Physiological Adaptation of Unicellular Microalgae To Environmental Stress and Their Potential For Biofuel Production

By

Halima Abdulsalam Alhani

MSc, University of Teesside, Middlesbrough, UK

The University Of Sheffield.

Thesis submitted in part fulfilment of the requirement for thedegree of Doctor of Philosophy

Department of Molecular Biology and Biotechnology

The University of Sheffield, UK

February 2019

Declaration

I hereby declare that this thesis is my own work and effort. Where other sources of information have been used, they have been acknowledged.

Signed By

Halima A. Alhani

Dedication

To

My Father "Abdullsalam"

My Mother "Fatama"

My Husband "Mustafa"

My daughters "Shahd and Shada"

My sons "Suhaib and Muhammed"

For Their Love, Encouragement and Support

Abstract

The crisis of producing enough liquid fuels in an environmentally friendly way is expected to increase in the future. At the moment most liquid fuels are produced from crude oil, which is a fossil fuel contributing to global warming. Microalgae offer a high potential for the production of biodiesel which is a reliable, non-toxic, and biocompatible liquid fuel that can replace the existing unsustainable fossil fuel sources of liquid fuels. In this study, *Nannochloropsis salina* was examined for its ability to produce neutral lipid, the precursor of biodiesel. *Nannochloropsis salina* was obtained from the Culture Centre of Algae and Protozoa, Oban, Scotland, UK and its identity was confirmed using molecular techniques (18S rDNA sequencing) and light microscopy. Two growth media (defined f/2 medium and AMCONA medium) were used to determine which medium is the most appropriate based on the highest growth potential. Effects of stress conditions, which included high salinity up to 1 M NaCl, pH values from pH 5 to pH10 and nitrogen depletion conditions (75%, 50%, 25% $NaNO_3$ as nitrogen source and nitrogen free) on neutral lipid accumulation in *N. salina* cells were investigated using Nile red technique to confirm their suitability for biofuel (biodiesel) production. It was found that 0.6 M NaCl, high pH values (pH10) and nitrogen free mediumtended to induce higher levels of neutral lipid in the algal cells (74.65%, 76.89% and 72.12% of total dry weight respectively). Total lipid content in *N. salina* cells grown under different treatments was also investigated using the gravimetric technique. Nitrogen free medium induces more total lipid content in cells. Fatty acid methyl ester (FAME) profiles of *N. salina* cells were analyzed by gas chromatography-mass spectrometry (GC-MS) after direct transesterification. Cells of *N. salina* grown in 0.6 M NaCl showedthe highest levels of fatty acids which were principally C16 and C18 - ideal for biodiesel production. Lipid content in *N. salina*

cells was also examined using ^{1}H-NMR and the results confirmed that the fatty acid content was much higher in the 0.6 M NaCl grown cells. Further analysis of neutral lipid in *N. salina* cells grown under different conditions was established using a flow cytometry technique.

Acknowledgements

First and foremost, all thanks to the Almighty Allah who makes this work possible. There are many people who I would like to thank for their help over the past four years, I can only apologise now if I manage to forget to name them personally. I blame it on my complete brain failure.

I would like to thank my supervisor Dr. Jim Gilmour for his invaluable help, guidance, encouragement and patience during the period of this study. I would also like to thank my advisors Professor Milton Wainwright and Dr. Dave Kelly for their advice.I would also like to take this opportunity to thank staff members in the university of Sheffield which have helped me to complete many of the parts of my study, including Simon Thorpe (Chemistry) for GC-MASS assistance, Professor Mike Williamson for processing NMR samples, Flow cytometry group (Medical school) for flow cytometry running. I would like to thank the laboratory technicians in the Department of Molecular Biology and Biotechnology in the Sheffield University for their help and all the others in the laboratory who were always there to help guide and assist me through all my experiments.

I would like to thank my colleagues in laboratory F3 in the Department of Molecular Biology and Biotechnology in the University of Sheffield.

Last and in the no way the last I would like to thank my parents for their emotional and standing by me through my every step in life. I would like to say a special thank you to my husbund Dr. Mustafa Alsull for his encouragement and support over the years. I also would like to thank my daughters: Shahd and Shada and my sons: Suhaib and Muhammed who offered me happiness.

Table of Contents

Chapter 1: Literature Review and Aims

Chapter 2: Materials and Methods

Chapter 4: Physiological Characterization of Unicellular *Nannochloropsis salina* Strain CCAP 849/2

Chapter 5: Determination and Quantification of Neutral Lipid Content and Fatty Acid Composition

List of Tables

List of Figures

Abbreviations

ACCase	**Cytoplasmic acetyl-CoA carboxylase**
acetyl-coA	**Acetyl coenzyme A**
ADP	**Adenosine diphosphate**
AMCONA	**Artificial Multipurpose Complement for the Nutrition of Algae**
ATP	**Adenosine triphosphate**
B_1	**Thiamine ·HCl**
B_{12}	**Cyanocobalamin**
BLAST	**Basic local alignment search tool**
bp	**Base pair (s)**
$CDCl_3$	**Deuterated chloroform**
CD_3OD	**Deuterated methanol**
CTAB	**Cetyltrimethylammonium bromide**
dH_2O	**Distilled water**
DMSO	**Dimethyl sulfoxide**
DNA	**Deoxyribonucleic acid**
dNTPs	**Deoxynucleoside triphosphates**
DW	**Dry weight**
EB	**Ethidium bromide**
EDTA	**Ethylene diamine tetraacetic acid**
EPA	**Eicosapentaenoic acid**
ER	**Endoplasmic reticulum**
FAMEs	**Fatty acid methyl esters**
FAs	**Fatty acids**
FSC	**Forward scatter**
F_v/F_m	**Quantum efficiency**
GC	**Gas chromatography**
GC-MS	**Gas chromatography-mass spectrometry**
gDNA	**Genomic DNA**
G3P	**Glycerate-3-phosphate**
h	**Hour (s)**
^{1}H-NMR	**Proton Nuclear Magnetic Resonance**
Hz	**Hertz**

kb	**Kilobase (s)**
kg	**Kilogram (s)**
L	**Litre (s)**
LD	**Lipid droplet**
LEDs	**Light-emitting diode**
M	**Molar**
m	**Metre (s)**
mg	**Milligram (s)**
min	**Minute (s)**
ml	**Millilitre (s)**
mM	**Millimole (s)**
mm	**Millimeter (s)**
NAD	**Nicotinamide adenine dinucleotide (oxidised form)**
NADH	**Nicotinamide adenine dinucleotide (reduced form)**
NF	**Nitrogen free**
NPQ	**Non-photochemical quenching**
NMR	**Nuclear Magnetic Resonance**
NR	**Nile red dye**
OD	**Optical density**
PAM	**Pulse Amplitude Modulation**
PBRs	**Photobioreactors**
PBS	**Phosphate buffer**
PC	**Phosphatidylcholine**
PCR	**Polymerase chain reaction**
PDC	**Pyruvate dehydrogenase complex**
PG	**Phosphatidylglycerol**
PSII	**Photosystem II**
PUFAs	**Polyunsaturated fatty acids**
rDNA	**Ribosomal Deoxyribonucleic acid**
RNA	**Ribonucleic acid**
rRNA	**Ribosomal Ribonucleic acid**
RNase	**Ribonuclease**
rpm	**Revolutions per minutes**

$	**American Dollar**
SP	**Special Saturation Pulse**
SSC	**Side scatter**
SQDG	**Sulfoquinovosyl diacylglycerols**
SSU	**Small subunit**
TAE	**Tris-acetate-EDTA (Ethylene diamine tetraacetic acid)**
TAG	**Triacylglycerides**
TG	**Triglyceride**
Tris	**Tris (hydroxymethyl) methylamine**
UV	**Ultra violet**
UV-A	**UV radiation with wavelength from380 nm to 400 nm**
UV-B	**UV radiation with wavelength from215 nm to 380 nm**
vit B_1	**Thiamine·HCl**
vit B_{12}	**Cyanocobalamin**
vit H	**Biotin**
V/V	**Volume per unit volume**
W/V	**Weight per unit volume**
16S rRNA	**gene in prokaryotes**
18S rRNA	**gene in eukaryotes**
µl	**Microlitre (s)**
µg	**Microgram (s)**
µM	**Millimolar (s)**
Mm	**Millimeter (s)**
%	**Percentage**
°C	**Centigrade**

Literature Review and Aims

Chapter One

1.1. Algae Background

Algae are known as one of the oldest life forms. They are often thought of as lower plants and generally defined as organisms that are non vascular in nature (i.e. lacking structures such as leaf system, roots and stems but containing chlorophyll *a* as their primary photosynthetic pigment) (Richmond, 2004; Brennan and Owende, 2010; Chu, 2012). However, in recent years, based on molecular phylogenetic systematics, algae have not been classified under the Kingdom Plantae, although some types of algae (e.g. Chlorophyta) do resemble higher plants (Graham and Wilcox, 2000).

Algae are present in all existing earth ecosystems and a large variety of species can be found in a wide range of environmental conditions (Mata *et al.*,2010). Moreover, algae are widely found in both aquaticand terrestrial habitats, including extreme environments such as snow and glaciers in the Arctic and Antarctic (Chu, 2012). The presence of algae in the air (airborne algae) has been known as they may be dislodged from the soil and splashed up by the rain (Sharma *et al*., 2007). The prokaryotic cyanobacteria are the major airborne algae, with the dominance of *Phormidium tenue* (Chu, 2012). Most algae can be found as phototrophic organisms and therefore require sunlight, water, carbon dioxide and nutrients including nitrogen and phosphorus for their growth (Mata *et al.,* 2010; Williams and Laurens, 2010; Chen*et al.*, 2011b; Zosel*et al.,* 2011; Drexler and Yeh, 2014). On the other hand, algae can be autotrophic, heterotrophic or mixotrophic organisms that can be cultivated with a variety of feedstocks (Liang *et al.*, 2009; Drexler and Yeh, 2014). Moreover, algae can be divided into two major categories of organisms; microscopic (unicellular or filamentous) microalgae and multicellular macroalgae, more commonly known as seaweed(Lundquist*et al.*, 2010). Algae cells can be found in a wide range of sizes for example from picoplankton of only 0.2 - 2.0 µm in diameter to giant kelps with fronds

up to 60 m in length (Chu, 2012). The number of algal species has been estimated to be one to ten million (although 50000 may be a more reliable number, even so only a limited number have been studied and investigated) (Richmond, 2004). However, most of these speciesare microalgae,cyanobacteria are an example of microalgae that were the first colonisers of Earth and they released oxygen through photosynthesis which enabled other life forms to live on this planet (Chu, 2012).

Based on a few studies, some airborne algae can cause unfavorable impacts on human health, such that they can produce allergic reactions in humans (Chu, 2012). Algae represent an important group of organisms for biotechnological exploitation, that are important in global commerce, with annual revenues of about $1.25 billion in the microalgae and $6 billion in the macroalgae industries (Pulz and Gross, 2004; Raja *et al.*, 2008; Drexler and Yeh, 2014).

The cells of algae are identified as sunlight driven factories and therefore capable to convert solar energy to chemical energy by photosynthesis (i.e. convert the carbon dioxide to potential biofuels, feeds and high value bioactives production) (Schwartz *et al.*,1990; Ghirardi *et al.,* 2000; Akkerman *et al.,* 2002; Banerjee *et al.,* 2002; Melis, 2002; Lorenz and Cysewski, 2003; Shimizu, 2003; Walter *et al.,* 2005; Spolaore *et al.,* 2006; Chisti, 2007; Bellou and Aggelis, 2012; Priyadarshani and Rath, 2012; Wei *et al.,* 2013; Hounslow *et al.,* 2017). Algae have long been accepted as important potential sources for biofuels (biodiesel) manufacture due to their ability to produce substantial amount of natural lipids (triacylglycerides, TAG) via biosynthesis processes (Meng *et al.,* 2009; Vicente *et al.,* 2009; Devi *et al.,* 2012; Bellou and Aggelis, 2012; Campos *et al.,* 2014; Drexler and Yeh, 2014). In addition, algae are increasingly recognised as potentially important organisms for biotechnological utilisation especially for significant products, processes and services, with important

impact in pharmaceutical industries and for food production and as well as in public health (Chisti, 2007; Ahmad *et al.,* 2011; Chu, 2012; Drexler and Yeh, 2014). Algae are also an important group of organisms for a variety of bioproducts such as nutritional supplements, animal/fish feed, fertilizers and cosmetics (Morineau-Thomas *et al.,* 2002; Grima *et al.,* 2003; Chisti, 2007; Harun *et al.,*2010). Algae are very effective at treating a variety of wastewater streams such as municipal, agricultural or industrial waste and the cultivated biomass can be an aerobically digested for biogas or converted to liquid fuels (Chisti 2007; Chu, 2012). Depending on the species, algae can also contain a high quantity of protein, carbohydrates, vitamins, minerals, polyunsaturated fatty acids and pigments (Morineau-Thomas *et al*., 2002; Drexler and Yeh, 2014; Demirbas, 2009).

Furthermore, photosynthetic microorganisms are useful in bioremediation applications (Mallick, 2002; Suresh and Ravishankar, 2004; Kalin *et al.,* 2005; Munoz and Guieysse, 2006) and nitrogen fixing biofertilisers (Vaishampayan *et al.,* 2001). Recent studies, however, suggest that a diverse range of metabolites with various bioactivities are produced in algae that are yet to be fully exploited (Chu, 2012).

1.2. Characteristics and Diversity of Microalgae

Microalgae are microscopic unicellular prokaryotic or eukaryotic photosynthetic organisms that are capable of growth in both marine and freshwater environments due to simplicity of their structures (Moazami *et al.,* 2012; Saifullah *et al.,* 2014). Microalgae also can form algal blooms (Graham and Wilcox, 2000).

Microalgae can be either heterotrophic or autotrophic microorganisms according to their energy pathways (Davis *et al.*, 2011). If microalgae are heterotrophs, they can use organic compounds (sugars) as organic carbon source and energysource for their growth (Dragone *et al*., 2010; Davis *et al.*, 2011). Moreover, organic carbon sources,

(for example fructose, glucose, mannose, galactose and lactic acid) can increase the growth of microalgae cultured in static flasks (Zhang *et al.*, 2011). If microalgae are autotrophs, they can use inorganic compounds, for example carbon dioxide (CO_2) as sole inorganic carbon source and light as energy source to support their growth and produce biomass rich in value-added products including lipids, carbohydrates, proteins and pigments such as carotenoids (Markou and Nerantzis, 2013; Minhas *et al.*, 2016). Autotrophic microalgae are able to convert solar energy into biomass via photosynthesis, according to the generic growth equation as below (Davis *et al.*, 2011).

$$CO_2 + \text{Nutrients} + \text{Sunlight} \longrightarrow O_2 + \text{Microalgal Biomass}$$

The microalgae biomass contains 20 - 30% carbohydrate, 10 - 20% lipid and 40 - 60% protein (Singh *et al.*, 2011).

Some photosynthetic microalgae are mixotrophic microorganisms by combining autotrophy and heterotrophy, where microalgae simultaneously use inorganic nutrients (CO_2) and organic compounds as carbon source in the presence of light as energy source to drive photosynthesis as shown in Table 1.1 (Kang *et al.* 2004; Dragone *et al.*, 2010; Chen *et al.*, 2011a; Perez-Garcia and Bashan, 2015; Smith *et al.*, 2015). Therefore, photoautotrophy and heterotrophy can occur simultaneously (Wang *et al.*, 2014b). This mode (mixotrophic cultivation) reduces dependency on light penetration, enabling higher cell densities than autotrophy, while using considerably less organic material per unit of biomass than dark heterotrophic growth (Boyle and Morgan, 2009; Smith *et al.*, 2015). Moreover, heterotrophic cultivation of microalgae by promoting aerobic respiration on an organic carbon source enables

biomass production to be maintained during the night hours and at higher cell densities due to light independent growth (Bjornsson *et al.*, 2013).

Growth mode	Energy source	Carbon source	Light availability requirements	Metabolism variability
Heterotrophic	Organic	Organic	No requirements	Switch between sources
Photoheterotrophic	Light	Organic	Obligatory	Switch between sources
Photoautotrophic	Light	Inorganic	Obligatory	No switch between sources
Mixotrophic	Light and organic	Inorganic and organic	Not obligatory	Simultaneous utilization

Table 1.1: Growth modes of microalgae cultivation and light requirements. Adapted from Perez-Garcia and Bashan (2015).

Therefore, microalgae cultivated under mixotrophic conditions synthesize compounds characteristic of both photosynthetic and heterotrophic metabolisms at high production rates (Abreu *et al.*, 2012). These characteristics are also a promising alternative strategy for microalgae biofuel production, especially when coupled with the use of waste organic carbon sources (Moon *et al.*, 2013). In addition, lower energy costs have been associated with mixotrophic cultivation systemsin comparison with photoautotrophic culture systems, due to their relatively lower requirements for high light intensities (Garcia *et al.*, 2005).

However, many unicellular algae are capable of converting solar energy to chemical energy via photosynthesis (Converti *et al.*, 2009; Priyadarshani and Rath, 2012). For autotrophic algae, photosynthesis is a key component of their survival, whereby they convert solar radiation (sunlight) absorbed by chloroplasts into adenosine triphosphate (ATP) the usable energy currency at cellular level, and

NAD(P)H, the source of reducing power(Brennan and Owende, 2010). Oxygen is evolved as a by-product and this makes microalgae one of the largest contributors to atmospheric oxygen supplies (Graham and Wilcox, 2000).

Currently, one emerging application of microalgae is for the fixation of CO_2 (Wang *et al.,* 2008; Ying *et al.,* 2015). Microalgae can fix CO_2 efficiently from different sources, including the atmosphere, industrial exhaust gases and soluble carbonate salts. Although, the fixation of CO_2 from the atmosphere is the major basic process, it is not an economically feasible option during commercial growth of microalgae due to the relatively small percentage of CO_2 in the air, which is approximately 0.036 % (Brennan and Owende, 2010). On the other hand, industrial exhaust gases such as flue gas contain up to 15% of CO_2 and therefore provide a rich source of CO_2 for cell growth and potentially more efficient process for bio-fixation of CO_2. Some species of microalgae are also able to use carbonate salts such as Na_2CO_3 and $NaHCO_3$ for microalgael cultivation. These cells typically have high extracellular carbonic anhydrase activities which is the enzyme responsible for the conversion of carbonate to free CO_2 to facilitate carbon dioxide assimilation. Moreover, the direct uptake of bicarbonate by an active transport system has also been found in several species of cyanobacteria (Wang *et al.,* 2008).

Growth medium must be provided containing the inorganic essential elements that constitute the microalgal cell including nitrogen, phosphorus, iron and in some cases silicon (Chisti, 2007). Nitrogen is typically supplied as nitrate (NO_3^-), but often as ammonium (NH_4^+) and urea may be used as a nitrogen source (Shia*et al.,* 2000). Phosphorus must be supplied in significant excess because the phosphates added complex with metal ions, therefore, not all the added phosphate is bioavailable (Chisti, 2007).

Furthermore, microalgae growth depends not only on a sufficient supply of essential macronutrient elements such as carbon, nitrogen, phosphorus, silicon and major ions such as Mg^{2+}, Ca^{2+}, Cl^{-}, and SO_4^{2-} but also depends on a number of micronutrient metals such as manganese, iron, cobalt, copper, zinc and molybdenum (Sunda *et al.*, 2005; Dragone *et al.*, 2010). The cells of microalgae can be motile with variable shapes, microalgal cells are capable to grow in clumps and to form filaments with sizes between 1 to >100 μm (Lee, 1999). Microalgae are one of the oldest living groups of organisms on our planet and recognized as thallophytes (plants lacking roots, stems and leaves) and lack a sterile covering of cells around the reproductive cells (Brennan and Owende, 2010). Microalgae are therefore a variety of microorganisms and represent a huge variety of species that are living in a wide range of environmental conditions included aquatic and terrestrial ecosystems. There are estimated to be more than 50,000 species with only a limited number of about 30,000 species that have been discovered and analysed to date (Mutanda *et al.*, 2011; Richmond, 2004).

Moreover, several microalgal species have oil (lipid) content up to 80% of their dry body weight (Oncel, 2013; Saifullah *et al.*, 2014; Ma *et al.*, 2016). Microalgae have been classified by several different systems over the last century, typically based on their coloured pigments, cell wall composition, chemical nature of storage products of the different strains, morphological and cytological characters such as the presence and spatial organization of flagella, the processes of nuclear and cell division, the presence of an endoplasmic reticulum envelope and if there is any connection with the nuclear membrane (van den Hoek *et al*., 1995). On the basis of their pigments, microalgae can be split into several major groups, namely green,

brown and red algae, but pigmentation is not always a reliable guide to taxonomy (Chu, 2012; Buono *et al.,* 2014).

Furthermore, microalgae can be divided into prokaryotic oxygenic photosynthetic organisms (cyanobacteria) and eukaryotic photosynthetic microalgae (all other groups). Many cyanobacteria and eukaryotic microalgae can grow rapidly and live in harsh conditions such as varying salinity, pH, temperature etc, due to their unicellular or simple multi-cellular structure (Li *et al.,* 2008; Brennan and Owende, 2010; Mata *et al.,* 2010; Parmar *et al*., 2011; Buono *et al.,* 2014; Unnithan *et al.,* 2014). Moreover, because of their simple cellular structure, microalgae are very efficient converters of solar energy (Saifullah *et al.,* 2014). Prokaryotic microalgae are grouped under the Domain Bacteria, whereas Eukaryotic microalgae are included in the Domain Eukarya (Chu, 2012). Prokaryotic cyanobacteria (*Cyanophyceae*), were formerly known as blue green algae (Mata *et al.,* 2010; Chu, 2012; Buono *et al.,* 2014). Although, cyanobacteria belong to the domain of bacteria being photosynthetic prokaryotes, often they are considered as microalgae due to their ability to carry out oxygenic photosynthesis (Chu, 2012; Buono *et al.,* 2014). Eukaryotic microalgae are for example green algae (*Chlorophyta*) and diatoms (*Bacillariophyta*) (Li *et al.,* 2008; Mata *et al.,* 2010). Eukaryotic microalgae have a nucleus containing genetic material and several other organelles (such as mitochondria, chloroplast, etc.) that are surrounded by membranes and also have a high degree of internal organization, while in prokaryotic microalgae (Cyanobacteria) there is no distinct nucleus or membrane bound organelles (van den Hoek *et al*., 1995; Dragone *et al*., 2010).

Since the beginning of rDNA sequencing and other molecular techniques, the molecular taxonomy has aided in recognising fundamental domains in the phylogeny of living organisms and classifying them into more natural groups (Burki, 2016). A

diagrammatic tree depicting the organisation of most eukaryotes into six major groups and their relationship with the Bacteria and Archaea is shown in Figure 1.1. The key information in this figure is that a more detailed classification of algae within the eukaryotic domain has been produced with wide separation of different algal groups. The green and red algae are grouped with the higher plants, but diatoms and dinoflagellates are widely separated and have little in common at the molecular level with green algae or higher plants (Simpson and Roger, 2004).

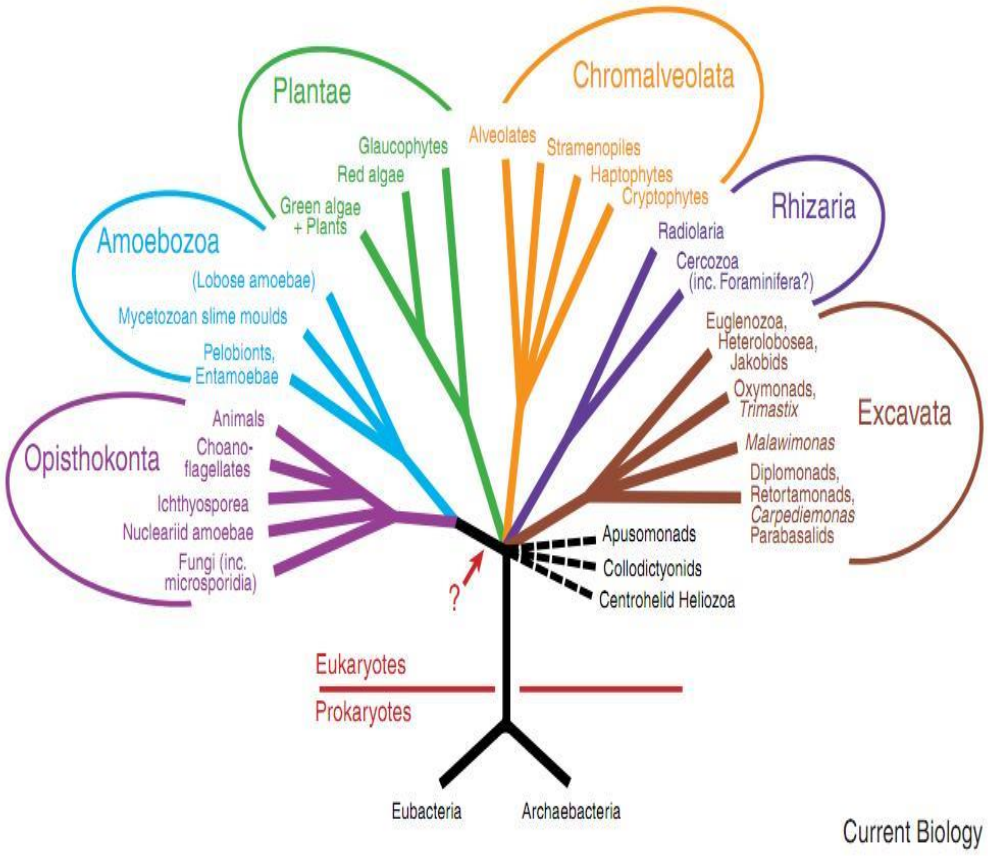


Figure 1.1: A diagrammatic representation of various groups of organisms in the eukaryotic tree of life. Eukaryotes can be divided into six main groups. Algae aregrouped within the Plantae and Chromalveolata (e.g. diatoms). The arrow shows a possible precise placement of the root. Taken from Simpson and Roger (2014).

1.3. Description of *Nannochloropsis*

Nannochloropsis is a genus of unicellular and nonmotile marine microalgae belonging to: Phylum: Heterokontophyta, Class: Eustigmatophyceae, Family: Eustigmataceae. *Nannochloropsis* was described by Hibberd as comprising six known species (*N.gaditana, N. granulata, N. limnetica, N. oceanica, N. oculata and N. salina*) (Ma *et al.,* 2016). In the past, *Nannochloropsis* was known as "marine Chlorella" This classification was based on its ultrastructure and named *Nannochloropsis* by Maruyama in 1986. *Nannochloropsis* cells are spherical to slightly ovoid and simple in morphology with diameters varying from 2 to 8 µm with plastids similar to plant cells (Maruyama *et al.,* 1986). *Nannochloropsis* cells have lipid droplets (LDs) that act as energy storage reservoirs for the cells, which can be augmented in size under environmental stressful conditions (Olmstead *et al.,* 2013; Ma *et al.,* 2016). The cell division of *Nannochloropsis* cells was investigated by electron microscopy in *N. oculata* cells, which revealed the existence of a continuum between both plastid and nucleus during the cell cycle (Murakami and Hashimoto, 2009).

Species of *Nannochloropsis* have recently been considered as feedstock for biodiesel production due to their ability to accumulate high amounts of lipids (Bongiovani *et al.,* 2014). For example, *Nannochloropsis* sp. (Rodolfi *et al.,* 2009; Pal *et al.* 2011; Bondioli *et al.,* 2012), *N. gaditana* (Simionato *et al.,* 2013), *N. oculata* (Vooren *et al.,* 2012), *N. oceanic* (Bongiovani *et al.,* 2013; Dong *et al*., 2013; Pal *et al*., 2013; Solovchenko *et al*. 2014) and *N. salina* (Sforza *et al*., 2012; Loira *et al.,* 2017). *Nannochloropsis salina* is a marine strain and has become a biotechnological target due to its high capacity to produce polyunsaturated fatty acids and triacylglycerols. It has been used as a source of biofuel, pigments and also food supplements, like omega -3 fatty acids (Loira *et al.,* 2017). *Nannochloropsis salina* is

a halotolerant species due to its ability to grow in salinities up to around 0.8 to 1 M NaCl (Griffiths and Harrison, 2009; Bartley *et al.*, 2013). However, *N. salina* has been reported to grow fastest at lower salinities of 20 to 40 PSU (Practical Salinity Unit is a unit based on the properties of seawater conductivity, it is equivalent to per thousand or to g/kg, and 20 to 40 PSU = approximately 0.35 to 0.7 M NaCl) (Abu-Rezq *et al.,* 1999). It can produce the highest amounts of total lipids per unit of biomass at a range of salinity between 30 to 35 PSU (Bartley *et al.,* 2013).

In general, the lipid productivity is increased when *N. salina* cells are grown under stressful conditions, because the cells are changing from actively dividing to storing energy (Wang *et al.,* 2009). *N. salina* cells can reach 60% of total lipid content by weight when cells are exposed to environmental stresses such as nitrogen starvation and high irradiances, see section 1.7. (Rodolfi *et al.,* 2009). Therefore, the main focus of this study is based around oleaginous green microalgal species such as *N. salina,* which have high a growth rate, wide environmental tolerance and high neutral lipid productivity. They also produce lipids that have similar properties to oils produced by first and second generation fuel crops (Chisti, 2007).

1.4. Lipids in Microalgae

Lipids produced by microalgae like those of higher plants consist of two main categories: structural lipids (polar lipids) and neutral storage lipids (non-polar lipids) (Sharma *et al*., 2012; Satpat and Pal, 2015; Minhas *et al.,* 2016). Structural lipids (polar lipids) include phospholipids and glycolipids, whereas neutral storage lipids (non-polar lipids) generally include triacylglycerides (TAGs), sterols, free fatty acids, hydrocarbons and wax esters (Hu *et al*., 2008; Huang *et al*., 2010; Yu *et al.,* 2011; Satpat and Pal, 2015). However, structural lipids mainly have a high content of

polyunsaturated fatty acids (PUFAs) such as omega-3 LC-PUFA (Ryckebosch *et al.,* 2014), which are important nutrients for both humans and aquatic animals. Moreover, polar lipids (phospholipids) and sterols are essential structural components for cellm embranes that act as selective permeable barriers for cell membrane and organelles of microalgae (structural function) (Sharma *et al*., 2012). In addition to a structural function, these lipids maintain specific membrane functions such as providing the matrix for a wide variety of metabolic processes, play a role in the cell membrane fusion and also participate in responding to changes in the environmental conditions (for example nitrogen starvation) (Sharma *et al*., 2012)**.**

On the other hand,neutral storage lipids (non-polar lipids) are energy-rich storage products, which can be simply catabolised to provide metabolic energy(Gurr *et al*., 2002; Sharma *et al*., 2012). In general, triacylglycerides (TAGs) or (triglyceride, TG) are made predominately of saturated fatty acids and some monounsaturated fatty acids (FAs), which can be transesterified to produce biodiesel(Sharma *et al*., 2012). Typically, triacylglyceride (TAG) is an ester derived from glycerol and three fatty acids with substantial amounts of oxygen in the structure as shown in Figure 1.2 (Simionato *et al.,* 2013).

The fatty acids differ in their carbon chain length and in the number of double bonds (Barnwal and Sharma, 2005). However, microalgal triacylglycerol (TAG) is mostly synthesized in the presence of light and thereafter stored in cytosolic lipid bodies and then used for polar lipid synthesis in the dark (Thompson, 1996). Triacylgylcerols (TAG) are neutral lipids (non-polar lipids) and mainly act as high energy storage compounds that accumulate in plants, animals and algae (Liu *et al.,* 2013), once extracted, can be easily converted into biofuels through transesterification reactions (Chisti, 2008; Fukuda *et al.,* 2001). Therefore, TAGs are of great interest to

the biofuels industry as they can be converted into fatty acid methyl esters (FAMEs) by series of enzymatic reactions called transesterification process (Hu *et al.*, 2008).

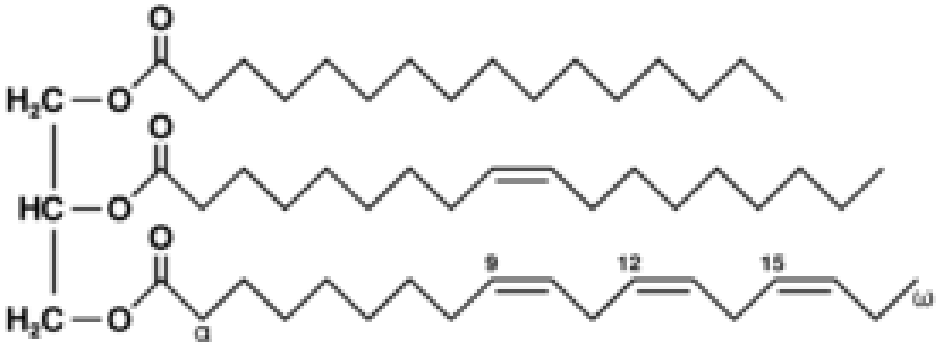


Figure 1.2: Example of a triacylgylcerols (TAG) structure, a type of lipid commonly found in microalgal cells. Taken from, Triglyceride-Wikipedia.

1.5. Metabolism of Microalgal Lipids and Environmental Stress

Although understanding the lipid metabolism pathway and its regulators during algal growth is very important for improving lipid production, algae have been little studied in their biochemistry compared with higher plants (Hu *et al.*, 2008; Khozin-Goldberg and Cohen, 2011; Liu and Benning, 2013; Jinkerson *et al.*, 2013; Bellou *et al.*, 2014). Current understanding of lipid pathways is based upon some shared biochemical characteristics of a range of genes and enzymes isolated from algae and selected higher plants that are involved in lipid metabolism (Hu *et al.*, 2008). Moreover, microalgae are known to store high amounts of oil in the form of lipids within internal organelles called lipid bodies in their cytoplasm (Hu *et al.*, 2008). Research efforts to improve the accumulation of lipid in microalgae by modifying the expression of enzymes in lipid synthesis usually fail, this is due to poor understanding

of the mechanisms that regulate and control lipid accumulation in microalgae (Bellou, *et al.,* 2014).

In general, the basic lipid metabolism pathways in algae are directly analogous to those demonstrated in higher plants, and some broad generalisations can be based on quite limited experimental data (Hu *et al.,* 2008). Recently, microalgae have been considered as significant alternative fuel sources due to their high growth rate and their ability to accumulate large quantities of triacylglycerol lipid (Hu *et al.,* 2008; Chen *at al.,* 2009; Budarin *et al.,* 2012; Satpati and Pal, 2015). Furthermore, it has been reported that lipid contents of most microalgae are affected by different environmental conditions due to both physical and chemical stresses (Renaud *et al.,* 2002; Li *et al.,* 2008; Pal *et al.,* 2011; Yeesang and Cheirsilp, 2011; Bellou and Aggelis, 2012; Msanne *et al.,* 2012; Bellou *et al.,* 2014). Moreover, microalgae can accumulate a large amount of neutral lipid especially at exponential phase or when the environmental conditions are stressful for their growth (Illman *et al.,* 2000). These stress conditions include light intensity (Kojima and Zhang, 1999; Ruangsomboon, 2012), nitrogen limitation (Illman *et al.,* 2000; Pal *et al.,* 2011; Juergens *et al.,* 2015) and phosphate limitation (Yeesang and Cheirsilp, 2011), silicon deficiency (Lynn *et al.,* 2000), high salinity (Rao *et al.,* 2007; Zhila *et al.,* 2011), cadmium stress (Guschina and Harwood, 2006), temperature, pH, culture age (Khozin-Goldberg *et al.,* 2002; Zhekisheva *et al.,* 2002; Guschina and Harwood, 2006; Solovchenko *et al.,* 2008a; Pal *et al.,* 2011) and iron content of the medium also affects algal growth (Liu *et al.,* 2008; Yeesang and Cheirsilp, 2011). It has been well reported that depletion of nitrate in the medium increases the lipid production (Feng *et al.,* 2012; Sun *et al.,* 2014). Nitrogen starvation is widely used for increasing lipid accumulation in some microalgae (Breuer *et al.,* 2012; Santos *et al.,* 2013; Wan *et al.,* 2013; Campos *et*

al.,2014). Triacylgylcerols can reach 40% to 70% of the total dry biomass in some oleaginous species (Hu *et al.,* 2008).

In nature, however, microalgae are exposed to different changes of the external environment (e.g. changes in climatic and geographical location of the habitat) over time during the growing season (Fanesi *et al.,* 2014), in which these microalgae are growing and different strains of the same species may respond differently. Therefore, strains resulting from different geographical locations are physiologically different and may have developed their mechanisms for growing and adaptation to these changes in the surrounding environments, and thus their use promises higher potential (Delgado *et al.*,1991; Bouaicha *et al*., 2001; Bellou *et al.*, 2014).

Specifically, under favorable environmental conditions, the microalgae can accumulate high levels of polar lipids such as phospholipids and glycolipids in their cell membrane (Satpati and Pal, 2015). Under unfavorable conditions, microalgal growth is arrested and therefore photosynthetic activity decreases and the excess energy may be stored in the form of neutral lipids and/or carbohydrates (Pal *et al.,* 2011). Thus, both management of the environmental conditions and strain selection are usually common approaches used for increasing lipid accumulation in cells of microalgae (Bellou *et al.,* 2014; Ma *et al.,* 2014). On the other hand, it is necessary to investigate the most suitable microalgal species that are able to produce high levels of neutral lipids of triacylglycerides (TAG) as a basis for biofuels production in order to move from laboratory to commercial cost effective biofuel production (Chisti, 2008; Devi *et al.,* 2012; Campos *et al.,* 2014; Drexler and Yeh, 2014). However, there are many microalgae that are excellent feedstock for biofuels especially because they produce valuable neutral lipids (triacylglycerols) exceeding in several cases 20% of

their dry biomass weight as storage products and then can easily be converted to biofuels, therefore are placed in the group of oleaginous microorganisms, as shown in Table 1.2 (Ratledge, 2004; Chisti, 2007 and 2008; Ratledge and Cohen, 2008; Bellou and Aggelis, 2012; Christophe *et al.*, 2012; Priyadarshani and Rath, 2012; Xu and Boeing, 2014).

Microalgae Type (Species name)	**Average Total Lipid (% Dry weight)**	**Habitat (Fresh water/marine)**
Botryococcus braunii	25–75	Fresh water/estuary
Chlorella sp	28–32	Fresh water
Crypthecodinium cohnii	20	Marine
Cylindrotheca sp.	16 – 37	Marine
Dunaliella primolecta	23	Marine
Isochrysis sp.	25 – 33	Marine
Monallanthus salina	> 20	Marine
Nannochloris sp.	20 – 35	Marine
Nannochloropsis sp.	31– 68	Marine
Neochloris oleoabundans	35 – 54	Fresh water
Nitzschia sp	45 – 47	Marine
Phaeodactylum tricornutum	20 – 30	Marine
Schizochytrium sp	50 –77	Marine
Tetraselmis sueica	15 – 23	Marine

Table 1.2: Habitats and lipid contents of different microalgae species. Modified from, Chisti (2007) and Rodolfi *et al.* (2009) and Saifullah *et al.* (2014).

Indeed, some oleaginous microalgae can synthesis polyunsaturated fatty acids (PUFAs) and other bioactive compounds such as pigments, antioxidants, antibiotics and toxins, so they are considered as competent sources of new products of interest

for pharmaceutical industries (Bellou and Aggelis, 2012). However, *Nannochloropsis* sp. for example are considered as industrial strains of microalgae for biofuels production because of their rapid growth rate, use of CO_2 and their ability to accumulate large amounts of neutral lipids (TAG) (Simionato *et al.,* 2011; Wang *et al.,* 2014a; Kang *et al.,* 2015), which can reach concentrations up to 65-70% of total dry weight (Simionato *et al.,* 2011).

Furthermore, *Nannochloropsis* sp. are particularly promising as oleaginous microalgae species due to the availability of genomic information which can be used to generate economically feasible biofuel production by improvements to strains by genetic engineering (Kang *et al.,* 2015). Genome and transcriptome data of *Nannochloropsis sp.* have been recently released and global regulators of biological pathways (by up or down regulation of related genes) have been identified (Wang *et al.,* 2014a; Li *et al.,* 2014a; Kaye *et al.,* 2015). Lipid accumulation of *Nannochloropsis sp.* can be improved by different ways including nitrogen limitation and other specific growth conditions such as osmotic stress in microalgae (Li *et al.,* 2014b; Kang *et al.,* 2015). The work by Rodolfi *et al.,* (2009) demonstrated that the alga *Nannochloropsis* can achieve up to 60% lipid content after nitrogen starvation, along with high productivity.

1.6. Biosynthesis of Microalgal Lipids

Both inorganic carbon (CO_2) and organic carbon sources (glucose, acetate, etc.) can be used by microalgae for lipid production (Huang *et al.,* 2010). Like higher plants and animals, microalgae are able to biosynthesise triglycerides to store carbon and energy, which are the main materials in biodiesel production (Huang *et al.,* 2010). In general, the synthesis of triglycerides in microalgae consists of three main steps as shown in Figure 1.3: (1) formation of acetyl coenzyme A (acetyl-coA), catalyzed by

acetyl-CoA carboxylase (ACCase) in the cytoplasm; (2) elongation and desaturation of carbon chain of the fatty acids; and (3) the biosynthesis of triglycerides (TAG) in microalgae (Huang *et al.*, 2010; Yen *et al.*, 2013).

During photosynthesis, CO_2 is converted to glycerate-3-phosphate (G3P). This molecule is the precursor for the synthesis of many storage materials in cells such as polysaccharides and lipids (Bellou and Aggelis, 2012).

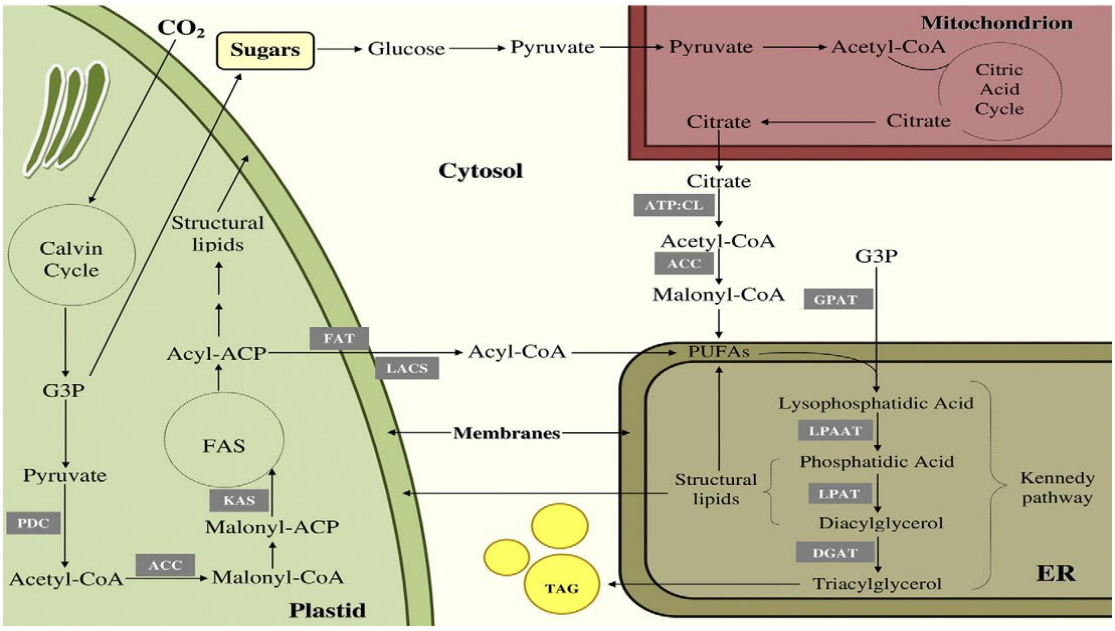


Figure 1.3: A simplified scheme showing lipid biosynthesis pathway in microalgae. Taken from, Bellou *et al.* (2014).

The conversion of glycerate-3-phosphate (G3P) to pyruvate and thereafter to acetyl-CoA, via a reaction catalyzed by the pyruvate dehydrogenase complex (PDC), initiates the lipid biosynthetic pathway and occurs in the plastid (Bellou and Aggelis, 2012; Bellou *et al.*, 2014). Acetyl-CoA can also be generated via a biochemical

pathway that permits the conversion of polysaccharides into lipids (Ratledge, 2004; Chatzifragkou *et al.*, 2010; Bellou and Aggelis, 2012; Bellou *et al.*, 2014).

Specifically, the breakdown of storage polysaccharides during their catabolism, which is occurring under specific growth conditions (e.g. light limitation) usually generates energy through the glycolysis process occurring in the cytoplasm followed by the citric acid cycle occurring in the mitochondrion (Bellou and Aggelis, 2012; Bellou *et al.*, 2014). However, several environmental stresses, such as nitrogen or phosphate limitation, may disturb the citric acid cycle due (i.e. to the inhibition of NAD^+ - isocitrate dehydrogenase enzyme that catalyzes the conversion of isocitrate to α-ketoglutarate). In this case, citrate is accumulated in the mitochondrion and subsequently excreted into the cytoplasm and then cytoplasmic ATP-dependent citrate lyase (ATP:CL) converts citrate into oxaloacetate and acetyl-CoA (Muhlroth *et al.*, 2013; Bellou *et al.*, 2014). This acetyl-CoA is converted into malonyl-CoA by cytoplasmic acetyl-CoA carboxylase (ACCase) and then becomes available for fatty acid elongation (fatty acid synthesis) in the membranes of the endoplasmic reticulum (ER) (Hu *et al.*, 2008; Mühlroth *et al.*, 2013; Bellou*et al.*, 2014). Besides acetyl-CoA, a supply of NADPH (generated from NADH via a small cycle in which a malic enzyme participates) is also required for the fatty acid synthesis (Bellou and Aggelis, 2012). In fact, the biochemical pathway converting polysaccharides to lipids during sugar assimilation is commonly used in oleaginous heterotrophs (Ratledge, 2004; Bellou and Aggelis, 2012).

Although, regulation of biosynthesis of storage materials in microalgae and potential conversions connecting the pools of polysaccharides and lipids are less understood (Bellou and Aggelis, 2012). Several approaches have been used aiming to direct carbon flux toward lipid rather than polysaccharides synthesis (Weselake *et*

al.,2008; Bellou and Aggelis, 2012). Bellou and Aggelis, (2012) confirmed that this mechanism has been shown in *N. salina* and *Chlorella* sp. cultivated in a lab scale open pond-simulating photobioreactor (PBR), it is probably common in oleaginous strains that are able to grow as heterotrophic microalgae (Bellou*et al.*, 2014).

1.7. Effect of Environmental Factors on Microalgal Lipids Induction

Microalgaecan grow fastunder favourable growth conditionsand subsequently produce a huge amount of biomass with a low amount of lipids in biomass which made are less ideal for biofuel (Sharma *et al*., 2012).

During photosynthesis, the algae are using only light and nutrients, thus producing intercellular lipids, proteins, and carbohydrates. The relative total of these metabolic products is strongly associated with environmental conditions such as the intensity of sunlight, CO_2concentration, temperature, external pH, nutrients available, osmotic stress and presence or absence of other organisms in surrounding environments as shown in Figure 1.4 (Takagi *et al.*, 2006; Chiu *et al.*, 2009; Juneja *et al.,* 2013). Therefore, the production of microalgal lipids, biomass and carotenoids can be enhanced using environmental stress factors as lipid induction techniques (Mata *et al.,* 2010; Mulders *et al.*, 2014; González *et al.*, 2015).

However, the ability of microalgae to grow and survive under extreme environmental conditions is reflected in the huge diversity and unusual type of cellular lipids obtained from these microalgae (Sato *et al.,* 2000). It's believed that under environmental stress factors, some of these microalgae have a huge ability to alter their metabolic pattern to maintain their growth rates as a response to changes in the external stress (Thompson, 1996; Guschina and Harwood, 2006; Markou and Nerantzis, 2013) and consequently modify their lipid biosynthetic pathways to

accumulate and produce neutral lipids, typically in the form of triacylglycerol (TAG) (Hu *et al.*, 2008).

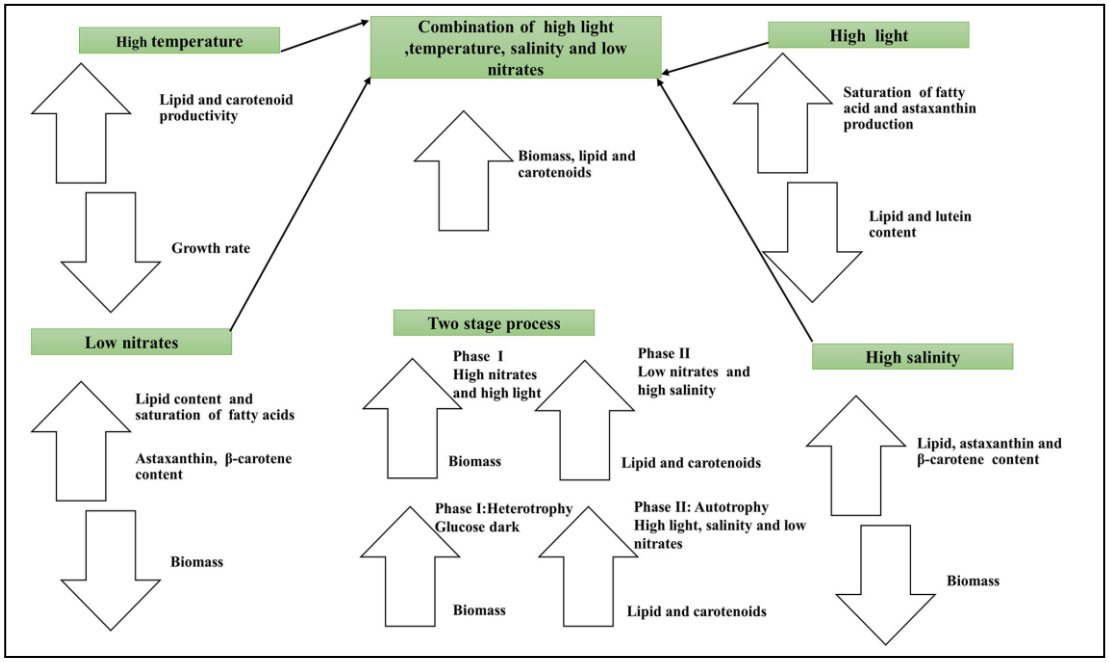


Figure 1.4: Schematic diagram showing the effect of environmental stresses on lipid and carotenoid production by microalgel cells. Taken from, Minhas *et al*., (2016).

The biosynthesis pathway of triacylglyceride may play a key role with the environmental stress response, and offers carbonand energy storage function in the cell that allows microalgae to tolerate environmental stress conditions (Hu *et al.* 2008; Fakhry and Maghraby, 2015). Under optimal growth conditions, the microalgae can produce huge amounts of microalgal biomass with relatively low neutral lipid contents which constitute about 5% - 20% of dry cell weight, including glycerol-based membrane lipids (Taggar *et al.*, 2014). On the other hand, under stress growth conditions, the microalgae change their lipid biosynthetic pathways towards the formation and accumulation of large amounts of neutral lipids which constitute about

20% - 50% of dry cell weight enabling these microalgae to tolerate these extreme conditions (Hu, 2004; Sharma *et al.,* 2012).

However, numerous studies have been extensively carried out on a number of microalgae species to identify and develop efficient lipid induction techniques using various physical and chemical stresses such as the use of nutrient starvation (nitrogen and phosphate), light intensity, temperature, salinity and/or pH of growth medium, heavy metals and other chemicals (Taggar *et al.,* 2014). In addition to these factors that have been used in lipid induction, growth phase and aging of the culture also affect both TAG content and fatty acid composition (Sharma *et al.,* 2012; Taggar *et al.,* 2014). The following sections present different neutral lipid induction techniques that can be used for enhancing lipid productivity in different microalgae species and give explanation of their potential (Sharma *et al.,* 2012).

1.7.1. Lipid Induction inMicroalgaeusing Nutrient Starvation Stress

Nutrient starvation is one of the most widely used and applied lipid induction techniques in triacylglycerides (TAG) production. For example, an increase in TAG accumulation and decrease of polar lipids was noticed in all of the nutrient-limited cultures when the diatom *Stephanodiscus minutulus was* grown under silicon, nitrogen or phosphorus limitation (Lynn *et al.,* 2000). Nitrogen and phosphate are two important nutrients for both growth and metabolism of microalgae. Nitrogen is an essential element for protein and nucleic acid formation. Nitrogen is also an integral part of essential molecules such as the energy carrier molecules in cells (ATP). Phosphate is also part of ATP and of the backbone of both DNA and RNA, which are fundamental molecules for all living cells. Phosphate is also important for the formation of phospholipids (Harris, 1986). However, limitation of nitrogen or

phosphorus changes the metabolic pathways in cells (i.e. starvation of these macronutrients modifies the metabolism of lipid from membrane lipid synthesis to neutral lipid storage in cells) (Juneja *et al.,* 2013).

Based on the literature reviewed, it is clear that nutrient starvation including nitrogen or phosphorus is one of the most widely used techniques to enhance TAG production from numerous species of microalgae that can be considered for biodiesel production (Sharma *et al.,* 2012). Numerous studies have extensively reviewed the impact of nutrient starvation (mainly nitrogen, phosphorus) on several diatoms, green algae, red algae, prymnesiophytes and eustimatophytes (Rodolfi *et al.,* 2009).

Nitrogen is the most critical nutrient which directly impacts on metabolism of lipids in eukaryotic microalgae and subsequently increases the lipid accumulation, mainly TAG as a response to nitrogen deficiency (Hsieh and Wu, 2009; Yeh and Chang, 2011; Praveenkumar *et al.,* 2012). These observation has been reported in several species of various microalgae such as green microalgae, cyanobacteria and diatoms and all tested species showed a significant rise in lipid production (Taggar *et al.*, 2014). The Nitrogen starvation technique is relatively easy to apply as a growth-limiting factor on microalgae by reducing or removing the nitrogen source from the media (Sharma *et al.,* 2012).

Widjaja *et al.* (2009) reported that high cellular lipid production under nitrogen deficiency could possibly take 2-5 incubation days and was accompanied by growth and low cell counts which subsequently affected the total biomass and lipid production. In addition, the effect of nitrogen stress conditions on cellular TAG production was successfully demonstrated and all the microalgae species grown under nitrogen stress, so far appear to increase their TAG production (Sharma *et al.,* 2012).

Phosphate directly impacts on metabolism of lipids in microalgal cells, which produce very long chain (C_{20}- C_{22}) polyunsaturated fatty acids such as arachidonic acid (20:4ω6, AA) and eicosapentaenoic acid (20:5ω3, EPA) (Khozin-Goldberg and Cohen, 2006). Phosphate limitation caused significant changes in the fatty acid and lipid composition of *Monodus subterraneus* strains with decreasing phosphate availability from 175 µM (K_2HPO_4) to 52.5, 17.5 and 0 µM (K_2HPO_4). An increase in the overall TAG production from 6.5% up to 39.3% of total lipids was observed along with a decrease in eicosapentaenoic acid (EPA) content. Moreover, the total lipid content was increased, essentially due to cellular TAG accumulation in *M.subterraneus* (Khozin-Goldberg and Cohen, 2006). Phosphate limitation also resulted in an increase in the lipid content, particularly TAG in some microalgal strains such as *Isochrysis galbana, Phaeodactylum tricornutum, Chaetoceros* sp. and *Pavlova lutheri*, while phosphate deprivation led to a decrease in cellular lipid concentration in other microalgae strains such as *Tetraselmis* sp. and *Nannochloropsis atomus* (Reitan *et al.*, 1994).

It is clear that the availabilities of nutrients in the surrounding environments have significant effects on both microalgal growth and fatty acid composition in microalgal cells. Furthermore, nutrient limitations invariably cause a steady decline in cell division rate (Taggar *et al.*, 2014). Under these growth conditions, active fatty acid biosynthesis is sustained in several microalgae species, provided there is enough light and CO_2 available for photosynthesis (Thompson, 1996). When there is no requirement for new membrane synthesis (i.e. the growth of microalgae slows down) the cells instead divert and deposit fatty acids into TAG, in this case the TAG production may act as a protective mechanism in cells (Taggar *et al.*, 2014).

In normal growth conditions, both ATP and NADPH produced by photosynthesis processes are consumed by generating biomass. When the growth and reproduction of microalgal cells are damaged due to growth-limiting conditions, the major electron acceptor ($NADP^+$) of photosynthesis processes is consumed (Taggar *et al.,* 2014). As photosynthesis processes are essentially controlled by light, they are not completely shut down, this may lead to unbalanced growth and subsequently the NADPH is consumed in fatty acid (FAs) biosynthesis. This increases the FAs production, which are converted to and stored in TAGs. This replenishes the pool of $NADP^+$ under growth limiting conditions (Thompson, 1996; Hu *et al*., 2008).

