v) of inocula was added into Conway media and TMRL Enrichment Medium. The culture media was prepared by mixing various nutrients in sea-water filtered through 0.22 to 1 micron. All media constituents were added aseptically after sterilization [104, 169].

3.2.4 Culture Maintenance

Culture maintenance was carried out with prudence to produce good quality algae. A total of five test tubes were maintained for each strain at a time but with different duration ranging from 0-week old strain to 3-week old as shown in Table 3.1.

Inoculation of strains was done under sterile conditions. Each tube was filled with about 20 ml of media solution and 2-5 ml of inoculums was transferred from a week old strain tube to new strain tubes.

Table 3.1 Culture maintenance in tubes and its importance

Age of strain	No. of test tubes	Importance
0-week	2	Newly innoculated
1-week	1	Strain maintained for 1 week and as back-up in case 0-week strain fails to grow
2-week	1	Strain growth observed for 2 weeks and as back-up for failure 1 week strain
3-week	1	As above and as back-up for 2 week old strain

Cultures were subcultured on a fortnightly basis, and grown on an orbital shaker at 80 rpm, at 28 ±2°C, under 24 h illumination of 90 µmol photons m^2 s^{-1} intensity from white fluorescent tube (Philips).

3.3 Batch Cultures

3.3.1 Static Culture in 250 ml Erlenmeyer Flasks

Sterile 250 ml Erlenmeyer flasks were used in all cultures. The flasks were filled with the specific culture media and above 120 ml of culture medium were poured into each flask aseptically. For cultivation, 10% (v/v) of inocula was added. The shake flasks were kept static on the illuminated shelf for 16-18 days to allow the algae to increase in density. Aeration was not provided for this culture.

3.3.2 Culture in 1 L and 5 L Containers

The 1-L and 5-L plastic containers were filled to two-thirds of their volume with microsterilized seawater. The plastic containers were disinfected by dilute solution of Chlorox (40 ppm) and dried in oven at 60°C for 30 mins. Aeration was provided non-aseptically and air was enriched with CO_2 to about 5%. Plastic containers were covered with plastic caps pierced with two holes for the air inlet and outlet. Nutrients were added at the time of inoculation and plastic containers were inoculated with 8-11 days old culture from 250 mL or 1-L flask, respectively. The plastic containers were kept on the illuminated shelf.

3.3.3 Culture in 30 L Spherical Fiber Glass Tanks

Thirty liter spherical fiber glass tanks were disinfected with Chlorox (40 ppm) and filled with 25-27 liters microfiltered seawater. Media enrichment was made at the time of inoculation and tanks were inoculated with 8-11 days old stock cultures from 5-L containers. Polyvinyl chloride (PVC) distribution pipes were fixed with a channel to allow condensing water to be purged. The distribution of air into the culture tanks was made through 6 mm diameter glass tubes and the air was enriched with CO_2 to about 5%.

3.3.4 Culture in 300 L Spherical Fiber Glass Tanks

A cylindrical tank with conical bottom was used for 300-L open tank cultivation. The tank was made from translucent 1 mm fiberglass sheet. The tanks were shaded with green sheets 4 m above to prevent overheating during long periods of strongest sunlight irradiation. Tanks were washed and filled with chlorinated hot water overnight, and thoroughly rinsed before use. The tanks were then filled with microfiltered seawater and disinfected by chlorination. Chlorox was used as the disinfectant at 10-20 ppm concentration and stirred for even distribution.

The oxidizing agent is often applied to situations where autoclaving is not practical, as in the case of large volume cultures. After 24 hours, chlorox was neutralized by the addition of sodium thiosulfate. Aeration was provided and air was enriched with CO_2 to about 5%. Media enrichment was made at the time of inoculation and 300 L tanks were inoculated with 8-11 days old stock cultures from 30 L tanks. Cultures were grown for 14-16 days and samples were taken from the top, bottom and middle of the tanks to ensure representative sample for analyses.

3.3.5 Culture in 5 L Photobioreactor

The vessels of bioreactor were filled to two-thirds of their volume with microsterilized seawater. The vessels of photobioreactor were disinfected by dilute solution of Chlorox (40 ppm). Aeration was provided aseptically by using air filters. Lighting system was in the form of external source of light supplied by white florescence tubes (Philips). Motor was aligned and connected to stirrer shaft coupling of the vessel. The preferred parameters were adjusted by SETPOINT. The sampling was done by sampling tube and the cultivation period was 14-16 days.

3.4 Cell Growth and Kinetics Studies

3.4.1 Cell Density

The growth of microalgae was measured through counting the number of cells by haemocytometer. Cell density was monitored using a haemocytometer. It contains a quantitative grid and is designed to be used with a microscope to determine bodily cell counts. When the required number of days of algae growth was passed, flasks were removed from the incubation tray and the flasks were swirled to ensure even distribution of algae throughout and then sample was lifted approximately 10μL by using capillary dropper. Sample was then transferred to the filling slide chamber and examined under high power microscope (10 x 40 MAG).

3.4.2 Determination of Cell Dry Weight (DW)

100 ml of algal suspension was filtered through a pre-dried and pre-weighed glass micro fiber filter (Whatman GF/C 0.47µ). The biomass was washed with de-mineralized water and dried at 105^{o}C in oven overnight, cooled in a desiccators and dry weight was measured. The formulations are as follows:

$$\text{Dry weight} = DW_A - DW_C / V \qquad (3.1)$$

where DW_A is avgrage dry weight retained on algal filter, DW_C is average dry weight retained on control filter and V is volume used.

3.4.3 Kinetics of Cell Growth in Batch Culture

For the scale-up of a production process, a number of batch culture experiments were performed to yield essential data like maximum biomass formation rate, specific growth rate and doubling time. The rate of increase in cell dry weight is proportional to the concentration of cells initially [170]. Cells undergo lag, exponential, stationary and death phase as shown in Figure 3.1. Calculation of kinetics parameters are shown in Table 3.2.

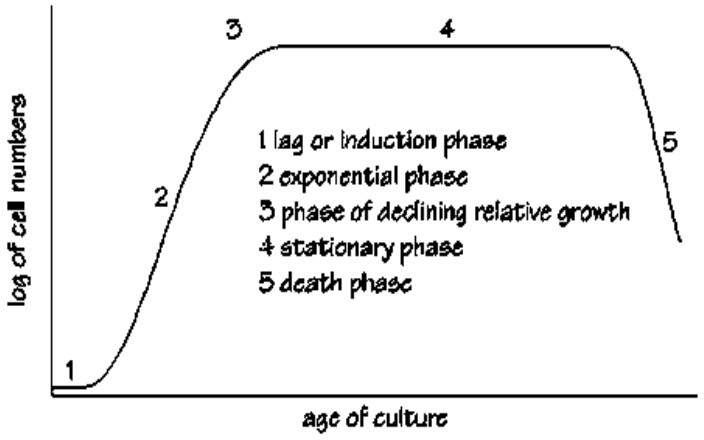


Figure 3.1 Growth phases of microalgal cultures [171].

Growth rate refers to the rate of increase in algal cell concentration, X with time as measured in batch culture:

$$r = dX/dt \qquad (3.2)$$

The specific growth rate, μ (day^{-1}) is the growth rate normalized by tha algal biomass concentration, X:

$$\mu = \frac{r}{X} = \frac{dX}{dt}\left(\frac{1}{X}\right) \qquad (3.3)$$

Equation (3) can be integrated to give:

$$X_t = X_o e^{\mu t} \qquad (3.4)$$

where X_o and X_t are initial and final cell concentrations, respectively.
The maximum specific growth rate, μ_{max} is the value of the specific growth rate that is measured during the exponential growth phase.

$$\frac{dX}{dt} = \mu_{max} X$$

$$\frac{dX}{dt} = \int_{x_1}^{x_2} \frac{1}{X} dX = \mu_{max} \int_{t_1}^{t_2} dt$$

$$ln\frac{X_2}{X_1} = \mu_{max}\,(t_2 - t_1) \qquad (3.5)$$

$$\mu_{max} = \frac{ln2}{t_d}$$

$$t_d = \frac{ln2}{\mu_{max}} \qquad (3.6)$$

The cell dry weight doubles from X_o and $2X_o$ at regular intervals, t_d known as doubling time.

Table 3.2 Calculation of kinetics parameters

Kinetics Parameters	Method of Calculation
Maximum biomass formation rate X'_{max} (g L^{-1} d^{-1})	$X'_{max} = \frac{\ln X2/X1}{t2-t1}$ X_2 = dry weight (g L^{-1}) at day 14 X_1 = dry weight (g L^{-1}) at day 4 t_2 = day 14 ; t_1 = day 4
Maximum specific growth rate μ_{max} (d^{-1})	$\mu_{max} = \frac{\ln X2/X1}{t2-t1}$ X_2 = No. of cells (millions cells mL^{-1}) at day 14 X_1 = No. of cells (millions cells mL^{-1}) at day 4 t_2 = day 14 ; t_1 = day 4
Doubling time t_d (day)	$t_d = \frac{\ln 2}{\mu max}$ $= \frac{0.6931}{\mu max}$
Biomass yield $Y\ x/s$ (g g^{-1})	$Y\ x/s = \frac{Xm - Xi}{S}$ X_m = Maximum cell concentration (g L^{-1}) at day 14 or 16 X_i = Initial cell concentration (g L^{-1}) at day 2 or 4 S = Concentration of nitrate (g L^{-1}) as a substrate
Lipid yield $Y\ p/s$ (g g^{-1})	$Y\ p/s = \frac{Pm - Pi}{S}$ P_m = Maximum lipid content (g L^{-1}) at day 14 or 16 P_i = Initial lipid content (g L^{-1}) at day 2 or 4 S = Concentration of nitrate (g L^{-1}) as a substrate

3.5 Harvesting and Lipid Extraction

Microalgae harvesting is referred to the process where fairly diluted microalgae have been concentrated until slurry or paste is acquired. In this experiment, cells were harvested after 14-16 days by centrifugation at 3500 rpm for 10 min.

The sample subjected to centrifugal forces and makes the solid particles separated from liquid supernatant. Large scale culture samples were flocculated by using appropriate quantity of alum. The lipid content analysis was conducted based on Bligh and Dyer method [136]. 200 ml of sample was taken, homogenized by using sonicater and centrifuged at 3500 rpm for 10 minutes. The pellet generated from this centrifugation was put into flasks containing distilled water, methanol, and chloroform with ratio 4:10:5. After overnight, 5 ml of distilled water and 5 ml of chloroform was added (final ratio 9:10:10) and the mixture was centrifuged again. The lower layer in the centrifuge tube that contained lipid and chloroform was extracted and put into pre-weighed vials using a dropper. All vials were placed into a water bath at 65°C until 1/3 left and put in an oven at 80 °C for 4 hours to evaporate the chloroform and methanol. The % extracted lipids from chloroform finally calculated. Soxhlet apparatus was also used for the extraction of lipid from dried algae. The dried percentage of lipid is calculated as follows.

$$\text{Lipid Content Analysis (\%)} = \frac{W2 - W1}{Wd} X\ 100 \qquad (3.7)$$

Where w_1 is previously weighed glass vessel, w_2 is weight of vessel alongwith lipid content and w_d is dry weight of algae.

3.5.1 Fatty Acids Analysis

The extracted lipid was first transesterified into fatty acid methyl esters (FAME) [172], where 20 mg of lipid sample was mixed with 2 ml of toluene, followed by addition of 2 ml of 1.5% of sulphuric acid in dry methanol. After mixing well, the mixture was incubated at 55^{o}C overnight. Four ml of saturated NaCl solution was added, vortexed and 2 ml of hexane (HPLC grade) added, followed by 3 ml of sodium hydrogen carbonate (2 % $NaHCO_3$). The mixture was vortexed and 180 µl of the upper phase was taken for gas chromatography analysis.

FAME were separated and quantified using gas chromatography (7890A GC System), and separation achieved by Supelcowax TM 10 fused silica capillary column (60 m x 0.32 mm x 0.25 μm film thickness; USA). The carrier gas was helium at 550 kpa. The temperature programme was as follows: initial column oven temperature of 100 ^{o}C held for 3 min, and increased to 170 ^{o}C at 20 ^{o}C/min for 0 min and 10 ^{o}C/min for 25 min. The detector temperature was kept constant at 280 ^{o}C and run time was 40.5 min.

3.6 Effects of Growth Factors

The effects of pH, salinity, photoperiod, nitrate, phosphate and iron on the biomass and lipid content of the four microalgal species were measured by using TMRL Enrichment medium. The maximum biomass formation rate, maximum specific growth rate and doubling time were also measured in response to different treatments of abiotic factors and nutrients. This study was conducted as batch cultures, cultured species were harvested at their stationary phase (16-18) days and subjected to analysis of dry weight and lipid content. The growth of the four microalgal species was measured using the Haemocytometer and the mean values from the three replicates were recorded for each of the treatments.

3.6.1 Effects of pH and Salinity

Unless stated otherwise, the standard conditions for culture were 24 hrs illumination, 30 ppt NaCl and initial pH 7.5. The effects of pH studied were pH 5, 6, 7, 8, 9 and 10.The effects of salinity were tested at 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 ppt of NaCl. For RSM, the medium was tested at pH 6, 7, 8 and 9 and salinity at 25, 30, 35 and 40 ppt of NaCl.

3.6.2 Effects of Photoperiod and Light intensity

All cultures were subjected to photoperiod of 6, 9, 12, 15, 18, 21 and 24 hrs and photon flux density of 66, 90, 130, 160, 198 and 250 µmol photons $m^{-2}s^{-1}$. Light intensity was supplied from white fluorescent tube (Philips). Culture was grown for 16-18 days, and removed at 3–4 days interval, and subjected to analysis of cell no., dry weight and lipid. Light intensity of 165–250 µmol photons $m^{-2}s^{-1}$ was supplied to 300-L by shaded sunlight. For RSM, the effects of photoperiod tested were 10, 14.7, 19.3 and 24 hours illuminations and the effects of light intensity tested were 90, 126, 162 and 198 µmol photons $m^{-2}s^{-1}$ where the cultures were grown at 30 ppt NaCl and initial pH 7.5. Light intensity was measured by Lux Meter (LX1330B).

3.6.3 Effect of Nutrient Deficiency Conditions

All four microalgal strains were subjected to nutrient stress under different nutrient conditions. Nitrate concentrations were tested at 0, 10, 40, 70,100,120 and 150 g L^{-1} KNO_3, phosphate concentration were tested at 0, 1, 4, 7, 10, 12 and 15 g L^{-1} $Na_2HPO_4.12H_20$ and iron at 0, 0.5, 1, 2, 3, 4 and 5 g L^{-1} $FeCl_3.6H_2O$. Culture was grown for 16-18 days, and removed at 3–4 days interval, and subjected to analysis of cell no., dry weight and lipid content. For RSM, nitrate concentration were tested at 10, 65 and 120 g L^{-1} KNO_3, phosphate concentration at 3, 7.5, 12 g L^{-1} $Na_2HPO_4.12H_20$ and iron at 1, 2.5 and 4 g L^{-1} $FeCl_3.6H_2O$ at standard culture conditions.

3.6.4 Response Surface Methodology

The culture conditions for biomass and lipid production were optimized by RSM based on multilevel factorial design. Experimental design, mathematical modeling and optimization were performed by using Statgraphics Centurion Version XVI. The experiments were performed independently based on multilevel factorial experimental

plan with seven conditions (i.e., photoperiod, light intensity, pH, salinity, nitrate, phosphate and iron).

Multilevel factorial design of 16 experimental runs were carried out, to optimize two independent variables: pH (x_1) and salinity (x_2); and photoperiod (x_3) and light intensity (x_4), with all possible combinations of values for each experimental factor at low level (x_1 = 6 h, x_2 = 25 ppt; x_3 = 10, x_4 = 90 µmol photons $m^{-2}s^{-1}$); and high level (x_1 = 9 h, x_2 = 40 ppt; x_3 = 24, x_4 = 198 µmol photons $m^{-2}s^{-1}$). Multilevel factorial design of 27 experimental runs were also carried out, to optimize three independent variables of nutrient deficiency conditions: nitrate (x_1), phosphate (x_2) and iron (x_3) with all possible combinations of values for each experimental factor at low (x_1 = 10 g L^{-1}, x_2 = 3 g L^{-1}, and x_3= 1 gL^{-1}) and high (x_1 = 120 g L^{-1}, x_2 = 12 gL^{-1}, and x_3= 4 g L^{-1}) levels. All evaluated levels were codified and combined in different experimental runs. Each experiment was run in duplicate in order to estimate experimental error, and carried out in randomized order to minimize the error.

A second-order polynomial regression model was used to predict the optimal point for both responses:

$$y = \beta 0 + \sum_{i=1}^{x} \beta ixi + \sum_{i=1}^{x} \beta ixi^2 + \sum_{i=1}^{x} \sum_{j=i+1}^{x} \beta ijxixj \qquad (3.8)$$

where y is the response variable, x_i and x_j are the independent coded variables, k is the number of independent values, ε is the random error, β_0, β_i, β_{ii}, β_{ij} are constant coefficient, coefficient of linear, interaction regression and quadratic term, respectively. The goodness of fit of the model was evaluated by the analysis of variance (ANOVA). The quality of fit of the polynomial model equation was expressed by the coefficient of determination, R^2. The model terms were selected or rejected based on the p-value with 95% confidence level ($p< 0.05$). The simultaneous interaction of the independent variables was investigated by constructing the response contour and surface plots and standardized Pareto charts for the interactive effects of photoperiod, light intensity, pH, salinity, nitrate, phosphate and iron.

3.7 Preparation of POME Medium

POME was filtered to remove sand and dust particles and then centrifuged (Avanti J-251 Centrifuge). The supernatant of the effluent which contains nutrient was taken for algal culture and the pellet was discarded. The supernatant was diluted with sea water to make 1%, 5%, 10%, 15% and 20% concentration levels and autoclaved at 121 ^{o}C for 30 minutes to eliminate bacterial and other contaminations. pH level of POME medium was adjusted to pH 7–8 and filtered again before use.

3.7.1 Chemical Analyses of POME

3.7.1.1 Chemical Oxygen Demand (COD)

COD measurement was carried out by using spectrophotometer DR2800 and 5000-Reactor Digested Method according to the standard method provided by HACH. The DR5000-Reactor was preheated to 150 ^{o}C. 1 ml of sample was diluted at ratio 1:50, 1:100, 1:250 of POME and distilled water, respectively. Diluted POME 2.0 ml of each standard was added to the corresponding high range COD Digestion Reagent vials. In the case of the "blank" 2 ml of distilled water was added. Each vial was mixed well and placed into the reactor block for two hours. After two hours, the vials are removed from block to a cooling rack for 20 minutes before reading. The stored HACH program 435 COD HR was recall for COD test. The blank vial was placed in the cell holder with the lightshied closed and set as zero. Then, the sample vial was placed in the cell holder for the test with the reading of COD in mg L^{-1} was displayed on the screen [173].

3.7.1.2 Biological Oxygen Demand (BOD)

Measurement of BOD with BOD track was carried out according to Standard Method provided by HACH. 1 ml of sample was diluted at ratio 1:100 and 1:250 of POME and distilled water, respectively.

The sample (95ml) was poured into the specialized 300 ml BOD trak designed to allow full filling with no air space and provide sample bottle an airtight seal are used. Four samples were prepared and 3.8 cm (1.5in) magnetic stir bar was placed in each sample bottle. BOD Nutrient Buffer Pillow was added to each sample and Lithium hydroxide Powder was added to the seal cup of each sample bottle. The instrument was placed in the incubator at temperature of 20 ^{o}C. The stored Hach program for 5.25 days and 0-700mg L^{-1} was selected for the BOD test. Then, the reading was taken after 5 days with the reading BOD in mg L^{-1} were displayed on the screen for each sample bottle [173].

3.7.1.3 Total Organic Carbon, Total Nitrogen (TOC & TN) and Oil and Grease

Measurement of TOC and TN was carried out by using TOC Analyzer (TOC-$V_{CSH\ SHIMADZU}$) according to the APHA Standard Method. The sample was diluted at ratio 1:50, 1:100 and 1:250. Oil and grease was measured by oil and grease analyzer (InfraCal TOG Model HATR-T2). Samples of POME were analyzed by adding hexane into bottles containing POME and shake vigorously for 2 minutes for complete mixing. After the two layers separated, 50 µl was extracted from the upper layer using a syringe and deposited in the center of the sample crystal. Oil concentration displayed was recorded.

Removal efficiencies of BOD, COD, TOC, TN and oil and grease were calculated using the following equation:

$$Removal\ efficiency\ (\%) = \frac{Ai - Af}{Ai} \times 100 \qquad (3.9)$$

Where Ai and Af are the initial and final parameter concentrations, respectively.

3.8 Molecular Identification of *Nannochloropsis oculata*

3.8.1 Genomic DNA Extraction

Genomic DNA was extracted from the harvested cells by CTAB method [174]. 20 mL CTAB isolation buffer was preheated in 50 mL Falcon tube to 65°C in water bath and 40 µL mercaptoethanol was added to extraction buffer just before use. 3 g fresh pellet of algae was grinded to a powder in liquid nitrogen in a chilled mortar and pastle. The powder was poured directly into preheated buffer and incubated at 65°C for 1-2 hrs. 20 mL (equal volume) of CIA (chloroform, Isoamylalcohol) was added and mixed gently and centrifuged at 4000 rpm for 15 mins at room temperature. The viscous aqueous phase was then transferred to a clean centrifuged tube and 2/3 volume of cold isopropanol was added, kept for overnight at -20°C and centrifuged at 5000 rpm for 20 mins. The pellet was separated and 1 mL of TE buffer was added and centrifuged at 12000 rpm for 10 mins. The DNA pellet was air dried, resuspended in 1 mL TE buffer and stored at 4°C. Some additional steps with 25 µL of 5 M Nacl, 50 µL phenol, 50 µL chloroform, 250 µL cold 70% ethanol and 10 µL sodium acetate were carried out to remove polysaccharide from genomic DNA. The DNA pellet obtained at the end of the protocol was used as a template in PCR.

3.8.2 DNA Quantification and Purity

The concentration and purity of the extracted DNA was determined by a nanophotometer. The readings were taken using the wavelengths of 260 nm and 280 nm. The ratio of the reading at 260 nm to 280 nm provides an estimate for the purity of the DNA. A pure DNA sample has a ratio of 1.7 to 2.0.

3.8.3 Amplification of Genomic DNA by Polymerase Chain Reaction (PCR)

Primers were synthesized by FBCO 1st Base Custom Oligos Malaysia according to the 18S rRNA, rbcL gene and ITS region sequences. The genes were amplified by denaturing at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s and extension at 72°C for 2min and a final extension at 72°C for 10min. To amplify the 18s rRNA region, rbcL gene and ITS region, PCR was performed using forward primers (F298- 18S rRNA, F1 rbcL, F3 ITS) and reverse primers (R948- 18S rRNA, R1 486-rbcL, R1049-ITS).

For the PCR mixture; 1 µL of DNA template was mixed with 0.5 µL of *Taq* polymerase, 1.6 µL of 2 mM $MgCl_2$, 1.6 µL of 0.2 mM dinucleotide triphospate (dNTP), 0.8 µL of 0.4 µM primer, 12.5 µL of doubled distilled water and 2 µL of 10X buffer to a total volume of 20 µl. The PCR reaction mixture was dispensed into a 0.5 ml thin-walled PCR tube. The samples were then placed in the thermal cycler for amplification.

3.2.2.4 Detection of PCR Products

DNA amplified was separated using agarose gel electrophoresis. A 1% agarose gel was prepared in a 250 mL conical flask by melting 1 g of agarose in 100 ml of 1X TBE electrophoresis buffer (0.1 mM Tris/HCL, 0.1 mM boric acid, 0.002 mM EDTA, pH 8.3) [175]. The agarose gel at 50-70°C was poured into the gel tray and the comb was inserted. After the gel has hardened completely, the comb was removed carefully and the gel placed in the electrophoresis chamber. The chamber was then filled with 1X TBE electrophoresis buffer. Four microliters of amplified PCR products were pre-mixed with 1 µl of 6X loading dye on a piece of parafilm and loaded into the gel well. 1kb DNA marker (Hyper ladder 1) was used as a standard.

The DNA was electrophoresed at 70 V supplied from a Power Supply for 30 mins. After electrophoresis, the gel was removed from the tray, stained with 0.5 µg/ml ethidium bromide for 5 min and then destained with distilled water. The bands on the gel were visualized on an ultraviolet light transilluminator and photographed.

3.8.4 Purification of Desired PCR Product

The desired PCR product was purified by adding 10 µL of 3M sodium acetate and 250µL absolute ethanol in 100 µL of PCR product. After waiting for 30 mins at room temperature the sample was centrifuged at 13000 rpm at 4°C. The supernatant was discarded and 100 µL ethanol (70%) was added and centrifuged again at 13000 rpm at 4°C for 10 mins. DNA pallet was then air dried by removing supernatant. The DNA pellet was stored at 4°C.

3.9 Statistical Analysis

The data were analyzed through one-way Analysis of Variance (ANOVA) by using Statgraphics Centurion Version XVI to determine significant difference among the treatment mean.

CHAPTER 4

ESTABLISHMENT OF BASIC CULTURE CONDITIONS AND IDENTIFICATION OF FACTORS FOR MICROALGAL CULTIVATION ON SMALL SCALE REACTORS USING COMMERCIAL MEDIA

4.1 Basic Culture Conditions

The basic conditions for all the four selected marine microalgae were 28 ±2°C, pH 7-8, salinity at 30 ppt, 24 hrs illumination of 90 µmol photons m^2 s^{-1} intensity from white fluorescent tube (Philips), and shake flask agitation at 80 rpm. For cultivation, 10 % (v/v) inocula was added under sterile conditions using Conway medium (for small-scale cultivation) and TMRL Enrichment medium (for large-scale cultivation). Media formulations are as shown in APPENDIX (B). Fig. 4.1 (a & b) show the growth curves of *N. oculata*, *T. suecica*, *I. galbana* and *P. lutheri* cultivated in 20 mL test tubes and 250 mL shake flasks. The highest cell density were achieved on day 20 in test tube and on day 16 in shake flask, respectively. From Table 4.1 the biomass of 0.58, 0.51, 0.44 and 0.45 g L^{-1}; X'_{max} of 0.116, 0.098, 0.101 and 0.093 g L^{-1} d^{-1}; μ_{max} of 0.15, 0.13, 0.13 and 0.14 d^{-1}; and t_d of 4.62, 5.41, 4.95 and 4.98 days; and lipid content of 26.4 ± 3.78%, 21.5 ± 2.01%, 18.9 ± 2.56% and 28.6 ± 1.88% were attained respectively in 250 mL shake flasks and these were comparable to cultures in 20 mL test tubes.

Natural seawater is a complex medium containing more than 50 known elements and a large and variable number of organic compounds [104]. Direct use of seawater for algal culture without the addition of further nutrients and trace metals, may result in low yield.

Enrichment of sea water with macronutrients, trace metals, and vitamins are normally made to enrich algal growth and productivity. The materials required for preparation

of seawater media and the formulations of stock solutions of macronutrients, trace elements, and vitamins have been described in Chapter 3. Conway [168] and TMRL Enrichment medium [104] were selected for the growth of marine microalgae. Comparing today's marine culture media with those of 40 years ago [104], most media show broad-based constituents indicating that most culturable algae can be grown with the following modifications:

1) addition of synthetic metal chelators such as Disodium EDTA to decrease metal precipitates;
2) addition of pH buffer such as Tris or glycylglycine (pH 7–8.5 range) as the pH may increase during autoclaving that may increase the precipitates;
3) replacement of $NaNO_3$ with more soluble KNO_3

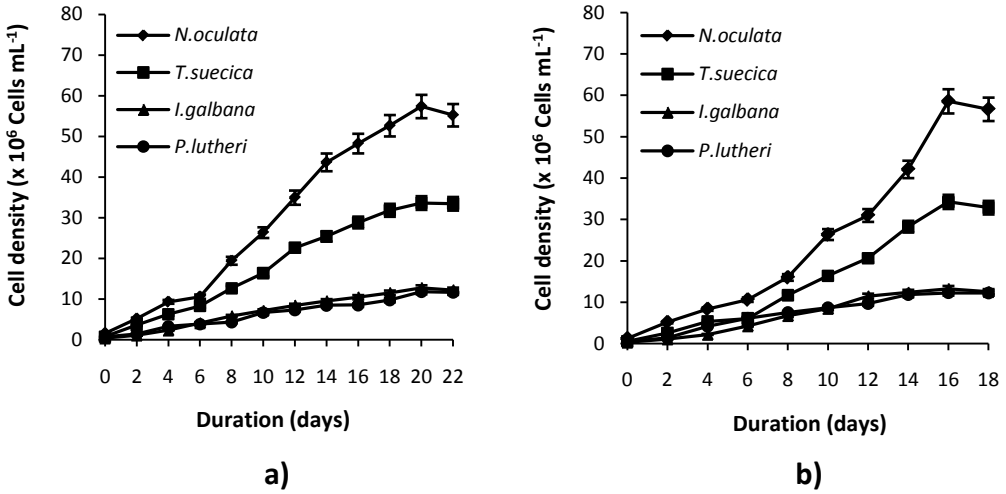


Fig. 4.1 Growth profile under basic culture conditions in
a) 20 mL test tubes and b) 250 mL shake flasks

Table 4.1 Growth kinetics under basic culture conditions in 20 mL test tubes and 250 mL shake flasks

Experimental condition		N_{max} (10^6 cells mL^{-1})	X_{max} (g L^{-1})	X'_{max} (g L^{-1} d^{-1})	μ_{max} (d^{-1})	t_d (day)	Lipid Content (%)
N. oculata	Test Tube	57.38	0.54	0.108	0.13	5.17	-
	Shake Flask	58.56	0.58	0.116	0.15	4.62	26.4 ± 3.78
T. suecica	Test Tube	33.64	0.48	0.075	0.12	5.77	-
	Shake Flask	34.25	0.51	0.098	0.13	5.41	21.5 ± 2.01
I. galbana	Test Tube	12.76	0.38	0.082	0.12	5.21	-
	Shake Flask	13.26	0.44	0.101	0.13	4.95	18.9 ± 2.56
P. lutheri	Test Tube	11.78	0.42	0.073	0.13	5.33	-
	Shake Flask	12.22	0.45	0.093	0.14	4.98	28.6 ± 1.88

4.2 Effects of pH and Salinity

4.2.1 Cell Growth and Lipid Productivity

The productivity of four microalgae strains was compared under the same cultivation conditions. Figs 4.2 (a–d) show the effects of salinity of *N. oculata*, *T. suecica*, *I. galbana* and *P. lutheri* with the highest cell density achieved at 75.5×10^6, 46.0×10^6, 15.1×10^6 and 14.8×10^6 cells mL^{-1}; and maximum biomass at 0.80, 0.66, 0.62 and 0.58 gL^{-1} attained at 35-40 ppt for *N. oculata*, *T. suecica*, *I. galbana* and *P. lutheri*, respectively. The results of pH effects (not shown) were comparable to salinity with optimal pH of 8. Table 4.2 shows the kinetics of cell growth and lipid production at pH range of 5-10 and salinity 15-60 ppt. Of the four strainsat pH 8 and 35 ppt, *T. suecica* had higher specific growth rate of 0.18 d^{-1} while *N. oculata* and *I. galbana* and *P. lutheri* were 0.15-0.17 d^{-1}.

The t_d in different pH media ranged from 3.85 days at pH 8 for *T. suecica* to 6.93 days at pH 5 for *N. oculata*. Generally, the shortest t_d was observed in pH 7–9 media while the longest was observed in pH 5-6 and10. At 15 ppt, the t_d was 6.11 days and achieving 3.89-5.41 between 25-40 ppt, for all species. *P. lutheri* and *N. oculata* attained the highest lipid contents of 31.3-36.5% at pH 8 and 35 ppt salinity while the lipid contents for *T. suecica* and *I. galbana* were relatively low, around 23.4-26.3%. Hence, only *N. oculata* showed consistent high productivity of cell growth and lipid production.

4.2.2 Optimal Conditions and Interaction between Factors

The optimum pH and salinity were pH 8 and salinity of 35 ppt to achieve cell dry weight of 0.82, 0.72 and 0.58 g L^{-1} and lipid content of 35.7, 33.5 and 37.3 % for *N. oculata*, *T. suecica* and *P. lutheri*, respectively. For *I. galbana* the cell dry weight of 0.68 g L^{-1} and lipid content of 27.4% was achieved at optimum pH 9 and salinity of 40 ppt as predicted by the RSM model (Appendix C1). The Pareto charts of *N. oculata* and *T. suecica* for pH and salinity (Fig 4.3 a,b and 4.4 a,b) show that salinity ($p< 0.0001$-0.0007) has the most significant positive effect on biomass and lipid, but the interactive effects of pH-pH and salinity-salinity may affect negatively both cell growth and lipid content. For *I. galbana* and *P. lutheri* pH ($p< 0.0028$-0.0005) and salinity ($p< 0.0095$-0.0001) both have the significant positive effects on biomass and lipid (Fig 4.3 c,d and 4.4 c,d). The second-order polynomial equations representing the dry weight and lipid content for all variables. For *N. oculata* showed the r^2 of 86.6% for biomass and 87.9% for lipid production; *T. suecica* showed the r^2 of 82.4% (biomass) and 90.3% (lipid). *I. galbana* 94.2% (biomass) and 91.20% (lipid) and *P. lutheri* showed the r^2 of 84.04% for biomass and 86.19% for lipid. The mean absolute error percentage between experimental and predicted values of 0.02-0.03% and 0.68-1.12%, for biomass and lipid content of all four tested species, suggest good agreements between experimental and predicted values (Appendix C3).