1.7.2. Lipid Induction in Microalgae using Temperature Stress

Temperature is one of the most important environmental stress factors that greatly influences microalgae cultivation and productivity of microalgae in a wide range of species (Ho *et al.,* 2014 a; Minhas *et al*., 2016). Thus temperature stress has been extensively used as a lipid induction technique to enhance intracellular FAs production in various microalgal strains that may be considered for biodiesel production (Juneja *et al.,* 2013; Taggar *et al.,* 2014). Different temperature regimes have been found to have a significant impact on growth and propagation of microalgae and on their lipid and FAs composition (Guschina and Harwood, 2006; Morgan-Kiss *et al.,* 2006; Juneja *et al.,* 2013). It has been reported that several lipid profile changes modify the physical properties of membranes following temperature shift so that usual functions continue unimpaired such as ion permeability, photosynthetic machinery and respiratory process (Somerville, 1995). However, a generally observed change in membrane lipids following temperature shift is alteration in the level of unsaturated FAs (Harwood and Jones, 1989). Due to the

geometry of FAs, those with carbon-carbon double bonds cannot be as densely packed as saturated fatty acids, therefore increasing the membrane fluidity of membranes containing unsaturated intracellular FAs (Taggar *et al.,* 2014).

Since the fluidity of membranes is decreased with lower temperatures, increased the unsaturated fatty acid (FA) provides an adaptation to the changing environmental conditions (Sharma *et al.,* 2012). Most microalgae species irrespective of their taxonomic status respond to the temperature modification by similar changes in their FA composition (Taggar *et al.,* 2014), that is increasing the content of their cellular unsaturated FAs with the decrease in the temperature and increasing content of their cellular saturatedFAs with increasing temperature (Sato and Murata, 1980; Lynch and Thompson, 1982; Renaud *et al.,* 2002).

*Dunaliella salina*was widely studied for low temperature modification of lipid composition, it was found that when temperature decreased from 30°C to 12°C, the content of unsaturated lipids (FAs) was significantly increased by 20% (Thompson, 1996; Sharma *et al.,* 2012). For an *Ochromonas danica* strain, when the culture temperature increased from 15°C to 30°C, the cell number was increased per unit volume of medium as a consequence increasing total content of their lipid (Aaronson, 2006). Furthermore, decreases of the relative content of unsaturated intracellular FAs were exhibited in both *Botryococcus braunii* and *Chlorella vulgaris* with an increase in incubation temperature (Sushchik *et al.,* 2003). In *Nannochloropsis salina*, increased culture temperature resulted in an increase of growth rate and total content of lipid (Sharma *et al.,* 2012). In contrast, there was no change in the content of cellular lipid found in *Chlorella sorokiniana* cells grown in various temperatures (Patterson, 1970). Furthermore, no effect of changing incubation temperature was exhibited on the level of the acidic thylakoid lipids, phosphatidylglycerol (PG) and

sulfoquinovosyl diacylglycerols (SQDG) in *Chlamydomonas reinhardtii* (Sato *et al.,* 2000). At present**,** no study has highlighted the effect of temperature to induce lipids on large-scale cultivation, but as lipid profiles clearly change with changing temperatures, the properties of algal-derived biodiesel should also change for different climates and seasons and therefore different microalgal strains or species may be used in different seasons for biodiesel production (Taggar *et al.,* 2014).

However, as reviewed above, only a limited information is available and all studies have been carried out at laboratory-scale where it is easy to maintain the incubation temperature. Thus maintaining, decreasing or increasing temperature is feasible only in closed system photobioreactors which are costly when compared to open systems (Sharma *et al.,* 2012).

1.7.3. Lipid Induction in Microalgae using Salinity Stress

Salinity is an intricate environmental stress factor that seriously influences biochemical composition. As a consequence, it alters the microalgal growth rate and overall biomass productivity (Renaud and Parry, 1994; Sharma *et al.,* 2012; Juneja *et al.,* 2013; Taggar *et al.,* 2014; Minhas *et al.,* 2016). Salinity refers mainly to sodium chloride concentration unless otherwise specified (Juneja *et al.,* 2013). Influences of salinity on intracellular FA composition in marine microalgae cells have been studied by several researchers (Bartley *et al.,* 2013; Martinez- Roldan *et al.,* 2014). However, only limited reports are available on fresh water microalgae (Minhas *et al*., 2016). Not surprisingly, some studies showed that marine microalgae can tolerate higher salinity rather than fresh water microalgae (Zhu *et al.,* 2003). Therefore, salinity as stress factor has been widely used as a lipid induction technique to improve intracellular

FAs production in a range of seawater microalgae strains that may be considered to produce biodiesel (Sharma *et al.,* 2012; Juneja *et al.,* 2013; Taggar *et al.,* 2014).

Microalgae have optimal growth salinity that is when salinity is higher or lower than optimal growth salinity the growth rate of microalgae will be reduced (Chunguang*et al.,* 2006). Zhila *et al.* (2011) reported that exposing microalgal cells to either higher or lower salinity levels changed their growth rate and altered their composition (e.g. lipid content), *Dunaliella* species offer the best example of microalgae which can tolerate high salinity stress and produce hugea mounts of lipid (Azachi *et al.,* 2002). Due to the ability of *Dunaliella* species to reproduce over a wide range of salinities, this makes them one of the preferred candidates to study salinity effects on microalgae (Xu and Beardall, 1997; Azachi *et al.,* 2002; Takagi *et al.,* 2006). *Dunaliella,* as a marine alga, can tolerate high salt concentration and showed an increase in both saturated and monounsaturated FAs with increase in NaCl concentration from 0.4 M to 4 M NaCl (Xu and Beardall, 1997).

Moreover, *D. tertiolecta* cells subjected to an increase in the initial NaCl concentration from 0.5 M NaCl (29 g/L) to 2.0 M NaCl (117 g/L) resulted in an increase in cellular lipid content with a higher amount of intracellular TAG (Takagi *et al.,* 2006). With a higher increase in salinity concentration from 0.4 M NaCl (23 g/L) to 4 M NaCl (234 g/L), *Dunaliella* sp. exhibited increased amounts of saturated FA and monounsaturated FA, while decreasing the percentage of PUFAs (Xu and Beardall, 1997). In another study with *D. tertiolecta,* an increase in cellular lipids from 60% to 67% with increasing TAG concentration from 40% to 56% were observed when NaCl concentration was increased from 0.5 NaCl to 1.0 M NaCl (Takagi *et al.,* 2006). Another study carried out with *D. salina* cells transferred from 0.5 M NaCl (29 g/L) to 3.5 M NaCl (205 g/L), observed a significantly higher ratio of

intracellular unsaturated FAs (C18) to intracellular saturated FAs (C16) in the cells grown in 3.5 M NaCl compared with cells grown at 0.5 M NaCl (Azachi *et al.,* 2002).

The effect of increasing the NaCl level on a fresh water microalgal species *Botryococcus braunii* showed an increase in growth rate, content of cellular lipid and carbohydrate content, although, the highest biomass concentration was achieved at lowest salinity concentration and the salinity increases were very small in the mM range (Rao *et al.,* 2007). In a different study, *B. braunii* grown in 0.5 M NaCl showed a higher total level of cellular lipid compared to cells grown in media without NaCl (Ben-Amotz*et al.,*1985). In contrast, when the same strain of *B. braunii* was exposed to higher salinities, the growth rate reduced, protein content was decreased and carbohydrate and lipid content were unchanged (Vazquez-Duhalt and Arredondo-Vega, 1991). This reduction in growth at high salinitymay have been due to an inability of the microalgae to adapt to high salinity (Juneja *et al.,* 2013).

1.7.4. Lipid Induction in Microalgae Using pH Stress

Collectively, pH is one of the most important environmental factors for microalgae cultivation that significantly influences the microalgal growth, cell size, biochemical composition, productivity of microalgae and nutrient requirements as it determines the solubility and availability of CO_2 and other essential nutrients (Chen and Durbin, 1994). Due to uptake of inorganic carbon by microalgae, pH can rise significantly in microalgal cultures (Hansen, 2002). Thus, the effect of changing pH has been extensively used as a lipid induction technique to improve intracellular FA production in a range of microalgal strains that can be considered as a biofuel source (Sharma *et al.,* 2012; Taggar *et al.,* 2014).

Most species of microalgae grow maximally at neutral pH (around 7 - 7.6), although optimum pH is the initial culture pH at which a microalgae is adapted to grow (Goldman *et al.,* 1982). Fluctuations in the pH of the media were observed to limit the microalgal growth by inhibition of metabolism (Goldman *et al.,* 1982). Furthermore, changing pH in the culture media also alters the microalgal lipid composition (Guckert and Cooksey, 1990; Sharma *et al.,* 2012). The effect of changing pH has been carried out on *Chlamydomonas applanata* grownwithin a wide range of pH from 1.4 to 8.4 with one point increments. It is clear that no growth was observed from pH 1.4 to 3.4, above which tolerance of pH was observed in *C.applanata*. Exponential growth was reported between pH 5.4 to 8.4 with optimum growth at pH 7.4 for up to five days incubation (Visviki and Santikul, 2000).

Alkaline pH leads to increases in the cell wall flexibility of microalgal cells, which prevents rupture and inhibits autospore release, therefore increasing the time for cell cycle completion (Juneja *et al.,* 2013). Moreover, alkaline pH has been reported to inhibit the growth of microalgae, as a result the microalgae are diverting energy to form TAG (Sharma *et al.,* 2012). For example, alkaline pH stress in media resulted in an increase in intracellular TAG accumulation, but caused a decrease in membrane associated polar lipids (Taggar *et al.,* 2014). Under alkaline pH, the extracellular pH of microalgal cells is higher than intracellular pH and consequently the cell has to rely on active transport of HCO_3^- and not on passive flux of CO_2^- for inorganic carbon accumulation (Moazami-Goudarzi and Colman, 2012). Furthermore, the effects of changing pH on carbon uptake of *Chlamydomonas reinhardtii* were successfully demonstrated in a detailed study and have been reported as an efficient utilization of CO_2 for photosynthesis at pH values below 6.9, but at high external pH from 6.9 to 9.5, the microalgae cannot efficiently accumulate carbon and therefore

required a high supply of carbonates to maintain their photosynthetic activity (Moroney and Tolbert, 1985).

Similar to alkaline pH influence, external acidic pH leads to altered nutrient uptake by microalgae and consequently affects their growth (Gensemer *et al.,* 1993). However, under acidic pH, where the internal pH exceeds the external pH and $CO_2$is themain portion of the total external inorganic carbon, the carbon accumulation is thought to be accomplished via passive movement of CO_2 along a pH gradient into the chloroplasts or microalgal cells (Colman *et al.,* 2002; Ramanan *et al.,* 2012). However, acidic stress conditions have been found to limit the growth, motility and also have effects on morphology of some microalgal species grown in low external pH between 1.3 to 1.5 including *Euglena mutabilis* and *Chlamydomonas applanata* (Juneja *et al.,* 2013). The effects of external pH were also observed on photosynthesis in microalgal cells and found to induce a significant reduction in both carbon accumulation and oxygen evolution in some cyanobacterial species such as *Coccochloris peniocystis* when cultivated in acidic conditions in growth media at pH 5.0 and 6.0 (Coleman and Colman, 1981).

In general, maintenance of neutral intracellular pH of microalgae at acidic external pH should require spending of energy to pump protons out of the microalgal cell (Terry and Abadía, 1985). For example, the cellular pH of 7.3 was maintained for an external pH range from 5.0 to7.5 in *Chlorella saccharophila* cells and decrease in the internal pH to 6.4 was observed when the external pH was decreased to pH 3.0 (Gehl and Colman, 1985). Furthermore, *Euglena mutabilis* cells maintained their internal pH at 5.0 under highly acidic conditions (pH less than 3.0) and exhibited an internal pH of 8.0 at high external pH of more than pH 9.0 (Lane and Burris, 1981). In addition, some microalgae species are able to adapt to acidic conditions via glycerol

accumulation for preventing the osmotic imbalance caused by high H_2SO_4 concentrations in growth media, and such a mechanism was successfully demonstrated in *Dunaleilla acidophila* (Fuggi *et al.,* 1988). While acidophilic species such as *Chlamydomonas* sp. and *Pinnilaria braunii* var. *amplicephala*are capable of increasing storage lipid accumulation, mainly TAG when grown at pH 1 and therefore, the relative percentage of triacylglycerides to the total lipid content was higher than that in the cells grown at higher external pH (Tatsuzawa *et al.,* 1996). Another study observed different adaptation under acidic conditions by increasing the saturated fatty acid content, which decreases the membrane fluidity and also inhibits high proton concentrations (Tatsuzawa *et al.,* 1996). Such adaptation was successfully confirmed in *Chlamydomonas* sp., and by this mechanism, the total content of FAs increased from 2% to 2.4% with decrease in the external pH from 7 to 2.7, although a modest percentage was statistically significant increase (Poerschmann *et al.,* 2004). However, this increase in total content of fatty acids in membrane lipids was reported to represent a lowing of pH to reduce membrane lipid fluidity (Tatsuzawa *et al.,* 1996; Sharma *et al.,* 2012).

1.7.5. Lipid Induction in Microalgae Using Light Irradiation

Light is the most important element for the photosynthesis process (without which no photoautotrophic life can be sustained or exist) and the potential lipid productivity of a commercial algae cultivation facility will depend on both light and temperature in outdoor pond culture systems (Wagenen *et al.,* 2012). However, the light with photoperiod can be makeing important changes in chemical composition of the microalgal cells and the growth rate and then lipid production in many microalgae strains (Wahidin *et al.,* 2013). Therefore, light stress conditions have been widely

used as lipid induction techniques to enhance intracellular FA production by various microalgal strains that can be considered as oleaginous microalgae species (Taggar *et al.,* 2014).

Organisms are using light as energy to convert carbon dioxide to organic compounds, mainly sugars during photoautotrophic growth phase (Juneja *et al.,* 2013). Microalgae can grow at various light intensities exhibiting remarkable changes in their chemical composition, pigment content and photosynthetic activity (Taggar *et al.,*2014). Furthermore, different light intensities have been reported to change the lipid metabolism in microalgae, altering their lipid profile (Sharma *et al.,* 2012; Taggar *et al.,* 2014). Study on the effects of light intensity in some microalgae have reported that high light intensity leads to oxidative damage of PUFAs and also alters the level of these FAs in microalgae (Taggar *et al.,* 2014). High light intensity also leads to a decrease in the total content of polar lipid with a simultaneous increase in the amount of neutral storage lipids (mainly TAG) while low light intensity encourages polar lipid formation, mainly membrane polar lipids associated with chloroplast formation (Napolitano, 1994; Brown *et al.,* 1996). The ability of microalgae to increase their TAG production under high light conditions may serve as a protective mechanism for the cell (Taggar *et al.,* 2014). The electron acceptor pool which is required for photosynthetic activity may by reduced under high light intensity, thus increased FA synthesis in microalgal cells, which in turn is stored as TAG, potentially helps microalgal cells to regenerate their electron acceptor pool (Sharma *et al.,* 2012).

There are strong indications that growth phase plays a variety of important roles in the accumulation of certain FAs under various light intensities. Higher amount of TAG with both saturated and monounsaturated FAs have been produced by

microalgal cells grown to stationary growth phase in strong continuous light or in 12:12 h high light/dark conditions compared with microalgal cells grown under less light (Sharma *et al.,* 2012; Taggar *et al.,* 2014). On the other hand, the highest amount ofPUFAs was obtained when a microalgal culture was grown to exponential growth phase under strong light conditions (Taggar *et al.,* 2014; Brown *et al.,* 1996).

At the onset of stationary growth phase, microalgal cells typically demonstrate an increased amount of saturated and monounsaturated FAs and reduced amount of PUFAs (Brown *et al.,* 1996). However, different light intensity effects on the composition and metabolism of microalgal lipid were successfully demonstrated in several microalgae strains such as *Chlorella vulgaris* and *Nitzschiapalea* (Hassan *et al.,* 2013). Moreover, in the filamentous green microalga (*Cladophora sp*.), exposure to strong continuous light resulted in a decreased level of cellular phospholipid and increased the total amounts of neutral lipids, namely in the form of TAG (Napolitano, 1994). Low light intensity exposure led to an increase in the total content of various cell membrane lipids mainly sulfoquinovosyl diacylglycerols (SQDG), phosphatidylcholine (PC) and phosphatidylglycerol (PG) in the red microalga *Tichocarpus crinitus*, but higher light intensities resulted in an increased amount of TAG (Khotimchenko and Yakovleva, 2005). In a *Pavlova lutheri* strain (haptophyte), high light intensities acted as a catalyst to increase the level of cellular lipid associated with increasing cell population and weight per cell (Carvalho and Malcata, 2005).

When *Nannochloropsis* sp. were exposed to different light intensities, the total content of cellular unsaturated FAs was lower with increasing light irradiance, but there was a significant decrease in the level of omega-3 (ω-3) PUFAs from 29% to 8% of total fatty acids (Fabregas *et al.,* 2004). On the other hand, dark treatment resulted in a decrease in the total content of both TAG and galactosyl glycerides with

slightly changed levels of phospholipids in dinoflagellate (*Prorocentrum minimum*) cells (McLarnon-Riches *et al.,* 1998).

Besidesthe effect of light intensity on FA synthesis in microalgae, light intensities also have a significant effect on pigment composition (Solovchenko *et al.,* 2008b; Sharma *et al.,* 2012). Microalgal cells grown under high light intensity showed higher growth and increase in the total content of carotenoids, especially β-carotene and lutein (Solovchenko *et al.,* 2008b). Furthermore, the green microalga *Parietochloris incise* showed slow growth rate and a relatively low carotenoid to chlorophyll ratio when grown under low irradiances (Solovchenko *et al.,* 2008b; Sharma *et al.,* 2012).

In general, as shown by examples reviewed above, light irradiation stress as a lipid induction technique to increase TAG production can only be controlled in either closed photobioreactors systems or in laboratory-scale cultures (Sharma *et al.,* 2012). This means the operational expense of controlled light irradiation leads to increased production costs of biofuels from microalgae, although there are many commercial techniques that can be used such as LEDs or diverted sunlight techniques in closed photobioreactors system (Sharma *et al.,* 2012). In conclusion, higher light irradiation generally stimulates fatty acid synthesis, growth and formation of membranes (particularly chloroplast) and subsequently the overall total content of microalgal lipid will be reflected in such morphological changes (Sharma *et al.*, 2012).

1.7.6. Lipid Induction in Microalgae Using Ultraviolet Light Irradiance

Ultraviolet (UV) radiation is another important environmental stress factor that has been extensively studied and suggested for microalgal lipid induction in large-scale cultivation systems (Bougaran *et al.,* 2012). On the other hand, there is concern that use of UV radiation will have significant physiologically and genetically harmful

effects on many microalgal processes (carbon assimilation mechanism and damaging DNA) and consequently affecting the composition and production of microalgal lipid as a result of inhibiting nutrient uptake, and possibly related to the radiation intensity (Guiheneuf *et al.,* 2009; Guiheneuf *et al.*, 2010). However, UV light with wavelengths ranging from 215 nm to 400 nm has been found to have especially inhibitory effects on microalgae primarily due to damage to the photosynthetic machinery (Pessoa, 2012). The UV radiation with wavelength from 215 nm to 380 nm is typically called UV-B radiation and causes higher damage to the cells than UV-A radiation that have wavelength from 380 nm to 400 nm at similar intensities (Pessoa, 2012).The UV-B radiation directly effects microalgal DNA, while UV-A radiation has indirect and limited inhibitory effects through enhanced production of reactive oxygen and hydroxyl radicals. Therefore, UV-A light could stimulate photosynthesis, while UV-B light will have negative effects at moderate levels of intensity (Pessoa, 2012; Rastog and Incharoensadk, 2013).

Some microalgal mechanisms, including gmigration and development of protective cell walls, are responses to reduce the damage caused by UV radiation and could lead to an increase in production of carotenoids and other pigments (Xue *et al,* 2005; Rastogi and Incharoensadki, 2013). The effect of UV irradiance in microalgae has been extensively studied (He and Hader, 2002; Xue *et al*, 2005; Holzinger and Lutz, 2006; Bhandari and Sharma, 2011), especially on photosynthesis and pigments accumulation (Fouqueray *et al.,* 2007). The effect of UV-A irradiance at different levels of exposure on total lipid accumulation was successfully demonstrated using *Nannochloropsis oculata* cells. The results showed UV-A treatments significantly increased the polyunsaturated fatty acid (chlorophyll-specific lipid concentration) (Srinivas and Ochs, 2012). The study indicated that, the intracellular lipid content of

eicosapentaenoic acid (EPA) and PUFAs increased, while lipid content of monounsaturated FA decreased when *P. tricornutum* cells were exposed to UV-A radiation in comparison with no UV irradiance or under combined UV-A with UV-B treatments (Liang *et al.*, 2006).

1.7.7. Lipid Induction in Microalgae Using Heavy Metals

Heavy metals are perhaps one of the most important environmental factors that significantly influences microalgal metabolism and their lipid and FA composition (Sharma *et al.,* 2012). Heavy metals such as zinc, cadmium and copper have been observed to increase the total content of intercellular lipids in some microalgal species (Einicker-Lamas *et al.,* 2002). Thus, some heavy metals have been extensively studied to see their effects on lipid induction to improve intracellular FAs production in a range of microalgal strains that may be considered as biodiesel sources (Sharma *et al.,* 2012). The effect of high cadmium concentration was successfully studied in *Euglena gracilis* cells grown in three culture systems, i.e. heterotrophic (dark), mixotrophic (light with organic carbon source) and autotrophic systems.It was observed that cadmium resulted in an increase in the total content of cellular lipid per cell in all three cultures, but membrane lipids and sterol content were lower in cells cultivated under illumination (Einicker-Lamas *et al.,* 2002). On the other hand, there were no changes in the total content of phospholipid, while there was an increase in phosphatidylglycerol. *E. gracilis* has been observed to display different sensitivity to both zinc and copper (Einicker-Lamas *et al.,* 2002). Furthermore, the effect of iron on growth rate and accumulation of lipid was successfully studied in *Chlorella vulgaris* cells grown till late exponential growth phase with different concentrations of iron, the cells showing an increase in the total content of lipid up to 56.6% biomass by dry weight (Liu *et al.,* 2008).

1.8. Microalgae Mass Agriculture Systems

It is clear thatboth microalgae and cyanobacteria can be cultivated either via photoautotrophic or heterotrophic methods (Ferrell and Sarisky-Reed, 2010). Under photoautotrophic methods, the microalgae require light for growing and generation of new biomass with either open or closed ponds, whereas under heterotrophic methods, the microalgae are grown without light and are fed a carbon source such as sugars to create new biomass (Ferrell and Sarisky-Reed, 2010). Macroalgae have different cultivation requirements that usually need open, off-shore or coastal facilities(Ferrell and Sarisky-Reed, 2010). The microalgal culture systems typically require enough exposure of light to allow the facilitation of mass transfer of CO_2 and O_2 for photosynthesis or respiration to allow the microalgal crop to effectively grow (Grima, *et al.,* 1999). Furthermore, the microalgal culture systems require to becontinual mixing in order to enable enough light exposure for the microalgae and also gas exchange consequently avoiding the stratification case that led to formation of dead flow zones and thereafter stagnation and predation (Becker, 1994). Microalgal cultivation systems are dependent on cost, final product, source of nutrients and CO_2 control (Suali and Sarbatly, 2012). However, choices made for the cultivation system are key to the affordability and sustainability of microalgae to biofuel systems (Ferrell and Sarisky-Reed, 2010). However, research on the mass cultivation of microalgal biomass successfully was not initiated until the late 1940s and early 1950s in Germany, U.S. and Japan (McBride and Merrick, 2014; Yun *et al.,* 2015).

In general, microalgae are usuallycultured in two types of system; open systems, which are known as open ponds and closed systems which are known as photobioreactors (PBRs) as shown in Figure 1.5 and Figure 1.6, respectively (Mata *et al.*,2010; Bahadar and Khan, 2013; Yun *et al.,* 2015).

Figure 1.5: A pair of algae open ponds at Sapphire Energy's Las Cruces, New Mexico R and D Facility. *Photo courtesy Sapphire Energy.* Taken from, Algae Basics at:allaboutalgae.com/open-pond

Figure 1.6: A close look at a photobioreactor operated by BioProcess Algae. *Photo courtesy Bioprocess Algae.* Taken from, Algae Basics at: allaboutalgae.com/open-pond

However, open and closedsystems havedisadvantages and advantagesas shown in Table 1.3 (Carvalho *et al.*, 2006).

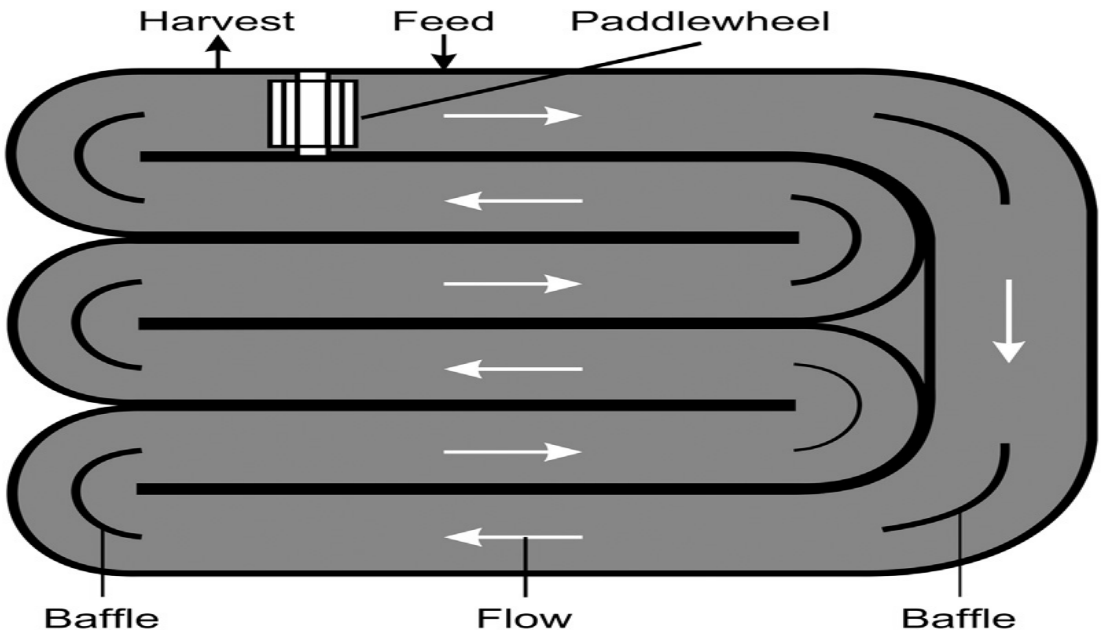
Feature of cultivation system	**Open cultivation system (Ponds)**	**Closed cultivation system (PBRs)**
Area-to-volume ratio	Large(4-10 times higherthanclosedcounterpart)	Small
Algal species	Restricted	Flexible
Main criteria for species selection	Growth competition	Shear-resistance
Population density	Low	High
Harvesting efficiency	Low	High
Cultivation period	Limited	Extended
Contamination	Possible	Unlikely
Water loss through evaporation	Possible	Prevented
Lightutilization efficiency	Poor/fair	Fair/excellent
Gas transfer	Poor	Fair/high
Temperature control	None	Excellent
Most costly parameters	Mixing	Oxygen and temperature control
Capital investment	Small	High

Table 1.3: Major design features of openand closed microalgal cultivation systems.Taken from, **Carvalho *et al.*, (2006).**

1.8.1. Microalgae open cultivation systems (pond systems)

The open pond culture system is the oldest system for mass cultivation of microalgae. It is the main type of culture system for the commercial-scale culture of microalgae cultivation since the 1970s (Terry and Raymond, 1985; Crowe *et al.,* 2012). Due to their relatively low cost they are the systems most likely to be used for the commercial production of microalgae for biofuels (Walker *et al.,* 2005; Borowitzka and Moheimani, 2012; Ugwa and Aoyagi, 2012; Yun *et al.,* 2015), and can be highly productive (up to 30 g DW/m^2/day) (Yun *et al.,* 2015). Open culture systems are usually located outdoors and rely on direct sunlight (Bahadar and Khan, 2013). The construction and operation of this system are simple, but it has poor light utilization and needs a large open land area and therefore there are evaporative losses (Meghnani, 2013). Moreover, microalgae cannot grow under controled conditions in open culture systems, because these systems are dependent on climatic conditions, and contamination by predators and heterotrophs not allow to use on a commercial scale (Meghnani, 2013). However, open systems can be broadly classified into: (1) shallow lagoons and ponds (2) inclined (cascade) systems (3) circular central-pivot ponds (4) simple mixed ponds (5) raceway ponds (Borowitzka and Moheimani, 2012). However, the raceway ponds are the culture system must used for microalgae production (Crowe *et al.,* 2012). The open cultivation systems typically consist of a series of closed loop flow channels that are directly open to the air. Moreover, in this system, the algal cultures are usually circulated around the pond or raceway circuit by a pump or paddlewheel to avoid algal flows forming on the liquid surface and provide enhanced aeration of the culture, as showin in Figure 1.7 (Bahadar and Khan, 2013). The open ponds are typically designed to be around 0.2 - 0.3 meters in depth to enable the light to penetrate to the bottom of the algal culture with areas covered ranging

from 0.5 to 200 hectares, depending on the microalgal crop, Figure 1.8 (Greenwell *et al.*, 2010).



Figuer 1.7: Schematics of a raceway pond (pond culture system). Taken from Bahadar and Khan, (2013).

Figure 1.8: Arial view of Cyanotech Algal Production Facility showing (raceway) growth ponds. Courtesy Cyanotech Corporation, Hawaii, USA. Taken from Milledge, (2011).

1.8.2. Microalgae closed cultivation systems (Photobioreactors, PBRs)

Unlike open culture system, closed culture systems can be either indoors or preferably outdoors to use free sunlight (Bahadar and Khan, 2013). They are designed to overcome the open culture systems limitations including contamination risks and also improveing regulation (Chisti, 2007; Wang *et al.*, 2008; Bahadar and Khan, 2013). Closed culture systems have shorter harvest times, higher biomass productivity, high surface-to-volume ratios and can cultivate a wider range of algal species than open systems (Chisti, 2007;Wang *et al.*, 2008). Additionally, wastewater or flue gases can be used from power plants providing additional environmental benefits to the use of closed culture systems (Bahadar and Khan, 2013; Zimmerman *et al.,* 2011). In comparison to construction and operation of open culture systems, the closed culture systems are much more expensive and there are concerns about the feasibility of scaling up photobioreactors (PBRs) (Kiran *et al*., 2014). However, closed culture systems comprise a thin panel of transparent tubes or plates supplied with CO_2 cylinders that are positioned either horizontally or vertically to maximize sun exposure (Bahadar and Khan, 2013). Closed systems come in a different range of designs for different microalgal species, including tubular, flat plate (flat panel), bubble-column and biofilm photobioreactors (Ugwa and Aoyagi 2012; Bahadar and Khan, 2013). Table 1.4 makes a comparison between advantages and limitations of closed culture systems. However,the most prominent photobioreactor designs are those with a tubular configuration that are the most widely used and considered the most promising (Chisti, 2007; Tababa *et al.,* 2012). Their design encompasses key operational conditions such as light supply and temperature control as well as gas supply and removal in order to achieve the most efficient and cost effective cultivation. More specifically, tubular photobioreactor consist of many straight tubes

with transparent walls that allow sunlight to penetrate, thus enabling algal biomass production as shown in Figures1.9 and 1.10.

Closed Culture systems	Advantages of systems	Limitations of systems
Tubular Photobioreactor	• Relatively cheap • Large illumination surface area • Suitable for outdoor cultures • Good biomass productivities	• Gradients of pH, dissolved oxygen and CO_2 along the tubes • Fouling • Some degree of wall growth • Requires large land space • Photo-inhibition
Flat Photobioreactor	• Relatively cheap • Easy to clean up • Large illumination surface area • Suitable for outdoor cultures • Low power consumption • Good biomass productivities • Good light path • Readily tempered • Low oxygen build-up • Shortest oxygen path	• Difficult scale-up • Difficult temperature control • Some degree of wall growth • Hydrodynamic stress to some algal strains •Low photosynthetic efficiency
Column Photobioreactor	• Low energy consumption • Readily tempered • High mass transfer • Good mixing • Best exposure to light-dark cycles • Low shear stress • Easy to sterilize • Reduced photo-inhibition • Reduced photo-oxidation • High photosynthetic efficiency	• Small illumination surface area • Sophisticated construction materials • Shear stress to algal cultures • Decrease of illumination surface area • Expensive compared to open ponds • Support costs • Modest scalability

Table 1.4: Advantages and disadvantages of various microalgae culture systems. Adapted from Dragone *et al*., (2010).

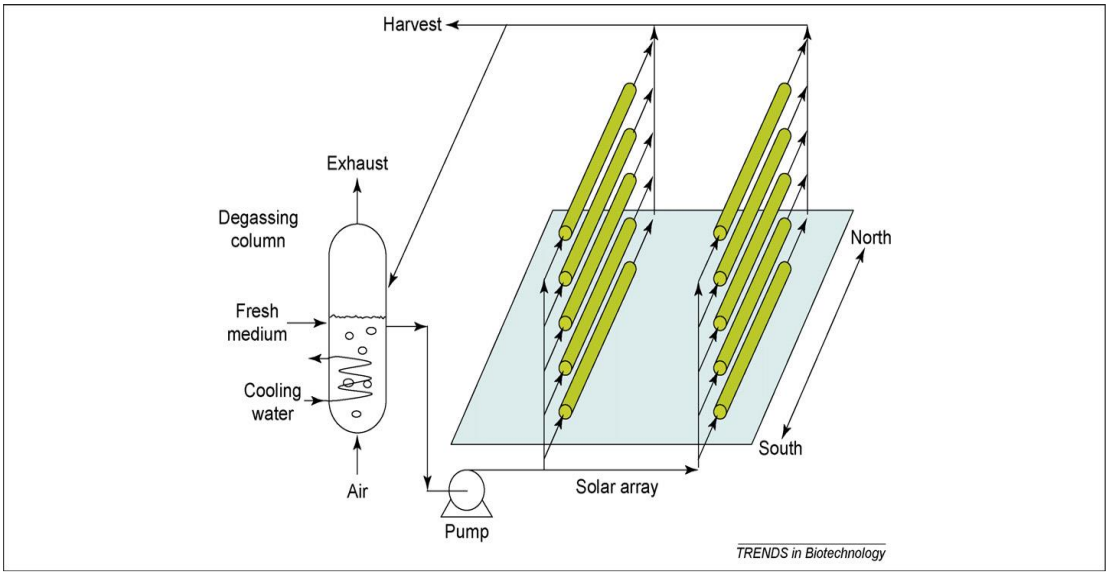


Figure 1.9: A tubular photobioreactor with fence-like solar collectors. Algal broth from the degassing column is continuously pumped through the solar array, where sunlight is absorbed, and back to the degassing column. Fresh culture medium is fed continuously to the degassing column during daylight and an equal quantity of the broth is harvested from the stream that returns to the degassing column. Cooling water pumped through a heat exchanger coil in the degassing column is used for temperature control. The degassing column is continuously aerated to remove the oxygen accumulated during photosynthesis and oxygen-rich exhaust gas is expelled from the degassing column. Taken from Chisti*et al*., (2008).

Figure 1.10: Pilot scale tubular photobioreactor. Taken fromKyndt (2010).

1.9. Industrial Applications of Microalgae

The idea of using microalgae by humans is not new. The first use of microalgae dates back 2000 years to the Chinese, who used *Nostoc* to survive during famine (Spolaore *et al.,* 2006). As a result, microalgae have received much attention for their commercial and industrial potential since the early 1950s when productivity and yield were first studied in mass culture (Burlew, 1953; Metting, 1969; Knothe *et al.,* 1997; Fukuda *et al.,* 2001; Barnwal and Sharma, 2005; Demirbas, 2005; Gerpen, 2005; Felizardo *et al.,* 2006; Meher *et al.,* 2006; Chisti, 2007; Wijffels and Barbosa, 2010). Due to the ability of microalgae to produce substantial large amounts of lipids such as triacylglycerides (TAG), their high growth rate and no compete for land with crops, the oleaginous microalgae species have been targeted as a source of bioactive compounds (such as pigments and vitamins) pharmaceuticals, speciality chemicals, health foods (food additives, feed and aquaculture feeds), waste treatment, agriculture and biodiesel production (Radmer, 1996; Metting, 1996; Olmos *et al.,* 2000; Razon and Tan, 2011; Abreu *et al.,* 2012; Gilmour and Zimmerman, 2012; Shields and Lupatsch, 2012; Lananan *et al.,* 2013; Simionato *et al.,* 2013; Yaakob *et al.,* 2014; Abinandan and Shanthakumar, 2015; Cavonius *et al.,* 2015).

However, most of the microalgae grown on a commercial scale are not consumed by humans or terrestrial animals directly (Cavonius *et al.,* 2015). Thus, microalgae have great potential to synthesize marine drugs such as antibiotics, vitamin and antioxidants for cosmetics, pharmacological and food industry (food and feed ingredient) (Hu *et al.,* 2008; Khatoon *et al.,* 2014). In aquaculture, microalgae species with valuable properties have high potential to produce lipid, carbohydrate and protein that determines its overall economic potential (Williams and Laurens, 2010; Camacho-Rodriguez *et al.,* 2015).

1.10. Potential of Microalgal Biofuel

Biofuel (biodiesel) refers to any fuel made from renewable biological material which usually needs a special process to transform it into a fuel. Biodiesel (fatty acid methyl ester) is a non-toxic renewable energy source (Kaisan *et al.*,2005; Kulkarni and Dalai, 2006; Saifullah *et al.,* 2014). Biodiesel is more specifically defined as the monoalkyl esters of long chain fatty acids derived from the chemical reaction (transesterification) of renewable feedstocks, such as vegetable oil or animal fats, and alcohol with or without a catalyst (Barnwal and Sharma, 2005; Knothe, 2010; Ahmad *et al.,* 2011; Saifullah *et al.,* 2014). The potential use of microalgae as feedstock for biofuels production is not a new idea (Khan *et al.,* 2009; Muthukumar *et al.,* 2012). The use of biofuels began first in prehistoric times when humans learnt how to ignite biological material to cook food (Gressel, 2008). Biofuel is produced mainly from vegetables and plants oils, such as the oils extracted from soybeans, sunflower, corn, rapeseed and palms (Jeong *et al.*,2004; Sanchez and Vasudevan 2006; Chisti, 2007; Hankamer *et al.,* 2007; Huang *et al.,* 2010; Amaro *et al.,* 2011; Stansell *et al.,* 2012; Ma *et al.,* 2016). However, in the United States, biofuel is typically produced from the oils extracted from soybeans (Chisti, 2007). Additionally, numerous studies confirmed that the production of biofuel from animal fats is available (Felizardo *et al.*,2006; Kulkarni and Dalai, 2006; Chisti, 2007; Bondioli *et al.,* 2012). The technology to produce biofuel from microalgae as a main alternative biofuel feedstock has recently received a great deal of attention worldwide and was one of the first alternative fuels to become known to the public (Chisti, 2007; Huang *et al.,* 2010; Priyadarshani and Rath, 2012; Bartley *et al.,* 2014; Ahmad *et al.,* 2011; Tang *et al.,* 2016). Useing fossil fuels as an energy source for a long time is unsustainable and the release of the CO_2 in the air, which contributes to global climate change, is related to global warming and

environmental problems (Friedlingstein and Solomon, 2005; Bartley *et al.,* 2014). On the other hand, biofuel appears to be an attractive energy resource for several reasons: (1) The biofuel is a renewable fuel that could be sustainably supplied. (2) The biofuel is highly biodegradable and has minimal toxicity (Mata *et al.,* 2010; Ahmad *et al.,* 2011; Rattanapoltee and Kaewkannetra, 2013). As a result, the use of biofuel can decrease 90% of air toxicity and 95% of cancers compared to common diesel sources (Huang *et al.,* 2010). (3) The biofuel appears to cause essential improvement of rural economic potential. (4) The biofuel is environmentally friendly, resulting in no net increased release of carbon dioxide and aromatic compounds or other chemical substances which are harmful to the environment. (5) The biofuel is better than diesel fuel due to its lower combustion emission profile and does not contribute to global warming because of its closed carbon cycle. (6) The biofuel decreases dependence on foreign crude oil. (7) The biofuel can be used in existing diesel engines with little or no modification and with good engine performance. (8) The biofuel can be blended in any ratio with first generation biodiesel feedstocks because they were the first crops to be used to produce biofuel (Huang *et al.,* 2010; Ahmad *et al.,* 2011). Furthermore, numerous research reports have described many advantages whenusing microalgae for biofuel production in comparison with other available feedstocks such as plants, since it will not compromise production of food and other feedstocks obtained from those plants (Tsukahara and Sawayama, 2005; Chisti, 2007; Hossain *et al.,* 2008; Rosenberg *et al.,* 2008; Mata *et al.,* 2010; Chu, 2012).

Algae offer many potential advantages such that algae can potentially produce biofuel at significantly higher levels than other oil crops or soybeans. Algae do not compete with traditional agriculture, because they are not traditional foods and feeds and they can be grown or cultivated in large open ponds or in closed photobioreactors located

on non arable land (Priyadarshani and Rath, 2012). However, in this case, extremophile microalgal species can grow under high salinity or high pH such as halotolerant microalgae *Dunaliella sp.* are used to avoid the contamination by other microalgae or bacteria (Li and Qi, 1997). Algae can be processed into a variety of products including biofuel by transesterification conversion or green diesel (gasoline replacements) by direct catalytic hydrothermal conversion or methane by anaerobic digestion or bioethanol by fermentation or biochar by thermochemical conversion or high protein animal feed (Priyadarshani and Rath, 2012). Algae can grow and survive under a wide range of climate and water conditions, so they can utilize and release CO_2 from different sources (Priyadarshani and Rath, 2012). Algae are very efficient to utilize the CO_2 directly from the atmosphere and sunlight as the sole carbon source and converting it into organic compounds through the process of photosynthesis, due to the ability of many algal species to grow like plants as photoautotrophs (Brennan and Owende, 2010; Zimmerman *et al.*, 2011; Priyadarshani and Rath, 2012). In many cases, microalgae have relatively simple nutrient requirements and they can be grown in saline water or waste water and do not compete for arable land and precious freshwater (Gilmour and Zimmerman, 2012). Furthermore, microalgae synthesize and accumulate large quantities of neutral lipids (20 - 50% dry cell weight) and grow at high rates (e.g. 1-3 doublings/day) (Khan *et al.*, 2009). It is estimated that the annual oil production from algae is in the range of 40,700 - 53,200 L ha^{-1} $year^{-1}$ (Weyer *et al.*,2010; Iwai *et al.*, 2015).

1.11. Microalgae Harvest and Lipid Extraction Technique

Figure 1.11 shows a diagram of biofuel production from microalgae in stages, starting with the selection of suitable microalgal species with the relevant properties for the

specific culture conditions and products (Pulz and Gross, 2004). The culture conditions that must be considered include light, temperature, pH, carbon dioxide and nutrient concentration (Ahmad *et al.*, 2011).

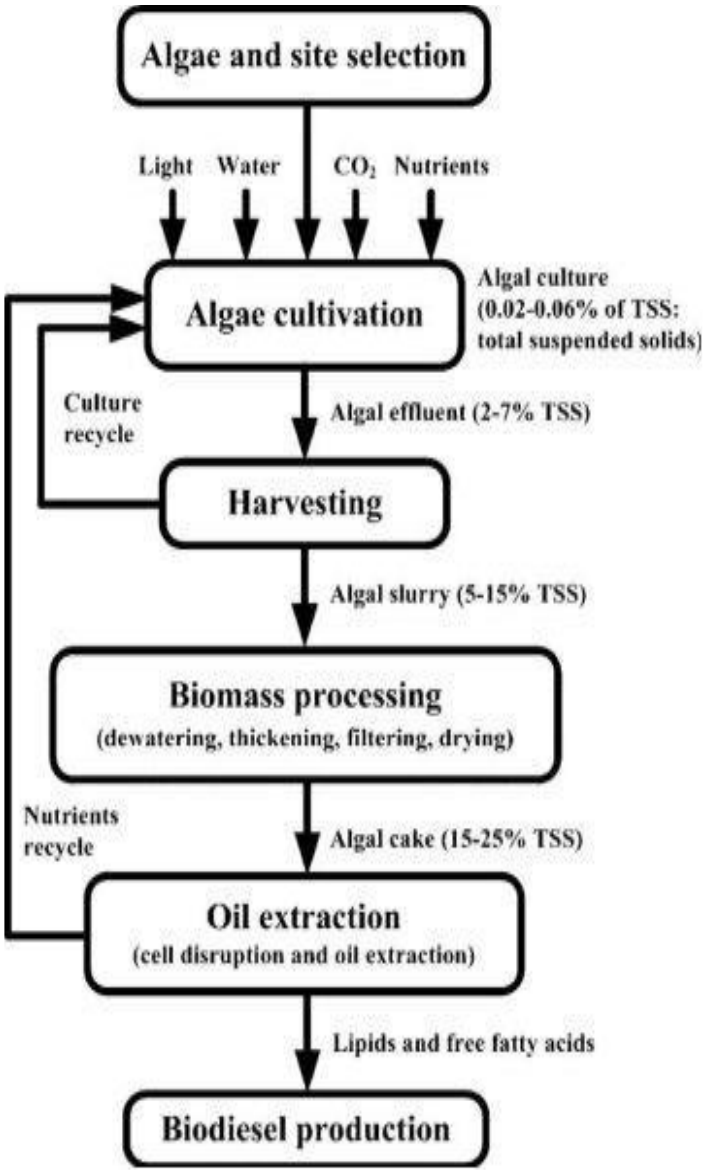


Figure 1.11: Microalgae biofuel value chain stages. Taken from, Priyadarshani and Rath (2012).

However, once the selected microalgal species has been grown to a sufficient biomass yield and has the required lipid levels, it needs to be harvested and the lipids extracted (Chisti, 2013). The biomass of microalgae can be harvested using different methods. The most widely adopted forms of harvest are traditionally methods based on technologies that were developed for water purification and treatment (Kim *et al.,* 2013).

In general, these techniques included sedimentation/flotation, centrifugation, flocculation or membrane filtration and microscreens. It has been reported that, the harvested biomass should be dried under vacuum to release water until it reaches a constant weight. The dried biomass is pulverised with a mortar and pestle before the lipid is extracted to supply the biofuel production unit (Mata *et al.,* 2010; Ahmad *et al.,* 2011). For extraction of microalgal lipid, there are three traditional methods that are well known to extract the lipid from microalgae; expeller/press, solvent extraction using chemicals and supercritical fluid extraction (Mata *et al.,* 2010; Ahmad *et al.,* 2011; Demirbas, 2009).

These traditional extraction methods that are used for lipid extraction usually involve solvent extraction through diffusion. These methods, however, are not ideal as they either involve the use of dangerous and environmentally harmful solvents or are not capable of extracting substantial quantities of lipids from the biomass. Lipids (TAG) are non-polar so therefore non polar or low polar solvents such as chloroform or hexane are traditionally used in extraction (Aranda-Burgos *et al.,* 2014). However, a simple process is to use a press to extract a large percentage (70% -75%) of the lipids out of algae (Demirbas, 2009). Nevertheless, the most popular extraction method is Soxhlet extraction (Soxhlet, 1879), which involves bringing an appropriate solvent such as hexane (which is relatively inexpensive) to boiling point and percolating

through the microalgal biomass. This then falls back into the evaporating flask and this is repeated until the solvent evaporates (Ranjan *et al.,* 2010). Other solvents such as petroleum ether and ethanol can be used (Ahmad *et al.,* 2011). Supercritical fluid extraction methods can be used as an alternative extraction by replacing traditional solvents with supercritical liquids. This method has proved to be more effective than traditional extraction methods, because it providing high purity and product concentrations, it can extract almost 100% of lipid. In the supercritical fluid carbon dioxide (CO_2) extraction, CO_2 is liquified under pressure and heated to the point that it has the properties of both a liquid and gas. This liquified fluid then acts as the solvent in extracting the lipid (Demirbas, 2009). After extraction, the lipids are converted to biodiesel using one of the four primary methods; direct use and blending of raw oils, microemulsions, thermal cracking (pyrolysis) and transesterification (Mata *et al.,* 2010; Ahmad *et al.,* 2011; Demirbas, 2009). Complete descriptions and comparisons of these methods have been provided by Ma and Hanna (1999), Leung *et al.* (2010) and Ahmad *et al.* (2011).

1.12.Transesterification Technologies in The Production of Biodiesel

Biofuel production from microalgal cellsis expected to use similar techniques for commercial biodiesel production from plant oils and animal fats (Chisti, 2007). In general, there are several ways to convert microalgal biomass to energy sources that can be classified into biochemical conversion, chemical reaction, direct combustion and thermochemical conversion (Priyadarshani and Rath, 2012). However, following the process of lipid extraction, the total lipids (normally containing triglycerides, polar lipids, free fatty acids and pigments) are usually separated using another method, as only triglycerides with three fatty acid molecules are suitable for production of biodiesel (Medina *et al.,* 1998). Once the microalgal lipids are purified they can be

converted into biofuelby thetransesterification process. For making biofuel, lipids (triglycerides) are reacted with a short chain alcohol as reagents (such as methanol) in a reaction known as transesterification (also called alcoholysisor ester exchange reaction) in the presence of a catalyst.However, the overall transesterification reaction is described in Figure1.12.

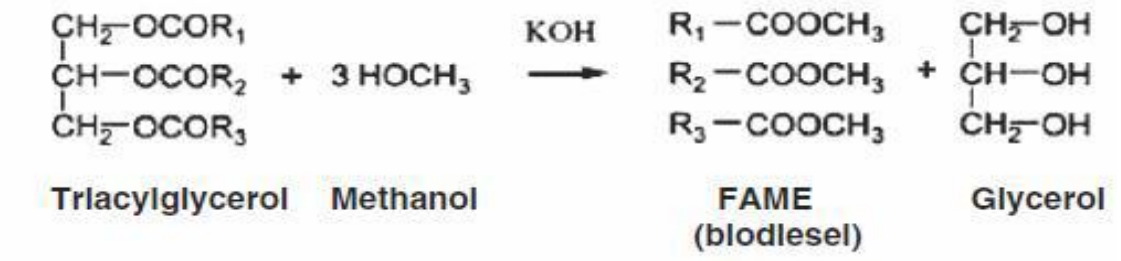


Figure 1.12: Transesterification reaction to convert triglycerides (TAG) to biofuel (FAMEs) and glycerol (overall reaction), using methanol alcohol as reagent and an alkali KOH catalyst. R_1, R_2 and R_3 are hydrocarbon groups. Taken from Halim *et al.*, (2012)

This reaction (transesterification) is a multiple step reaction, including three reversible steps in series where triglycerides are first converted to diglycerides then diglycerides are converted to monoglycerides, and monoglycerides are then converted to fatty acid methyl esters (FAME) or esters that are biofuel and glycerol as by-product (Chisti, 2007; Mata *et al.*, 2010). However, the transesterification reaction requires 3 mol of alcohol for each mole of triglyceride to produce 1 mol of glycerol and 3 mol of methyl esters, the reaction to be in equilibrium. Industrial processes use 6 mol of methanol for each mole of triglyceride (Fukuda *et al.*, 2001). The relationship between the feedstock mass input and biodiesel mass output is about 1:1, which means that theoretically, 1 kg of lipid results in about 1 kg of biodiesel (Mata *et al.*, 2010). This large excess of methanol ensures that the reaction is driven in the

direction of methyl esters (i.e. towards biofuel). Yield of methyl esters exceeds 98% on a weight basis (Fukuda *et al.*, 2001).

Furthermore, transesterification reaction can be catalysed by many different acids, alkalis (Fukuda *et al.*, 2001; Meher *et al.*, 2006), and lipase enzymes (Sharma *et al.*, 2001; Kim *et al.*, 2013). Alkali catalysed transesterification is about 4000 times faster than the acid catalysed reaction (Fukuda *et al.*, 2001). Consequently, alkaline catalysts such as sodium and potassium hydroxide are commonly used as commercial catalysts at a concentration of about 1% by weight of lipid. Alkaline catalysts are typically used in the chemical industry for the conversion of plant and animal lipids due to their superior reaction rates and conversion efficiencies compared to the acid catalysts (Huang *et al.*, 2010). Alkoxides such as sodium methoxide are better catalysts than sodium hydroxide and are being increasingly used. However, lipase enzymes offer important advantages but currently are not feasible due to the relatively high cost (Fukuda *et al.*, 2001). Alkali catalysed transesterification is carried out at approximately 60°C at atmospheric pressure, as methanol boils off at 65°C at air pressure. However, under these conditions, the transesterification reaction takes about 90 min to complete. However, a higher temperature can be used in combination with higher pressure, but this is expensive. Lipid and methanol do not mix and hence the reaction mixture contains two liquid phases. Furthermore, other alcohols can be used, but methanol is the least expensive. To prevent yield loss due to saponification reactions (i.e. soap formation), both lipid and alcohol must be dry and the lipid should have a minimum of free fatty acids. Once the reaction is completed, the products undergo purification to remove the glycerol, catalyst and excess solvents, which typically involves some form of bi-phasic fractionation separation (Griffiths *et al.*,

2010). Therefore, the biofuel is recovered by repeated washing with water to remove glycerol and methanol (Chisti, 2007).

1.13. Aims of Project

The aims of this study were as follows:

1. To compare the growth of *Nannochloropsis salina* strain in both the modified f/2 Guillard's culture medium and artificial AMCONA growth medium.

2. To examine the ability of *Nannochloropsis salina* strain to grow and adapt at different salinities.

3. To examinethe ability of *Nannochloropsis salina* strain for lipid production using Nile Red staining technique.

4. To improve photosynthetic efficiency of *Nannochloropsis salina* and biomass production to optimize overall lipid production.

5. To investigate the influence of stress growth conditions on the growth, neutral lipids (TAG) accumulation, lipid profile and lipid metabolism.

6. To attempt to enhance neutral lipid content of *Nannochloropsis salina* via flow cytometric cell sorting.

Materials and Methods

Chapter Two

2.1. Provenance of Microorganisms

Unicellular *Nannochloropsis salina* strain (CCAP 849/2, a marine microalga, Phylum: *Heterokontophyta* / *Ochrophyta*, Order: *Eustigmatales* and Class: *Eustigmatophyceae*) was obtained from the Culture Centre of Algae and Protozoa, Oban, Scotland, UK. This strain was isolated near the Isle of Cumbrae in the Firth of Clyde by Hibberd in 1981.

2.2. Media Used for Microalgae Growth

All culture media were prepared using distilled water and unless stated otherwise sterility was achieved by autoclaving for 20 min at 121°C (15 lbs in^{-1}).

2.2.1. F/2 Growth Medium (Ultramarine Synthetica Sea Salt)

This medium was prepared using a slightly modified method as described by Guillard and Ryther (1962) and Guillard, (1975), using a series of stock solution combinations as shown in Table 2.1 below:

Component	Stock solution g /100 ml dH_2O	Volume of stock solution added to final f/2medium (Quantity used in 1000 ml of dH_2O)
$NaNO_3$	7.5 g	1 ml
$NaH_2PO_4.H_2O$	0.5 g	1 ml
f/2 Trace metal solution	see table 2.2	1 ml
f/2 Vitamin solution	see table 2.3	0.5 ml

Table 2.1: Composition of f/2 normal growth medium for *Nannochloropsis salina* cells.

Originally, f Medium was a common and widely used general enriched seawater medium designed for growing coastal marine algae, especially diatoms. The final concentration

of the original formulation was termed "f Medium" (Guillard and Ryther, 1962; Guillard, 1975). The major nutrients of this medium were reduced by half and therefore termed f/2 medium.

Normally, f/2 growth medium is prepared using filtered natural seawater, but in Sheffield this is not really an option. Instead, a commercial product called Ultramarine Synthetica Sea Saltwas used to prepare f/2 growth medium. Furthermore, *N. salina* used in this study does not require the silicate component ($Na_2SiO_3 \cdot 9H_2O$) and therefore the modified f/2 growth medium was prepared without silica to reduce precipitation, according to Guillard and Ryther, (1962) and Borowitzka and Siva (2007).

In this study, the modified f/2 Guillard's culture medium was prepared by dissolving 33.6 g of Ultramarine Synthetica Sea Salt in 950 ml of distilled water in a 1 litre Duran bottle by stirring and then the volume was made up to 1000 ml with distilled water.

Two separate stock solutions were prepared by adding 7.5 g of Sodium nitrate ($NaNO_3$) and 0.5g of Sodium dihydrogen phosphate ($NaH_2PO_4. H_2O$) to 100 ml of distilled water using two Duran bottles.

Both trace metals and vitamins solutions are usually prepared as primary stock solutions of high concentrations to permit weighing of reasonable amounts. These stock solutions are used to make working solutions from which the final medium is made.

To produce 100 ml of f/2 final trace metal stock solution (trace elements), all the ingredients shown in Table 2.2 were separately added into 90 ml of distilled water using a 100 ml Duran bottle and gently inverted to mix. The volume was made up to

100 ml using distilled water to give 100 ml of f/2 final trace metal stock solution and then stored in the lab at room temperature until required.

Component	Sub-stock solution g /100 ml dH_2O	Quantity of component or substock added to final trace metals stock solution (made up to 100 ml with dH_2O)
$FeCl_3 \cdot 6H_2O$	-	0.315 g
$Na_2EDTA. 2H_2O$	-	0.436 g
$MnCl_2 \cdot 4H_2O$	1.8	0.1 ml
$ZnSO_4 \cdot 7H_2O$	2.2	0.1 ml
$CoCl_2 \cdot 6H_2O$	1	0.1 ml
$CuSO_4 \cdot 5H_2O$	0.98	0.1 ml
$Na_2MoO_4 \cdot 2H_2O$	0.63	0.1 ml

Table 2.2: Composition of the f/2 Trace Metal stock solution for *Nannochloropsiss alina* cells.

To produce 100 ml of f/2 final vitamin stock solution, all the components shown in Table 2.3 were separately added into 90 ml of distilled water using a 100 ml Duran bottle and gently inverted to mix. The volume was made up to 100 ml with distilled water to give 100 ml of f/2 final vitamin stock solution. To sterilise, the f/2 final vitamin stock solution was filtered and covered in foil and then stored in the fridge until required.

Component	Sub-stock solution g /100 ml dH_2O	Quantity of component or substock added to final vitamin stock solution (made up to 100 ml with dH_2O)
Cyanocobalamin (vit B_{12})	0.1	0.1 ml
Biotin (vit H)	0.1	0.1 ml
Thiamine $\cdot HCl$ (vit B_1)	-	20 mg

Table 2.3: Composition of the f/2 Vitamin stock solution for *Nannochloropsis salina* cells.

Finally, to produce 1 litre of the final f/2 growth medium using Ultramarine Synthetica Sea Salt, 950 ml of Ultramarine synthetica sea salt stock solution, that already had been prepared as mentioned above, was transferred to a fresh 1 litre Duran bottle and then 1ml from each Sodium nitrate stock solution ($NaNO_3$), Sodium dihydrogen phosphate stock solution (NaH_2PO_4. H_2O) and trace metal stock solution were added and gently inverted to mix. The pH was adjusted to between 7.6 and 7.8 as necessary using either 1 M H_2SO_4 or 1 M NaOH. The volume was made up to 1000 ml with ultramarine synthetic sea salt stock solution and then autoclaved. After autoclaving, the final f/2 medium was allowed to cool at room temperature and 0.5 ml of filtered vitamin stock solution was added and gently inverted to mix. The pH of final f/2 medium was checked after autoclaving and shown not to alter by more than 0.05 pH units. The f/2 medium (Ultramarine synthetica sea salt) was stored in the lab at room temperature until required.

The same procedure mentioned above was employed when 1 litre of modified f/2 Guillard's culture solid medium was required, but in this case 10g of bacteriological agar No.1 was added to the main solution before making it up to 1000 ml with distilled water as described by Simionato *et al.* (2011). After autoclaving, the medium was allowed to cool in a water bath to approximately 50ºC. Then plates were poured and allowed to solidify at room temperature. For slopes, the medium was poured into sterile test tubes with cotton bungs and then the test tubes were rested at an angle so that the setting agar formed a slope. Both plates and test tubes were stored in the fridge until required. All chemicals were "Analar" grade and obtained from different companies mainly Sigma and Fisher.

2.2.2. The Artificial AMCONA Growth Medium

The artificial AMCONA growth medium (Artificial Multipurpose Complement for the Nutrition of Algae) was selected as the most promising marine culture medium for growth and biomass production, according to Fanesi *et al.* (2014) and Giordano *et al.* (2014).

To produce 1 litre of AMCONA medium, all the ingredients shown in Table 2.4 were added and dissolved in 900 ml of distilled water using a 1 litre Duran bottle. Two Metal stock solutions I, II and Vitamin stock solution were separately prepared using 100 ml of distilled water as shown in Tables 2.5, 2.6 and 2.7, respectively. To sterilise, the vitamin stock solution was filtered using 0.22 μm pore size and covered in foil and stored in the fridge until required.

1 ml from both Metal stock solutions I and II were added to the main medium and gently mixed and the pH was adjusted to between 7.6 and 7.8 as necessary using either 1 M H_2SO_4 or 1 M NaOH before the total volume was made up to 1000 ml with distilled water and autoclaved. After autoclaving and cooling to room temperature, 1 ml of vitamin stock solution was added aseptically to the medium. The pH value of liquid medium was checked after autoclaving and shown not to alter by more than 0.05 pH units. The medium was stored in the lab at room temperature until required. All chemicals were "Analar" grade and obtained from different companies mainly, Sigma and Fisher.

When 1 litre of solid artificial growth AMCONA medium was required, 10g of bacteriological agar No.1 was added to the main solution before making it up to 1000 ml with distilled water. After autoclaving, the medium was allowed to cool in a water bath to approximately 50ºC. Then plates were poured and allowed to solidify at room temperature. For slopes, the medium was poured into sterile test tubes with cotton

bungs and then the test tubes were rested at an angle so that the setting agar formed a slope. Both plates and test tubes were stored in the fridge until required.

Medium component	Stock concentration	Final concentration	Volume of stock solution for 1 L growth medium
NaCl	-	363 mM	21.2 g
Na_2SO_4	1 M	25 mM	25 ml
KCl	2 M	8.04 mM	4.02 ml
$NaHCO_3$	1 M	2.07 mM	2.07 ml
KBr	1 M	725 µM	725 µl
H_3BO_3	0.5 M	372 µM	744 µl
NaF	0.5 M	65.7 µM	131.4 µl
$MgCl_2$	2 M	41.2 mM	20.6 ml
$CaCl_2$	1 M	9.14 mM	9.14 ml
$SrCl_2$	0.5 M	82 µM	164 µl
$NaNO_3$	1 M	549 µM	549 µl
NaH_2PO_4	100 mM	21 µM	210 µl
Na_2SiO_3	105 mM	205 µM	2 ml
$CuSO_4$	40 µM	40 nM	1 ml
TRIS-HCl. pH 8.1	1 M	10 mM	10 ml
Metal Stock I	-	-	1 ml
Metal Stock II	-	-	1 ml
Vitamin Stock	-	-	1 ml

Table 2.4: Composition of the growth AMCONA medium for *Nannochloropsis salina* cells.

Component	Final concentration of metals stock solution I (made up to 100 ml with dH_2O)
$FeCl_3$	6.56 µM
Na_2EDTA	6.56 µM

Table 2.5: Recipe of the Metal stock I for the AMCONA medium for *Nannochloropsis salina* cells.

Component	Final concentration of metals stock solution II (made up to 100 ml with dH_2O)
$ZnSO_4$	254 nM
$CoSO_4$	5.69 nM
$MnSO_4$	2.42 μM
Na_2MoO_4	6.1 nM
Na_2SeO_3	1 nM
$NiCl_2$	6.3 nM
Na_2EDTA	8.29 μM

Table 2.6: Recipe of the Metal stock II for the AMCONA medium for *Nannochloropsis salina* cells.

Component	Final concentration of vitamin stock solution (made up to 100 ml with dH_2O)
Tiamine-HCL	297 nM
Biotine	4.09 nM
Vitamine B12	1.47 nM

Table 2.7: Recipe of the Vitamin Stock for the AMCONA medium for*Nannochloropsis salina* cells.

2.2.3. Defined f/2 Growth Medium (Seawater)

f/2 defined medium was prepared using a series of stock solution combinations as shown below:

•Stock Solution of Artificial seawater hydrated salts (1)

1. Magnesium sulphate	($MgSO_4$)	2.92g	$MgSO_4\,7H_2O$	5.98g
2. Magnesium Chloride	($MgCl_2$)	4.66g	$MgCl_2\,6H_2O$	9.95g
3. Calcium Chloride	($CaCl_2$)	1.17g	$CaCl_2\,2H_20$	1.55g
4. Strontium Chloride	($SrCl_2$)	0.02g	$SrCl_2\,6H_20$	0.034g

All the ingredients shown above were separately added and dissolved into 400 ml of distilled water using a 500 ml Duran bottle before adding the next salt and gently inverted to mix. The volume was made up to 500 ml using distilled water to give 500 ml of Artificial seawater (hydrated salts) stock solution (1) and then autoclaved and stored in the lab at room temperature until required.

•Stock Solution of Artificial seawater anhydrated salts (2)

1. Sodium Chloride	(NaCl)	23.9g
2. Potassium Chloride	(KCl)	0.62g
3. Sodium Bicarbonate	($NaHCO_3$)	0.18g
4. Potassium Bromide	(KBr)	0.024g
5. Boric Acid	(H_3BO_3)	0.020g
6. Sodium Thiosulphate	$Na_2S_2SO_3$)	0.004g

All the anhydrous salts shown above were separately added and dissolved into 400 ml of distilled water using a 500 ml Duran bottle before adding the next salt and gently inverted to mix. The volume was made up to 470 ml using distilled water to give 470 ml of Artificial seawater (hydrated salts) stock solution (2) and then autoclaved and stored in the lab at room temperature until required.

•Stock Solution of Nitrate (3)

Nitrate stock solution was prepared by adding and dissolving 0.75g of Sodium Nitrate ($NaNO_3$) into 100 ml of distilled water.

•Stock Solution of Phosphate (4)

Phosphate stock solution was prepared by adding and dissolved 0.057g of Sodium Dihydrogen Phosphate ($Na_2H_2PO_42H_2O$) into 100 ml of distilled water.

•Stock Solution of Trace Elments (5)

Trace metal (Elments) stock solution was prepared as shown in Table 2.2 in section 2.2.1.

•Stock Solution of Vitamin Mix(6)

Vitamin Mix stock solution was prepared as shown in Table 2.3 in section 2.2.1.

•Stock Solution of Tris Buffer (7)

Tris Buffer stock solution was prepared as 1M Tris Buffer pH 7.8.

To prepare 1 litre of f/2 defined growth medium, both stock solution (1) and stock solution (2) were aseptically transferred into a fresh autoclaved 1 litre Duran bottle and then 10 ml of Nitrate stock and 10 ml of Phosphate stock were added using filter sterilisation. 1 ml of Trace element stock and 1 ml of Vitamin mix stock and 10 ml of 1M Tris Buffer pH 7.8 were added using filter sterilisation and then gently inverted to mix. The pH of the final f/2 defined growth medium was checked and shown not to alter by more than 0.05 pH units. The f/2 defined growth medium was stored in the lab at room temperature until required.

2.3. Growth Conditions of Microalgal Strain

Unicellular *Nannochloropsis salina* (strain CCAP 849/2) was cultured in three different media including modified f/2 growth medium without silica (Guillard and Ryther 1962; Borowitzka and Siva, 2007), artificial AMCONA medium and f/2 defined medium to observe which medium was the most appropriate based on the highest growth potential. Growth was measured using a Unicam Helisα spectrophotometer at 595 nm. OD_{595} readings (using the medium as the blank) were taken every day to measure the growth rate of *N. salina.* All experiments were conducted in an incubation room (25°C) located in H floor at Department of Molecular Biology and Biotechnology, Sheffield University, UK.

Nannochloropsis salina was cultivated by adding 5 ml of active stationary phase cultures grown in the f/2 medium at 25°C for 14 - 21 days aseptically into 4 x 250 ml

conical flasks plugged with foam bungs containing 100 ml of each modified f/2 growth medium and artificial AMCONA medium and f/2 defined medium. Cultures were illuminated with constant light of 50 - 70 µmol photons m^{-2} s^{-1} supplied by daylight fluorescent lamps. Cultivation was carried out for 14 days at 25°C as described by Converti *et al.* (2009) and Bondioli *et al.* (2012) and Muthukumar *et al.* (2012). All flasks were shaken manually, usually every two days.

2.4. Cleaning and Sterile Techniques

To ensure sterile conditions and avoid any contamination, all culturing equipment used in experiments was autoclaved at 121°C. Also, inoculation and sub-culturing were carried out with a flame after cleaning the bench with 70% ethanol. Moreover, the glassware was soaked for 2 hours in concentrated sulphuric acid to ensure that all residual material from previous cultures was removed.

2.5. Maintenance of the Microalgae Strains

Sub-culturing of the *Nannochloropsissalina*was performed routinely as mentioned above once every two weeks to minimize contamination over time by adding 5 ml of active stationary phase culture of *N.salina* cells into 250 ml conical flasks plugged with foam bungs containing 100 ml of f/2 growth medium and incubated as mentioned above and then stored at room temperature until required. In addition, microalgal cultures of strain CCAP 849/2 were maintained by streaking on three plates of f/2 growth medium and incubated once every three months and when pure colonies had developed on the plates, a piece of parafilm was stretched around each plate's edge and then one set of plates was stored at room temperature and the other set in the fridge until required. When required, a single colony was used as an

inoculum for 100 ml of f/2 growth medium and incubated under the same conditions as mentioned above.

2.6. Purity of the Microalgae Strains

The purity of *Nannochloropsissalina* strain (CCAP 849/2) was monitored by streaking a loopful of the *N.salina* culture on agar plates of f/2 growth medium. After incubation, the plates were first examined for colony morphology and then single colonies were picked off and observed as a wet mount using a phase contrast microscope.

2.7. Comparison of F/2 Medium and AMCONA Medium

N. salina cells were cultured in two different media (f/2 growth medium and artificial growth AMCONA medium) to observe which medium is the most appropriate based on the highest growth potential. To produce a growth curve of *N. salina* cells in different media, 5 ml of active stationary phase cultures grown in the f/2 growth medium were inoculated into 4 x 250 ml conical flasks containing 100 ml of each f/2 growth medium and artificial growth AMCONA medium. The optical density (OD) at 595 nm was measured using the Unicam Helisα spectrophotometer against a distilled water blank in 1 ml plastic cuvettes immediately after inoculation and then every day over an incubation period at 25ºC and all flasks were shaken manually. The growth rate was plotted against time of incubation.

2.8. Effect of Defined F/2 Medium on The Growth of *N. salina* Cells

The effect of defined f/2 medium on the growth of *N. salina* cells in comparison with the effect of normal f/2 growth medium was determined by inoculating 5 ml of active stationary phase cultures of *N. salina* cells aseptically into 4 x 250 ml conical flasks

containing 100 ml of each f/2 growth medium and f/2 defined growth medium. To produce a growth curve, the defined f/2 medium was prepared in the laboratory as described in section 2.2.3 and then the optical density (OD) at 595 nm was measured using the Unicam Helisα spectrophotometer against a distilled water blank in 1 ml plastic cuvettes immediately after inoculation and then every day over an incubation period at 25ºC and all flasks were shaken manually. The growth rate was plotted against time of incubation.

2.9. Effect of Vitamin B12 Concentration on The Growth of *N. salina* Cells

In the first set of experiments, vitamin free f/2 growth medium was prepared as described in section 2.2.1, but in this case the vitamin B12 was omitted from the f/2 growth medium.

To produce a growth curve of *N. salina* cells in different media, 5 ml of active stationary phase cells were inoculated into 4 x 250 ml conical flasks containing 100 ml of each f/2 growth medium and vitamin free growth f/2 medium. The optical density (OD) at 595 nm was measured using the Unicam Helisα spectrophotometer against a distilled water blank in 1 ml plastic cuvettes immediately after inoculation and then every day over an incubation period at 25ºC for 25 days and all flasks were shaken manually. The growth rate was plotted against time of incubation.

2.10. Effect of Adaptation to Different Salinity Concentrations on *N. salina* Growth

The salinity tolerance of *Nannochloropsis* species is variable with an optimum around seawater salinity (0.5 to 0.6 M NaCl) and a salinity growth range of 0.1 to 1 M NaCl (Pal *at al.*, 2011). Resistance to salinity stress is important, because salinity level may

vary in outdoor pond cultures due to evaporation or rain (Pal *at al.*, 2011). However, this experiment was carried out to find the upper salinity limit for *N. salina* growth.

To produce a growth curve of *Nannochloropsis salina* cells adapted to grow at different salinities, modified f/2 growth medium was prepared as described in section 2.2.1 and enriched with different concentrations of NaCI (0.2 M, 0.4 M, 0.6 M, 0.8 M and 1M NaCl). Application of a sub-culture technique was used to inoculate salinity medium by aseptically adding 5 ml of active inocula from previous salinity concentrations to the next highest salinity concentration when the growth reached at least 0.5 at OD_{595}.

Three 250 ml conical flasks containing 100 ml of f/2 growth medium with salinity concentration of 0.2 M NaCl were inoculated with 5 ml of appropriate active stationary phase cultures grown in normal f/2 growth medium at 25°C for 14-21 days. The OD at 595nm was measured using a spectrophotometer against a distilled water blank in 1 ml plastic cuvettes immediately after inoculation and then every day over an incubation period at 25ºC using un-inoculated medium as a blank. Once an OD at 595 nm reached 0.5, the same procedure mentioned above was employed for the next highest salinity concentration using 5 ml of active inoculums adapted cells from the previous salinity concentration of NaCl to inoculate the next highest salinity concentration until the growth of *N. salina* cells was no longer possible. The growth curves of adapted cells grown at different salinity values were plotted against time of incubation.

2.11. Effect of pH on Growth of *N. salina* and Growth Curve Determination

The effect of pH on the growth of *N. salina* cells was determined every day using the optical density (OD) at 595 nm measurements of microalgal cultures in f/2 growth

medium with spectrophotometer against a distilled water blank in 1 ml plastic cuvettes.

To produce a growth curve of *N. salina* cells, f/2 growth medium was prepared as described in section 2.2.1 with six different pH levels of pH 5, 6, 7, 8, 9 and 10 in three replicates, based on the work described by Bartley *et al.* (2014).

Three 250 ml conical flasks containing 100 ml of each pH value f/2 growth medium were each inoculated with 5 ml of appropriate active stationary phase culturesgrown in normal f/2 medium at 25°C for 14-21 days. The OD_{595} was measured using a spectrophotometer against a distilled water blank in 1 ml plastic cuvettes immediately after inoculation and then every day over an incubation period at 25ºC using un-inoculated medium as a blank. The growth curves of cells grown at different pH values were plotted against time of incubation.

2.12. Effect of Nitrogen Concentration on Cell Growth of *N. salina*

The effect of nitrogen concentration on cell growth was examined in *N. salina* cells grown in f/2 growth medium using the optical density (OD) at 595 nm measurements. To run this experiment, the same procedure described in section 2.11 was employed, but in this case, the f/2 growth medium was prepared with vastly different nitrogen concentrations (0.0%, 25%, 50% and 75% of $NaNO_3$) in three replicates.

Three 250 ml conical flasks containing 100 ml of each nitrogen concentration f/2 growth medium were each inoculated with 5 ml of appropriate active stationary phase cultures grown in normal f/2 medium at 25°C for 14-21 days. The OD_{595} was measured using a spectrophotometer against a distilled water blank in 1 ml plastic cuvettes immediately after inoculation and then every day over an incubation period

at 25ºC using un-inoculated medium as a blank. The growth curves of cells grown at different nitrogen concentration were plotted against time of incubation.

2.13. Relationship Between OD_{595} and Cell Number of *N. salina* Cells

To investigate the relationship between optical density measurement and cell count of algal cells, an optical density (OD) against cell count standard curve was prepared according to Madigan *et al*. (2003) and Skoog *et al*. (2007).

To produce the standard curve, the OD595 of a well grown algal culture was adjusted to 1 using fresh f/2 growth medium using the Unicam Helisα spectrophotometer against a distilled water blank in 1 ml plastic cuvettes. A serial dilution of the cell culture from 0% to 100% was prepared in 15 ml Falcon tubes using fresh f/2 growth medium as shown in Table 2.8.

Tube (Number)	Concentration (%)	Culture (ml)	Media (ml)
12	0	0.00	5.50
11	5	0.50	5.00
10	10	1.00	4.50
9	20	1.50	4.00
8	30	2.00	3.50
7	40	2.50	3.00
6	50	3.00	2.50
5	60	3.50	2.00
4	70	4.00	1.50
3	80	4.50	1.00
2	90	5.00	0.50
1	100	5.50	0.00

Table 2.8: Dilution scheme used to produce a range of cell concentrations (12 culture dilutions) for cell count determination.

Then 1 ml from each dilution was transferred to 1 ml plastic cuvettes and the OD at 595nm measurements were taken using the Unicam Helisα spectrophotometer with fresh medium as the blank. At the same time, 900 µl from each dilution was transferred to 1.5 ml Eppendorf tube and 100 µl of Gram's Iodine was added and mixed well (to stop cell motility). After that, 20 µl of the stained algal culture was placed into the counting chamber of a Neubauer improved haemocytometer and viewed using a Nikon microscope with the x40 objective (x400 magnification). Five replicates were carried out for each dilution and the nnumber of microralgal cells were counted in four large squares – one hundred small squares in total. The cell count was repeated ten times for each sample. Using the mean number of cells per small square, the number of cells in each dilution was calculated using the following equation:

Depth of counting chamber = 0.02 mm

Area of small square= $1/400\text{mm}^2$ = 2.5×10^{-3} mm^2

∴ Volume of small square = $2.5 \times 10^{-3} \times 0.02 = 5.0 \times 10^{-5}$ mm^3

∴ Volume of culture small square= 2.5×10^{-8} $\text{mm}^3 (\equiv \text{mL})$

Cell no. per small square $\times \frac{1}{5 \times 10^{-8}}$ = cell mL^{-1} sample

$\frac{\text{Cells ml sample}}{9} \times 10$ = cells mL^{-1} original culture

2.14. Microalgal Dry Weight (DW) Determination

The relationship between optical density (OD) measurement and the microalgal cell dry weight (DW) was measured based on the work described by Storms *et al.* (2014). To produce the standard curve of dry weight in biomass of *N. salina* cells, a well grown algal culture (14 day old algal culture) was taken aseptically and adjusted with fresh medium to an OD of 1 at 595 nm using the Unicam Helisα spectrophotometer

against a distilled water blank in 1 ml plastic cuvettes. A serial dilution of the cell culture was then prepared by combining the adjusted culture with fresh medium in 12×50 ml Falcon tubes to give 30 ml of final volume for each dilution as shown in Table 2.9. For every culture dilution tube, 1 ml was transferred to 1 ml plastic cuvettes and then OD at 595 nm was measured using the spectrophotometer against a distilled water blank. To keep the amount of dilution, the sample was returned to the original dilution tube after measurement.

Tube (Number)	Concentration (%)	Culture (ml)	Medium (ml)
12	0.0	0	30
11	8.3	2.5	27.5
10	16.6	5	25
9	33.3	10	20
8	41.6	12.5	17.5
7	50	15	15
6	58.3	17.5	12.5
5	66.6	20	10
4	75	22.5	7.5
3	83.3	25	5
2	91.6	27.5	2.5
1	100	30	0.0

Table 2.9: Microalgal culture concentrations (12 culture dilutions)for preparation of dry weight (DW) versus optical density (OD).

All 12×50 ml Falcon tubes were harvested by centrifugation at 3000 *g* for 10 minutes. The supernatant was poured off immediately and then each pellet was resuspended in 5 ml of distilled water and then transferred to 12×15 ml Falcon tubes. The 12×15 ml Falcon tubes were centrifuged at 3000 *g* for 10 minutes and then the supernatant was removed. Each pellet was again resuspended in 1 ml of distilled water and then transferred to pre-weighed Eppendorf tubes (on a fine balance). To seal the Eppendorf tubes containing the samples, tops from 12 other Eppendorf tubes were removed and a hole was put in each top. The tubes with pellets were frozen overnight at -80°C and

then freeze-dried for 24 to 48 hours until samples were completely dried. After freeze drying, the tops with holes were discarded and the tubes were reweighed using an analytical balance that was sensitive to 0.0001 g to determine the amount of dry biomass present in each tube. The microalgal dry weight present in each tube was calculated by subtracting the tubes weight after drying from the original weight of tubes to give g dry weight per 1ml of cells (g ml^{-1} dry weight). The concentration curve was plotted on Excel using the optical density (OD) readings against dry weight of each sample.

2.15. Quantification of Neutral Lipid Content Using Nile Red Method

To measure natural lipid concentration in *N. salina* cells, a number of experiments were done. Firstly, droplets of neutral lipids were visualised using a fluorescence microscope. Secondly, optimisation of Nile Red Fluorescence emission using the 96 well microplate method involved optimisation of cell concentration, time of staining and Nile Red concentration. Finally, the Nile Red fluorescence method was quantified using Triolein as the standard.

2.15.1. Nile Red Fluorescence Microscopy for Lipid Comparison

2.15.1.1. Microscope and Settings Used

The sample imaging was performed using a Nikon Eclipse E400 Microscope equipped with a Nikon DXM1200 Digital Camera for visualisation of lipid bodies.

Sample excitation light was provided by a Nikon Super High Pressure Mercury Lamp via 450 - 490 nm monochromatic optical filter. General illumination was achieved using the microscopes built-in lighting system. All images were taken using a Nikon 100x oil immersion lens (Phase 3). Image acquisition was performed using LUCIA G

Software with the following capture settings; Red: 48, Green: 31, Blue: 68, Gain: 25, Gamma: 0.40, Offset: 23, Fine Preview: on, Exposure: 72ms.

2.15.1.2. Sample Preparation and Method Used

The visualising of microalgal neutral lipid in lipid bodies which appear as oil droplets was examined by staining the *N. salina* cells grown under different stress conditions with the Nile Red method based on the work described by Cooksey *et al.* (1987).

5 ml of *N. salina* cultures grown until in stationary phase (14-21 days old) were taken aseptically from each flask and added to a 1.5 ml Eppendorf tube. The sample was then adjusted to an optical density of 0.2 at 595 nm using fresh media to avoid self shading. 1 ml was then taken and added to a 1.5 ml Eppendorf tube along with 5 µl of Grams Iodine and shaken to kill the cells and stop motility. 10 µl of 100 µg ml^{-1} Nile red fluorescent dye dissolved in 100% dimethyl sulfoxide (DMSO) were then added to the sample. A timer was then started to check the progress of the dye penetration. 10 µl of the sample was then added to a clean microscope slide and a cover slip was added gently. The edges of the cover slip were then sealed with nail varnish to stop evaporation. Once the varnish had dried (and after 4-5 minutes of dye penetration), the slide was then placed in the microscope. To view the cells, a single drop of mineral oil was added onto the cover slip and the image was brought in to focus using excitation light only. Two sets of photos were taken for each cell of interest, one using white light, one using excitation light only. White light intensity was controlled using the microscope iris and the fluorescence using the inbuilt shutter. To avoid light bleaching and quenching effects, samples were only illuminated when the photos were being captured.

2.15.2. Determination of Optimum Cell Concentration

This experiment was performed according to Chen *et al.* (2009) and Simionato *et al.* (2011). To determine the optimum cell concentration to use, 5 ml of well grown algal culture of *N. salina* (active stationary phase culture) were taken aseptically and adjusted with fresh medium to an OD of 1 at 595 nm against a distilled water blank using a 1 ml plastic cuvette and spectrophotometer. The following series of 8 dilutions of algal culture were then made in 1 ml from this adjusted culture using fresh medium at 12.5, 25, 37.5, 50, 62.5, 75, 87.5 and 100% in 8 x autoclaved 1.5 ml Eppendorf tubes as shown in Table 2.10.

Percentage (%)	100	87.5	75	62.5	50	37.5	25	12.5	Total (ml)
Culture (µl)	1000	875	750	625	500	375	250	125	4.5
Medium (µl)	0	125	250	375	500	625	750	875	3.5

Table 2.10: Dilutions scheme to produce a range of cell concentration for Nile Red peak fluorescence test.

The ODs were then taken at 595nm for each of these dilutions, in order to provide ODs at a given culture concentration. These tubes were then centrifuged at 3000 *g* for 10 minutes in a bench top centrifuge. The supernatant was discarded immediately and then each pellet was resuspended in 20 µl of distilled water. Two 2 ml screw top microfuge tubes were then prepared for each of the 8 culture concentrations, one for stained and one for unstained cells. Then 10 µl of the each resuspended algal pellet was transferred to each of the labelled stained and unstained screw top microfuge tubes. Then 50 µl of dimethyl sulfoxide (DMSO) was transferred to each screw capped microfuge tube. The caps of the tubes were then tightened loosely and all tubes were heated for

50 seconds at full power in a Matsui microwave. The tubes were then removed from the microwave and then 930 µl of distilled water was added to tubes labelled stained and 940 µl of distilled water to all unstained labelled tubes. 10 µl of 100 µg ml^{-1} Nile-red fluorescent dye dissolved in 100% dimethyl sulfoxide (DMSO) were then added to each tube labeled stained. All tubes were microwaved again at full power for 60 seconds and a timer was started as soon as heating was completed. The entire 1 ml content of each stained tube was transferred to a multi-pipette reservoir. Then 200 µl aliquots of each cellular concentration were transferred to rows A - D in a 96 well plate, providing 4 technical replicates at each concentration. The same was employed for the unstained tubes in rows E - H. The plate was then put in the plate reader after removing the lid of the plate as shown in Figure 2.1. The details of the plate reader settings for peak fluorescence test were achieved as shown in Figure 2.2. To track the fluorescence, Gen5 2.05 software was used for 30 minutes at 5 minute intervals.

The results were exported to Excel and the unstained fluorescence values subtracted from stained values in order to remove any cellular background fluorescence. Average fluorescence values for the 4 technical replicates at each cellular concentration were obtained, allowing for optimum cell concentration and thus optical density and peak time to be established.

Dilution (%)		100	87.5	75	62.5	50	37.5	25	12.5	Empty Wells			
		1	2	3	4	5	6	7	8	9	10	11	12
A	R1	Stain1	Stain5	Stain9	Stain13	Stain17	Stain21	Stain25	Stain29	BLK	BLK	BLK	BLK
B	R2	Stain2	Stain6	Stain10	Stain14	Stain18	Stain22	Stain26	Stain30	BLK	BLK	BLK	BLK
C	R3	Stain3	Stain7	Stain11	Stain15	Stain19	Stain23	Stain27	Stain31	BLK	BLK	BLK	BLK
D	R4	Stain4	Stain8	Stain12	Stain16	Stain20	Stain24	Stain28	Stain23	BLK	BLK	BLK	BLK
E	R1	unsta1	Unsta5	Unsta9	unsta13	unsta17	Unsta21	Unsta25	Unsta29	BLK	BLK	BLK	BLK
F	R2	Unsta2	Unsta6	unsta10	unsta14	unsta18	Unsta22	Unsta26	Unsta30	BLK	BLK	BLK	BLK
G	R3	Unsta3	Unsta7	unsta11	unsta15	unsta19	Unsta23	Unsta27	Unsta31	BLK	BLK	BLK	BLK
H	R4	Unsta4	Unsta8	unsta12	unsta16	Unsta20	Unsta42	Unsta28	Unsta32	BLK	BLK	BLK	BLK

Figure 2.1: 96 Well plate layout for peak fluorescence test (cell concentration experiment). Note: Rows R1 to R4 are technical replicates from the same concentration.

Procedure details			
Plate Type	96 Well Plate		
Read	Fluorescence end point		
	Full plate		
	Filter Set 1		
	Excitation 485/20, Emission 580/50		
	Optics Top, Gain:60		
	Read Speed: Normal		

Figure 2.2: Plate reader settings for peak fluorescence test (optimum concentration of microalgal cell experiments).

2.15.3. Determination of Optimum Nile Red Concentration Method

This experiment was performed according to Chen *et al.* (2009). This protocol will give the optimum stain concentration needed for *N. salina* strain to produce the clearest fluorescence signal achievable. In this procedure, the Nile Red stock was diluted to give concentrations of 0.05, 0.1, 0.2, 0.3, 0.4 and 0.6 μmol ml^{-1} by

dissolving in DMSO from a 1 mg ml^{-1} stock of Nile Red as shown in Table 2.11 below. NB 0.3 μmol ml^{-1} = 100 μg ml^{-1} (used in section 2.15.2).

Concentration of Nile Red (μmol ml^{-1})	Primary Stock 1mg ml^{-1} (μl)	DMSO (μl)
0.05	16	984
0.1	32	968
0.2	64	936
0.3	100	900
0.4	128	872
0.6	192	808

Table 2.11: Series of 6 dilutions of Nile Red stock concentration were prepared by dissolving in 100% dimethyl sulfoxide (DMSO).

The same procedure described in section 2.15.2 was established to prepare algal suspensions at optimum OD at 595 nm and stained and unstained 2 ml screw top microfuge tubes. The samples were also prepared as mentioned above for each Nile Red concentration using each of the 6 Nile Red concentrations separately in place of the 100 μg ml^{-1} dilution used in section 2.15.2. The 96 well plate was then prepared and run as described in section 2.15.2, ensuring that fluorescence values are read at the optimum time after microwaving established in section 2.15.2, as shown in Figure 2.3.

The results were exported to Excel and the unstained fluorescence values subtracted from stained values and average of the 4 technical replicates at each concentration were obtained in order to ascertain which concentration of Nile Red was optimum.

Nile Red Concentration (µmol ml^{-1})			0.05	0.1	0.2	0.3	0.4	0.6	Empty Wells					
			1	2	3	4	5	6	7	8	9	10	11	12
Stained Cells	R1	A	200	200	200	200	200	200	0	0	0	0	0	0
	R2	B	200	200	200	200	200	200	0	0	0	0	0	0
	R3	C	200	200	200	200	200	200	0	0	0	0	0	0
	R4	D	200	200	200	200	200	200	0	0	0	0	0	0
Unstained Cells	R1	E	200	200	200	200	200	200	0	0	0	0	0	0
	R2	F	200	200	200	200	200	200	0	0	0	0	0	0
	R3	G	200	200	200	200	200	200	0	0	0	0	0	0
	R4	H	200	200	200	200	200	200	0	0	0	0	0	0

Figure 2.3: 96 Well plate layout for optimum Nile Red concentration experiment. Note: Rows R1 to R4 are technical replicates from the same concentration.

2.15.4. Triolein Calibration Curve Method

This experiment was performed based on the work described by Chen *et al.* (2009) and Bertozzini *et al.* (2011). This protocol will allow the Nile red fluorescence signal to be converted to Triolein equivalents.

To allow quantification of neutral lipid within algal cells, a concentration curve of Nile Red fluorescence versus concentration of Triolein (a model neutral lipid) was set up. The Triolein was added to algal samples so that the Nile Red fluorescence due to Triolein was measured in the presence of algal cells. 10 mg ml^{-1} Triolein lipid standard stock was made by adding 50 mg of Triolein (Sigma T7140) to 5 ml isopropanol and dissolved. Then 1 ml aliquots were transferred to five 1.5 ml Eppendorf tubes labeled 1 to 5. Eight algal samples were prepared under the conditions optimised in section 2.15.2 and taken through the procedure up to the addition of 50 µl DMSOand microwaving the samples. At this point, 910 µl of distilled water was added to tubes labelled stained and 920 µl of distilled water to all unstained labelled tubes. Then 20 µl of triolein and isopropanol was added to each stained and

unstained tubes as shown in Table 2.12 below to give concentrations of 0.2, 0.16, 0.12, 0.08, 0.06, 0.04, 0.02 and 0.0 mg ml^{-1}.

Conc. Triolein (mg ml^{-1})	0.2	0.16	0.12	0.08	0.06	0.04	0.02	0.0
Triolein (µl)	20	16	12	8	6	4	2	0
Isopropanol (µl)	0	4	8	12	14	16	18	20

Table 2.12: Dilutions of Triolein mixture for Nile Red fluorescence calibration curve.

10 µl of 100 µg ml^{-1} Nile Redfluorescence dye dissolved in DMSO was then added to each tube labelled stained. All tubes were microwaved again at full power for 60 seconds and a timer was started as soon as heating was completed. The method was then continued as described in section 2.15.2 as shown in Figure 2.4.

Triolein Concentration (mg/ml)			**0.02**	**0.16**	**0.12**	**0.08**	**0.06**	**0.04**	**0.02**	**0.0**	**Empty Wells**			
			1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Stained Cells	R1	A	200	200	200	200	200	200	200	200	0	0	0	0
	R2	B	200	200	200	200	200	200	200	200	0	0	0	0
	R3	C	200	200	200	200	200	200	200	200	0	0	0	0
	R4	D	200	200	200	200	200	200	200	200	0	0	0	0
Unstained Cells	R1	E	200	200	200	200	200	200	200	200	0	0	0	0
	R2	F	200	200	200	200	200	200	200	200	0	0	0	0
	R3	G	200	200	200	200	200	200	200	200	0	0	0	0
	R4	H	200	200	200	200	200	200	200	200	0	0	0	0

Figure 2.4. 96 Well plate layout of different concentration of triolein mixture for the calibration curve. Rows R1 to R4 are technical replicates from the same concentration

2.15.5. Lipid Quantification of *N. salina* Cells (Nile Red sample measurement test)

Neutral lipids were quantified for *N. salina* cells grown until stationary phase (14-21 days old) under normal growth conditions. The protocol was followed using the optimum conditions defined including optimum cell concentration, peak time and optimum Nile Red concentration as described in section 2.15.2 and 2.15.3, respectively. As three flasks of culture were set up under normal growth conditions, results from one plate from each flask were averaged. Results of the experiments were then converted to quantify lipid using the calibration curves to give the concentration as well as the dry weight percentage according to Bertozzini *et al.* (2011)

2.16. Determination of Neutral Lipid of *N. salina* Cells Grown at Different Salinities

A common observation is that an increase in salinity can increase the lipid content in microalgal cells while lowering the growth rate of cells. Therefore, the effect of increasing external salinity on the growth and lipid content of microalgal species was investigated in *N. salina* cells grown in f/2 cultre medium by Nile Red method over a significant time period with a range of different salinity levels (0.2, 0.4, 0.6, 0.8 and 1 M NaCl) at 25ºC for 4 weeks incubation in comparison with normal growth conditions.

To produce a growth rate, 4x250 ml conical flasks containing 50 ml of each salinity concentration in f/2 growth medium were inoculated with 5 ml of active stationary and all flasks were incubated at 25ºC for 4 weeks incubation and then optical density (OD) at 595 nm was measured using the Unicam Helisα spectrophotometer against a distilled water blank immediately after inoculation, then every day over an incubation period at 25ºC and all flasks were shaken manually and then the growth rate was

plotted against time of incubation. The lipid accumulation in *N. salina* cells was determined every week using Nile Red dye staining with optimum conditions of Nile Red method including 75% cell concentration of *N. salina* which is equivalent to an OD_{595} = 0.818 and Nile Red concentration of 0.2 μmol ml^{-1} with 15 minutes staining. The lipid content in *N. salina* cells was plotted against concentration of salinity used over incubation time for 4 weeks.

2.17. Determination of Neutral Lipid Accumulation by *N. salina* Cells Grown at Different pH Values

This experiment was achieved based on the work described by Bartley *et al.* (2014) to examine the effect of inducing stress with varying levels of pH change (pH 5 - 10) on the growth and lipid content of microalgae cells using *N. salina* grown in f/2 growth medium by Nile Red method over a significant time period for 4 weeks incubationin comparison with normal growth conditions. To generate this experiment, *N. salina* cultures were grown at six different pH levels (pH 5, 6, 7, 8, 9, and 10) in three replicates using 250 ml conical flasks containing 100 ml of each f/2 growth medium. All cultures were inoculated by 5 ml of active stationary phase cultures grown in the f/2 growth medium at 25°C for 14-21 days. Then optical density (OD) at 595 nm was measured using the Unicam Helisα spectrophotometer against a distilled water blank immediately after inoculation, then every day over an incubation period at 25ºC and all flasks were shaken manually and then the growth rate was plotted against time of incubation.The lipid accumulation in *N. salina* cells was determined every week using Nile Red dye staining with optimum conditions of Nile Red method including 75% cell concentration of *N. salina* which is equivalent to an OD_{595} = 0.818 and Nile Red concentration of 0.2 μmol ml^{-1} with 15 minutes staining. The lipid content in *N. salina* cells was plotted against different pH levels over incubation time for 4 weeks.

2.18. Determination of Neutral Lipid of *N. salina* Cells Grown Under Nitrogen Starvation

The effect of nitrogen limitation on the grow th and lipid content of microalgal species was examined in *N. salina* cells grown in f/2 growth medium by the Nile Red method. To procedure this experiment, the same procedure described in section 2.17 was employed, but in this case the culture f/2 medium was prepared with vastly different nitrogen concentration (Nitrogen free, 25%, 50% and 75% of $NaNO_3$) and then growth experiments were carried out for four weeks incubation period at 25°C in comparison with normal growth conditions.The lipid content in *N. salina* cells was plotted against nitrogen concentrations used over incubation time.

2.19. Quantification of Total Lipid Content Using Gravimetric Method

This experiment was undertaken to investigate the effect of different treatments including 0.6 M NaCl, nitrogen starvation and high pH on the total lipid production by *N. salina* cells compared to normal growth conditions using the Gravimetric technique. For gravimetric measurements, algal lipid extraction was performed based on the methods first described by Bligh and Dyer (1959) and adapted from Chiu *et al.* (2009). 4 x 30 ml of well grown cultures of algae (approximately OD_{595} = 1) were aseptically transferred to 4 x 50 ml Falcon tubes and harvested by centrifugation in a bench top centrifuge at 3000 *g* for 10 minutes. The supernatant fluid was discarded and each pellet was re-suspended in 5 ml of distilled water and then transferred to 15 ml Falcon tubes. The samples were then centrifuged again for 5 minutes at 3000 *g* and the supernatant fluid discarded. Each pellet was re-suspended in 1ml of distilled water and the samples transferred to labelled pre-weighed 1.5 ml Eppendorf tube (fitted with

a separate, pierced lid). Samples were then frozen at -80°C overnight and then lyophilized (freeze dried to remove the water) for 48 hours. The samples were then weighed to estimate the weight of biomass and then 500 µl of methanol/chloroform (2:1 v/v) was added to each tube. The samples were then sonicated on ice for 1 minute in 15 second bursts and then centrifuged at full speed (3000 *g*) in the microfuge for 5 min. The volume of supernatant fluid was estimated using a Gilson pipette and the pellet was discarded. Chloroform and 1% NaCl (1 g NaCl in 100 ml) were added to achieve a volume of 1.0 ml in the ratio 2:2:1of methanol: chloroform: water.The samples were mixed, centrifuged at full speed for 2 minutes and the chloroform phase (top phase) was transferred into fresh pre-weighed 1.5 ml Eppendorf tubes. The solvent was allowed to evaporate in a fume cupboard for 24 hours, or until dry, and the sample weighed to calculate the weight of total lipids recovered. This procedure was carried out in triplicate. The amount of culture used depended on the growth rate and total volume, and no more than 50 ml was typically harvested per sample.

2.20. Quantification of Neutral Lipid by Direct Transesterification Method

A Gas chromatography-mass spectrometry (GC-MS) technique was used to determine and identify total lipids as fatty acid methyl ester (FAME) profiles of the algal cultures across the varying salinities. However, the fatty acid profile of total lipids in algal samples was analysed based on an *in-situ* direct transesterification gas chromatography (GC) method described by Griffiths *et al*. (2010) and Van Wychen and Laurens, (2013). However, in this procedure the whole biomass lipids (including phospholipids and galactoglycerolipids) was transesterified via an acid catalysed reaction to fatty acid methyl esters (FAME).

To prepare cells for measurements, samples were first prepared by lyophilisation and therefore, well grown cultures of algae were aseptically adjusted to an OD_{595} = 1 in final volume of 30 ml in a 50 ml Falcon tube. Tubes were centrifuged at 3000 *g* for 10 minutes. The supernatant fluid was discarded and the remaining pellet was resuspended in 5 ml of distilled water. The culture was centrifuged again at the same speed and for the same time. The supernatant fluid was discarded and the pellet was resuspended in 1 ml of distilled water. Samples were then transferred into pre-weighed 1.5 ml Eppendorf tube (fitted with a separate, pierced lid) and then frozen at -80°C for 48 hours and then samples were lyophilised (freeze dried to remove the water) for 24 - 48 hours until samples were completely dry. The samples were then re-weighed using a fine balance after freeze-drying to estimate the weight of biomass and then 7 - 10 mg of biomass was then transferred into a fresh glass crimped vial (11 mm, 2 ml, 12x30 mm Kinesis CRV12-02). 190 µl of chloroform/methanol (2:1, v/v) were then added to each vial for lipid solubilisation. 10 µl (0.1 mg) of tridecanoic acid (C13 lipid) dissolved in chloroform/methanol (2:1, v/v) was added as an internal standard to check for reaction efficiency. 0.3 ml HCl/Methanol (MeOH) (5%, v/v) catalyst was then added to each vial and crimp-sealed immediately with a PTFE lined cap (Kinesis CRC11-02 B2). The vials were mixed gently by hand and placed on a hot plate set at 85°C for 1 hour to start transesterifying the lipids. When completed, the vials were removed and allowed to cool to room temperature for 10 min.The caps were then removed and 975 µl of Hexane added for fatty acid methyl ester (FAME) extraction. The tubes were then resealed (closed again), mixed well and left at room temperature for 1 hour. After extraction, the caps were removed again (opened) and 487.5 µl of the top hexane phase were transferred into a fresh vial. 12.5 µl (0.125 mg) of the 10 mg/ml methyl nonadecanoate (C19 FAME) in hexane as an internal standard

were then added to check the efficiency of GC-MS analysis and the vials were sealed (re-closed).

The extracted FAMEs from the transesterification reactions were then identified and quantified by gas chromatography mass spectroscopy (GC-MS) using a Perkin Elmer-Auto System XL Gas Chromatograph (CHM-100-790) and Perkin Elmer-Turbo Mass Mass Spectrometer (13657). The machine was fitted with a Zebron - ZB-5MS -30 m 0.25 mm ID and 0.25 μm FT (7HG-G010-11) GC Capillary column and run using the following settings; Autosampler Method: Injection volume: 5 μl, Preinjection solvent washes: 2, Post injection solvent washes: 6. Temperature Program (FAME03-100mins): 100- 300°C, Ramp 1: 2ºC/min to 300, 20 ml/ min He constant carrier gas flow. MS Scan: El+, Start mass: 50, End mass: 600, Scan time: 0.5 s, Interscan time: 0.1 s, Start time: 0, End time: 100 min. The resultant peaks were identified and integrated using Perkin Elmer's Turbomass software linked to a NIST database.

2.21. Nuclear Magnetic Resonance (NMR) Analysis of Compatible Solutes

A Nuclear Magnetic Resonance (NMR) technique was used in order to identify the compatible solutes produced by the cells to establish tolerance under both salt and nitrogen stress conditions, respectively, in comparison with normal growth conditions. However, Proton Nuclear Magnetic Resonance (^{1}H-NMR) Spectroscopic Lipid Analysis was performed on micreoalgal samples with help from Prof Mike Williamson, MBB department, Sheffield University based on the methods adapted from Kumar *et al*. (2014) and Sarpal *et al.* (2015) for nuclear magnetic resonance (NMR) analysis.

To prepare cells, samples were first prepared by lyophilisation and therefore, duplicate 20 ml of a well cultures of algae (OD_{595} =1) was aseptically removed from a

relevant growth device and transferred to a 50 ml Falcon tube and then centrifuged at 3000 *g* for 10 minutes and the supernatant fluid was discarded. The pellets were then re-suspended in 1 ml of distilled water and sonicated on ice for 2 × 20 seconds with a 10 second cooling period between sonication steps. After that samples were transferred into 1.5 ml Eppendorf tubes and centrifuged for 5 minutes in the microfuge at full speed. The supernatants were then transferred into fresh pre-weighed 1.5 ml Eppendorf tubes (fitted with a separate, pierced lid) and frozen at -80°C for 48 hours followed by freeze drying (lyophilisation) for 48 hours until the samples were completely dry. Samples were then re-weighed using a fine balance after freeze-drying to estimate the weight of biomass.

400 µl of deuterated chloroform ($CDCl_3$) and 100 µl of deuterated methanol (CD_3OD) were added to each sample for solubilisation of the biomass. The samples were then mixed and transferred to a 5 mm NMR tube and then 5 µl of Chloroform ($CHCl_3$) was added as an internal standard. NMR spectra were obtained on a BrukerAvance 600 equipped with a cryoprobe. Data were recorded into 16k complex data points with simple pulse-acquire pulse program and a 3 s recycle time. Fourier transformation was applied using a 1 Hz line broadening followed by manual baseline correction. All spectra were acquired using 8 scans (with 4 dummy background scans). Processing and integration were performed using Bruker Topspin v1.3 software.

2.22.The Use of PAM Fluorescence Technique to Examine the Effect of Environmental Stress On Photosynthesis

Pulse Amplitude Modulation (PAM) fluorometers techniques are designed specifically to measure chlorophyll fluorescence yield at a high degree of sensitivity. Pulse Amplitude Modulation (PAM) measurements also are capable of differentiating between fluorescence from photochemical quenching as opposed to heat dissipation

or non-photochemical quenching (NPQ) (Schreiber, 2004). The maximum quantum efficiency (F_v/F_m) of *N. salina* was measured under different treatment conditions to elucidate the impact of environmental stress conditions including salinity concentration of 0.6 M, nitrogen starvation and f/2 growth medium with pH10 compared to normal f/2 medium as control on integrity of photosynthetic apparatus in comparison to the influence of normal growth medium. However, a difference in fluorescence at this point (F_o) and F_m gives the value F_v. The ratio of (F_v/F_m) gives a measure of the intrinsic maximum efficiency of photosystem II (PSII). In this experiment. a Pulse Amplitude Modulation (PAM) fluorescence technique was utilised to measure chlorophyll a fluorescence. PAM fluorometers apply pulses (μseconds) of weak measuring light (ML <0.1 μmols photon m^{-2} s^{-1}) that induce fluorescence but not photosynthesis, therefore enabling dark acclimated F_o measurement.

In order to determine the influence of these factors on the carbon metabolism of *N. salina* cells, a parallel series of cultures were grown using the treatments and growth conditions using 3 x 250 ml conical flasks containing 100 ml of each f/2 medium with 0.6 M of NaCl, nitrogen free (NF) f/2 medium, pH10 f/2 medium in comparison with normal growth conditions as control. The experiment was carried out according to the method described by Goodenough *et al*. (2014).

Samples were taken immediately before resuspension (LOG), followed by 24, 48, 96 and 144 hours after resuspension period. A sample of 2.2 x 10^8 cells was needed for two technical replicates to achieve the minimum steady state fluorescence signal needed (150 mV).Cell density (OD) at 595 nm at these time points was measured and an appropriate quantity of sample was aseptically taken from each culture and transferred to 50 ml Falcon tubes.

The tubes were centrifuged (1250*g* for 5 minutes at 15°C) and supernatant was poured into a fresh 50 ml Falcon tube. The cell pellet was resuspended to a volume of 4 ml at a cell density of 5.67 x 10^7 cells using the separated supernatant.The 4 ml concentrate was separated into 2 x 2 ml aliquots, which were transferred to 2 wells of a 6 well plate (Costar®, Corning®). After transferring 3 biological replicates, the plate was placed in a Maxi IMAGING-PAM chlorophyll fluorimeter, fitted with an IMAG-MAX/LR measuring head (Heinz Waltz GmBh). The plate was covered with a dense thread black cloth and the fluorimeter hood was closed. The cells were left to acclimate to the dark for 30 minutes. Once dark acclimated, the cloth was removed and the measuring light was turned on. Saturation pulse was initiated and the F_o and Fm were recorded using Imaging Win GigE software. The equipment settings used for analysis were; Special Saturation Pulse (SP) routine settings, ML 3, Gain 20, Frequency 2, Damping 2.

2.23. Flow Cytometry Analysis of Neutral Lipid Content

The Flow Cytometry Technique was used for the analysis of neutral lipid in *Nannochloropsis salina* cells grown under different growth conditions included f/2 medium containing 0.6 M NaCl, nitrogen starvation f/2 medium and pH 10 of f/2 growth medium in comparison with normal f/2 growth medium as control according to the method described by Satpati and Pal (2015).

2.23.1. Determination of DMSO Concentration

Different concentrations of DMSO ranging from (10-60%) were prepared using distilled water and used to identify the best concentration of DMSO to be used in the experiment. An aliquot of 500 µl of *N. salina* cells grown under normal and stress growth conditions as mentioned above for different time periods (high OD =1.5 after

14-21 days culture) was transferred to a 2 ml Eppendorf tube, 7 of these tubes were labelled as stained and 7 were labelled as unstained. Then 5 µl of 100 µg ml^{-1} of Nile Red solution was added to stained tubes. From each concentration of DMSO, 295 µl was added to the one of the stained tubes and 300 µl was transferred to unstained tubes and the final concentrations of DMSO were (3%,7%,11%,15%,18% and 22%). All the tubes were vortexed and left in the dark at room temperature for 10 min and then the stained and unstained tubes were washed 2 to 3 times with 1 ml of phosphate buffer (PBS). The samples were then transferred to a multi-pipette reservoir (Thermo Sceintific). Using a multi-channel pipette, 200 µl aliquots of each stained tube were transferred to the 96 well plate rows from A to D, to give 4 technical replicates of each concentration. The same step was done for the unstained tubes in rows E to H as shown in Figure 2.5.

DMSO (%)			10	20	30	40	50	60	Empty wells					
			1	2	3	4	5	6	7	8	9	10	11	12
Stained Cells	R1	A	200	200	200	200	200	200	0	0	0	0	0	0
	R2	B	200	200	200	200	200	200	0	0	0	0	0	0
	R3	C	200	200	200	200	200	200	0	0	0	0	0	0
	R4	D	200	200	200	200	200	200	0	0	0	0	0	0
Unstained Cells	R1	E	200	200	200	200	200	200	0	0	0	0	0	0
	R2	F	200	200	200	200	200	200	0	0	0	0	0	0
	R3	G	200	200	200	200	200	200	0	0	0	0	0	0
	R4	H	200	200	200	200	200	200	0	0	0	0	0	0

Figure 2.5: 96 well plate layout of different concentrations of DMSO mixture for determination of the best concentration of DMSO that was used in the flow cytometry.R1-R4 are technical replicates from the same concentration.

2.23.2. Optimising Nile Red Concentration

After determination of the optimal concentration of DMSO as described in section 2.23.1, the Nile Red concentration was optimised by preparing serial concentrations of Nile Red with DMSO at 0.05, 0.1, 0.2, 0.3, 0.4 and 0.6 mg/ml^{-1} as final concentration. The same steps were carried out except adding 5 µl of each concentration of Nile Red to one of the stained tubes. After the cell washing wit hphosphate buffer (PBS), the stained and unstained tubes were transferred to run through the flow cytometer.

2.23.3. Determination of Neutral Lipid in *N. salina* Cells Grown Under Different Growth Conditions

The neutral lipid contents of the *N.salina* cells grown in f/2 medium with 0.6 M NaCl, f/2 growth medium with nitrogen starvation and f/2 growth medium with pH 10 in comparison with normal f/2 growth medium as control were measured using the flow cytometry analysis.

2.23.4. Flow Cytometry Analysis

The analysis of *N. salina* cells staining with Nile Red were carried out based on the method described by Velmurugan *et al.* (2013) using a high speed flow cytometer BD LSRII (BD Bioscience) as shown in Figure 2.6.

To obtain the fluorescence readings, the Nile Red stained cells at different concentrations were excited with a 475-nm laser, and then the emission was 568/42 nm while the unstained cells were employed as an auto-fluorescence control. The features of the *N. salina* cells including cell size and granularity using flow cytometer were obtained by Forward Scatter (FSC) and Side Scatter (SSC) signals. Flow

cytometry data were analysed depending on many parameters including the stained cells grandparents' percentage and median of the stained cells using Flow Jo software.

Figure 2.6: High speed flow cytometer (BD LSRII Flow cytometer).

2.24. Molecular Identification Techniques

2.24.1. Extraction of Genomic DNA (gDNA)

In this study molecular identification techniques were used to confirm the identity of the unicellular *Nannochloropsis salina* strain received from the Culture Centre of Algae and Protozoa, Oban, Scotland, UKusing 18S rRNA gene sequencing. However, the efficacy of two nucleic acid extraction methods was investigated using *Nannochloropsis salina* cells grown in f/2 growth medium as described below:

2.24.1.1. DNA Extraction Using CTAB Method

Genomic DNA of *N. salina* cells was extracted for 18S rDNA analysis using the commercially available CTAB method as described by Chen *et al*. (2001).