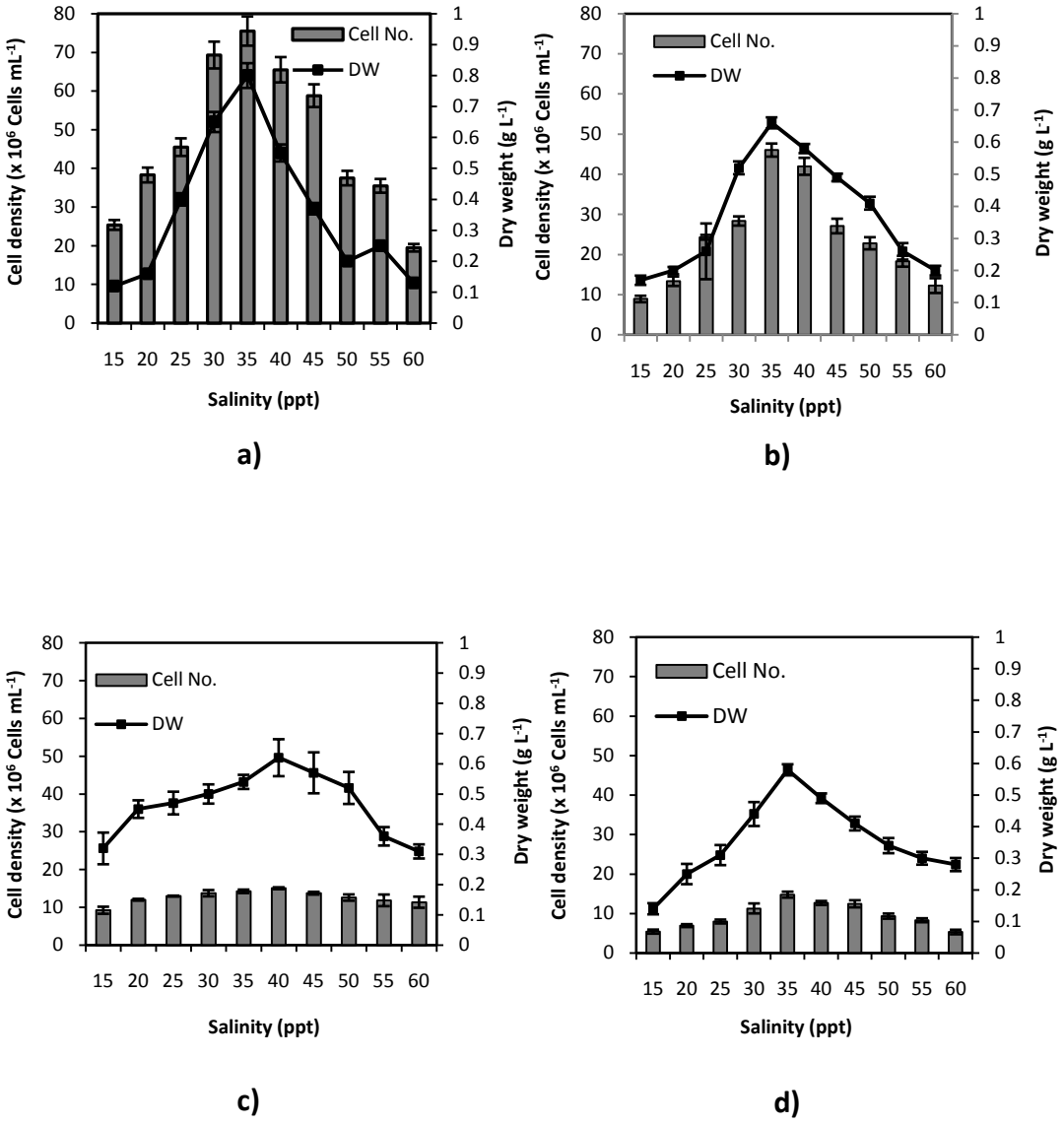


Fig. 4.2 Effects of salinity on cell density and dry weight of a) *N. oculata*; b) *T. suecica*; c) *I. galbana* and d) *P. lutheri*

Table 4.2 Kinetics of cell growth and lipid production of *N. Oculata*, *T. suecica*, *I. galbana* and *P. lutheri*

Media Conditions		*N. oculata*				*T. suecica*				*I. galbana*				*P. lutheri*			
		X'_{max} ($gL^{-1}d^{-1}$)	μ_{max} (d^{-1})	t_d (day)	Lipid (%)	X'_{max} ($gL^{-1}d^{-1}$)	μ_{max} (d^{-1})	t_d (day)	Lipid (%)	X'_{max} ($gL^{-1}d^{-1}$)	μ_{max} (d^{-1})	t_d (day)	Lipid (%)	X'_{max} ($gL^{-1}d^{-1}$)	μ_{max} (d^{-1})	t_d (day)	Lipid (%)
pH	5	0.103	0.10	6.93	15.9 ± 1.62	0.075	0.12	5.77	12.3 ± 1.06	0.141	0.11	6.30	14.3 ± 0.94	0.097	0.12	5.77	22.5 ± 3.05
	6	0.133	0.13	5.33	23.9 ± 1.20	0.096	0.15	4.62	15.3 ± 0.91	0.167	0.13	5.18	18.8 ± 1.03	0.110	0.13	5.33	25.4 ± 0.96
	7	0.137	0.15	4.50	28.4 ± 1.64	0.139	0.16	4.33	18.2 ± 1.94	0.168	0.14	4.92	21.4 ± 1.97	0.117	0.13	5.33	31.2 ± 2.46
	8	0.150	0.17	4.07	31.3 ± 0.78	0.152	0.18	3.85	23.4 ± 2.04	0.176	0.17	4.10	24.8 ± 1.97	0.131	0.16	4.38	35.2 ± 0.92
	9	0.123	0.15	4.62	26.2 ± 1.32	0.140	0.15	4.68	20.8 ± 1.02	0.157	0.15	4.62	22.8 ± 1.07	0.130	0.14	4.95	33.8 ± 0.82
	10	0.121	0.13	5.33	18.1 ± 1.30	0.120	0.14	5.02	16.8 ± 0.96	0.144	0.14	5.06	19.0 ± 0.51	0.110	0.12	5.77	23.3 ± 1.95
Salinity	15	0.091	0.12	5.77	10.3 ± 0.78	0.062	0.11	6.11	13.6 ± 0.85	0.127	0.13	5.29	13.4 ± 1.03	0.071	0.12	5.54	17.4 ± 1.05
	20	0.096	0.13	5.33	15.3 ± 2.28	0.075	0.13	5.45	17.3 ± 0.99	0.143	0.14	4.78	17.4 ± 0.90	0.085	0.12	5.68	21.5 ± 1.19
	25	0.100	0.13	5.21	26.4 ± 0.78	0.098	0.13	5.41	18.9 ± 2.01	0.147	0.13	5.17	18.5 ± 0.76	0.110	0.13	5.13	24.6 ± 1.83
	30	0.134	0.14	4.95	28.7 ± 1.13	0.146	0.14	4.81	21.4 ± 0.93	0.152	0.15	4.66	21.5 ± 1.94	0.123	0.14	4.81	29.6 ± 1.88
	35	0.145	0.15	4.62	32.3 ± 1.01	0.157	0.18	3.85	24.4 ± 1.94	0.159	0.16	4.45	22.5 ± 1.11	0.131	0.17	4.00	36.5 ± 1.93
	40	0.126	0.14	4.95	30.6 ± 1.14	0.146	0.18	3.89	20.2 ± 1.04	0.170	0.17	4.12	26.3 ± 1.06	0.124	0.15	4.74	33.9 ± 2.48
	45	0.117	0.12	5.54	29.9 ± 1.21	0.141	0.16	4.33	18.4 ± 0.99	0.153	0.15	4.66	23.4 ± 0.61	0.117	0.14	4.95	30.3 ± 1.06
	50	0.100	0.12	5.77	26.5 ± 1.11	0.136	0.13	5.29	15.7 ± 1.90	0.167	0.14	4.78	21.4 ± 0.44	0.120	0.13	5.46	25.4 ± 3.16
	55	0.118	0.12	5.77	18.8 ± 1.13	0.098	0.13	5.33	14.5 ± 1.76	0.136	0.14	4.95	20.6 ± 1.95	0.110	0.12	5.63	23.6 ± 1.83
	60	0.098	0.11	6.03	13.1 ± 1.98	0.087	0.11	6.30	12.7 ± 2.67	0.123	0.13	5.21	17.4 ± 3.03	0.095	0.12	5.77	18.1 ± 3.07

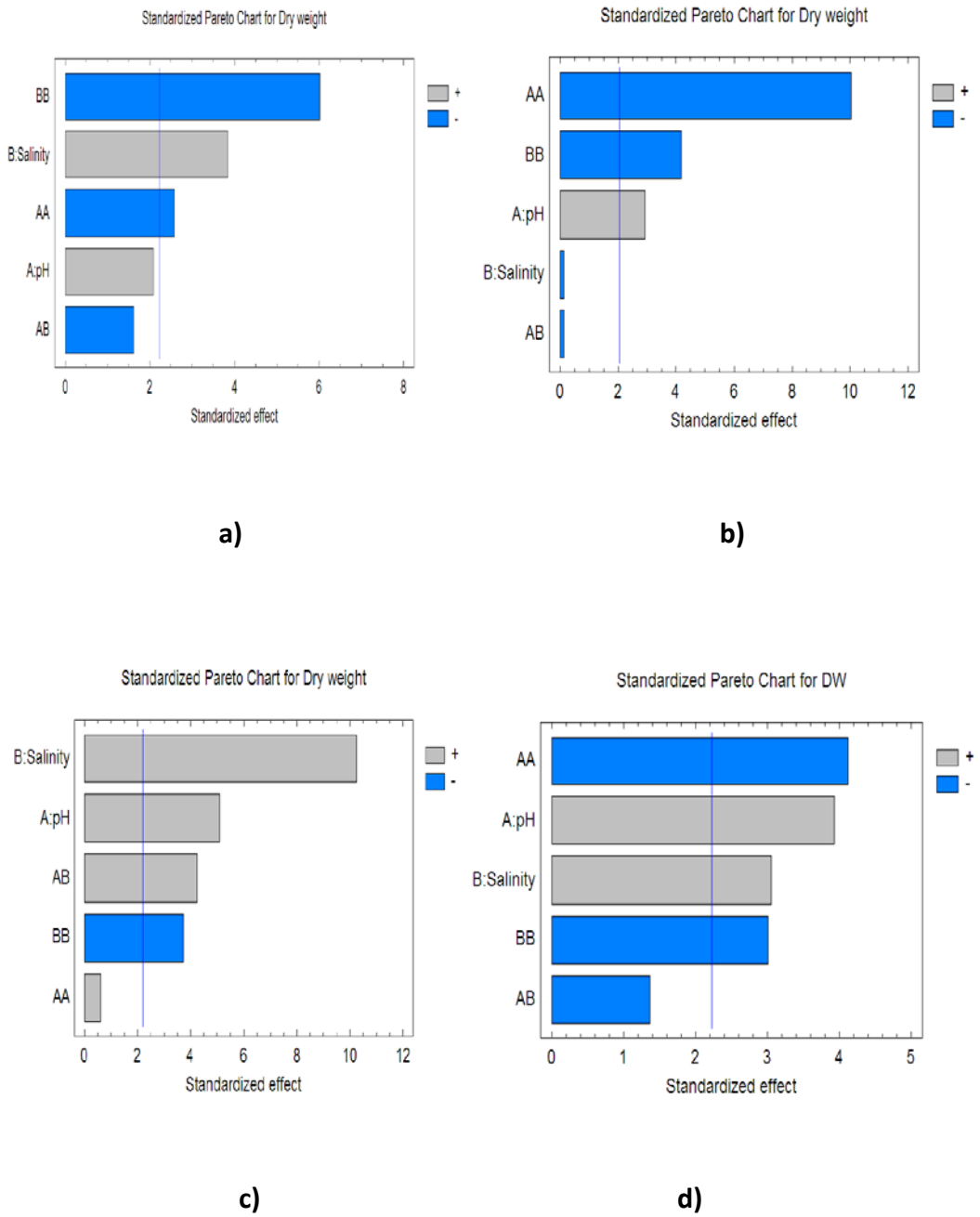


Fig. 4.3 Standardized Pareto chart for interactions between pH and salinity on cell dry weight of a) *N. oculata*; b) *T. suecica*; c) *I. galbana* and d) *P. lutheri*

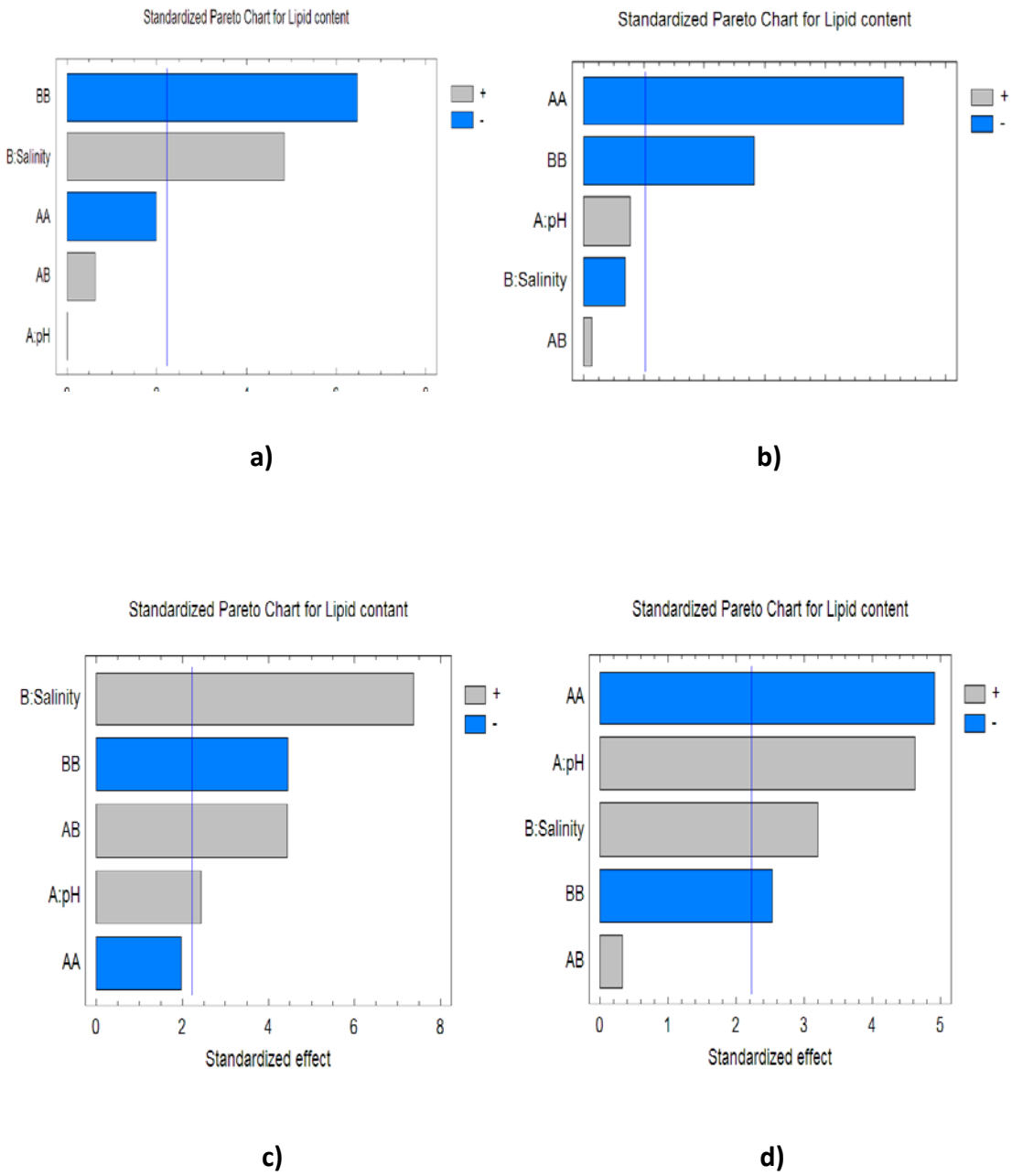


Fig. 4.4 Standardized Pareto chart for interactions between pH and salinity on lipid content of a) *N. oculata*; b) *T. suecica*; c) *I. galbana* and d) *P. lutheri*

4.3 Effects of Photoperiod and Light Intensity

At pH 8 and 35 ppt NaCl, the highest cell density of *N. oculata*, *T. suecica*, *I. galbana* and *P. lutheri* at 68.7 × 10^6, 42.8 × 10^6, 15.9 × 10^6 and 15 × 10^6 cells mL^{-1} and maximum biomass production of 0.78, 0.67, 0.71 and 0.61 gL^{-1} attained respectively, under 21-24 hrs illumination (results not shown) were comparable and reproducible from previous study. Fig 4.5 (a–d) show the effects of light intensity with the highest cell density and maximum biomass attained at 160-198 µmol photons $m^{-2}s^{-1}$. As shown in Table 4.3 *T. suecica* had higher specific growth rate of 0.18-0.19 d^{-1} at 198 µmol photons $m^{-2}s^{-1}$ while *N. oculata* and *P. lutheri* showed 0.16-0.17 d^{-1}. *I. galbana* had specific growth rate of 0.18-0.19 d^{-1} under 21 hrs photoperiod at 160 µmol photons $m^{-2}s^{-1}$. *P. lutheri* and *N. oculata* attained maximum lipid content of 31-35.5% as compared to *I. galbana* (26-27%) and *T. suecica* (22-23%).

RSM model predicted and verified experimentally the maximum cell dry weight of 0.84, 0.72, 0.67, 0.66 and 0.84 g L^{-1}; and lipid content of 36.6, 23.9, 28.8 and 36.4 % at optimum conditions of 19.3-24 h illumination and light intensity of 162-198 µmol photons $m^{-2}s^{-1}$ for *N. oculata*, *T. suecica*, *I. galbana* and *P. lutheri*, respectively (Fig 4.6 a-d & 4.7 a-d). The photoperiod ($p< 0.0001$) has the most significant positive effect on biomass, while light intensity ($p< 0.0043$) has the most significant positive effect on lipid content for *N. oculata*. For *T. suecica*, both photoperiod and light intensity ($p< 0.0001$) have the significant positive effect on biomass and lipid. For *I. galbana* and *P. lutheri* the photoperiod ($p< 0.0001$) and ($p< 0.003$) has the most significant positive effect on biomass, while light intensity ($p< 0.0001$-0.0003) has the most significant positive effect on lipid content (Pareto charts not shown). The mean absolute error percentage between experimental and predicted values of 0.02-0.04% and 0.61-1.66%, for biomass and lipid content of all four tested species, suggest good agreements between experimental and predicted values.

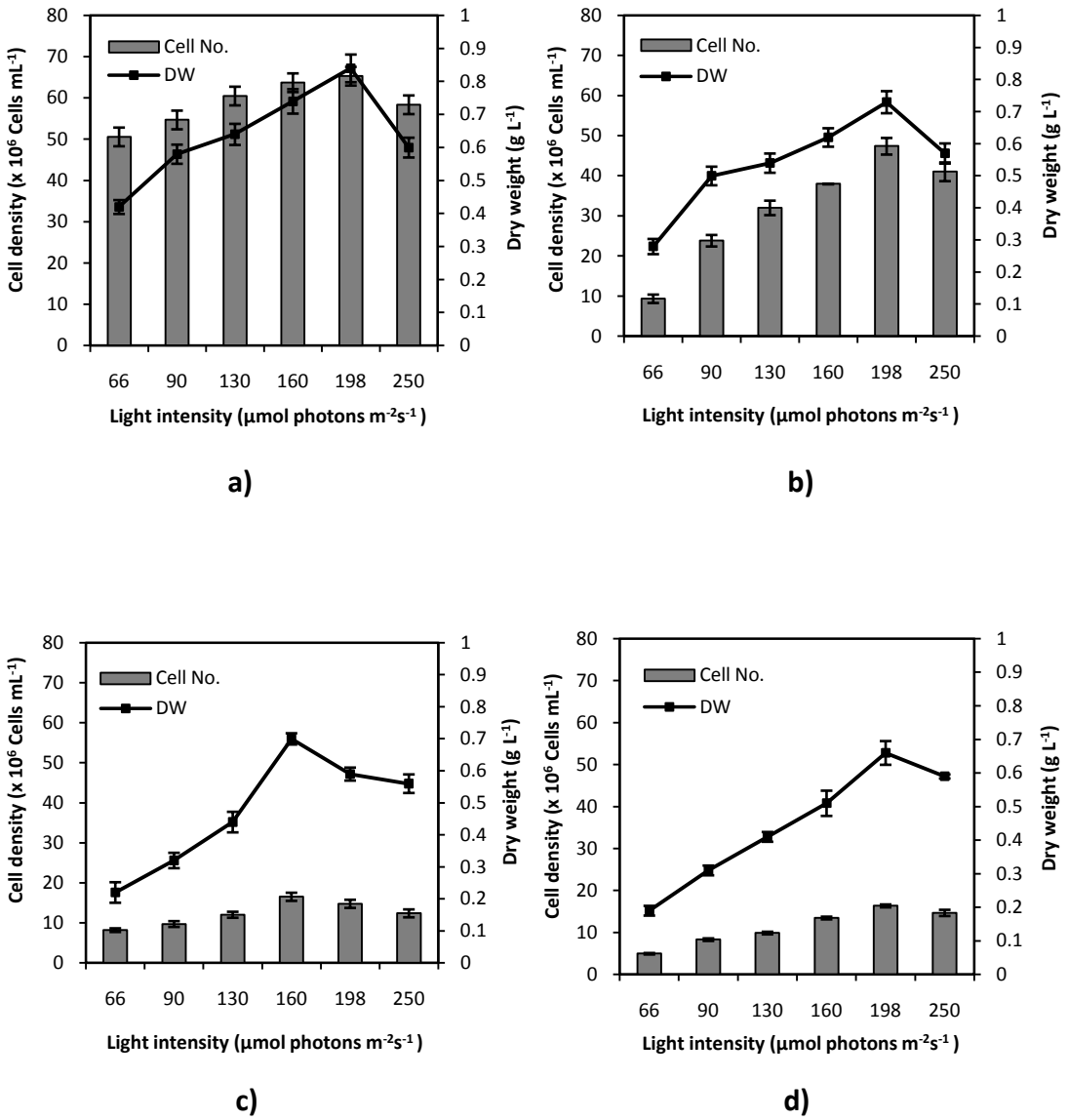


Fig. 4.5 Effects of light intensity on cell density and dry weight of a) *N. oculata*; b) *T. suecica*; c) *I. galbana* and d) *P. lutheri*

Table 4.3 Kinetics of cell growth and lipid production of *N. oculata, T. suecica, I. galbana* and *P. lutheri*

Media Conditions		*N. oculata*				*T. suecica*				*I. galbana*				*P. lutheri*			
		X'_{max} ($gL^{-1}d^{-1}$)	μ_{max} (d^{-1})	t_d (day)	Lipid (%)	X'_{max} ($gL^{-1}d^{-1}$)	μ_{max} (d^{-1})	t_d (day)	Lipid (%)	X'_{max} ($gL^{-1}d^{-1}$)	μ_{max} (d^{-1})	t_d (day)	Lipid (%)	X'_{max} ($gL^{-1}d^{-1}$)	μ_{max} (d^{-1})	t_d (day)	Lipid (%)
Photoperiod (hrs)	6	0.115	0.12	5.63	14.6 ± 1.60	0.083	0.12	6.02	9.5 ± 0.58	0.104	0.11	6.07	14.5 ± 1.20	0.091	0.11	6.30	18.4 ± 1.64
	9	0.117	0.12	5.63	18.5 ± 1.58	0.106	0.13	5.22	11.4 ± 1.93	0.127	0.14	5.02	16.6 ± 0.28	0.085	0.12	5.82	21.8 ± 1.35
	12	0.142	0.14	4.95	20.6 ± 1.29	0.122	0.14	5.04	13.4 ± 2.05	0.128	0.15	4.71	19.7 ± 0.97	0.121	0.13	5.46	30.1 ± 1.46
	15	0.143	0.14	4.83	21.9 ± 1.35	0.136	0.14	4.82	14.3 ± 0.98	0.149	0.15	4.56	20.8 ± 2.08	0.126	0.13	5.33	30.9 ± 2.54
	18	0.143	0.15	4.62	25.4 ± 1.62	0.137	0.15	4.62	18.3 ± 1.88	0.158	0.15	4.50	21.5 ± 0.94	0.130	0.14	4.84	31.8 ± 1.97
	21	0.152	0.15	4.55	28.4 ± 1.55	0.140	0.16	4.25	20.4 ± 1.92	0.172	0.17	4.03	26.3 ± 0.94	0.128	0.14	4.84	34.4 ± 0.76
	24	0.156	0.16	4.33	31.1 ± 1.61	0.158	0.18	3.94	22.8 ± 2.05	0.149	0.16	4.25	25.4 ± 0.54	0.143	0.16	4.36	35.5 ± 1.02
Light Intensity (µmol photons $m^{-2}s^{-1}$)	66	0.111	0.12	5.97	18.4 ± 1.68	0.104	0.13	5.45	11.9 ± 2.00	0.108	0.13	5.50	15.7 ± 2.18	0.095	0.12	5.87	16.8 ± 2.34
	90	0.146	0.14	4.92	24.3 ± 0.73	0.143	0.16	4.43	16.7 ± 0.92	0.139	0.14	4.95	18.9 ± 0.56	0.110	0.12	5.63	19.0 ± 2.12
	130	0.147	0.15	4.62	30.2 ± 1.27	0.149	0.17	3.98	19.4 ± 1.91	0.142	0.15	4.26	20.5 ± 0.44	0.117	0.13	5.37	26.5 ± 0.96
	160	0.151	0.15	4.62	32.5 ± 0.77	0.152	0.18	3.76	21.8 ± 1.98	0.170	0.18	3.85	27.4 ± 0.91	0.128	0.14	4.85	30.8 ± 1.56
	198	0.162	0.17	4.03	35.2 ± 0.84	0.184	0.19	3.66	19.6 ± 0.57	0.156	0.16	4.28	24.3 ± 0.92	0.149	0.17	3.98	35.3 ± 1.06
	250	0.143	0.17	4.03	23.3 ± 1.68	0.164	0.18	3.85	17.8 ± 1.08	0.143	0.15	4.59	23.3 ± 0.47	0.132	0.16	4.33	31.9 ± 1.43

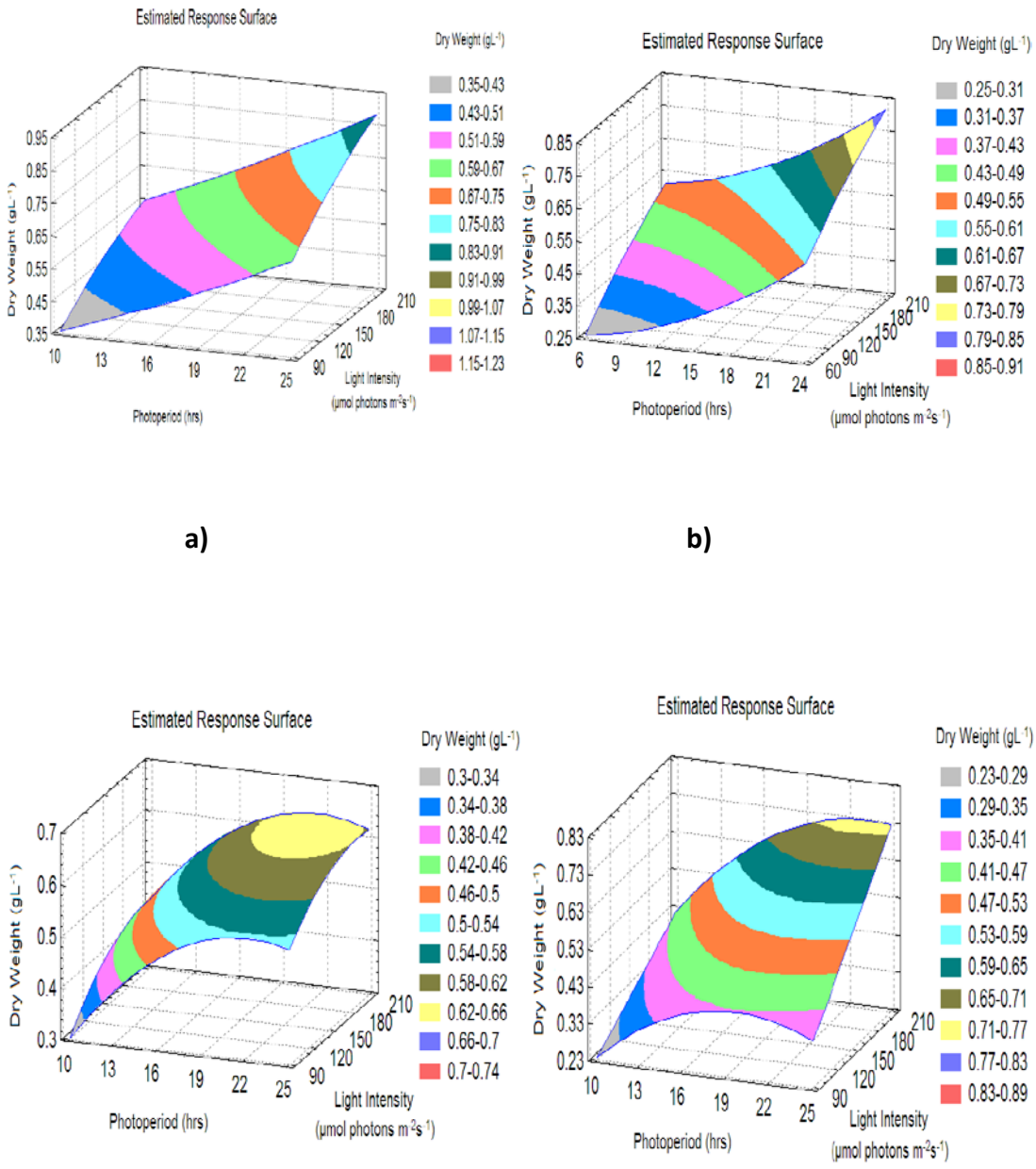


Fig. 4.6 Estimated response surface plot of interactions between photoperiod and light intensity on dry weight of a) *N. oculata*; b) *T. suecica*; c) *I. galbana* and d) *P. lutheri*

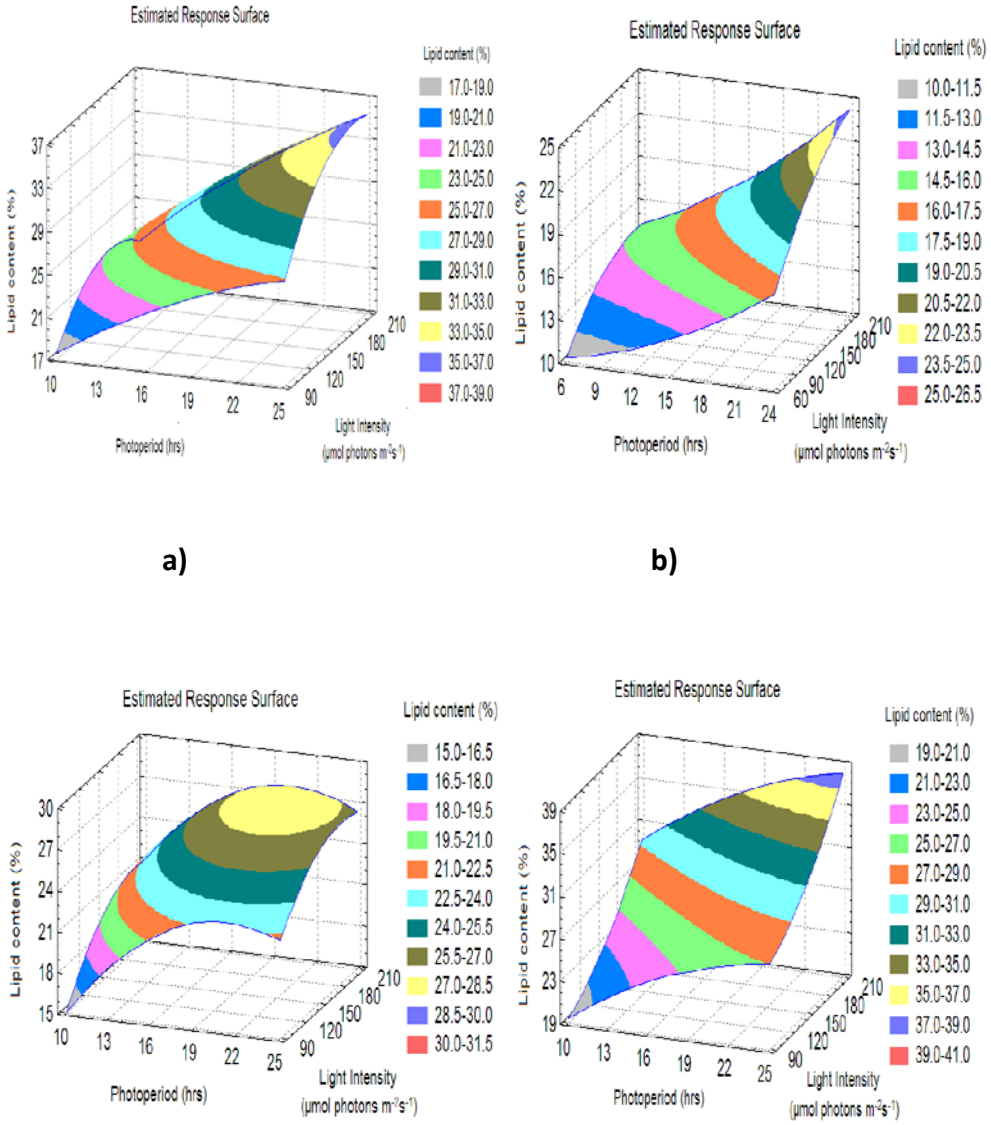


Fig. 4.7 Estimated response surface plot of interactions between photoperiod and light intensity on lipid content of a) *N. oculata*; b) *T. suecica*; c) *I. galbana* and d) *P. lutheri*

4.4 Discussion

In the present study, green and brown microalgae were isolated from marine environment that thrive at high salinity. Different geographical strains have different preferences towards salinity and optimal salinity may be a function of immediate conditions from which the strain is initially isolated. Species isolated at higher salinities will grow better at higher salinities and may not be as good at lower salinities [24]. Higher lipid (60.6% DW) has been reported for the genus *Dunaliella* in salt concentration as high as 0.5 M NaCl (equivalent to seawater, or 35 psu) [26] which increases as NaCl increases. Optimal pH 8 agrees well with that reported for *Navicula acceptata*, *Navicula pelliculosa*, *Navicula saprophila* [16] and *Chlorella vulgaris* at pH 7-8 [176]. In the present study, biomass, μ_{max} and t_d of all the four strains are comparable to *Spirulina platensis* at 0.82 g L^{-1}, 0.14 d^{-1} and 5.11 days [177], respectively and *Scenedesmus dimorphus* and *Scenedesmus quadricauda* at 0.141 g L^{-1} and 0.358 L^{-1}, 0.202 d^{-1} and 0.516 d^{-1} and 4.91 and 1.93 days with lipid content of 34% and 31%, respectively [178]. Several strains such as *Nannochloropsis*, *Nannochloris*, *P. lutheri* and *Phaeodactylum tricornutum* have reported lipid contents around 26-36% [16].

Irrespective of pH tested (pH 6-9), the lipid content remained below 25% and biomass below 0.50 g L^{-1} at 25 ppt NaCl. This may suggest the role of osmotic stress in regulating influx or efflux of nutrients and ions for improved cell growth and lipid accumulation. At 40 g L^{-1} NaCl, *Nannochloropsis* sp. cultivated has reportedly achieved the dry weight of 3.3 ± 0.002 pg/cell and total fatty acid (TFA) of 0.9 ± 0.066 pg/cell. *Nannochloropsis* sp. accumulates TAG as a means of storing the excessive photosynthetic carbon under unfavorable conditions such as increased salinity (35-40 ppt), and increased pH (7.5-8.5). Maximum lipid productivities of about 200 g L^{-1} day^{-1} by *Nannochloropsis* sp. [13] and 140 g L^{-1} day^{-1} by *N. oculata* NCTU-2 [4] have been reported under increased salinity and increased pH.

Light intensity influences algal lipid synthesis, with strong illumination inducing their accumulation [26]. Under 6 hrs illumination, the lowest cell density of 3.93–21.58 x 10^6 cells mL^{-1}, dry weight of 0.18-0.28 g L^{-1} and lipid of 9.5-18.4% were obtained.

The lowest biomass of 0.23-0.38 g L^{-1} and lipid of 10.1-17.4% achieved under 10 hrs photoperiod and 90 µmol photons $m^{-2}s^{-1}$ light intensity indicates the importance of photoperiod and light intensity. As shown in Fig. 4.5-4.7 (a-d), the transition from low to high photoperiod and light conditions enhanced both cell growth and lipid accumulation by 2-fold. This is in agreement with a study on *Nannochloropsis salina* which has the lipid enhanced from 10-70% when the light intensity is increased from 150 µE m^{-2} s^{-1} to 350 µE m^{-2} s^{-1} [179], and *Ochromonas danica* where the cell growth and lipid content increases with increasing light intensity [22]. Light is the main source of photosynthesis, as well as cell photo-acclimatization for microalgal growth. The quantity of photon energy absorbed by each cell is a combination of several factors such as cell density, photoperiod, length of optical path, thickness of layers, photon flux density and rate of agitation [96]. The physiological properties of phytoplankton and photosynthetic organisms can be changed upon exposure to light intensity. *Rhodomonas sp., Cryptomonas sp.,* and *Isochrysis sp.* show increasing lipid content with increase in photoperiod by 15.5, 12.7, and 21.7% respectively [180].

The responses of microalgal strains to different light intensities are indication of accessibility of light antenna on cell surface. Cell responds to this by modulating the composition of its photosynthetic apparatus, a response called acclimation [181]. At good penetration of light, the individual cells are exposed to a larger quantity of light energy, resulting in more metabolic flux generated from photosynthesis to be channelled to lipid accumulation on a unit biomass basis. Light and temperature plays an important role in algal lipid synthesis and accumulation [182]. The high lipid content at high light exposure is measured as total global radiation (TGR) where light and photoperiod may also actually, influence the cultivation temperature. Increased microalgal lipid productions to 26-36% at temperatures 25-30^{o}C for several microalgal species have been reported. However, reduced lipid production to 15-20% at extremes of low (15^{o}C) and high temperature (30^{o}C) has been observed in *Isochrysis galbana and Nanochloropsis* species [183]. Low temperatures reduce enzyme activity in glycolysis and the Krebs cycle and consequently the metabolism of carbon sources. Metabolic engineering of pathways could assist to evaluate the activities of enzymes in the metabolic network that can be upregulated or downregulated for enhanced lipid productivity.

4.5 Summary

The basic culture conditions and identification of factors for microalgal cultivation and growth kinetics were developed in 20 mL test tubes and 250 mL shake flasks. Effcts of pH, salinity, photoperiod and light intensity were decribed and optimized by Response Surface Methodology (RSM). The cell densities, cell dry weight and lipid content increased with the increase of pH, salinity, photoperiod and light intensity for all the four tested species. The optimum pH and salinity were pH 8-9 and salinity of 35-40 ppt to achieve cell dry weight of 0.82, 0.72, 0.68 and 0.58 g L^{-1}; and lipid content of 35.7, 33.5, 27.4 and 37.3% for *N. oculata*, *T. suecica*, *I. galbana*, and *P. lutheri* respectively. The maximum cell dry weight of 0.84, 0.72, 0.66 and 0.67 g L^{-1}; and lipid content of 36.6, 23.9, 28.8 and 36.4 % were achieved, respectively at optimal conditions of 19.3-24 h illumination and light intensity of 162-198 µmol photons $m^{-2}s^{-1}$. The interactions of pH, salinity, photoperiod and light intensity significantly influenced the biomass and lipid content positively. The second-order polynomial equations represent the dry weight and lipid content for all variables with the r^2 of 82.4-95.1% for biomass and 84.8-97.5% for lipid. The mean absolute error percentage between experimental and predicted values of 0.02-0.04% and 0.61-1.66% suggest good agreements between experimental and predicted values.