To produce this procedure, 2 x 5 ml of algal cells grown at stationary phase were centrifuged for 5 minutes at 3500 rpm on the bench centrifuge using 15 ml centrifuge tubes. The supernatant was poured off immediately and then each pellet was resuspended in 500 µl CTAB buffer [2% (w/v) cetyltrimethylammonium bromide (CTAB), 2% (v/v) ß-mercaptoethanol**,** 0.1 M Tris-HCl pH 8.0**,** 1.4 M NaCl**,** 20 mM EDTA (ethylene diamine tetraacetic acid)] and then sonicated for 30 seconds at full power using the Bead Bug machine to break open the cells. Both samples were then incubated in a bench hot block at 65°C for 1 hour.

The genomic DNA was extracted using an equal volume (500 µl) of phenol-chloroform-isoamylalcohol (24:25:1, v/v), the bottom layer was taken and vortexed and then centrifuged for 5 minutes at full speed at the room temperature. The aqueous layer (top layer) was carefully transferred into a fresh 1.5 ml Eppendorf tube and 500 µl of chloroform was added into the same tube and vortexed briefly. The sample was then centrifuged for 5 minutes at full speed in a microfuge at room temperature.The top layer was carefully transferred into a fresh Eppendorf tube and then 1/10 total

volume of 3 M sodium acetate (pH 5.2) was added into the same tube. DNA was precipitated with 2.5 volumes of 100% (v/v) cold ethanol (More is OK, but not less) and incubated on ice for 15 minutes (can be longer) and then pelleted by centrifugation at 13000 rpm for 15 minutes at 4°C and the supernatant was carefully poured off immediately. The pellet was gently washed with 1 ml of 70% cold ethanol and precipitated by centrifugation for 5 minutes at full speed at 4°C and the supernatant (ethanol) was carefully poured off immediately. Centrifugation was carried out again for the same time at full speed and pipette off any remaining liquid carefully without disturbing the pellet. The genomic DNA pellet was air dried for 5 - 10 minutes (No longer or pellet will not resuspend) and then genomic DNA pellet was dissolved (resuspended) in 30 - 40 µl of MilliQ water (Ultrapure water) and genomic DNA pellet was left on bench to resuspend overnight. In the morning the genomic DNA pellet was incubated in a hot block at 50ºC for 60 minutes to ensure solubilisation of the DNA. Successful extraction of genomic DNA was verified by resolving 10 µl of genomic DNA with 2 µl of 6×DNA loading dye by gel electrophoresis against 6 µl of 1 kb DNA ladder.1.5 ml Eppendorf tubes containing genomic DNA were labelled and stored at – 20°C until required.

2.24.1.2. DNA Extraction Using ZR Soil Microbe DNA MicroPrep kit

Genomic DNA extraction of *N. salina* cells grown in f/2 medium was performed using ZR Soil Microbe DNA MicroPrep™ kit according to the manufacturer's protocol except instead of the kit tubes (Bashingbead™), Banchmark tubes that fit in the Bead Bug machine were used.

To produce this process, 20 ml of algal cells grown at stationary phase were centrifuged at 3000 g for 10 minutes and then supernatant was discarded. 750 µl of Lysis solution was added to each pellet and then transferred to a Banchmark prefilled

tube (0.1, 0.5 or 1 mm zirconium beads). The tubes were then shaken in the Bead Bug machine for 1 to 3 minutes at full speed (3000 rpm) to break open the cells (Optimum time to give best breakage without damaging DNA). The Benchmark Bead Bug tubes were then centrifuged at 10,000 g for 1 minute and the procedure continued as described below using ZR Soil kit. 400 µl of the supernatant was transferred to Zymo-spin™ filter in a collection tube and then centrifuged at 7000 g for 1 minute and then 1,200 µl of Soil DNA binding buffer was added to filtrate in the collection tube from the last step. After that 800 µl of the mixture was transferred to Zymo-spin™ IC Column in a collection tube and then centrifuged at 10,000 g for 1 minutes. The flow through was discarded from the collection tube and the last step was repeated. Then 200 µl of DNA Pre-wash Buffer was added to the Zymo-spin™ IC Column in a new collection tube and then centrifuged at 10,000 g for 1 minute. 500 µl of Soil DNA Wash Buffer was added to the Zymo-spin™ IC Column in a collection tube and centrifuged at 10,000 g for 1 minute. The Zymo-spin™ IC Column was transferred to clean 1.5 ml microcentrifuge tube and 50 µl of DNA Elution Buffer was added directly to the column matrix and then centrifuged at 10,000g for 30 seconds. The genomic DNA pellet was left on the bench to resuspend overnight and then incubated in a hot block at 50ºC for 60 minutes to ensure solubilisation of the DNA.

Successful extraction of genomic DNA was verified by resolving 10 µl of genomic DNA with 2 µl of 6×DNA loading dye by gel electrophoresis against 6 µl of 1 kb DNA ladder. 1.5 ml Eppendorf tubes containing genomic DNA were labelled and stored at – 20°C until required.

2.24.2. Agarose Gel Electrophoresis

To detect the presence of DNA in the extracted samples, all DNA samples were analysed (separated) by gel electrophoresis using 1% gels. To prepare 1% agarose

gels, 0.6 g low melting point agarose powder (Roche) were added to 60 ml of distilled water with 1.2 ml of 50 X TAE buffer (Appendix A) in a specially designated 250 ml conical flask labelled “Toxic” containing a stirrer bar. To dissolve the agarose, the gel mixture was heated in the microwave oven at power 6 for 90 seconds until the agarose had melted.

The solution was allowed to cool before 5 µl aliquot of ethidium bromide (BioRad # 161-0.433) was added and then the gel poured to set in the BioRad Subcell GT electrophoretic tank with 14, 20 or 30 well comb. Once the gel had set, it was covered with 1 X TAE running buffer (20 ml of 50 X TAE made up to 1000 ml distilled water, using TAE measuring cylinder). To load the gel, 10 µl of each DNA sample was mixed with 2 µl of 6×DNA loading dye and carefully loaded into the agarose gel. Also, 6 µl of 1 kb DNA ladder was loaded into the agarose gel and then the voltage (electrophoresis) was set at 80 V and left to run for 45 minutes using the BioRad Power Pack 300. The bands of genomic DNA were visualized and photographed under ultra violet light using the Uvitec “Uvidoc machine” mounted camera system to ensure the genomic DNA was extracted successfully, presence of high molecular weight gDNA and that RNA was absent. If the gel showed smearing, this indicates the presence of RNA. It was removed by adding 1 µl of RNAse to the genomic DNA preparation with incubation at 4°C overnight.

2.24.3. Polymerase Chain Reaction (PCR) Amplification of 18S rDNA

Following extraction of total genomic DNA from a particular microalga, 18S rRNA gene was amplified from the total DNA extracted using the polymerase chain reaction (PCR) with two universal 18S rRNA primers according to Si *et al.* (2011). The sequences of universal 18S rRNA primers are shown in Table 2.13 and the details for the PCR mixture were provided in Table 2.14.

Primer (Gene) Name	Primer Sequence (5'- 3')
rbcl Forward	5'-GAT GCA AAC TAC ACA ATT AAA GAT ACT G-3'
rbcl Reverse	5'-ATT TTG TTC GTT TGT TAA ATC CG-3'
18S rDNA Forward	5'-CAA GTT TCT GCC CTA TCA GCT-3'
18S rDNA Reverse	5'-GCT TTC GCA GTA GTT CGT CTT-3'

Table 2.13: Sequences of universal 18S rRNA primers. Taken fromSi *et al.* (2011).

Sample	18S rDNA (µl)			Control (µl)
	1	2	3	
Master Mix	20	20	20	20
For Primer	7	6	8	6
Rev Primer	7	6	8	6
Genomic DNA	10	10	8	0
MilliQ water	6	8	6	18
NB: Total volume is 50 µl for all samples				

Table 2.14: Preparation for PCR using master mixture (contents of tubes for PCR amplification).

The PCR amplifications were performed in a Mycycler thermal cycler (Bio-Rad) and the cycle parameters included an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 2 min and a final 10 min elongation step at 72°C (Table 2.15). These conditions for PCR reaction using 18S rRNA primers were a pre-set programme and especially saved for *N. salina* cells in the Mycycler memory. The PCR products were then detected using 1% agarose gel as described in section2.24.2 to confirm the correct sized product had been amplified and positive results were purified using a keyPrep PCR clean-up kit.

	Cycle Parameters	Cycle Temperature	Cycle Time
1	Initial Denature	94°C	3 min
2	Denature	94°C	30 sec
3	Anneal	55°C	30 sec
4	Elongation	72°C	2 min
5	Final Elongation	72°C	10 min

30 cycles

Table 2.15: PCR cycle conditions using 18S rRNA primers. Modified fromSi *et al.* (2011).

2.24.4. Purification of PCR Products

After the PCR, amplified PCR product was visualised on an agarose gel using the protocol outlined in Section (2.24.2). Visualisation demonstrated the quality and size of the amplified regions. The amplified PCR products were purified using a keyPrep PCR Purification kit,which was designed to purify single or double stranded DNA fragments from PCR and other enzymatic reactions (to remove excess primers). The volume of the samples were determined and adjusted to 100 µl with sterile distilled water. Five volumes of Buffer PCR (PBI) were added to 1 volume of PCR reaction (sample) in sterile 1.5 ml tube (250 µl of PBI buffer were pipetted into 50 µl of the DNA) and the samples were mixed thoroughly by vortexing or inverting several times. The samples were pipetted into a QIAquick spin column and then placed in a 2 ml clean collection tube which was then centrifuged at 10,000 g for 1 minute and the flow through was discarded. The QIAquick spin columns were washed with 750 µl Wash Buffer and centrifuged at 10,000 g for 1 minute and the flow through was discarded. The QIAquick spin columns were centrifuged at 10,000 g for 1 minute to remove residual ethanol. The QIAquick spin columns were placed into clean

microcentrifuge tube. About 80 µl of Elution Buffer was added onto the column membrane and allowed to stand for 2 minutes. The eluted DNA was collected by centrifuging the column at 10,000 g for 60 seconds. The product of eluted DNA was run on a 0.6% agarose gel against 1 µl of 1 kb GeneRuler as a marker ladder to ensure that the correct sized product had been purified the rest of DNA was stored at 4°C.

2.24.5. DNA Quantification

The purified DNA was quantified using a Nanodrop spectrophotometer (Jenway Genova Nano, Bibby Scientific). A 2 µl aliquot of TE buffer was used to blank the instrument at wavelengths of 260 nm, 280 nm and 320 nm.

2.24.6. DNA Sequencing

DNA samples were sent out for sequencing to Eurofins/MWG. The obtained sequences were then compared against sequences in the GenBank nucleotide collection using the Basic Local Alignment Search Tool (BLAST).

2.24.7. Phylogenetic Analysis

The sequences were compared against sequences in the GenBank nucleotide collection using the Basic Local Alignment Search Tool, BLAST.

2.25. Statistical Analysis

Most of the experiments in this study were carried out in triplicate and error bars represent standard errors of the means. If no error bars are shown, they were smaller than the symbol used to represent the mean. For experiments carried out in duplicate, both values plus the average are shown. The statistical significance of differences between measurements taken at different time points within the same treatment was analysed using a paired two sample T-test.

Molecular and phylogenetic identification of an oil-producing strain of *Nannochloropsis salina* (CCAP 849/2) Using 18S rDNA Sequencing

.

Chapter Three

3.1. Introduction

The study of microbial biodiversity has been severely limited and therefore delayed for many years by relying on microorganisms that can be cultured in the laboratory, which represent only a tiny fraction of the microbial biodiversity in the environment. It has been estimated that less than 0.1% of the soil microbial community can be successfully isolated as pure culture (Hill*et al.,* 2000). However, this situation has arisen because the identification of microorganisms in most microbiology laboratories has usually been achieved by traditional identification techniques including, Gram staining, morphology, culture requirements and biochemical reactions (Woo *et al*., 2003). The conventional identification techniques have two major disadvantages: First, these techniques cannot be used for identification of non-cultivable or slow growing microorganisms, that are characteristics of many anaerobic microorganisms, and second, microorganisms which exhibit unusual biochemical characteristics tend to be difficult to identify (Lau *et al*., 2002; Woo *et al*., 2003).

For microalgal cells, both light microscopy and transmission electron microscopy procedures have been used as standard methods for identification and characterization of microalgal lineages, however these methods are not sufficient to provide accurate identification with various microalgae species (Karlson *et al.,* 1996).

However, for example a correct specific identification for *Nannochloropsis* cells is critical due to many reasons: the small cell size and simple structure (Maruyama *et al.*,1986; Brown,1987; Gladu *et al.*,1995), the species in this genus are often found offshore and are usually 2-5 μm in size (Si *et al.,* 2011). Therfore also the difficulties in fixing *Nannochloropsis* cells for transmission electronmicroscopy and the *Nannochloropsis* cells are lacking of sexual reproduction (Bongiovani *et al.,* 2014). Even although meiosis-related genes were found in the genome of *N. gaditana*, no

transcripts weredetected (Radakovits *et al.,* 2012). In addition, the taxonomic identification of *Nannochloropsis* species is challenging due to their ability to accumulate high amounts of specific lipids (Bongiovani *et al.,* 2014; Hu *et al*., 2008). Thus, the phylogenetic species identification becomes particularly useful (Andersen *et al*., 1998). Therefore, the characterisation of *Nannochloropsis* species has been defined using DNA sequence analysis (Fawley and Fawley, 2007), based on the nuclear indication included 18S rDNA sequence analysis and chloroplastic (*rbcL*) markers and also sets of orthogonal genes (Andersen *et al*., 1998; Vieler *et al*., 2012; Wang *et al*., 2014a).

Over the last decade, recombinant DNA and molecular phylogenetic identification procedures have provided sufficient techniques for analysis of microbial diversity without the need for cultivation. Molecular identification techniques overcome the problems that may arise from the isolation and culturing of microorganisms such as time consumption and uncertainty (Spratt, 2004; Janda and Abbott, 2007). Since the discovery of the polymerase chain reaction, the DNA sequencing process has been used as an important tool for phylogenetic characterisation and classification of microorganisms using conserved and variable regions of 16S rDNA or 18S rDNA (Hejazi *et al.,* 2010). It has been shown that the small subunit (SSU) ribosomal RNA gene (16S rRNA gene) is highly conserved in a species and among species of the same genus in prokaryotic microorganisms (Woo *et al.,* 2001; Cai *et al.,* 2003**;** Mignard and Flandrois, 2006). On the other hand, the 18S rDNA sequences (18S rRNA gene) is available in GenBank for *Nannochloropsis* and a very promising method for taxonomy studies, identification and classification of living eukaryoticorganisms (Hejazi *et al.,* 2010; Fawley *et al.,* 2015). Thus, 18S rDNA gene sequences have been used for molecular identification of different species of

Dunaliella as a eukaryotic microorganism (Olmos *et al.,* 2000; Raja *et al.,* 2007). Given the importance of *Nannochloropsis* as feedstocks for biodiesel production, a number of genomic and transcriptomic studies on many species of the genus have been recently published (Radakovits *et al*., 2012; Vieler *et al*., 2012; Carpinelli *et al*., 2013; Wei *et al*., 2013; Hu *et al*., 2014; Starkenburg *et al*., 2014).

The aim of this chapter was to compare classical morphological characters of *Nannochloropsis salina* cells grown in f/2 medium with modern molecular analysis. To achieve this aim,*N. salina* cells grown in f/2 medium was identified to the species level by 18S rRNA gene sequencing. The 18S rRNA gene sequence analysis was used for this initial study because it is the only gene with an extensive data set for green algae. However, the 18S rRNA gene was amplified by the polymerase chain reaction (PCR), purified and thereafter quantified using a Nanodrop spectrophotometer. The sequence of the PCR product (~ 7.5 kb) was compared with known 18S rRNA gene sequences in the MicroSeq database and the EMBL public database by multiple sequence alignment. Phylogenetic trees were also constructed for *N. salina* cells to predict their genetic relatedness.

3.2. Results and Discussion

3.2.1. Morphologic Identification of *N. salina* Strain Using A Light Microscope

Figure 3.1 presents the basic characteristicsof *Nannochloropsis salina* cells grown inthe f/2 growth medium at 25°C for 14-21 days using a light microscope. It was found that cells of *N. salina* were nonmotile cells and spherical shape with a smooth cell wall. In this strain, a single parietal chloroplast and an eyespot structure were not observed. This is because the cells of *Nannochloropsis salina* are a very small unicellular marine microalga (Brown,1987).However, the eyespot, which was constantly present in the algal cells of *N. oceanica* var. *sinensis* (Cao *et al.* 2013), was rarely observed by Suda *et al*. (2002). Furthermore, these observations do not agree with the results reported for *N. oceanica* cell dimensions under exponential and stationary growth phases that were 2-3 µm and 3-5 µm diameters, respectively, and showed nucleus, parietal chloroplast, eyespot and thick cell wall (Bongiovani *et al.,* 2014).

On the other hand, these findings are in good agreement with those of Suda *et al*. (2002) and Cao *et al.,* (2013) who did not observe the cell structure such as wall papilla and pyrenoid-like in *Nannochloropsis oceanica* and other species of *Nannochloropsis*. Furthermore, these studies did not find any morphological distinct to distinguish between the *Nannochloropsis* species including *N. granulata, N. salina, N. gaditana* and *N. oceanica* by either light microscopy or transmission electron microscopy. However, the characterisation of *Nannochloropsis* species is mainly completed using the *rbcL* gene and 18S rDNA sequence analysis (Vieler *et al.,* 2012; Andersen *et al.,* 1998; Suda *et al.,* 2002; Cao *et al*., 2013).

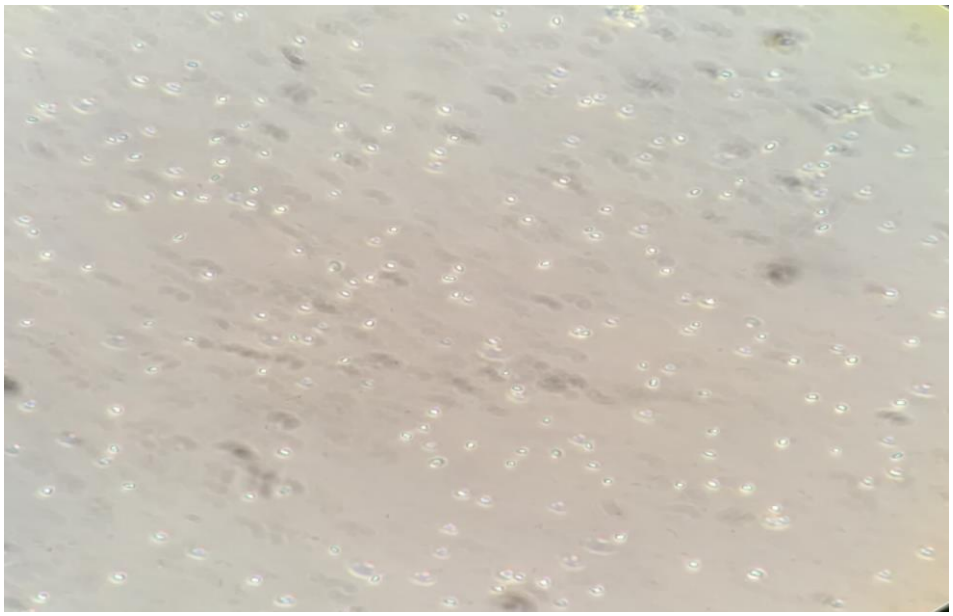


Figure 3.1: *Nannochloropsis salina* cells viewed under a light microscope.

3.2.2. Molecular and Phylogenetic Identification of *N. salina* Strain Using 18S rDNA Sequencing

3.2.2.1. Extraction of Genomic DNA for 18S rDNA Analysis

Sequences for 18S rDNA and *rbcL* genes were used to identify several microalgal species and in particular to differentiate species of *Nannochloropsis* (Karlson *et al*., 1996; Krienitz *et al*., 2000). Therefore, in this study, the molecular procedure based on two genes allowed confirmation of the identification of the isolated strain of CCAP 849 as *N. salina.* The gene sequences for this strain were highly similar (100% for 18S rDNA and 99.7% for *rbcL*) to other strains of *Nannochloropsis* isolated from different marine habitats as shown in Table 3.1.

Species	Strain	Collection Site	GenBank Accession Number	
			rbcL	18S rDNA
N. oceanica	CCALA 978	Southwestern Ocean Atlantic Cost	KF010153 (this study)	KF010154 (this study)
N. oculata	CCAP 849	Skate Point,Isle of Cumbrae, Scotland, UK	AB052286	-
N. oculata	CCMP525	Skate Point, Isle of Cumbrae, Scotland, UK	HQ710609	AF045044
N. oculata	CCMP533	Lake of Tunis, Tunesia, North Africa	-	AY045045
N. granulata	MBIC10054	Pacific Ocean, 32°12' N, 147°23' E	AB052280	AB052272
N. granulata	BDH02	Unknown	KC 128502	KC128500
N. granulata	CCMP1662	Skagerrak, North Sea	-	AF045041
N. granulata		Skagerrak, North Sea		NGU38903
N. limnetica	KR1998	Arrowodd National Wildlife Refuge, Itasca Lake Park, Minessota, USA	DQ977729	-
N. limnetica	JL1125	Arrowodd National Wildlife Refuge, Itasca Lake Park, Minessota, USA	DQ977730	-
N. limnetica	DML1114	Arrowodd National Wildlife Refuge, Itasca Lake Park, Minessota, USA	DQ977740	-
N. limnetica	AS39	Arrowodd National Wildlife Refuge, Itasca Lake Park, Minessota, USA	DQ977741	DQ977726
N. limnetica	AS2168	Arrowodd National Wildlife Refuge, Itasca Lake Park, Minessota, USA	DQ977739	-
N. limnetica	SAG1899	Lake Roter See, Mecklenburg-Vorpommern, Germany	AM421006	AF251496
N. limnetica	JL24	Arrowodd National Wildlife Refuge, Itasca Lake Park, Minessota, USA	-	DQ977727
N. gaditana	MBIC10118	Shell Beach, Australia	AB052279	AB052269
N. gaditana	MBIC10123	Monkey Mia, Australia	AB05273	AB052271
N. gaditana	MBIC10418	Atlantic Ocean, Cape Town, South Africa	AB052735	AB052271
N. gaditana	Ferrara & Andreoli 2004	Comachio Lagoon, Ferrara, Italy	-	AF133819
N. gaditana	IVP	Unknown	-	AB473733
N. gaditana	CCAP849/5-CCMP1775	Cadiz Bay, Cadiz, Spain	-	AF067957
N. gaditana	CCMP526	Lagune di Quadilia, Morocco	-	AF045037
N. gaditana	B	Unknown	-	JF444989
N. gaditana	CCAP849/5-CCMP1775	Cadiz Bay, Cadiz, Spain	-	AF045036
N. salina	CCAP849/2	Skate Point, Isle of Cumbrae, Scotland, UK	AB052288	AF045046
N. salina	MBIC10063	Pacific Ocean, 41°28'N, 146°57'E	AB052287	AB052278
N. oceanica	MBIC10179	Red Sea, Eilat, Israel	AB052283	AB052275
N. oceanica	MBIC10176	Mediterranean Sea, Haifa, Israel	AB052272	AB052274
N. oceanica	MBIC10426	Red Sea, Eilat, Israel	AB052284	AB052276
N. oceanica	MBIC10440	Red Sea, Eilat, Israel	AB052285	AB052277
N. oceanica	MBIC10090	Pacific Ocean, Off Sanriku, Japan	AB052281	AB052273
N. oceanica	LAMB001	Unknown	HQ201773	-
N. oceanica	EUS001	Unknown	-	HQ710567
N. oceanica	CCAP211/46	Kuwait	-	AF045034
N. oceanica	CCAP211/78	Unknown	-	AF045035
Nannochloropsis sp.1	CCMP531	Qingdao, China	-	U41094
Nannochloropsis sp.2	CCMP505	Morehead City, USA	-	U41050
Nannochloropsis sp.	UTEX2379	Unknown	-	AY560119

Table 3.1: Taxonomic data, collection site and GenBank accession number of taxa in the *rbcL* or 18S rDNA alignment. Taken from Bongiovani *et al.* (2014).

Therefore, the DNA extraction protocol was successfully established in order to obtain high molecular weight genomic DNA from *N. salina* cells grown in f/2 medium. The DNA extraction process generally involves cell breakage by digesting cell walls, centrifugation to remove the cell fragments and debris, and then nucleic acid precipitation and purification. Cell breakage is normally achieved using detergents such as sodium dodecylsulphate (SDS) or cetylmethylammonium bromide (CTAB) (Puchooa, 2004).

However, the efficacy of two commercially available nucleic acid extraction methods was investigated using *N. salina* cells, these are the CTAB protocol and the ZR Soil Microbe DNA MicroPrep kit as described in sections 2.24.1.1 and 2.24.1.2, respectively.

After a number of experiments, the total genomic DNA was successfully extracted from *N. salina* cells with the ZR Soil Microbe DNA MicroPrep kit and therefore was superior in comparison to the CTAB method.

Evidently, the CTAB method did not extract DNA that showed there are clearly no DNA bands (lanes 2 and 3), whereas the ZR Soil Microbe DNA MicroPrep kit protocol showed a clear bright DNA band with a size over 10,000 base pairs on the gel at lanes 4 and 5 compared with standard molecular weight marker (1 kb DNA ladder) at lanes 1 and 6, as shown in Figure 3.2, and therefore this gDNA was selected for PCR amplification. However, the purity of the genomic DNA is evidence that the ZR Soil Microbe DNA MicroPrep protocol effectively removed proteins, polysaccharides and other contaminating molecules. This result also showed the absence of RNA and presence of high molecular weight DNA in large enough amounts for conventional PCR.

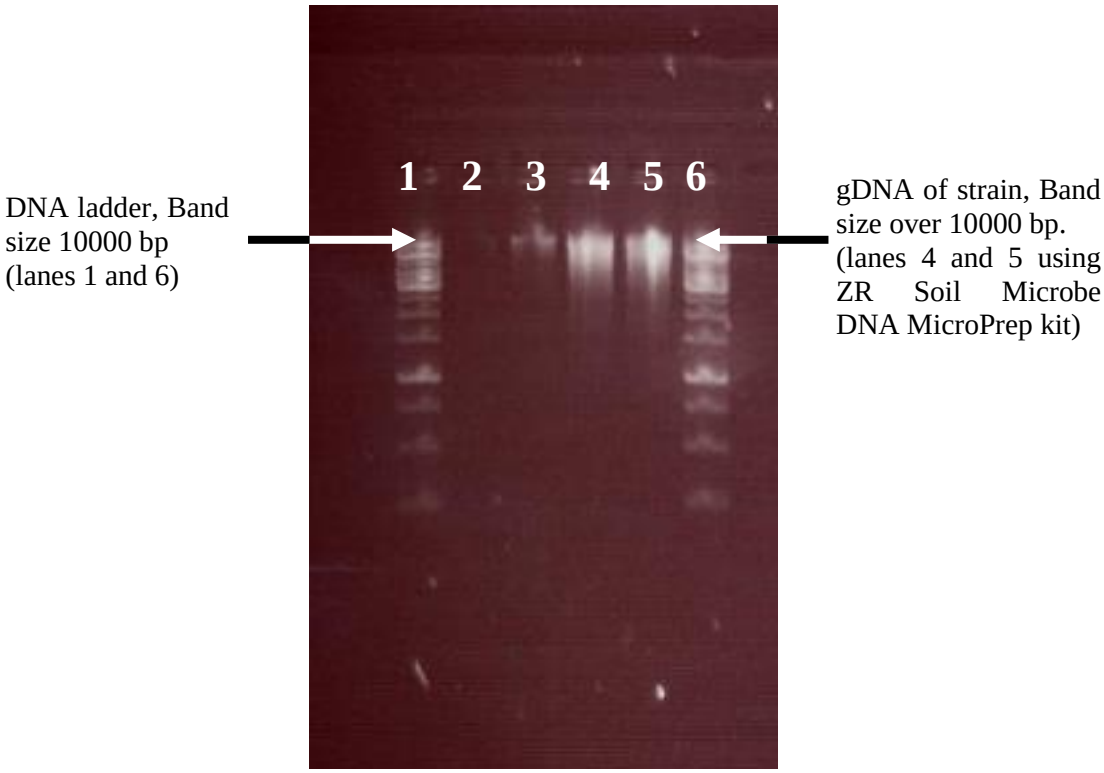


Figure 3.2: Agarose gel electrophoresis showing 13 spaced bands of molecular weight marker(lanes 1 and 6) and total genomic DNA with a size more 10000 bp from *Nannochloropsis salina*strain (lanes 4 and 5) using ZR Soil Microbe DNA MicroPrep kit and no clearly gDNA with CTAB method (lanes 2 and 3).

Figure 3.3 represents the standard molecular weight marker (1 kb DNA ladder) that was used in this study as molecular weight marker. The standard molecular weight marker (1 kb DNA ladder) produces a pattern of 13 regularly spaced bands, ranging from 250 to 10,000 bp. The 1,000 and 10,000 bp bands have the highest intensity to allow easy identification. The standard DNA ladder has been specially designed for easy quantification and as a convenient marker for determining the size of double stranded DNA from 250 to 10,000 bp. The size of each band is an exact multiple of 100 bp. The standard molecular weight marker was used in all further experiments to allow the size of DNA molecules to be determined.

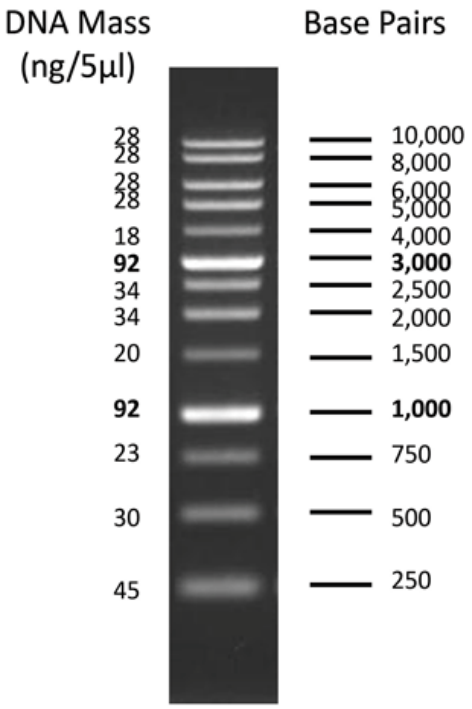


Figure 3.3: Standard molecular weight marker (1 kb DNA Ladder) 250-10,000 bp contains DNA fragments ranging from 250bp to 10,000bp that can be used as molecular weight standards for agarose gel electrophoresis. DNA Ladder 250-10,000 bp produces a pattern of 13 regularly spaced bands (250, 500, 750, 1000, 1500, 2000, 2500, 3000, 4000, 5000, 6000, 8000 and 10000).

3.2.2.2. Polymerase Chain Reaction (PCR) Amplification of Small Subunit (SSU) rDNA

After extraction, the PCR amplification protocol was successfully established to amplify the 18S ribosomal RNA gene (18S rRNA gene) from total genomic DNA extract as described in see section 2.24.3, that was used as template DNA in the polymerase chain reaction (PCR) assay using two universal 18S rRNA primers specific for *N. salina*. The sequences of both universal 18S rRNA primers (forward and reverse primers as shown in Table 2.13) are designed to target the conserved regions of the 18S rRNA gene that were utilized (Si *et al.,* (2011).Therefore, these primers should allow the amplification of the 18S rRNA gene from the *N. salina* strain.

Figure 3.4 shows the result of 18S rDNA PCR amplification experiment with the *N. salina* strain at lanes 2, 3 and 4 compared with standard molecular weight marker (1 kb DNA ladder) at lanes 1 and 5.It is clear from these findings that the 18S rRNA genes were successfully amplified from the genome of microalgaal strain *N. salina*, with the size of 18S rRNA gene as expected around 750 bp. Therefore this genome was cleaned up using a PCR purification kit to obtain the correct size (750 bp).

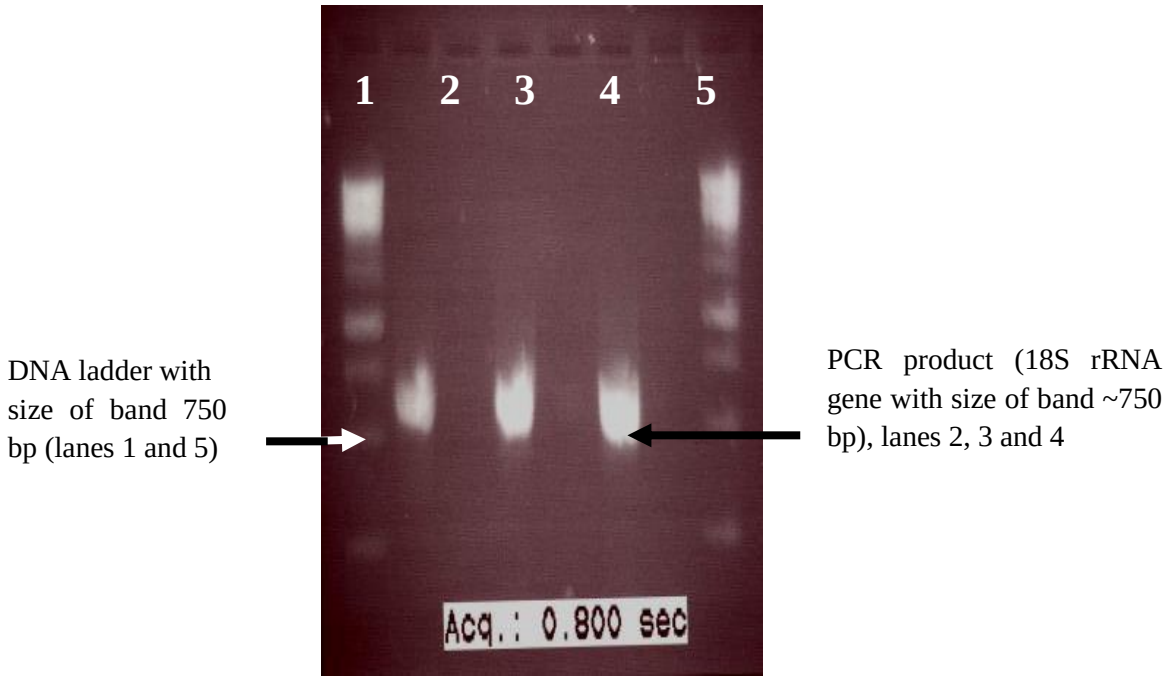


Figure 3.4: Agarose gel electrophoresis showing resolution of an approximate 750 base pair from the PCR involving two universal 18S rRNA primers using 1 µl of total genomic DNA from the *Nannochloropsis salina* strain (lanes 2, 3 and 4) compared with the standard molecular weight marker (1 kb DNA ladder) (lanes 1 and 5).

3.2.2.3. PCR Product Purification and Quantification

A purification step was also successfully performed on PCR products (18S rRNA gene) using keyPrep PCR Purification kit protocol as described in section 2.24.5.

The PCR purification process removes compounds that may be present in the PCR products such as primers that may inhibit subsequent sequencing reactions. Furthermore, the purified 18S PCR product was quantified using a Nanodrop spectrophotometer with the wavelength of 260 nm to improve the quantity of the 18S rRNA gene. The result of this experiment indicated that PCR clean up was successfully completed and inhibitory compounds present in the PCR products were completely removed and the unpurified PCR product produced the sharpest band with

the correct size (approximately 750 bp) as shown in Figure 3.5 at lane 2, 3 and 4 against the standard molecular weight marker (1 kb DNA ladder) at lanes 1 and 5 and was subsequently used for sequencing.

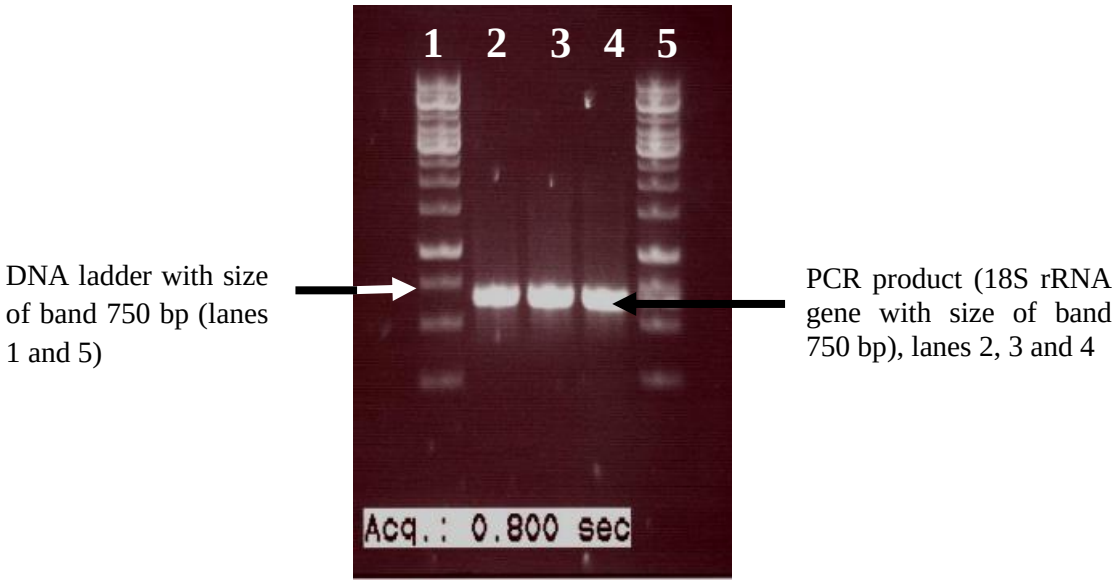


Figure 3.5: Showing the resolution of 750 bp purified of 18S rRNA gene from the polymerase chain reaction (PCR) involving the universal 18S rRNA primers using 1 µl of genomic DNA from the *Nannochloropsis salina* strain (lanes 2, 3 and 4) compared with the standard molecular weight marker ((lanes 1 and 5).

3.2.2.4. DNA Sequencing

18S rDNA gene sequencing is a powerful tool that has been used to identify phylogenetic relationships between microalgae species. However, the 18S rRNA gene sequences of the *N. salina* strain were aligned with the highly similar 18S rDNA sequences of other microalgae available in the GenBank nucleotide collection using the Basic Local Alignment Search Tool (BLAST) on the NCBI website using the MicroSeq database and the EMBL public database. However, the sequencing of

purified 18S PCR product returned from Eurofine/MWG were successfully sequenced and produced a partial forward and reverse sequence of 591 bp and 596 bp as shown in Figures 3.6 and 3.7, respectively.

Sequences of the 18S rRNA genes obtained in this work were deposited with the GenBank nucleotide collection in Basic Local Alignment Search Tool (BLAST) on the NCBI website under entry name (Order ID: 6219391) for *Nannochloropsis salina* strain CCAP 849/2.

CTACATGGCTCTACGGGTAACGGAGAATTGGGGTTCGATTCCGGAGAGGG
AGCCTGAGAGACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGTAAAT
TACCCAATCCTGACACAGGGAGGTAGTGACAATAAATAACAATGCCGGG
GTTTAACTCTGGCTATTGGAATGAGAACAATTTAAATCCCTTATCGAGGAT
CAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAA
TAGCGTATACTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTCTGG
CGGGGACGGCTGGTCGGTCTCGAAAGGGGCTGTACTGTTGTTGGTTCCCG
TCATCCTTGGGGAGAGCGGCTCTTACATTAAGTTGTCGGCGTCGGGATCCC
TATCTTTTACTGTGAAAAAATTAGAGTGTTCAAAGCAGGCTTAGGCCCTGA
ATACATTAGCATGGAATAATAAGATACGACCTTGGTGGTCTATTTTGTTGG
TTTGCACGCCAAGGTAATGATTAATAGGGATAGTTGGGGGTATTCGTATTC
AATTGTCAGAGGTGAAATTCTTGGATTTATGGAAACAC

Figure 3.6: Sequences of the 18S rRNA gene from *Nannochloropsis salina* strain (entry name ID: 6219391) using rbcl forward primer.

ACCTCTGACATTGAATACGAATACCCCCAACTATCCCTATTAATCATTACC
TTGGCGTGCAAACCAACAAAATAGACCACCAAGGTCGTATCTTATTATTC
CATGCTAATGTATTCAGGGCCTAAGCCTGCTTTGAACACTCTAATTTTTTC
ACAGTAAAAGATAGGGATCCCGACGCCGACAACTTAATGTAAGAGCCGCT
CTCCCCAAGGATGACGGGAACCAACAACAGTACAGCCCCTTTCGAGACCG
ACCAGCCGTCCCCGCCAGAAATCCAACTACGAGCTTTTTAACTGCAACAA
CTTTAGTATACGCTATTGGAGCTGGAATTACCGCGGCTGCTGGCACCAGA
CTTGCCCTCCAATTGATCCTCGATAAGGGATTTAAATTGTTCTCATTCCAA
TAGCCAGAGTTAAACCCCGGCATTGTTATTTATTGTCACTACCTCCCTGTG
TCAGGATTGGGTAATTTACGCGCCTGCTGCCTTCCTTGGATGTGGTAGCCG
TCTCTCAGGCTCCCTCTCCGGAATCGAACCCCAATTCTCCGTTACCCGTTA
GAGCCATGGTAGGCCAATACCCTACCATCCAAAGCTGTCGA

Figure 3.7: Sequences of the 18S rRNA gene from *Nannochloropsis salina* strain (entry name ID: 6219391) using rbcl reverse primer.

3.2.2.5. Phylogenetic Analysis

BLAST analysis of this sequence produced over 100 different highly matched sequences (99%), all associated with the genus of *Nannochloropsis*. As shown in Table 3.2 the top 10 matches were associated with either *Nannochloropsis gaditana* strain or *Nannochloropsis salina* strain with a 99 % sequence similarity. Evidently, the 18S rRNA gene sequencing work was successfully established and identified that the microalgal isolate used in this study was indeed a species of green marine microalgal genus *Nannochloropsis* with correct identification at species level of *Nannochloropsis salina* strain. However, this isolate was obtained from the Culture Centre of Algae and Protozoa (CCAP), Oban, Scotland which was isolated near the Isle of Cumbrae in the Firth of Clyde by Hibberd in 1981 and classified belonging to Phylum: *Ochrophyta*,Order: *Eustigmatales* and Class: *Eustigmatophyceae*). Moreover, a phylogenetic tree and sequence alignment of 18S rRNA gene sequences

of *Nannochloropsis salina* strain CCAP 849/2 were already constructed and deposited with the GenBank nucleotide collection in Basic Local Alignment Search Tool (BLAST) on the NCBI websiteunder sequence ID: KJ756828.1as shown in Figures 3.8 and 3.9, respectively. Therefore, thephylogenetic information was used in comparison with sequence alignment of the 18S rRNA gene for the isolate from this study which was deposited under entry name (Order ID: 6219391) (Figure 3.10).

Description (Suggested identification from GenBank)	**Total score**	**Query match (18S rRNA % Similarity)**	**Accession number**
*Nannochloropsis sp.*RCC565	1077	99%	KT860976.1
*Nannochloropsis sp.*RCC562	1077	99%	KT860975.1
Nannochloropsis gaditana strain CCAP 849/6	1077	99%	KJ756832.1
Nannochloropsis salina strain CCAP 849/4	1077	99%	KJ756830.1
Nannochloropsis salina strain CCAP 849/2	1077	99%	KJ756828.1
Nannochloropsis gaditana strain CCMP526	1077	99%	KF040086.1
Nannochloropsis salina isolate D12	1077	99%	JX185299.1
Nannochloropsis gaditana isolate B	1077	99%	JF444989.1
Nannochloropsis gaditana strain IVP	1077	99%	EF473733.1
Nannochloropsis gaditana strain	1077	99%	AF133819.1

Table 3.2: Similarity between the 18S rRNA gene sequences of *Nannochloropsis salina* CCAP 849/2 strain and other related *Nannochloropsis* species based on MicroSeq database and EMBL public database by Eurofins/MWG.

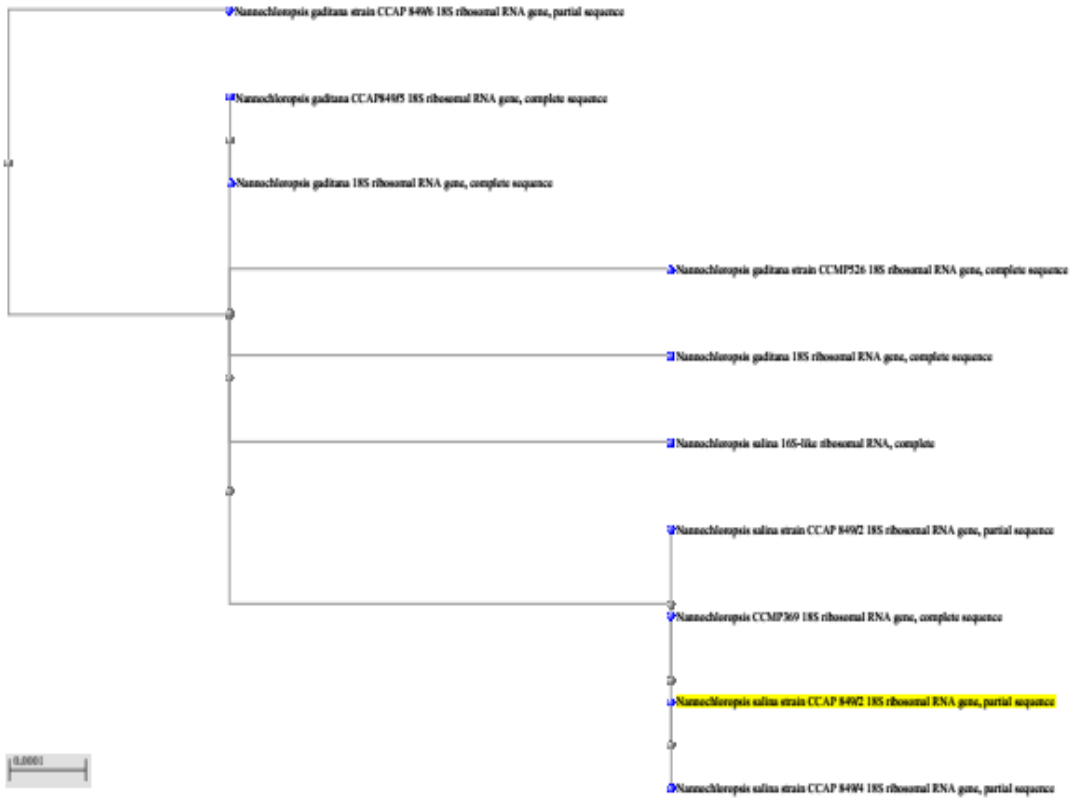


Figure 3.8: Phylogram (Phylogenetic tree) based on the 18S rDNA gene sequence for species of *Nannochloropsis salina* CCAP 849/2 associated with other members of the genus *Nannochloropsis* sp.

Nannochloropsis salina strain CCAP 849/2 18S ribosomal RNA gene, partial sequence.

Sequence ID: KJ756828.1 and Length: 1733 and Number of Matches: 1

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
3201 bits(1733)	0.0	1733/1733(100%)	0/1733(0%)	Plus/Plus

```
Query  1     TGGTTGATCCTGCCAGTAGTCATACGCTTGTCTCAAAGATTAAGCCATGCACGTCTGAGA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1     TGGTTGATCCTGCCAGTAGTCATACGCTTGTCTCAAAGATTAAGCCATGCACGTCTGAGA  60

Query  61    ATAAAGAGTTTTCTCTGAATCTGCGAATGGCTCATTATATCAGTTATAGTTTATTTGATA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61    ATAAAGAGTTTTCTCTGAATCTGCGAATGGCTCATTATATCAGTTATAGTTTATTTGATA  120

Query  121   GTCCTTTACTACATGGATAACCGTAGTAATTCTAGAGCTAATACATGCATCAAATCCCAA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121   GTCCTTTACTACATGGATAACCGTAGTAATTCTAGAGCTAATACATGCATCAAATCCCAA  180

Query  181   CTGCTTGTCGGAAGGGATGTATTTATTAGATAGAAACCAATGCGGGGCAACCCGGTATTG  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181   CTGCTTGTCGGAAGGGATGTATTTATTAGATAGAAACCAATGCGGGGCAACCCGGTATTG  240

Query  241   TGGTGAATCATGATAACTTTGCGGATCGCCGGCCTAGCCAGCGACGAATCATTCAAGTTT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241   TGGTGAATCATGATAACTTTGCGGATCGCCGGCCTAGCCAGCGACGAATCATTCAAGTTT  300

Query  301   CTGCCCTATCAGCTTTGGATGGTAGGGTATTGGCCTACCATGGCTCTAACGGGTAACGGA  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301   CTGCCCTATCAGCTTTGGATGGTAGGGTATTGGCCTACCATGGCTCTAACGGGTAACGGA  360

Query  361   GAATTGGGGTTCGATTCCGGAGAGGGAGCCTGAGAGACGGCTACCACATCCAAGGAAGGC  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361   GAATTGGGGTTCGATTCCGGAGAGGGAGCCTGAGAGACGGCTACCACATCCAAGGAAGGC  420

Query  421   AGCAGGCGCGTAAATTACCCAATCCTGACACAGGGAGGTAGTGACAATAAATAACAATGC  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421   AGCAGGCGCGTAAATTACCCAATCCTGACACAGGGAGGTAGTGACAATAAATAACAATGC  480

Query  481   CGGGGTTTAACTCTGGCTATTGGAATGAGAACAATTTAAATCCCTTATCGAGGATCAATT  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481   CGGGGTTTAACTCTGGCTATTGGAATGAGAACAATTTAAATCCCTTATCGAGGATCAATT  540

Query  541   GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATACTAAAG  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541   GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATACTAAAG  600

Query  601   TTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTCTGGCGGGGACGGCTGGTCGGTCTCGAA  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601   TTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTCTGGCGGGGACGGCTGGTCGGTCTCGAA  660

Query  661   AGGGGCTGTACTGTTGTTGGTTCCCGTCATCCTTGGGGAGAGCGGCTCTTACATTAAGTT  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  661   AGGGGCTGTACTGTTGTTGGTTCCCGTCATCCTTGGGGAGAGCGGCTCTTACATTAAGTT  720

Query  721   GTCGGCGTCGGGATCCCTATCTTTTACTGTGAAAAAATTAGAGTGTTCAAAGCAGGCTTA  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  721   GTCGGCGTCGGGATCCCTATCTTTTACTGTGAAAAAATTAGAGTGTTCAAAGCAGGCTTA  780

Query  781   GGCCCTGAATACATTAGCATGGAATAATAAGATACGACCTTGGTGGTCTATTTTGTTGGT  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  781   GGCCCTGAATACATTAGCATGGAATAATAAGATACGACCTTGGTGGTCTATTTTGTTGGT  840

Query  841   TTGCACGCCAAGGTAATGATTAATAGGGATAGTTGGGGGTATTCGTATTCAATTGTCAGA  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  841   TTGCACGCCAAGGTAATGATTAATAGGGATAGTTGGGGGTATTCGTATTCAATTGTCAGA  900

Query  901   GGTGAAATTCTTGGATTTATGGAAGACGAACTACTGCGAAAGCATTTACCAAGGATGTTT  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  901   GGTGAAATTCTTGGATTTATGGAAGACGAACTACTGCGAAAGCATTTACCAAGGATGTTT  960
```

```
Query  961   TCATTAATCAAGAACGAAAGTTAGGGGATCGAAGATGATTAGATACCATCGTAGTCTTAA  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  961   TCATTAATCAAGAACGAAAGTTAGGGGATCGAAGATGATTAGATACCATCGTAGTCTTAA  1020

Query  1021  CCATAAACTATGCCGACTAGGGATCGGTGGGTGCATTTTAAGGCCTCATCGGCACCTTAT  1080
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1021  CCATAAACTATGCCGACTAGGGATCGGTGGGTGCATTTTAAGGCCTCATCGGCACCTTAT  1080

Query  1081  GAGAAATCAAAGTCTTTGGGTTCCGGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGAA  1140
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1081  GAGAAATCAAAGTCTTTGGGTTCCGGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGAA  1140

Query  1141  ATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGGA  1200
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1141  ATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGGA  1200

Query  1201  AACTTACCAGGTCCAGACATAGTAAGGATTGACAGATTGAGAGCTCTTTCTTGATTCTAT  1260
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1201  AACTTACCAGGTCCAGACATAGTAAGGATTGACAGATTGAGAGCTCTTTCTTGATTCTAT  1260

Query  1261  GGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGTTAA  1320
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1261  GGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGTTAA  1320

Query  1321  CGAACGAGACCCCCGCCTGCTAAATAGTACTGGGAATGCTTAGCATTGTCAGAGACTTCT  1380
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1321  CGAACGAGACCCCCGCCTGCTAAATAGTACTGGGAATGCTTAGCATTGTCAGAGACTTCT  1380

Query  1381  TAGAGGGACTTTCGGCGCTAGGCCGAAGGAAGTTGGGGGCAATAACAGGTCTGTGATGCC  1440
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1381  TAGAGGGACTTTCGGCGCTAGGCCGAAGGAAGTTGGGGGCAATAACAGGTCTGTGATGCC  1440

Query  1441  CTTAGATGTCCTGGGCCGCACGCGCGCTACACTGATGCGTTCAACGAGTTTATAACCTTG  1500
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1441  CTTAGATGTCCTGGGCCGCACGCGCGCTACACTGATGCGTTCAACGAGTTTATAACCTTG  1500

Query  1501  TCCGGAAGGACCGGGTAATCTTGAAATGCGCATCGTGATAGGGATAGATTATTGCAACTA  1560
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1501  TCCGGAAGGACCGGGTAATCTTGAAATGCGCATCGTGATAGGGATAGATTATTGCAACTA  1560

Query  1561  TTAATCTTGAACGAGGAATTCCTAGTAAACGCGAGTCATCAGCTCGCATTGATTACGTCC  1620
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1561  TTAATCTTGAACGAGGAATTCCTAGTAAACGCGAGTCATCAGCTCGCATTGATTACGTCC  1620

Query  1621  CTGCCCTTTGTACACACCGCCCGTCGCACCTACCGATTGAATGATTCGGTGAAGCTTTCG  1680
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1621  CTGCCCTTTGTACACACCGCCCGTCGCACCTACCGATTGAATGATTCGGTGAAGCTTTCG  1680

Query  1681  GATTACGCCATCAGCCTCGGCCGACGGCGTGAGAAGTTATCTAAACCTCATCA  1733
             ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1681  GATTACGCCATCAGCCTCGGCCGACGGCGTGAGAAGTTATCTAAACCTCATCA  1733
```

Figure 3.9: Sequence alignment of the 18S rRNA gene of *Nannochloropsis salina* CCAP 849/2 strain (Accession number: KJ756828.1) in Gen Bank database.

Nannochloropsissalina strain CCAP 849/2 18S ribosomal RNA gene, partial sequence. Sequence ID: ID: 6219391 and Length: 589 Number of Matches: 1 Range.

Score	Expect	Identities	Gaps	Strand
1077 bits (583)	0.0	588/590(99%)	2/590 (0%)	Plus/Plus

```
Query  2    CTA-CATGGCTCT-ACGGGTAACGGAGAATTGGGGTTCGATTCCGGAGAGGGAGCCTGAG  59
            ||| ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  335  CTACCATGGCTCTAACGGGTAACGGAGAATTGGGGTTCGATTCCGGAGAGGGAGCCTGAG  394

Query  60   AGACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGTAAATTACCCAATCCTGACACAGG  119
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  395  AGACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGTAAATTACCCAATCCTGACACAGG  454

Query  120  GAGGTAGTGACAATAAATAACAATGCCGGGGTTTAACTCTGGCTATTGGAATGAGAACAA  179
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  455  GAGGTAGTGACAATAAATAACAATGCCGGGGTTTAACTCTGGCTATTGGAATGAGAACAA  514

Query  180  TTTAAATCCCTTATCGAGGATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAAT  239
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  515  TTTAAATCCCTTATCGAGGATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAAT  574

Query  240  TCCAGCTCCAATAGCGTATACTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTCT  299
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  575  TCCAGCTCCAATAGCGTATACTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTCT  634

Query  300  GGCGGGGACGGCTGGTCGGTCTCGAAAGGGGCTGTACTGTTGTTGGTTCCCGTCATCCTT  359
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  635  GGCGGGGACGGCTGGTCGGTCTCGAAAGGGGCTGTACTGTTGTTGGTTCCCGTCATCCTT  694

Query  360  GGGGAGAGCGGCTCTTACATTAAGTTGTCGGCGTCGGGATCCCTATCTTTTACTGTGAAA  419
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  695  GGGGAGAGCGGCTCTTACATTAAGTTGTCGGCGTCGGGATCCCTATCTTTTACTGTGAAA  754

Query  420  AAATTAGAGTGTTCAAAGCAGGCTTAGGCCCTGAATACATTAGCATGGAATAATAAGATA  479
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  755  AAATTAGAGTGTTCAAAGCAGGCTTAGGCCCTGAATACATTAGCATGGAATAATAAGATA  814

Query  480  CGACCTTGGTGGTCTATTTTGTTGGTTTGCACGCCAAGGTAATGATTAATAGGGATAGTT  539
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  815  CGACCTTGGTGGTCTATTTTGTTGGTTTGCACGCCAAGGTAATGATTAATAGGGATAGTT  874

Query  540  GGGGGTATTCGTATTCAATTGTCAGAGGTGAAATTCTTGGATTTATGGAA  589
            |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  875  GGGGGTATTCGTATTCAATTGTCAGAGGTGAAATTCTTGGATTTATGGAA  924
```

Figure 3.10: Comparison sequence alignment of the 18S rRNA gene of*Nannochloropsis salina* CCAP 849/2 strain (Accession number: KJ756828.1) and *Nannochloropsis salina* strain (Order ID: 6219391) in Gen Bank database.

3.3. Conclusion

In this study, the basic characterisation of *Nannochloropsis salina*, which belongs to class Eustigmatophyceae grown in the f/2 growth medium to stationary growth phase was observed using the light microscope. The result found was that cells of *N. salina* were non motile cells and with a spherical shape and smooth cell wall. However, other cell structures including parietal chloroplast, eyespot and nucleus were not observed.

The molecular identification of *Nannochloropsis* cells was successfully achieved using molecular and phylogenetic analyses of 18S rDNA gene sequences and *rbcL* genes. The genomic DNA of *Nannochloropsis* species was successfully extracted by the ZR Soil Microbe DNA MicroPrep kit which was superior in comparison to the CTAB method. The DNA extracted was used to amplify the sequences of the 18S ribosomal RNA gene using a PCR protocol and purified to completely remove contaminating compounds. The gene sequences for the studied strain were highly similar to the genus *Nannochloropsis* with a 99 % sequence similarity associated with the *Nannochloropsis salina* strain which was isolated from near the Isle of Cumbrae in the Firth of Clyde in Scotland, UK by Hibberd in 1981.

Physiological Characterization of Unicellular *Nannochloropsis salina* Strain CCAP 849/2

Chapter Four

4.1. Introduction

Microalgae are autotrophic organism that can consume light energy and inorganic compounds to produce microalgal biomass containing high value products including carbohydrates, lipids, proteins and pigments (Markou and Nerantzis, 2013). In addition, microalgae cultures can also produce metabolite products including some carotenoids such as lutein, zeaxanthin, and astaxanthin and long chain polyunsaturated fatty acids (PUFA) and vitamins (Pal *et al.,* 2011). These value-added bioactive compounds are widely used in nutraceutical industries such as food additives (Priyadarshani and Rath, 2012). Moreover, microalgae cultures are commonly used as live feed for fish, crustaceans and molluscs with all growth stages (Meireles *et al.,* 2003).

Recently, microalgae have been receiving significant attention due to their key characteristics for using solar light as the main energy source and their ecological role in phytoremediation (Xue *et al.,* 2011; Yoshida *et al.,* 2006). Microalgae are receiving increased interest as potential feedstock for production of biofuels to solve the global energy crisis due to their high photosynthetic rate and their ability to produce large amounts of lipid (Triacylglycerides, TAGs) under different growth conditions and be cultured on an industrial scale (Demirbas, 2011; Huerlimann *et al.,* 2010; Malcata, 2011; Suali and Sarbatly, 2012; Liu and Benning, 2013). Furthermore, the cultivation of microalgae can be optimized by regulating the environmental parameters included such as salinity, temperature and pH (Moheimani and Borowitzka, 2011; Moazami *et al.,* 2012; Bartley *et al.,* 2013).

However, the growth performance of microalgae is typically determined by the quality of medium used for their cultivation (Lam and Lee, 2012; Li *et al.,* 2012).The organic fertilizer that is produced from derivatives of food waste and composted sea

weed are widely used as an alternative nutrient for the preparation of cultivation media for microalgae in outdoor cultures (Lam and Lee, 2012; Alvarado *et al.*, 2008). On the other hand, a more stringent composition of nutrients is required in growth media to produce cultures of pure microalgal strains. Therefore, special formulation of cultivation media such as f/2 medium and Conway medium are typically used to generate microalgae monocultures at the laboratory scale. Enough supplement of nutrients for microalgae growth is a key step to produce the volume and quantity of high value microalgae biomass (Xin *et al.*, 2010; Lananan *et al.*, 2013).

Hence, the present study was performed to evaluate the effect of three cultivation media including and f/2 Medium, Artificial Multipurpose Complement for the Nutrition of Algae (AMCONA) medium and defined f/2 medium on the growth performance of *Nannochloropsis salina* cells. The *N. salina* strain was further studied based on its ability to produce lipid under different environmental conditions including pH, different salinity concentrations and nitrogen starvation using f/2 medium.

4.2. Results and Discussion

4.2.1. Effect of AMCONA Medium on the Growth of *N. salina* Cells

The *Nannochloropsis salina* strain was cultured in two different media including f/2 Guillard's culture medium and Artificial Multipurpose Complement for the Nutrition of Algae (AMCONA) medium (Fanesi *et al.,* 2014) as the nutrient supplement to observe which medium was the most appropriate based on the highest growth potential. Three replicate OD_{595} measurements for each medium were performed each day with initial OD_{595} for both cultures being 0.045 and 0.046, respectively, for a period of 25 days at 25°C in order to reach a growth plateau.From the growth curves shown in Figure 4.1, it was found that the growth of *Nannochloropsis salina* cells in f/2 medium was higher than its growth in AMCONA medium. However, in the f/2 medium, the optical density of *N. salina* cells at 595 nm reached 0.849 and 1.384 after 14 and 25 days incubation, respectively. For the AMCONA medium, the optical density of *N. salina* cells at 595 nm reached 0.219 and 0.421 after 14 and 25 days incubation respectively and therefore the AMCONA medium could not support the growth of *N. salina* cells. It was clear that these findings contrasted with the results reported for *Dunaliella salina* cells grown with the AMCONA medium that showed good growth with different NaCl concentrations (Giordano *et al.,* 2014).

This investigation showed that the f/2 medium was the optimum growth medium for growing *N. salina* and therefore was accepted and used as growth medium for the current studies. Moreover, this medium is a common and widely used general enriched seawater medium designed for growing coastal marine algae, especially diatoms (Guillard and Ryther, 1962; Guillard, 1975). It can be made using filtered natural seawater or by adding commercially available seawater salt mixture (Ultramarine Synthetica Sea Salt) as in the present work. However, in either case, the

f/2 medium is not recognised as a defined medium due to the concentration of all salts not being known. On a confidential basis, the manufacture of the synthetic seawater salt mix provided a recipe and this was adapted to produce the defined f/2 medium. Thus the defined f/2 medium was examined as a culture medium for growing the *N. salina* cells in comparison with f/2 medium to observe which medium was the best for supporting *N. salina* cell growth under stress conditions such as effects of salinity on growth and lipid accumulation in the next experiments.

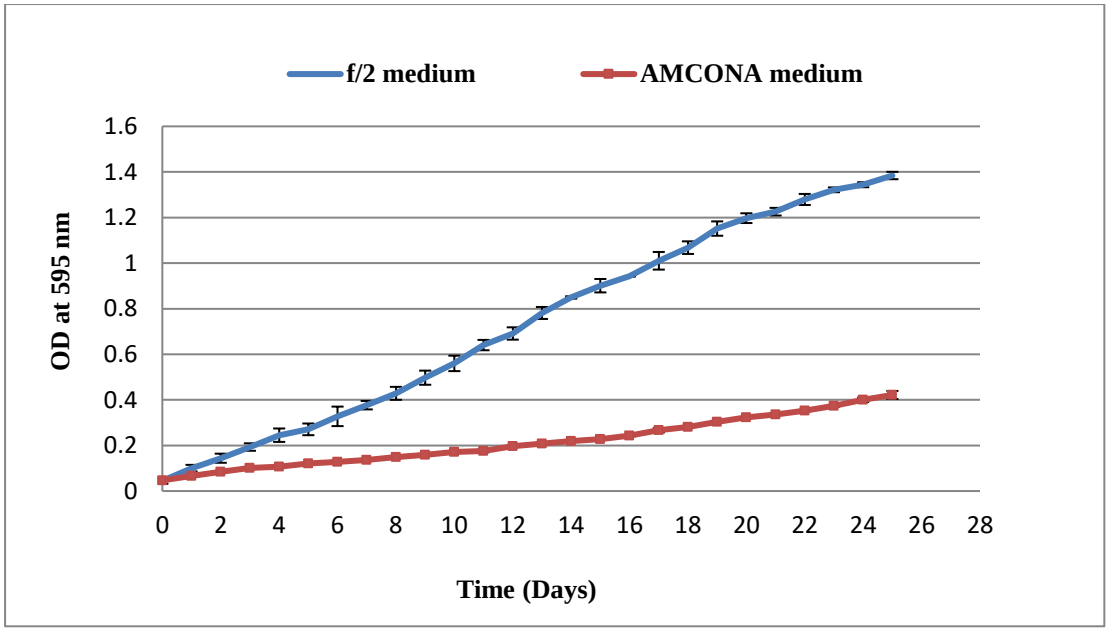


Figure 4.1: Growth curves for *N. salina*cells grown in f/2 medium and AMCONA medium, incubated in a 25ºC constant temperature room for 25 days. Data points are the means of three replicates plus or minus the standard error.

4.2.2. Comparison of F/2 Medium and Defined F/2 Medium

One of the advantages of using microalgae for biofuel production is the ability to control their accumulation and secretion of biofuel precursors by changing their growth conditions or by metabolic engineering. However, the idea was to identify a defined medium (all components known) to replace the rich f/2 medium which normally uses the commercial seawater salt mixture. This led to the development of a

defined f/2 medium where the quantity of the chemicals added to the media such as N, P, and NaCl can be adjusted. However, after the development of f/2 defined medium, a comparison with the growth rate of *N. salina* cells in normal f/2 medium was carried out to observe which medium is the most appropriate based on the highest growth potential. Figure 4.2 shows the growth curves of *N. salina* cells grown in normal f/2 medium and defined f/2 medium at 25ºC over 25 days of incubation with initial OD_{595} for both cultures being 0.045 and 0.047, respectively. It was found that, *N. salina* cells grew well in both f/2 medium and defined f/2 medium and no significant differences were detected between the measurements of optical density at 595 nm where the optical density of *N. salina* cells reached 0.849 and 0.778 in f/2 medium and defined f/2 medium, respectively, over the first 14 days of incubation but, there was slight differences between the measurements of optical density over the next 10 days of incubation, where the optical density of *N. salina* cells reached 1.384 and 1.240 at 25 days of incubation in f/2 medium and defined f/2 medium, respectively. The t-test was done at level p (α) 0.05 and showed that the results at day 25 were statistically significant. It was clear that the normal f/2 medium produced a higher biomass than defined f/2 medium and supported good growth of *N. salina* cells. Overall, f/2 medium is a common and widely used general enriched seawater medium designed for growing coastal marine algae, especially diatoms (Guillard and Ryther, 1962; Guillard, 1975). Therefore, f/2 medium was selected as the growth medium for further study in this work. However, in this study, the modified f/2 Guillard's culture medium was prepared by dissolving 33.6 g of Ultramarine Synthetica Sea Salt in 950 ml of distilled water in a 1 litre Duran bottle by stirring and then the volume was made up to 1000 ml with distilled water.

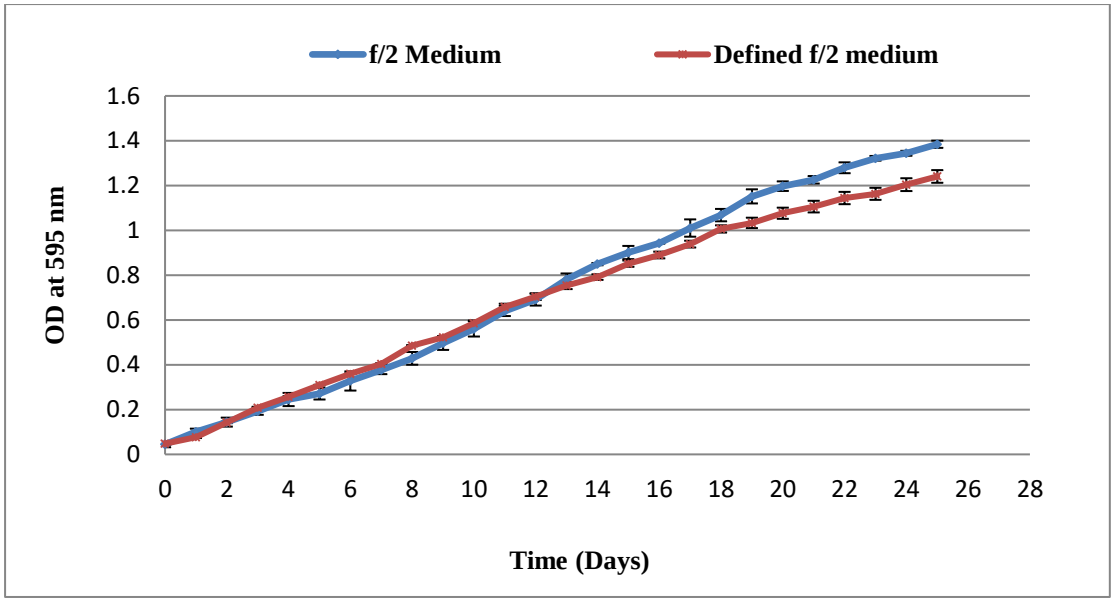


Figure 4.2: Comparison of growth of *N. salina* cells in normal f/2 medium and defined f/2 medium. Cells were incubated in a 25ºC constant temperature room for 25 days. The OD for each medium was measured at 595 nm. Each point represents the mean of three readings plus/minus the standard error.

4.2.3. Effect of Vitamin B12 Concentration on The Growth of *N. salina* Cells

Many species of microalgae require an exogenous source of vitamin B12 for their growth as a cofactor in vitamin B12-dependent enzymes, which are regularly acquired during symbiosis with bacteria (Croft *et al.*, 2005). It was necessary, therefore, to investigate the requirement of vitamin B12 for growth of *N. salina* cells. To produce the growth curve, cells were grown in both normal f/2 medium for marine microalgae and vitamin B12 free f/2 medium (vitamin B12 was omitted) under the same growth conditions in a 25ºC constant temperature room for 25 incubation days with initial OD_{595} for both cultures being 0.045 and 0.065, respectively.

Figure 4.3 represents the growth rates of *N. salina* cells in both media that mentioned above. It was clear that the cells of *N. salina* exhibited similar growth over the first 12 incubation days, where the optical density of *N. salina* cells reached 0.681 and

0.659 in normal f/2 medium and vitamin B12 free f/2 medium, respectively. There were, however, slight but significant differences observed between the growth rates of *N. salina* cells in both media over the next 13 incubation days, where the optical density of *N. salina* cells at OD_{595} reached 1.384 in normal f/2 medium and 1.181 in f/2 medium without vitamin B12. The t-test was done at level p (α) 0.05 and showed that the results at day 25 were statistically significant.

The results very clearly indicate that f/2 medium successfully supported the growth of *N. salina* cells through out the incubation period when the vitamin B12 was removed. These results indicated that the vitamin B12 was not an essential compound for maintaining growth of *N. salina* cells. However, these findings are in good agreement with those of Jinkerson *et al.,* (2013) who observed that *Nannochloropsis* cells do not require an exogenous vitamin B12 supplemented media for growth and as they contain several vitamin B12 dependent enzymes, the addition of this micronutrient can augment its metabolic capabilities.

In general, it appears that many species of microalgae can only generate methionine with vitamin B12-dependent methionine synthase, as the methionine synthesis can occur in the absence of vitamin B12 via vitamin B12-independent methionine synthase (Croft *et al.,* 2005). *Nannochloropsis* cells contain both vitamin B12 dependent and vitamin B12-independent methionine synthase. However, the vitamin B12-dependent enzyme is often preferred when vitamin B12 is available due to higher catalytic efficiency (Gonzalez *et al*., 1992).

Further studies are required to investigate if addition of this vitamin can enhanced growth or improve the production of biofuel in *Nannochloropsis salina* cells. Therefore, the normal f/2 medium for marine microalgae containing vitamin B12 was considered as growth medium for further development study in this work.

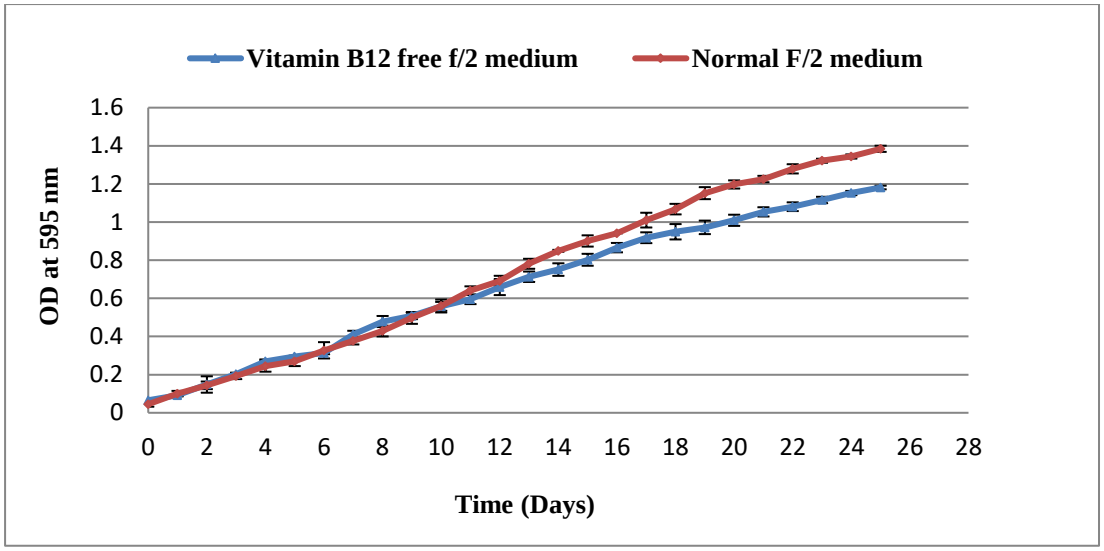


Figure 4.3: Growth rate of *N. salina* cells grown in f/2 medium in comparison with growth rate of *N. salina* cells grown in vitamin B12 free f/2 medium. Cells were incubated in a 25ºC constant temperature room for 25 days. Growth rate was determined by measuring the OD at 595 nm every day. Each point represents the mean of three readings plus or minus the standard error.

4.2.4. Relationship Between OD_{595} and Cell Number of *N. salina* Cells

To allow cell number to be calculated from OD values of *N. salina* cells, a concentration curve of *N. salina* cells grown in normal f/2 growth medium for marine microalgae was produced using 12 dilutions of microalgal culture. The standard concentration curve of cell number was plotted on Excel using optical density (OD) reading (X axis) against cell count measurements of each sample (Y axis) as shown in Figure 4.4. These observations showed a strong correlation between optical density (OD) reading increases at 595 nm and cell numbers of *N. salina* cells grown in normal f/2 growth medium for each dilution.

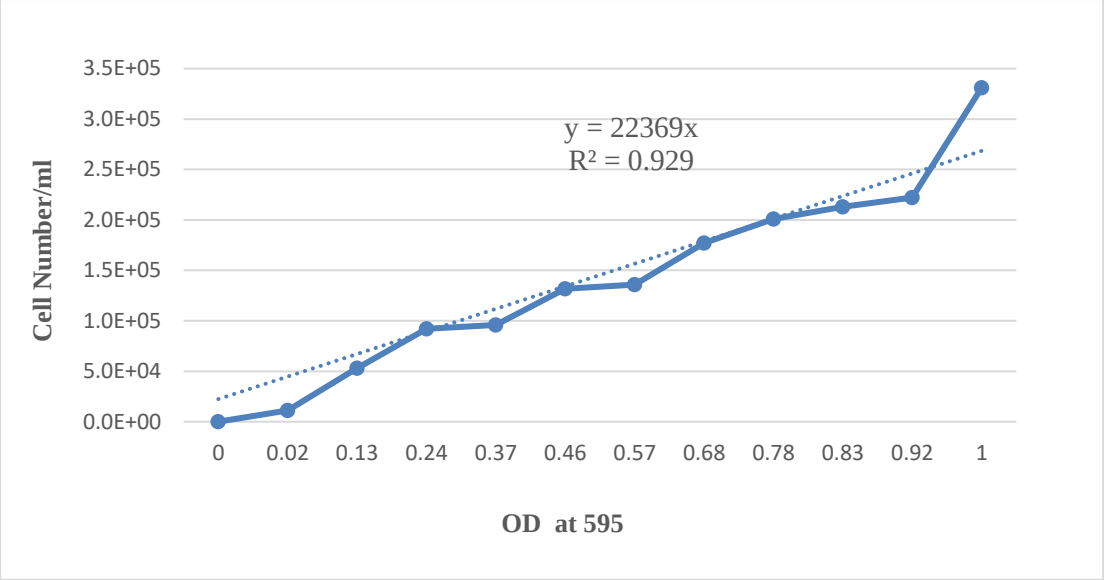


Figure:4.4: Average of cell number for *N. salina* cells grown in normal f/2 growth medium. The concentration curve of *N. salina* cellswas plotted using optical density (OD) at 595 reading against range of cell number in each dilution (Relationship between OD_{595} and cell count of *N. salina* cells).

4.2.5. Microalgal Dry Weight Determination for *N. salina* Cells

The biomass dry weight of *N. salina* cells grown in f/2 growth medium was determined at different cell concentrations using 12 dilutions of microalgal culture. Table 4.1 shows the result of the biomass dry weight of *N. salina* cells which was calculated as mg ml^{-1} of cells and the optical density measurements at 595 nm for each dilution. It is clear that from these findings, the biomass dry weight of *N. salina* cells increased with increasing the concentration of sample (OD_{595}). However, the main purpose of this experiment was to establish the relationship between optical density at 595 nm and dry weight biomass of *N. salina* cells to use it as standard dry weight curve of *N. salina* cells, in order to convert the levels of Nile Red fluorescence readings (lipid measurements in Nile Red procedure) obtained from the Triolein calibration curve into percentage of lipid for algal cells (% dry weight) using the graph equation. The standard concentration curve of dry weight in *N. salina* cells was

plotted using optical density (OD_{595}) reading (X axis) against dry weight biomass measurements of each concentration (Y axis) as shown in Figure 4.5.

Tube number	Concentration cells (Dilutions)	Culture (ml)	Medium (ml)	OD_{595}	DryWeight (mg/30 ml)	Dry Weight (mg/ ml)	Dry Weight (mg/L)
12	0.0	0	30	1.050	18.6	0.62	620
11	8.3	2.5	27.5	0.995	17.1	0.57	570
10	16.6	5	25	0.944	16.1	0.54	540
9	33.3	10	20	0.810	13.4	0.45	400
8	41.6	12.5	17.5	0.729	10.8	0.36	360
7	50	15	15	0.661	9.4	0.31	310
6	58.3	17.5	12.5	0.595	8.4	0.28	280
5	66.6	20	10	0.503	6.6	0.22	220
4	75	22.5	7.5	0.409	5.0	0.16	170
3	83.3	25	5	0.271	1.6	0.05	50
2	91.6	27.5	2.5	0.097	0.8	0.03	30
1	100	30	0	0	0	0	0

Table 4.1: Microalgal culture concentrations (12 culture dilutions), optical density at 595 nm reading and amount of dry weight calculated as mg/30ml, mg/ml and mg/L of microalgal biomass dry weight, respectively.

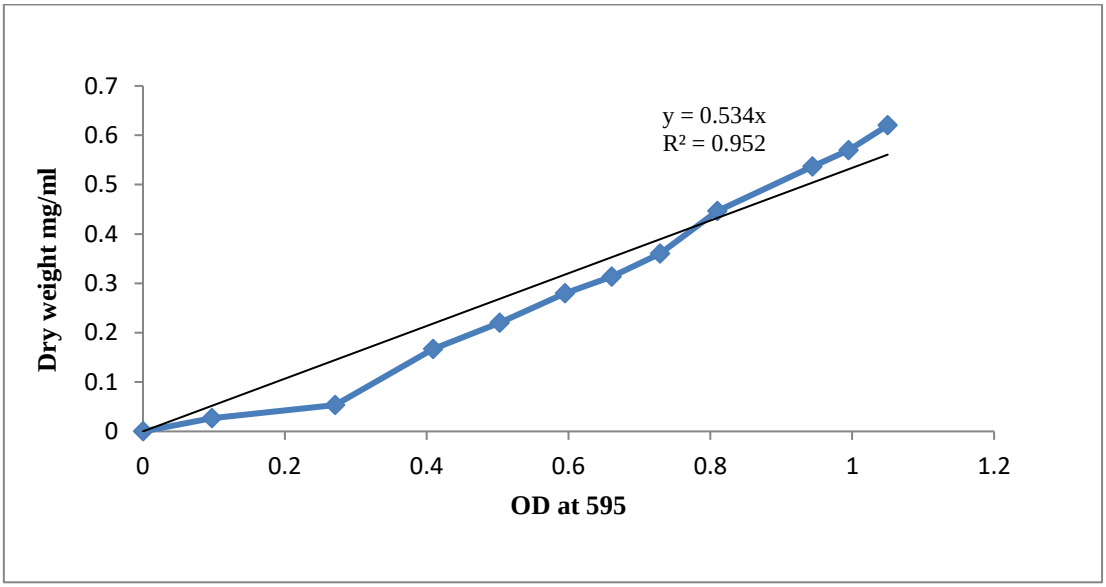


Figure 4.5: Linear relationship between OD and dry weight of *N. salina* cells. The concentration curve was plotted using optical density (OD) reading against dry weight of each sample.

4.2.6. Effect of Adaptation to Different Salinity Concentrations on *N. salina* Growth

In this experiment, the effect of adaptation at five different salinity concentrations on the *N. salina* cells growth was evaluated every day using a spectrophotometer to measure the cell density (OD) at 595 nm during the incubation period.

The cell density measurements at 595 nm of *N. salina* cells adapted to grow at different salt concentration values (0.2 M, 0.4 M, 0.6 M, 0.8 M and 1M NaCl) in f/2 medium was shown in Figure 4.6. It was found that the specific growth rate and cell growth productivity revealed growth of *N. salina* cells was higher at 0.2 M, 0.4 M, 0.8 M and 1M salinity. However, the highest specific growth rate of *N. salina* cellswas oserved at 0.2 M salinity and was lowest at 0.6 M NaCl. Generally, *N. salina* cells grew well at 0.2 M and 0.8 M salinity with the cell density reading reaching 0.5 at 595 nm after 14 days incubation at 25°C compared with their growth at 0.4 M, 0.6 M and 1 M of NaCl which the cell density readin greached 0.5 after 16, 29 and 19 days incubation at 25°C, respectively.

However, the findings of the experiment demonstrated the ability of *N. salina* cells to fully adapt to grow well across salinities from 0.2 M to 1M with different times of incubation. These results for *N. salina* cells are in agreement with the results which reported that the salinity tolerance of *Nannochloropsis* sp. is changeable with an optimum around seawater salinity range of 0.5 M to 0.6 M NaCl and a salinity growth range of 0.1M to 1M NaCl (Pal *et al.*, 2011). Conversely,these findings do not agree with the results of earlier work that confirmed a growth-permitting range (0.1M - 1.0 M) and optimal growth for *N. salina* cells at 0.6 M NaCl (Boussiba *et al.*, 1987).

The Observation of optimal salinity, will inform the stress conditions for lipid induction by these strains in the next experiments. Moreover, further studies must

consider application of higher salinity concentrations up to 1M NaCl to find the upper salinity limit for *N. salina* cell growth in next experiments.

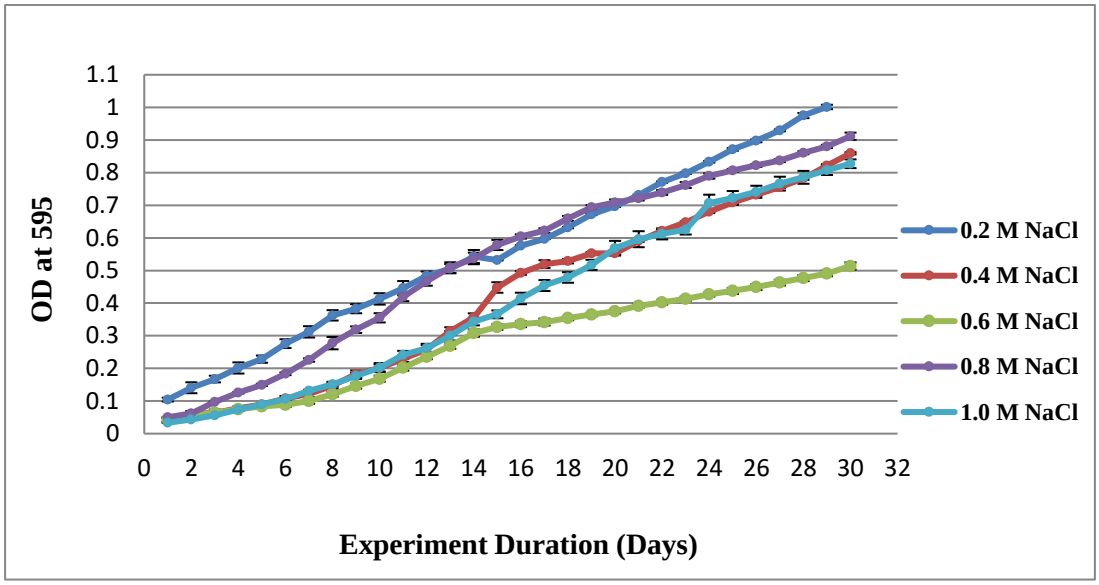


Figure 4.6: Growth curves for *N. salina* cells grown in f/2 medium adapted to grow at different salinity values (0.2, 0.4, 0.6, 0.8 and 1M NaCl) incubated for a period of 30 days incubation at 25ºC constant temperature room. The OD for each medium was measured at 595 nm. Data points are the means of three replicates plus or minus the standard error.

4.2.7. Effect of Nitrogen Concentration on Cell Growth of *N. salina*

Nitrogen concentration in the culture medium is considered as a principal factor directly affecting cell growth, biochemical composition of microalgae and determining the carbohydrate or lipid productivity for biofuels (Yeh and Chang, 2011; Kim *et al.,* 2016). Nitrogen starvationis well reported to trigger a high quantity of lipid accumulation in many microalgae (Illman *et al.,* 2000; Converti *et al.,* 2009), and consequently, poor cell growth could lead to an overall decreased microalgal lipid production (Piorreck *et al.,* 1984; Li *et al.,* 2008). Nitrogen limitation is thus one of the key factors widely used to generate lipid synthesis in microalgae (Chu *et al.,* 2014; Mandotra *et al.,* 2014). Therefore, it is most important to establish the right culture

medium and cultivation conditions to obtain optimum lipid production (Wan *et al.,* 2013).

In this study, the effect of different nitrogen concentrations on the cell growth of *N. salina* was estimated using a spectrophotometer at 595 nm for a period of 30 days incubation to investigate the appropriate culture medium to obtain optimum lipid production. Figure 4.7 illustrates the growth curves of *N. salina* cells grown at different nitrate concentrations (Nitrogen free, 25%, 50% and 75% of $NaNO_3$) in f/2 medium cultivated for 30 days.The relationship between cell growth and nitrogen limitation in the medium indicated that the *N. salina* cells grew better at 25%, 50% and 75% of $NaNO_3$ and although the highest biomass was found under the highest nitrate concentration, there was no significant difference. Whereas the lowest specific growth rate of *N. salina* cells was found under nitrogen-deficient condition with nitrogen free f/2 medium.These findings are in good agreements with those of Yeesang and Cheirsilp (2011) who observed that the cell growth of four green microalgae isolated from freshwater was increased with increased nitrogen concentration in culture medium. Similar results were found for *C. vulgaris,* where the growth rate was lower on low nitrogen medium but the final cell numbers were a little higher (Illman *et al.,* 2000). The same observation was reported for green microalga *Dunaliella tertiolecta,*when the organism was grown in modified Erdschreiber's medium with various concentrations of nitrate and ammonium (Chen *et al.,* 2011b).

It was reported by Ahlgren and Hyenstrand (2003) that microalgae grown under nitrogen-deficient conditions mainly accumulate a surplus of carbon metabolites as lipids. It was also confirmed that cells of microalgae respond to the nitrogen limitation condition by degrading the nitrogen containing macromolecules and then

accumulating carbon reserve compounds such as polysaccharides and fats (Banerjee *et al.*, 2002; Dayananda *et al.*, 2005).

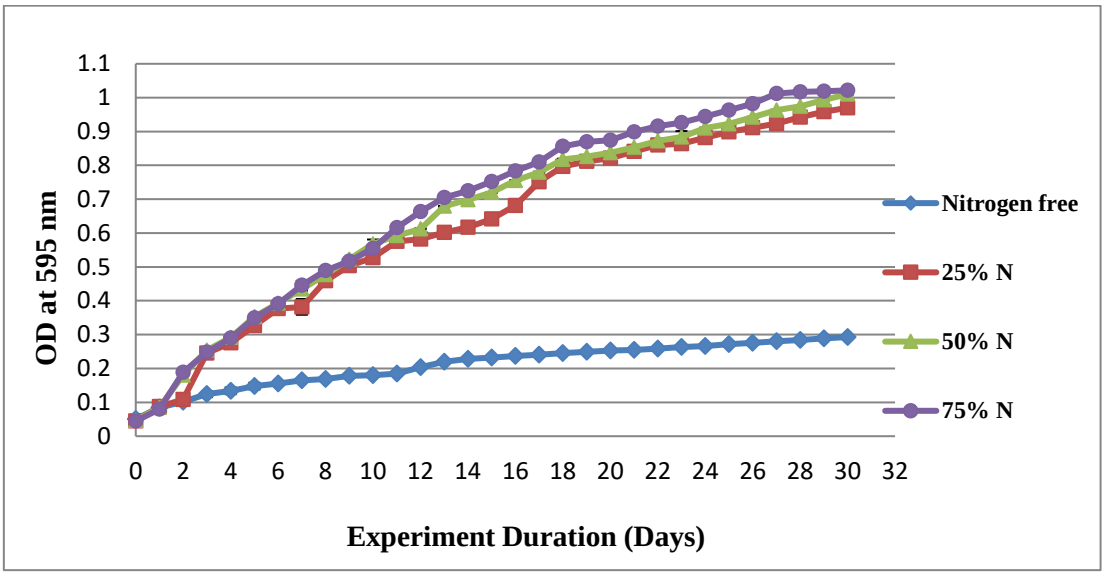


Figure 4.7: Effect of nitrogen concentrations (Nitrogen free, 25%, 50% and 75% of $NaNO_3$) on cell growth of *N. salina* grown in f/2 medium. Cells were incubated in a 25ºC constant temperature room for 30 days. The OD for each medium was measured at 595 nm. Data points are the means of three replicates plus or minus the standard error.

4.2.8. Effect of pH on Growth of *N. salina* and Growth Curve Determination

To further investigate the effect of environmental parameters on *N. salina* cells, the effect of pH on growth rates was evaluated every day using the optical density (OD) at 595 nm of *N. salina* cells growin in f/2 growth medium prepared at different pH levels of pH 5, 6, 7, 8, 9 and 10 using a spectrophotometer against a distilled water blank in 1 ml plastic cuvettes. Three replicate OD_{595} measurements for each pH were performed each day for 25 days incubation with initial OD_{595} about 0.045. Figure 4.8presents the influence of different pH levels on the growth of *N. salina* cells. It can be seen from this figure that the pH can be used to predict the growth rates of *N. salina* cells.

However, the highest growth rate wasfound in *N. salina* cells grown at pH 8, while the optical density at 595 nm reached 1.301 after 25 days incubation followed by the growth rate of *N. salina* cells grown at pH 7 while the optical density at 595 nm reached 1.117 after the same time of incubation. On the other hand, the lowest growth rates were seen at pH 5, while the optical density at 595 nm reached 0.536 after 25 days incubation. Furthermore, the growth rate of *N. salina* cells was decreased when the pH was increased up to pH 10. It appears that both low and high pH will not support optimum growth of *N. salina* cells. These findings are in close agreement with Bartley *et al.*, (2014) who measured the growth rates of *N. salina* cells at different pH and found that the highest growth was found in the cells grown at pH 9.Similar results were found when *Nannochloropsis* sp. and *Tetraselmis* sp. were examined under different pH of 5.5, 7.5, 8.5 and 9.5, where high cell densitywas obtained when cultured at pH 8.5 and 7.5 for both species (Khatoon *et al.*, 2014).

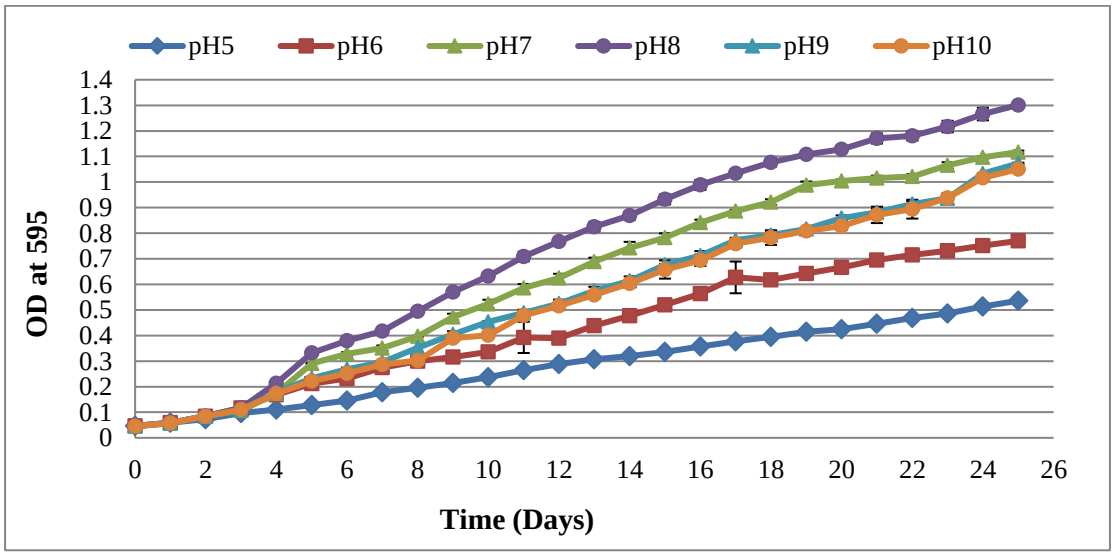


Figure 4.8: Effect of pH on growth of *N. salina* cells grown in f/2 medium with different pH values of pH 5, pH 6, pH 7, pH 8, pH 9, and pH 10. Cells were incubated in a 25°C constant temperature room for 25 days. The OD for each medium was measured at 595 nm. Data points are the means of three replicates plus or minus the standard error.

4.3. Conclusion

Different species of *Nannochloropsis* have recently been considered as appealing feedstocks for biodiesel production due to their ability to accumulate high amounts of lipids. Morever, some of these species are used in marine aquaculture as an important tsource of eicosapentaenoic acid (Bongiovani *et al.*, 2014). Three different culture media including modified f/2 growth medium without silica, artificial AMCONA medium and f/2 defined medium were used to confirm the optimum growth medium for unicellular *Nannochloropsis salina* cells based on the highest growth potential and this was shown to be F/2 medium, which is a very widely used seawater medium that can be made using filtered natural seawater or by adding commercially available seawater salt mixtures. However, in comparison with f/2 defined medium, the results were showed that *N. salina* cells grew well in both f/2 medium and defined f/2 medium and no significant differences were detected between the measurements of optical density at 595 nm over the first 14 days of incubation (Figures 4.2). However, the growth of *N. salina* was highest in f/2 growth medium with optical density of 1.384 at 595 nm over the next 10 days of incubation. It was clear that the normal f/2 medium produced a higher biomass than defined f/2 medium and supported good growth of *N. salina* cells. On the other hand, poorest growth was found in the AMCONA medium and this did not support the growth of *N. salina* cells with optical density of 0.421 nm over the incubation period (Figures 4.1). The requirement of vitamin B12 for *N. salina* cells growth was investigated using vitamin B12 free f/2 medium and normal f/2 medium under the same growth conditions.The results very clearly indicated that f/2 medium successfully supported the growth of *N. salina* cells through incubation period of 25 days when the vitamin B12 was removed and these results indicated that the vitamin B12 was not an essential compound for maintaining growth of *N. salina* cells (Figure

4.3). At an early stage in the adaptation process, the ability of *N. salina* cells to adapt to grow over a range of salinities (0.2 M, 0.4 M, 0.6 M, 0.8 M and 1M NaCl) was examined and the growth curves were repeated using f/2 growth medium. It was found that *N. salina* cells grew well at salinities values of 0.2 M, 0.4 M, 0.8 M and 1M with lowest specific growth rate at 0.6 M NaCl over 30 days incubation at 25ºC (Figure 4.6).

To investigate the effect of different nitrogen concentrations on the cell growth of *N. salina*, the cells was cultured and incubated in a 25ºC constant temperature room for 30 day susing f/2 growth medium combined with different nitrate concentrations of 75%, 50%, 25% of $NaNO_3$ compared with nitrogen free f/2 growth medium. However, it was found that the lowest specific growth rate of *N. salina* cells was under nitrogen-deficient conditions with nitrogen free f/2 growth medium. Moreover, *N. salina* cells grew well at other $NaNO_3$ concentrations tested and there was no significant difference in specific growth rate (Figure 4.7). The next step was to examine the ability of *N. salina* cells to grow and survive at external pH values 5, 6, 7, 8, 9 and 10 in f/2 growth medium. The results of the experiments showed thatthe highest growth rate were found in *N. salina* cells grown at pH 8, with OD_{595} 1.301 nm over 25 days incubation followed by the growth rate of *N. salina* cells grown at pH 7 with OD_{595} 1.117 nm. While, the lowest growth rates were found at pH 5, with OD_{595} 0.536 nm over the same incubation time(Figure 4.8). From these results, it was concluded that either low or high pH will not support optimum growth of *N. salina* cells.However, based on the results of the experiments, it was decided to apply stress conditions for lipid induction in the *Nannochloropsis salina* strain in the next experiments.

Determination and Quantification of Neutral Lipid Content and Fatty Acid Composition

Chapter Five

5.1. Introduction

Microalgal biofuel biotechnology exploits microalgal photosynthesis and biosynthesis processes to produce microalgal lipid using only sunlight, CO_2, water and limited nutrients (Iwai *et al.,* 2015). The potential for using some marine microalgal species as feedstock for the production of various renewable fuels, such as biodiesel and bioethanol have been receiving increased attention (Chen *et al.,* 2009; Iwai *et al.,* 2015; Kim *et al.,* 2015; Satpati and Pal, 2015). *Nannochloropsis* sp., for example, are considered as significant industrial strains for both their high growth rate and their ability to accumulate large quantities of neutral lipids (triacylglyceride, TAG) (Wang *et al.,* 2014a; Kang *et al.,* 2015). However, production of microalgal biomass and biofuel precursors is usually affected by various environmental parameters including light supply, temperature, pH, salinity and nutrients limitation (Hu *etal*., 2008; Kim *et al.,* 2014; Yen *et al.,* 2014; Kim *et al.,* 2016; Bartley *et al.,* 2016; Zhu *et al*., 2016). Therefore, even a minor fluctuation in those parameters can cause changes in the growth and composition of microalgal cells either by decreasing or increasing the nutritional value and subsequently, the overall commercial value of the microalgae will be affected (Khatoon *et al.,* 2011). These physicochemical factors also impact on invading organisms of *Nannochloropsis* cultures and their overall effect on lipid accumulation has been less well studied (Bartley *et al.,* 2014).

Numerous studies have extensively demonstrated that final culture densities of microalgal cultivation and lipid productivity by marine microalgae can be improved in different ways by regulating environmental factors (Rocha *et al.,* 2003; Doan *et al.,* 2011; Moazami *et al.,* 2012; Wagenen *et al.,* 2012; Bartley *et al.,* 2013; Kang *et al.,* 2015). For example, the variations in salinity concentration in the culture medium directly influence the optimal growth and biochemical composition of microalgal

cultures (e.g. decreasing salinity has been reported to alter starch metabolism) (Yao *et al.,* 2013; Khatoon *et al.,* 2014). On the other hand, high salinity mostly leads to inhibition of growth and increased lipid accumulation of microalgal cells (Takagi *et al.,* 2006). Notably, hydrogen ion concentration (pH) of the surrounding medium an essential factor which seriously affectes the optimal growth of microalgal cultures and any changes in external pH can lead to significant change in the biochemical compositions of microalgae or it can cause cell death (Khalil *et al.,* 2010). Moreover, microalgae are able to grow well when the pH is within a range of 6 to 9, not taking into account other growth factors (Wang *et al.,* 2011). Nitrogen is particularly identified as one of the most critical nutrients which is required for the organism's growth. Nitrogen is a constituent in structural and functional proteins (such as enzymes, peptides), chlorophylls, energy transfer molecules (ATP, ADP) and also genetic materials included DNA and RNA molecules in cells (Cai *et al.,* 2013). The nitrogen concentration in the medium considerably affects cell growth and biochemical composition in the microalgal cells (Wang *et al.,* 2013). However, the cell growth rate of microalgae decreases and there is an increase in their neutral lipid content which can be used to produce biodiesel (when the nitrogen concentration is limited in culture medium) (Ho *et al.,* 2014b). Accordingly, there are a number of ways to measure lipids including the Nile Red (NR) staining method, Gravimetric techniques, Gas Chromatography-Mass Spectrometry (GC-MS) techniques and Nuclear Magnetic Resonance (NMR) techniques.

The Gravimetric technique is a conventional method for microalgal lipid quantified by cell disruption and solvent extraction of microalgal biomass lipids. However, the total microalgal lipids are measured by drying and weighing the total lipid extract through a gravimetric technique (Kumari *et al.,* 2011). The Gravimetric technique is

an inaccurate technique for quantification of microalgal lipid due to loss of some of the lipid content after additional steps required to differentiate various types of lipid fractions such as transesterification and chromatographic separation, which are time consuming and also require labour-intensive manipulation (Bertozzini *et al.*, 2011). Furthermore, the gravimetric method is not suitable for determination of the total neutral lipid content for biodiesel production because this protocol not only assesses total lipid but also other non-fatty acids containing lipids such as pigments or sterols and consequently these non-fatty acids will increase the total lipid content (Breuer *et al.*, 2013).

On the other hand, screening of microalgal lipid content using spectrophotometry has been recommended as the preferred protocol to assess lipid content. Moreover, fluorescence spectroscopy is considered as the most easy, rapid and cheapest analysis tool for assessment of lipid content based on a fluorescent dye, mainly using Nile Red dye. Also the excitation and emission filters of spectroscopy are using to read the fluorescence of the dye which is associated indirectly with the lipid content (Cirulis *et al.*, 2012). Nuclear Magnetic Resonance (NMR) is widely used as a tool for the compositional evaluation of the lipid profile such as saturated and unsaturated fatty acids, directing the production and characteristics of feed stock and their conversion to biodiesel (Bharti and Roy, 2012).

However, lipid-soluble fluorescent dye Nile Red staining (NR, 9-diethylamino-5H-Benzo[α] phenoxazine-5-one) is simple, rapid, sensitive, accurate and widely specific method utilised to assess the lipid content in different organisms when compared with other conventional methods including solvent extraction and the gravimetric method which are time consuming and only allow low level throughput of neutral lipid measurements (Chisti, 2008; Govender *et al.*, 2012; Huang *et al.*, 2009; Sitepu *et*

al.,2012). Nile Red dye combined with organic solvents such as dimethyl sulfoxide (DMSO) have the ability to exceed through the cell wall and cytoplasmic membrane and then dissolve in the intracellular neutral lipid which show a yellow colour fluorescence for neutral lipids, and a red colour for chlorophyll auto fluorescence and polar lipids (Pancha *et al.*, 2014; Wu *et al.*, 2014).

This study was aimed to determine the optimum salinity, pH, nitrogen limitation and culture condition that can result in higher growth and proximate composition (protein, lipid and carbohydrate) of *N. salina* cells. These results can be applied by farmers and industry in culturing microalgae with a targeted growth and proximate composition that can only be achieved under certain culture conditions.

However, the neutral lipid in *N. salina* cells was detected and then quantified using a Nile Red staining (NR) technique and the free fatty acids profiles of *N. salina* were analysed by a Gas Chromatography-Mass Spectrometry (GC-MS) technique after direct transesterification with hydrochloric acid in methanol and also by using a Nuclear Magnetic Resonance (NMR) technique.

5.2. Results and Discussion

5.2.1. Quantification of Neutral Lipid Using Nile Red Method

5.2.1.1. Nile Red Fluorescence Microscopy for Visualisation of Lipid Droplets

The Nile red (NR) technique is accepted as a fluorescent indicator to visualize lipid bodies in intact cells and has been extensively used to monitor triglyceride (TG) accumulation in cell of microalgae (Pick and Rachutin-Zalogin, 2012). Normally, the quantification technique for neutral lipid from microalgal cells using Nile Red fluorescence varies depending on the microalgae species and consequently, different parameters are required to be optimized for neutral lipid quantification including cell concentration of microalgal cells, Nile Red concentration, solvent type and staining time (Rumin *et al.*, 2015). To enhance the ability of Nile Red dye penetration to *N. salina* cells a protocol was established in two steps (Chen *et al.*, 2009), using organic solvent carrier (dimethyl sulfoxide), because Nile Red dye is quenched in water (Greenspan *et al*., 1985) and microwaving the cells.

The accumulation of neutral lipid in *N. salina* cells was first monitored by examining Nile Red fluorescence under the fluorescence microscope to check the Nile Red dye that dissolved in 100% dimethyl sulfoxide (DMSO) was able to penetrate into the lipid droplets within the *N. salina* cells. However, one of the negative impact of using the Nile Red dye for neutral lipid determination was due to the dye failure to exceed into the microalgal cells because of the rigidity of the microalgal cell wall and cytoplasmic membrane which might affect the penetration of Nile Red dye and therefore it will not reach the intracellular lipid (Chen *et al*., 2011c).

Numerous studies have confirmed that the DMSO is a powerful organic solvent to be used as a stain carrier with Nile Red to enhance the fluorescence intensity of the

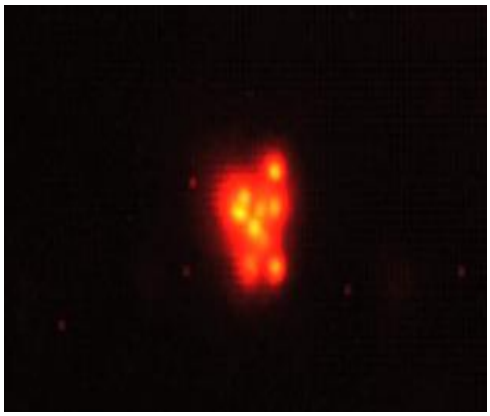
microalgal cells (Chen *et al.*, 2009; Frick *et al*., 2014) compared with other solvents that ma ybe used such as acetone, ethanol, ethylene glycol and diaminotetraacetic acid (Cooksey *et al.*, 1987; Doan and Obbard, 2011; Siegler *et al.*, 2012; Wong *et al*., 2014).

Moreover, the fluorescent intensity of *N. salina* cells was lower when using organic solvent alone even at a high concentrations of DMSO, because of the fluorescence quenching of Nile Red under high concentrations of DMSO. Therefore, the *N. salina* cells were microwaved two times in combination with DMSO to increase the fluorescent intensity of cells. However, this association was in agreement with the results reported by earlier experiments (Chen *et al.*, 2009). The influence of two microwave steps on the fluorescence intensity of *N. salina* cells may be related to the effect on the molecular collision speed enhancing and therefore simplifying the entrance and interaction of Nile Red dye with neutral lipids in cells. Moreover, the acetone stain was used instead of dimethyl sulfoxide (DMSO) as a stain carrier with Nile Red dye, but poor results were found, probably because acetone was not a good solvent carrier.

The microscope images in Figure 5.1 clearly show microalgal cells appeared red in colour (due to chlorophyll autofluorescence) and internal lipids (TAG) fluoresced in a yellow colour. The staining shows that neutral lipids are present as discrete droplets within the cells. However, the images on the left are taken under normal light and the images on the right show the same cell under fluorescent light conditions. The key finding is that Nile Red in DMSO successfully entered the *N. salina* cells and that the stained neutral lipid droplets were clearly visualized under the fluorescence microscope. Moreover, these results have offered indirect indication of the marked increase in the amount of neutral lipid in the *N. salina* cells grown under normal

growth conditions. Based on the observations it was decided to examine Nile Red fluorescence under the fluorescence microscope to check the accumulation of neutral lipid in *N. salina* cells growing under various stress growth conditions including high salinity concentrations, range of pH values and nitrogen starvation in further experiments using the Nile Red method to investigate the influence of these stress growth conditions on the growth rate and total neutral lipid content of *N. salina* cells.

Taken under normal light condition (left image)



Taken under fluorescent condition (right image)

Figure 5.1. Fluorescence microscopy images of *Nannochloropsis salina* cells stained with NR at final concentration of 0.2 µmol ml^{-1}. The cells were grown in f/2 growth medium as normal growth conditions and incubated in a 25ºC constant temperature room. The image on the left is taken under normal light and the image on the right shows the same cell under fluorescent light conditions.

5.2.1.2. Determination of Optimum Cell Concentration

To optimize cell concentrations for determination of cellular neutral lipid, several cell concentrations were used as described in section 2.15.2. Table 5.1 presents the fluorescence intensities for different cell concentrations of *N. salina* strain ranging from 12.5% to 100% (where 100% was OD_{595} = 1) at different staining times (10, 15, 20, 25 and 30 minutes). It was found that the highest fluorescence intensities were associated with cell concentrations of 100%, 87.5% and 75%, respectively. The other lower cell concentrations gave low fluorescence intensities which decreased as the cell concentration decreased. However, based on higher normalized value of 5.87 and the smallest standard error value of stain average (0.9) to avoid the overlapping between the stained and unstained average, the cell concentration of 75% (which is equivalent to an OD_{595} = 0.818) with 15 minutes staining has been selected as optimum cell concentration to use for determination of optimum Nile red concentration.

However, the cell normalized value is the ratio of Nile Red/concentration cells and the time of staining. In this study, the normalization procedure was used because it is an accurate technique to compare the fluorescent intensity readings of either the same sample or different samples established at the same cell density (Chen *et al.*, 2009). Furthermore, the ratio between NR and cell concentration is playing a crucial role in precise measurement of neutral lipid in cells. The Nile Red molecules will be stacking at high NR/cells ratios and consequently increases the possibility of quenchers in the cells (Pick and Rachutin-Zalogin, 2012). Based on the pre-scan of both excitation and emission characteristics of neutral lipid standards, the excitation and emission wavelengths for the fluorescence determination were selected to be 490 nm and 580

nm, respectively (Chen *et al*., 2011c). In addition, the staining time of cells by Nile Red dye was considered to be a crucial factor to achieve the highest fluorescence intensity and however this is mainly dependent on the cell wall structure of strain (Balduyck *et al.*, 2015). However, 15 minutes staining was selected to be an optimal staining time for *N.salina* cells because the results of this study showed the maximum fluorescence intensity was achieved at 15 minutes and then the fluorescence intensity was slightly decreased with 20 mins incubation. These findings are similar to those of Pick and Rachutin-Zalogin, (2012) who observed that the fluorescence intensity values will be deceased between 50% to 90% after 20 min staining with Nile Red due to the quenching with long staining time because of the stacking of Nile Red molecules, partial solubility of different concentration of Nile Red or quenchers of the sample.

Concentration of cells (%)	100	87.5	75	62.5	50	32.5	25	12.5
Stained Average (StDev)	665.75 (2.2)	585 (1.4)	574.75 (0.9)	456.25 (1.7)	362 (4.2)	349.5 (6.4)	290.25 (4.9)	283 (1.8)
Unstained Average (StDev)	152.5 (3.8)	140.75 (3.9)	134.5 (1.2)	130 (1.6)	124.75 (4.8)	117.5 (3.3)	113.25 (4.3)	108.5 (6.1)
Stained-Unstained	513.25	444.25	440.25	326.25	237.25	232	177	174.5
Normalised per % dilution	5.1325	5.0771	5.87	5.22	4.745	7.1384	7.08	13.96

Table 5.1: Optimization of Nile Red staining time and cell concentration on fluorescence intensity of the green alga *N. salina* strain. The optimum conditions were at 75% of cell concentration after 15 min staining. Data points are the means of four replicates.