CHAPTER 5

EFFECTS OF MACRONUTRIENTS, IRON AND ECONOMICAL PALM OIL MILL EFFLUENT MEDIA ON SMALL SCALE REACTORS

5.1 Effects of Nitrate, Phosphate and Iron

5.1.1 Cell Growth and Lipid Production

Fig 5.1–5.3 shows the effects of nitrate, phosphate and iron on cell density and dry weight of *N. oculata, T. suecica, I. galbana* and *P. lutheri*. The highest cell density of 72.3 × 10^6 cells mL^{-1} with maximum biomass of 0.81 g L^{-1} were achieved by *N. oculata*, followed by 46.5 × 10^6 cells mL^{-1} density with 0.70 g L^{-1} biomass by *T. suecica* at 120–150 gL^{-1} KNO_3, 12–15 g L^{-1} Na_2HPO_4 and 4–5 g L^{-1} $FeCl_3$. The maximum specific growth rates were 0.16–18 d^{-1} with doubling time of 3.78–4.62 days. Both *I. galbana* and *P. lutheri* only achieved 17 × 10^6 cells mL^{-1} though almost comparable dry weight at 0.7 g L^{-1}.

Although cell growth appears to require excesses of nitrate, phosphate and iron, lipid production was induced under conditions of nutrients limitation (Table 5.1). The lipid content were highest in *P. lutheri* at 37.1 ± 1.39, 34.5 ± 1.11 and 34.2 ± 1.02% (in 40 g L^{-1} KNO_3, 2 g L^{-1} $FeCl_3$ and 4 g L^{-1} Na_2HPO_4) and *N. oculata* at 35.3 ± 1.65, 33.5 ± 1.54 and 33.1 ± 2.44% (in 10 g L^{-1} KNO_3, 4 g L^{-1} Na_2HPO_4 and 2 g L^{-1} $FeCl_3$), respectively. *I. galbana* and *T. suecica* only attained around 23.8- 26.8% lipid. *N. oculata* was therefore the highest lipid titre of 0.286 g L^{-1}.

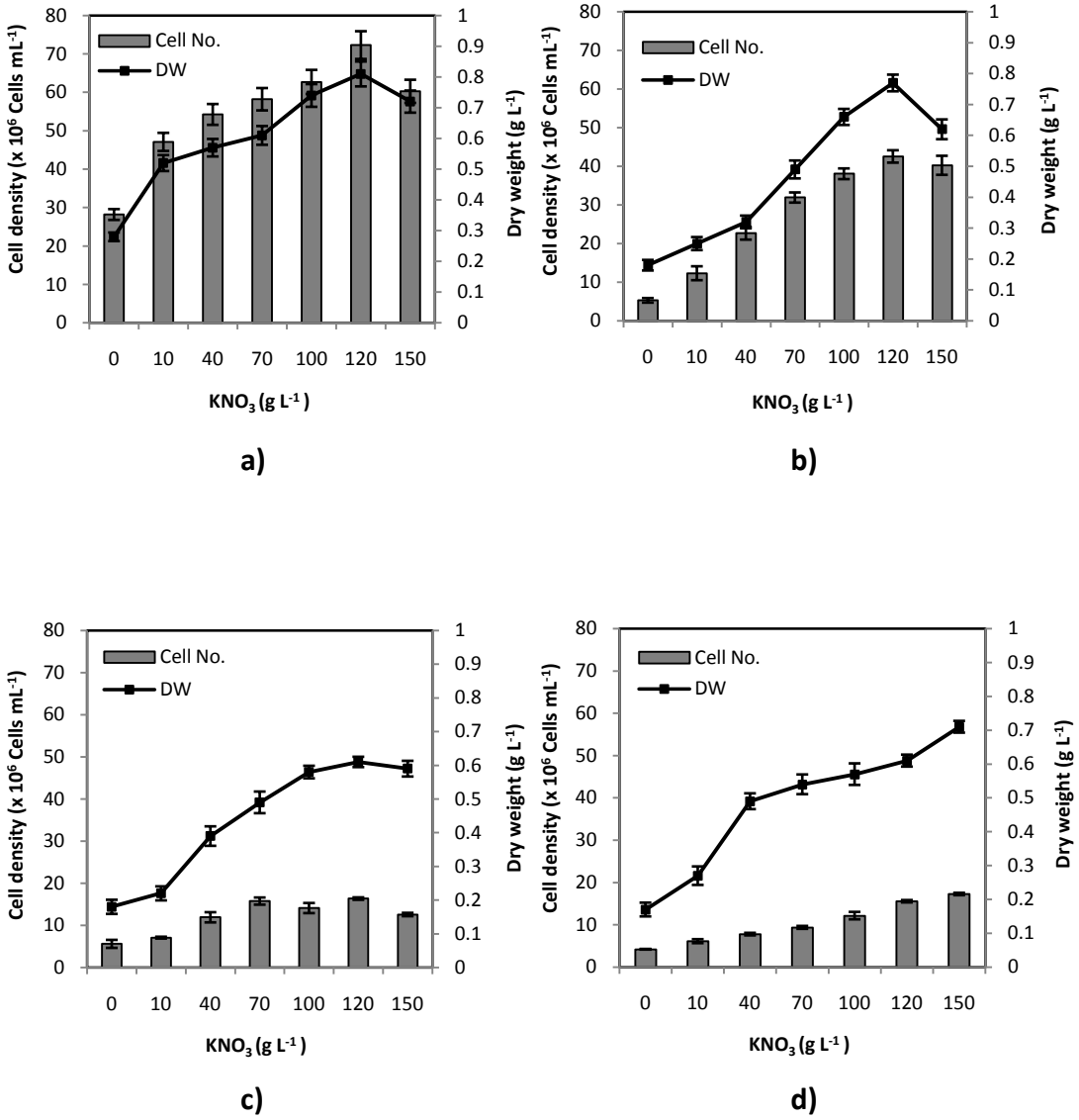


Fig. 5.1 Effects of KNO_3 on cell density and dry weight of a) *N. oculata*; b) *T. Suecica*; c) *I. galbana* and d) *P. lutheri*

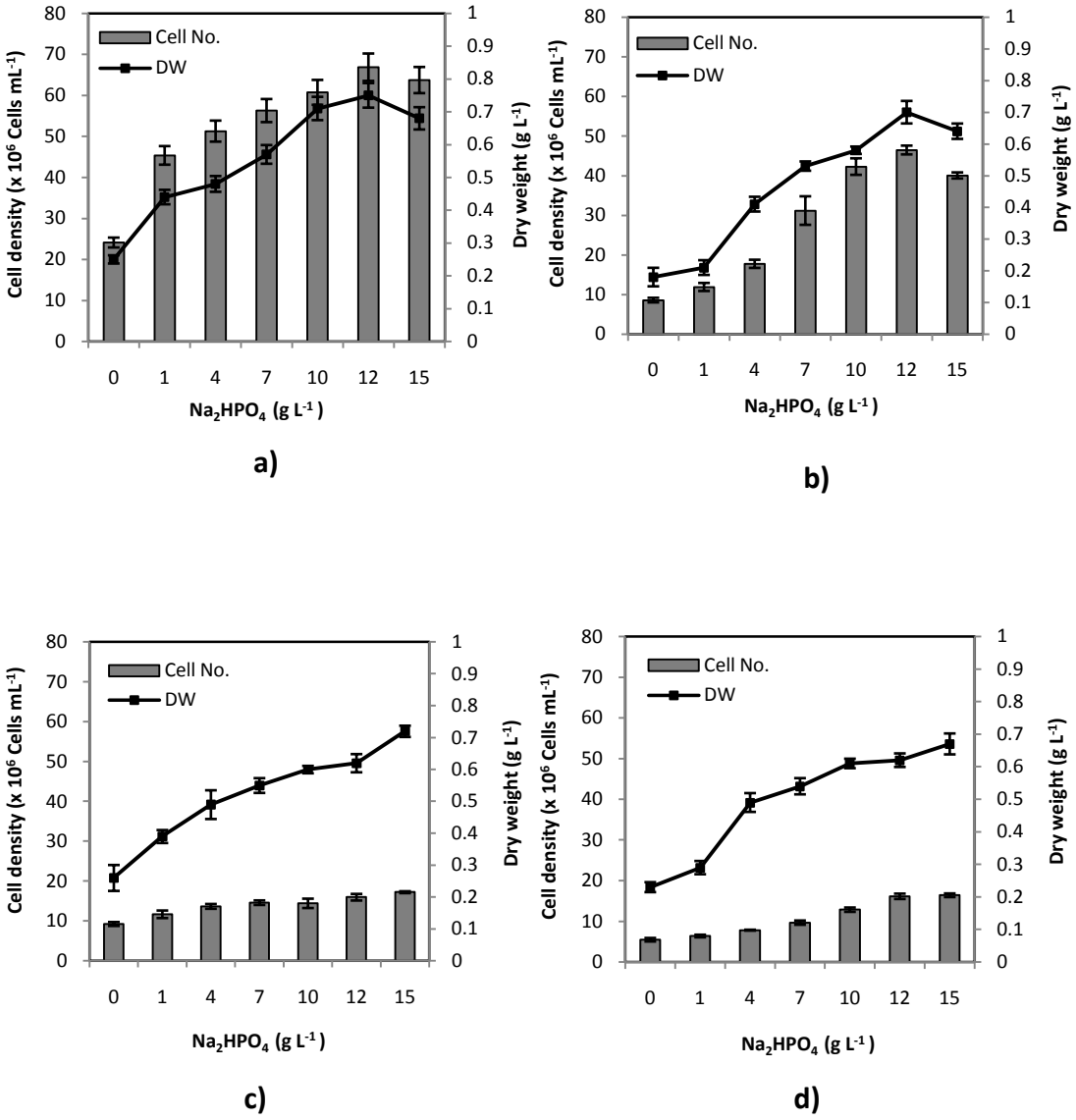


Fig. 5.2 Effects of Na_2HPO_4 on cell density and dry weight of a) *N. oculata*; b) *T. Suecica*; c) *I. galbana* and d) *P. lutheri*

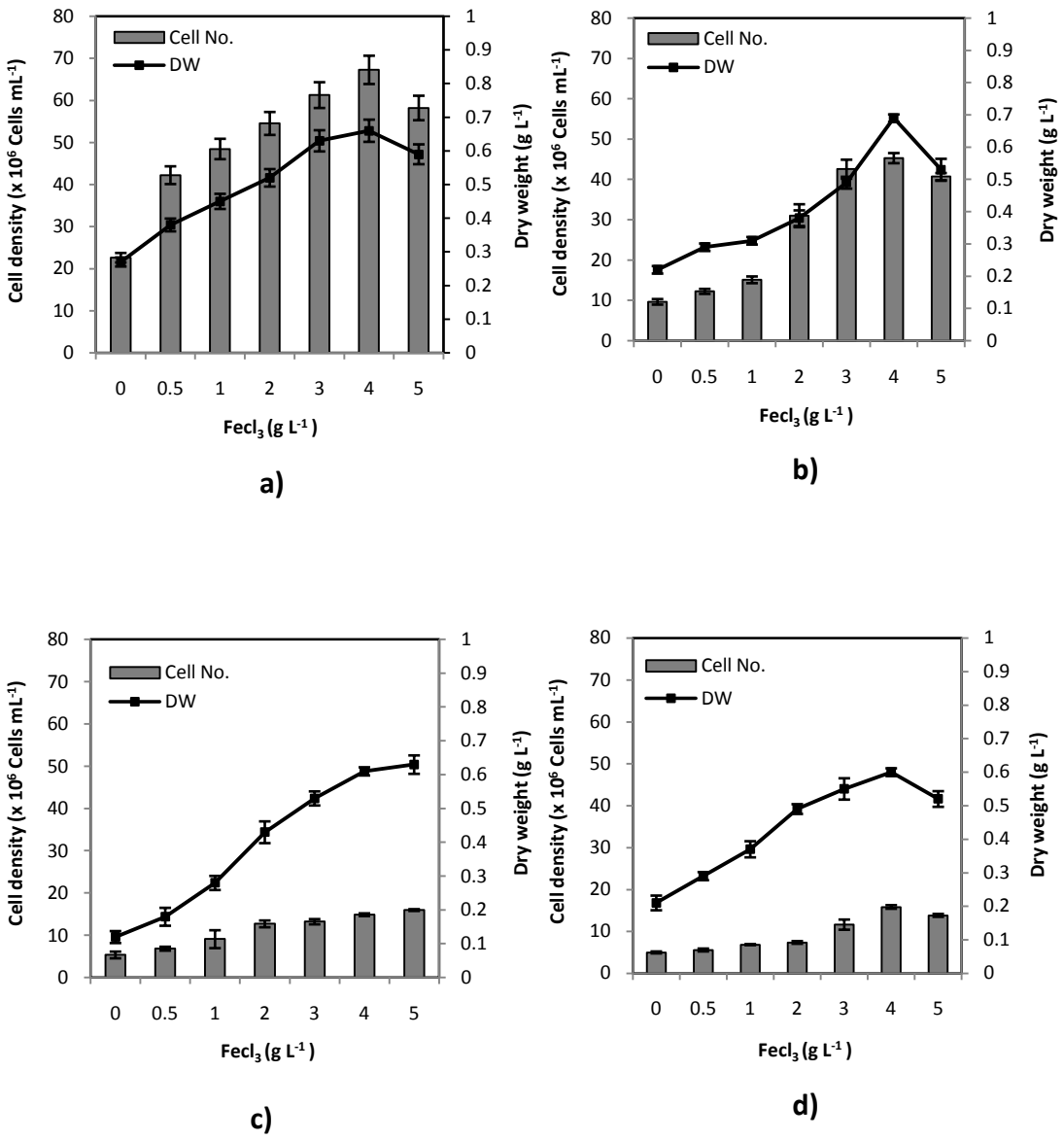


Fig. 5.3 Effects of $FeCl_3$ on cell density and dry weight of a) *N. oculata*; b) *T. suecica*; c) *I. galbana* and d) *P. lutheri*

Table 5.1 Kinetics of cell growth and lipid production of *N. Oculata*, *T. suecica*, *I. galbana* and *P. lutheri*

Media Conditions		*N. Oculata*				*T. suecica*				*I. galbana*				*P. lutheri*			
		X'_{max} ($gL^{-1}d^{-1}$)	μ_{max} (d^{-1})	t_d (day)	Lipid (%)	X'_{max} ($gL^{-1}d^{-1}$)	μ_{max} (d^{-1})	t_d (day)	Lipid (%)	X'_{max} ($gL^{-1}d^{-1}$)	μ_{max} (d^{-1})	t_d (day)	Lipid (%)	X'_{max} ($gL^{-1}d^{-1}$)	μ_{max} (d^{-1})	t_d (day)	Lipid (%)
KNO_3 (g L^{-1})	0	0.115	0.11	6.25	29.3 ± 0.65	0.078	0.12	5.68	19.8 ± 0.95	0.106	0.10	6.93	16.6 ± 0.70	0.087	0.11	6.24	19.6 ± 2.93
	10	0.137	0.13	5.21	35.3 ± 1.65	0.106	0.14	4.77	25.3 ± 2.09	0.108	0.12	5.77	18.5 ± 0.95	0.091	0.12	5.77	28.9 ± 3.18
	40	0.145	0.14	4.95	29.2 ± 0.73	0.115	0.15	4.72	18.3 ± 1.11	0.131	0.15	4.47	19.4 ± 1.84	0.124	0.13	5.21	37.1 ± 1.39
	70	0.143	0.15	4.62	30.3 ± 0.37	0.141	0.16	4.25	17.2 ± 0.41	0.141	0.15	4.47	26.4 ± 1.08	0.132	0.14	5.06	34.2 ± 0.84
	100	0.151	0.15	4.54	30.4 ± 1.58	0.149	0.18	3.93	16.4 ± 1.03	0.146	0.16	4.34	22.6 ± 0.89	0.137	0.15	4.74	30.1 ± 1.45
	120	0.152	0.16	4.30	27.2 ± 1.13	0.170	0.18	3.87	15.6 ± 0.86	0.150	0.17	4.06	21.1 ± 0.96	0.143	0.16	4.30	27.5 ± 1.05
	150	0.156	0.17	4.07	24.5 ± 0.85	0.152	0.17	4.00	15.7 ± 0.97	0.147	0.15	4.50	17.8 ± 2.05	0.148	0.16	4.25	29.4 ± 1.87
Na_2HPO_4 (g L^{-1})	0	0.084	0.10	6.86	25.6 ± 0.53	0.075	0.12	5.92	14.2 ± 0.26	0.098	0.13	5.21	16.8 ± 1.84	0.078	0.12	5.54	17.6 ± 2.94
	1	0.101	0.13	5.33	25.5 ± 0.91	0.091	0.14	4.95	16.4 ± 1.93	0.113	0.13	5.33	17.5 ± 1.31	0.089	0.13	5.21	26.0 ± 1.51
	4	0.108	0.13	5.17	28.6 ± 1.45	0.136	0.14	4.95	18.3 ± 0.97	0.124	0.14	4.85	19.7 ± 1.95	0.125	0.13	5.12	34.2 ± 1.02
	7	0.116	0.14	4.95	33.5 ± 1.54	0.147	0.15	4.47	23.8 ± 1.92	0.126	0.14	4.81	26.8 ± 0.98	0.125	0.14	4.82	30.3 ± 0.97
	10	0.135	0.15	4.54	30.5 ± 0.73	0.155	0.17	3.96	20.9 ± 2.04	0.134	0.14	4.81	25.1 ± 1.21	0.128	0.15	4.53	29.6 ± 1.24
	12	0.140	0.16	4.30	27.7 ± 1.45	0.170	0.17	4.00	17.9 ± 0.97	0.130	0.15	4.50	23.2 ± 0.48	0.130	0.15	4.53	26.7 ± 1.35
	15	0.137	0.18	3.85	26.4 ± 1.60	0.154	0.17	4.07	13.7 ± 2.02	0.164	0.18	3.92	18.5 ± 1.79	0.131	0.16	4.33	24.0 ± 3.59
$FeCl_3$ (g L^{-1})	0	0.091	0.12	5.77	22.3 ± 3.26	0.095	0.12	5.77	12.4 ± 0.53	0.091	0.10	6.66	15.4 ± 0.95	0.080	0.12	5.77	15.4 ± 2.07
	0.5	0.119	0.14	4.95	25.5 ± 2.37	0.107	0.12	5.72	17.6 ± 1.19	0.106	0.12	5.63	18.4 ± 2.84	0.088	0.12	5.77	20.1 ± 2.17
	1	0.117	0.14	4.89	27.4 ± 1.87	0.113	0.13	5.37	19.3 ± 0.74	0.094	0.13	5.21	19.8 ± 1.10	0.109	0.14	4.95	27.0 ± 2.49
	2	0.122	0.15	4.62	33.1 ± 2.44	0.120	0.17	4.17	24.4 ± 0.55	0.121	0.16	4.36	25.7 ± 0.94	0.132	0.13	5.33	34.5 ± 1.11
	3	0.145	0.16	4.30	29.5 ± 0.84	0.141	0.17	4.00	22.7 ± 0.98	0.130	0.16	4.33	22.4 ± 1.30	0.127	0.14	4.81	29.4 ± 0.63
	4	0.150	0.16	4.30	26.4 ± 3.00	0.160	0.18	3.78	17.4 ± 0.76	0.128	0.17	4.12	21.0 ± 1.14	0.134	0.15	4.62	28.9 ± 0.51
	5	0.139	0.15	4.62	23.2 ± 2.61	0.147	0.17	4.10	16.8 ± 1.01	0.138	0.17	4.12	19.4 ± 1.92	0.122	0.14	4.95	26.5 ± 0.84

5.1.2 Optimal Conditions and Interactions between Factors

Response surface analysis was carried out in this study to determine the second-order behaviour of the factors, to model the relationship between the factors and the response, as well as the factor settings that produce the best response. The predictive model used to generate response surface graphs and contour plots. When the problem involves the data that are subjected to experimental errors, statistical methods measure the effects of change in operating variables and their mutual interactions on the process performance through factorial experimental designs. The data collected from the batch runs were used to develop empirical models to describe the experimental results (APPENDIX C). Estimated response surface plots of interaction between nitrate, phosphate and iron for biomass and lipid (Fig 5.4 a-d and Fig 5.6 a-d) show that all the four microalgae had higher biomass and lower lipid at elevated levels of nitrate, phosphate and iron while higher lipid and lower biomass was observed under nutrient deficiency. *P. lutheri* showed the highest lipid accumulation of 37.2% with reduced cell growth of 0.38 g L^{-1} when cultures were grown under deficiency conditions of KNO_3 (65 g L^{-1}), Na_2HPO_4 (3 g L^{-1}) and $FeCl_3$ (2.5 g L^{-1}). The highest lipid accumulation of 37.3 and 23.6% with slightly reduced cell growth (0.64 g L^{-1} and 0.49 g L^{-1}) were achieved for *N. oculata* and *T. suecica*, respectively when cultures were grown under deficiency conditions at 10 g L^{-1} KNO_3, 7.5 g L^{-1} Na_2HPO_4 and 2.5 g L^{-1} $FeCl_3$. The highest lipid accumulation for *I. galbana* was 28.3% with a slightly reduced cell growth of 0.54 g L^{-1} achieved under deficiency conditions of KNO_3 (65 g L^{-1}), Na_2HPO_4 (7.5 g L^{-1}) and $FeCl_3$ (2.5 g L^{-1}). *T. suecica, I. galbana* and *P. lutheri* showed the highest cell dry weight (0.81 g L^{-1}, 0.63 g L^{-1} and 0.68 g L^{-1}) but low lipid content (18.5 %, 25.4 % and 25.2 %), respectively at 120 g L^{-1} KNO_3, 12 g L^{-1} Na_2HPO_4 and 4 g L^{-1} $FeCl_3$, while *N. oculata* showed the cell dry weight of 0.78 g L^{-1} and 25.6% lipid at 120 g L^{-1} KNO_3, 7.5 g L^{-1} Na_2HPO_4 and 4 g L^{-1} $FeCl_3$.

Figure 5.5 (a-d) and Fig 5.7 (a-d) show that all nutrients affected cell growth positively, though the interaction of phosphate-phospate for *N. oculata* and interaction of nitrate-nitrate for *I. galbana* may affected negatively the growth. Nitrate (p< 0.0001), iron (p< 0.0001-0.1019), and phosphate (p< 0.0268-0.1649) have the most significant positive effects on biomass for all the four tested species.

However, nitrate, phosphate, phosphate-phosphate and ferum-ferum interactions may all affect lipid content, negatively, which explains the need for nutrient deficiency to achieve high lipid content. The second-order polynomial equations representing the dry weight and lipid content for all variables show that the r^2 were 78.7-90.8% for biomass and lipid. The mean absolute error percentage between experimental and predicted values of 0.02-0.04% and 0.67-1.33%, for biomass and lipid content suggest a good agreement between experimental and predicted values for all the four tested species.

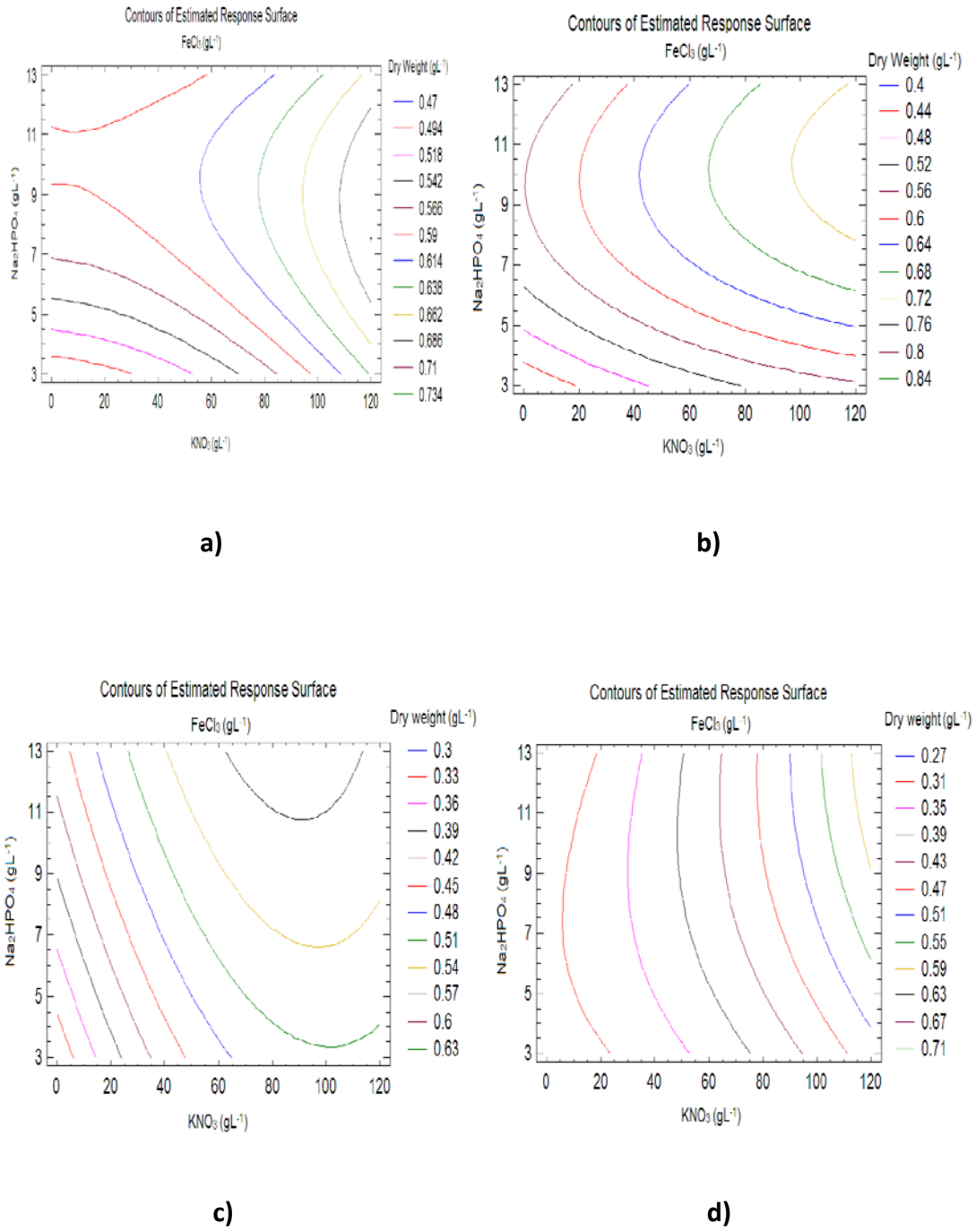


Fig. 5.4 Estimated response surface plot of interactions between KNO_3, Na_2HPO_4 and $FeCl_3$ on dry weight of a) *N. oculata*; b) *T. suecica*; c) *I. galbana* and d) *P. lutheri*

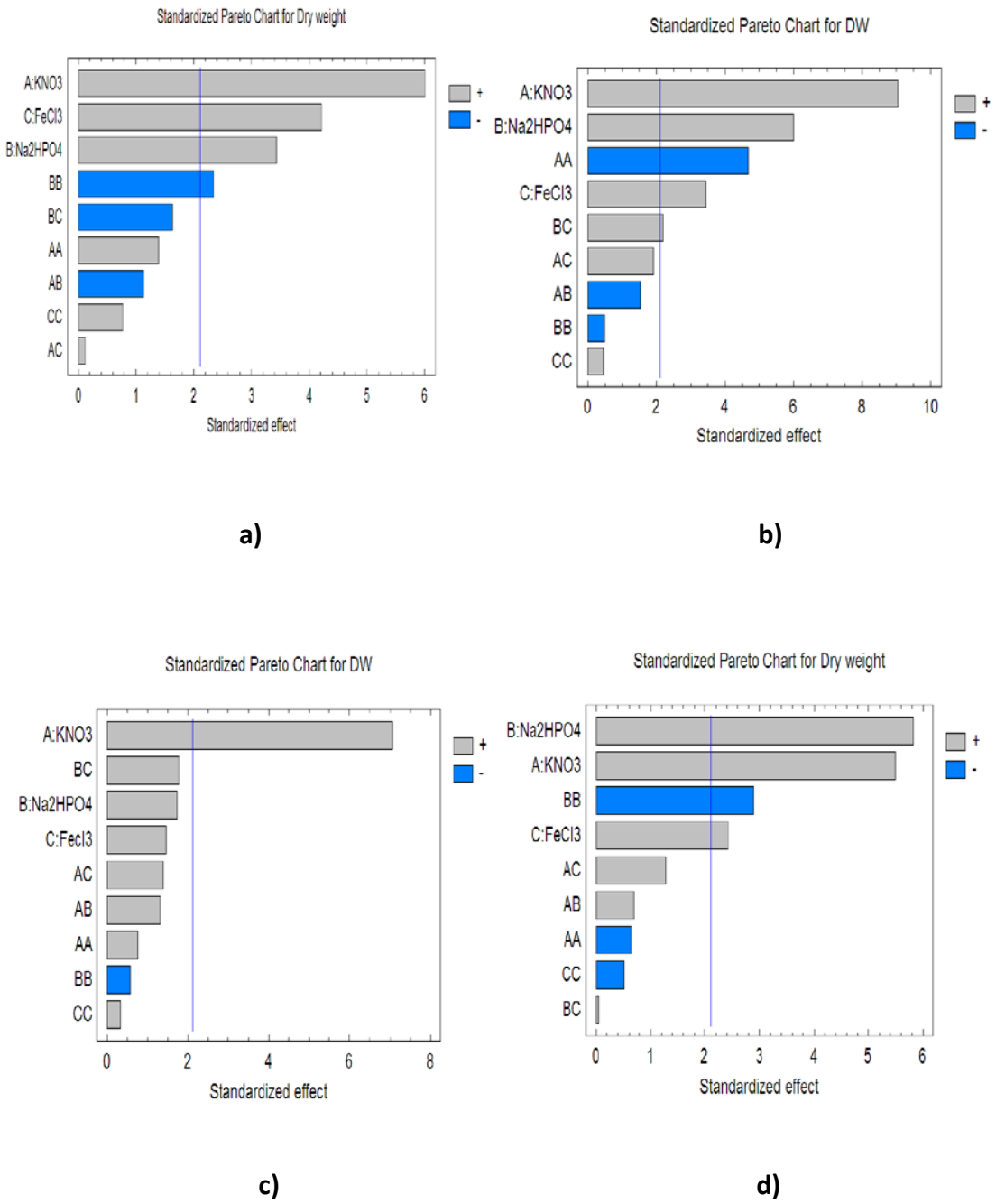


Fig. 5.5 Standardized Pareto chart for interactions between KNO_3, Na_2HPO_4 and $FeCl_3$ on dry weight of a) *N. oculata*; b) *T. suecica*; c) *I. galbana* and d) *P. lutheri*

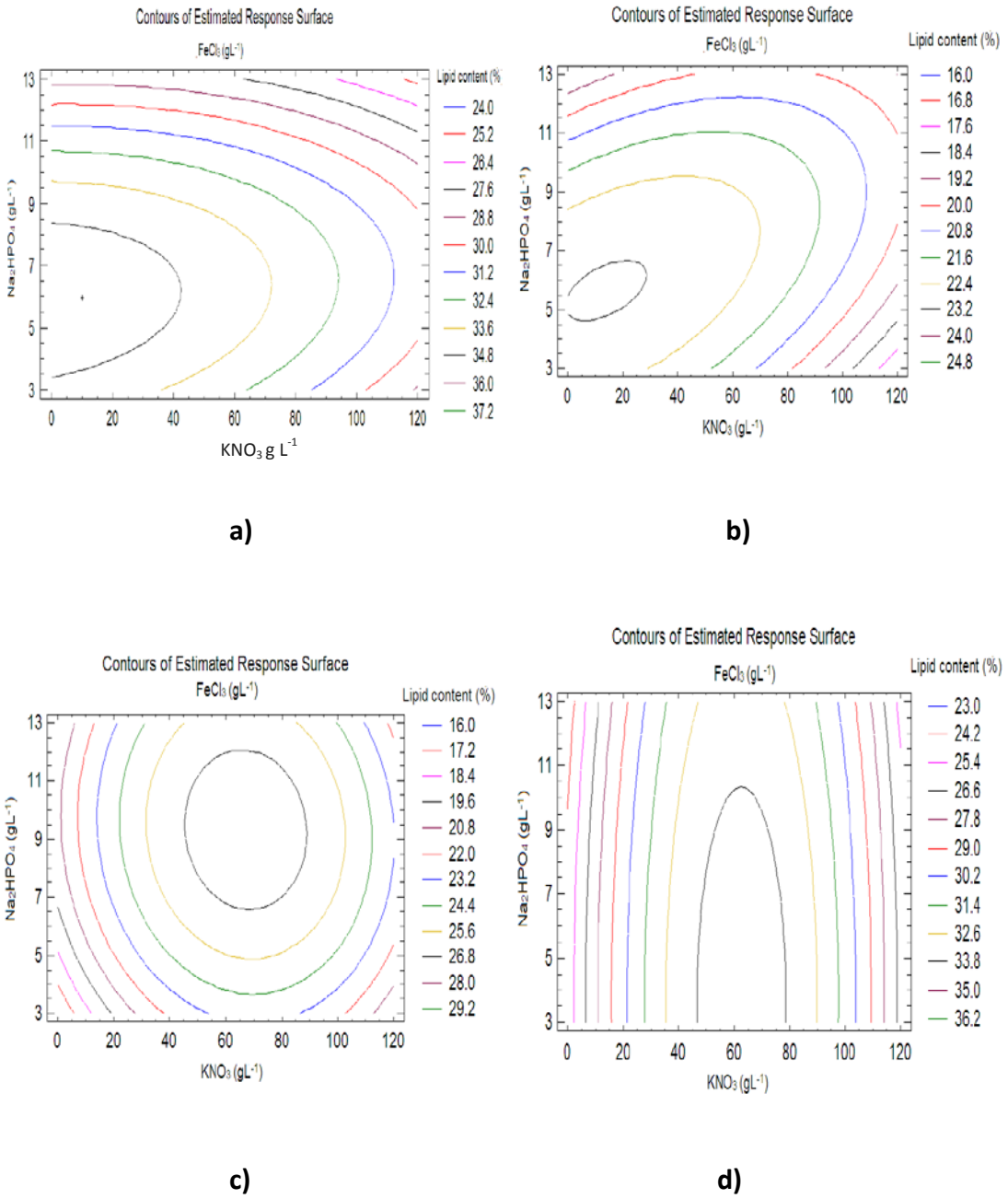


Fig. 5.6 Estimated response surface plot of interactions between KNO_3, Na_2HPO_4 and $FeCl_3$ on lipid content of a) *N. oculata*; b) *T. suecica*; c) *I. galbana* and d) *P. lutheri*

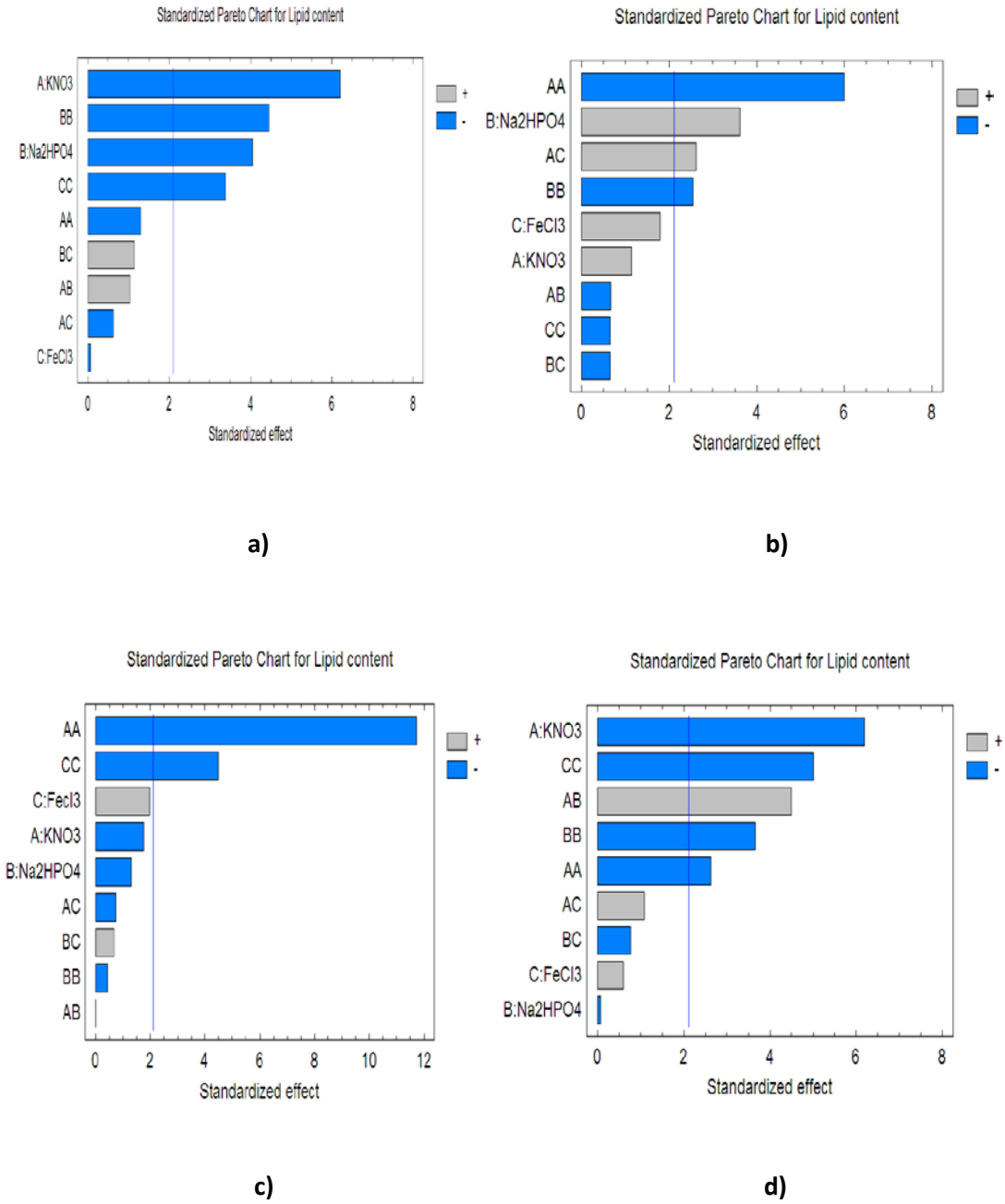


Fig. 5.7 Standardized Pareto chart for interactions between KNO_3, Na_2HPO_4 and $FeCl_3$ on lipid content of a) *N. oculata*; b) *T. suecica*; c) *I. galbana* and d) *P. lutheri*

5.2 Effects of Palm Oil Mill Effluent (POME)

5.2.1 Cell Growth and Lipid Production

Raw POME had high concentrations of chemical and inorganic components suitable as nutrients for the culturing of microalgae. Fig. 5.8 shows the growth curve of *N. oculata, T. suecica, I. galbana* and *P. lutheri* cultivated under control, 1%, 5%, 10%, 15% and 20% POME media. The highest cell density of 66.2 x 10^6 cells mL^{-1} and dry weight of 0.84 g L^{-1} was obtained under 10% POME composition for *N. oculata*. This corresponds to the rate X'_{max} of 0.151 g L^{-1} d^{-1}, t_d of 3.3 day, μ_{max} of 0.21 d^{-1} and lipid content of 39.1 ± 0.73%. The cell growth with POME media was comparable to control which showed cell density of 68.9 x 10^6 cells mL^{-1} and dry weight of 0.88 g L^{-1}, but with lower lipid content of 31.5 ± 1.47%. *T. suecica* showed the highest cell density of 38.7 x 10^6 cells mL^{-1}, dry weight of 0.74 g L^{-1} and lipid content of 27.0 ± 0.61% under 10% POME. With *I. galbana* and *P. lutheri*, the highest cell density of 15.4 x 10^6 and 14.2 x 10^6 cells mL^{-1} were lower than control but had higher lipid content (26.3 ± 0.31, 34.5 ± 0.82%) under 15% POME, respectively. There is a statistically significant difference between the mean dry weight and lipid content from one level of POME composition to another ($P < 0.05$). This shows the potential of POME as economical media to culture algae with enhanced lipid content.