5.2.1.3. Determination of Optimum Nile Red Concentration Methed

The optimization of Nile Red concentration was conducted as described in section 2.15.3. Table 5.2 shows the effect of using different concentrations of Nile Red ranging from 0.05 to 0.6 μmol ml^{-1} dissolved in 100% dimethyl sulfoxide (DMSO) with the 75% cell concentration (OD_{595} = 0.818) of the *N. salina* strain for different staining times (10, 15, 20, 25 and 30 minutes). It was found that the highest fluorescence intensities were observed when Nile Red concentration was 0.05, 0.1 and 0.2 μmol ml^{-1}, respectively. However, higher Nile Red concentrations of 0.3, 0.4 and 0.6 μmol ml^{-1} were shown to have low fluorescence intensities, respectively. This is likely due to self shading effects when using higher concentrations of dye. Based on the smallest standard deviation value of stain average (2.9) and balance between high and low Nile Red concentrations, because of the drawbacks of relying on the lowest or highest concentration, the Nile Red concentration of 0.2 μmol ml^{-1} with 15 minutes staining has been chosen as optimum Nile Red concentration for use in neutral lipid determination experiments with a cell concentration of 75%.

However, numerous studies have reported that the fluorescence intensity is dependent on the Nile Red dye concentrations (Chen *et al.*, 2009; Huang *et al.*, 2009; Xu *et al.*, 2013). Thus, at low concentration of Nile Red, the interaction between the Nile Red stain and hydrophobic core will be increased and, consequently, the quenching of Nile Red dye was decreased when compared with excess of Nile Red (Greenspan *et al.*, 1985). Besides, the interaction between the dye and lipid droplets will be affected and therefore decrease the ability of the Nile Red dye to access through hydrophilic quenchers if using low concentration of Nile Red (Pick and Rachutin-zalogin, 2012). Moreover, using high Nile Red concentration may be reacting not only with neutral

lipids but also with phosphorlipidic coat and hydrophobic surface of protein (Sackett and Wolff, 1987).

Concentration of Nile Red μmol ml^{-1}	0.05	0.1	0.2	0.3	0.4	0.6
Stained Average (StDev)	865 (29.2)	672 (13.6)	567 (2.9)	441.25 (13.7)	377.5 (34.1)	274.75 (22.8)
Unstained Average (StDev)	113.6 (13.3)	103.8 (8.3)	100.4 (7.8)	128.2 (35.3)	122.4 (31.3)	111.2 (21.9)
Stained-Unstained	751.4	568.2	466.6	313.05	255.1	163.55

Table 5.2: Optimization of Nile Red staining concentration for the green alga N. *salina* strain. The optimum conditions were at 75% of cell concentration after 15 min staining. Data points are the means of four replicates.

5.2.1.4. Determination of Triolein Calibration Curve (Triolein Concentration Test)

The final part of the Nile Red set-up experiments was to produce the Triolein calibration curve (Triolein concentration curve) in the range of 0.2, 0.16, 0.12, 0.08, 0.06, 0.04, 0.02 and 0.0 mg ml^{-1} using Triolein as the neutral lipid standard with algal cell concentration of 75% and 0.2 μmol ml^{-1} Nile Red dye concentration for 15 minutes staining. This will allow the conversion of fluorescence in arbitrary units to Triolein equivalents. The Nile Red fluorescent intensity values were then converted to concentrations of neutral lipid (mg ml^{-1}). The Triolein calibration curve was plotted from Triolien concentration as neutral lipid for each dilution against the Nile Red

fluorescent intensity for each dilution. The resulting concentration curve is shown in Figure 5.2.

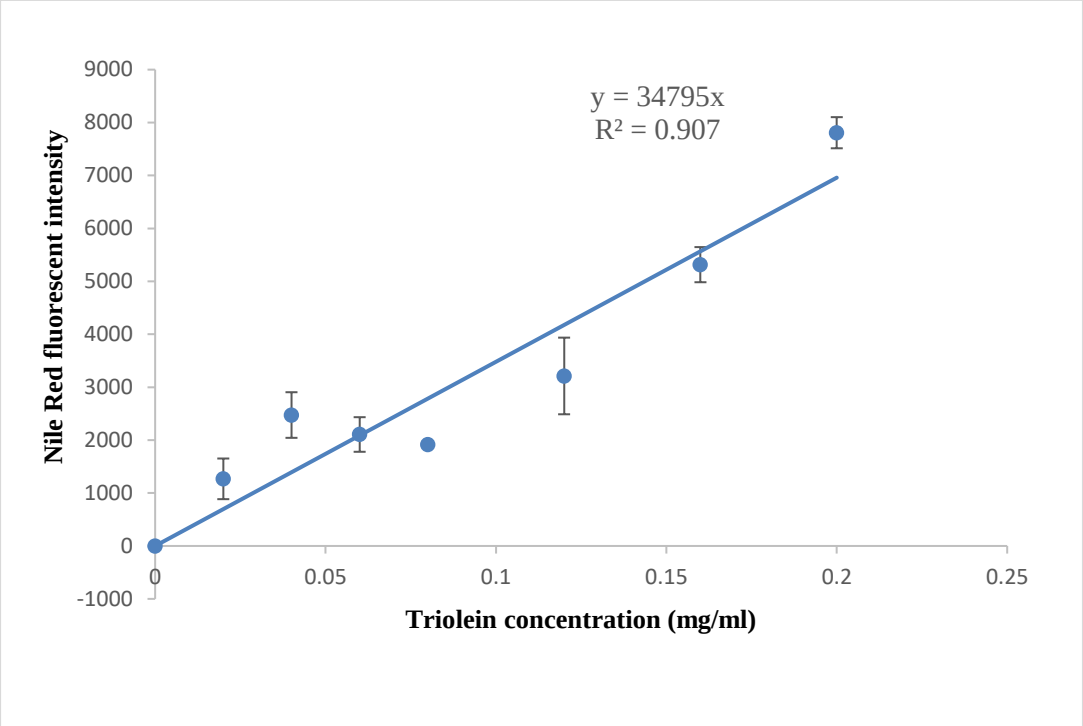


Figure 5.2: Linear correlation between fluorescence intensity and Triolein concentration of *N. salina* cells to allow the conversion of fluorescence reading to Triolein equivalents. Each measurement represents the difference between the average of four stained and four unstained readings. Data points are the means of four replicates plus or minus the standard error.

5.2.1.5. Lipid Quantification in *N. salina* Cells Grown Under Normal Growth Conditions

Neutral lipid measurements in *N. salina* cultures grown under normal growth conditions (without environmental stress) using f/2 medium were performed using optimum conditions for the Nile Red method including cell concentration of 75% which is equivalent to an OD_{595} = 0.818 and Nile Red concentration of 0.2 μmol ml^{-1} with 15 minutes staining as determined in section 5.2.1.2 and 5.2.1.3, respectively. The results were calculated as shown in Figure 5.3 using both dry weight calibration curve at OD_{595} and a Triolein calibration curve as neutral lipid standard produced in the preliminary work.

From the results shown in Figure 5.3, it is clear that the neutral lipid accumulation in *N. salina* cells was 71.78% of total dry weight mass. There are, however,some microalgal species are able to accumulate up to 50 - 70% of lipid per dry weight when growing under suitable culture conditions (Rattanapoltee and Kaewkannetra, 2013). Morever, the results of this study are in agreement with the results reported for *Nannochloropsis* species that showed some oleaginous microalgae have the ability to accumulate large amounts of neutral lipids within cells up to 65-70% of total dry weight under normal growth conditions (Simionato *et al.,* 2011). Therefore, the *N. salina* strain appears from these initial results to be an oleaginous microalga and further studies will be carried out on this strain with different environmental stresses conditions such as nitrogen and phosphate limitation, high salinity and temperature or pH of culture to improve lipid accumulation in *N. salina* cells to consider it as a potential industrial strain of microalgae for biofuel production (Li *et al.,* 2014b; Kang *et al.,* 2015).

T° 485/20,580/50	75%	75%	75%	75%	75%	75%	75%	75%	75%
A	10259	10229	10302	10243	10240	11031	10304	10322	11022
B	10266	10260	10299	10268	10287	10287	11012	10244	10278
C	10260	10259	10321	10236	10312	11011	10299	10269	10296
D	10256	11002	10243	10302	10406	10199	10302	10288	10289
E	402	398	369	387	360	364	361	395	368
F	379	378	398	386	355	358	360	388	388
G	401	403	395	392	356	358	361	356	357
H	399	388	396	389	367	355	365	368	353
St ave	10260.25	10437.5	10291.25	10262.25	10311.25	10632	10479.25	10280.75	10471.25
St stdev	4.193249	376.6081	33.60927	29.84823	69.86356	450.6869	355.1726	32.8773	367.2414
Unst ave	395.25	391.75	389.5	388.5	359.5	358.75	361.75	376.75	366.5
Unst stdev	10.90489	11.08678	13.72346	2.645751	5.446712	3.774917	2.217356	17.95132	15.67376
ST-Unst	9865	10045.75	9901.75	9873.75	9951.75	10273.25	10117.5	9904	10104.75

av st	10380.64
av stdev	188.8175
av unst	376.4722
av unst stdev	5.922566
av st-unst	10004.17

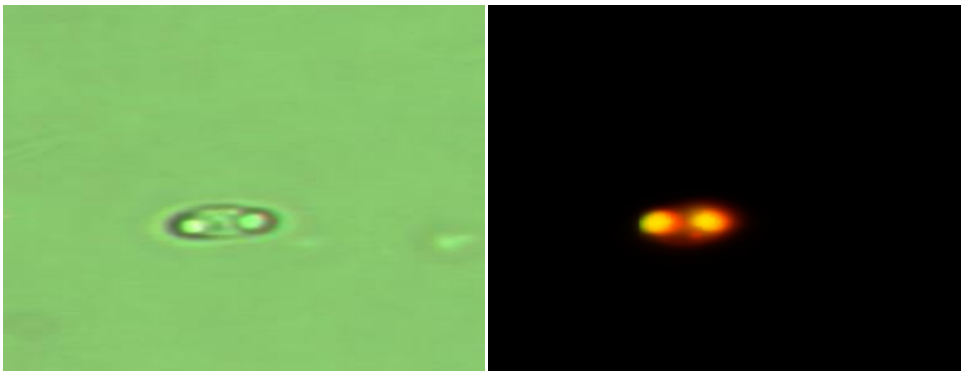
Figure 5.3: The calculation of real experiment for neutral lipid determination in *N. salina* cells grown under normal growth conditions using optimum conditions including cell concentration of 75% and Nile Red concentration of 0.2 µmol ml^{-1} with 15 minutes staining. The results were calculated using the dry weight calibration curve at OD_{595} and Triolein calibration curve as the neutral lipid standard that was produced in the preliminary work.

5.2.2. Neutral Lipid Content of *N. salina* Cells Grown at Different Salinities

Numerous studies have suggested that the quantity and quality of lipids in the cell can be affected as a result of changes in growth conditions such as temperature, light intensity and nutrient concentration in media including nitrogen and phosphates (Illman, *et al.,* 2000; Liu *et al.,* 2008; Converti, *et al.,* 2009). Salinity is also an intricate stress factor that affects lipid production in microalgal cells, and microalgal species can tolerate salinity stress up to an extent that is species dependent (Bartley *et al.,* 2013; Minhas *et al.,* 2016). Particularly, *Nannochloropsis* species are marine microalgae and have been reported to have an optimum around seawater salinity (0.5 to 0.6 M NaCl) and a salinity growth range of 0.1 to 1.0 M NaCl (Pal *et al.,* 2011). Resistance to salinity stress is important because salinity levels may vary in outdoor pond cultures due to evaporation or rain (Pal *et al.,* 2011).

The aim of the study was to investigate the relationship between increasing the salinity concentrations (NaCl) in the culture medium and neutral lipid production (triacylglycerides, TAG) in *N. salina* cells over a significant time period. The growth experiments were carried out by cultivating 5 ml of *N. salina* cells in 250 ml conical flasks containing 100 ml of f/2 medium with five different salinity levels (0.2, 0.4, 0.6, 0.8 and 1 M NaCl) at 25ºC for 4 weeks of incubation period in comparison with normal growth conditions (normal f/2 medium). The neutral lipid accumulation in *N. salina* cells grown under 0.6 M NaCl was monitored by examining Nile Red fluorescence under the fluorescence microscope to check the ability of Nile Red dye to penetrate into the lipid droplets within the *N. salina* cells as mentioned in section 5.2.1.1. However, the microscope images in Figure 5.4 clearly show microalgal cells appeared red in colour and internal lipids fluoresced as a yellow colour. The staining

shows that neutral lipids are present as discrete droplets within the cells. The images on the left are taken under normal light and the images on the right show the same cell under fluorescent light conditions.



Taken under normal light condition (left image) **Taken under fluorescent condition (right image)**

Figure 5.4: Fluorescence microscopy images of *Nannochloropsis salina* cells stained with NR at final concentration of 0.2 µmol ml^{-1}. The cells were grown in f/2 growth medium with 0.6 M NaCl and incubated in a25ºC constant temperature room. The image on the left is taken under normal light and the image on the right show the same cell under fluorescent light conditions.

The lipid accumulation in *N. salina* was measured across each salinity over various time periods every week for four weeks of incubation using Nile Red dye staining with optimum conditions for the Nile Red method as optimised in the previous section including 75% cell concentration of *N. salina* which is equivalent to an OD_{595} = 0.818 and Nile red concentration of 0.2 µmol ml^{-1} with 15 minutes staining.

Figure 5.5 presents the levels of neutral lipids (TAG) accumulated in *N. salina* cells grown across different salinity concentrations over four weeks of incubation. The results very clearly indicated that the highest levels of neutral lipids were observed with the cells grown in 0.6 M NaCl and the consequent highest level of neutral lipids was reported, while the total content of neutral lipid reached 74.65% of total dry

weight followed by normal growth f/2 medium, while the total content of neutral lipid reached 71.78% of total dry weight after four weeks incubation. Similar results were recorded with the microalga species of *Chlorella* that showed highest accumulation around 21.4% (Rai *et al.*, 2015). However, the correlation between increasing the salt concentration and TAG accumulation was not straight forward. On the other hand, the lowest levels of triacylglycerides were found in the 1 M NaCl grown cells. Overall, the amount of neutral lipids was increased with increasing the incubation time from first week to fourth week for each salinity. It is clear that the age of culture is also important when considering the optimum conditions for TAG accumulation. These findings are in good agreement with those of Natunen *et al.* (2015), who reported that the amount of neutral lipids is typically low during exponential growth, but may start to accumulate rapidly when cell division slows down in the stationary growth phase.

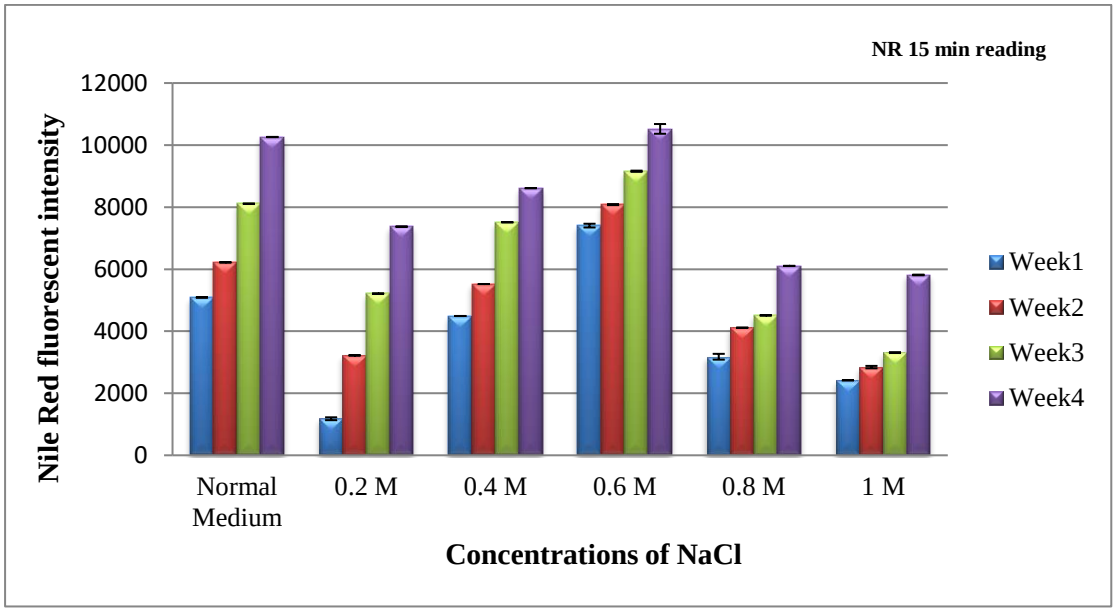


Figure 5.5: Determination of neutral lipid of *N. salina* cells grown in f/2 medium with different concentrations of NaCl in comparison with normal growth conditions using Nile Red fluorescence with optimum conditions for the Nile Red method including 75% cell concentration of *N. salina* which is equivalent to an OD_{595} = 0.818 and Nile Red concentration of 0.2 µmol ml^{-1} with 15 minutes staining. Each column represents the mean of four readings plus or minus the standard deviation.

5.2.3.Effect of pH on Lipid Accumulation by the Biofuel Microalgae *N. salina*

The influence of pH on the neutral lipid content of *N. salina* was examined across six different pH values (pH 5, pH 6, pH 7, pH 8, pH 9, and pH 10) in comparison with normal growth conditions (normal f/2 medium) over four weeks incubation using Nile Red dye staining as optimised in the previous section with optimum conditions for the Nile Red method including 75% cell concentration of *N. salina* which is equivalent to an OD_{595} = 0.818 and Nile Red concentration of 0.2 μmol ml^{-1} with 15 minutes staining. The experiment was performed each week from 1 to 4 weeks after inoculation to examine the effect of pH stress over a long period.

Figure 5.6 shows that, interestingly, the highest neutral lipid levels were observed in *N. salina* cells grown at pH10 for four weeks, while the total content of neutral lipid reached 76.89 % of total dry weight mass followed by cells grown at pH9. However, the results indicated that there was no significant difference between the neutral lipid levels in *N. salina* cells grown at pH8 and normal growth conditions, while the total content of neutral lipid reached 71.92 % and 71.78 % of total dry weight, respectively, after four weeks incubation. In contrast, the lowest levels of neutral lipid were found at low external pH of pH5 and pH6, and there was no difference in the neutral lipid levels in cells grown at both pH values after four weeks growth. However, these findings contrast with the results reported for *Chlorella vulgaris*, that showed maximum total lipid content of 53.43% of total dry weight at pH 7.5 (Sakarika and Kornaros, 2016). These results also disagreed with those reported by Bartley *et al.* (2014) where the lipid accumulation appeared to be unaffected by pH and the greatest mean accumulation occurred in the pH 8 treatment with an average of 24.75% by

mas. Further results were recorded with *Chlorella* sp. that showed maximum lipid production of 0.1995 g L^{-1} with lipid accumulation of 23% at pH 8 (Rai *et al.*, 2015).

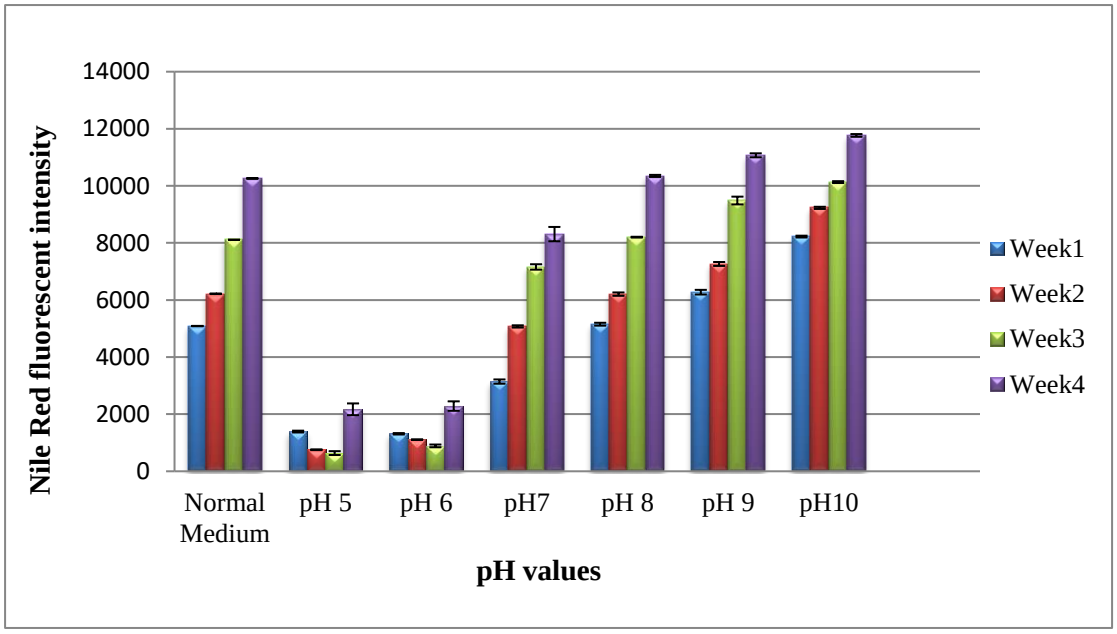
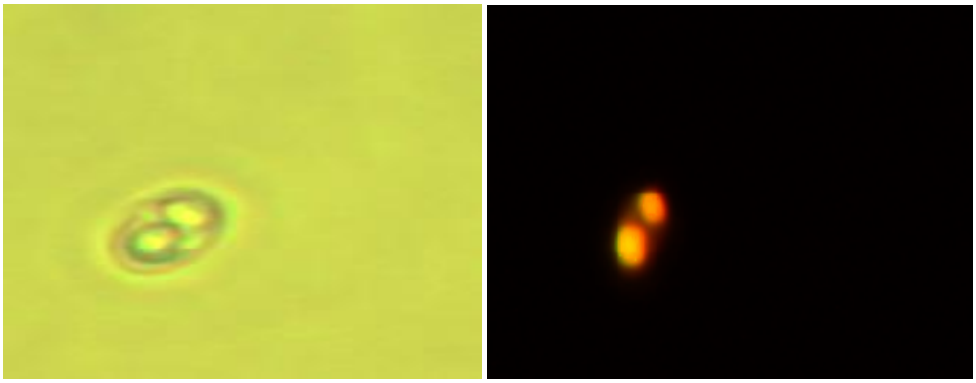


Figure 5. 6: Determination of neutral lipid of *N. salina* cells grown in f/2 medium with different pH values compared to normal growth medium using Nile Red fluorescence with optimum conditions for the Nile Red method including 75% cell concentration of *N. salina* which is equivalent to an OD_{595} = 0.818 and Nile Red concentration of 0.2 µmol ml^{-1} with 15 minutes staining. Each measurement represents the difference between the average of four stained readings and four unstained readings plus or minus the standard deviation.

5.2.4. Effects of Nitrogen Concentration on Lipid Production by *N. salina*

The effect of nitrogen depletion on the neutral lipid accumulation in *N. salina* was examined as described in section 2.18 every week for four weeks of incubation period. The cells were grown in 25%, 50%, 75% and absence of $NaNO_3$ that was used as the source of Nitrogen in f/2 medium compared to normal growth conditions as control. The neutral lipid accumulation in *N. salina* cells grown in nitrogen free (NF) f/2 medium was monitored by examining Nile Red fluorescence under the fluorescence microscope to check the ability of Nile Red dye to penetrate into the

lipid droplets within the *N. salina* cells as mentioned in section 5.2.1.1. However, the microscope images in Figure 5.7 clearly show that microalgal cells appeared red in colour and internal lipids fluoresced as a yellow colour. The staining shows that neutral lipids are present as discrete droplets within the cells. The images on the left are taken under normal light and the images on the right show the same cell under fluorescent light conditions.



Taken under normal light condition (left image) **Taken under fluorescent condition (right image)**

Figure 5.7: Fluorescence microscopy images of *Nannochloropsis salina* cells stained with NR at a final concentration of 0.2 µmol ml^{-1}. The cells were grown in nitrogen free (NF) f/2 medium and incubated in a 25ºC constant temperature room. The image on the left is taken under normal light and the image on the right shows the same cell under fluorescent light conditions.

The accumulation of lipids in *N. salina* was assessed using NR dye staining as optimised in earlier work using 0.2 µmol ml^{-1} of NR dye concentration with the concentration of *N. salina* cells set at OD595 = 0.818 (75% cell concentration) and the measurement of fluorescence intensities 15 minutes after staining the algal cells with NR dye. Figure 5.8 shows the percentage of lipid content accumulation over four weeks for *N. salina* cells subjected to nitrogen limitation and starvation. It can be seen that there was no significant difference in the percentage of lipid content of *N. salina*

cells grown under nitrogen free f/2 medium and normal growth f/2 medium. But there were significant differences between the 25%, 50% and 75% environments from that found under the nitrogen free condition. It can be seen also that the highest percentage of lipid content of *N. salina* was in the nitrogen free environment after four weeks of incubation (72.12%) of total dry weight followed by *N. salina* cells grown in normal growth f/2 medium (71.78% of total dry weight). Overall, the amount of neutral lipids was increased with increasing the incubation time from first week to fourth week for each salinity. It is clear that the age of culture is also important when considering the optimum conditions for lipids accumulation. These results agreed with Rodolfi *et al.* (2009) and Martin *et al.* (2014) who demonstrated that nitrogen deprivation induces lipid accumulation and lipid droplet formation in *Nannochloropsis* strains (up to 60 % of dry weight), because the neutral lipid content of *N. salina* cells was enhanced when the concentration of $NaNO_3$ was decreased over a significant time period. However, nitrogen (N) deprivation induces high accumulation of starch and TAG in a range of microalgae (Juergens *et al.*, 2015). These findings are in good agreement with those of Boussiba*et al.* (1987) who observed that the highest concentration of lipids in *N. salina* cells up to 70% of dry weight, occurred when it was deprived of nitrogen for nine days. The same observation was reported for *Chlamydomonas* cells when grown under stress conditions such as nitrogen starvation, where their growth nearly stops and cells accumulate large amounts of TAGs (Grossman, 2000; Zhang *et al.*, 2004). These results are in agreement with those of Iwai *et al.* (2014), who observed that neutral lipid content was highest in *C.reinhardtii* cells grown under nitrogen or phosphate deprivation at logarithmic growth phase. These results are also in agreement with those of Xia *et al*. (2016) who confirmed that the neutral lipid content of *Scenedesmus* cells was enhanced when the concentration of $NaNO_3$ is decreased.

Moreover, similar investigations found the highest lipid productivity was recorded in three *Chlorella* strains grown in 3% nitrogen compared to 10% nitrogen (Ordog *et al.*, 2016). It is clear that when microalgal cells grow under nitrogen limitation, the protein synthesis required for growth will be affected and therefore excess carbon derived from the photosynthesis process is accumulated in the storage molecule that is triglyceride (Scott *et al.*, 2010; Miller *et al.*, 2010).

In contrast, these findingsdisagreed with these reported by Monshupanee and Incharoensakdi, (2014) who observed that nitrogen starvation did not significantly increase the lipid content of *Synechocystis* in comparison with a significant increase of glycogen and polyhydroxybutyrate. It is known that cyanobacteria do not increase their lipid content in response to Nitrogen limitation.

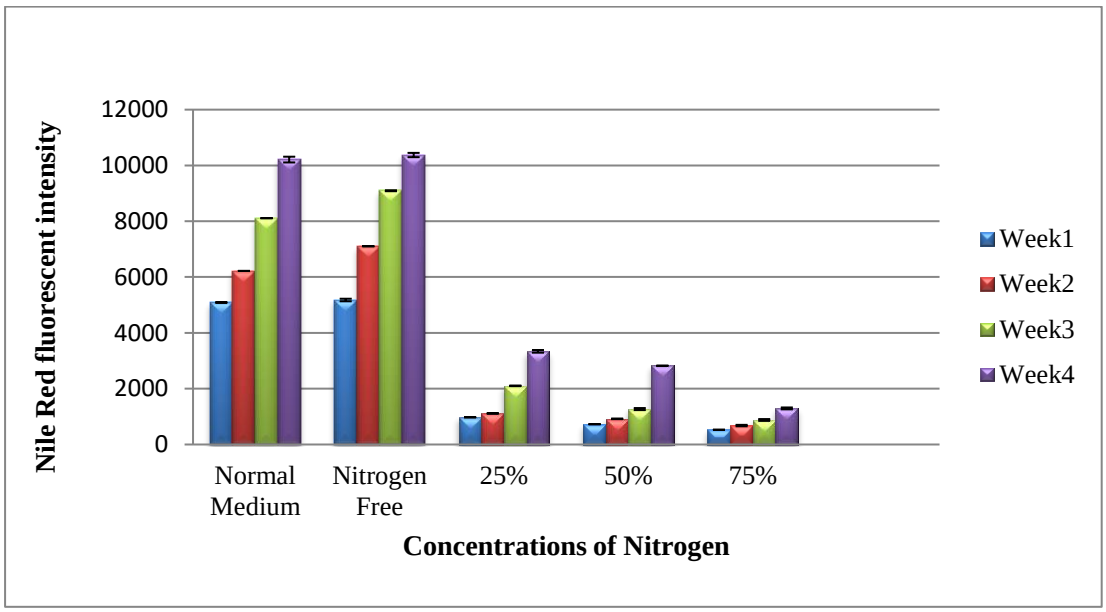


Figure 5.8: Determination of neutral lipid of *N. salina* cells grown in f/2 medium with different Nitrogen concentrations (Nitrogen free, 25%, 50% and 75% of $NaNO_3$) compared to normal growth medium using Nile Red fluorescence with optimum conditions of Nile Red method including 75% cell concentration of *N. salina* which is equivalent to an OD_{595} = 0.818 and Nile Red concentration of 0.2 µmol ml^{-1} with 15 minutes staining. Each measurement represents the difference between the average of four stained readings and four unstained readings plus or minus the standard deviation.

5.2.5. Quantification of Total Lipid Content Using Gravimetric Method

In order to produce microalgal lipids that can be transformed to biodiesel fuel, effects of high pH, nitrogen starvation and salinity (0.6 M of NaCl) on the biomass production and total lipid accumulation of *N. salina* cells in f/2 medium culture were investigated using the gravimetric technique adapted from Chiu *et al.* (2009) compared to normal growth conditions in this study.

The Gravimetric technique mainly uses cell disruption and solvent extraction of microalgal lipids to quantify the total lipids that accumulate in algal cells by drying and weighing the lipid extract (Kumari *et al.,* 2011). However, a huge amount of biomass is required for this technique so the *N. salina* cells were grown for 4 weeks and the whole flask used to generate sufficient biomass (section 2.17).

Figure 5.9 presents the percentage of lipid content of the dry weight biomass of *N. salina* cells grown under different growth conditions. It was found that there was no significant difference between the percentage of total lipid in *N. salina* cells grown under normal growth treatment and nitrogen free stress after four weeks incubation. However, the highest level of total lipid content was found in cells grown under the nitrogen free treatment, where the percentage of total lipid content reached 42%, followed by *N. salina* cells grown in normal growth conditions, where the percentage of total lipid content reached 41.7%, and *N. salina* cells grown in 0.6 M of NaCl with 35.2% of total lipid content. On the other hand,the lowest percentage of lipid content was found in *N. salina* cells exposed to pH10 with 31.5%. These results very clearly indicate that similar trends were found when the Nile Red technique was used for neutral lipid determination under the same growth treatments, which observed that the highest levels of neutral lipid accumulation were found in *N. salina* cells grown in

nitrogen free (NF) f/2 medium and *N. salina* cells grown in normal growth f/2 medium.

However, the conclusion to be drawn is that using either normal growth conditions or nitrogen starvation in the *N. salina* culture can induce increased total lipid accumulation and may be the best conditions to use for TAG synthesis in a commercial biofuel process based on *N. salina* cells as the feedstock. This has the additional advantage that contamination of *N. salina* cell cultures will be more easily controlled under nitrogen starvation than normal growth conditions.

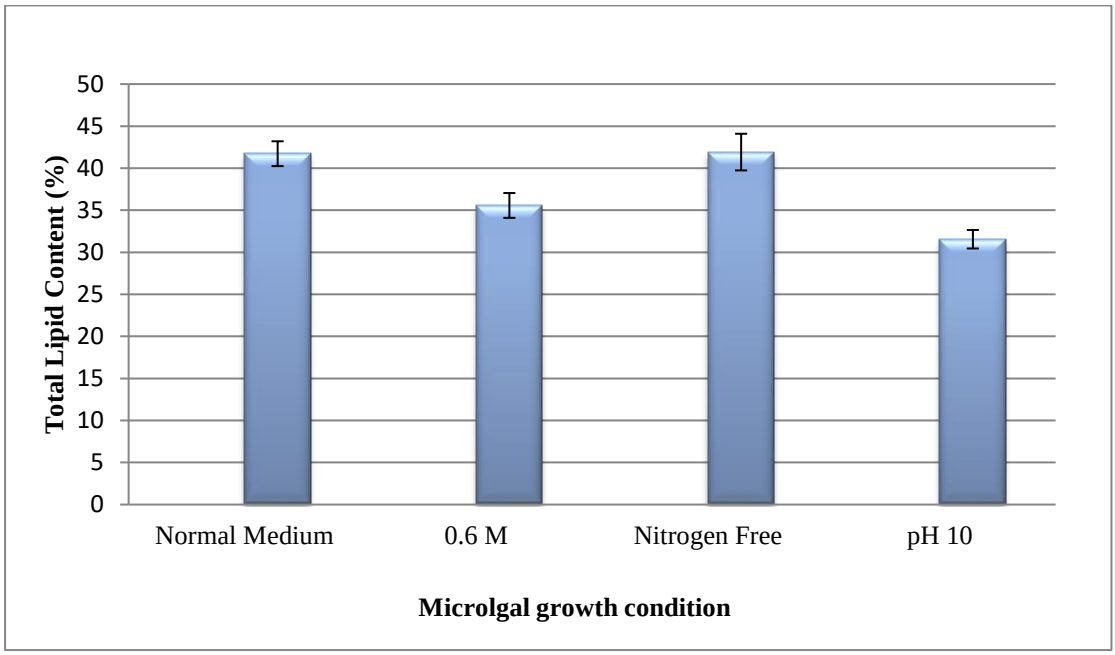


Figure 5.9: Gravimetric measurement of total lipid content (Total Lipid Content percentage) in *N. salina* cultures grown at different growth conditions included 0.6 M NaCl, pH10 and nitrogen starvation compared to normal growth conditions. Each column represents the mean of four reading plus / minus the standard deviation.

5.2.6. Determination of Fatty Acid Methyl Esters (FAMEs) Using Direct Transesterification Methods (GC-MS)

Fatty acid methyl esters (FAMEs), which can be derived from vegetable oil and animal fats by transesterification reaction with alcohol, show great potential applications as diesel substitutes, known as biodiesel fuel (Jeong *et al.*, 2004). Compared to land plants, microalgae can exhibit large biomass productivities and do not require high quality agricultural conditions to grow and consequently microalgae do not directly compete with food crops for arable land (Ahmad *et al.*, 2011; Tang *et al.*, 2016).

The aim of the study was to obtain high quality biodiesel from *N. salina* cells through transesterification under both normal and stress growth conditions. However, to carry out this experiment, the fatty acid methyl esters (FAME) profiles of the TAG accumulated by *N. salina* cells grown in normal growth conditions using normal f/2 medium (Figure 5.10), stress growth conditions using f/2 medium containing 0.6 M NaCl (Figure 5.11) and nitrogen free f/2 medium (Figure 5.12) were analyzed by gas chromatography–mass spectrometry (GC-MS) after direct transesterification with hydrochloric acid in methanol when cells were in late phase growth.

The results very clearly indicated that similar fatty acid methyl esters were observed in all growth conditions examined. There was some indication that the amount of total neutral lipid accumulated by cells grown under the salinity concentration of 0.6 M NaCl was higher than the amount of neutral lipid in cells grown under normal growth and nitrogen free concentrations.

Table 5.3 illustrates the measurement and identification of the fatty acid methyl ester (FAME) profiles of the TAG accumulated by *N. salina* cells in response to the changes in external growth conditions.The relative fatty acid methyl ester profiles

accumulated were identified as**:** Hexadecane pentadecane acid methyl ester (C15:0, n-3), Tridecanoic acid methyl ester (C13:0), Dodecanoic acid methyl ester (C12:0), Palmitioleinate acid methyl ester (C16:2), Palmitic acid methyl ester (C16:1), Oleic acid methyl ester (C18:1) and Eicosapentaenoic acid methyl ester (C20:0). The fatty acid composition of microalgal cell extracts was found to be similar to vegetable and fish oils, mostly rich in C16:0, C18:N (N = 0 to 3), and n-3 omega polyunsaturated fatty acids (PUFAs). The mixture of all lipids present does however have applicability to industry as a base for biofuels generation from the C16 and C18 FAMEs and should meet the requirements for cold-flow, ignition properties, viscosity and oxidative stability (Stansell *et al*., 2012), while omega-3 PUFAs (long-chain polyunsaturated fatty acids)such as Eicosapentaenoic acid (EPA) could feed into the health industries (food supplements) (Tang *et al.*, 2016).

However, this established is similar to that made by Ryckebosch *et al.* (2014) who confirmed that microalgae are an alternative source of omega-3 LC-PUFA.

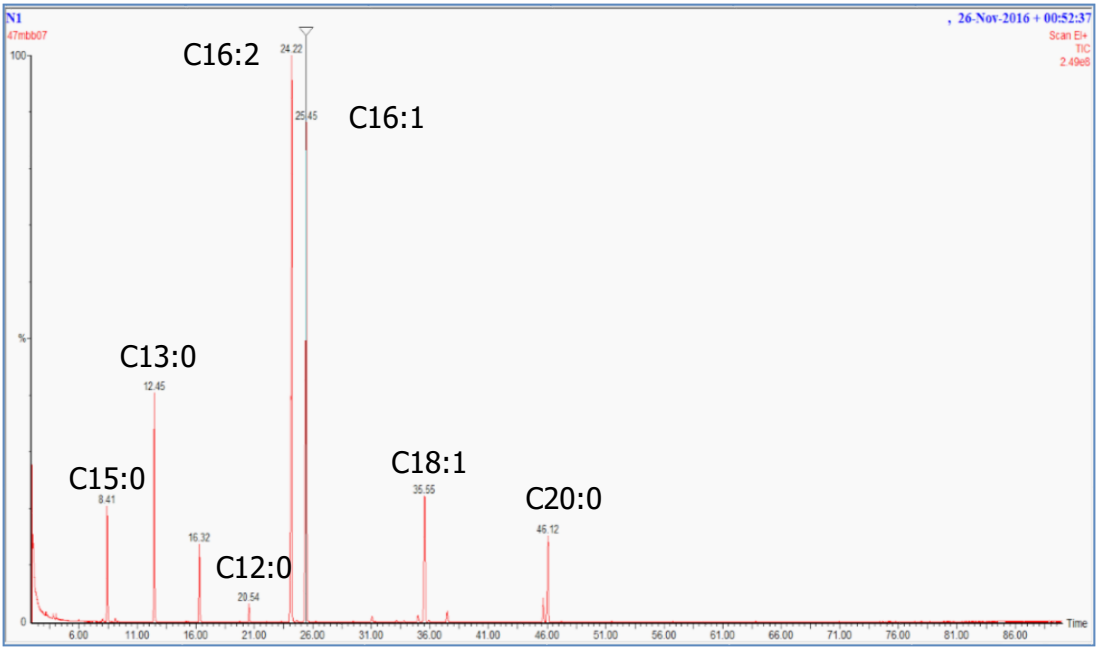


Figure 5.10: Fatty acid methyl esters (FAMEs) found in *Nannochloropsis salina* cells grown in normal conditions using f/2 medium.

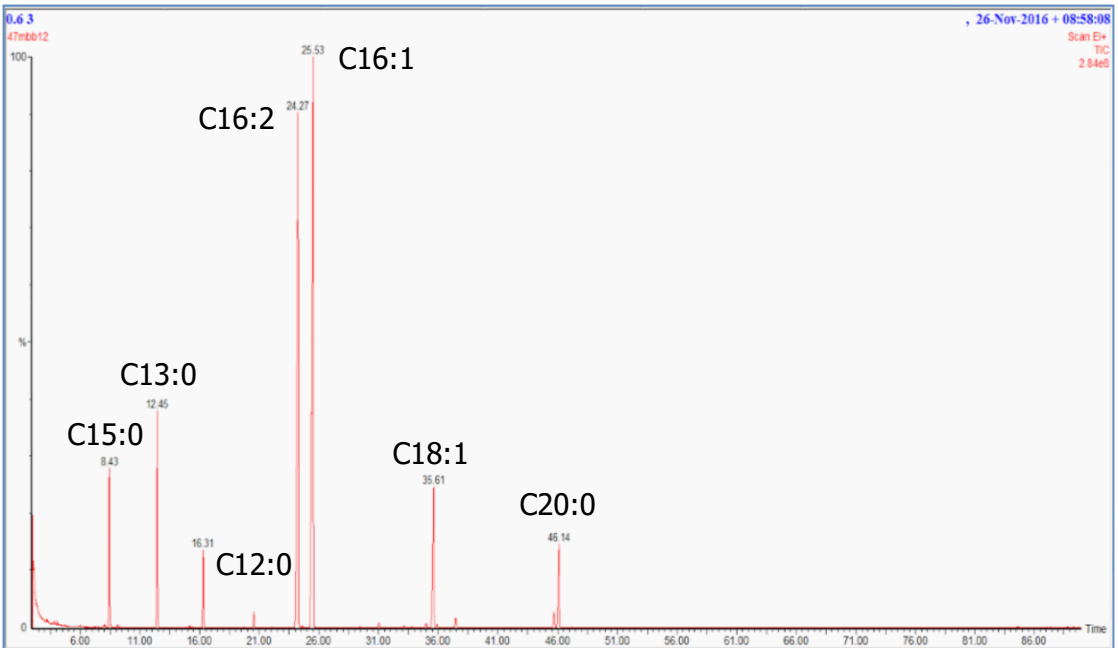


Figure 5.11: Fatty acid methyl esters (FAMEs) found in *Nannochloropsis salina* cells grown in 0.6M NaCl using f/2 medium.

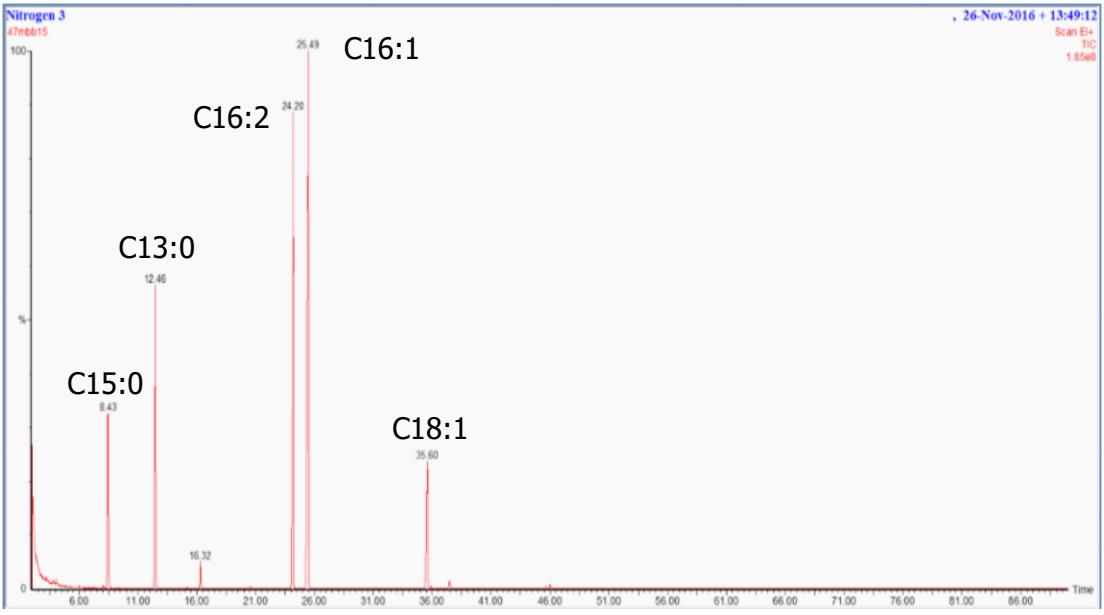


Figure 5.12: Fatty acid methy esters (FAMEs) found in *Nannochloropsis salina* cells grown in free nitrogen f/2 medium. The fatty acids accumulated were identified as: Hexadecane pentadecane acid methyl ester, C15:0/n-3, Peak 8.41 (1n-cetane, 2n-Hexodecone and 3n-cetane). Tridecanoic acid methyl ester acid, C13:0 (Peak 12.45). Dodecanoic acid methyl ester, C12:0 (Peak 20.54). Palmitioleinate acid methyl ester, C16:2 (Peak 24.22). Palmitic acidmethyl ester, C16:1(Peak 25.45). Oleic acid methyl ester C18:1 (Peak 35.55). Eicosapentaenoic acid methyl ester, C20:0 (Peak 46.12).The data represents the means ± the standard error of the mean for 3 samples.

Methyl Ester	C:D (Position)	Growth Condition		
		Normal f/2 medium	0.6 M NaCl	nitrogen starvation
Hexadecane Pentadecane Acid	C15:0, n-3	✓	✓	✓
Tridecanoic Acid	C13:0	✓	✓	✓
Dndeconoic Acid	C12:0	✓	✓	
Palmitioleinate Acid	C16:2	✓	✓	✓
Palmitic Acid	C16:1	✓	✓	✓
Oleic Acid	C18:1	✓	✓	✓
Eicosapentaenoic Acid	C20:0	✓	✓	

Table 5.3:The fatty acid profiles of the TAG accumulated by *N. salina* cells cultured in varying stress conditions.

5. 2.7. Nuclear Magnetic Resonance (NMR) Analysis of Compatible Solutes

This investigation was developed for the identification and determination of the presence lipids including triglycerides and free fatty acid profiles accumulated by oleaginous microalgal biomass cultivation of *Nannochloropsis salina* cells grown in normal f/2 medium for marine microalgae and f/2 medium containing 0.6 M NaCl and nitrogen starvation f/2medium. Freeze dried extracts were analysed by a Nuclear Magnetic Resonance (^{1}H-NMR) spectroscopic technique according to Kumar *et al.,* (2014) and Sarpal *et al.* (2015).

^{1}H-NMR spectroscopic techniques provide direct, rapid and convenient methods for the determination of glycerides, free fatty acids (FAs) and polar lipids in the solvent extracts of microalgal biomass without any sample pretreatment and prior separation (Sarpal *et al.,* 2015; Sarpal *et al.,* 2016). ^{1}H-NMR spectra technique allows the quantification of the compounds present in samples which give rise to well resolved

and assigned peaks (Kumar *et al.*, 2014). However, ^{1}H-NMR spectra were plotted at high and low intensity, with an extra one labelled with the assignments. The detail assignments of structural moieties from compound classes such as lipids, free fatty acids (FAs) and other components of microalgae are given in Table 5.4. However, results of extraction in *Nannochloropsis salina* by ultasonication in the ^{1}H-NMR tube and soxhlet extraction indicate that, the three replicates were all roughly the same in each of the samples, thus one replicate only has been shown. It is clear that there was a considerable difference in lipid content between the three growth conditions that used in this study. There seems to be the most lipid (compared to sugars, i.e. normal cellular metabolites) in *Nannochloropsis salina* cells grown under nitrogen starvation (R); and the smallest amount of polyunsaturated fatty acids in *Nannochloropsis. salina* cells grown under normal f/2 medium (N). In addition, the highest levels of neutral lipids were observed with 0.6 M NaCl, while the neutral lipid content of TAG reached 93% of total dry weight as shown in Figure 5.13.

Peak	Compound	Functional Group	Chemical Shift (ppm)
1	All FA except PUFA	-CH3	0.85
2	All PUFA	-CH3	0.95
3	All FA	-(CH2) n	1.3
4	All FA	-OOC-CH2-CH2 -	1.62
5	Unsaturated FA	-CH2-CH=CH-	2.04
6	All FA	-OOC-CH2-CH2-	2.37
7	PUFA	=CH-CH2-CH=	2.84
8	Sugars		3.8
9	Glycerol in TAG	-CH2-OOC-	4.2
10	Glycerol in TAG	-CH2-OOC-	4.4
11	Unsaturated FA	-CH=CH-	5.35

Table 5.4: Compounds accumulated by *Nannochloropsis salina* cells cultured in varying stress conditions and determined by the Nuclear Magnetic Resonance (^{1}H-NMR) spectroscopic technique with their functional group and Chemical shift (ppm).

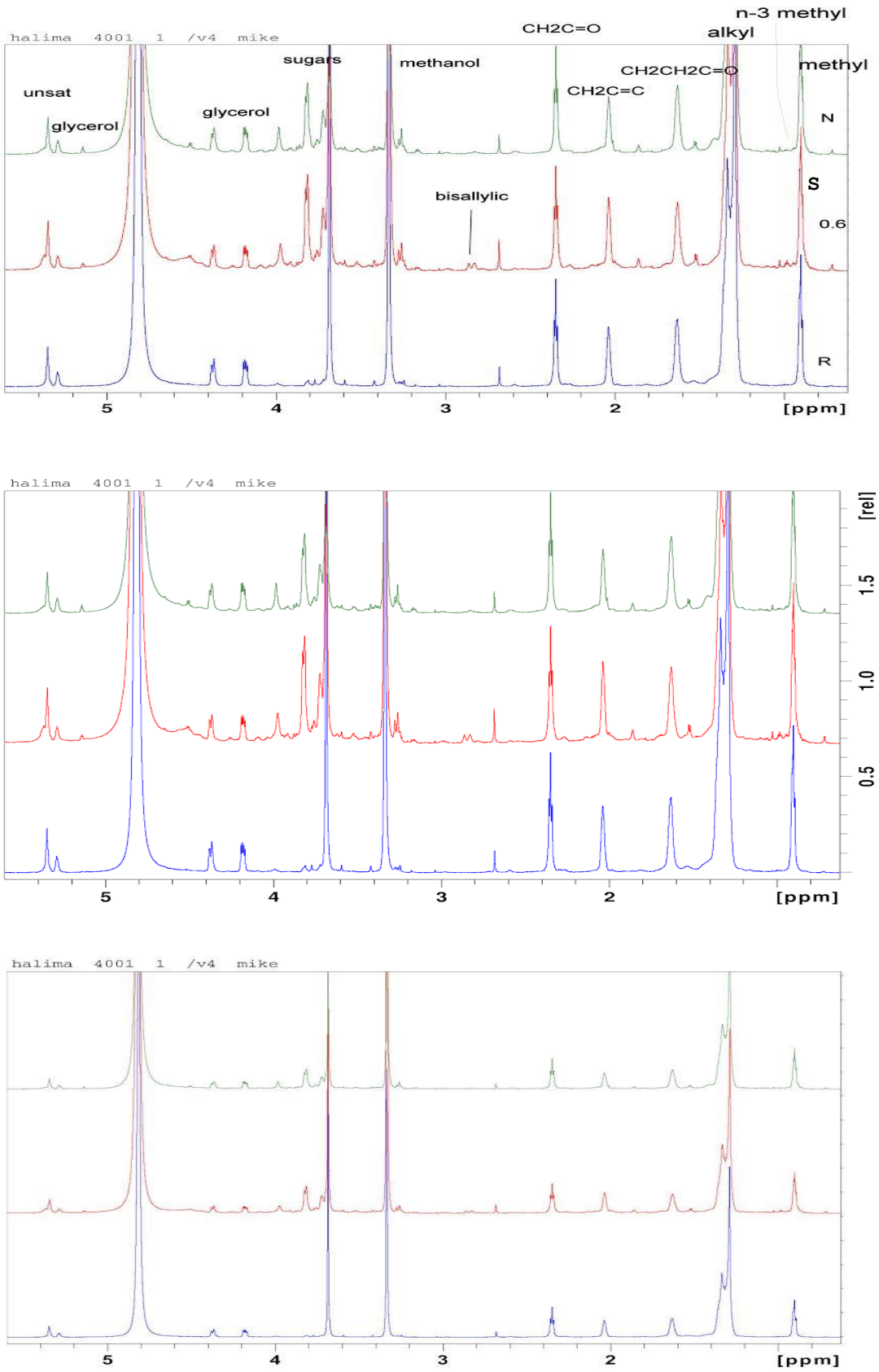


Figure 5.13: NMR spectral analyses of *N. salina* cells grown in normal f/2 medium appeared green in colour (N), f/2 medium supplemented with 0.6 M NaCl appeared red in colour (S) and nitrogen starvation f/2 medium appeared blue in colour (R).

However, most of the compounds in the microalgae contained a dominant aliphatic signal. The most intense peak 1 (0.85 ppm) corresponds to terminal methyls (-CH2CH2-CH3) at the end of long chain aliphatic structures. Peak 2 (0.95 ppm) corresponds to terminal methyl of PUFA. Peak 3 (1.3 ppm) indicates mid-chain methylenes (-CH2-). Peaks labeled 4 (1.62 ppm) are characteristic of -CH2- groups β to a carbonyl group (-OOC-CH2 CH2. Peak 5 (2.04 ppm) results from a mid-chain methylene α to a double bond (-CH2-CH=CH-) nearby to an ester functional group. All these structural moieties can be part of the phytyl structure associated with chlorophyll but are most likely associated with hydrocarbons, FFA and lipids. Peak 6 (2.37 ppm) mainly results from methylenes attached to carboxyl groups (-CH2COOR) as in fatty acid esters. However, signals at 2.84 ppm (peak 7) from methylene-interrupted olefins (-CH=CH-CH2-CH=CH-) are key indicators for PUFA as seen in linoleic and linolenic acid, respectively. Peak 9 (4.2 ppm) and 10 (4.4 ppm) indicate a methylene of a glycerol backbone (-OCCH2-) as in triacylglycerols associated with lipids. Peak 11 (5.35 ppm) is assigned to unsaturated protons (-CH=CH-) in lipids and FFA, see Table 5. 4.

5.3. Conclusion

The present work was carried out to maintain an axenic culture of the microalga *Nannochloropsis* sp. for high biomass and enhanced lipid accumulation. The *N. salina* strain was chosen due to its faster growth rate. The important growth parameters such as pH, salinity, nitrogen starvation were studied for greater production of *Nannochloropsis* biomass.

In this chapter, the Nile red fluorescence method has been successfully applied to the determination of lipids in certain microalgae. It was shown to be a suitable method for visualising lipid droplets inside *N. salina* cells (Figure 5.1). However, the accumulation of neutral lipids by *N. salina* cells was monitored by examining Nile red fluorescence under the fluorescence microscope to confirm the NR dye that dissolved in 100% DMSO was able to penetrate into the lipid droplets within the *N. salina* cells. The microscope images clearly show the *N. salina* cells appeared red in colour and internal lipids fluoresced as a yellow colour and the staining shows that neutral lipids were present as discrete droplets within the cells.

The Nile red fluorescence process was then optimised for a quantitative assay in a 96 well plate reader format by setting the time required for peak fluorescence and both of *N. salina* cells and Nile red dye concentration were optimal. Therefore, a range of cell concentrations of *N. salina* strain from 12.5% to 100% at different staining time (10, 15, 20, 25 and 30 min) were used to optimize cell concentrations and staining time for determination of cellular neutral lipid using the Nile red method. However, the cell concentration of 75% with 15 minutes staining has been chosen as the optimum cell concentration to use for determination of the optimum Nile red concentration. This was based on a higher normalized value of 5.87 and the smallest standard error value of stain average (0.9) to avoid the overlapping between the stained and unstained

average (Table 5.1). To optimize the Nile red concentration, different concentrations of Nile red ranging from 0.05 to 0.6 μmol ml^{-1} dissolved in 100% DMSO with 75% cell concentration with 15 min staining were used. However, based on the smallest standard deviation value of stain average (2.9) and balance between high and low Nile Red concentrations, the Nile red concentration of 0.2 μmol ml^{-1} was selected as the optimum Nile Red concentration to use for neutral lipid determination experiments with a cell concentration of 75% (Table 5.2) using triolein concentration curve which was conducted as described in section 5.2.14. However, using these parameters, the amount of neutral lipid (TAG) accumulated by *N. salina*cells was visually confirmed by examining the fluorescence of Nile Red in cells grown under stress conditions included higher salinities, different pH values and nitrogen depletion in comparison with normal growth conditions using fluorescence microscopy. Under normal growth conditions, the amount of neutral lipid accumulated by *N. salina* cells grown in normal f/2 growth medium was assessed using optimum conditions for the Nile Red technique including cell concentration of 75% and Nile Red concentration of 0.2 μmol ml^{-1} with 15 minutes staining. The concentration of neutral lipid content and lipid percentage (%) were calculated based on the triolein calibration curve and the relationship between OD and dry weight of the cells that offered the optimum concentration of biomass. It was found that, the percentage of lipid accumulated by *N. salina* cells under normal growth was 71.78% of total dry weight (Figure 5.3).