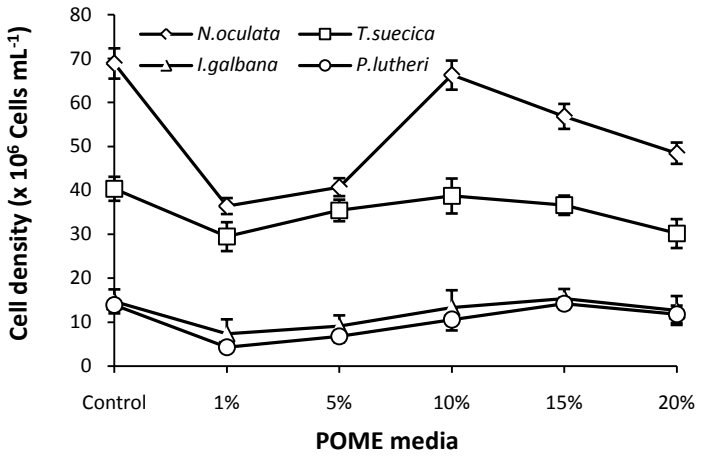


Fig. 5.8 Growth curve of *N. oculata*, *T. suecica*, *I. galbana* and *P. lutheri* cultivated under control, 1%, 5%, 10%, 15% and 20% POME media

Table 5.2 Kinetics of cell growth and lipid production of microalgae cultivated under control and different composition of POME in sea water

Media conditions		X'_{max} ($gL^{-1}d^{-1}$)	μ_{max} (d^{-1})	t_d (day)	Lipid (%)
N. oculata	Control	0.152	0.18	3.85	31.5 ± 1.47
	1 %	0.133	0.17	4.07	30.9 ± 1.53
	5 %	0.141	0.18	3.85	34.1 ± 1.20
	10 %	0.151	0.21	3.30	39.1 ± 0.73
	15 %	0.134	0.20	3.46	27.7 ± 1.12
	20 %	0.123	0.15	4.62	26.2 ± 1.32
T. suecica	Control	0.153	0.19	3.78	24.4 ± 0.28
	1 %	0.121	0.17	4.07	20.7 ± 0.38
	5 %	0.125	0.18	3.85	22.9 ± 0.42
	10 %	0.144	0.20	3.62	27.0 ± 0.61
	15 %	0.124	0.18	3.76	24.3 ± 0.46
	20 %	0.136	0.14	4.81	21.4 ± 0.93
I. galbana	Control	0.152	0.15	4.66	22.3 ± 1.94
	1 %	0.104	0.11	6.07	15.4 ± 1.20
	5 %	0.108	0.12	5.77	17.3 ± 0.95
	10 %	0.128	0.15	4.71	19.7 ± 0.97
	15 %	0.142	0.16	4.26	26.3 ± 0.31
	20 %	0.094	0.13	5.21	19.8 ± 1.10
P. lutheri	Control	0.137	0.15	4.74	29.7 ± 1.45
	1 %	0.097	0.12	5.77	19.5 ± 3.05
	5 %	0.110	0.12	5.63	22.8 ± 1.83
	10 %	0.122	0.14	4.95	27.5 ± 0.84
	15 %	0.130	0.14	4.95	34.5 ± 0.82
	20 %	0.117	0.13	5.37	26.3 ± 0.96

5.2.2 POME Characterization

Due to the nature of POME and its dark colour, the suitable percentage based on Table 5.2 for optimum growth and lipid content of the marine *N. oculata* and *T. suecica* was 10% and for *I. galbana* and *P. lutheri* was 15%. The raw POME collected from FELCRA Nasaruddin Bota, Perak, Malaysia had initial temperature of $80^{o}C \pm 3$ when collected (Table 5.3). With increasing POME ratio from 1−20% before inoculation of microalgal species, the decreasing trend of pH (7 to 4.7) could be observed. The highest average COD of 11129 mg L^{-1} and lowest average COD of 627 mg L^{-1} and the highest average BOD of 2985 mg L^{-1} and lowest average BOD of 183 mg L^{-1} were recorded at 20% and 1% POME before algal inoculation.

5.2.3 POME Remediation

At the end of culture on day 16, pH becoming more basic around 7−8.7 with *N. oculata*, 6.5-8.1 with *T. suecica*, 7.3-8.8 with *I. galbana* and 6.8-8.4 with *P. lutheri*. The removal efficiencies varied with different dilutions of wastewater. Higher removal of COD (90-95%) and TOC (64-75%) were achieved for 1–20% POME dilution after addition of *N. oculata*, and removal of COD (86.6-93.6%) and TOC (43.1-71.1%) were achieved after addition of *T. suecica*. Lower removal of COD (74.5-80.1%) and TOC (43.1-62.3%) were achieved after addition of *I. galbana* and *P. lutheri*. The BOD removal (88.5- 97%, 81.5- 96%) were highest for 1–20% POME after addition of *N. oculata* and *T. suecica* while only 68.3-82.5% removal achieved after *I. galbana* and *P. lutheri* addition. The highest removal of TN (90.8%) was achieved in 1% POME after the addition of *N. oculata*. *T. suecica* achieved 78.8% removal at 10% POME but *I. galbana* (57.5%) at 20% POME and *P. lutheri* (61.3%) at 15% POME were clearly ineffective. The 94.9% removal of oil and grease was achieved in 15% POME with *N. oculata* and 92% removal was obtained in 10% POME with *T. suecica*. Both *I. galbana* and *P. lutheri* were again poor oil and grease remover at 60.6-68.8 at 10% and 15% POME, respectively.

Table 5.3 Chemical characteristics of POME (mg L^{-1}) and removal efficiency (%) in different dilutions with sea water before and after inoculation of *N. oculata*, *T. suecica. I. galbana* and *P. lutheri*

POME: Sea Water (v/v)	pH	COD	BOD	TOC	TN	Oil and grease
1. Raw						
1 %	6.5-7	627	183	33.4	5.4	30.3
5 %	6.2	2974	843	153.1	28.6	144.5
10 %	5.5	5839	1642	285.5	56.7	285.1
15 %	4.7	8947	2448	456.8	80.3	364.5
20 %	3.8	11129	2985	618.4	98.5	438.2
2. *N. oculata*						
1 %	7	63 (90%)	21 (88.5%)	12 (64%)	0.5 (90.8%)	5.3 (82.5%)
5 %	7.5	145 (95%)	32 (96.2%)	53 (65.3%)	4 (86%)	9.8 (93.2%)
10 %	7.8	375 (93.6%)	47 (97%)	87 (69.5%)	7 (87.7%)	15.6 (94.5%)
15 %	8.3	558 (93.8%)	94 (96%)	115 (74.8%)	13 (83.8%)	18.7 (94.9%)
20 %	8.7	876 (92.1%)	152 (94.9%)	174 (71.7%)	20 (79.6%)	24.2 (94.4%)
3. *T. suecica*						
1 %	7	84 (86.6%)	34 (81.5%)	19 (43.1%)	2 (62.3%)	9.3 (69.3%)
5 %	6.5	196 (93.4%)	47 (94.4%)	67 (56.2%)	7 (75.5%)	16.7 (88.4%)
10 %	7.3	463 (93.6%)	62 (96%)	104 (63.3%)	12 (78.8%)	22.8 (92%)
15 %	7.2	638 (92.8%)	114 (95.3%)	132 (71.1%)	18 (77.6%)	31.7 (91.3%)
20 %	8.1	825 (92.6%)	178 (94%)	183 (70%)	23 (76.6%)	43.6 (90.1%)
4. *I. galbana*						
1 %	7.3	148 (76.4%)	58 (68.3%)	21.3 (43.1%)	2.8 (48.7%)	12.4 (59.1%)
5 %	7.5	716 (75.9%)	251 (70.2%)	78.4 (48.8%)	13.3 (53.5%)	55.7 (61.4%)
10 %	7.8	1394 (76%)	440 (73.2%)	130.2 (54.4%)	25.5 (55%)	112.2 (60.6%)
15 %	8.4	2018 (77.4%)	605 (75.3%)	197.5 (56.8%)	34.2 (57.4%)	143.8 (60.5%)
20 %	8.8	2833 (74.5%)	753 (74.8%)	260.3 (57.9%)	41.9 (57.5%)	171.2 (60.9%)
5. *P. lutheri*						
1 %	6.8	128 (79.6%)	47 (74.3%)	18.3 (45.2%)	2.5 (53.7%)	10.2 (66.3%)
5 %	7.2	666 (77.6%)	213 (74.7%)	73.1 (52.2%)	12.4 (56.6%)	51.8 (64.1%)
10 %	7.5	1274 (78.2%)	337 (79.5%)	121.2 (57.5%)	22.7 (59.9%)	94.3 (66.9%)
15 %	7.9	1784 (80.1%)	484 (80.2%)	172.3 (62.3%)	31.1 (61.3%)	113.7 (68.8%)
20 %	8.4	2451 (78%)	523 (82.5%)	236.3 (61.8%)	39.8 (59.6%)	138.5 (68.4%)

Note: Microalgae inoculated at 1-20% (POME: Sea water v/v) and grown for two weeks before analyses.

5.3 Discussion

5.3.1 Effects of Macronutrients and Iron

Three constituents of TMRL enrichment medium (KNO_3, Na_2HPO_4 and $FeCl_3$) were adopted for stress conditions to enhance biomass and lipid production. The levels at 120–150 g L^{-1} KNO_3, 12–15 g L^{-1} Na_2HPO_4 and 4–5 g L^{-1} $FeCl_3$ are the best to promote biomass. For enhanced lipids, the levels were 10–70 g L^{-1} KNO_3, 4–7 g L^{-1} Na_2HPO_4 and 2 g L^{-1} $FeCl_3$. Lipid production is highly affected by nitrate and phosphate concentration where excesses could inhibit lipid production while iron shows little effect.

The biomass productivities achieved with *N. oculata* at 0.08-0.170 g L^{-1} day^{-1} were lower than the reported values of *Chlorella* sp. at 0.28, 0.54 and 0.30 g L^{-1} day^{-1} (initial pH 7.2, ammonium 17 mM, phosphate 1.2 mM), *D. salina* DCCBC2 (initial pH 8.0, nitrate 3.3 mM, phosphate 0.0375 mM) and *Dunaliella* sp. (initial pH 8.0, nitrate 3.7 mM, phosphate 0.17 mM), respectively [184]. Despite these low biomass of *N. oculata* and *P. lutheri*, the lipid accumulation at 35−37.2% were relatively higher than the total lipid contents of 29.8, 35.9 and 32.1% respectively, reported for *T. subcordiformis* SHOU-S05, *N. oculata* SHOU-S14 and *P. viridis* SHOU-S16 cultured in different amounts of nitrogen level [185]. The lower biomass accumulation and increased lipid yields (40-46%) have also been reported in nitrogen-deficient *C. minutissima* [186]. The increase of lipid contents in *N. oculata* from 7.9% to 15.31% and *C. vulgaris* from 5.9% to 16.41% have been reported with reduced nitrogen as low as 75% [48]. Nitrogen stress on *N. oculata* (75 gL^{-1}, 37.5 g L^{-1} and 150 g L^{-1}) and *C. vulgaris* (75 g L^{-1}, 150 g L^{-1} and 300 g L^{-1}) with nitrate in the form of $NaNO_3$ have been reported under light intensity of 70 μE m^{-2} s^{-1} and temperature 20ºC. Low nitrogen in the growth medium increases the lipid production because higher nitrogen extends growth cycle which results in lower lipid production per day [48].

The present results may suggest specific metabolic pathways related to the lipid accumulation being turned on under nutrient deficient media. At low nutrient

concentrations, protein synthesis is low and most of fixed carbon is converted to carbohydrate or lipid.

Thus, concentration of biomass decreases, while accumulation of lipid increases. High lipid production when algae are starved with nitrogen leads to longer duration than the normal time to yield the same amount from non-starved microalgae. The cell division is possibly delayed and the biochemical structure of cell is altered from the original chemical composition due to the stress and converted to oil or starch for survival [48]. The increase in TAG levels from 6.5% up to 39.3% of total lipids has been reported under the condition of phosphorus limitation [179]. Phosphate deficiency changes the lipid accumulation as reported for *Monodus subterraneus* where the cellular contents of digalactosyldiacylglycerol (DGDG) and diacylglyceroltrimethylhomoserine (DGTS) increase sharply, respectively, from 0.29 and 0.19 to 0.60 and 0.38 fg $cell^{-1}$ [187]. The reduced or oxidized form of Fe ion may also influence cell productivity where cell density increases by adding chelated Fe^{3+} but behaves negatively with the addition of chelated Fe^{2+} at high concentrations [18]. Increasing Fe^{2+} levels from 2.5 to 4 g L^{-1} enhances the biomass production, while Fe^{2+} limitation increases lipid content upto 56.6% in *C. vulgaris*. The marine diatom *Amphiprora paludosa* accumulates high lipid content of 65.6%, 63.2% and 57.8% at 0.026, 0.023 and 0.009 mM Fe EDTA, respectively [188] while the maximum biomass production of 3.56 g L^{-1} is reported at 18 μmol L^{-1} iron [189]. On the basis of fluorescence measurements taken over 12 years, it has been demonstrated that iron has a key function in regulating phytoplankton biomass in both high nitrate low-chlorophyll and oligotrophic waters near the Equator and further south. However, the ‘‘bioavailable’’ iron deficiency and some biochemical components such as lipids in response to iron have not been well documented in microalgae [119].

5.3.2 Effects of POME Media

The biomass formation rate (0.144−0.151 g L^{-1} d^{-1}) and specific growth rates (0.14−0.21 d^{-1}) in the present study were lower than those reported values of 0.27 g $L^{-1}d^{-1}$ and μ_{max} of 0.49 d^{-1} with *Auxenochlorella protothecoides* UMN 280, but

the lipid accumulation at 26−39% were relatively higher than the reported 28.9% total lipid content of the latter, cultured in concentrated municipal wastewater [190].

The higher biomass and lipid production for the 10-15% POME in sea water was likely due to higher and balanced nutrient concentrations. A study on a mixture of green algae and diatoms show increase in biomass from 0.5-0.9 g L^{-1} and lipid content from 14-29% when the waste water composition is increased from 10-25% [138]. The higher biomass accumulation has also been reported for *Chlorella* sp. grown on concentrated municipal wastewater [152]. A study on marine *Isochrysis* sp. utilizing 5% POME-fortified medium achieves maximum biomass of 91.7 mg m^{-2} day^{-1} and lipid content of 52.8 ± 2.4% under 10 L outdoor culture system [191]. Another report suggests that algae can grow optimally at 14% POME, followed by 10%, 20% and 30% [192]. Raw POME has been reported previously at 60-65 ^{o}C at the time of discharge to collection pit with an acidic pH between 4 to 5 and biological oxidation demand (BOD) between 10250 to 43750 mg L^{-1} [193, 194]. These may not be suitable for mesophilic methanogens without preconditioning should biogas or biomethane production is the aim. POME contains 50000 mg L^{-1} chemical oxygen demand (COD), 750 mg L^{-1} total nitrogen (TN), 95–96% water, 4–5% total solids and 0.6–0.7% oil and grease and typically the oil and grease mean value is 6000 mg L^{-1} [195].

The more basic pH was most probably due to the absorption of nitrogen and lack of CO_2 sparging. The highest growth rate of *Scenedesmus obliquus* is reportedly achieved at a constant pH of 7 [196]. In microalgal cultivation, pH value usually increases because of the photosynthetic CO_2 assimilation and affects the availability of inorganic carbon. Some species such as *A. protothecoides* UMN280 tolerates high pH in concentrated municipal wastewater suggesting that pH variation may not be the major limiting factor for microalgae in wastewater [197]. The COD removal was enhanced when the POME concentration was increased to 10% and 15% which was in agreement with the 76% COD removal from piggery wastewater associated with microbe in the high rate algal ponds [198]. A study with *A. protothecoides* UMN280 achieves the removal efficiency of 88% COD and 96% TOC when the algae is grown in concentrated municipal wastewater [190].

Different algal strain could utilize the different organic compounds as carbon sources at different efficiency. The organic substances may function directly as an essential organic nutrient or act as an accessory growth factor.

However microalgae could utilize the available dissolved oxygen to break down organic material. Low COD removal of 41.8% has been reported in the axenic culture condition of *Desmodesmus* sp.CHX1 [199]. *Synechocystis* sp. achieves 98% BOD removal from treated wastewater under hydraulic residence time of 24 h [200]. The algae-based sewage treatment plant (STP) has reportedly achieved total BOD removal of 82 % [201]. A three-stage aquaculture of certain macrophytes and algae, such as *Eichhornia crassipes, Microcystis aeruginosa, Scenedesmus falcatus, Chlorella vulgaris* and *Chlamydomonas mirabilis* involving a water hyacinth culture in the first stage, followed by an algal culture, and finally a second water hyacinth also achieved BOD reductions around 96.9% when tested in the laboratory conditions [202].

TN is the sum of organic nitrogen, ammonia (NH_3), and ammonium (NH_4^+) in the chemical analysis of wastewater. Total Kjeldahl nitrogen (TKN) removal of 36%, ammonium N (NH_4 -N) removal efficiency of 18%, nitrate (NO_3 -N) removal efficiency of 22%, and nitrite (NO_2 -N) removal efficiency of 57.8% have been reported for algae-based STP where the predominant algae are euglenoides and chlorophycean [201]. Green *Chlorella* sp. when grown in different wastewaters from municipal wastewater treatment plant achieve 50.8%– 82.8% TN removal [176]. The relative constancy of uptake, irrespective of nitrogen source, is considered to be due to the saturation of the assimilator to the production of amino groupings for entry into nitrogenous metabolism. Nitrite is generated in the process of nitrate being reduced to ammonium and it is possible that part of the nitrite produced is excreted into the media [203]. When ammonia is utilized as N-source, pH could decrease significantly during active growth because of the release of H^+ ions, but with nitrate uptake pH could increase [107].

Oil and grease are poorly soluble in water due to their tendency to separate out from the aqueous phase. Although this characteristic is advantageous in facilitating the separation of oil and grease by the use of floatation devices, it does complicate the transportation of wastes through pipelines, the possible destruction in the biological

treatment unit, and disposal into receiving waters. The high removal of oil and grease content by the four algal strains suggest big potential to be considered for handling and treatment of the waste material for disposal. These can be co-cultivated with bacterial consortium.

Free Gram-negative bacteria (*Pseudomonas* sp., *P. diminuta* and *P. pseudoalcaligenes*) are effective for oil and grease removal from contaminated industrial effluents and are able to degrade the palm oil completely utilizing the free fatty acids (FFA) as a carbon source [204]. Besides carbon, nitrogen and phosphorus, other macro-nutrients (e.g potassium, calcium, and magnesium), micro-nutrients (manganese, molybdenum, copper, iron, zinc, boron, chloride and nickel) and some trace elements are important for microalgal cultivation. Many of trace elements are important in enzyme reaction and for the biosynthesis of value-added compounds [205]. Sea water used to make up POME media constituent, itself contains many natural macro and micro nutrients to fulfil microalgal growth requirements. Changes in all chemical parameters of the waste media after the culture of microalgal species, could pave the way for more environmentally-friendly method to treat wastes whilst benefiting from the algal cultivation for value-added products.

5.4 Summary

The highest lipid accumulation of 37.3, 23.6, 28.3 and 37.2% with slightly reduced cell growth of 0.64, 0.49, 0.54 and 0.38 g L^{-1} were achieved for *N. oculata*, *T. suecica*, *I. galbana* and *P. lutheri* when cultures were grown under deficiency conditions at 10-65 g L^{-1} KNO_3, 3-7.5 g L^{-1} Na_2HPO_4 and 2.5 g L^{-1} $FeCl_3$. All nutrients affected cell growth positively, though the interaction of phosphate-phospate for *N. oculata* and interaction of nitrate-nitrate for *I. galbana* may affect negatively the growth. The interaction of nitrate, phosphate, phosphate-phosphate and ferum-ferum interactions may all affect lipid content, negatively. The $r^2$of 78.7-90.5% for biomass and 80.1-90.8% for lipid, and mean absolute error of 0.02-0.042% and 0.67-1.33% for biomass and lipid content, respectively suggest a good agreement between experimental and predicted values. Lipid contents of 26.3-39.1% were attained under 10-15% POME.

The COD, TOC, BOD, TN and oil and grease removal efficiencies varied with different composition of wastewater. The removal of COD (76.1-95%), TOC (43.1-74.8%), BOD (68.3- 97%), TN (48.7-90.8%) and oil and grease (59.1-94.9%) were achieved for 1–20% POME after addition of microalgal strains.

CHAPTER 6

CELL GROWTH AND FATTY ACIDS PROFILES FOR ALGAL CULTIVATION IN PHOTOBIOREACTOR AND LARGE-SCALE REACTORS

6.1 Establishment of Kinetics in Shake-Flask and 1-30 L Batch Cultures

The cultivation was carried out at optimized conditions as shown in Table 6.1. The highest cell density of 64.6 x 10^6, 40.8 x 10^6, 15.5 x 10^6 and 14.5 x 10^6 cells mL^{-1} were achieved in 250 mL flask for *N. oculata*, *T. suecica*, *I. galbana* and *P. lutheri*, respectively (Fig. 6.1 a-d). In 30 L tank, the cell densities were lower at 52.6 x 10^6 and 10.7 x 10^6 cells mL^{-1} for *N. oculata* and *P. lutheri*, and were 36.4 x 10^6 and 13.4 x 10^6 cells mL^{-1} for *T. suecica* and *I. galbana* respectively in 5 L container. Only *N. oculata* and *P. lutheri* showed higher lipid content at 32-37%. The highest μ_{max} of 0.15 d^{-1}, t_d of 4.62 days and lipid content of 36.8 ± 1.68% were achieved for *N.oculata* in 250 ml shake-flask. Except for *T. suecica*, most μ_{max} were 0.13-0.15 d^{-1} and lipid at 24-28%. The highest μ_{max} achieved for *T. suecica* in 30 L tank was 0.17 d^{-1} with doubling time of 3.98 days and lipid content of 27.2 ± 2.67% (Table 6.1).

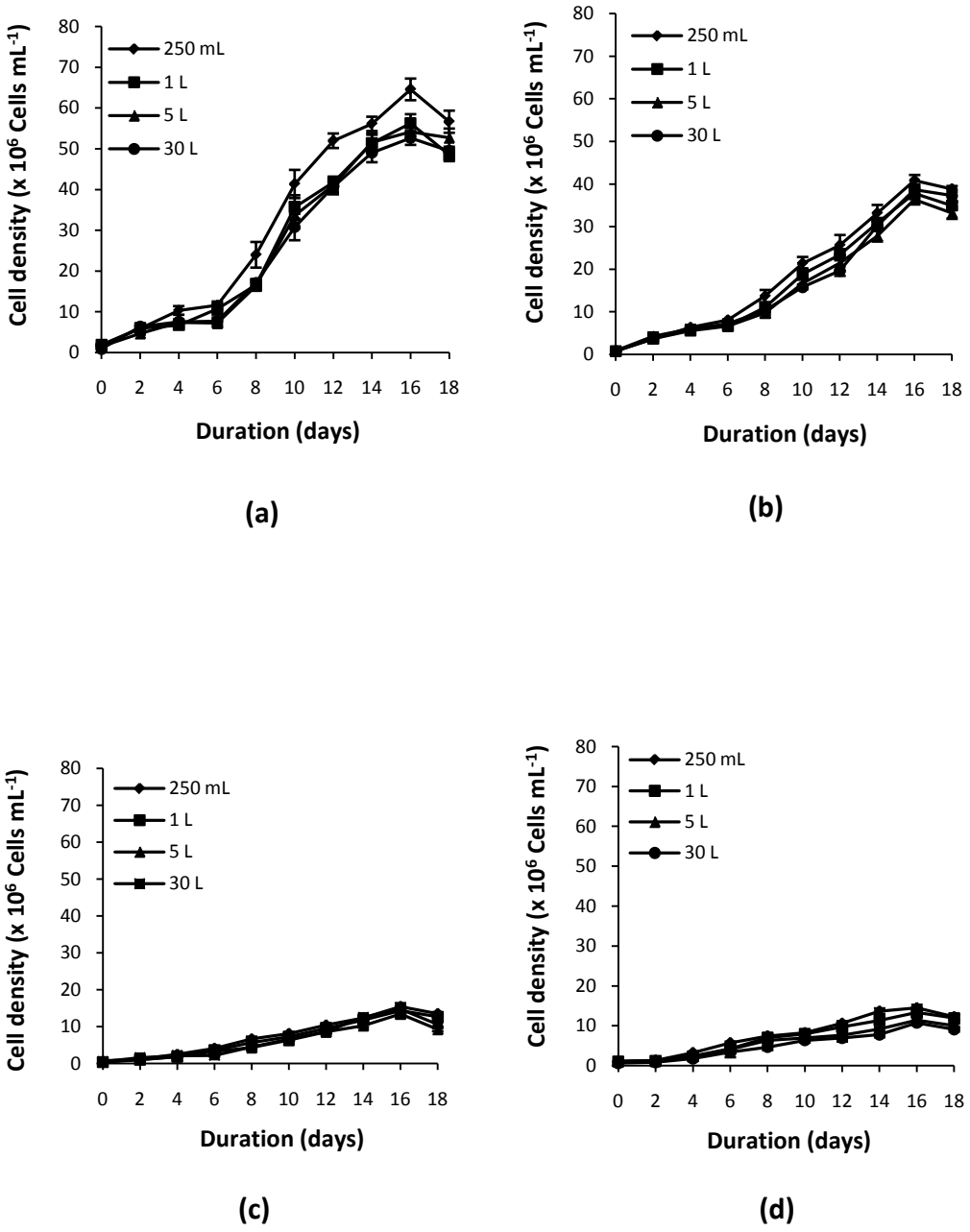


Fig. 6.1 Batch growth profile of a) *N. oculata*; b) *T. suecica*; c) *I. galbana* and d) *P. lutheri* cultivated in 250 mL flask, 1 L, 5 L and 30 L tank

Table 6.1 Kinetics of cell growth and lipid production in 250 mL shake flask and 1-30 L batch cultures at optimized conditions

Culture Parameters	*N. Oculata* [a]				*T. suecica* [b]				*I. galbana* [c]				*P. lutheri* [d]			
	X'_{max} ($gL^{-1}d^{-1}$)	μ_{max} (d^{-1})	t_d (day)	Lipid (%)	X'_{max} ($gL^{-1}d^{-1}$)	μ_{max} (d^{-1})	t_d (day)	Lipid (%)	X'_{max} ($gL^{-1}d^{-1}$)	μ_{max} (d^{-1})	t_d (day)	Lipid (%)	X'_{max} ($gL^{-1}d^{-1}$)	μ_{max} (d^{-1})	t_d (day)	Lipid (%)
250 mL	0.134	0.15	4.62	36.8 ± 1.68	0.120	0.16	4.33	26.8 ± 1.98	0.128	0.15	4.72	26.3 ± 1.06	0.124	0.15	4.74	37.1 ± 1.83
1 L	0.117	0.14	4.83	35.3 ± 1.35	0.115	0.15	4.72	25.4 ± 1.93	0.136	0.14	4.77	28.2 ± 1.20	0.120	0.14	4.77	35.8 ± 1.93
5 L	0.126	0.14	4.83	34.7 ± 0.88	0.113	0.14	4.77	25.1 ± 2.09	0.108	0.13	5.21	24.4 ± 3.03	0.110	0.13	5.21	34.3 ± 0.76
30 L	0.115	0.13	5.21	32.4 ± 1.43	0.136	0.17	3.98	27.2 ± 2.67	0.134	0.14	4.81	25.7 ± 0.94	0.085	0.13	5.33	34.5 ± 1.11

[a]*N. oculata* : pH 8, Salinity (35 ppt), photoperiod (24 hrs), light intensity (188 µmol photons m^{-2} s^{-1}) KNO3 (10 gL^{-1}), Na_2HPO_4 (6 gL^{-1}) and $FeCl_3$ (2.53 gL^{-1})

[b]*T. suecica* : pH 7.9 Salinity (32 ppt), photoperiod (24 hrs), light intensity (196.5 µmol photons m^{-2} s^{-1}), KNO3 (13.7 gL^{-1}), Na_2HPO_4 (5.6 gL^{-1}) and $FeCl_3$ (2.50 gL^{-1})

[c]*I. galbana* : pH 9, Salinity (39.2 ppt), photoperiod (20.5 hrs), light intensity (188.7 µmol photons m^{-2} s^{-1}), KNO3 (75.4gL^{-1}), Na_2HPO_4 (8.9gL^{-1}) and $FeCl_3$ (2.8gL^{-1})

[d]*P. lutheri* : pH 7.9, Salinity (35.5 ppt), photoperiod (24 hrs), light intensity (198 µmol photons m^{-2} s^{-1}), KNO3 (62.5gL^{-1}), Na_2HPO_4 (3.92gL^{-1}) and $FeCl_3$ (2.63gL^{-1})

6.2 Establishment of Kinetics and Fatty Acids Profiles in 5 L Photobioreactor and 300 L Open Tank

6.2.1 Optimum pH and Salinity

Fig 6.2 and 6.3 (a-d) show the cell growth of *N. oculata, T. suecica, I. galbana* and *P. lutheri* in 5 L photobioreactor and 300 L open tank at optimum pH and salinity. The highest cell density and biomass were achieved by *N. oculata* at 78.8×10^6 in 5 L PBR and 55.5×10^6 cells mL^{-1} in 300 L tank with 0.93 and 0.65 gL^{-1} biomass respectively. This was followed by *T. suecica* at 53.8×10^6 and 39.2×10^6 cells mL^{-1} density with 0.68 and 0.56 gL^{-1} biomass respectively. The highest μ_{max} of 0.21-0.24 d^{-1} with t_d of 2.92-3.30 days were in 5 L PBR and slightly lower in 300 L open tank with μ_{max} of 0.19-0.23 d^{-1} but slightly higher t_d of 2.98-3.61 days shown by all species. The lipid content were higher in 5 L PBR at 38.6 ± 1.54 and 40.2 ± 3.31% for *N. oculata* and *P. lutheri*, respectively (Table 6.2). The biomass and lipid yield by using nitrate as a substrate were also higher in 5 L PBR while slightly lower in 300 L open tank for all the four species (Table 6.4).

Table 6.3 shows that pentadecanoic acid C15:0 (6.1%, 14.7%); palmitic acid C16:0 (38.2%, 25.2%); palmitoleic acid C16:1 (11.0%, 5.2%); oleic acid C18:1 (6.1%, 7.9%); and eicosanoic acid C20:0 (6.0%, 3.7%) were major components of the oil derived from *N. oculata* cultivated in PBR and open tank, respectively. The total saturated fatty acids (63.8%, 55.03%); monounsaturated fatty acids (17.1%, 13.1%); and PUFA (15.7%, 9.4%) were obtained for *N. oculata*, respectively. The major components of *P. lutheri* were palmitic acid C16:0 (32.3%, 28.4%); palmitoleic acid C16:1 (22.4%, 19.4%); oleic acid C18:1 (12.1%, 8.3%); and docosahexaenoic acid DHA C22:6 (5.7%, 4.8%) with the total saturated fatty acids (46.9%, 38.1%); monounsaturated fatty acids (34.5%, 27.6%); and PUFA (14.5%, 11.4%) respectively, for cultivation in PBR and open tank.

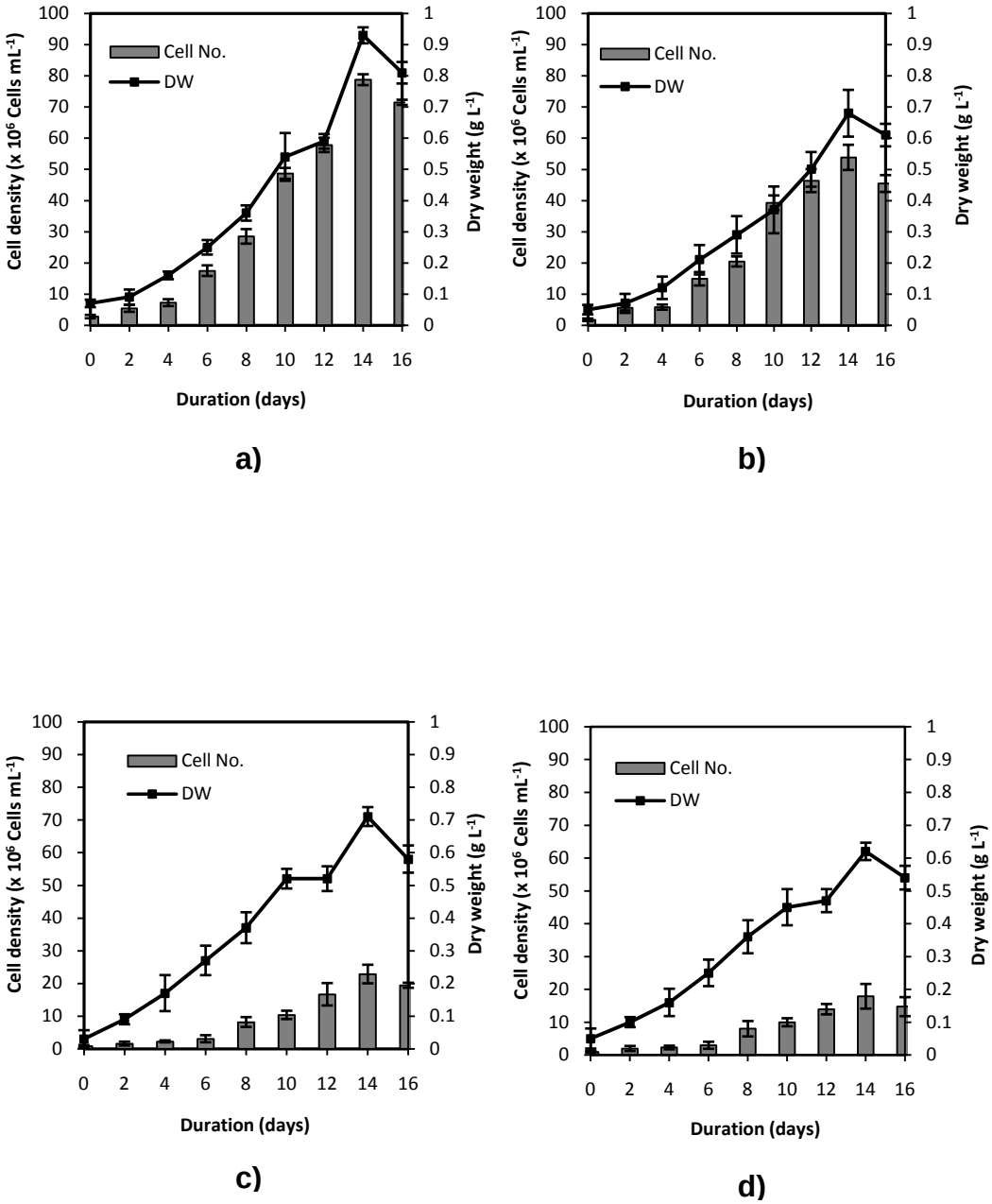


Fig 6.2 Cell growth at optimum pH and salinity of a) *N. oculata*; b) *T. suecica*; c) *I. galbana* and d) *P. lutheri* in 5L PBR.

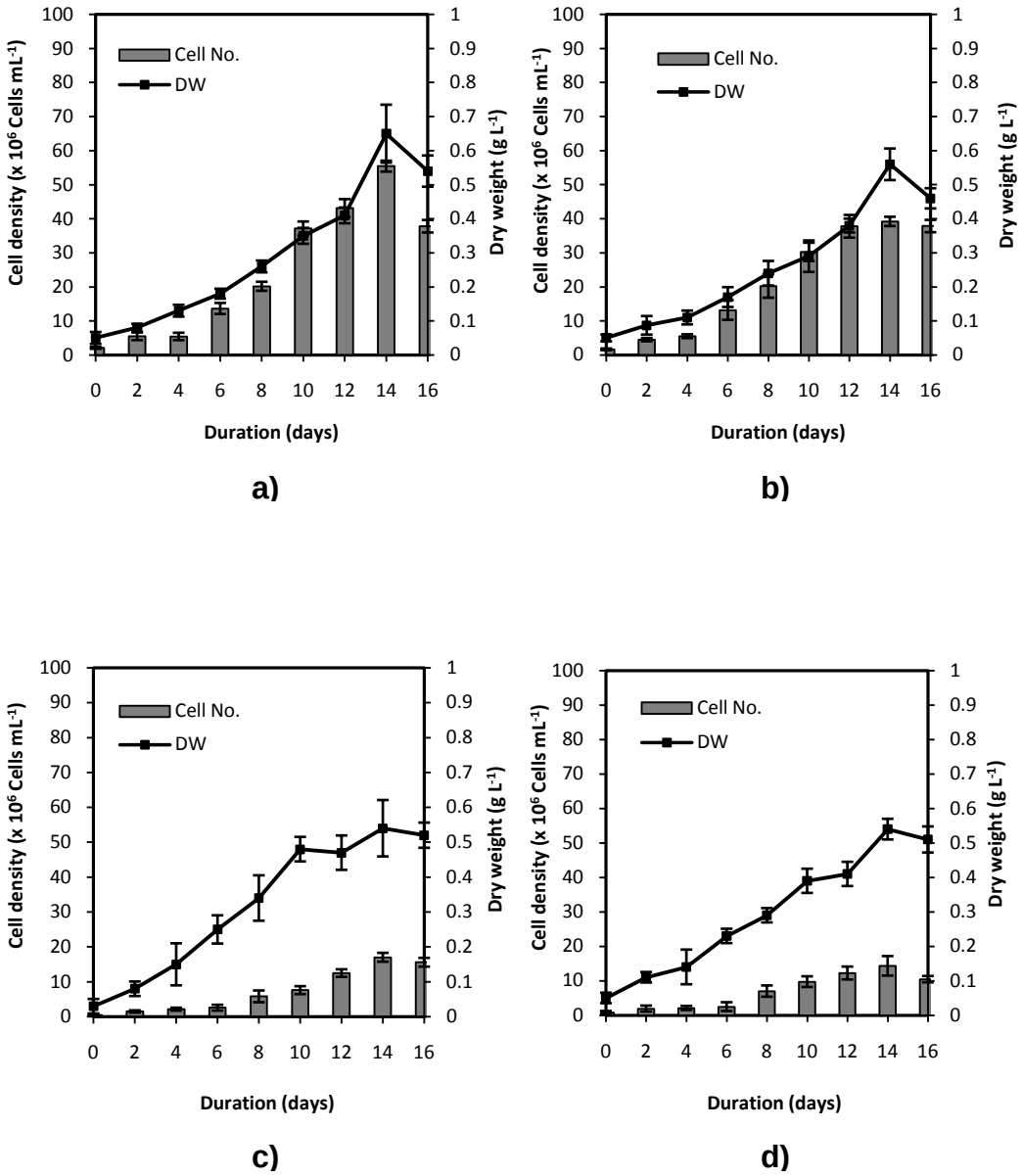


Fig 6.3 Cell growth at optimum pH and salinity of a) *N. oculata*; b) *T. suecica*; c) *I. galbana* and d) *P. lutheri* in 300 L open tank.

Table 6.2 Establishment of kinetics between 5 L PBR and 300 L open tank cultures at optimized conditions as predicted by RSM

Media Conditions			*N. Oculata* [a]				*T. suecica* [b]				*I. galbana* [c]				*P. lutheri* [d]			
			X'_{max} ($gL^{-1}d^{-1}$)	μ_{max} (d^{-1})	t_d (day)	Lipid (%)	X'_{max} ($gL^{-1}d^{-1}$)	μ_{max} (d^{-1})	t_d (day)	Lipid (%)	X'_{max} ($gL^{-1}d^{-1}$)	μ_{max} (d^{-1})	t_d (day)	Lipid (%)	X'_{max} ($gL^{-1}d^{-1}$)	μ_{max} (d^{-1})	t_d (day)	Lipid (%)
pH and Salinity		PBR	0.176	0.24	2.92	38.6 ± 1.54	0.173	0.22	3.15	30.3 ± 4.38	0.143	0.23	2.99	32.2 ± 2.11	0.135	0.21	3.30	40.2 ± 3.31
		Open Tank	0.161	0.23	2.98	35.5 ± 1.81	0.162	0.20	3.53	27.5 ± 2.98	0.131	0.21	3.36	28.6 ± 2.75	0.134	0.19	3.61	36.1 ± 3.65
Photoperiod and Light Intensity		PBR	0.185	0.24	2.92	40.1 ± 2.77	0.172	0.22	3.16	30.8 ± 2.87	0.148	0.23	2.98	32.8 ± 3.44	0.142	0.22	3.11	41.8 ± 0.78
		Open Tank	0.171	0.20	3.40	30.7 ± 2.52	0.166	0.21	3.32	25.6 ± 3.90	0.120	0.20	3.45	24.8 ± 3.13	0.120	0.18	3.82	32.1 ± 2.57
Nitrate, Phosphate and Iron		PBR	0.172	0.21	3.27	42.2 ± 3.78	0.157	0.21	3.25	31.6 ± 4.33	0.134	0.21	3.30	33.5 ± 2.27	0.137	0.19	3.65	41.2 ± 1.92
		Open Tank	0.168	0.19	3.63	36.2 ± 2.47	0.152	0.20	3.48	30.5 ± 1.84	0.126	0.18	3.75	32.4 ± 1.47	0.126	0.17	3.96	38.5 ± 0.76

[a]*N. oculata* : pH 8, Salinity (35 ppt), photoperiod (24 hrs), light intensity (188 µmol photons m^{-2} s^{-1}) KNO3 (10 gL^{-1}), Na_2HPO_4 (6 gL^{-1}) and $FeCl_3$ (2.53 gL^{-1})

[b]*T. suecica* : pH 7.9 Salinity (32 ppt), photoperiod (24 hrs), light intensity (196.5 µmol photons m^{-2} s^{-1}), KNO3 (13.7 gL^{-1}), Na_2HPO_4 (5.6 gL^{-1}) and $FeCl_3$ (2.50 gL^{-1})

[c]*I. galbana* : pH 9, Salinity (39.2 ppt), photoperiod (20.5 hrs), light intensity (188.7 µmol photons m^{-2} s^{-1}), KNO3 (75.4gL^{-1}), Na_2HPO_4 (8.9gL^{-1}) and $FeCl_3$ (2.8gL^{-1})

[d]*P. lutheri* : pH 7.9, Salinity (35.5 ppt), photoperiod (24 hrs), light intensity (198 µmol photons m^{-2} s^{-1}), KNO3 (62.5gL^{-1}), Na_2HPO_4 (3.92gL^{-1}) and $FeCl_3$ (2.63gL^{-1})

Note: For 300 L open tank photoperiod was 12 hrs and light intensity was 165-250 µmol photons $m^{-2}s^{-1}$ (shadded from direct sunlight).

Table 6.3 Fatty acid profile at optimized pH and salinity

	Experimental Conditions	Total fatty acids composition (%)														
		C14:0	C15:0	C16:0	C16:1	C17:0	C18:0	C18:1	C18:2	C18:3	C20:0	C20:2	C20:3	C20:4	C20:5	C22:6
N. oculata	PBR	4.60	6.10	38.23	10.96	3.12	5.80	6.10	3.28	2.1	5.96	ND	ND	5.8	4.3	2.34
	Open Tank	3.36	14.73	25.23	5.23	1.67	6.35	7.86	3.13	2.4	3.69	1.08	ND	ND	ND	2.79
T. suecica	PBR	4.16	5.20	22.95	5.98	12.67	5.07	2.49	6.45	0.54	3.70	ND	ND	ND	ND	4.20
	Open Tank	6.02	15.51	8.91	2.75	3.61	5.33	1.44	2.36	ND	3.98	ND	ND	ND	ND	4.87
I. galbana	PBR	3.32	15.68	13.94	2.61	3.15	4.72	11.67	ND	ND	3.50	2.38	ND	8.43	2.23	2.66
	Open Tank	2.72	9.49	11.43	3.67	1.92	2.64	8.35	ND	ND	2.21	1.54	ND	5.43	1.32	2.37
P. lutheri	PBR	2.69	3.52	32.34	22.42	4.04	0.41	12.10	0.34	1.36	3.86	ND	ND	ND	7.14	5.69
	Open Tank	1.54	2.62	28.38	19.37	2.87	ND	8.28	ND	1.25	2.64	ND	ND	ND	5.27	4.84

Table 6.4 Yield of biomass and lipid at optimized conditions

Media Conditions		*N. Oculata*		*T. suecica*		*I. galbana*		*P. lutheri*	
		$^{*}Y\ x/s$ ($g\ g^{-1}$)	$^{+}Y\ p/s$ ($g\ g^{-1}$)	$^{*}Y\ x/s$ ($g\ g^{-1}$)	$^{+}Y\ p/s$ ($g\ g^{-1}$)	$^{*}Y\ x/s$ ($g\ g^{-1}$)	$^{+}Y\ p/s$ ($g\ g^{-1}$)	$^{*}Y\ x/s$ ($g\ g^{-1}$)	$^{+}Y\ p/s$ ($g\ g^{-1}$)
pH and Salinity	PBR	0.08	0.03	0.04	0.02	0.005	0.002	0.006	0.003
	Open Tank	0.05	0.02	0.03	0.01	0.007	0.001	0.007	0.002
Photoperiod and Light Intensity	PBR	0.08	0.03	0.04	0.02	0.006	0.003	0.007	0.003
	Open Tank	0.06	0.02	0.03	0.009	0.004	0.001	0.005	0.002
Nitrate, Phosphate and Iron	PBR	0.05	0.02	0.03	0.01	0.005	0.002	0.006	0.003
	Open Tank	0.04	0.01	0.03	0.008	0.004	0.001	0.004	0.002

*Y x/s (gram cells per gram nitrate): yield of biomass on the basis of potassium nitrate as a substrate
$^{+}$Y p/s (gram lipid per gram nitrate): yield of lipid on the basis of potassium nitrate as a substrate

6.2.2 Optimum Photoperiod and Light Intensity

The highest cell density and biomass (Fig 6.4 and 6.5 a-d) in 5 L PBR and 300 L open tank were again shown by *N. oculata* at 82.6 × 10^6, 63.7 × 10^6 cells mL^{-1} density with 0.96, 0.72 gL^{-1} biomass followed by *T. suecica* at 59 × 10^6, 42.7 × 10^6 cells mL^{-1} density with 0.73, 0.58 gL^{-1} biomass respectively. The cell growth of *I. galbana* and *P. lutheri* remained at 19.6-21.2 × 10^6, 15.1-15.9 × 10^6 cells mL^{-1} density, respectively with 0.52-0.66 biomass. The lipid content were higher in 5 L PBR at 40.1 ± 2.77 and 41.8 ± 0.78% as compared to 30.7 ± 2.52 and 32.1 ± 2.57% in 300 L open tank for *N. oculata* and *P.* lutheri, respectively (Table 6.2). The higher biomass and lipid yield were obtained in 5 L PBR while lower biomass and lipid yield were observed in 300 L open tank (Table 6.4).

Table 6.5 suggests that palmitic acid C16:0 (22.1%) and palmitoleic acid C16:1 (9.9%) were reduced but heptadecanoic acid C17:0 (13.7%) and oleic acid C18:1

(7.4%) for *N. oculata* were enhanced in PBR. Although the total saturated fatty acids (57.0%) and monounsaturated fatty acids (17.7%) were comparable to previous results at optimum pH and salinity, PUFA (22.3%) was enhanced for *N. oculata* in PBR. For *P. lutheri* in PBR, palmitic acid C16:0 (34.4%) remained high, while both eicosapentaenoic acid (EPA) C20:5 (8.4%) and docosahexaenoic acid (DHA) C22:6 (6.9%) slightly increased with the total saturated fatty acids (47.9%) and monounsaturated fatty acids (30.9%) remained comparable but with PUFA (18.9%) elevated.

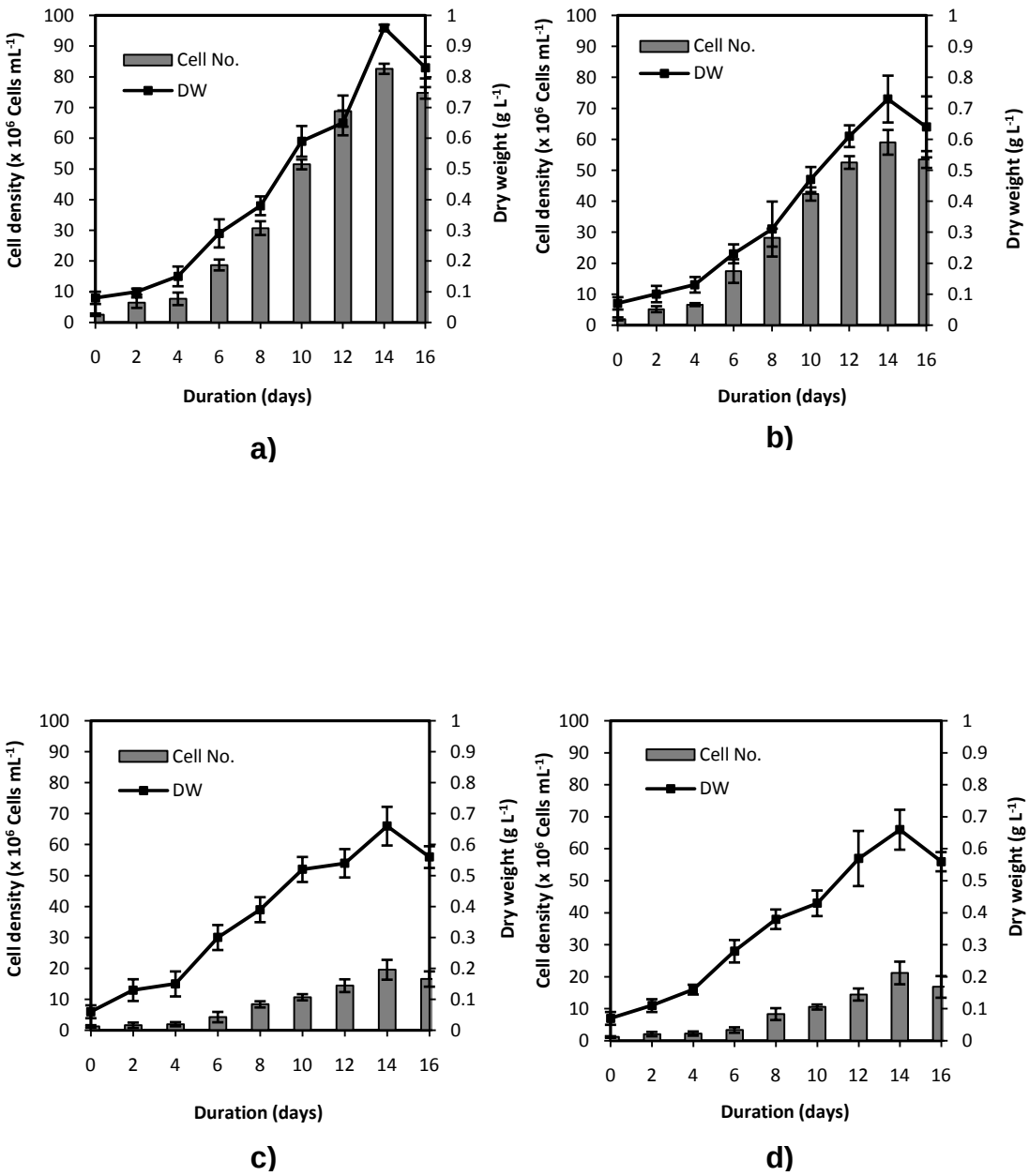


Fig 6.4 Cultivation at optimum photoperiod and light intensity of a) *N. oculata*; b) *T. suecica*; c) *I. galbana* and d) *P. lutheri* in 5 L PBR

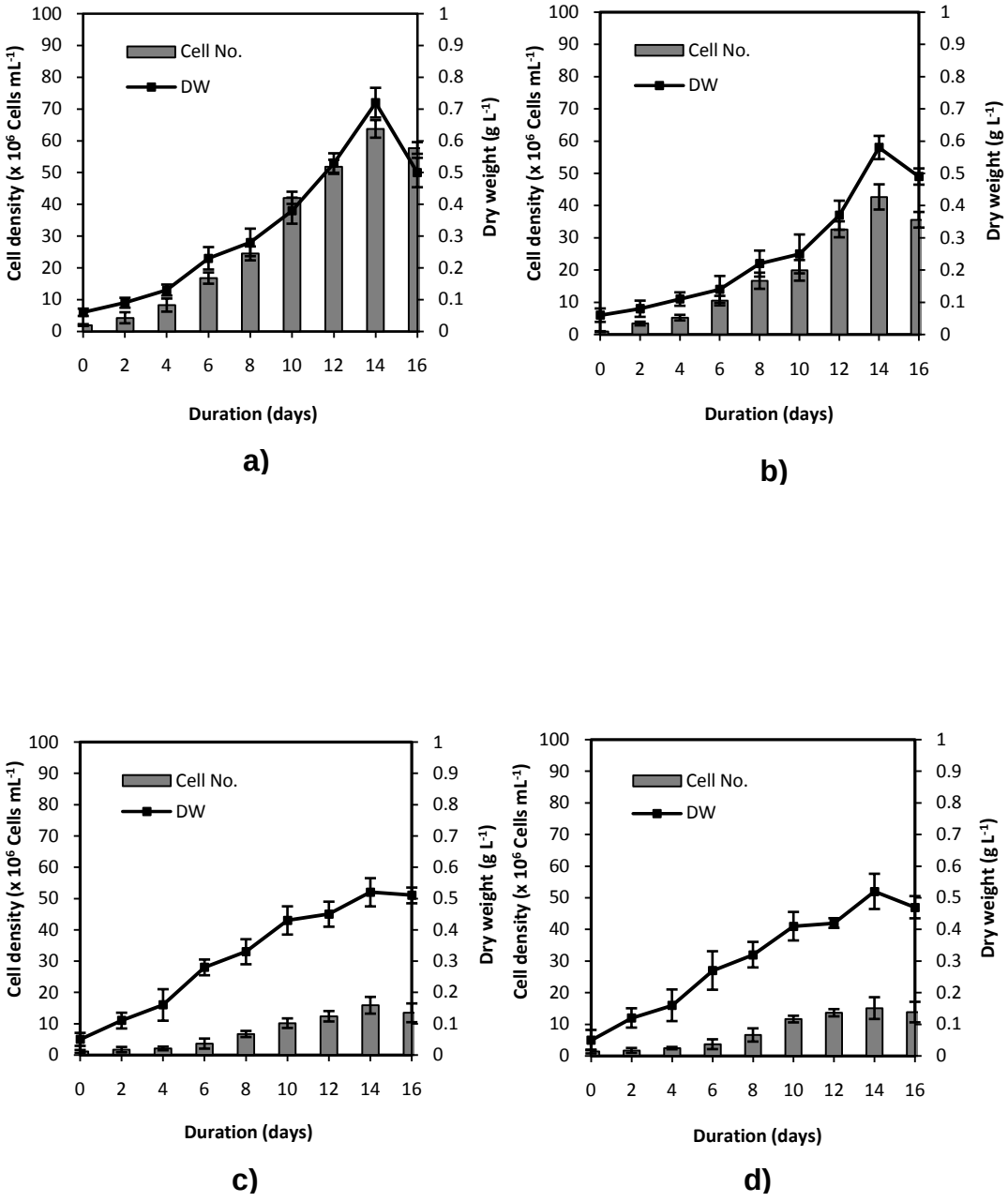


Fig 6.5 Cultivation at optimum photoperiod and light intensity of a) a) *N. oculata*; b) *T. suecica*; c) *I. galbana* and d) *P. lutheri* in 300 L open tank

Table 6.5 Fatty acid profile at optimized photoperiod and light intensity

	Experimental Conditions	Total fatty acids composition (%)														
		C14:0	C15:0	C16:0	C16:1	C17:0	C18:0	C18:1	C18:2	C18:3	C20:0	C20:2	C20:3	C20:4	C20:5	C22:6
N. oculata	PBR	4.47	7.15	22.08	9.89	13.74	5.50	7.38	4.6	3.2	4.07	ND	ND	3.33	6.65	4.56
	Open Tank	3.01	13.08	21.23	7.44	5.27	5.92	5.16	2.8	ND	3.24	ND	ND	ND	ND	4.45
T. suecica	PBR	4.29	17.8	14.89	2.82	2.66	5.48	3.27	5.38	1.65	1.88	1.17	0.45	ND	6.80	3.13
	Open Tank	4.08	16.93	12.60	4.97	3.80	5.64	2.71	2.44	ND	4.21	0.72	ND	ND	ND	0.07
I. galbana	PBR	10.31	8.20	19.18	5.05	9.74	5.45	14.56	2.2	0.6	8.09	2.71	ND	ND	4.13	5.56
	Open Tank	8.99	2.69	13.21	4.52	5.13	3.38	11.52	1.4	ND	2.42	2.12	ND	ND	3.42	1.83
P. lutheri	PBR	2.86	3.76	34.42	21.26	3.48	0.74	9.67	1.46	2.11	2.68	ND	ND	ND	8.44	6.95
	Open Tank	1.58	2.26	26.38	18.77	2.37	ND	7.28	ND	1.22	2.46	ND	ND	ND	7.78	5.44

6.2.3 Optimum Nitrate, Phosphate and Iron

At optimum nitrate, phosphate and iron (Fig 6.6 and 6.7 a-d) the highest cell density and biomass in 5 L PBR and 300 L open tank were much reduced for *N. oculata* at 63.5×10^6, 51.4×10^6 cells mL^{-1} density with 0.62, 0.54 gL^{-1} biomass and *T. suecica* at 42.8×10^6, 34.8×10^6 cells mL^{-1} density with 0.58, 0.47 gL^{-1} biomass respectively. The μ_{max} was also reduced to 0.19-0.21 d^{-1} with t_d of 3.25-3.65 days in 5 L PBR and 0.17-0.20 d^{-1} with t_d of 3.48-3.96 days for 300 L open tank attained for all species. The lipid content remained higher in 5 L PBR at 42.2 ± 3.78% and 41.2 ± 1.92% as compared to 300 L open tank at 36.2 ± 2.47% and 38.5 ± 0.76% for *N. oculata* and *P. lutheri*, respectively (Table 6.2). The biomass and lipid yield were also higher in 5 L PBR while slightly reduced yield of biomass and lipid was obtained in 300 L open tank (Table 6.4).

Comparing Table 6.3 and 6.6 only pentadecanoic acid C15:0 (8.2%), and oleic acid C18:1 (11.3%) were elevated in *N. oculata* cultivated in PBR, while palmitic acid C16:0 (18.4%) and heptadecanoic acid C17:0 (4.7%) were much reduced. The total saturated fatty acids (46.4%), monounsaturated fatty acids (16.8%) and PUFA (8.3%) for *N. oculata* in PBR were much reduced. *P. lutheri* in PBR on the other hand showed slightly elevated palmitic acid C16:0 (35.2%), palmitoleic acid C16:1 (23.3%) and oleic acid C18:1 (13.8%) though EPA C20:5 (5.2%) was reduced. These were translated into increased total saturated fatty acids (50.1%) and monounsaturated fatty acids (37.0%) but lower PUFA (11.3%).

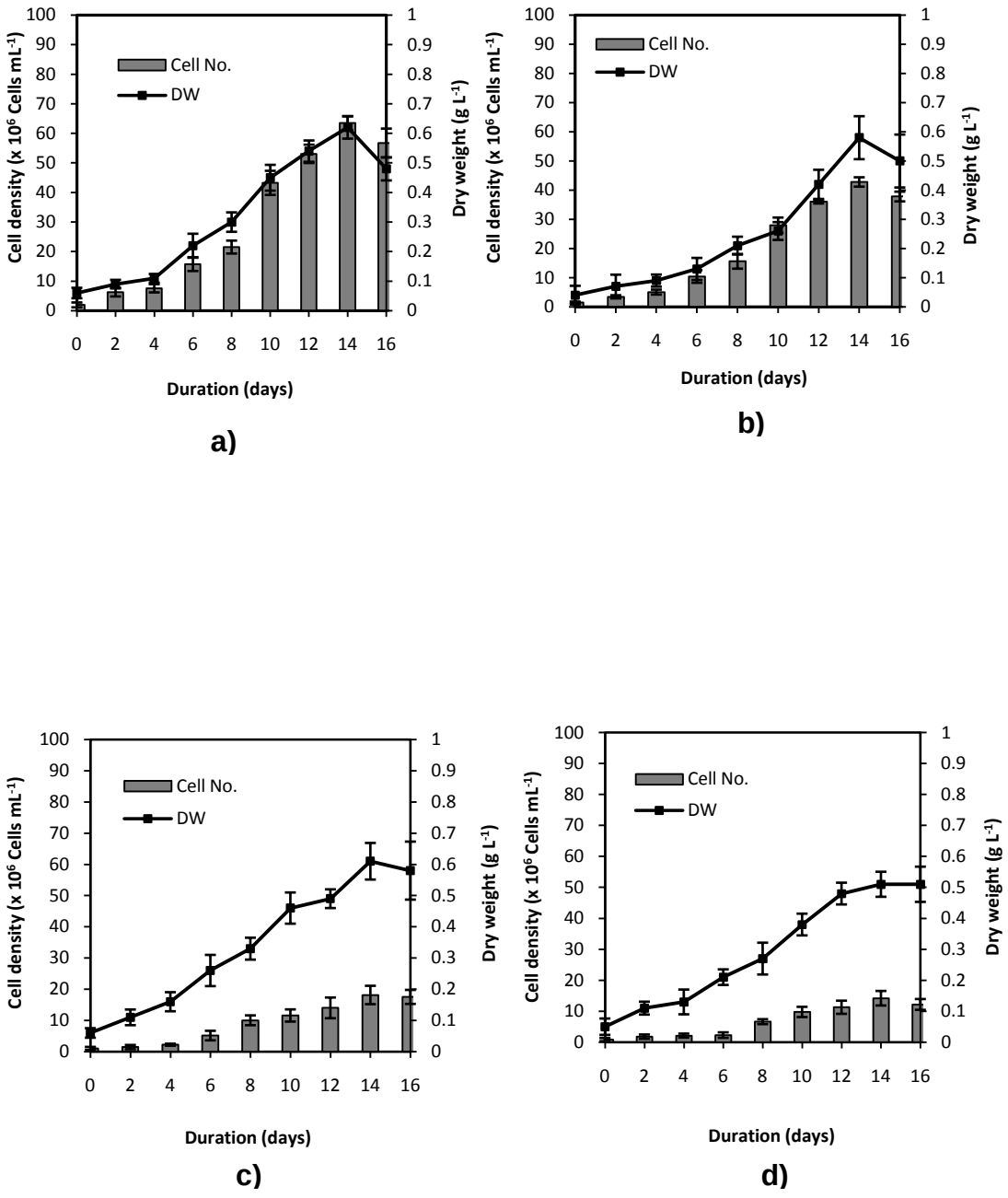


Fig 6.6 Cultivation at optimum macro-nutrients of a) *N. oculata*; b) *T. suecica*; c) *I. galbana* and d) *P. lutheri* in 5 L PBR

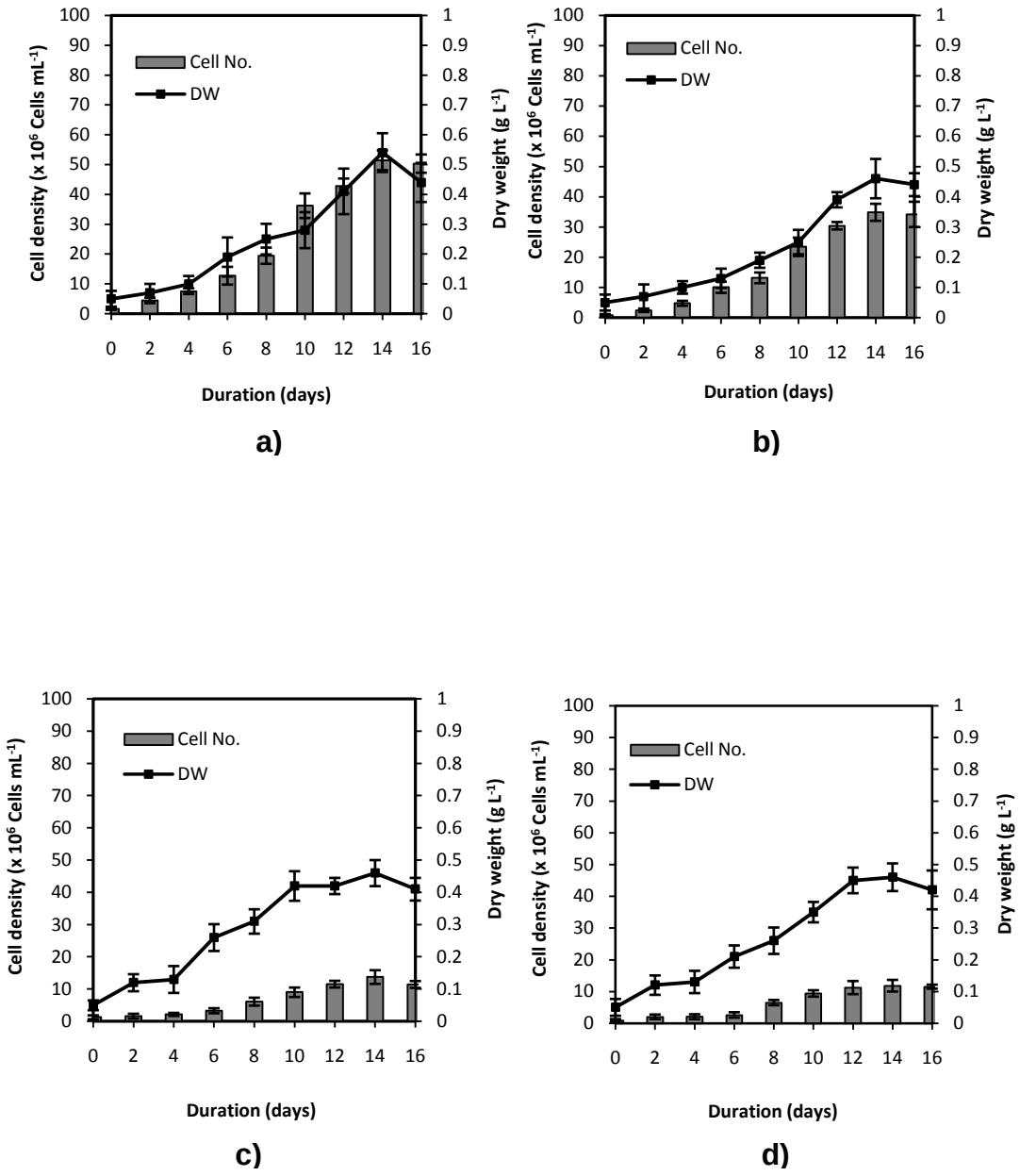


Fig 6.7 Cultivation at optimum macro-nutrients of a) *N. oculata*; b) *T. suecica*; c) *I. galbana* and d) *P. lutheri* in 300 L open tank

Table 6.6 Fatty acid profile at optimized nitrate, phosphate and iron

	Experimental Conditions	Total fatty acids composition (%)														
		C14:0	C15:0	C16:0	C16:1	C17:0	C18:0	C18:1	C18:2	C18:3	C20:0	C20:2	C20:3	C20:4	C20:5	C22:6
N. oculata	PBR	4.75	8.16	18.43	5.47	4.73	5.97	11.34	3.34	ND	4.40	2.89	0.20	1.26	ND	0.63
	Open Tank	3.80	4.98	14.84	4.05	11.86	4.79	3.50	4.68	ND	1.62	1.07	0.61	2.43	ND	2.86
T. suecica	PBR	4.22	6.40	15.16	6.12	13.36	12.43	8.32	0.86	0.45	3.78	3.15	2.55	ND	ND	4.65
	Open Tank	3.17	13.81	13.95	2.28	2.63	4.64	3.62	ND	ND	3.46	1.02	ND	ND	ND	ND
I. galbana	PBR	12.17	11.49	14.45	3.13	8.46	8.11	15.38	3.52	2.64	3.79	3.44	ND	ND	5.42	4.68
	Open Tank	8.84	6.88	10.21	2.52	4.13	3.56	7.24	1.82	ND	2.11	3.63	ND	ND	4.21	2.33
P. lutheri	PBR	3.64	4.62	35.23	23.26	3.48	0.44	13.78	1.24	0.32	2.68	ND	ND	ND	5.24	4.53
	Open Tank	1.88	3.26	29.82	19.77	2.73	ND	10.81	ND	ND	4.64	ND	ND	ND	8.65	6.54

6.3 Discussion

The biochemical compositions of microalgae can change with their growth rates and environmental conditions and with the phase of their life cycle. The present study achieved cell densities, biomass, μ_{max} and t_d in shake flasks and 1-30 batch cultures comparable to *P. lutheri* culture at 250 mL-300 L with biomass reported at 0.45 g L^{-1} (in 250 mL), μ_{max} at 0.14 day^{-1} (in 30 L) and t_d at 4.95 days (in 30 L) [206]. However both *N. oculata* and *T. suecica* attained higher biomass at 0.68-0.93 g L^{-1} in 5 L PBR while the biomass was comparable for *I. galbana* and *P. lutheri* at 0.62-0.71 g L^{-1}, the cell densities were 3-4 fold lower. The reason being that both brown *I. galbana* and *P. lutheri* showed bigger cell size than green *N. oculata* and *T. suecica*. This lower in cell density can be compensated by accumulation of nutrients making cellular components, leading to comparable biomass and dry weight.