To investigate the effect of increasing external salinity in the medium on the neutral lipid accumulation as a stress condition, *N. salina*cells was grown in normal F/2 medium, it was then grown under stress conditions of higher salinities by adding NaCl to give concentrations of 0.2 M, 0.4 M, 0.6 M, 0.8 M and 1.0 NaCl. The neutral lipid measurements of *N. salina* strain were evaluated once a week for 4 weeks using an

optimum concentration of *N. salina* cells (OD 595= 75% concentration of cells) and 0.2 μmol ml^{-1} concentration of Nile Red. It is clear that the highest Nile Red fluorescent intensity of *N. salina* cells was observed at 0.6 M NaCl, with percentage of lipid found as 74.65% followed by *N. salina*cells grown in normal growth f/2 medium, with percentage of lipid as 71.78% of total dry weight after four weeks incubation (Figure 5.5). To better understand the effect of external pH on the neutral lipid accumulation in *N. salina*, cells were incubated in f/2 medium containing different pH values of pH 5, pH 6, pH 7, pH 8, pH 9, and pH 10 for four weeks. The results of this study demonstrated that the highest neutral lipid content was observed in *N. salina* cells grown at pH10 where the percentage of microalgal lipid was 76.89 % of total dry weight after four weeks incubation (Figure 5.6).

To further investigate, the effect of nitrogen depletion on the content of neutral lipid production by *N. salina* cells was examined. Cells were grown in normal f/2 medium and in f/2 medium including different nitrogen concentrations (0.0%, 25%, 50% and 75% of $NaNO_3$). It can be seen that the percentage of neutral lipid content increased with increasing nitrogen starvation from 75% nitrogen to absolutely free nitrogen for *N. salina.* It can be seen also that the maximum lipid production of 72.12% of total dry weight was achieved by growing the cells in nitrogen free (NF) f/2 medium followed by *N. salina* cells grown in normal growth f/2 medium, while the total content of neutral lipid reached 71.78% of total dry weight mass after four weeks incubation (Figure 5.8). A one-off experiment was carried out to examine the effect of pH and salinity stress, where nitrogen free conditions on the total lipid production by *N. salina* cells compared with normal conditions using the Gravimetric method. The *N. salina* cells were grown in normal and under stress conditions until sufficient biomass was generated to conduct assessment since large amounts of biomass are

needed to quantify the total lipids by this method. As shown in Figure 5.9, the percentage of total lipid content of the dry weight biomass was found to be around 42%, 41.7%, 35.2% and 31.5% for nitrogen free, normal, 0.6 M NaCl and pH 10 conditions, respectively. To analyse and identify fatty acid methyl esters (FAMEs) that occur in *N. salina* neutral lipid, a gas chromatography mass spectrometry (GC-MS) technique was used using *N. salina* cells grown in normal f/2 medium (Figure 5.10), 0.6 M NaCl f/2 medium (Figure 5.11) and nitrogen free f/2 medium (Figure 5.12). The results very clearly indicated that similar fatty acid methyl esters were observed at all growth conditions examined. It can bee see that the neutral lipid of *N. salina* cells contained several FAMEs such as Hexadecane pentadecane acid methyl ester (C15:0, n-3), Tridecanoic acid methyl ester (C13:0), Dodecanoic acid methyl ester (C12:0), Palmitioleinate acid methyl ester (C16:2), Palmitic acid methyl ester (C16:1), Oleic acid methyl ester (C18:1) and Eicosapentaenoic acid methyl ester (C20:0). However, palmitic acid methyl ester (C16:0) and oleic acid methyl ester (C18:1) are saturated or monounsaturated fatty acids and therefore they are suitable for biofuel production.

An NMR technique was used in order to identify the compatible solutes produced by the microalgal cells to establish tolerance under both high salinity and nitrogen stress conditions, respectively, in comparison with typical growth conditions. The extraction was carried out by bead beating of the cells and then using chloroform and methanol as solvents. The NMR spectra results are shown in Figure 5.13. Table 4.5 shows the results of chemical shift and functional group analysis of the neutral lipids (TAG) and free fatty acids (FFAs), and polar lipids and their fatty acid content. It is clear that the fatty acid content of cells grown in 0.6 M of NaCl f/2 medium was higher than for cells grown in normal f/2 medium and Nitrogen free f/2 medium.

Enhancement of Intracellular Lipid Production by *N. salina* Via Flow Cytometric Cell Sorting

Chapter Six

6.1. Introduction

Microalgae have become a worldwide key point of powerful research activity due to their potential for generating high yields of intracellular lipid that can provide alternative, renewable and environmentally-friendly feedstock for biodiesel and nutritional lipid production (Chisti, 2007; Chisti, 2008; Doan and Obbard, 2012; Tang *et al.,* 2016). This is could be an ideal option to overcome the common, worldwide problems and global warming that are associated with fossil fuel exhaustion and greenhouse gas emissions (Gavrilescu and Chisti, 2005; Chisti, 2007). Unlike other oil crops, microalgae can be harvested nearly all year-round and many species of these unicellular organisms are capable of robust growth in wastewater, thereby reducing freshwater usage (Pires *et al.,* 2013). On the other hand, the high capital costs of biofuel production from microalgal yields using intracellular lipid is one of the major bottlenecks that require hard efforts to overcome to be economically feasible. Therefore, in main factors, some improvements in the microalgal yields production and the rate of lipid biosynthesis are required to reduce the cost of biodiesel production. However, it is recognised that increasing lipids ynthesis almost invariably deceases biomass levels (Sharma *et al.,* 2012). *Nannochloropsis* sp., for example, is one of the most ubiquitous types of marine microalgae that has been utilised widely in aquaculture practices for derivation of nutraceuticals and food supplements (Lubian *et al.,* 2009) and has also been accepted as a promising source of lipid feedstock for biodiesel production (Gouveia and Oliveira, 2009; Rodolfi *et al.,* 2009; Umdu *et al.,* 2009). However, to become a really viable biodiesel feedstock, *Nannochloropsis* lipid yield requires improvement with respect to lipid productivity and reduced total content of polyunsaturated fatty acid (PUFA) (Doan and Obbard, 2012).

At present, however, there are two main possible strategies for enhanced lipid yield in microalgal strains that can be used: biochemical engineering approaches and genetic engineering approaches (Courchesne *et al.,* 2009). The biochemical engineering strategy depends on creating environmental (physiological) stresses such as nutrient starvation or high salinity to channel metabolic fluxes to microalgal lipid accumulation.The genetic engineering strategy relies on overexpressing of rate-limiting enzymes to create a channeling of metabolic pathways to lipid biosynthesis in recombinant microalgal strains (Courchesne *et al.,* 2009).

The flow cytometry technique has produced a helpful tool to sort out problems related to selection of the mutant cells. By flow cytometry technique, it is possible to examine up to thousands of cells per second and therefore this technique begins a new period of quick cell mutant generation (Davey and Kell, 1996). Moreover, the flow cytometry procedure is currently considered as a powerful tool to analyse cells at high flow rate and can be able to differentiate between low signals that make it a good way to inspect several features and physiological factors within a short time (Hyka *et al.*, 2013; Velmurugan *et al.*, 2013). For microalgae, the flow cytometry procedure has been utilized as a desired technique to separate a mutant cell from complex populations based on an individual characteristic of microalgal photosynthetic pigments such as chlorophyll and carotenoids that exhibit strong fluorescence intensity in combination with lipid binding dyes such as Nile Red (Cirulis *et al.*, 2012).

This chapter aimed to determine the neutral lipid content of *N. salina cells* using flow cytometry technique in combination with Nile red staining as an improved t procedure for cell selection.

6.2. Results and Discussion

6.2.1. Flow Cytometry Analysis of Neutral Lipid Content

6.2.1.1. Determination of The Influence of DMSO

The optimum concentration of dimethyl sulfoxide (DMSO) as an organic solvent for the dye was optimised as described in section 2.23.1 to enhance the effectiveness and efficiency of NR to stain the intracellular neutral lipid. However, different concentrations DMSO ranging from (10 - 60%) were prepared with distilled water to dissolve Nile Red dye which was to be used with the flow cytometry.

From the results shown in Figure 6.1, it was clearly found that the 10% DMSO (final concentration) was the best concentration to be used as a stain carrier for determination of lipid content in *N.salina* cells using flow cytometry in combination with Nile Red. These findings are contrast to those of Satpati and Pal, (2015) who reported that 40% DMSO in 15 min incubation was an optimum concentration for achieving high fluorescent intensity of Nile Red using two green microalgae of *Chlorella ellipsoidea* and *Chlorococcum infusionum*.

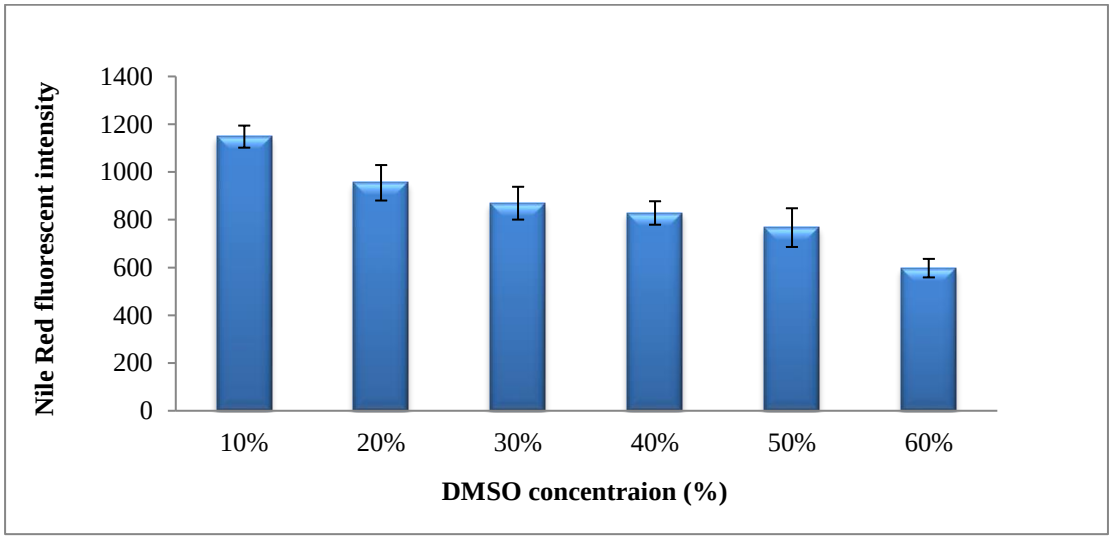


Figure 6.1: Optimization of DMSO concentration for using as a stain carrier for the green alga *N.salina* strain. Different concentrations of DMSO ranging from (10-60%) were prepared with distilled water andused to find out the best concentration of DMSO to be used in the experiment using the flow cytometry. Data points are the means of four replicates plus or minus the standard error.

6.2.1.2. Optimising The Concentration of Nile Red

The proper use of Nile Red staining solution is crucial to achieving the highest fluorescence and consequently produce the ideal staining of target clones. Serial concentrations of Nile Red dissolved in 10% DMSO were prepared to optimise an appropriate concentration of the dye to be used in the flow cytometry technique as described in section 2.23.2.

Table 6.1 shows the analysis of flow cytometry results using flow Jo software in which it is obvious that the concentration 0.2 $\mu g/ml^{-1}$ was the ideal concentration of Nile Red in terms of percentage of stained cells. However, the optimum concentration of Nile Red was selected based on the highest fluorescent intensity of *N. salina* cells (% of stained cells) which was 92 % and lowest cell population of *N. salina* after flow cytometry analysis.

These findings were in close agrement with Chen *et al.*, (2009) and Cabanelas *et al.* (2016) who observed that 0.5 μg/ml and 0.8 μg/ml, respectively, were the optimum concentrations of Nile red dye. In contrast, these observations do not agree with Satpati and Pal, (2014) who reported that 5μg/ml Nile red dye was the optimum concentration used to stain different strains of green microalgae.

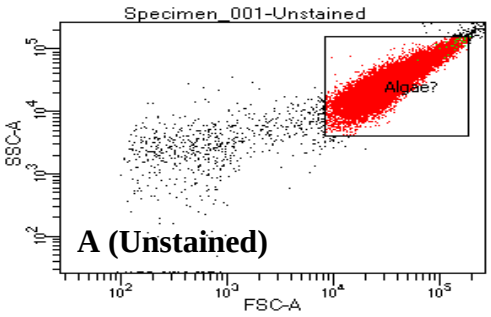
In general, the selection of the optimum concentration of Nile red dye varies between different microalgal species, because each strain has a different response to Nile red fluorescence due to their cell wall composition which affects the penetration of Nile red dye through the cells and the organic solvent used as a stain carrier (Rumin *et al.*, 2015).

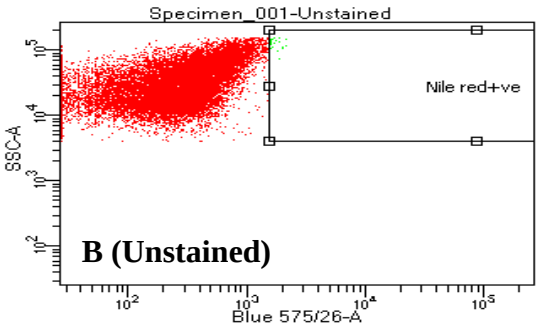
Specimen number	Nile Red concentration (μg/ml^{-1})	Cell population	Stained cells population	Stained cells (%)
1	Unstained	2.22	1.600	0.1
2	0.05	65.50	72.86	92.7
3	0.1	18.52	21.74	89.3
4	**0.2**	**17.64**	**19.83**	**92.0**
5	0.3	28.46	32.30	91.1
6	0.4	31.28	33.21	95.7
7	0.6	194.89	201.03	97.6

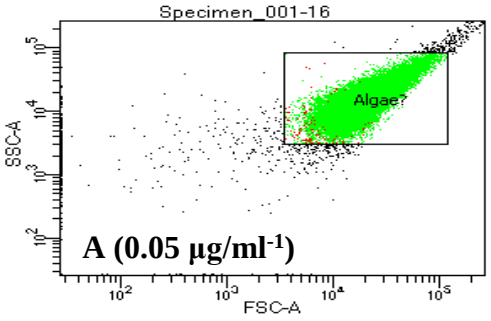
Table 6.1: Flow cytometry analysis of *N. salina* strain. Cells stained with different concentration of Nile Red using 10% of DMSO as best concentration (final concentration) for 10 min staining in comparison with unstained cells as control. Each value is the average of three technical repeats.

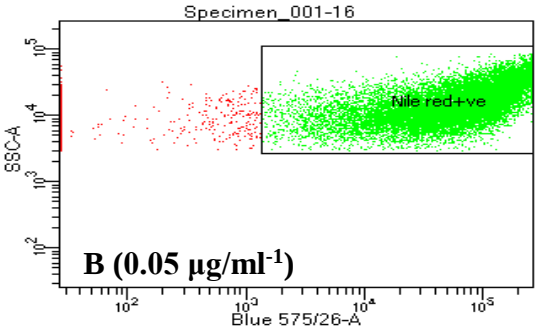
As shown in Figure 6.2 revealed the FSC vs (A) and SSC (B) area dot plot of scattered unstained and stained *N.salina* cells with different concentrations of Nile Red (0.05, 0.1, 0.2, 0.3, 0.4, 0.6 μg/ml^{-1}).

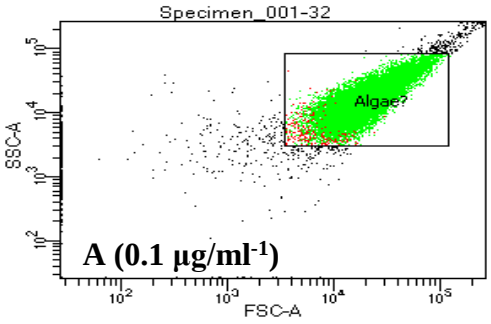
The unstained cells were utilised to evaluate the auto fluorescence pigments of the microalgae and therefore create the gate that distinguishes between the stained and unstained cells. The stained cells showed uniform scatter.

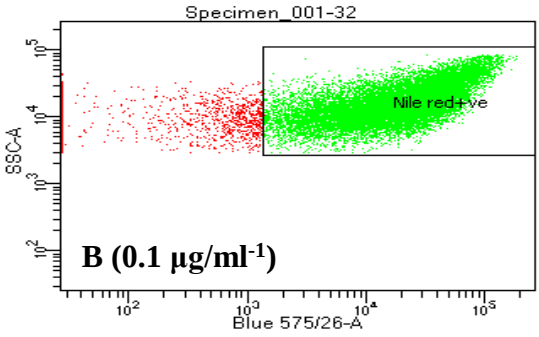

Specimen_001-Unstained
Algae?
SSC-A
FSC-A
A (Unstained)

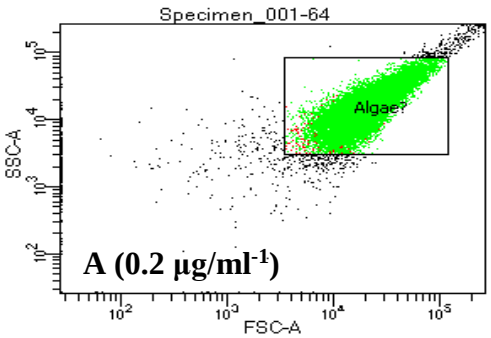

Specimen_001-Unstained
Nile red+ve
SSC-A
Blue 575/26-A
B (Unstained)

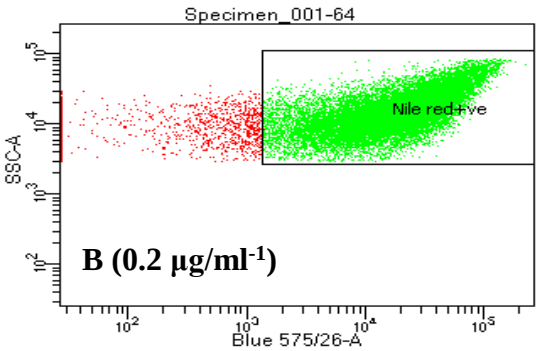

Specimen_001-16
Algae?
SSC-A
FSC-A
A (0.05 µg/ml-1)


Specimen_001-16
Nile red+ve
SSC-A
Blue 575/26-A
B (0.05 µg/ml-1)


Specimen_001-32
Algae?
SSC-A
FSC-A
A (0.1 µg/ml-1)


Specimen_001-32
Nile red+ve
SSC-A
Blue 575/26-A
B (0.1 µg/ml-1)


Specimen_001-64
Algae?
SSC-A
FSC-A
A (0.2 µg/ml-1)


Specimen_001-64
Nile red+ve
SSC-A
Blue 575/26-A
B (0.2 µg/ml-1)

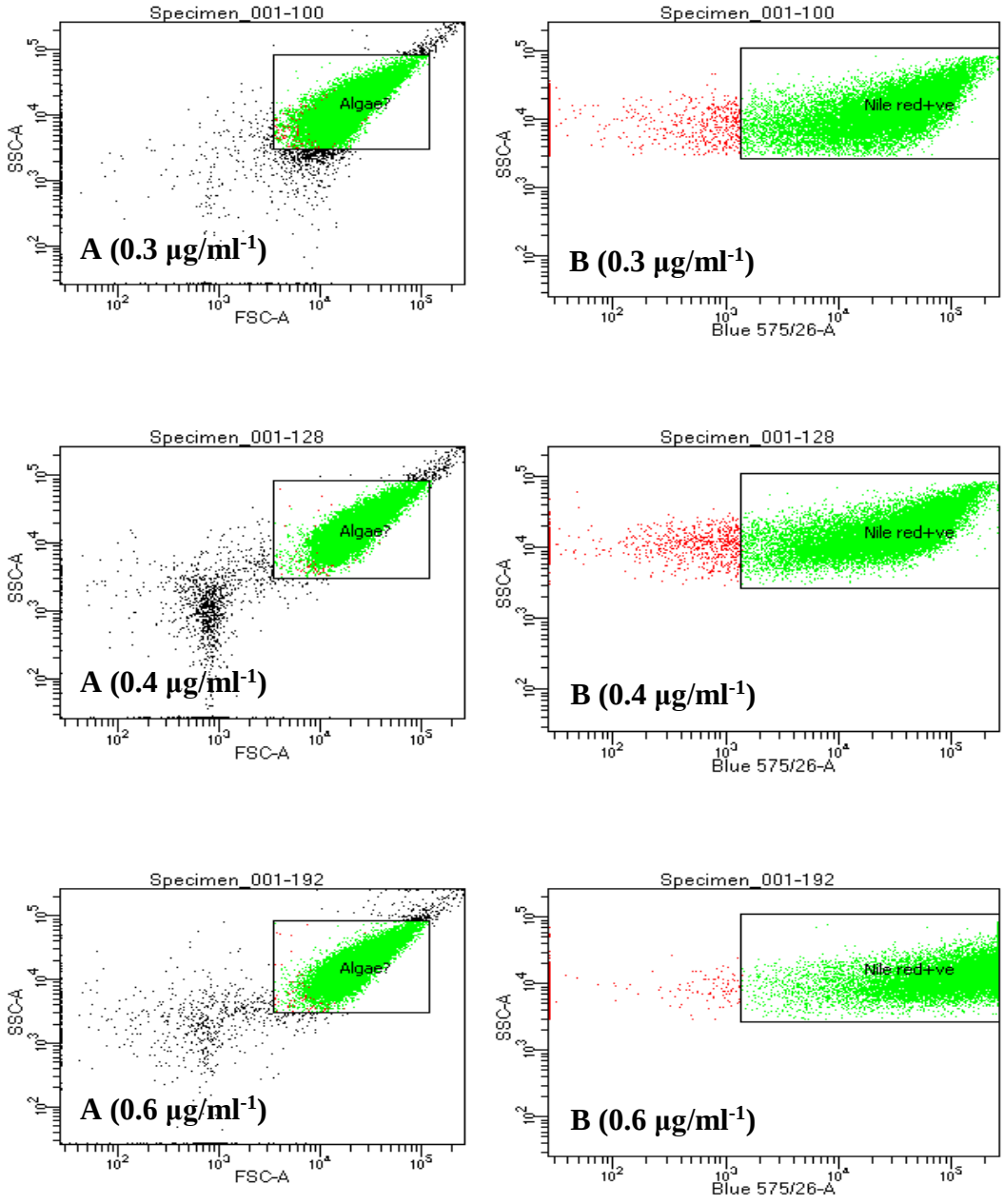


Figure 6.2: Flow cytometry analysis of *N.salina* cells unstained and stained, revealed the FSC vs (A) and SSC (B) area plot of scattered cells with different concentrations of Nile Red (0.05, 0.1, 0.2, 0.3, 0.4, 0.6µg/ml^{-1}) with 10% DMSO as the final concentration for 10 min staining to determine the optimum concentration of Nile Red to be used in the experiment using the flow cytometry. Each value is the average of three technical repeats.

6.2.1.3. Determination of The Neutral Lipid in *N. salina* Cells Grown Under Different Growth Conditions

The neutral lipid contents of the *N.salina* cells grown under different growth conditions included f/2 medium supplemented 0.6 M NaCl, f/2 growth medium with nitrogen starvation and f/2 growth medium with pH 10 in comparison with normal f/2 growth medium as control using 10% DMSO and 0.2 µg/ml^{-1}of Nile Red as final concentration for 10 min stainingwere measured using the flow cytometry analysis as as described in section 2.23.3. Table 6.2 illustrates the effect of different growth conditions on the neutral lipid contents in *N.salina* cells. The results very clearly indicated that the highest percentage of neutral lipids were observed with the cells grown in f/2 medium with pH 10 stress condition, this is based on the highest value of cell median. Morover, unstained cells (control) were used to show the level of auto fluorescence of the chlorophyll and other photosynthetic pigments, Figure 6.3.

Sample	% of Stained Cells	Cells Median
Unstained (Control)	0.1	2.22
Normal f/2 medium	92.8	47.90
0.6 M NaCl stress	99.8	43.80
Nitrogen starvation	98.3	92.37
pH 10 of f/2 medium	97.6	107.88

Table 6.2: Flow cytometry analysis of *Nannochloropsis salina* cells grown under different growth conditions including f/2 medium containing 0.6 M NaCl, f/2 medium with nitrogen starvation and f/2 growth medium with pH 10 in comparison with normal f/2 growth medium as control, with 10% DMSO and 0.2 µg/ml^{-1}of Nile Red as optimum concentration (final concentration). Each value is the average of three replicates for 10 min staining.

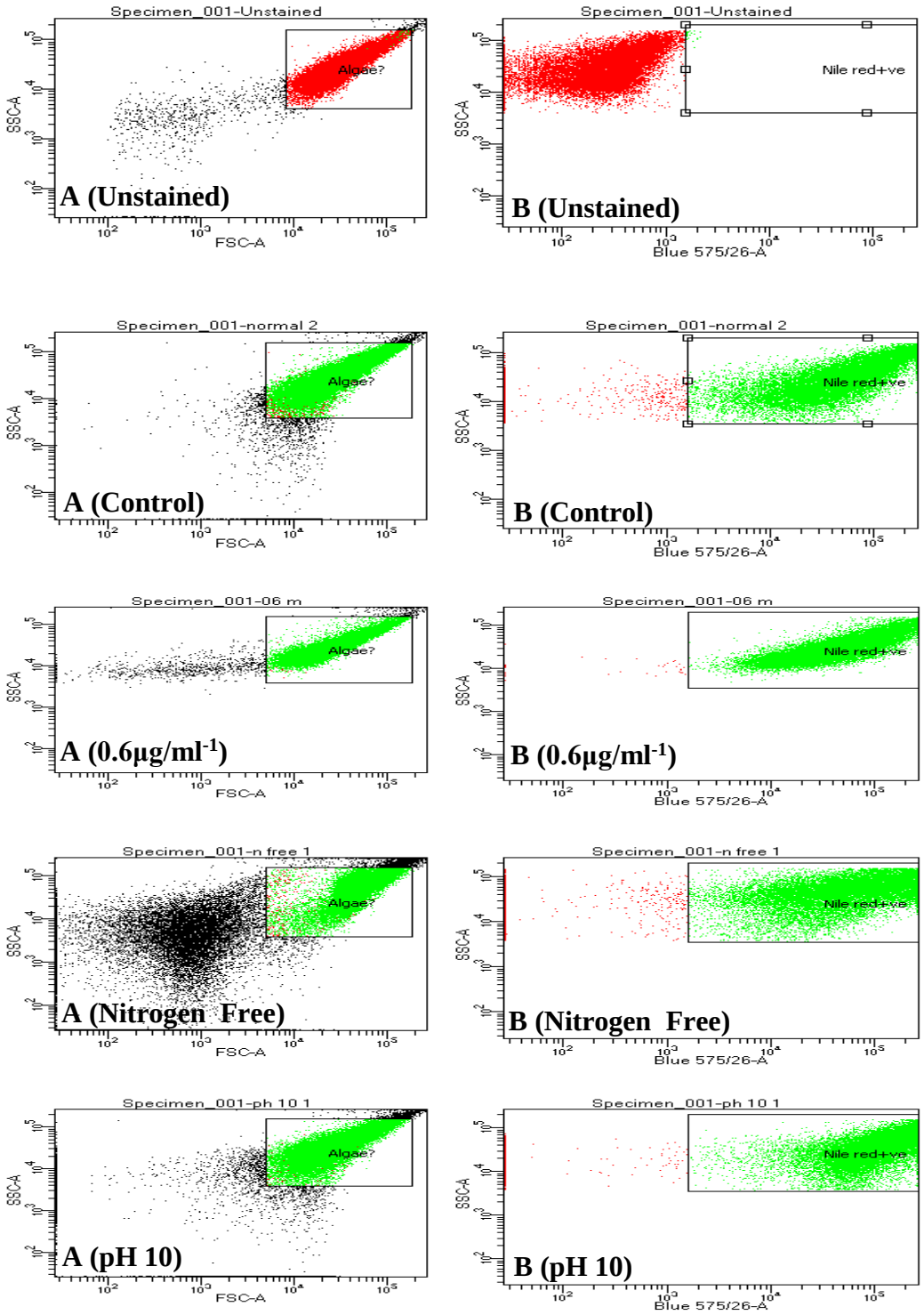


Figure 6.3: Flow cytometry analysis of neutral lipid content in *N.salina* cells grown under different growth conditions included f/2 medium supplemented with 0.6 M NaCl, f/2 medium with 100% nitrogen starvation and f/2 growth medium with pH 10 comparing with normal f/2 growth medium as control, using 10% DMSO and 0.2 µg/ml^{-1} of Nile Red as optimum concentration. Cells unstained and stained, revealed the FSC vs (A) and SSC (B) area plot of scattered cells each value is the average of three replicates.

6.2.2. Use of PAM Fluorimeter to Examine in Detail The Effect of Environmental Stress on Photosynthesis

In particular, Chlorophyll fluorescence is often used as an indicator of inhibition of photosynthetic energy conversion caused by environmental conditions in plants, algae and bacteria (Consalvey *et al.*, 2005).

Since the association was made between fluorescence and photosynthesis through illumination of dark adapted leaves and their correlation with CO_2 assimilation, chlorophyll analysis has been used as the essential technique to understand the reaction mechanics of photosynthesis processes and thereafter assessing plant stress physiology, ecophysiology and phytopathology (Maxwell and Johnson, 2000; Enriquez and Borowitzka, 2010).

However, the Pulse Amplitude Modulation (PAM) fluorometry technique operates on a basic signal modulation in which light is delivered by a series of signal pulses. PAM fluorometers can be used in photobiology to provide a fast assessment of photosynthetic state based on fluorescence and be able to obtain spectrofluoromic measurements of the kinetics of fluorescence by measuring the increases and decay of the light harvesting antennae of the thylakoid membranes (Maxwell and Johnson, 2000).

In order to determine the integrity of photosynthetic machinery (particularly PSII), the maximum quantum efficiency of PSII (F_v/F_m) of *N. salina* was tracked under different growth conditions to elucidate the impact of environmental stress on integrity of photosynthetic apparatus using 0.6 M NaCl, nitrogen starvation (NF) and f/2 growth medium with pH10 compared to normal f/2 growth medium as control treatment as shown in Figure 6.4.

The results very clearly indicate that the maximum quantum efficiency (F_v/F_m) of the control treatment and all of the stress conditions decreased after 24 hours. However, the decrease was more significant for nitrogen limited cells and to a lesser extent for cells exposed to 0.6 M NaCl. There was little effect of pH 10 on the F_v/F_m value. There was little or no change to this pattern of effects over the next 120 hours (Figure 6.4).

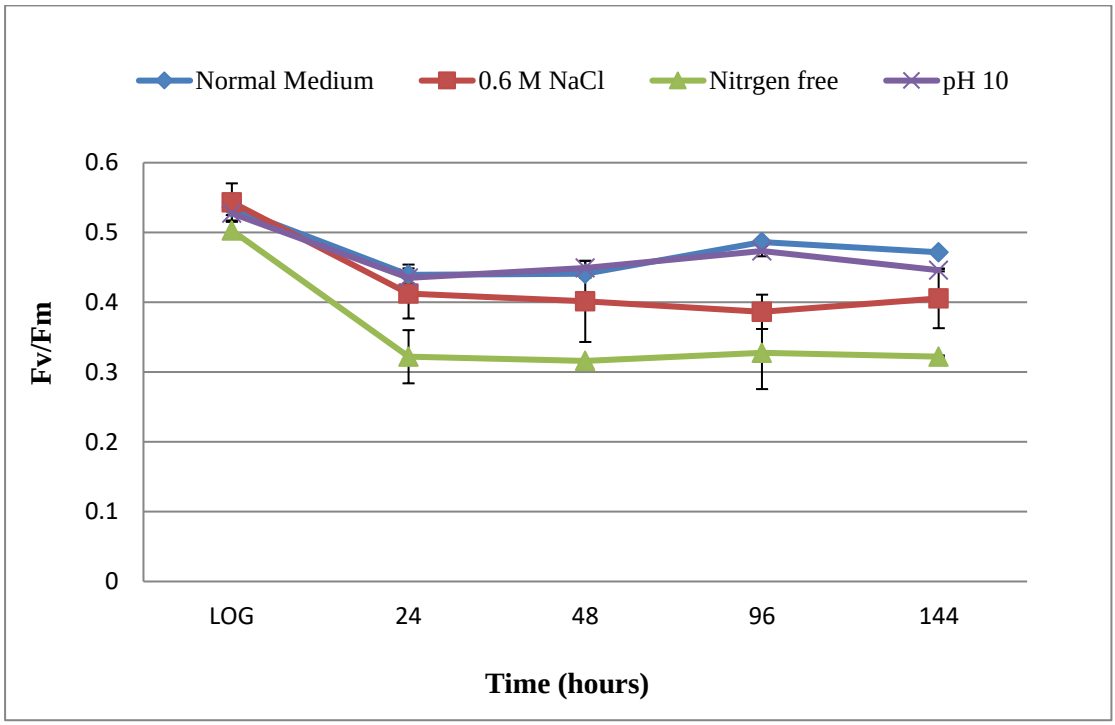


Figure 6.4: Maximum quantum yield of PSII (Fv/Fm) of *N. salina* cells before resuspension in different treatments (LOG) and after cell resuspension in different treatments including 0.6 M NaCl, nitrogen starvation and f/2 growth medium with pH10 compared to normal f/2 medium as control. Each value is the average of three replicates. plus or minus the standard error.

6.3. Conclusion

In this chapter the flow cytometry technique was considered as a favourable approach for determining the microalgal lipid accumulated by *N. salina* cells grown in f/2 medium supplemented with 0.6 M NaCl, f/2 growth medium with nitrogen starvation and f/2 growth medium at pH 10 in comparison with normal f/2 growth medium as control using 10% DMSO and 0.2 $\mu g/ml^{-1}$ of Nile red as final concentration for 10 min staining.

As part of the process of examining internal lipid, the DMSO was prepared using different serial concentrations of DMSO ranging from 10% to 60% using distilled water, and different serial concentrations of Nile red dye was dissolved in DMSO at 0.05, 0.1, 0.2, 0.3, 0.4 and 0.6 $\mu g/ml^{-1}$ as final concentration to optimise an appropriate concentration to be used in the flow cytometry technique. The results very clearly indicated that the 10% DMSO showed the best concentration to be used as a stain carrier for determination of lipid content in *N. salina* cells in combination with 0.2 $\mu g/ml^{-1}$ of Nile Red as the ideal concentration of Nile Red in terms of percentage of stained cells, as shown in Figure 6.1 and Figure 6.2 respectively. The cellular lipid contents of *N. salina* cells were analysed at different stress conditions. As a result, the maximum percentage of accumulated TAG was observed in cells grown in f/2 medium with pH 10 (Table 6.2).

Further observations, the maximum quantum efficiency (Fv/Fm) of *N. salina* cells was measured under the same treatment conditions mentioned above using a Pulse Amplitude Modulation (PAM) fluorometer technique to elucidate the impact of these treatments on integrity of photosynthetic apparatus. As shown in Figure 6.4, the maximum quantum efficiency (F_v/F_m) of the control treatment and all of the stress conditions decreased after 24 hours. However, the decrease was more significant for

nitrogen limited cells and to a lesser extent for cells exposed to 0.6 M NaCl. There was little effect of pH 10 on the F_v/F_m value. There was little or no change to this pattern of effects over the next 120 hours.

General Conclusions and Future Work

Chapter Seven

7.1. General Conclusions

Microalgae are important biological resources that have a wide range of biotechnological applications. Due to their high growth rates and a variety of high-value metabolic products, microalgae are considered promising candidates for polyunsaturated fatty acids (PUFA), triacylglycerols, pigments, such as carotenoids and phycobiliproteins, food supplements and biodiesel production. In terms of environmental biotechnology, microalgae are beneficial for bioremediation of agro-industrial wastewater and as biological tool for assessment and monitoring of environmental toxicants such as heavy metals, pesticides and pharmaceuticals. In recent years, microalgae have attracted much attention due to their potential use as a feedstock for biodiesel production.

As stated in section 1.15, one of the main aims of this project was to confirm the genomic identification and characterisation of a locally isolated green microalgal species obtained from the Culture Centre of Algae and Protozoa (CCAP) using 18S rRNA gene sequencing and light microscopy. The rDNA sequencing work confirmed that the algal isolate was indeed a species of the green algal genus *N. salina* with lengths of the partial forward and reverse sequences achieving 591 bp and 596 bp respectively (Chapter 3).

Some physiological characterization of unicellular *Nannochloropsis salina* strain CCAP 849/2 were investigated (Chapter 4). They included growth patterns, comparison of three different media for growing *N. salina*, vitamin B12 requirement, salinity adaptation, total biomass dry weight content, relationship between optical density and cell numbers, effect of nitrogen starvation and external pH on the cell growth. Optimum growth for *N. salina* cells was found for modified f/2 growth medium without silica followed by the growth rate of *N. salina* cells grown in f/2

defined medium. *N. salina* cells, however, showed lowest growth in AMCONA medium. There was no significant difference in the level of growth for normal f/2 medium and vitamin B12 free f/2 medium grown cells. The normal condition showed a higher growth rate and final biomass level. The ability of *N. salina* cells was observed to be fully adapted to grow and survive across a salinity range from 0.2 M to 1 M NaCl. However, the highest specific growth rate and cell growth productivity revealed growth of *N. salina* cells was reported at 0.2 M with lowest growth at 0.6 M NaCl over incubation period at 25ºC. Furthermore, the *salina* cells were grown in f/2 medium combined with different concentrations of nitrogen (0.0%, 25%, 50% and 75% of $NaN0_3$) and the effect on the growth was investigated. It could be seen that the growth rate and final biomass content reached after 30 days were decreased with nitrogen starvation. Under pH levels of pH 5, 6, 7, 8, 9 and 10, *salina* cells were grown to investigate the influence of pH on the growth rate biomass level. It was found that maximum cell densities were observed at pH 8 over 25 days incubation followed by the cell densities of *N. salina* cellsgrown at pH 7, although cells grown under pH 5 showed lowest growth rates.However, there was no significant difference in the level of growth for pH9 and pH10. In this study, the relationship between optical density (OD_{595}) and dry weight biomass of *N. salina* cells was established using a standard dry weight curve of *N. salina* cells, in order to convert the levels of Nile Red fluorescence reading obtained from the Triolein calibration curve into percentage of lipid for*N. salina* (% dry weight) under normal and stress conditions of salinity, external pH and nitrogen starvation using the graph equation.

In Chapter 5, the neutral lipid accumulated by *N. salina* cells grown under different growth conditions using f/2 growth medium was detected and then quantified using the Nile Red technique, and the free fatty acids profiles of *N. salina* were analysed by

a Gas Chromatography - Mass Spectrometry (GC-MS) technique after direct transesterification with hydrochloric acid in methanol and also by using a Nuclear Magnetic Resonance (NMR) technique.

As part of the process of examining potential neutral lipid production by *N. salina* cells using Nile Red technique, the cell concentrations of *N. salina* strain, the Nile red dye concentration and triolein concentration curve were optimised. It was found 75% *N. salina* cell concentration and 0.2 μmol ml^{-1} Nile red dye concentration for 15 minutes staining were optimum conditions. After that, the amount of neutral lipid accumulated by *N. salina* grown in normal growth conditions was assessed as a real experiment using the optimum conditions of Nile Red mentioned above. It was found that, the percentage of lipid accumulated by *N. salina* cells under normal was 71.78% of total dry weight mass. Furthermore, the percentage of lipid content accumulation over four weeks (week1- week 4) for *N. salina* cells was measured using the Nile red fluorescence at different salinity stresses. It could be seen that the highest percentage of lipid content was accumulated by cells grown at 0.6 M NaCl, with 74.65% of total dry weight after four weeks incubation. On the other hand, the percentage of lipid content accumulated over a period of four weeks which was determined using the Nile red fluorescence method was increased with increasing pH stress for *N. salina* cells, it was 76.89 % of total dry weight mass after four weeks incubation at pH 10. The percentage of lipid content accumulation over four weeks for *N. salina* cells subjected to nitrogen limitation and starvation was assessed. It was found that the percentage of neutral lipid content increase with increasing nitrogen starvation from 75% to 0.0% $NaNO_3$. It was clear that the highest percentage of lipid content of *N. salina* cells was in the nitrogen free environment after four weeks of incubation (72.12%).

In comparison with the Nile red method, the neutral lipid content accumulated by *N. salina* cells grown under pH 10, 0.6 M NaCl and absolutely free nitrogen condition was examined using the Gravimetric method. It could be seen also that, the percentage neutral lipid accumulation in the nitrogen free environment, normal, 0.6 M NaCl and pH10 were 42%, 41.7%, 35.2% and 31.5% respectively as the cells entered stationary phase in week four of incubation.

To further investigates, the fatty acid methyl esters (FAMEs) that occur in *N. salina* neutral lipid grown in normal f/2 medium, 0.6 M NaCl f/2 medium and nitrogen free f/2 medium were identified using gas chromatography mass spectrometry (GC-MS) technique. The results very clearly indicated that similar fatty acid methyl esters were observed in all three growth conditions examined. However, these fatty acids were identified as Hexadecane pentadecane acid methyl ester (C15:0, n-3), Tridecanoic acid methyl ester(C13:0), Dodecanoic acid methyl ester (C12:0), Palmitioleinate acid methyl ester (C16:2), Palmitic acidmethyl ester (C16:1), Oleic acid methyl ester (C18:1) and Eicosapentaenoic acid methyl ester (C20:0). However, palmitic acid methyl ester (C16:0) and oleic acid methyl ester (C18:1) are saturated or monounsaturated fatty acids and therefore they are suitable for biofuel production.

In this study, the NMR spectra were used to identify the compatible solutes produced by the *N. salina* cells to survive extreme conditions under both high salinity and nitrogen stress conditions. It was found that the fatty acids accumulated by cells grown in 0.6 M of NaCl f/2 medium were higher than for cells grown in normal f/2 medium and Nitrogen free f/2 medium.

In Chapter 6, a flow cytometry technique was used to determine the cellular lipid accumulated by *N. salina* cells grown in f/2 medium supplemented with 0.6 M NaCl, f/2 growth medium with nitrogen starvation and f/2 growth medium at pH 10 in

comparison with normal f/2 growth medium as control using 10% DMSO and 0.2 $\mu g/ml^{-1}$ of Nile red as final concentration for 10 min staining. It was found that the maximum percentage of accumulated cellular lipid was found in cells grown in f/2 medium at pH 10. The same growth conditions were used to measure the maximum quantum efficiency (Fv/Fm) of *N. salina* cells using the PAM technique. It can be seen that the maximum quantum efficiency (F_v/F_m) was decreased after 24 hours at all growth conditions.

7.2. Future Work

Based on the results of this study, the following future work is recommended. It is proposed to continue to work on environmental stress effects on TAG production by *N. salina* to make it a commercially viable strain to produce biodiesel using higher salinity concentrations up to 1 NaCl, different nitrogen sources, light irradiation, ultraviolet light irradiance (UV-A radiation and UV-B radiation), and heavy metals. Compare the lipid yield obtained using NR dye with other fluorometric approaches using lipophilic fluorescent BODIPY dye or other techniques such as Raman spectroscopy, and Fourier transform infrared spectroscopy to assess the optimum method of microalgal lipid quantification using *N. salina*. Effort to use various mutagens including heavy carbon ions, or chemical mutagens such as ethyl methane sulfonate or other mutagens to induce the lipid content of microalgae to reduce the cost of producing biodiesel from *N. salina*. The observation of good culture of *N. salina* in larger fermenters (up to 200) litres needs further work to explain this phenomenon. This will allow a full investigation of the properties of *N. salina* which can be used to test biofuel production in *N. salina* in a several thousand litre capacity outdoor fermenter.

References

Chapter Eight

Aaronson, S. (2006). Effect of incubation temperature on the macromolecular and lipid content of the phytoflagellate *Ochromonas danica*. *Journal of Phycology,* 9 (1): 111-113.

Abinandan, S. And Shanthakumar, S. (2015). Challenges and opportunities in application of microalgae (*Chlorophyta*) for wastewater treatment: Areview. *Renewable and Sustainable Energy Reviews,* 52: 123 -132.

Abreu, A. P., Fernandes B., Vicente, A. A., Teixeira, J. and Dragone. G. (2012). Mixotrophic cultivation of *Chlorella vulgaris* using industrial dairy wasteas organic carbon source. *Bioresource Technology*, 118: 61- 66.

Abu-Rezq, T. S., Al-Musallam, L., Al-Shimmari, J. and Dias, P. (1999). Optimum production conditions for different high-quality marine algae. *Hydrobiologia,* 403: 97-107.

Ahlgren, G. and Hyenstrand, P. (2003). Nitrogen limitation effects of different nitrogen sources on the nutritional quality of two freshwater organisms, *Scenedesmus quadricauda* (Chlorophyceae) and *Synechococcus* sp. (Cyanophyceae). *Journal of Phycolog*y, 39: 906 - 917.

Ahmad, A. L., Yasin, N. H., Derek, C. J. and Lim, J. K. (2011). Microalgae as a sustainable energy source for biodiesel production: A review. *Renewable and Sustainable Energy Reviews,* 15: 584 - 593.

Akkerman, I., Janssen, M., Rocha, J., Wijffels, R. H. (2002). Photobiological hydrogen production: photochemical efficiency and bioreactor design. *International Journal of Hydrogen Energy*, 27:1195 -1208.

Alvarado, D., Buitrago, E., Sole, M. and Frontado, K. (2008). Experimental evaluation of a composted seaweed extract as microalgal culture media. *Aquaculture International* 16 (1): 85 - 90.

Amaro, H. M., Guedes, A. C. and Malcata, F. X. (2011). Advances and perspectives in using microalgae to produce biodiesel. *Applied Energy*, 88: 3402-3410.

Andersen. R. A., Brett, R. W., Potter, D. and Sexton, J. P. (1998). Phylogeny of the Eustigmatophyceae based upon 18S rDNA, with emphasis on *Nannochloropsis*. *Protist* 149 (1) : 61-74.

Azachi, M., Sadka, A., Fisher, M., Goldshlag, P., Gokhman, I. and Zamir, A. (2002). Salt induction of fatty acid elongase and membrane lipid modifications in the extreme halotolerant alga *Dunaliella salina*.*Plant Physiology*,129 (3):1320 -1329.

Bahadar, A. And Khan, M. B. (2013). Progress in energy from microalgae:Areview. *Renewable and Sustainable Energy Reviews,* 27: 128 -148.

Balduyck, L., Veryser, C., Goiris, K. Bruneel, C. Muylaert, K. Foubert, I. (2015). Optimization of a Nile Red method for rapid lipid determination in autotrophic, marine microalgae is species dependent. *Journal of Microbiological Methods*,152 - 158.

Banerjee, A., Sharma, R., Chisti, Y. and Banerjee, U. C. (2002).*Botryococcus braunii*: a renewable source of hydrocarbons and other chemicals. *Critical Reviews in Biotechnology*, 22 (3) : 245 - 279.

Barnwal, B. K. and Sharma, M. P. (2005). Prospects of biodiesel production from vegetable oils in India. *Renewable and Sustainable Energy Reviews*. 9:363-378.

Bartley, M. L., Boeing, W. J., Corcoran, A., Holguin, F.O., Schaub, T. (2013). Effects of salinity on growth and lipid accumulation of biofuel microalga *Nannochloropsis salina* and invading organisms. *Biomass and Bioenergy*, 54: 83 - 88.

Bartley, M. L.,Boeing, W. J. Dungan, B. N., Holguin, F. O. and Schaub, T. (2014). pH effects on growth and lipid accumulation of the biofuel microalgae *Nannochloropsis salina* and invading organisms. *Journal of Applied Phycology*, 26: 1431-1437.

Bartley, M. L., Boeing, W. J., Daniel, D., Dungan, B. N. and Schaub, T. (2016). Optimization of environmental parameters for *Nannochloropsis salina* growth and lipid content using the response surface method and invading organisms. *Journal of Applied Phycology*, 28:15-24.

Becker, E. W. (1994). Microalgae: Biotechnology and Microbiology. Cambridge Univ. Press, Cambridge, NY, 178.

Bellou, S. and Aggelis, G. (2012). Biochemical activities in *Chlorella* sp. and *Nannochloropsis salina* during lipid and sugar synthesis in a lab-scale open pond simulating reactor. *Journal of Biotechnology*, 164: 318- 329.

Bellou, S., Baeshen, M. N., Elazzazy, A. M., Aggeli, D., Sayegh, F., Aggelis, G. (2014). Microalgal lipids biochemistry and biotechnological perspectives. *Biotechnology Advances,* 32: 1476-1493.

Ben-Amotz, A., Tornabene, T. G. and Thomas, W. H. (1985). Chemical profile of selected species of microalgae with emphasis on lipids. *Journal of Phycology*, 21: 72-81.

Bertozzini, E., Galluzzi, L., Penna, A. and Magnani, M. (2011). Application of the standard addition method for the absolute quantification of neutral lipids in microalgae using Nile red. *Journal of Microbiological Methods*, 87**:** 17-23.

Bhandari, R. R. and Sharma, P. K. (2011). Photosynthetic and biochemical characterization of pigments and UV-absorbing compounds in *Phormidium tenue* due to UV-B radiation. *Journal of Applied Phycology*, 23 (2): 283 - 292.

Bharti, S. K. and Roy, R. (2012). Quantitative H NMR spectroscopy. *Trends in Analytical Chemistry*, 35: 5 - 26.

Bjornsson, W. J., Nicol, R. W., Dickinson, K. E. and McGinn, P. J. (2013). Anaerobic digestates are useful nutrient sources for microalgae cultivation: functional coupling of energy and biomass production. *Journal of Applied Phycology*, 25: 1523 - 1528.

Bligh, E. G. and Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, 37(8) 911- 917.

Bondioli, P., Bella, L. D., Rivolta, G., Zittelli, G. C. b, Bassi, N., Rodolfi, L., Casini, D., Prussi, M., Chiaramonti, D. and Tredici, M. R. (2012). Oil production by the marine microalgae *Nannochloropsis sp*. F&M-M24 and *Tetraselmis suecica* F&M-M33. *Bioresource Technology*, 114: 567- 572.

Bongiovani, N., Popovich, C., Martínez, A. M., Freije, H., Constenla, D. and Leonardi, P. (2013). *In vivo* measurements to estimate culture status and neutral lipid accumulation in *Nannochloropsisoculata* CCALA 978: implications for biodiesel oil studies. *Algological Studies*, 142: 3 - 16.

Bongiovani, N., Sanchez-Puerta, V., Popovich, C. and Leonardi, P. (2014). Molecular and phylogenetic identification of an oil producing strain of *Nannochloropsis oceanic* (Eustigmatophyceae) isolated from the southwestern Atlantic coast (Argentina). *Revista de Biología Marina y Oceanografía*, 49: 615- 623.

Borowitzka, M. A. and Moheimani, N. R. (2012). Open Pond Culture Systems. *Developments in Applied Phycology*,5: 133-152.

Borowitzka, M. A. and Siva, C. J. (2007). The taxonomy of the genus *Dunaliella* (Chlorophyta, Dunaliellales) with emphasis on the marine and halophilic species. *Journal of Applied Phycology*, 19: 567- 590.

Bouaicha, N., Chezeau, A., Turquet, J., Quod J. P. and Puiseux-Dao, S. (2001). Morphological and toxicological variability of *Prorocentrum lima* clones isolated from four locations in the south-west Indian Ocean. *Toxicon*, 39 (8): 1195-11202.

Bougaran, G., Rouxel, C., Dubois, N., Kaas, R., Grouas, S., Lukomska, E., Coz, J. L. Cadoret, J. (2012). Enhancement of Neutral Lipid Productivity in the Microalga *Isochrysis* Affinis Galbana (T-Iso) by a Mutation-Selection Procedure. *Biotechnology and Bioengineering*, 109: 2737- 2745.

Boussiba, S., Vonshak, A., Cohen, Z., Avissar, Y. and Richmond, A. (1987). Lipid and biomass production by the halotolerant microalga *Nannochloropsis salina*. *Biomass*, 12: 37- 47.

Boyle, N. R. and Morgan, J. A. (2009). Flux balance analysis of primary metabolism in *Chlamydomonas Reinhardtii. BMC Systems Biology*, 1-14.

Brennan, L. and Owende, P. (2010). Biofuels from microalgae-A review of technologies for production, processing, and extractions of biofuels and co-products. *Renewable and Sustainable Energy Reviews*, 14: 557-577.

Breuer, G., Lamers, P. P., Martens, D. E., Draaisma, R. B. and Wijffels, R. H. (2012). The impact of nitrogen starvation on the dynamics of triacylglycerol accumulation in nine microalgae strains. *Bioresource Technology*, 124: 217-226.

Breuer, G., Evers, W. A., de Vree, J. H., Kleinegris, D. M., Martens, D.E., Wijffels, R. H. and Lamers, P. P. (2013). Analysis of fatty acid content and composition in microalgae. *Journal of Visualized Experiments*, 5: 1- 9.

Brown, M. R., Dunstan, G. A., Norwood, S. J. and Miller, K. A. (1996). Effects of harvest stage and light on the biochemical composition of the diatom *Thalassiosira pseudonana*. *Journal of Phycology*, 32: 64 -73.

Brown, J. S. (1987). Functional organization of chlorophyll a and carotenoids in the alga, *Nannochloropsis salina*. *Plant Physiology,* 83, 434 - 437.

Budarin, V., Ross, B. A., Biller, P., Riley, R., Clark, J. H, Jones, J. M., Gilmour, D. J. and Zimmermand, W.(2012). Microalgae biorefinery concept based on hydrothermal microwave pyrolysis, *Green Chemistry,* 14: 3251- 3254.

Buono, S., Langellotti, A. L., Martello, A., Rinnaa, F. and Fogliano, V. (2014). Functional ingredients from microalgae. *Food and Function* - Royal Society of Chemistry, 5: 1669-1685.

Burki, F. (2016). The Eukaryotic Tree of Life from a Global Phylogenomic Perspective. *Cold spring harbor perspectives in biology*, 4: 01-17.

Burlew, J. S. (1953). Algal culture from laboratory to pilot plant. This book first published 15 July1953.

Cabanelas, I. T. D., Zwart, M. V. Z., Kleinegris, D. M. M., Wijffels, R. H. and Barbosa, M. J. (2016). Sorting cells of the microalga *Chlorococcum littorale* with increased triacylglycerol productivity. *Biotechnol Biofuels*, 9 (183): 1- 12.

Cai, H., Archambault, M., Prescott, J. F. (2003). 16S ribosomal RNA sequence-based identification of veterinary clinical bacteria. *Journal of Veterinary Diagnostic Investigation*,15: 465 - 469.

Cai, T., Park, S. Y. and Li, Y. (2013) Nutrient recovery from wastewater streams by microalgae: status and prospects. *Renewable and Sustainable Energy Reviews*, 19:360 - 369.

Camacho-Rodriguez, J., Ceron-García M. C., Fernandez-Sevilla, J. Molina-Grima, M. E. (2015). The influence of culture conditions on biomass and high value product generation by *Nannochloropsis gaditana* in aquaculture. *Algal Research*, 11: 63 -73.

Campos, H., Boeing, W, J., Dungan, B. N. and Schaub, T. (2014). Cultivating the marine microalga *Nannochloropsis salina* under various nitrogen sources: Effect on biovolume yields, lipid content and composition, and invasive organisms. *Biomass and bioenergy*, 66: 301 - 307.

Cao, S., Zhang, X., Fan, X., Qiao, H., Liang, C., Xu, D., Mou, S., Wang, W. and Ye, N. (2013). Phylogeny and characterization of *Nannochloropsis oceanica* var. *sinensis* var. nov. (Eustigmatophyceae), a new oleaginous alga from China. *Phycologia*, 52: 573 - 577.

Carpinelli, E. C., Telatin, A., Vitulo, N., Forcato, C., D'Angelo, M., Schiavon, R., Vezzi, A., Giacometti, G. M., Morosinotto, T. and Valle, G. (2013). Chromosome scale genome assembly andtranscriptome profiling of *Nannochloropsis gaditana* innitrogen depletion. *Molecular Plant*, 6: 323 - 335.