In present study high lipid was obtained at high pH because high pH can increase TAG accumulation for green algae [207], while the salinity of 30-35 ppt has been recommended for algal species [208] but may be the opposite for structural lipids [209]. At pH 7.5 and 10, *P. tricornutum* CCAP achieves high growth rate of 0.3 day^{-1}, though the lipid content at pH 10 is 28% lower than at pH 7.5 [210]. The effects of low salinity level on growth retardation has been observed in *N. gregari* [25]; and on lower lipid contents (18-19% DW) in nutrient replete conditions in *Chlamydomonas applanata* and *C. reinhardtii* [16]. Previous studies on *Rhodomonas* sp., *Cryptomonas* sp., and *Isochrysis* sp. indicate that lipid content increase with increasing photoperiod by 15.5, 12.7, and 21.7%, respectively [180]. Light intensity as high as 400 μmol photons m^{-2} s^{-1} has reportedly resulted in the highest biomass yield of *Scenedesmus* sp. (3.88 g L^{-1}) with equally high lipid (41.1%), neutral lipid content (32.9%) and the major fatty acids, oleic acid (43-52%), palmitic acid (24-27%) and linoleic acid (7-11%) [211]. Although this may be good in terms of productivity, utilizing high light intensity or prolonged photoperiod may defeat the purpose of developing green-alternatives whilst reducing additional energy operating cost. A study on *Chlorella vulgaris* utilizing 16:8 h light/dark photoperiod achieves maximum biomass of 2 g L^{-1} under 62.5 μmol photons m^{-1} s^{-1} with maximum percentage of total saturated fatty acids (SFA) of 33.4% obtained under 100 μmol

photons $m^{-2}s^{-1}$ intensity [212]. Nutrient limiting conditions such as nitrate, phosphate, silicon and iron are the main factors that influence lipid content [18] and nitrogen deprivation has been the most studied. Culture medium with low nitrogen increases the lipid content of *Chlorella vulgaris* to 40% which is about two times more than control (18%) [30].

In a typical process of biofuels production, trans-esterification (alcoholysis) produces esters of fatty acids and glycerol. The synthesized fatty acids in algae are commonly in medium length, ranging from 16 to 18 carbons, despite the great variation in fatty acid composition. Specifically, the major fatty acids are C16:0, C16:1, C18:0, C18:1 and C18:2 in green algae and C16:0 and C16:1 in brown algae. In present study, FAME analyses revealed that the major components in all the four microalgal species were tetradecanoic acid (C14:0), pentadecanoic acid (C15:0), palmitic acid (C16:0), palmitoleic acid (C16:1), heptadecanoic acid (C17:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic (C18:3), eicosanoic acid (C20:0), eicosadienoic acid (C20:2), eicosatrienoic acid ETE (C20:3), eicosatetraenoic acid ETA (C20:4), eicosapentaenoic acid EPA (C20:5) and docosahexaenoic acid DHA (C22:6) with different composition (All chromatograms shown in Appendix D). The predominant fatty acids were pentadecanoic acid (C15:0), palmitic acid (C16:0), palmitoleic acid (C16:1), heptadecanoic acid (C17:0), oleic acid (C18:1), eicosanoic acid (C20:0), EPA (C20:5) and DHA (C22:6). Our results of fatty acid analyses are comparable to the lipid classes of *P. lutheri* cultivated in semicontinuous mode, where neutral lipids and glycolipids, as the major constituents, accounted for 57 and 24% of the total fatty acids residues (TFA), respectively, with emphasis on eicosapentaenoic (C20:5n-3, EPA) and docosahexaenoic (C22:6n-3, DHA) acids [213]. Another study with *P. lutheri* undergoing UV-R treatment has reported total saturated fatty acids of 33.8%, monounsaturated fatty acids of 18.7% and PUFA of 46.1% [214].

The relative proportion of nutrients can modify the fatty acid profile of the microalgae, increasing SFA and MUFA proportion and in smaller amount PUFA content. The percentage of phosphorus was found to be the limiting nutrient related to the synthesis of phospholipids. Nevertheless, fatty acid biosynthesis and proportion may vary according to the microalgae species [215].

The main fatty acids present in the lipids of *Chlorella* sp. are normally short-chain fatty acids (C14-C18) [216]. *Tetraselmis* sp. and *Chlorella* have been cultivated in industrial-scale bioreactors, which produce 2.33 and 2.44 % (w/w) lipid (calculated as the sum of fatty acid methyl esters) in dry biomass, respectively. These lipids contain higher amount of neutral lipids and glycolipids plus sphingolipids, than phospholipids. Lipids of *Tetraselmis* sp. are characterized by the presence of eicosapentaenoic acid (that is located mainly in phospholipids), and octadecatetraenoic acid (that is equally distributed among lipid fractions), but these fatty acids are completely absent in *Chlorella* lipids. Lipids produced by 16 newly isolated strains from Greek aquatic environments (cultivated in flask) have reported the highest percentage of lipids in *Prorocentrum triestinum* (3.69% w/w) while the lowest in *Prymnesium parvum* (0.47% w/w). Several strains produce lipids rich in EPA and DHA where the later is found in high percentage in lipids of *Amphidinium* sp. S1 and *Prorocentrum minimum*, while EPA high in lipids of *Asterionella* sp. S2. These lipids, containing ω-3-long-chain PUFA, have important applications in the food and pharmaceutical industries and in aquaculture [217].

The composition vary greatly when algal cells are exposed to different environmental or nutritional conditions such as temperature, pH, light intensity, or nitrogen concentration [218]. During the nitrogen starvation period, the proportion of oleic acid (C18:1) increases, whereas that of the linoleic acid (C18:2) and linolenic acid (C18:3) decrease [7]. Generally, saturated fatty esters possess high cetane number and superior oxidative stability; whereas unsaturated, especially polyunsaturated fatty esters have improved low-temperature properties [219]. Modification of fatty esters, such as the enhanced proportion of oleic acid (C18:1) ester, can provide a compromise between oxidative stability and low-temperature properties and therefore promote the quality of biodiesel [219].

Thus, microalgae with high oleic acid are suitable for biodiesel production. Over 65% of fatty acids are saturated and monounsaturated fatty acids (C16:0, C18:0 and C18:1) which are suitable for application in biodiesel production [1].

European biodiesel standards limit the contents of FAMEs with four and more double bonds to 1 mol% [3].

In the present study, 300 L open tank showed that the growth and lipid content of all the four algal strains at all times were lower than in 5 L PBR. Cultivation in open-pond tank faces challenges such as overheating, fouling, accumulation of oxygen to toxic levels [120] and contamination with bacterial species. Most commercial, large-scale outdoor microalgae cultivation is in artificial open pond as it is cheap to build and easy to operate and scale-up [59]. The raceway pond has relatively low capital and maintenance costs while circular ponds are less attractive because of expensive concrete construction, high energy consumption of stirring, the mechanical complexity of supplying CO_2 and inefficient land use [10]. Apart from disadvantages such as low productivity and biomass yield, high harvesting cost, water loss through evaporation, and the limited number of species that can be grown in ponds due to contamination, there may be low carbon dioxide consumption efficiency and temperature fluctuations due to diurnal variations, as they are difficult to control in open ponds [10].

Estimations of the production of algal oil as being able to reach over 100000 L per hectare per year (compared with just 450 L for soybean oil and 6000 L for palm oil) seems attractive [220]. However, what is feasible in the laboratory may not be achievable outdoors on a very large scale. With current technology, microalgae are too expensive to be viable alternatives to the major commodity plant oils. The challenge to make algal oils more economical will be in the form of hybrid PBR or open-pond cultivation with robust, fast growing algae that can withstand adventitious predatory protozoa or contaminating bacteria, whilst attaining an oil content of at least 40 % of the biomass [221]. If the prices of the major plant oils and crude oil continue to rise in the future, algal lipids may become a realistic alternative with better prospects focusing on algae as sources of PUFA and a major genetic redesign of algal metabolic processes [221].

6.4 Summary

Scale-up kinetics and fatty acids profiles for algal cultivation in 5 L PBR and 300 L open tank were developed at optimized conditions. Comparison between 250 mL-30 L batch cultures showed that *N. oculata* and *P. lutheri* in 250 mL, *T. suecica* in 30 L and *I. galbana* in 1 L, attained the maximum specific growth rate of 0.15-0.17 d^{-1} and lipid content of 27.2-37.1%. The biomass and lipid content were higher in 5 L PBR as compared to 300 L open tanks. The highest biomass of 0.62-0.96 gL^{-1} and lipid content of 31.6-42.2% in 5 L PBR and the highest biomass of 0.45-0.72 gL^{-1} and lipid content of 24.4-38.5 in 300 L open tank were achieved for all four tested species. The total saturated fatty acids (44.3-63.8% and 30.4-55.03%); monounsaturated fatty acids (6.1-37.0% and 4.2-13.1%); and PUFA (8.3-22.3% and 1.02-15.2%) were obtained, with pentadecanoic acid (C15:0), palmitic acid (C16:0), palmitoleic acid (C16:1), heptadecanoic acid (C17:0), oleic acid (C18:1), eicosanoic acid (C20:0), EPA (C20:5) and DHA (C22:6) as predominant fatty acids.

CHAPTER 7

MOLECULAR IDENTIFICATION OF NANNOCHLOROPSIS OCULATA

7.1 DNA Purity and PCR Products

Nannochloropsis is a genus of marine eukaryotic unicellular algae, generally regarded as picoeukaryotic plankton which belongs to class Eustigmatophyceae. Traditional morphological observation is difficult as the sizes are usually 2-5μm in size and morphologically similar. DNA sequences such as 18S ribosomal RNA gene, internal transcribed spacer (ITS) region of ribosomal RNA transcription unit and *rbcL* gene have been widely used to identify species [222].

Table 7.1 shows 18S ribosomal RNA gene, *rbcL* gene and ITS region of ribosomal RNA. DNA of *Nannochloropsis* was extracted and the quality was determined by nano-spectrophotometer analysis. The intensity of absorbance of the DNA solution at wavelength 260 nm and 280 nm was used as a measure of DNA purity. DNA absorbs UV light at 260 nm and protein absorbs UV light at 280 nm. Pure sample of DNA has the 260/280 ratio at 1.7 to 2.0 and is relatively free from protein contamination. In this study, the 260/280 ratio at 1.8-1.9 was recorded, suggesting that pure DNA was obtained.

Based on gel electrophoresis analysis, ~0.8 kb, ~2.0 kb and ~1.5 kb bands of genomic DNA were seen as shown in Figure 7.1. This corresponds to the 18S ribosomal RNA gene, *rbcL* gene and ITS region of ribosomal RNA. The digestion results indicated that the DNA extracted was qualified for molecular analyses.

Table 7.1 18S ribosomal RNA gene, *rbcL* gene and ITS region of ribosomal RNA

Oligo Name	Primer sequence (5`− 3`)	GenBank Accession No.	Position
F298-18S rRNA	CAAGTTTCTGCCCTATCAGCT	AF045045	298-318
R948-18S rRNA	GCTTTCGCAGTAGTTCGTCTT		928-948
F1-*rbcL*	GATGCAAACTACACAATTAAAGATACTG	AB280614	1-28
R1486-*rbcL*	ATTTTGTTCGTTTGTTAAATCCG		1464-1486
F3-ITS	GTCGCACCTACCGATTGA	DQ069777	3-20
R1049-ITS	CGGGTAGCCTTGCTTGAT		1032-1049

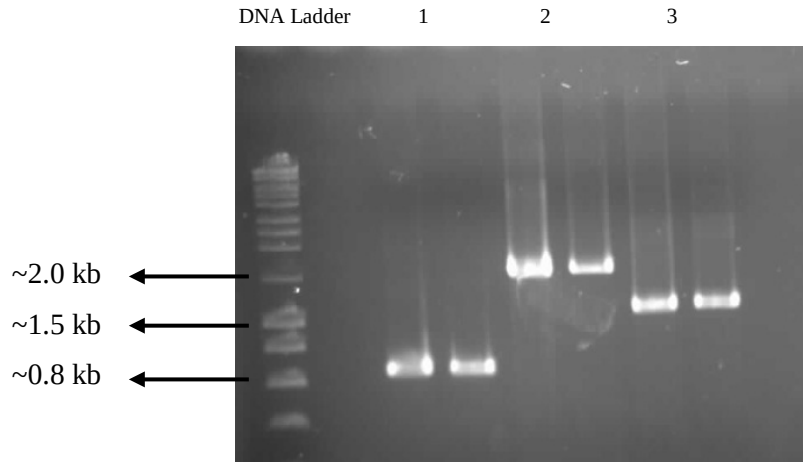


Fig.7.1 Genomic DNA with Lane 1: 18S ribosomal RNA gene (~0.8 kb), Lane 2: *rbcL* gene (~2.0 kb) and Lane 3: ITS region of ribosomal RNA (~1.5 kb)

7.2 DNA Sequencing and Phylogenetic Analysis

In this study, partial 18S rRNA sequence, partial *rbcL* gene, and the ITS region of the *Nannochloropsis* sp. were determined and deposited into Gen- Bank with accession numbers HQ201714, HQ201713 and HQ201712, respectively. Appendix E showed the DNA sequences and organism profile matching and percentage of similiraty.

The 18S rRNA, *rbcL* gene and ITS region of *Nannochloropsis* sp. were aligned with the homologous sequences of other algae with the Clustal W software. The phylogenetic analysis was carried out with neighbour joining tree of other species in genus *Nannochloropsis*. The consensus tree was calculated by 1000 permutation with bootstrap values of >50% as shown in Figs. 7.1, 7.2 and 7.3. The bootstrap consensus maximum parsimony tree of algae in class Eustigmatophyceae based on 18S rRNA sequences showed identical phylogenetic relationship brought out by the Neighbour-Joining tree, which proved the accuracy of the phylogenetic analysis. Fig. 7.2 suggests that *Nannochloropsis* sp. is grouped with a branch containing *N. oceanica*, *N. granulata* and *N. oculata* with the outgroups *Coccoid pelagophyte* and *Chrysosaccus* sp. Fig. 7.3 suggests that *Nannochloropsis* sp. is grouped with a branch containing *N. garditana*, *N. salina*, *N. oceanica*, *N. maritime* and *N. oculata* with the outgroups *Vischeria helvetica*, *Eustigmatos magna*, *N. oceanica* (LAMB0001) and *Nannochloropsis* (EU165325). Fig. 7.4 suggests that *Nannochloropsis* sp. is grouped with a branch containing *Nannochloropsis* (JX 913539) and *N. oceanica* with the outgroups *Rhizosolenia setigera* and *N. oceanica* (LAMB0001). The PCR product was sequenced using 18S rRNA, *rbcL* gene and ITS region, forward and reverse universal primers on both strands. Based on a BLAST search of GenBank and complete sequences, the 18S rRNA of *Nannochloropsis* sp. is 99 % identical to that of *N. oculata* (AF045044) and *N. oceanica* (LAMB0001). The partial sequence of *rbcL* gene of *Nannochloropsis* sp. is 97% similar to that of *N. oculata* (AB052286) (APPENDIX E). The ITS region of *Nannochloropsis* sp. gave the identification until genus level. The phylogenetic tree based on the ITS region of rRNA showed that the species belong to *Nannochloropsis* while the Neighbor-Joining tree based on partial sequence of 18S rRNA and *rbcL* gene confirmed that the species is *Nannochloropsis oculata*.

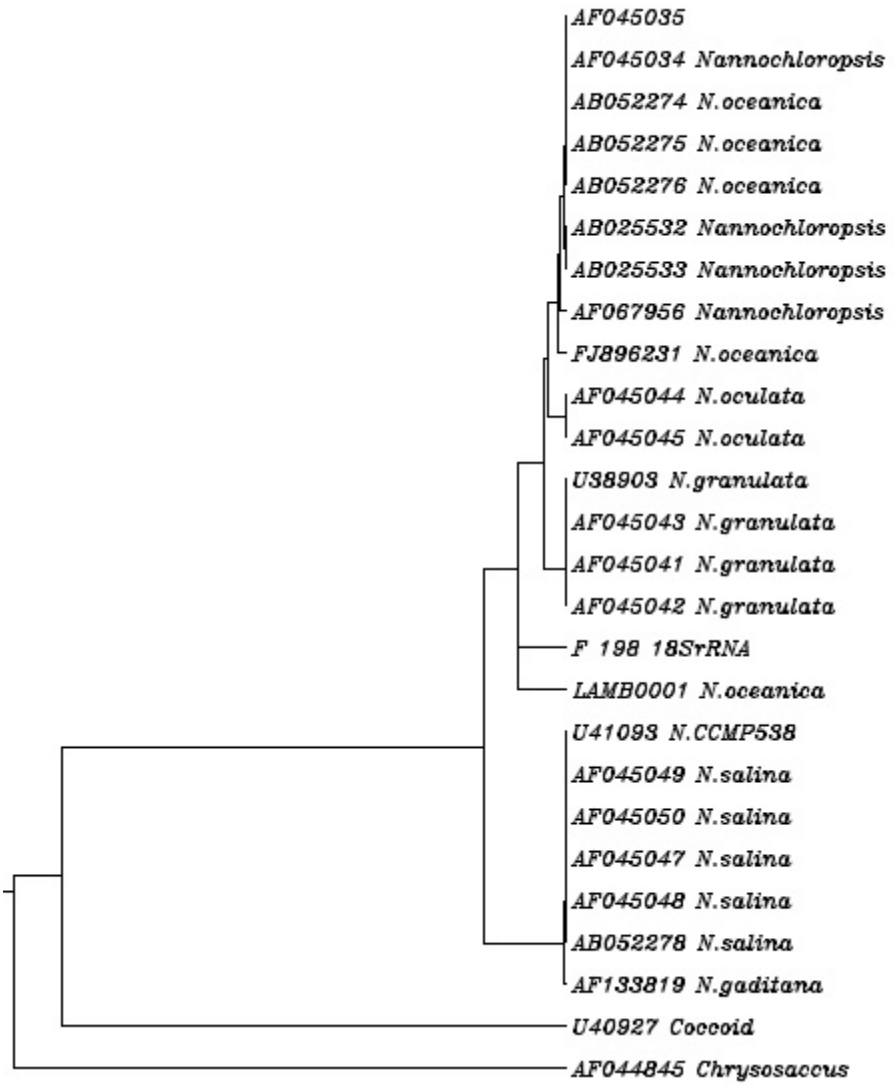


Fig. 7.2 Neighbour-Joining tree of Eustigmatophyceae and other chromophyte algae based on 18S rRNA sequence analysis

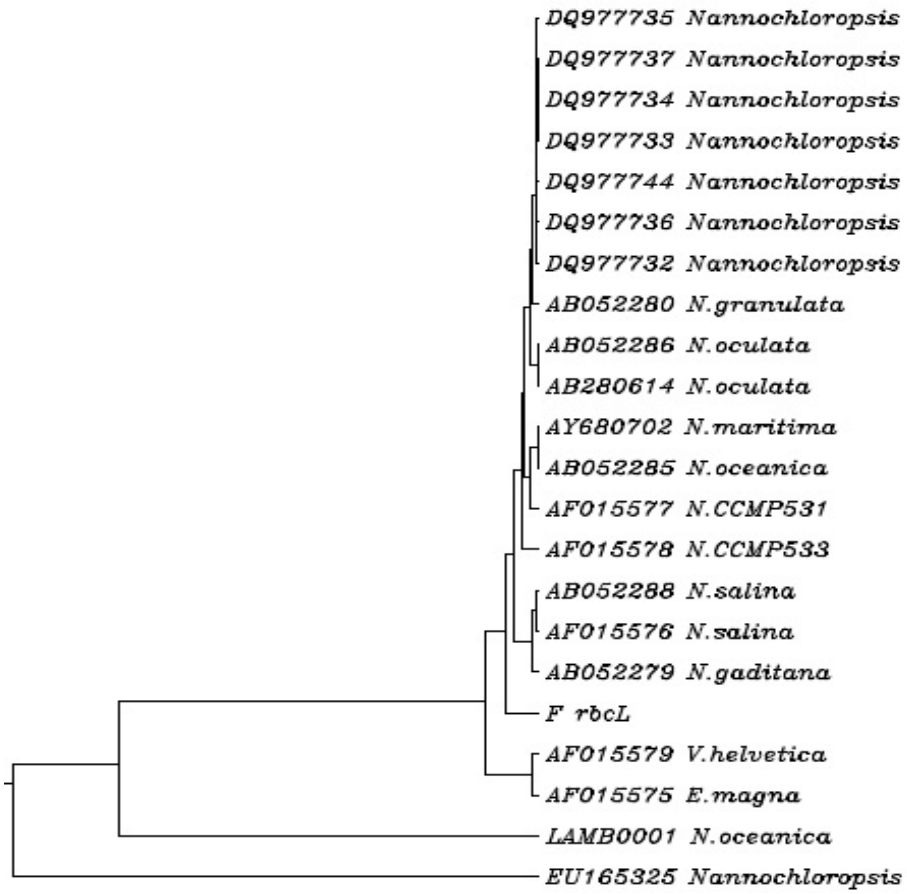


Fig. 7.3 Neighbour-Joining tree of algae in class Eustigmatophyceae based on *rbcL* gene sequence analysis

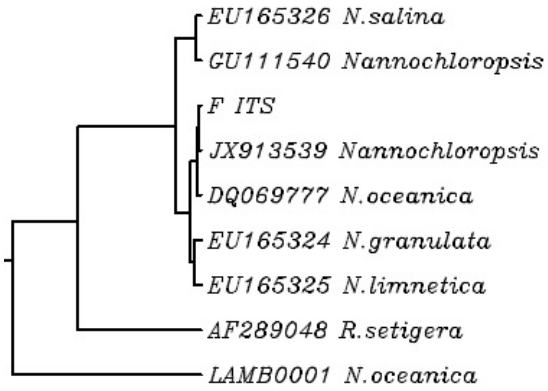


Fig. 7.4 Neighbour-Joining tree of algae in class Eustigmatophyceae based on ITS region of ribosomal RNA sequence analysis

7.3 Discussion

Molecular approach identifies the species to be *Nannochloropsis oculata*. The 18S rRNA, *rbcL* gene and ITS region sequences were selected as identifying tools, which have been widely applied in the identification of many species on different taxonomical levels. The 18S rRNA has been used in the phylogenetic analysis of Eustigmatophyceae [223]. The *rbcL* gene which encodes the large unit of ribulose -1, 5-bisphate carboxylase/oxygenase (RUBISCO) has been cloned from a large number of plant species [224]. The ITS of ribosomal RNA transcription unit is a desirable nuclear marker due to its high evolution rate and moderate length [225]. For higher plants, chloroplast DNA (cpDNA) variation is commonly used for molecular phylogenic analysis [226]. However, the low evolutionary rate of cpDNA limits its use in identifying an organism to genus or species. In our study *rbcL* gene is able to distinguish microalgal species. However the ITS region is more varied than 18S rRNA [227]. The combination of 18S rRNA and ITS region could identify an organism to the taxa below species, which has been applied in identification of two populations of *Ditylum brightwellii* [228].

In our study, the gene sequences were used successfully to identify the species. However, the molecular markers should be developed so that the strains or the varieties of a species could be distinguished further. The DNA finger printing technique based on molecular markers such as microsatellite DNA and Single Nucleotide Polymorphism (SNP) are among alternatives to be explored. Using microsatellite DNA, molecular identification can tell the differences among the varieties or strains within a species. The toxic dinoflagellate *Alexandrium minutum* has been investigated with microsatellite DNA where Global and Pacific clades are distinguished clearly. SNP has been applied in deepening the identification of species in habitat types as well as in eelgrass [229]. The drawback is the development of both microsatellite DNA and SNP markers is time-consuming and labour-intensive. Microsatellite DNA can be amplified only in a specific species or its closely related species (cross species amplification). SNP markers are powerful only when they are associated with high throughput-genotyping techniques (e.g., DNA chips) [222].

7.4 Summary

A species in genus *Nannochloropsis* has been identified based on18S ribosomal RNA gene, ITS region of ribosomal RNA transcription unit and *rbcL* gene. DNA purity, PCR products, DNA sequencing and phylogenetic analysis carried out to identify the species belonging to genus *Nannochloropsis*. BLAST search of GenBank showed that the complete sequences of the species shared 97-99% similarity with *N. oculata* as confirmed by sequence alignment and phylogenetic tree analysis.

CHAPTER 8

CONCLUSION AND RECOMMENDATIONS FOR FUTURE WORK

8.1 Conclusion

The biomass and lipid productivities of microalgal cultures can be increased by optimizing the growth parameters using Response Surface Methodology. The cell density, cell dry weight and lipid content increased with the increase of pH, salinity, photoperiod and light intensity for all the four tested species. The optimum pH and salinity were pH 8-9 and salinity of 35-40 ppt to achieve high cell dry weight and lipid content. The maximum cell dry weight and lipid content were achieved at optimum conditions of 19.3-24 h illumination and light intensity of 162-198 µmol photons $m^{-2}s^{-1}$. Estimated response surface plots of interaction between nitrate, phosphate and iron for biomass and lipid confirmed that, at nutrient deficient conditions, cells grow slowly but continue synthesizing lipids, resulting in enhanced lipid content. At nutrient sufficient conditions, cellular growth outpaces lipid production resulting in reduced lipid content. At intermediate nutrient deficient conditions, however, cell growth rate reaches a maximum without compromising the lipid content. A characteristic behaviour of microalgal cells is their great capacity to uptake nitrate, as well as other limiting nutrients, from the media and to store it intracellularly. Changes in the cell physiology occur when the intracellular nutrient concentration increases. It is expected that these physiological changes are accompanied with variations in the cell metabolism. Nitrate supply was considered as substrate utilization, which affected the biomass and lipid yield.

The interactions of pH, salinity, photoperiod and light intensity and macronutrients significantly influenced the biomass and lipid content positively.

All nutrients affected cell growth positively, though the interaction of phosphate-phospate for *N. oculata* and interaction of nitrate-nitrate for *I. galbana* may affect negatively the growth. The interaction of nitrate, phosphate, phosphate-phosphate and ferum-ferum interactions may all affect lipid content, negatively. The $r^2$of 78.7-95.1% for biomass and 80.1-97.5% for lipid and mean absolute error percentage between experimental and predicted values of 0.02-0.042% and 0.61-1.66% for biomass and lipid content of all four tested species, suggest good agreements between experimental and predicted values. An alternative and economical medium developed based on palm oil mill effluent (POME), with added POME remediation achieved high algal growth and lipid production. POME characterization suggested the presence of macronutrients which were suitable for the growth of microalgae. High lipid content was attained under 10-15% POME composition. The COD, TOC, BOD, TN and oil and grease removal efficiencies varied with different levels of wastewater.

Kinetics studies were established and fatty acids profiles were developed for algal cultivation in 5 L photobioreactor and 300 L open tanks at optimized conditions. Cultivation in 250 mL- 30 L batch cultures showed the maximum specific growth rate of 0.15-0.17 d^{-1} and lipid content of 27.2-37.1%. The biomass and lipid content were higher in 5 L photobioreactor and lower in 300 L open tanks, attributable possibly to more representative sampling and culture harvesting method. The fatty acid profile of the algal lipid extracted from the cells cultured under the optimized conditions indicated that the algae is a suitable feedstock for biodiesel production. Fast growth and increased lipid productivity were associated with an increased lipid quality. The final aim was to identify a species in genus *Nannochloropsis.* Identification was done based on18s ribosomal RNA gene, ITS region of ribosomal RNA transcription unit and *rbcL* gene.

8.2 Recommendations for Future Work

As the field of microalgal biotechnology is still in its infancy, there are plenty of opportunities for improvement. Further research should explore the effect of temperature and osmotic pressure and determine the functional relationships of the growth and lipid production rate. Low-cost microalgal bioenergy and high-value biocompounds can be produced optimally by improving the algal biology through genetic and metabolic engineering. The key enzymatic steps in microalgal fatty acid, biosynthetic and metabolic pathways should be identified for enhancement of lipid production.

Lipid production can be modelled as the coupling of a few series of reactions that generate all the fatty acids found in the cells: de-novo synthesis, elongation, and denaturation. A mechanistic model can be proposed starting with the current understanding of fatty acid profile, and moving to close any gap that results between model prediction and experimental observations. It is necessary to evaluate how the transitions between different cell cycle stages are regulated and what is the effect of growth promoters and inhibitors on microalgae at the different stages of the cell cycle.

To increase the efficiency of the extraction process, it is desirable to ensure that all the cells that are subjected to lipid extraction contain maximum amount of lipids. Extracting the lipid from the wet algae biomass using solvent is very energy intensive. Hence alternative processes of extracting the lipid from the wet biomass must be developed to make algal biofuel feasible. Wet algal biomass can be treated with sonication or high pressure to break the algae cell wall and release the intrinsic lipid into the algae slurry. Once the lipid part comes outside the cell wall, it would make a separate layer at the top and then it can be separated and converted into biodiesel using transesterification reaction.

Biorefinery concept and advances in photobioreactor engineering could further lower the cost of algal production, especially the use of hybrid tubular photobioreactors to enhance microalgal biomass productivity for biodiesel production. POME media should be used to produce the cost-effective microalgal biofuel. The consumption of CO_2 by microalgae should be investigated for better understanding of microalgal species to fix CO_2.

Further commercialization of microalgae as an integrated method for CO_2 removal, bioenergy generation and wastewater treatment could be implemented.

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APPENDIX A

INSTRUMENTS AND GLASSWARE USED

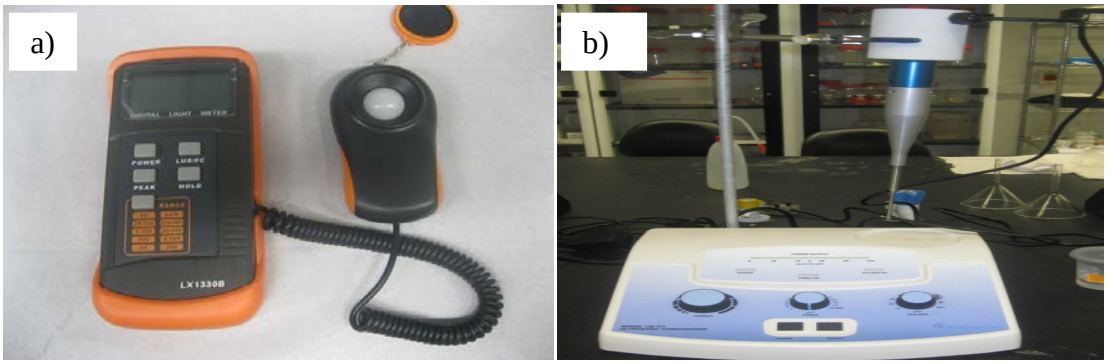


Fig A1: a) Lux Meter b) Ultrasonic homogenizer (Model 150/VT)

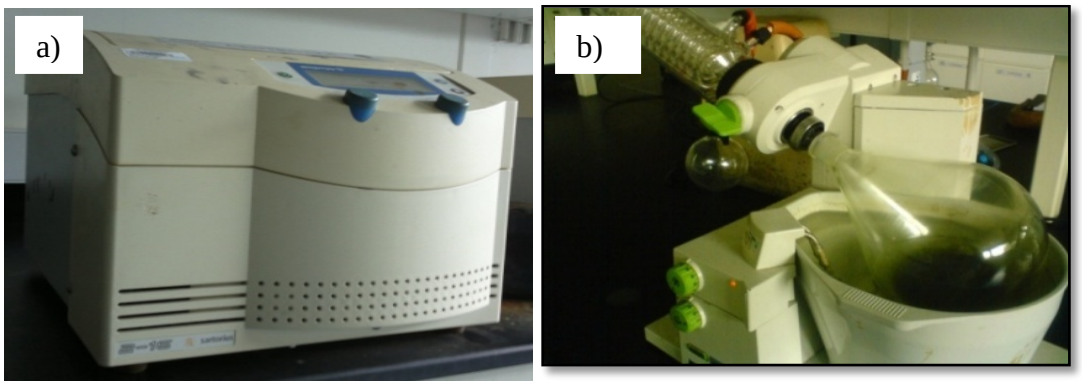


Figure A2: a) Centrifuge b) Rotary Evaporator

Figure A3: Gas Chromatography (7890A GC System)

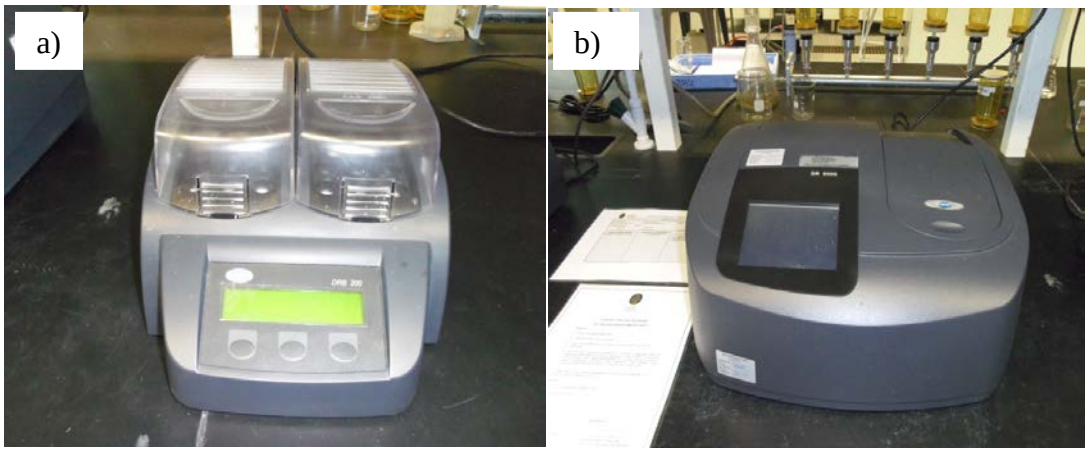


Figure A4: a) COD Thermoreactore DRB 200 b) Spectrophotometer HACH DR 500 for COD absorbance measurement

Figure A5: BOD track

Figure A6: 250 mL microalgae cultivations in laboratory

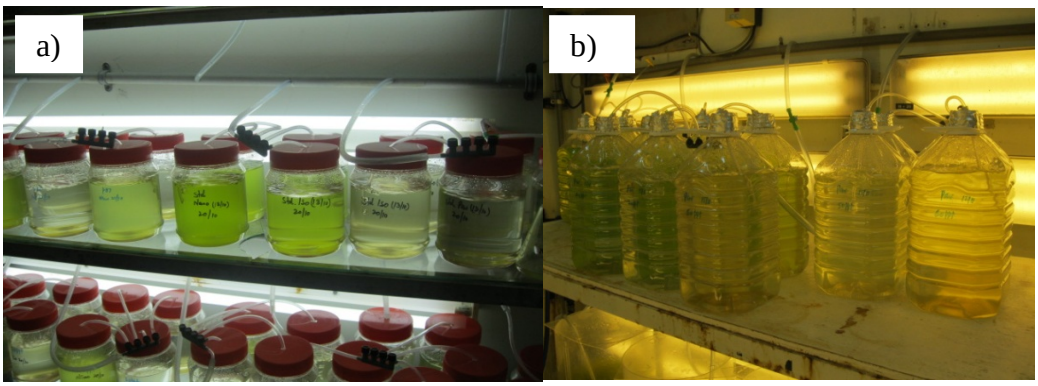


Figure A7: Cultivation of microalgae in a) 1-L and b) 5-L plastic container

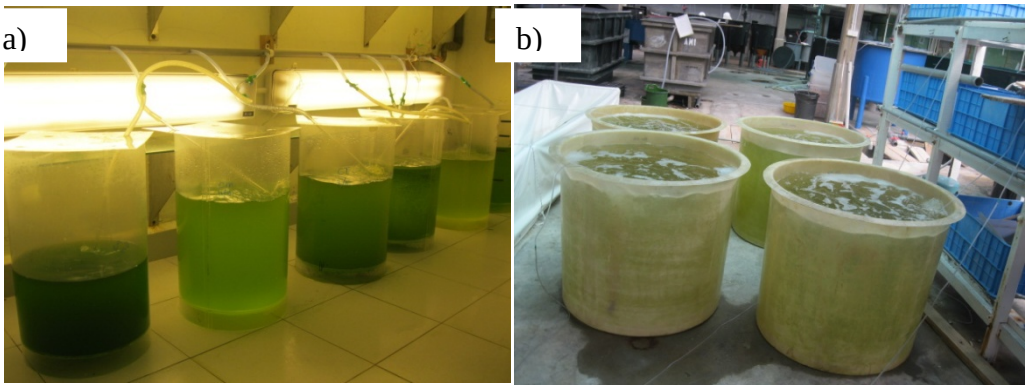


Figure A8: Cultivation of microalgae in a) 30 L tanks and b) 300 L tanks

Figure A9: Cultivation of microalgae in photobioreactor (Biosys 7-L x 6 Plus)

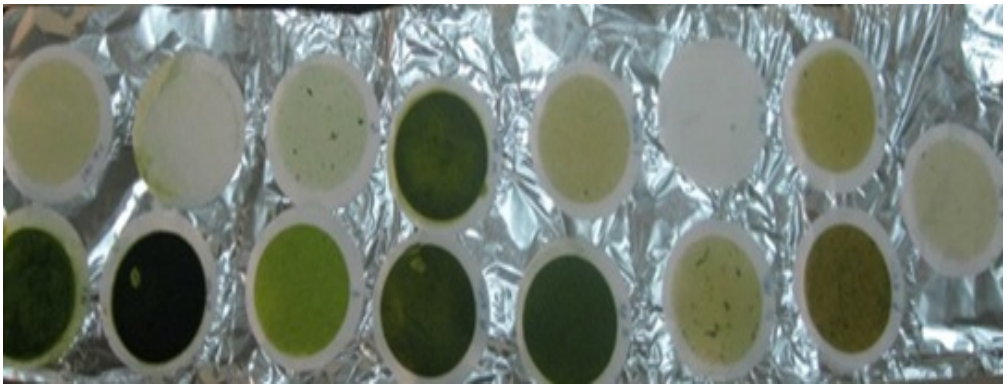


Figure A10: Dry weight by glass micro fiber filter (Whatman GF/C 0.47 µ)

APPENDIX B

MEDIA PREPARATIONS

Table B1: Composition of Conway media

Stock Solution	Constituents	Amount
Main Mineral Solution	$NaNO_3$ or KNO_3	100.00 g or 116 g
	Disodium EDTA	45.00 g
	H_3BO_3	33.60 g
	$Na_2HPO_4.4H_2O$	20.00 g
	$FeCl_3.6H_2O$	1.30 g
	$MnCl_2.4H_2O$	0.36 g
	Trace metal solution	1.00 mL
	Distilled water	1000 mL
Trace Metal Solution	$ZnCl_2$	2.10 g
	$CoCl_2.6H_2O$,	2.00 g
	$(NH_4)_6MO_7O_4.4H_2O$	0.90 g
	$CuSO_4.5H_2O$, 2.0;	2.00 g
	Distilled water	100 mL
Vitamin Solution	Thyamine chlorohydrate, B_1	200 mg
	Cyanocobalamin, B_{12}	10 mg
	Distilled water	100 mL
Nitrate Solution	KNO_3	100 g
	Distilled water	1000 mL

Table B2: Composition of TMRL Enrichment Medium

Constituents	Amount	Distilled water
KNO_3	100 g	1000 mL
$Na_2HPO_4.12H_2O$	10 g	1000 mL
$FeCl_3.6H_2O$	3 g	1000 mL
$Na_2SiO_3.9H_2O$	1 g	1000 mL

APPENDIX C

RSM RESULT REPORT

Table C1: Multilevel factorial design and responses for pH and salinity

Independent Variables			Responses															
			Dry Weight (g L^{-1})								Lipid (%)							
			N. oculata		*T. suecica*		*I. galbana*		*P. lutheri*		*N. oculata*		*T. suecica*		*I. galbana*		*P. lutheri*	
Run	pH	Salinity (ppt)	Experimental Value	Predicted Value	Experimental Value	Predicted Value	Experimental Value	Predicted Value	Experimental Value	Predicted Value	Experimental Value	Predicted Value	Experimental Value	Predicted Value	Experimental Value	Experimental Value	Experimental Value	Experimental Value
1	8	35	0.82	0.78	0.72	0.65	0.56	0.59	0.58	0.57	35.7	33.5	24.6	23.7	24.4	26.7	37.3	35.1
2	7	25	0.61	0.60	0.61	0.57	0.37	0.39	0.55	0.54	24.4	24.1	23.2	21.1	18.4	18.8	33.7	33.4
3	9	25	0.59	0.58	0.62	0.63	0.38	0.39	0.54	0.53	21.5	21.7	20.3	20.2	15.3	15.2	33.7	34.3
4	7	30	0.73	0.72	0.51	0.59	0.51	0.49	0.52	0.52	30.3	31.6	22.1	22.4	24.5	23.6	33.5	32.9
5	6	40	0.68	0.63	0.40	0.39	0.48	0.49	0.49	0.55	29.7	27.4	14.8	14.5	18.5	19.8	34.4	33.9
6	9	40	0.65	0.63	0.61	0.59	0.68	0.69	0.42	0.41	28.5	28.5	20.1	20.1	27.4	27.8	27.6	29.3
7	9	30	0.71	0.72	0.63	0.66	0.60	0.54	0.46	0.43	27.5	29.7	21.9	21.9	24.3	22.5	28.6	28.4
8	7	40	0.65	0.68	0.50	0.53	0.57	0.55	0.47	0.46	26.8	29.5	20.3	19.9	26.3	23.8	32.4	32.2
9	6	30	0.59	0.64	0.49	0.46	0.48	0.48	0.48	0.50	28.7	30.1	17.5	17.3	21.9	22.1	29.2	31.6
10	6	25	0.48	0.47	0.41	0.44	0.42	0.40	0.52	0.51	23.3	22.7	15.3	16.1	19.1	18.5	31.4	32.3
11	8	25	0.55	0.59	0.68	0.63	0.36	0.38	0.34	0.38	24.4	23.8	21.6	22.5	16.4	17.7	27.6	26.9
12	9	35	0.74	0.74	0.67	0.65	0.62	0.64	0.47	0.46	33.3	31.9	22.8	21.9	26.2	26.7	32.4	30.7
13	6	35	0.69	0.70	0.45	0.45	0.49	0.51	0.51	0.51	31.1	31.6	16.1	16.7	22.1	22.5	30.9	32.5
14	7	35	0.78	0.77	0.63	0.58	0.55	0.55	0.51	0.42	33.9	33.4	22.6	22.1	25.9	25.3	26.8	27.9
15	8	40	0.64	0.68	0.55	0.59	0.64	0.62	0.36	0.41	29.1	29.9	20.6	21.8	26.5	26.5	28.1	26.6
16	8	30	0.77	0.75	0.63	0.66	0.52	0.51	0.27	0.27	32.8	31.5	22.3	23.9	23.7	23.7	24.4	24.0

Table C2: Multilevel factorial design and responses for photoperiod and light intensity

Independent Variables			Responses															
			Dry Weight (g L^{-1})								Lipid (%)							
			N. oculata		*T. suecica*		*I. galbana*		*P. lutheri*		*N. oculata*		*T. suecica*		*I. galbana*		*P. lutheri*	
Run	Photoperiod (hrs)	Light Intensity (µmol $m^{-2}s^{-1}$)	Experimental Value	Predicted Value	Experimental Value	Predicted Value	Experimental Value	Predicted Value	Experimental Value	Predicted Value	Experimental Value	Predicted Value	Experimental Value	Predicted Value	Experimental Value	Experimental Value	Experimental Value	Experimental Value
1	19.3	198	0.78	0.73	0.67	0.65	0.62	0.63	0.65	0.67	33.5	31.5	11.4	12.9	26.7	27.8	34.6	34.5
2	10	198	0.57	0.53	0.42	0.43	0.48	0.43	0.47	0.40	24.4	22.0	14.2	14.7	22.4	21.1	27.5	27.8
3	24	198	0.84	0.85	0.72	0.71	0.61	0.63	0.67	0.70	36.6	35.0	23.9	24.1	26.6	27.1	36.4	36.2
4	24	90	0.63	0.62	0.51	0.52	0.53	0.53	0.36	0.37	25.4	26.5	18.4	18.4	23.3	22.5	26.6	26.9
5	10	90	0.38	0.36	0.26	0.27	0.33	0.30	0.23	0.24	17.4	17.6	11.0	10.1	16.4	15.0	18.5	19.5
6	24	162	0.74	0.78	0.65	0.67	0.64	0.62	0.62	0.59	31.6	34.5	22.0	23.5	28.1	26.9	31.7	32.1
7	14.7	90	0.42	0.44	0.42	0.39	0.46	0.45	0.38	0.36	18.7	21.4	14.0	14.3	20.3	20.2	23.8	23.0
8	19.3	126	0.67	0.61	0.56	0.56	0.58	0.58	0.51	0.49	29.5	29.1	19.6	20.2	25.5	25.8	27.3	27.4
9	14.7	198	0.58	0.63	0.55	0.56	0.54	0.56	0.58	0.57	22.9	27.2	19.3	19.3	25.3	25.8	32.6	31.7
10	10	162	0.48	0.49	0.47	0.41	0.36	0.41	0.25	0.34	22.4	22.9	15.8	14.5	18.2	20.4	23.8	24.0
11	10	126	0.43	0.43	0.31	0.35	0.35	0.37	0.29	0.29	21.3	21.4	11.4	12.9	17.4	18.4	22.3	21.2
12	14.7	126	0.51	0.52	0.50	0.47	0.51	0.51	0.49	0.43	29.4	25.6	18.5	17.3	23.4	23.4	25.7	24.8
13	14.7	162	0.55	0.58	0.49	0.53	0.57	0.55	0.53	0.50	26.0	27.6	17.6	18.9	27.4	25.3	26.7	27.7
14	19.3	162	0.71	0.68	0.61	0.62	0.66	0.62	0.56	0.58	31.5	31.5	22.9	21.9	28.8	27.5	29.3	30.4
15	19.3	90	0.49	0.52	0.45	0.47	0.49	0.52	0.33	0.40	26.7	24.4	16.4	17.1	21.4	22.7	25.1	25.5
16	24	126	0.72	0.71	0.64	0.61	0.59	0.59	0.52	0.48	32.5	31.7	22.7	21.6	24.4	25.4	29.8	28.9

Table C3: Multilevel factorial design and responses for nitrate, phosphate and iron

Independent Variables				Responses															
				Dry Weight (g L^{-1})								Lipid (%)							
				N. oculata		*T. suecica*		*I. galbana*		*P. lutheri*		*N. oculata*		*T. suecica*		*I. galbana*		*P. lutheri*	
Run	KNO_3 (g L^{-1})	Na_2HPO_4 (g L^{-1})	$FeCl_3$ (g L^{-1})	Experimental Value	Predicted Value	Experimental Value	Predicted Value	Experimental Value	Predicted Value	Experimental Value	Predicted Value	Experimental Value	Predicted Value	Experimental Value	Predicted Value	Experimental Value	Experimental Value	Experimental Value	Experimental Value
1	10	12	1	0.58	0.58	0.59	0.54	0.43	0.43	0.28	0.27	29.4	27.3	18.4	18.3	21.2	22.5	23.3	22.3
2	120	12	4	0.72	0.72	0.81	0.78	0.63	0.62	0.68	0.72	26.4	24.6	18.5	17.8	25.4	23.7	25.2	23.1
3	120	12	2.5	0.67	0.68	0.74	0.74	0.56	0.56	0.63	0.61	27.4	26.6	20.1	19.7	23.4	22.3	24.2	25.3
4	10	7.5	4	0.61	0.63	0.61	0.56	0.42	0.42	0.33	0.32	33.0	33.3	19.4	20.6	22.7	20.8	26.2	25.5
5	65	7.5	4	0.71	0.67	0.71	0.67	0.58	0.56	0.47	0.45	31.6	31.5	22.4	20.5	26.4	27.5	31.5	32.1
6	10	12	4	0.64	0.62	0.58	0.57	0.48	0.49	0.29	0.34	28.6	28.9	17.4	17.4	18.5	20.5	25.7	25.1
7	10	3	1	0.43	0.43	0.37	0.40	0.32	0.36	0.49	0.35	31.9	32.4	20.7	20.6	15.3	17.8	24.4	23.9
8	65	12	1	0.64	0.59	0.63	0.62	0.58	0.53	0.34	0.38	24.7	26.6	17.8	18.9	24.9	25.9	28.4	29.5
9	10	12	2.5	0.56	0.58	0.51	0.56	0.44	0.46	0.39	0.30	28.7	30.3	20.3	20.0	23.8	22.0	26.4	26.6
10	65	3	2.5	0.62	0.53	0.57	0.50	0.45	0.48	0.38	0.37	32.6	32.4	19.8	21.0	23.7	23.6	37.2	34.4
11	65	12	2.5	0.59	0.61	0.71	0.66	0.57	0.56	0.38	0.43	27.5	29.3	22.2	20.9	25.9	26.8	33.9	33.4
12	65	3	4	0.64	0.61	0.38	0.52	0.47	0.49	0.35	0.38	30.4	29.6	19.7	19.2	24.4	24.2	32.4	32.0
13	65	3	1	0.47	0.48	0.50	0.46	0.51	0.48	0.26	0.40	29.4	30.0	18.4	18.5	23.4	21.9	31.6	31.1
14	65	7.5	2.5	0.53	0.62	0.68	0.65	0.54	0.53	0.43	0.42	35.4	33.7	23.0	22.5	28.3	27.2	34.3	34.2
15	10	7.5	1	0.55	0.55	0.52	0.54	0.44	0.40	0.21	0.33	32.1	32.8	21.1	21.0	23.3	22.1	22.2	23.4
16	10	3	2.5	0.48	0.48	0.43	0.42	0.38	0.34	0.24	0.30	33.9	34.3	22.4	22.8	17.5	18.0	25.1	27.6
17	10	3	4	0.52	0.56	0.48	0.42	0.35	0.33	0.28	0.26	31.5	31.9	21.0	20.7	16.5	17.2	25.5	25.4
18	65	7.5	1	0.51	0.58	0.61	0.61	0.45	0.51	0.54	0.40	33.0	31.6	19.9	20.2	26.7	25.9	30.6	30.6
19	120	3	4	0.70	0.72	0.62	0.59	0.52	0.53	0.54	0.52	25.5	25.6	16.3	15.5	23.1	21.9	23.7	23.5
20	120	12	1	0.67	0.67	0.64	0.67	0.48	0.50	0.53	0.52	24.4	24.2	17.1	17.3	21.2	19.9	22.4	21.7
21	120	7.5	2.5	0.73	0.71	0.70	0.71	0.54	0.53	0.56	0.57	28.4	30.5	19.4	19.9	19.4	23.0	27.5	26.1
22	120	3	2.5	0.61	0.64	0.55	0.55	0.50	0.50	0.51	0.49	29.8	28.7	15.9	17.0	18.6	19.8	24.1	26.2
23	65	12	4	0.65	0.64	0.61	0.69	0.61	0.61	0.58	0.51	28.4	27.6	17.4	18.7	25.9	26.8	28.9	31.6
24	10	7.5	2.5	0.64	0.58	0.49	0.56	0.40	0.41	0.29	0.31	37.3	35.2	23.6	22.9	24.0	22.0	28.3	27.3
25	120	7.5	1	0.72	0.67	0.63	0.65	0.49	0.50	0.53	0.52	28.9	28.7	17.8	17.3	18.3	20.3	22.4	22.8
26	120	7.5	4	0.78	0.77	0.77	0.75	0.59	0.58	0.62	0.64	25.6	28.0	16.7	18.2	24.4	24.8	22.6	23.6
27	120	3	1	0.58	0.58	0.48	0.49	0.51	0.48	0.49	0.48	27.8	27.3	15.1	14.1	18.4	16.6	23.4	23.3

Table C4: List of polynomial equations for pH, Salinity, photoperiod and light intensity

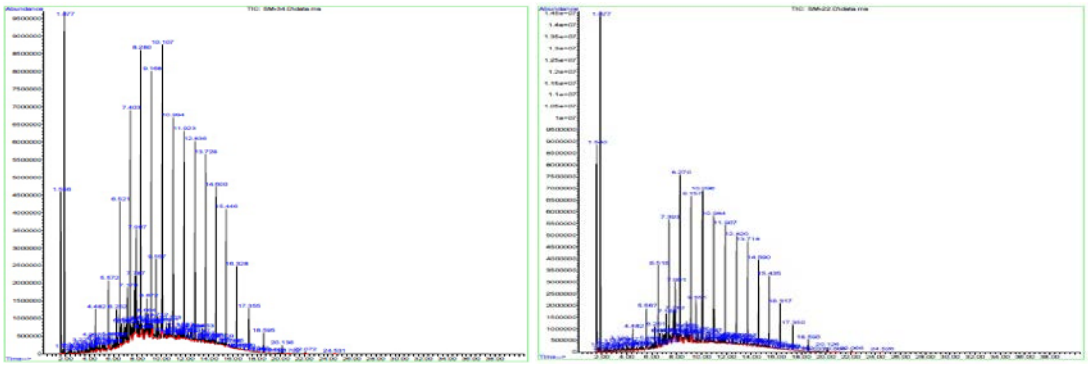
Experimental Conditions	Responses	Second Order Polynomial Equations				r^2			
		N. oculata	*T. suecica*	*I. galbana*	*P. lutheri*	*N. oculata*	*T. suecica*	*I. galbana*	*P. lutheri*
pH and Salinity	DW	$(x_1,x_2) = -4.301 + 0.50\,x_1 + 0.19\,x_2 - 0.03\,x_1^2 - 0.0026\,x_1 x_2 - 0.0024\,x_2^2$	$(x_1,x_2) = -2.553 + 0.57\,x_1 + 0.052\,x_2 - 0.034\,x_1^2 - 0.0003\,x_1 x_2 - 0.00087 x_2^2$	$(x_1,x_2) = 0.164 - 0.19\,x_1 + 0.046\,x_2 + 0.0044\,x_1^2 + 0.0049\,x_1 x_2 - 0.00107\,x_2^2$	$(x_1,x_2) = -4.08 + 0.74\,x_1 + 0.101\,x_2 - 0.042\,x_1^2 - 0.0022\,x_1 x_2 - 0.00122\,x_2^2$	86.60	82.45	94.21	84.04
	Lipid	$(x_1,x_2) = -137.73 + 11.70\,x_1 + 7.45\,x_2 - 0.87\,x_1^2 + 0.04422 x_1 x_2 - 0.11\,x_2^2$	$(x_1,x_2) = -115.09 + 27.46\,x_1 + 1.88\,x_2 - 1.80\,x_1^2 + 0.035\,x_1 x_2 - 0.034\,x_2^2$	$(x_1,x_2) = -39.75 + 3.045\,x_1 + 2.66\,x_2 - 0.69\,x_1^2 + 0.25\,x_1 x_2 - 0.063\,x_2^2$	$(x_1,x_2) = -130.05 + 29.79\,x_1 + 2.65\,x_2 - 1.92\,x_1^2 + 0.021\,x_1 x_2 - 0.040\,x_2^2$	87.46	90.34	91.20	86.19
Photoperiod and Light Intensity	DW	$(x_3,x_4) = -0.0029 + 0.0084\,x_3 + 0.0031\,x_4 + 0.00023\,x_3^2 + 0.000029\,x_3 x_4 - 0.0000058\,x_4^2$	$(x_3,x_4) = -0.41 + 0.042\,x_3 + 0.0045\,x_4 - 0.00077\,x_3^2 + 0.000020\,x_3 x_4 - 0.000011\,x_4^2$	$(x_3,x_4) = -0.53 + 0.071\,x_3 + 0.0042\,x_4 - 0.0015\,x_3^2 - 0.0000202\,x_3 x_4 - 0.00000964\,x_4^2$	$(x_3,x_4) = -0.31 + 0.060\,x_3 + 0.00017\,x_4 - 0.0018\,x_3^2 + 0.00011\,x_3 x_4 + 9.645\text{E-}7\,x_4^2$	94.51	95.07	93.08	88.18
	Lipid	$(x_3,x_4) = -10.62 + 1.05\,x_3 + 0.27\,x_4 - 0.02\,x_3^2 + 0.0027\,x_3 x_4 - 0.00089\,x_4^2$	$(x_3,x_4) = -15.91 + 1.67\,x_3 + 0.18\,x_4 - 0.034\,x_3^2 + 0.00077\,x_3 x_4 - 0.00050\,x_4^2$	$(x_3,x_4) = -20.40 + 2.74\,x_3 + 0.21\,x_4 - 0.062\,x_3^2 - 0.00095\,x_3 x_4 - 0.00052\,x_4^2$	$(x_3,x_4) = 9.45 + 1.29\,x_3 - 0.048\,x_4 - 0.024\,x_3^2 + 0.00069\,x_3 x_4 + 0.00041\,x_4^2$	84.81	94.38	90.25	97.53

Table C5: List of polynomial equations for nitrate, phosphate and iron

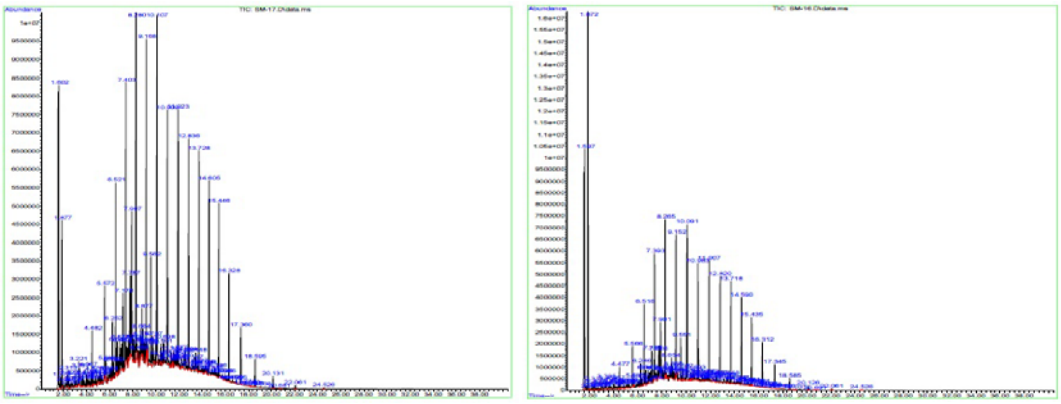
Experimental Conditions	Responses	Second Order Polynomial Equations				r^2			
		N. oculata	*T. suecica*	*I. galbana*	*P. lutheri*	*N. oculata*	*T. suecica*	*I. galbana*	*P. lutheri*
Nitrate, Phosphate and Iron	DW	$(x_1,x_2,x_3) = 0.27 + 0.00046 x_1 + 0.053 x_2 + 0.021 x_3 + 0.0000086 x_1^2 - 0.000061 x_1x_2 + 0.000020 x_1x_3 - 0.0022 x_2^2 - 0.0032 x_2x_3 + 0.0064 x_3^2$	$(x_1,x_2,x_3) = 0.197 + 0.00103 x_1 + 0.065 x_2 + 0.0313 x_3 + 0.0000051 x_1^2 - 0.000047 x_1x_2 + 0.00026 x_1 x_3 - 0.0034 x_2^2 - 0.00012 x_2 x_3 + 0.0054321 x_3^2$	$(x_1,x_2,x_3) = 0.32 + 0.0035 x_1 + 0.01043 x_2 - 0.0304 x_3 - 0.0000191 x_1^2 - 0.000054 x_1x_2 + 0.00020 x_1 x_3 - 0.000302 x_2^2 + 0.0028 x_2 x_3 + 0.0025 x_3^2$	$(x_1,x_2,x_3) = 0.41 - 0.00053 x_1 - 0.0021 x_2 - 0.074 x_3 + 0.0000079 x_1^2 + 0.00012 x_1x_2 + 0.00037 x_1 x_3 - 0.00088 x_2^2 + 0.0058 x_2 x_3 + 0.0047 x_3^2$	81.98	82.68	90.55	78.75
	Lipid	$(x_1,x_2,x_3) = 25.55 - 0.012 x_1 + 1.49 x_2 + 4.52 x_3 - 0.00028 x_1^2 + 0.0019 x_1x_2 - 0.0035 x_1x_3 - 0.143 x_2^2 + 0.079 x_2x_3 - 0.980 x_3^2$	$(x_1,x_2,x_3) = 14.96 - 0.031 x_1 + 0.88 x_2 + 4.85 x_3 - 0.00037 x_1^2 + 0.0055 x_1x_2 - 0.0039 x_1x_3 - 0.077 x_2^2 + 0.034 x_2 x_3 - 0.95 x_3^2$	$(x_1,x_2,x_3) = 10.15 + 0.18 x_1 + 2.06 x_2 + 0.95 x_3 - 0.0015 x_1^2 - 0.00145 x_1x_2 + 0.017 x_1x_3 - 0.098 x_2^2 - 0.053 x_2 x_3 - 0.23 x_3^2$	$(x_1,x_2,x_3) = 15.48 + 0.32 x_1 - 0.0053 x_2 + 6.81 x_3 - 0.0025 x_1^2 + 0.000023 x_1x_2 - 0.0041 x_1x_3 - 0.0143 x_2^2 + 0.044 x_2 x_3 - 1.28 x_3^2$	84.17	86.20	80.10	90.80

APPENDIX D

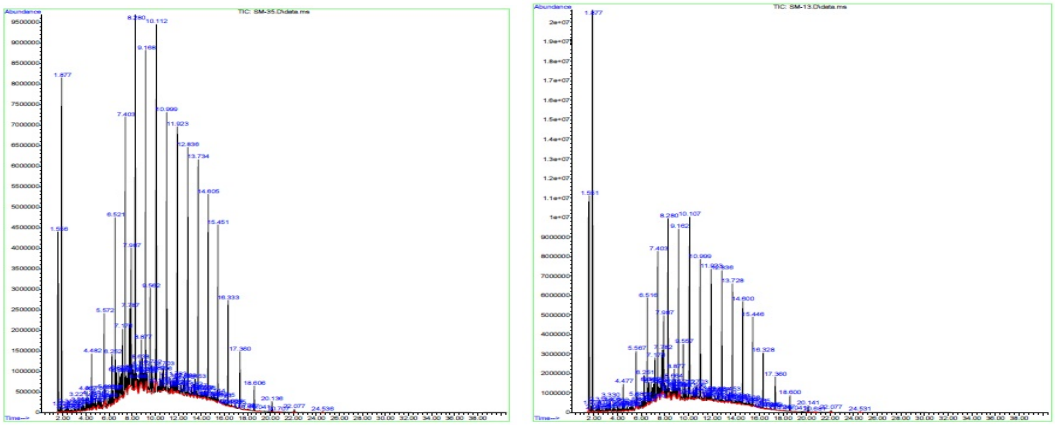
FATTY ACIDS CHROMATOGRAMS



a) d)

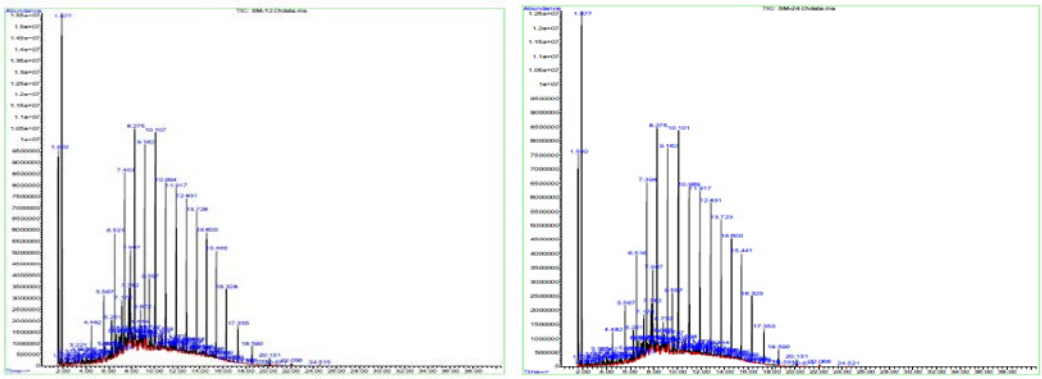


b) e)

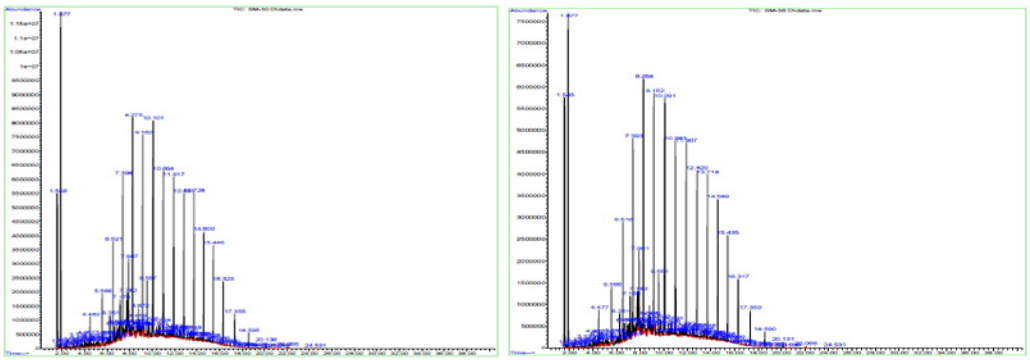


c) f)

Figure D1: Fatty acid analysis of *N. oculata* at optimum pH/salinity, photoperiod/light intensity and macronutrients a-c) in 5 L PBR and d-f) in 300 L open tank



a) d)



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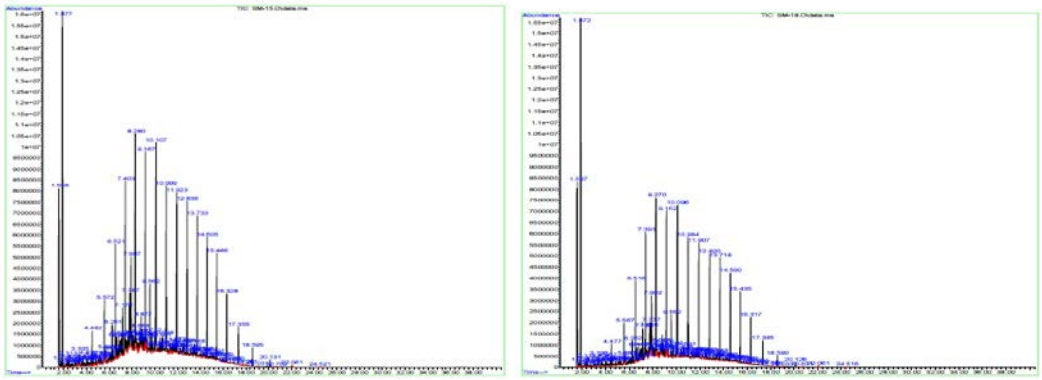
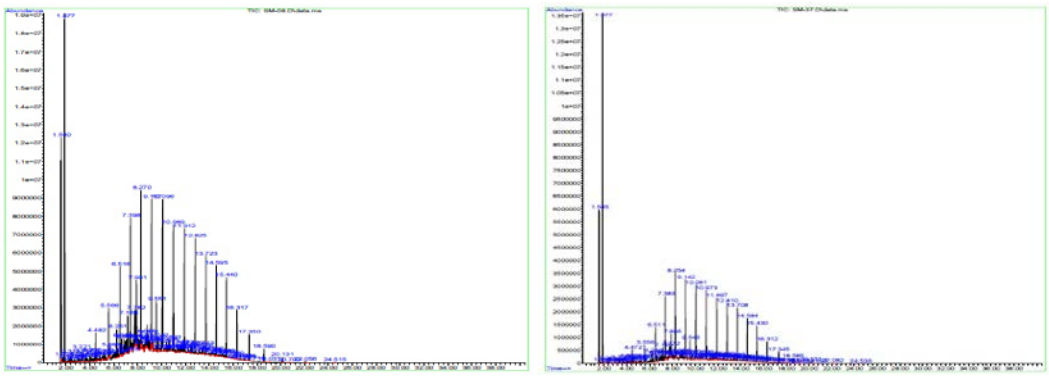
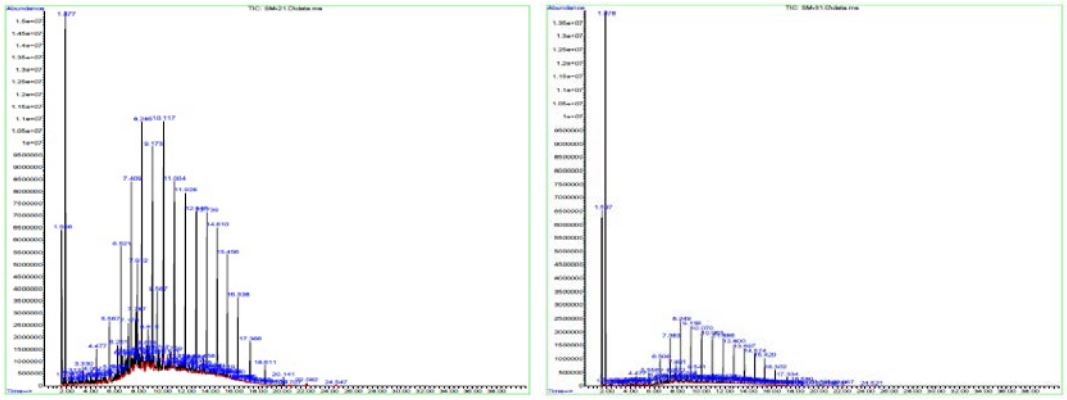


c) d)

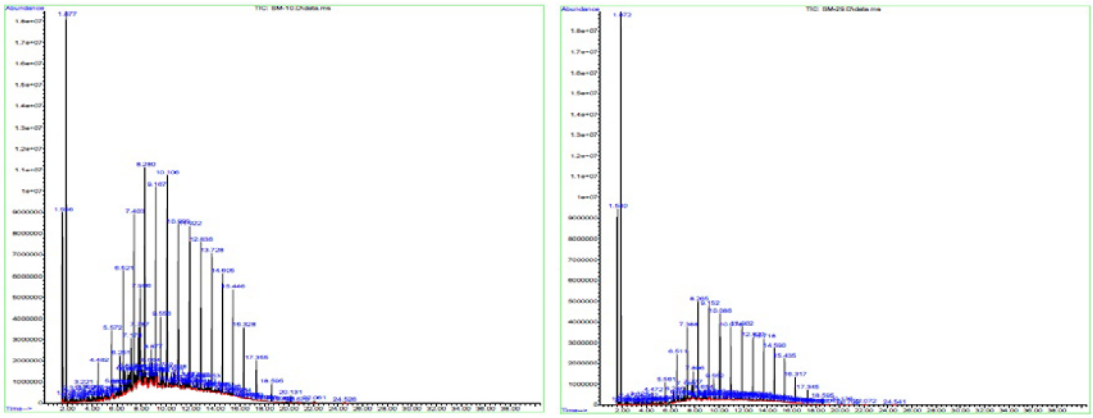
Figure D2: Fatty acid analysis of *T.suecica* at optimum pH/salinity, photoperiod/light intensity and macronutrients a-c) in 5 L PBR and d-f) in 300 L open tank



a) d)



b) e)



c) f)

Figure D3: Fatty acid analysis of *I. galbana* at optimum pH/salinity, photoperiod/light intensity and macronutrients a-c) in 5 L PBR and d-f) in 300 L open tank

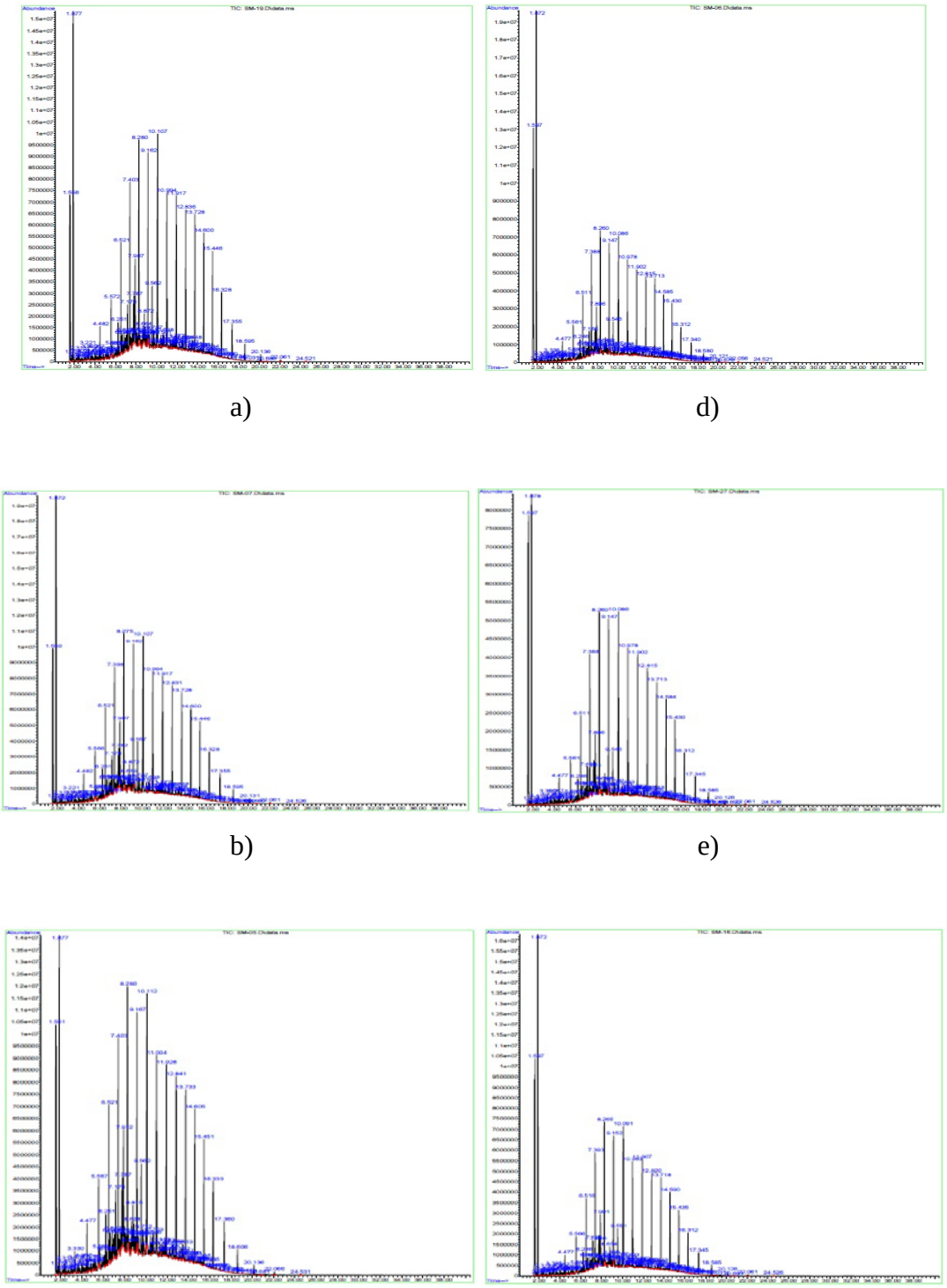


Figure D4: Fatty acid analysis of *P. lutheri* at optimum pH/salinity, photoperiod/light intensity and macronutrients a-c) in 5 L PBR and d-f) in 300 L open tank

APPENDIX E

BLAST (%) MATCH SEQUENCES

BLAST (%) Match Sequences for 18S rRNA

LAMB0001_*N.oceanica*

Sequence ID: lcl|44483Length: 650Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
1170 bits(633)	0.0	636/637(99%)	1/637(0%)	Plus/Plus

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Query  7    TTTTCTCCCTATCAGCTTTGGATGGTAGGGTATTGGCCTACCATGGCTCTAACGGGTAAC  66
            |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2    TTTTCT-CCTATCAGCTTTGGATGGTAGGGTATTGGCCTACCATGGCTCTAACGGGTAAC  60

Query  67   GGAGAATTGGGGTTCGATTCCGGAGAGGGAGCCTGAGAGACGGCTACCACATCCAAGGAA  126
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Sbjct  61   GGAGAATTGGGGTTCGATTCCGGAGAGGGAGCCTGAGAGACGGCTACCACATCCAAGGAA  120

Query  127  GGCAGCAGGCGCGTAAATTACCCAATCCTGACACAGGGAGGTAGTGACAATAAATAACAA  186
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  GGCAGCAGGCGCGTAAATTACCCAATCCTGACACAGGGAGGTAGTGACAATAAATAACAA  180

Query  187  TGCCGGGGTTTAACTCTGGCAATTGGAATGAGAACAATTTAAATCCCTTATCGAGGATCA  246
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Sbjct  181  TGCCGGGGTTTAACTCTGGCAATTGGAATGAGAACAATTTAAATCCCTTATCGAGGATCA  240

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Sbjct  241  ATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATACTA  300

Query  307  AAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTCTGGCAGGGACGGCTGGTCGGTTCC  366
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  AAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTCTGGCAGGGACGGCTGGTCGGTTCC  360

Query  367  GATAAGGGGCCGTACTATTGTTGGTTCCTGTCATCCTTGGGGAGAGCGATTCTGGCATTA  426
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  GATAAGGGGCCGTACTATTGTTGGTTCCTGTCATCCTTGGGGAGAGCGATTCTGGCATTA  420



Query  427  AGTTGTTGGGGTCGGGATCCCTATCTTTTACTGTGAAAAAATTAGAGTGTTCAAAGCAGG  486
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  AGTTGTTGGGGTCGGGATCCCTATCTTTTACTGTGAAAAAATTAGAGTGTTCAAAGCAGG  480
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Query  487  CTTAGGCCCTGAATACATTAGCATGGAATAATAAGATACGACCTTGGTGGTCTATTTTGT  546
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  CTTAGGCCCTGAATACATTAGCATGGAATAATAAGATACGACCTTGGTGGTCTATTTTGT  540

Query  547  TGGTTTGCACGCCAAGGTAATGATTAATAGGGATAGTTGGGGGTATTCGTATTCAATTGT  606
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  TGGTTTGCACGCCAAGGTAATGATTAATAGGGATAGTTGGGGGTATTCGTATTCAATTGT  600

Query  607  CAGAGGTGAAATTCTTGGATTTATGGAAGACGAACTA  643
            |||||||||||||||||||||||||||||||||||||
Sbjct  601  CAGAGGTGAAATTCTTGGATTTATGGAAGACGAACTA  637
```

AF045044_*N.oculata*

Sequence ID: lcl|44484Length: 1791Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
1170 bits(633)	0.0	649/656(99%)	3/656(0%)	Plus/Plus

```
Query  1    TTCAAGTTTTCT-CCCTATCAGCTTTGGATGGTAGGGTATTGGCCTACCATGGCTCTAAC  59
            |||||| ||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  296  TTCAAG-TTTCTGCCCTATCAGCTTTGGATGGTAGGGTATTGGCCTACCATGGCTCTAAC  354

Query  60   GGGTAACGGAGAATTGGGGTTCGATTCCGGAGAGGGAGCCTGAGAGACGGCTACCACATC  119
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  355  GGGTAACGGAGAATTGGGGTTCGATTCCGGAGAGGGAGCCTGAGAGACGGCTACCACATC  414

Query  120  CAAGGAAGGCAGCAGGCGCGTAAATTACCCAATCCTGACACAGGGAGGTAGTGACAATAA  179
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  415  CAAGGAAGGCAGCAGGCGCGTAAATTACCCAATCCTGACACAGGGAGGTAGTGACAATAA  474

Query  180  ATAACAATGCCGGGGTTTAACTCTGGCAATTGGAATGAGAACAATTTAAATCCCTTATCG  239
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  475  ATAACAATGCCGGGGTTTAACTCTGGCAATTGGAATGAGAACAATTTAAATCCCTTATCG  534

Query  240  AGGATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCG  299
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  535  AGGATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCG  594

Query  300  TATACTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTCTGGCAGGGACGGCTGGT  359
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  595  TATACTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTCTGGCAGGGACGGCTGGT  654

Query  360  CGGTTCCGATAAGGGGCCGTACTATTGTTGGTTCCTGTCATCCTTGGGGAGAGCGATTCT  419
            |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  655  CGGTTCCGATAAGGGGCCGTACTGTTGTTGGTTCCTGTCATCCTTGGGGAGAGCGATTCT  714
```

```
Query  420  GGCATTAAGTTGTTGGGGTCGGGATCCCTATCTTTTACTGTGAAAAAATTAGAGTGTTCA  479
            |||||||  |||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  715  GGCATTAGTTTGTTGGGATCGGGATCCCTATCTTTTACTGTGAAAAAATTAGAGTGTTCA  774

Query  480  AAGCAGGCTTAGGCCCTGAATACATTAGCATGGAATAATAAGATACGACCTTGGTGGTCT  539
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  775  AAGCAGGCTTAGGCCCTGAATACATTAGCATGGAATAATAAGATACGACCTTGGTGGTCT  834

Query  540  ATTTTGTTGGTTTGCACGCCAAGGTAATGATTAATAGGGATAGTTGGGGGTATTCGTATT  599
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  835  ATTTTGTTGGTTTGCACGCCAAGGTAATGATTAATAGGGATAGTTGGGGGTATTCGTATT  894

Query  600  CAATTGTCAGAGGTGAAATTCTTGGATTTATGGAAGACGAACTACATGCGAAAGCA  655
            |||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  895  CAATTGTCAGAGGTGAAATTCTTGGATTTATGGAAGACGAACTAC-TGCGAAAGCA  949
```

AF045049_*N.salina*

Sequence ID: lcl|44485Length: 1790Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
1114 bits(603)		0.0639/656(99%)	4/656(0%)	Plus/Plus

```
Query  1    TTCAAGTTTTCT-CCCTATCAGCTTTGGATGGTAGGGTATTGGCCTACCATGGCTCTAAC  59
            |||||| ||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  296  TTCAAG-TTTCTGCCCTATCAGCTTTGGATGGTAGGGTATTGGCCTACCATGGCTCTAAC  354

Query  60   GGGTAACGGAGAATTGGGGTTCGATTCCGGAGAGGGAGCCTGAGAGACGGCTACCACATC  119
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  355  GGGTAACGGAGAATTGGGGTTCGATTCCGGAGAGGGAGCCTGAGAGACGGCTACCACATC  414

Query  120  CAAGGAAGGCAGCAGGCGCGTAAATTACCCAATCCTGACACAGGGAGGTAGTGACAATAA  179
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  415  CAAGGAAGGCAGCAGGCGCGTAAATTACCCAATCCTGACACAGGGAGGTAGTGACAATAA  474


Query  180  ATAACAATGCCGGGGTTTAACTCTGGCAATTGGAATGAGAACAATTTAAATCCCTTATCG  239
            |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  475  ATAACAATGCCGGGGTTTAACTCTGGCTATTGGAATGAGAACAATTTAAATCCCTTATCG  534

Query  240  AGGATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCG  299
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  535  AGGATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCG  594
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Query  300  TATACTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTCTGGCAGGGACGGCTGGT  359
            ||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  595  TATACTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTCTGGCGGGGACGGCTGGT  654

Query  360  CGGTTCCGATAAGGGGCCGTACTATTGTTGGTTCCTGTCATCCTTGGGGAGAGCGATTCT  419
            ||||  ||| |||||||| ||||| |||||||||||| ||||||||||||||||||  |||
Sbjct  655  CGGTCTCGA-AAGGGGCTGTACTGTTGTTGGTTCCCGTCATCCTTGGGGAGAGCGGCTCT  713

Query  420  GGCATTAAGTTGTTGGGGTCGGGATCCCTATCTTTTACTGTGAAAAAATTAGAGTGTTCA  479
              |||||||||||  || ||||||||||||||||||||||||||||||||||||||||||
Sbjct  714  TACATTAAGTTGTCGGCGTCGGGATCCCTATCTTTTACTGTGAAAAAATTAGAGTGTTCA  773

Query  480  AAGCAGGCTTAGGCCCTGAATACATTAGCATGGAATAATAAGATACGACCTTGGTGGTCT  539
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  774  AAGCAGGCTTAGGCCCTGAATACATTAGCATGGAATAATAAGATACGACCTTGGTGGTCT  833

Query  540  ATTTTGTTGGTTTGCACGCCAAGGTAATGATTAATAGGGATAGTTGGGGGTATTCGTATT  599
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  834  ATTTTGTTGGTTTGCACGCCAAGGTAATGATTAATAGGGATAGTTGGGGGTATTCGTATT  893

Query  600  CAATTGTCAGAGGTGAAATTCTTGGATTTATGGAAGACGAACTACATGCGAAAGCA  655
            |||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  894  CAATTGTCAGAGGTGAAATTCTTGGATTTATGGAAGACGAACTAC-TGCGAAAGCA  948
```

BLAST (%) Match Sequences for rbcL Gene

AB052286_*N.oculata*

Sequence ID: lcl|27355Length: 1431Number of Matches: 1

Score	**Expect**	**Identities**	**Gaps**	**Strand**
2080 bits(1126)	0.0	1210/1252(97%)	1/1252(0%)	Plus/Plus

```
Query  134   CAAAAGCTTACAAAGTTGATTCAGTTCCAGGTACTAGCGACCAATACTTCGGTTACGTTG  193
             |||||||||||||||||||||||||||||||||||| |||||||||||||||| ||||
Sbjct  181   CAAAAGCTTACAAAGTTGATTCAGTTCCAGGTACTAGTGACCAATACTTCGGTTATGTTG  240

Query  194   CATATGAATGTGATCTTTTTGAAGAAGGTTCAATTGCTAACTTAACAGCTTCAATTATCG  253
             |||| |||||||||||||| |||||||||||||||||||||||||||||||||||||||
Sbjct  241   CATACGAATGTGATCTTTTCGAAGAAGGTTCAATTGCTAACTTAACAGCTTCAATTATCG  300
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Query  254   GTAACGTATTTGGATTCAAAGCTGTAAAAGCATTACGTCTTGAAGATATGCGTATGCCTT  313
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301   GTAACGTATTTGGATTCAAAGCTGTAAAAGCATTACGTCTTGAAGATATGCGTATGCCTT  360

Query  314   ACGCTTACTTAAAAACATTCCAAGGTCCAGCTACTGGTGTGATTGTTGAACGTGAGCGTT  373
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361   ACGCTTACTTAAAAACATTCCAAGGTCCAGCTACTGGTGTGATTGTTGAACGTGAGCGTT  420

Query  374   TAGACAAATTCGGACGTCCTTTATTAGGTGCAACTGTAAAACCTAAACTTGGTTTATCAG  433
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421   TAGACAAATTCGGACGTCCTTTATTAGGTGCAACTGTAAAACCTAAACTTGGTTTATCAG  480

Query  434   GTAAAAACTATGGACGTGTTGTATACGAAGGTTTAAAAGGTGGTTTAGACTTCTTAAAAG  493
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481   GTAAAAACTATGGACGTGTTGTATACGAAGGTTTAAAAGGTGGTTTAGACTTCTTAAAAG  540


Query  494   ATGACGAAAACATTAACTCTCAACCATTCATGCGTTGGCGTGAACGTTTCTCGTACGTAA  553
             |||| |||||||||||||||||||||||||||||||||||||||||||| || ||||
Sbjct  541   ATGATGAAAACATTAACTCTCAACCATTCATGCGTTGGCGTGAACGTTTCTCTTATGTAA  600

Query  554   TGGAAGGTGTTAATAGATCAGCTGCATCTTCTGGTGAAGTTAAAGGTTCTTACCTTAACG  613
             |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  601   TGGAAGGTGTTAATAGATCAGCTGCAGCTTCTGGTGAAGTTAAAGGTTCTTACCTTAACA  660

Query  614   TTACTGCAGCAACTATGGAAGAAATGTACGAACGTGCTGAATTTGCTAAACTTGTTGGTT  673
             ||||||| || |||||||||||||||||||||||||||||||||||||||||||| ||||
Sbjct  661   TTACTGCTGCTACTATGGAAGAAATGTACGAACGTGCTGAATTTGCTAAACTTGTAGGTT  720

Query  674   CAGTAATTATCATGATCGACTTAGTAANTGGTTATACTGCAATTCAATCGATGGCTGTTT  733
             |||||||||||||||||||||||||| |||||| |||||||||||||||||||| |||
Sbjct  721   CAGTAATTATCATGATCGACTTAGTAATTGGTTACACTGCAATTCAATCGATGGCTATTT  780

Query  734   GGTCTCGTAACGAATGATATGATCCTTCACTTACACCGTGCAGGTAACTCAGCATATGCT  793
             |||||||||| |||||| ||||| ||||||||||||||||||||||||||||||||||||
Sbjct  781   GGTCTCGTAA-GAATGACATGATTCTTCACTTACACCGTGCAGGTAACTCAGCATATGCT  839

Query  794   CGTCAAAAGAACCATGGTATTAACTTCCGTGTAATTTGTAAATGGATGCGTATGGCTGGT  853
             |||||||||||| |||||||||||||||||||||| |||||||||||||||||||||||
Sbjct  840   CGTCAAAAGAATCATGGTATTAACTTCCGTGTAATCTGTAAATGGATGCGTATGGCTGGT  899

Query  854   GTTGACCACATCCATGCAGGTACAGTTGTAGGTAAATTAGAAGGTGACCCTCTAATGGTT  913
             |||||||| |||||||| ||||||||||||||||||||||||||||||| ||||||||
Sbjct  900   GTTGACCATATCCATGCTGGTACAGTTGTAGGTAAATTAGAAGGTGACCCTTTAATGGTT  959
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Query  914   AAAGGTTTCTACAACGTATTATTACAAACATCACTAGATATTAACTTACCACAAGGTATC  973
             ||||||||||||||||||||||||||||||| ||||||| |||||||||||| |||||||||
Sbjct  960   AAAGGTTTCTACAACGTATTATTACAAACAACACTAGACATTAACTTACCTCAAGGTATC  1019

Query  974   TTCTTCGAACAAGACTGGGCTTCTTTAAGAAAAACACTACCTGTAGCTTCTGGTGGTATC  1033
             ||||||||||||||||||||||||||||||||||  |||||||||| ||||||||||
Sbjct  1020  TTCTTCGAACAAGACTGGGCTTCTTTAAGAAAAACTTTACCTGTAGCGTCTGGTGGTATT  1079

Query  1034  CATTGTGGACAAATGCACCAGTTACTTAACTATCTAGGTGAAGACTGTGTATTACAATTT  1093
             |||||||||||||||| ||||||||||||| |||||||||||||||||||||||||
Sbjct  1080  CATTGTGGACAAATGCATCAGTTACTTAACTACTTAGGTGAAGACTGTGTATTACAATTC  1139

Query  1094  GGTGGTGGTACAATTGGTCACCCTGATGGTATCGCTTCTGGTGCGACTGCTAACCGCGTA  1153
             |||||||||||||||||||||||||||||| |||||||||| |||||||||| |||
Sbjct  1140  GGTGGTGGTACAATTGGTCACCCTGATGGTATTGCTTCTGGTGCAACTGCTAACCGTGTA  1199

Query  1154  GCTATGGAGTCAGTGCTTTTAGCTAAATATGAAGGTAAAGATTACATTAACGAAGGACCA  1213
             ||||||||||||||| ||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  1200  GCTATGGAGTCAGTACTTTTAGCTAAATACGAAGGTAAAGATTACATTAACGAAGGACCA  1259

Query  1214  AAAATTTTACGTGCGGCGGCAGAAAGTTGTGCGCCATTACGTTCTGCTTTAGATCTTTGG  1273
             |||||| |||||||||| ||||||| ||||||||||||||||||||||||||||||||
Sbjct  1260  AAAATTCTACGTGCGGCTGCAGAAACTTGTGCGCCATTACGTTCTGCTTTAGATCTTTGG  1319

Query  1274  AAAGATATTGCTTTCAACTATACATCAACAGATACTGCTGATTACATTGAAACTGCAACT  1333
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1320  AAAGATATTGCTTTCAACTATACATCAACAGATACTGCTGATTACATTGAAACTGCAACT  1379

Query  1334  AAACAGTAATCGTATAAAAACAATATCAATCAGTTTAACATACTAAAGGAGT  1385
             ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1380  AAACAGTAATCGTATAAAAACAATATCAATCAGTTTAACATACTAAAGGAGT  1431
```

AF015576_*N.salina*

Sequence ID: lcl|27356Length: 1183Number of Matches: 2

Score	Expect	Identities	Gaps	Strand
941 bits(509)	0.0	713/812(88%)	26/812(3%)	Plus/Plus

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Query  531   GCG-TGAACGTTTCTCGTACGTAATGGAAGGTGTTAATAGATCAGCTGCATCTTCTGGTG  589
             ||| |||||||||||||  |||||||||||||||| |||||||| ||| |||||||||
Sbjct  395   GCGTTGAACGTTTCTCGTNNGTAATGGAAGGTGTTAACAGATCAGCAGCAGCTTCTGGTG  454
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Query  590   AAGTTAAAGGTTCTTACCTTAACGTTACTGCAGCAACTATGGAAGAAATGTACGAACGTG  649
             ||||||||||||||||||||||| ||||| ||||| |||||||||||||||||||||||||
Sbjct  455   AAGTTAAAGGTTCTTACCTTAATGTTACAGCAGCCACTATGGAAGAAATGTACGAACGTG  514

Query  650   CTGAATTTGCTAAACTTGTTGGTTCAGTAATTATCATGATCGACTTAGTAANTGGTTATA  709
             |||||||||| |||||| |||||||||||||||||||||||||||||| | |||||| |
Sbjct  515   CTGAATTTGCAAAACTTATTGGTTCAGTAATTATCATGATCGACTTAGTGATTGGTTACA  574

Query  710   CTGCAATTCAATCGATGGCTGTTTGGTCTCGTAACGAATGATATGATCCTTCACTTACAC  769
             |||||||||||||| ||||| ||||||||||||||  ||||| ||||||||||||||||||
Sbjct  575   CTGCAATTCAATCAATGGCAGTTTGGTCTCGTAA-AAATGACATGATCCTTCACTTACAC  633

Query  770   CGTGCAGGTAACTCAGCATATGCTCGTCAAAAGAACCATGGTATTAACTTCCGTGTAATT  829
             |||||||||||||| |||||||||||||||| || |||||||||||||||||||||||||
Sbjct  634   CGTGCAGGTAACTCGGCATATGCTCGTCAAAAAAATCATGGTATTAACTTCCGTGTAATT  693

Query  830   TGTAAATGGATGCGTATGGCTGGTGTTGACCACATCCATGCAGGTACAGTTGTAGGTAAA  889
             |||||||||||||||||||||||||||||||| || || || ||||| ||||||||||||
Sbjct  694   TGTAAATGGATGCGTATGGCTGGTGTTGACCATATTCACGCGGGTACTGTTGTAGGTAAA  753

Query  890   TTAGAAGGTGACCCTCTAATGGTTAAAGGTTTCTACAACGTATTATTACAAACATCAC-T  948
              |||||||||| ||| ||||||||||||||||||  ||   |||||| ||||| |    |
Sbjct  754   CTAGAAGGTGATCCTTTAATGGTTAAAGGTTTCTATAATACATTATTGCAAAC-TAGTTT  812

Query  949   AGATATTAACTTACCACAAGGTATCTTCTTCGAACAAGACTGGGCTTCTTTAAGAAAAAC  1008
             ||| |||||||||||||||||| ||||| ||||| |||||||||||||||| ||||||
Sbjct  813   AGACATTAACTTACCACAAGGTATTTTCTTTGAACAGGACTGGGCTTCTTTAACAAAAAC  872

Query  1009  ACTACCTGTAGCTTCTGGTGGTATCCATTGTGGACAAATGCACCAGTTACTTAACTATCT  1068
              |||||||||||| |||||||||||||| |||| |||||||||||| |||||||||||  ||
Sbjct  873   TCTACCTGTAGCCTCTGGTGGTATCCACTGTGCACAAATGCACCAATTACTTAACTA-CT  931

Query  1069  AGGTGAAGACTGTGTATTACAATTTGGTGGTGGTACAATTGGTCACCCTGATGGTATCGC  1128
                 | | |   |||          |||||||||    |||||| ||||||||| |||||
Sbjct  932   ---T-A-G---GTG----------GGTGGTGGTNNNNTTGGTCTCCCTGATGG-ATCGC  971

Query  1129  -TTCTGGTGCGACTGCTAACCGCGTAGCTATGGAGTCAGTGCTTTTAGCTAAATATGAAG  1187
              ||||||||| || || || || || |||||||| |  || |||||||||||||| ||||
Sbjct  972   TTTCTGGTGCAACAGCGAATCGTGTGGCTATGGAATGTGTACTTTTAGCTAAATACGAAG  1031

Query  1188  GTAAAGATTACATTAACGAAGGACCAAAAATTTTACGTGCGGCGGCAGAAAGTTGTGCGC  1247
             |||||||||| |||||||||||||||||| |||||||| || |||||||| ||||| |
Sbjct  1032  GTAAAGATTATATTAACGAAGGACCAAAAATCTTACGTGCTGCTGCAGAAAGCTGTGCTC  1091
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Query  1248  CATTACGTTCTGCTTTAGATCTTTGGAAAGATATTGCTTTCAACTATACATCAACAGATA  1307
             |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1092  CATTACGTACTGCTTTAGATCTTTGGAAAGATATTGCTTTCAACTATACATCAACAGATA  1151

Query  1308  CTGCTGATTACATTGAAACTGCAACTAAACAG  1339
             |||| |||||| |||||||  ||||||||||||
Sbjct  1152  CTGCAGATTACGTTGAAACAACAACTAAACAG  1183
```

AB052279_*N.gaditana*

Sequence ID: lcl|27357Length: 1439Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
1775 bits(961)	0.0	1152/1247(92%)	3/1247(0%)	Plus/Plus

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Query  134   CAAAAGCTTACAAAGTTGATTCAGTTCCAGGTACTAGCGACCAATACTTCGGTTACGTTG  193
             |||||||||| |||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  195   CAAAAGCTTATAAAGTTGATTCAGTTCCAGGTACTAGCGACCAATTCTTCGGTTACGTTG  254

Query  194   CATATGAATGTGATCTTTTTGAAGAAGGTTCAATTGCTAACTTAACAGCTTCAATTATCG  253
             |||||||||||||| |||||||||||||||||||| |||||||||||| |||||||| ||||
Sbjct  255   CATATGAATGTGACCTTTTTGAAGAAGGTTCAATCGCTAACTTAACGGCTTCAATCATCG  314

Query  254   GTAACGTATTTGGATTCAAAGCTGTAAAAGCATTACGTCTTGAAGATATGCGTATGCCTT  313
             |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  315   GTAACGTATTTGGATTCAAAGCTGTAAAAGCGTTACGTCTTGAAGATATGCGTATGCCTT  374

Query  314   ACGCTTACTTAAAAACATTCCAAGGTCCAGCTACTGGTGTGATTGTTGAACGTGAGCGTT  373
             | |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  375   ATGCTTACTTAAAAACATTCCAAGGACCAGCTACTGGTGTGATTGTTGAACGTGAGCGTT  434

Query  374   TAGACAAATTCGGACGTCCTTTATTAGGTGCAACTGTAAAACCTAAACTTGGTTTATCAG  433
             |||||||||||||||||| |||||||||||||||||||| ||| | ||||||||||
Sbjct  435   TAGACAAATTCGGACGTCCTCTATTAGGTGCAACTGTAAAACCAAAATTAGGTTTATCAG  494

Query  434   GTAAAAACTATGGACGTGTTGTATACGAAGGTTTAAAAGGTGGTTTAGACTTCTTAAAAG  493
             |||||||||||||| |||||||| || |||||||||||||||| ||||||||||||||||
Sbjct  495   GTAAAAACTATGGGCGTGTTGTTTATGAAGGTTTAAAAGGTGGATTAGACTTCTTAAAAG  554
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Query  494   ATGACGAAAACATTAACTCTCAACCATTCATGCGTTGGCGTGAACGTTTCTCGTACGTAA  553
             |||||||||||||| |||||||||||||||||||| ||||| |||||||||||| || ||||
Sbjct  555   ATGACGAAAACATCAACTCTCAACCATTCATGCGCTGGCGCGAACGTTTCTCTTATGTAA  614

Query  554   TGGAAGGTGTTAATAGATCAGCTGCATCTTCTGGTGAAGTTAAAGGTTCTTACCTTAACG  613
             |||||||||||||| |||||||| ||| ||||||| ||||||||||||||||||||||| |
Sbjct  615   TGGAAGGTGTTAACAGATCAGCAGCAGCTTCTGGCGAAGTTAAAGGTTCTTACCTTAATG  674

Query  614   TTACTGCAGCAACTATGGAAGAAATGTACGAACGTGCTGAATTTGCTAAACTTGTTGGTT  673
             |||| || || |||||||||||||||||| |||||||||||||||| |||||| ||||||
Sbjct  675   TTACAGCGGCTACTATGGAAGAAATGTATGAACGTGCTGAATTTGCGAAACTTATTGGTT  734

Query  674   CAGTAATTATCATGATCGACTTAGTAANTGGTTATACTGCAATTCAATCGATGGCTGTTT  733
             |||||||||||||||| |||||||| | |||||| |||||||||||||| |||||  |||
Sbjct  735   CAGTAATTATCATGATTGACTTAGTGATTGGTTACACTGCAATTCAATCAATGGCAATTT  794

Query  734   GGTCTCGTAACGAATGATATGATCCTTCACTTACACCGTGCAGGTAACTCAGCATATGCT  793
             ||||||||||  ||||| |||||||||||| |||||||||||||||||| |||||||||
Sbjct  795   GGTCTCGTAA-AAATGACATGATCCTTCACCTACACCGTGCAGGTAACTCGGCATATGCT  853

Query  794   CGTCAAAAGAACCATGGTATTAACTTCCGTGTAATTTGTAAATGGATGCGTATGGCTGGT  853
             |||||||| || ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  854   CGTCAAAAAAATCATGGTATTAACTTCCGTGTAATTTGTAAATGGATGCGTATGGCTGGT  913

Query  854   GTTGACCACATCCATGCAGGTACAGTTGTAGGTAAATTAGAAGGTGACCCTCTAATGGTT  913
             |||||||| || || || ||||| |||||||||||| |||||||||| ||| ||||||||
Sbjct  914   GTTGACCATATTCACGCGGGTACTGTTGTAGGTAAACTAGAAGGTGATCCTTTAATGGTT  973

Query  914   AAAGGTTTCTACAACGTATTATTACAAACATCAC-TAGATATTAACTTACCACAAGGTAT  972
             |||||||||||||   | |||||||||| |  | || | ||||||||||||||||||||
Sbjct  974   AAAGGTTTCTACAATACACTATTACAAAC-TAGCTTAAACATTAACTTACCACAAGGTAT  1032

Query  973   CTTCTTCGAACAAGACTGGGCTTCTTTAAGAAAAACACTACCTGTAGCTTCTGGTGGTAT  1032
              ||||| ||||| |||||||||||||||||||| |||||||||| ||||||||||
Sbjct  1033  TTTCTTTGAACAGGACTGGGCTTCTTTAAGAAAAACTCTACCTGTAGCCTCTGGTGGTAT  1092

Query  1033  CCATTGTGGACAAATGCACCAGTTACTTAACTATCTAGGTGAAGACTGTGTATTACAATT  1092
             ||| |||||||||||||||| ||||||||||  |||||||||||||||||||||||||
Sbjct  1093  CCACTGTGGACAAATGCACCAATTACTTAACTACTTAGGTGAAGACTGTGTATTACAATT  1152

Query  1093  TGGTGGTGGTACAATTGGTCACCCTGATGGTATCGCTTCTGGTGCGACTGCTAACCGCGT  1152
             |||||||||||||||||||||||||||||||||||||||||||| || || || || ||
Sbjct  1153  TGGTGGTGGTACAATTGGTCACCCTGATGGTATCGCTTCTGGTGCAACAGCGAATCGTGT  1212
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Query  1153  AGCTATGGAGTCAGTGCTTTTAGCTAAATATGAAGGTAAAGATTACATTAACGAAGGACC  1212
              ||||||||  |  ||  ||||||||||||||  ||||||||||||||||||||||||||||
Sbjct  1213  GGCTATGGAATGTGTACTTTTAGCTAAATACGAAGGTAAAGATTACATTAACGAAGGACC  1272

Query  1213  AAAAATTTTACGTGCGGCGGCAGAAAGTTGTGCGCCATTACGTTCTGCTTTAGATCTTTG  1272
              ||||||||||||||| || ||||||||  |||||  |||||||||  ||||||||||||||||
Sbjct  1273  AAAAATTTTACGTGCTGCTGCAGAAAGCTGTGCTCCATTACGTACTGCTTTAGATCTTTG  1332

Query  1273  GAAAGATATTGCTTTCAACTATACATCAACAGATACTGCTGATTACATTGAAACTGCAAC  1332
              |||||||||||||||||||||||||||||||||||||||||  ||||||  |||||||  ||||
Sbjct  1333  GAAAGATATTGCTTTCAACTATACATCAACAGATACTGCAGATTACGTTGAAACAACAAC  1392

Query  1333  TAAACAGTAATCGTATAAAAACAATATCAATCAGTTTAACATACTAA  1379
              ||||||||||||||  ||||||||||||||||||||||||||||||
Sbjct  1393  TAAACAGTAATCGTAAAAAAACAATATCAATCAGTTTAACATACTAA  1439
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Biodata

Syed Muhammad Usman Shah was born on 14 August 1980 in Chiniot, Punjab Pakistan. He graduated with BSc (Hons) in Plant Breeding and Genetics from NWFP Agricultural University, Peshawar, Pakistan. He obtained a Master of Philosophy (M.Phil) in Biotechnology and Genetic Engineering from the Institute of Biotechnology and Genetic Engineering (IBGE), Peshawar, Pakistan.

He was appointed as a Research Assistant at NWFP Agricultural University, Peshawar, Pakistan (2003-2005) and Research Officer at IBGE (2005-2007) by the Higher Education Commision of Pakistan. He served Qarshi Research International Industry as a Scientific Officer from 2007-2008. He was then appointed as a lecturer at Sarhad University of Science and Information Technology (SUIT), Peshawar, Pakistan where he taught the subjects of Biotechnology and Genetic Engineering from 2008-2009.

In August 2009, he enrolled as a Doctor of Philosophy candidate under the kind supervision of Assoc. Prof. Dr. Mohd Azmuddin Abdullah, in Chemical Engineering Department at Universiti Teknology PETRONAS and was awarded with graduate assistantship scheme (GA).

Publications

1. Journals

1. **S. M. U. Shah**, C. C. Radziah, S. Ibrahim, F. Latiff, M. F. Othman, & M. A. Abdullah (2013). Effects of photoperiod, salinity and pH on cell growth and lipid content of *Pavlova lutheri*. Annals of Microbiology, (4) 645–656.
2. **S. M. U. Shah**, A. Ahmad, M. F. Othman, & M. A. Abdullah (2013). Enhancement of lipid content in *Nannochloropsis oculata* and *Tetraselmis suecica* using palm oil mill effluent as an alternative medium. Submitted to International Journal of Green Energy (IJGE-2013-0242, 2013).
3. **S. M. U. Shah**, A. Ahmad, M. F. Othman, & M. A. Abdullah (2013). Optimization of biomass and lipid content of *Nannochloropsis oculata* through abiotic stressors and nutrient limitations. Submitted to Marine Biology Research (MBR-2013-0108).
4. A. Ahmad, **S. M. U. Shah**, M.F. Othman, M.A. Abdullah. Aerobic and anaerobic co-cultivation of *Nannochloropsis oculata* with Oil palm empty fruit bunch for enhanced biomethane production and palm oil mill effluent treatment. Submitted to Biomass and Bioenergy (JBB-D-13-00016, 2012).
5. A. Ahmad, **S. M. U. Shah**, M.F. Othman, M.A. Abdullah. Enhanced palm oil mill effluent treatment and biomethane production by aerobic and anaerobic co-cultivation of *Chlorella* sp., Submitted Canadian Journal of Chemical Engineering (CJCE-13-0400).

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1. M. A. Abdullah, **S. M. U. Shah**, A. Ahmad, H. El-Sayed. (2013). Algal biotechnology for bioenergy, environmental remediation and high-value biochemicals. Indian Society of Applied Biotechnology.

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1. **S. M. U. Shah**, A., C.M.Z. Che Radziah, N. Mansor, S. Ibrahim, F. Latiff, M. A. Abdullah (2011). Enhancement of Carotenoids in Green Microalgae under Different Growth Conditions and Different Solvent Extracts. International Conference on Materials for Advanced Technologies Singapore (2011) ICMAT (11)-Reg-1875.
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3. **S. M. U. Shah**, A. Ahmad, M. F. Othman, M. A. Abdullah. "Enhancement of biomass and lipid production by light irradiation in the laboratory and outdoor cultivation of *Nannochloropsis oculata*" Annual Post graduate Conference (APC), 3-5 July, 2013. Universiti Teknologi PETRONAS.
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5. A. Ahmad, **S. M. U. Shah**, M.F. Othman, A.B. Buang, M.A. Abdullah. (2013). Evaluation of aerobic and anaerobic co-digestion of *Tetraselmis suecica* and palm oil mill effluent by using response surface methodology. Joint International Conference on Nanoscience, Engineering and Management (BOND21) 19-21 August, 2013. Penang, Malaysia
6. A. Ahmad, **S. M. U. Shah**, M. F. Othman and M. A. Abdullah. (2013). Optimization of Biomethane Production and Palm Oil Mill Effluent Treatment by Aerobic & Anaerobic co-cultivation of *Chlorella* sp. International

Conference of Chemical Engineering and Industrial Biotechnology. 28-29 August 2013. Kuantan, University Malaysia Pahang

Awards

1. Bronze Medal in 30th Science and Engineering Design Exhibition UTP (8-9 August 2012).
2. Bronze Medal in "Citrawarna Inovasi 2012" Competition UTP (19-21 October 2012).
3. Bronze Medal in 31st Science and Engineering Design Exhibition UTP (21-22 August 2013).