Carvalho, A. P. and Malcata, F. X. (2005). Optimization of ω-3 fatty acid production by microalgae: Crossover effects of CO_2 and light intensity under batch and continuous cultivation modes. *Marine Biotechnology*, 7 (4): 381- 388.

Carvalho, A. P., Meireles, L. A. and Malcata, F. X. (2006). Microalgal Reactors: A Review of Enclosed System Designs and Performances. *Biotechnology Progress*, 22 (6): 1490 - 1506.

Cavonius, L. R., Albers, E. and Undeland, I. (2015). pH-shift processing of *Nannochloropsis oculata* microalgal biomass to obtain a protein-enriched food or feed ingredient. *Algal Research*, 11: 95 -102.

Chatzifragkou, A., Fakas, S., Galiotou-Panayotou, M., Komaitis, M., Aggelis, G. and Papanikolaou, S. (2010). Commercial sugars as substrates for lipid accumulation in *Cunninghamella echinulata* and *Mortierella isabellina* fungi. *European Journal of Lipid Science and Technology*, 112: 1048 -1057.

Chen, C. Y. and Durbin, E. G. (1994). Effects of pH on the growth and carbon uptake of marine phytoplankton. *Journal: Marine Ecology Progress Series,*109: 83 - 94.

Chen, Y., Wamg, Y., Sun, Y., Zhang, L. and Li, W. (2001). Highly efficient expression of rabbit neutrophil peptide-1 gene in *Chlorella ellipsoidea* cells. *Current Genetics*, 39: 365 -370.

Chen, W., Zhang, C., Song, L., Sommerfeld, M. andHu, Q. (2009). A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. *Journal of Microbiological Methods*, 77: 41- 47.

Chen, C., Yeh, K., Aisyah, R., Lee, D. and Chang, J. (2011a). Cultivation, photobioreactor design and harvesting of microalgae for biodieselproduction: A critical review. *Bioresource Technology*, 102: 71-81.

Chen, M., Tang, H., Ma, H., Holland, T. C., Ng, K. Y. S. and Salley, S. O. (2011b). Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*. *Bioresource Technology*, 102:1649-1655.

Chen, W., Sommerfeld, M. and Hu, Q. (2011c). Microwaveassisted Nile red method for *in vivo* quantification of neutral lipids in microalgae, *Bioresource Technology*, 102 (1): 135 - 141.

Chiu, S., Kao, C., Tsai, M., Ong, S., Chen, C., Lin, C. (2009). Lipid accumulation and CO_2 utilization of *Nannochloropsis oculata*in response to CO_2 aeration. *Bioresource Technology*, 100: 833 - 838.

Chisti, Y. (2007). Biodiesel from microalgae. Research review paper. *Biotechnology Advances*, 25: 294 - 306.

Chisti, Y. (2008). Biodiesel from microalgae beats bioethanol. *Trends in Biotechnology*, 26 (3): 126 - 131.

Chisti, Y. (2013). Constraints to commercialization of algal fuels. *Journal of Biotechnology*, 167: 201- 214.

Christophe, G., Kumar, V., Nouaille, **R., Gaudet**, **G., Fontanille**, **P., Pandey**, **A., Soccol**, **C. R. and Larroche, C. (2012).** Recent Developments in Microbial Oils Production: a Possible Alternative to Vegetable Oils for Biodiesel Without Competition with Human Food? *Brazilian Archives of Biology and Technology*, 55: 29- 46.

Chu, F. F., Chu, P. N., Shen, X. F., Lam, P. K. S and Zeng, R. J. (2014). Effect of phosphorous on biodiesel production from *Scenedesmus obliquus* under nitrogen-deficiency stress. *Bioresource Technology*, 152: 241 - 246.

Chu, W. L. (2012). Biotechnological applications of microalgae-Review Article. *IeJSME*, 6 (Suppl 1): S24 - S37.

Chunguang, L. , Hongwen, S. , Lin, Z., Jian, L. , Qian, N., Yuanyi, Z. and Shugui, A. (2006). Effects of salinity formed with two inorganic salts on freshwater algae growth. *Acta Scientiae Circumstantiae*, 26 (1): 157-161.

Cirulis, J. T., Strasser, B. C., Scott, J. A. And Ross, G. M. (2012). Optimization of staining conditions for microalgae with three lipophilic dyes to reduce precipitation and fluorescence variability. *Cytometry Part A*, 7: 618 - 626.

Colman, B., Huertas, I. E., Bhatti, S. and Dason, J. S. (2002). The diversity of inorganic carbon acquisition mechanisms in eukaryotic microalgae. *Functional Plant Biology*, 29 (3): 261- 270.

Coleman, J. R. and Colman, B. (1981). Inorganic carbon accumulation and photosynthesis in a blue-green alga as a function of external pH. *Plant Physiology*, 67 (5): 917- 921.

Consalvey, M., Perkins, R. G., and Patterson, D. M., and Underwood, G. J. C. (2005). PAM Fluorescence: a beginners guide for benthic diatomists. *Diatom Research*, **20** (1): 1- 22.

Converti, A., Casazza, A. A., Ortiz, E. Y., Perego, P. and Borghi, M. D. (2009). Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. *Chemical Engineering and Processing*, 48: 1146 - 1151.

Cooksey, K. E., Guckert, J. B., Williams, S. A. and Callis, P. R. (1987). Fluorometric determination of the neutral lipid content of microalgal cells using Nile Red. *Journal of Microbiological Methods*, 6 (6): 333 - 345.

Courchesne, N. M. D., Parisien, A., Wang, B. and Lan, C. Q. (2009). Enhancement of lipid production using biochemical, genetic and transcription factor engineering approaches. *Journal of Biotechnology*, 141(1-2): 31- 41.

Croft, M. T., Lawrence, A. D., Raux-Deery, E., Warren, M. J. and Smith, A. G. (2005). Algae acquire vitamin B12 through a symbiotic relationship with bacteria. *Nature,* 438: 90 - 93.

Crowe, B., Attalah, S., Agrawa, S., Wallerl, P., Ryan, R., VanWagenen, J., Chavis, A., Kyndt, J., Kacira, M., Ogden, K. and Michael, H. (2012). A Comparison of *Nannochloropsis salina* growth performance in two outdoor pond designs: conventional raceways versus the ARID pond with superior temperature management. *International Journal of Chemical Engineering,* 2012: 1- 9.

Davey, H. M. and Kell, D. B. (1996). Flow cytometry and cell Sorting of heterogeneous microbial populations: the importance of single-cell analyses. *Microbiological Reviews,* 60 (4): 641 - 696.

Davis, R., Aden, A. and Pienkos, P. T. (2011). Techno-economic analysis of autotrophic microalgae for fuel production**.** *Applied Energy,* 88: 3524 -3531.

Dayananda, C., Sarada, R., Bhattacharya, S. and Ravishankar, G. A. (2005). Effect of media and culture conditions on growth and hydrocarbon production by *Botryococcus braunii.Process Biochemistry,* 40: 3125- 3131.

Delgado, M., Jonge, V.N. d. and Peletier, H. (1991). Experiments on resuspension of natural mierophytobenthos populations. *Marine Biology,* 108: 321- 328.

Demirbas, A. (2005). Biodiesel production from vegetable oils via catalytic and non-catalytic supercritical methanol transesterification methods. *Progress in Energy and Combustion Science,* 3: 466 - 487.

Demirbas, A. (2009). Production of Biodiesel from Algae Oils. *Energy Sources, Part A: Recovery, Utilization and Environmental Effects,* 31:163 - 168.

Demirbas, A. and Demirbas, M. F. (2011). Importance of algae oil as a source of biodiesel, *Energy Conversion and Management,* 52: 163 - 170.

Devi, S., Santhanam, A. P., Rekha, V.Ananth1, S., Prasath, B. B., Nandakumar, R., Jeyanthi, S. and Kumar, S. D. (2012). Culture and biofuel producing efficacy of marine microalgae *Dunaliella salina* and *Nannochloropsis* sp. *Journal of Algal Biomass Utilization,* 3: 38 - 44.

Doan, T. T., Sivaloganathan, B. and Obbard, J. P. (2011). Screening of marine microalgae for biodiesel feedstock. *Biomass and Bioenergy,* 35: 2534 -2544.

Doan, T. Y. and Obbard, J. P. (2011). Improved Nile Red staining of *Nannochloropsis* sp. *Journal of Applied Phycology,* 23: 895 - 901.

Doan, T. Y. and Obbard, J. P. (2012). Enhanced intracellular lipid in Nannochloropsis sp. via random mutagenesis and flow cytometric cell sorting. *Algal Research,* 1: 17 - 21.

Dong, H., Williams, E., Wang, D., Xie, Z., Hsia, R., Jenck, A., Halden, R., Li, J., Chen, F. and Place, A. R. (2013). Responses of *Nannochloropsis oceanica* IMET1 to Long-Term Nitrogen Starvation and Recovery. *Plant Physiology,* 162: 1110 -1126.

Dragone, G., Fernandes, B., Vicente, A. A., and Teixeira, J. A. (2010). Third generation biofuels from microalgae. Editors: *A. Méndez-Vilas,* 1355 - 1366.

Drexler, I. L. C. and Yeh D. H. (2014). Membrane applications for microalgae cultivation and harvesting: review. *Reviews in Environmental Science and Biotechnology*, 13:487 - 504.

Edwards, C. (1990). Microbiology of Extreme Environments. Milton Keynes, *Open University Press.*

Einicker-Lamas, M., Mezian, G. A., Fernandes, T. B.; Silva, F. L. S.; Guerra, F., Miranda, K., Attias, M., Oliveira, M. M. (2002). *Euglena gracilis* as a model for the study of Cu^{2+} and Zn^{2+} toxicity and accumulation in eukaryotic cells. *Environmental Pollution*, 120 (3): 779 -786.

Enriquez, S. and Borowitzka, M. A. (2010). The Use of the Fluorescence Signal in Studies of Seagrasses and Macroalgae. Chlorophyll a fluorescence in aquatic sciences methods and applications. Dordrecht: *Springer*, 187-208.

Fabregas, J., Maseda, A., Domínguez, A. and Otero, A. (2004). The cell composition of *Nannochloropsis* sp. changes under different irradiances in semicontinuous culture. *World Journal of Microbiology and Biotechnology*, 20 (1) 31 - 35.

Fakhry, E. M. and Dahlia Maghraby, M. (2015). Lipid accumulation in response to nitrogen limitation and variation of temperature in *Nannochloropsis salina*. *Botanical Studies*, 56 (6): 1- 8.

Fanesi, A., Raven, J. A. and Giordano, M. (2014). Growth rate affects the responses of the green alga *Tetraselmis suecica* to external perturbations. *Plant, Cell and Environment,* 37 (2): 512- 519.

Fawley, K. P. and Fawley, M. W. (2007). Observations on the diversity and ecology of freshwater *Nannochloropsis* (Eustigmatophyceae) with descriptions of new taxa. *Protist,*158: 325 - 336.

Fawley, M. W., Jameson, I., Fawley, K. (2015). The phylogeny of the genus Nannochloropsis (Monodopsidaceae, Eustigmatophyceae), with descriptions of *N. australis* sp. nov. and *Microchloropsis gen. nov*. *Phycologia*, 54 (5): 545 - 552.

Felizardo, P., Correia, M. J. N., Raposo, I., Mendes, J. F., Berkemeier, R., Bordado, J. M. (2006). Production of biodiesel from waste frying oils. *Waste Management*, 26: 487- 494.

Feng, P., Deng, Z., Fan, L. and Hu, Z. (2012). Lipid accumulation and growth characteristics of *Chlorella zofingiensis* under different nitrate and phosphate concentrations. *Journal of Bioscience and Bioengineering*, 114 (4): 405- 410.

Ferrell, J, and Sarisky-Reed, V. (2010). National Algal Biofuels Technology Roadmap. U.S. Department of Energy, Biomass Program: *Energy Efficiency and Renewable Energy*, 1- 126.

Frick, A. A., Busetti, F. and Lewis, S. W. (2014). Aqueous Nile blue: a simple versatile and safe reagent for the detection of latent fingermarks. *Chemical Communications*, pp. 3341-3343. doi: 10.1039/c3cc49577a.

Friedlingstein, P. and Solomon, S. (2005). Contributions of past and present human generationsto committed warming caused by carbon dioxide. *PNAS*, 102:10832-10836.

Fuggi, A., Pinto, G., Pollio, A. and Taddei, R. (1988). The role of glycerol in osmoregulation of the acidophilic alga *Dunaliella acidophila* (Volvocales, Chlorophyta): effect of solute stress on photosynthesis, respiration and glycerol synthesis. *Phycologia*, 27: 439 - 446.

Fukuda, H., Kondo, A. and Noda, H. (2001). Biodiesel fuel production by transesterification of oils. *Journal of Bioscience and Bioengineering*, 92 (5): 405 - 416.

Fouqueray, M., Mouget, J. L., Morant-Manceau, A. and Tremblin, G. (2007). Dynamics of short-term acclimation to UV radiation in marine diatoms. *Journal of Photochemistry and Photobiology B: Biology*, 89 (1): 1- 8.

Garcia, M.C. C. Miron, S. A., Sevilla, J. M. F., Grima, E. M. and Camacho, F. G. (2005). Mixotrophic growth of the microalga *Phaeodactylum tricornutum* Influence of different nitrogen and organic carbon sources onproductivity and biomass composition. *Process Biochemistry*, 40: 297 - 305.

Gavrilescu M, Chisti Y (2005). Biotechnology- a sustainable alternative for chemical industry. *Biotechnology Advances*, 23 (7-8): 417- 419.

Gehl, K. A. and Colman, B. (1985). Effect of external pH on the internal pH of *Chlorella saccharophila*. *Plant Physiology*, 77 (4): 917- 921.

Gensemer, R. W., Smith, R. E.H. and Duthie, H. C. (1993). Comparative effects of pH and aluminum on silica limited growth and nutrient uptake in *Asterionella ralfsii* var. Americana (*Bacillariophyceae*). *Journal of Phycology*, 29: 36 - 44.

Gerpen, J. V. (2005). Biodiesel processing and production. *Fuel Processing Technology*, 86: 1097-1107.

Ghirardi, M. L., Zhang, L., Lee, J. W., Flynn, T., Seibert, M, Greenbaum, E. and Melis, A. (2000). Microalgae: a green source of renewable H_2. *Elsevier Science Ltd*, 18: 506-511.

Gilmour, D. J, and Zimmerman, W. B. (2012). Can algal biofuels play a major role in meeting future energy needs?*Biofuels***,** 3 (5): 511-513.

Giordano, M., Bargnesi, F., Mariani, P. and Ratti, S. (2014). *Dunaliella salina* (Chlorophyceae) affects the quality of NaCl crystals. *Cryptogamie, Algologie*, 35 (3): 285 - 302.

Gladu, P. K., Patterson, G. W., Wikfors, G. H. and Smith, B. C. (1995). Sterol, fatty acid and pigment characteristics of UTEX 2341, a marine Eustigmatophyte identified previously as *Chlorellaminutissima* (Chlorophyceae). *Journal of Phycology*, 31: 774-777.

Goldman, J. C., Azov, Y., Riley, C. B. and Dennett, M. R. (1982). The effect of pH in intensive microalgal cultures. I. biomass regulation. *Journal Experimental Marine Biology and Ecology*, 57: 1- 13.

Gonzalez, L. E., Díaz, G. C., Aranda, D. A. G., Cruz, Y. R., and Fortes, M. M. (2015). Biodiesel production based in microalgae: a biorefinery approach. *Natural Science*, 7: 358 - 369.

Gonzalez, J. C., Banerjee, R.V., Huang, S., Sumner, J. S. and Matthews, R. G. (1992). Comparison of cobalamin-independent and cobalamin-dependent methionine synthases from *Escherichia coli*: two solutions to the same chemical problem. *Biochemistry*, 31: 6045 - 6056.

Goodenough, U., Blaby, I., Casero, D., Gallaher, S. D, Goodson, C., Johnson, S., Lee, J. H., Merchant, S. S., Pellegrini, M., Roth, R., *et al.* (2014). The Path to Triacylglyceride Obesity in the sta6 Strain of *Chlamydomonas reinhardtii*. *Eukaryotic Cell*, 13: 591- 613.

Gouveia, L. and Oliveira, A.C. (2009). Microalgae as a raw material for biofuels production,*Journal of Industrial Microbiology and Biotechnology*, 36 (2): 269 - 274.

Govender, T., Ramanna, L., Rawat, I. and Bux, F. (2012). BODIPY staining, an alternative to the Nile Red fluorescence method for the evaluation of intracellular lipids in microalgae. *Bioresource Technology*. 114: 507 - 511.

Graham, L. E. and Wilcox, L.W. (2000). Algae. USA: *Prentice-Hall Inc*.,640 pp.

Greenspan, P., Mayer, E. P. and Fowler, S. D. (1985). Nile Red: A selective fluorescent stain for intracellular lipid droplets. *The Journal of Cell Biology*, 100 (10): 965 - 973.

Greenwell, H. C., Lauens, L. M., Shields, R. J. and Flynn, K. J. (2010). Placing microalgae on the biofuels priority list: a review of the technological challenges. *Journal of the Royal Society Interface*, 7 (46) : 703 -726 .

Gressel, J. (2008). Transgenics are imperative for biofuel crops. *Plant Science*, 174: 246-263.

Grima, E. M., Belarbi, E. H., Fernandez, F. G. A., Medina, A. R. and Chisti, Y. (2003). Recovery of microalgal biomass and metabolites: process options and economics. *Biotechnology Advances*, 20: 491-515.

Grima, E. M., Fernandez, F. G. A. Camacho, F.G. and Chisti, Y. (1999). Photobioreactors: Light regime, mass transfer, and scaleup. *Journal of Biotechnology*, 70 (1-3): 231 - 247.

Griffiths, M. J. and Harrison, S. T. L. (2009). Lipid productivity as a key characteristic for choosing algal species for biodiesel production. *Journal of Applied Phycology*, 21: 493 -507.

Griffiths, M. J., Hille, R. P. V. and Harrison, S. T. L. (2010). Selection of direct transesterification as the preferred method for assay of fatty acid content of microalgae. *Lipids* 45:1053 - 1060.

Grossman, A. (2000). Acclimation of *Chlamydomonas reinhardtii* to its nutrient environment. *Protist*, 151: 201 - 224.

Guckert, J. B. and Cooksey, K. E. (1990). Triglyceride accumulation and fatty acid profile changes in *Chlorella* (*Chlorophyta*) during high pH induced cell cycle inhibition. *Journal of Phycology*, 26: 72 - 79.

Guiheneuf, F., Mimouni, V., Ulmann, L., Tremblin, G. (2009). Combined effects of irradiance level and carbon source on fatty acid and lipid class composition in the

microalga *Pavlova lutheri* commonly used in mariculture. *Journal of Experimental Marine Biology and Ecology*, 369 (2): 136 -143.

Guiheneuf, F., Fouqueray, M., Mimouni, V., Ulmann, L., Jacquette, B. and Tremblin, G. (2010). Effect of UV stress on the fatty acid and lipid class composition in two marine microalgae *Pavlova lutheri* (pavlovophyceae) and *Odontella aurita* (bacillariophyceae). *Journal of Applied Phycology*, 22 (5): 629 - 638.

Guillard, R. R. L. and Ryther, J. H. (1962). Studies of marine planktonic diatoms. *Cyclotella nana* Hustedt and Detonula confervacea Cleve. *Canadian Journal of Microbiology*, 8: 229 - 239.

Guillard, R. R. L. (1975). Culture of phytoplankton for feeding marine invertebrates, "Culture of Marine Invertebrate Animals." (eds: Smith W.L. and Chanley M.H.), Plenum Press, New York, USA. 26 - 60.

Gurr, M. I., Harwood, J. L. and Frayn, K. N. (2002). Lipid Biochemistry: An Introduction, 5th ed.; Blackwell: Oxford, UK, p. 320.

Guschina, I. A. and Harwood, J. L. (2006). Lipids and lipid metabolism in eukaryotic algae. *Progress in Lipid Research*, 45:160 - 186.

Halim, R., Danquah, M. K., Webley, P. A. (2012). Extraction of oil from microalgae for biodiesel production: A review. *Biotechnology Advances*, 30: 709 - 732.

Hankamer, B., Lehr, F., Rupprecht, J., Mussgnug, J. H., Posten, C. and Kruse, O. (2007). Photosynthetic biomass and H2 production by green algae: from bioengineering to bioreactor scale-up. *Physiologia Plantarum*, 131: 10 - 21.

Hansen, P. J. (2002). Effect of high pH on the growth and survival of marine phytoplankton: Implications for species succession. *Aquatic Microbial Ecology*, 28: 279- 288.

Harun, R., Singh, M., Forde, G. M. and Danquah, M. K. (2010). Bioprocess engineering of microalgae to produce a variety of consumer products. *Renewable and Sustainable Energy Reviews*, 14: 1037-1047.

Harris, G. P. (1986). Phytoplankton Ecology: Structure, Function and Fluctuation, *Chapman and Hall:* New York, NY, USA.

Harwood, J. L. and Jones, A. L. (1989). Lipid Metabolism in Algae. *In Advances in Botanical Research*; Callow, J.A., Ed.; Academic Press: *Waltham, MA, USA*, 16: 1-53.

Hassan, F. M., Aljbory, I. F and Al- Jumaily, E. F. (2013). Stimulation of biodiesel production from two algae: *Chlorella vulgaris* Berjerinck and *Nitzschiapalea* (Kütz.) Smith, and study some growth parameters under different light intensity. *Journal of environmental science, Toxicology and food technology* (IOSR-JESTFT), 6 (2): 31-42.

He, Y. Y. and Hader, D. P. (2002). UV-B-induced formation of reactive oxygen species and oxidative damage of the cyanobacterium Anabaenasp.: Protective effects of ascorbic acid and *n*-acetyl-cysteine. *Journal of Photochemistry and Photobiology BBiology*, 66 (2): 115- 124.

Hejazi, M. A., Barzegari, A., Gharajeh, N. H. and Hejazi, M. S. (2010). Introduction of a novel 18S rDNA gene arrangement along with distinct ITS region in the saline water microalga *Dunaliella*. *Saline Systems*, 6(4): 1- 10.

Hibberd, D. J. (1981). Notes on the taxonomy and nomenclature of the algal classes Eustigmatophyceae and Tribophyceae (synonym Xanthophyceae). *Journal of the Linnean Society of London, Botany*, 82: 93-119.

Ho, S. H., Chen, C. N., Lai, Y. Y., Lu, W. B. and Chang J. S. (2014a). Exploring the high lipid production potential of a thermotolerant microalga using statistical optimization and semi-continuous cultivation. *Bioresource Technology*, 163: 128 - 135.

Ho, S. H., Ye, X., Hasunuma, T., Chang, J. S. and Kondo, A. (2014b). Perspectives on engineering strategies for improving biofuel production from microalgae: a critical review. *Biotechnology Advances*, 32: 1448-1459.

Holzinger, A. and Lutz, C. (2006). Algae and UV irradiation: Effects on ultrastructure and related metabolic functions. *Micron*, 37: 190 - 207.

Hossain, A. B. M. S. and Salleh, A. (1008). Biodiesel Fuel Production from Algae as Renewable Energy. *American Journal of Biochemistry and Biotechnology*, 4 (3): 250-254.

Hounslow, E., Noirel, J., Gilmour, D. J, and Wright, P. C. (2017). Lipid quantification techniques for screening oleaginous species of microalgae for biofuel production. *European Journal of Lipid Science and Technology*, 119: 1-24.

Hsieh, C. H. and Wu, W. T. (2009). Cultivation of microalgae for oil production with a cultivation strategy of urea limitation. *Bioresource Technology*, 100: 3921 - 3926.

Hu, J., Wang, D., Li, J., Jing, G., Ning, K. and Xu, J. (2014). Genome wide identification of transcription factors and transcription-factor binding sites in oleaginous microalgae *Nannochloropsis*. *Scientific Report*, 4: 5454 - 5464.

Hu, Q. (2004). Environmental effects on cell composition. In Handbook of Microalgal Culture (Richmond, A, ed.). Oxford: Blackwell, pp. 83 - 93.

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi M., Posewitz, M., Seibert, M and Darzins. A. (2008). Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *The Plant Journal,* 54: 621- 639.

Huang, G., Chen, G. and Chen, F. (2009). Rapid screening method for lipid production in alga based on Nile red fluorescence. *Biomass and Bioenergy*, 33(10): 1386 - 1392.

Huang, G., Chen, F., Wei, D., Zhang, Xue, W. and Chen, G. (2010). Biodiesel production by microalgal biotechnology. *Applied Energy*, 87: 38 - 46.

Huerlimann, R., de Nys, R., and Heimann, K. (2010). Growth, lipid content, productivity, and fatty acid composition of tropical microalgae for scale-up production. *Biotechnology Bioengineering*, 107 (2): 245- 257.

Hyka, P., Lickova, S., Pribyl, P. Melzoch, K. and Kovae, K.(2013). Flow cytometry for the development of biotechnological processes with microalgae. *Biotechnology Advances*, 31 (1): 2 -16.

Illman, A. M., Scragg, A. H. and. Shales, S. W. (2000). Increase in *Chlorella* strains calorific values when grown in low nitrogen medium. *Enzyme and Microbial Technology*, 27: 631- 635.

Iwai, M., Hori, K., Sasaki-Sekimoto, Y., Shimojima, M. and Ohta, H. (2015). Manipulation of oil synthesis in *Nannochloropsis* strain NIES 2145 with a phosphorus starvation–inducible promoter from *Chlamydomonas reinhardtii*. *Frontiers in Microbiology*, 6: 1- 16.

Iwai, M., Ikeda, K., Shimojima, M. and Ohta, H. (2014). Enhancement of extraplastidic oil synthesis in Chlamydomonas reinhardtii using a type-2 diacylglycerol acyltransferase with a phosphorus starvation-inducible promoter. *Plant Biotechnol Journal,* 12 (6): 808 - 819.

Janda, J, M. and Abbott, S. L. (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Pluses, perils, and pitfalls. *Journal of Clinical Microbiology*, 9: 2761-2764.

Jeong, G. T., Park, D. H., Kang, C. H., Lee, W. T., Sunwoo, C. S., Yoon, C. H., Choi, B. C., Kim, H. S., Kim, S. W. and Lee, U. T. (2004). Production of biodiesel fuel by transesterification of rapeseed oil.*Applied Biochemistry and Biotechnology*, 114:747-758.

Jinkerson, R. E., Radakovits, R. and Posewitz, M. C. (2013). Genomic insights from the oleaginous model alga *Nannochloropsis gaditana.Bioengineered*, 4: (1) 37-43.

Juergens, M. T., Deshpande, R. R. and Lucker, B. F. (2015). The Regulation of Photosynthetic Structure and Function during Nitrogen Deprivation in *Chlamydomonas reinhardtii*. *Plant Physiology*, 167: 558 - 573.

Juneja, A., Ceballos, R. M. and Murthy, G. S. (2013). Effects of environmental factors and nutrient availability on the biochemical composition of algae for biofuels production. *Energies*, 6: 4607- 4638.

Kaisan, M. U., Pam, G. Y., Kulla, D. M. (2013). Physico-chemical properties of bio-diesel from wild grape seeds oil and petro-diesel blends, *American Journal of Engineering Research*, 2: 291- 297.

Kalin, M., Wheeler, W. N. and Meinrath, G. (2005). The removal of uranium from mining waste water using algal/microbial biomass. *Journal of Environmental Radioactivity*, 78: 151-177.

Kang, N. K., Jeon, S., Kwon, S., Koh, H. G., Shin, S. E., Lee, B., Choi, G., Yang, J., Jeong, B. and Chang, Y. K. (2015). Effects of overexpression of a bHLH transcription factor on biomass and lipid production in *Nannochloropsis salina. Biotechnology for Biofuels*, 8 (200): 1-13.

Kang, R., Wang, J., Shi, D., Cong, W., Cai, Z. and Ouyang, F. (2004). Interactions between organic and inorganic carbon sources during mixotrophic cultivation of *Synechococcus* sp. *Biotechnology Letters*, 26: 1429-1432.

Karlson B., Potter, D., Kuylenstierna, M. and Andersen, R. A. (1996). Ultrastructure, pigment composition, and 18S rRNA gene sequence for *Nannochloropsis granulata* sp. nov. (Monodopsidaceae, Eustigmatophyceae), a marine ultraplankter isolated from the Skagerrak, northeast Atlantic Ocean. *Phycologia*, 35: 253 - 260.

Kaye, Y., Grundman, O., Leu, S., Zarka, A., Zorin, B., Cohen, D. S., Goldberg, I. K. and Boussuba, S. (2015). Metabolic engineering toward enhanced LC-PUFA biosynthesis in *Nannochloropsisoceanica*: Overexpression of endogenous Δ12 desaturase driven by stress-inducible promoter leads to enhanced deposition of polyunsaturated fatty acids in TAG. *Algal Research*, 11: 387- 398.

Khan, S., Rashmi, A, Hussain, M. Z., Prasad, S. and Banerjee, U. C. (2009). Prospects of biodiesel production from microalgae in India. *Renewable and Sustainable Energy Reviews*, 13: 2361- 2372.

Khalil, Z., Asker, M.M., El-Sayed, S. and Kobbia, I. (2010). Effect of pH on growth and biochemical responses of *Dunaliella bardawil* and *Chlorella ellipsoidea*. *World Journal Microbiology and Biotechnology*, 26: 1225 - 1231.

Khotimchenko, S.V. and Yakovleva, I .M. (2005). Lipid composition of the red alga *Tichocarpus crinitus* exposed to different levels of photon irradiance. *Phytochemistry*, 66, 73 - 79.

Khatoon, H., Rahman, N. A., Banerjee, S., Harun, N., Suleiman, S. S., Zakaria, N. H., Lananan, F., Abdul Hamid, S. H., Endut, A. (2014). Effects of different salinities and pH on the growth and proximate composition of *Nannochloropsis* sp. and *Tetraselmis* sp. isolated from South China Sea cultured under control and natural condition. *International Biodeterioration and Biodegradation,* 95: 11-18.

Khozin-Goldberg, I., Bigogno, C., Shrestha, P. and Cohen, Z. (2002). Nitrogen starvation induces the accumulation of arachidonic acid in the freshwater green alga *Parietochloris incisa* (Trebouxiophyceae). *Journal of Applied Phycology,* 38: 991-994.

Khozin-Goldberg, I. and Cohen, Z. (2006). The effect of phosphate starvation on the lipid and fatty acid composition of the fresh water eustigmatophyte *Monodus subterraneus*. *Phytochemistry*, 67: 696 -701.

Khozin-Goldberg, I. and Cohen, Z. (2011). Unraveling algal lipid metabolism: Recent advances in gene identification. *Biochimie,* 93: 91-100.

Kiran, B., Kumar, R. and Deshmukh, D. (2014). Perspectives of microalgal biofuels as a renewable source of energy. *Energy Conversion and Management,* 88: 1228 -1244.

Kim, G., Mujtaba, G. and Lee, K. (2016). Effects of nitrogen sources on cell growth and biochemical composition of marine chlorophyte *Tetraselmis* sp. for lipid production. *Algae,* 31(3): 257-266.

Kim, G., Mujtaba, G., Rizwan, M. and Lee, K. (2014). Environmental stress strategies for stimulating lipid production from microalgae for biodiesel. *Applied Chemical Engineering,* 25: 553 - 558.

Kim, J., Yoo, G., Lee, H., Lim, J, Kim, K., Kim, C. W., Park, M. S. and Yang, J.W. (2013). Methods of downstream processing for the production of biodiesel from microalgae. *Biotechnology Advances*, 31: 862-876.

Knothe, G., Dunn, R. O. and Bagby, M. O. (1997). Biodiesel: the use of vegetable oilsand their derivatives as alternative diesel fuels. *ACS Symposium Series, eBooks*, Vol. 666: Chapter 10, pp 172-208.

Knothe, G. (2010). Biodiesel and renewable diesel: A comparison. *Progress in Energy and Combustion Science*, 36: 364- 373.

Kojima, E and Zhang, K. (1999). Growth and hydrocarbon production of microalga botryococcus braunii in bubble column photobioreactors. *Journal of Bioscience and Bioengineering*, 87 (6): 811-815.

Krienitz L, Hepperle, D., Stich, H. B. and Weiler, W. (2000). *Nannochloropsis limnetica* (Eustigmatophyceae), a new species of picoplankton from freshwater. *Phycologia*, 39: 219 - 227.

Kulkarni, M. G. and Dalai, A. K. (2006). Waste cooking oils an economical source for biodiesel: A review. *Industrial and Engineering Chemistry Research*, 45: 2901-2913.

Kumari, P., Reddy, C. R. K. and Jha, B. (2011). Comparative evaluation and selection of a method for lipid and fatty acid extraction from macroalgae. *Analytical Biochemistry*, 415 (2): 134 -144.

Kumar, R., Bansal, V., Patel, M. B. and Sarpal, A. S. (2014). Compositional analysis of algal biomass in a nuclear magnetic resonance (NMR) tube. *Journal of Algal Biomass Utilization,* 5 (3): 36 - 45.

Kyndt, J. (2010). Algae cultivation system- open ponds vs PBR. *Alge Man*, (Online) Available: http://algaeforbiofuels.com/algae-cultivation-systems-open-ponds-vs-pbr/

Lam, M. K. and Lee, K. T. (2012). Potential of using organic fertilizer to cultivate *Chlorella vulgaris* for biodiesel production. *Applied Energy*, 94, 303 - 308.

Lananan, F., Jusoh, A., Ali, N., Lam, S. S. and Azizah, E. (2013). Effect of Conway Medium and f/2 Medium on the growth of six genera of South China Sea marine microalgae. *Bioresource Technology*, 141: 75 - 82.

Lane, A.E. and Burris, J. E. (1981). Effects of environmental pH on the internal pH of *Chlorella pyrenoidosa*, *Scenedesmus quadricauda*, and *Euglena mutabilis*. *Plant Physiology*, 68: 439 - 442.

Lau, S. K., Woo, P. C., Teng, J. L., Leung, K.W. and Yuen, K. Y. (2002). Identification by 16S ribosomal RNA gene sequencing of *Arcobacter butzleri* bacteraemia in a patient with acute gangrenous appendicitis. *Journal of Clinical Pathology*, 55: 182 - 185.

Lee, R.E. (1999). Phycology. ThirdEdition.Cambridge University Press.

Leung, D. Y.C., Wu, X., Leung, M. K. H. (2010). A review on biodiesel production using catalyzed transesterification. *Applied Energy*, 87: 1083-1095.

Li, D. M. and Qi, Y. Z. (1997). *Spirulina* industry in China: Present status and future prospects. *Journal of Applied Phycology*, 9: 25-28.

Li, Y., Horsman, M., Wang, B., Wu, N. and Lan C. Q. (2008). Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans*. *Applied Microbiology and Biotechnology*, 81: 629- 636.

Li, Y., Fei, X. and Deng, X., (2012). Novel molecular insights into nitrogen starvation induced triacylglycerols accumulation revealed by differential gene expression analysis in green algae *Micractinium pusillum*. *Biomass and Bioenergy*, 42: 199 - 211.

Li, F., Gao, D. and Hu, H. (2014a). High-efficiency nuclear transformation of the oleaginous marine *Nannochloropsis* species using PCR product. Bioscience, Biotechnology, and Biochemistry, 78 (5): 812 - 817.

Li, J., Han, D., Wang D., Ning, K, Jia, J., Wei, L., Jing, X., Huang, S., Chen, J., Li,Y., Hu, Q. and Xu, J. (2014b). Choreography of Transcriptomes and Lipidomes of *Nannochloropsis* Reveals the Mechanisms of Oil Synthesis in Microalgae. *The Plant Cell*, 26: 1645-1665.

Liang, Y., Beardall, J. and Heraud, P. (2006). Effect of uv radiation on growth, chlorophyll fluorescence and fatty acid composition of *Phaeodactylum tricornutum* and *Chaetoceros muelleri* (bacillariophyceae). *Phycologia*, 45: 605- 615.

Liang, Y., Sarkany, N. and Cui, Y. (2009). Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions. *Biotechnology Letters*, 31:1043 - 1049.

Liu, Z. Y., Wang, G. C. and Zhou, B. C. (2008). Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*. *Bioresource Technology*, 99 (11): 4717- 4722.

Liu, B. and Benning, C. (2013). Lipid metabolism in microalgae distinguishes itself. *Current Opinion in Biotechnology*, 24 (2): 300- 309.

Liu, B., Vieler, A., Li, C., Jones, A. D. and Benning, C. (2013). Triacylglycerol profiling of microalgae *Chlamydomonas reinhardtii* and *Nannochloropsis oceanic*. *Bioresource Technology,* 146: 310-316

Loira, N., Mendoza, S., Cortes, M., Rojas, N., Travisany, D., Genova, A., Gajardo, N., Ehrenfeld, N. and Maass, A. (2017). Reconstruction of the microalga *Nannochloropsis salina* genome-scale metabolic model with applications to lipid production, *BMC Systems Biology*, 11(66): 2 -17.

Lorenz, R. T., and Cysewski, G. R. (2000). Commercial potential for Haematococcus microalga as a natural source of astaxanthin. *Elsevier Science Ltd*, 18: 160 - 167.

Lubian, L. M., Montero, O., Moreno-Garrido, I., Huertas, I. E., Sobrino, C., Gonzalez-del V., M. and Pares G., (2000). *Nannochloropsis* (Eustigmatophyceae) as source of commercially valuable pigments, *Journal of Applied Phycology*, 12 (3-5): 249 - 255.

Lundquist, T. J., Woertz, I.C., Quinn, N.W.T. and Benemann, J.R. (2010). A Realistic Technology and Engineering Assessment of Algae Biofuel Production, *Energy Biosciences Institute: University of California, Berkeley, California*.

Lynch, D. V. and Thompson, G. A. (1982). Low temperature-induced alterations in the chloroplast and microsomal membranes of *Dunaliella salina*. *Plant Physiology*, 69 (6): 1369 - 1375.

Lynn, S. G. Kilham, S. S. Kreeger, D. A. and Interlandi S. J. (2000). Effect of nutrient availability on the biochemical and elemental stoichiometry in the freshwater diatom *stephanodiscus minutulus* (Bacillariophyceae). *Journal of Applied Phycology*, 36 (3) : 510 - 522.

Ma, F. and Hanna, M. A. (1999). Biodiesel production: a review. *Bioresource Technology*, 70: 1-15.

Ma, Y., Wang, Z., Yu, C., Yin, Y. and Zhou, G. (2014). Evaluation of the potential of 9 *Nannochloropsis strains* for biodiesel production. *Bioresource Technology*, 167: 503-509.

Ma, X., Chen, T., Yang, B., Liu, J. and Chen, F. (2016). Lipid Production from Nannochloropsis. *Marine drugs*, 14 (61): 1-18.

Madigan, M. T., Martinko, **J. M. and Parker,** J. **(2003).** Book, Biology of Microorganisms. Prentice Hall international editions.10th ed/ ed. Upper Saddle River, N. J., Prentice Hall.

Malcata, F. X. (2011). Microalgae and biofuels: a promising partnership? Trends in Biotechnology, 29: 542 - 549.

Mallick, N. (2002). Biotechnological potential of immobilized algae for wastewater N, P and metal removal: A review. *BioMetals*, 15: 377-390.

Mandotra, S. K., Kumar, P., Suseela, M. R. and Ramteke, P.W. (2014). Fresh water green microalgae Scenedesmus abundans: A potential feedstock for high quality biodiesel production. *Bioresour Technology*, 156: 42 - 47.

Martin, G. J., Hill, D. R., Olmstead, I. L., Bergamin, A., Shears, M. J., Dias, D. A., Dias, A. D., Kentish, S. E., Scales, P. J., Botte, C. Y., Callahan, D. L. (2014). Lipid profile remodeling in response to nitrogen deprivation in the microalgae *Chlorella* sp. (Trebouxiophyceae) and *Nannochloropsis* sp. (Eustigmatophyceae). *Plos One*, 1- 10.

Maruyama, I., Nakamura, T., Matubayashi, T., Ando, Y. And Maeda, T. (1986). Identification of the alga known as "marine *Chlorella*" as a member of the Eustigmatophyceae. *Japanese Journal of Phycology,* 34 (4): 319 -325.

Mata, T. M., Martins, A. A. and Caetano, N. S. (2010). Microalgae for biodiesel production and other applications: A review. *Renewable and Sustainable Energy Reviews*, 14: 217-232.

Markou, G., and Nerantzis, E. (2013). Microalgae for high-value compounds and biofuels production: A review with focus on cultivation under stress conditions. *Biotechnology Advances*, 31: 1532- 1542.

Martinez-Roldan, A. J., Perales-Vela, H. V., Canizares-Villanueva, R. O. and Torzillo, G. (2014). Physiological response of *Nannochloropsis* sp. to saline stress in laboratory batch cultures. *Journal Applied Phycology*, 26:115 -121.

Maxwell, K., and Johnson, G. N. (2000). Chlorophyll fluorescence – a practical guide. *Journal of Experimental Botany*, 51 (345): 659 - 668.

McBride, R. C. and Merrick, D. S.(2014). Innovations in open pond algae agriculture for biofuel production. *Industrial Biotechnology*, 10 (3):162 -163.

McLarnon-Riches, C. J., Rolph, C. E., Greenway, D.L. A. and Robinson, P. K. (1998). Effects of environmental factors and metals on *Selenastrum capricornutum* lipids. *Phytochemistry*, 49 (5): 1241 - 1247.

Medina, A. R. Grima, E. M., Giménez, A. G., González, M. J. I. (1998). Downstream processing of algal polyunsaturated fatty acids. *Biotechnology Advances*, 16 (3): 517-580.

Meghnani, R. (2013). Microalgal Biofuels: A review. *Recent Research in Science and Technology,* 5 (5): 59 - 60.

Meher, L. C., Sagar, D. V. and Naik, S. N. (2006). Technical aspects of biodiesel production by transesterification-a review. *Renewable and Sustainable Energy Reviews,* 10: 248 - 268.

Meireles, L. A., Guedes, A. C. and Malcata, F. X. (2003). Lipid class composition of the microalga *Pavlova lutheri*: eicosapentaenoic and docosahexaenoic acids. *Journal of Agricultural and Food Chemistry,* 51 (8): 2237- 2241.

Meng, X., Yang, J., Xu, X., Zhang, L., Nie, Q. and Xian, M. (2009). Biodiesel production from oleaginous microorganisms. *Renewable Energy,* 34: 1- 5.

Melis, A. (2002). Green alga hydrogen production: progress, challenges and prospects. *International Journal of Hydrogen Energy*, 27: 1217- 1228.

Metting, F., J. (1996). Biodiversity and application of microalgae. *Journal of Industrial Microbiology*, 17: 477- 489.

Mignard, S., and Flandrois, J. P. (2006). 16S rRNA sequencing in routine bacterial identification: a 30-month experiment. *Journal of Microbiological Methods*, 67: 574 - 581.

Milledge, J. J. (2011). Commercial application of microalgae other than as biofuels: a brief review. *Reviews in Environmental Science and Biotechnology*, 10:31- 41.

Miller, R., Wu, G. and Deshpande, R. R. (2010). Changes in transcript abundance in *Chlamydomonas reinhardtii* following nitrogen deprivation predict diversion of metabolism. *Plant Physiology,* 154: 1737-1752.

Minhas, A. K., Hodgson, P., Barrow, C. J. and Adholeya, A. (2016). A review on the assessment of stress conditions for simultaneous production of microalgal lipids and carotenoids. *Frontiers in Microbiology,*7: 1-19.

Moazami-Goudarzi, M. and Colman, B. (2012). Changes in carbon uptake mechanisms in two green marine algae by reduced seawater pH. *Journal of Experimental Marine Biology and Ecology,* 413 : 94 - 99.

Moazami, N., Ashori, A., Ranjbar, R., Tangestani, M., Eghtesadi, R. and Nejad, A. S. (2012). Large-scale biodiesel production using microalgae biomass of *Nannochloropsis*. *Biomass and Bioenergy,*39: 449 - 453.

Moheimani, N. R, Borowitzka M. A. (2011). Increased CO_2 and the effect of pH on growth and calcification of *Pleurochrysis carterae* and *Emiliania huxleyi* (Haptophyta) in semicontinuous cultures. *Applied Microbiology and Biotechnology,* 90:1399 -1407.

Monshupanee, T. and Incharoensakdi, A. (2014). Enhanced accumulation of glycogen lipids and polyhydroxybutyrate under optimal nutrients and light intensities in the *cyanobacterium Synechocystis* sp. PCC 6803. *Journal of Applied Microbiology,* 116: 830 - 838.

Moon, M., Kim, C., Park, W., Yoo, G., Choi, Y. and Yang, J. (2013). Mixotrophic growth with acetate or volatile fatty acids maximizes growth and lipid production in *Chlamydomonas reinhardtii*. *Algal Research,* 2: 352 - 357.

Morgan-Kiss, R. M., Priscu, J. C., Pocock, T., Gudynaite-Savitch, L. and Huner, N. P. A. (2006). Adaptation and acclimation of photosynthetic microorganisms to permanently cold environments. *Microbiology and Molecular Biology Reviews,* 70: 222 - 252.

Morineau-Thomas, O., Jaouen, P. and Legentilhomme, P. (2002). The role of exopolysaccharides in fouling phenomenon during ultrafiltration of microalgae (*Chlorella sp. and Porphyridium purpureum*): Advantage of a swirling decaying flow. *Bioprocess and Biosystems Engineering,* 25: 35- 42.

Moroney, J. V. and Tolbert, N. E. (1985). Inorganic carbon uptake by *Chlamydomonas reinhardtii*. *Plant Physiology,* 77: 253 - 258.

Msanne, J., Xu, D., Konda, A. R., Casas-Mollano, J. A., Awada, T., Cahoon, E. B., Cerutti, H. (2012). Metabolic and gene expression changes triggered by nitrogen deprivation in the photoautotrophically grown microalgae *Chlamydomonas reinhardtii* and *Coccomyxa* sp. C-169. *Phytochemistry,* 75: 50 - 59.

Muhlroth, A., Li, K., Rokke, G., Winge, P., Olsen, Y., Hohmann-Marriott, M. F., Vadstein, O. and Bones, A. M. (**2013).** Pathways of lipid metabolism in marine algae, co-expression network, bottlenecks and candidate genes for enhanced production of EPA and DHA in Species of chromista. *Marine Drugs,*11: 4662- 4697.

Mulders, K. J. M., Lamers, P. P., Martens,D. E., and Wijffels, R. H. (2014). Phototrophic pigment production with microalgae: biological constraints and opportunities. *Journal Phycolo*gy, 50: 229 - 242.

Munoz, R. and Guieysse, B.(2006). Algal-bacterial processes for the treatment of hazardous contaminants: A review. *Water Research,* 40: 2799 - 2815.

Murakami, R. and Hashimoto, H. (2009). Unusual nuclear divisionin *Nannochloropsis oculata* (Eustigmatophyceae, Heterokonta) which may ensure faithful transmission of secondary plastids. *Protist*, 160: 41- 49.

Mutanda, T., Ramesh, D, Karthikeyan, S., Kumari, S., Anandraj, A. and Bux, F. (2011). Bioprospecting for hyper-lipid producing microalgal strains for sustainable biofuel production. *Bioresource Technology*, 102: 57 - 70.

Muthukumar, A., Elayaraja, S., Ajithkumar, T. T., Kumaresan, S. and Balasubramanian, T. (2012). Biodiesel production from marine microalgae *Chlorella marina* and *Nannochloropsis salina. Journal of Petroleum Technology and Alternative Fuels*, 3(5): 58 - 62.

Napolitano, G. E. (1994). The relationship of lipids with light and chlorophyll measurements in freshwater algae and periphyton. *Journal of Phycology*, 30: 943 - 950.

Natunen, K., Seppala, J., Schwenk, D., Rischer, H., Spilling, K. and Tamminen, T. (2015). Nile Red staining of phytoplankton neutral lipids: species-specific fluorescence kinetics in various solvents. *Journal of Applied phycology*, 27:1161-1168.

Olmos, J., Paniagua, J. and Contreras, R. (2000). Molecular identification of *Dunaliella* sp. utilizing the 18S rDNA gene. *Letters in Applied Microbiology*, 30, 80 - 84.

Olmstead, I. L.D., Hill, D. R.A., Dias, D. A. and *et al*., (2013). A Quantitative analysis of microalgal lipids for optimization of biodiesel and omega-3 production.*Biotechnology and Bioengineering*, 8 (110) : 2096- 2104.

Oncel, S. S. (2013). Microalgae for a macroenergy world. *Renewable and Sustainable Energy Reviews*, 26: 241-264.

Ordog, V, Stirk, W. A., Bálint, P., Aremu, A. O., Okem, A., Lovasz, C., Molnar and Z., Staden, J. (2016). Effect of temperature and nitrogen concentration on lipid productivity and fatty acid composition in three Chlorella strains. *Algal Research*, 16: 141-149.

Pal, D., Goldberg, I., Cohen, S., Solovchenko, A., Batushansky, A., Kaye, Y., Sikron, N., Samani, T., Fait, A. and Boussiba, S. (2013). Growth, lipid production and metabolic adjustments in the euryhaline eustigmatophyte *Nannochloropsis oceanic* CCALA 804 in response to osmotic downshift, *Applied Microbiology and Biotechnology*, 97: 8291- 8306.

Pal, D., Khozin-Goldberg, I., Cohen, Z. and Boussiba, S. (2011). The effect of light, salinity, and nitrogen availability on lipid production by *Nannochloropsis* sp. *Applied Microbiology and Biotechnology*, 90: 1429-1441.

Pancha, I., Chokshi, K., George, B., Ghosh, T., Paliwal, C., Maurya, R. , Mishra, S.(2014). Nitrogen stress triggered biochemical and morphological changes in the microalgae *Scenedesmus* sp. CCNM 1077. *Bioresource Technology*, 156: 146 - 154.

Parmar, A., Singh, Niraj, K., Pandey, A., Gnansounou, E. and Madamwar, D. (2011). Cyanobacteria and microalgae: A positive prospect for biofuels. *Bioresource Technology*, 102: 10163-10172.

Patterson, G. (1970). Effect of culture temperature on fatty acid composition of *Chlorella sorokiniana*. *Lipids*, 5: 597- 600.

Perez-Garcia, O. and Bashan, Y. (2015). Microalgal heterotrophic and mixotrophic culturing for bio-refining: from metabolic routes to techno-economics. *Algal Biorefineries*, 61-131.

Pessoa, M. F. (2012). Harmful effects of UV radiation in algae and aquatic macrophytes- A review. *Emirates Journal of Food and Agriculture*, 24: 510 - 526.

Pick, U. and Rachutin-Zalogin, T. (2012). Kinetic anomalies in the interactions of Nile red with microalgae. *Journal of Microbiological Methods*, 88 (2): 189 - 96.

Piorreck, M., Baasch, K., Pohl, P. (1984). Biomass production, total protein, chlorophylls, lipids and fatty acids of freshwater green and blue-green algae under different nitrogen regimes. *Phytochemistry*, 23: 207 - 216.

Pires, J. C. M., Alvim-Ferraz, M. C. M., Martins, F. G. and Simoes, M. (2013). Wastewater treatment to enhance the economic viability of microalgae culture. *Environmental Science and Pollution Research*, 20: 5096 - 5105.

Poerschmann, J., Spijkerman, E. and Langer, U. (2004). Fatty acid patterns in *Chlamydomonas* sp. as a marker for nutritional regimes and temperature under extremely acidic conditions. *Microbial Ecology*, 48 (1): 78 - 89.

Praveenkumar, R., Shameera, K., Mahalakshmi, G., Akbarsha, M. A. and Thajuddin, N. (2012). Influence ofnutrient deprivations on lipid accumulation in a dominant indigenous microalga *Chlorella* sp., BUM11008: Evaluation for biodiesel production. *Biomass and Bioenergy*, 37: 60 - 66.

Priyadarshani, I. and Rath, B. (2012). Commercial and industrial applications of micro algae - A review. *Journal of Algal Biomass Utilization*, 3: 89-100.

Puchooa, D. (2004). A simple, rapid and efficient method for the extraction of genomic DNA from lychee (*Litchi chinensis* Sonn.). *African Journal of Biotechnology*, 3: 253 -255.

Pulz, O. and Gross, W. (2004). Valuable products from biotechnology of microalgae *Applied Microbiology and Biotechnology*, 65: 635 - 648.

Radakovits, R., Jinkerson, R. E., Fuertenberg, S. I., Tae, H., Settlage, R. E., Boore, J. L. and Posewitz, M. C. (2012). Draft genomesequence and genetic transformation of the oleaginous alga *Nannochloropsis gaditana*. *Nature Communication*, 3: 686- 696.

Radmer, R. J. (1996). Algal diversity and commercial algal products. BioScience, *Marine Biotechnology*, 46 (4): 263-270.

Rai, M. P., Gautom, T. and Sharma, N. (2015). Effect of salinity, pH, light intensity on growth and lipid production of microalgae for bioenergy application. *Journal of Biological Sciences*, 15 (40): 260 - 267.

Raja, R., Hemaiswarya, S., Kumar, N. A., Sridhar S. and Rengasamy, R. (2008). A Perspective on the biotechnological potential of microalgae. *Critical Reviews in Microbiology*, 34: 77- 88.

Raja, R., Iswarya, S. H., Balasubramanyam, D. and Rengasamy, R. (2007). PCR-identification of *Dunaliella salina* (Volvocales, Chlorophyta) and its growth characteristics**.** *Microbiological Research*, 162: 168 - 176.

Ramanan, R., Vinayagamoorthy, N., Sivanesan, S. D., Kannan, K. and Chakrabarti, T. (2012). Influence of CO_2 concentration on carbon concentrating mechanisms in cyanobacteria and green algae: a proteomic approach. *Algae*, 27 (4): 295 -301.

Ranjan, A., Patil, C. and Moholkar, V. S. (2010). Mechanistic assessment of microalgal lipid extraction. *Industrial and Engineering Chemistry Research*, 49: 2979-2985.

Rao, A. R., Dayananda, C., Sarada, R., Shamala, T. R. and Ravishankar, G.A., (2007). Effect of salinity on growth of green alga *Botryococcus braunii* and its constituents. *Bioresource Technology*, 98: 560-564.

Rastogi, R.P. and Incharoensadki, A. (2013). UV radiation-induced accumulation of photoprotective compounds in the green alga *Tetraspora* sp. CU2551. *Plant Physiology and Biochem*istry, 70:7 - 13.

Ratledge, C. (2004). Fatty acid biosynthesis in microorganisms being used for single cell oil production. *Biochimie*, 86: 807- 815.

Ratledge, C. and Cohen, Z. (2008). Microbial and algal oils: Do they have a future for biodiesel or as commodity oils? *Lipid Technology*, 20 (7): 155- 160.

Rattanapoltee, P. and Kaewkannetra, P. (2013). Quantification of neutral lipids in microalgae. *International Journal of Biological, Food, Veterinary and Agricultural Engineering*, 7 (9): 546 - 550.

Razon, L. F. and Tan, R. R.(2011). Net energy analysis of the production of biodiesel and biogas from the microalgae: *Haematococcus pluvialis* and *Nannochloropsis*. *Applied Energy*, 88: 3507- 3514.

Reed, R. D. (1998). Practical Skills in Biomolecular Sciences. Harlow, Prentice Hall.

Reitan, K. I., Rainuzzo, J. R. and Olsen, Y. (1994). Effect of nutrient limitation on fatty acid and lipid content of marine microalgae. *Journal of Phycology*, 30: 972- 979.

Renaud S. M., Thinh, L., Lambrinidis, G. and Parry, D. L. (2002). Effect of temperature on growth, chemical composition and fatty acid composition of tropical Australian microalgae grown in batch cultures. *Aquaculture*, 211: 195-214.

Renaud, S. M. and Parry, D. L. (1994). Microalgae for use in tropical aquaculture II: Effect of salinity on growth, gross chemical composition and fatty acid composition of three species of marine microalgae. *Journal of Applied Phycology*, 6: 347-356.

Richmond, A. (2004). Handbook of microalgal culture: *Biotechnology and Applied Phycology*. Blackwell Science Ltd.

Rocha, J. M., Garcia, J. E. and Henriques, M, H. (2003). Growth aspects of the marine microalga *Nannochloropsis gaditana*. *Biomolecular Engineering*, 20: 237 - 242.

Rodolfi, L., Zittelli, G. C., Bassi, N., Padovani, G., Biondi, N., Bonini, G.and Tredici, M. R. (2009). Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. *Biotechnology and Bioengineering*, 102 (1): 100 - 112.

Rosenberg, J. N., Oyler, G. A., Wilkinson, L. and Betenbaugh, M. J. (2008). A green light for engineered algae: redirecting metabolism to fuel a biotechnology revolution. *Current Opinion in Biotechnology*,19: 430-436.

Ruangsomboon, S. (2012). Effect of light, nutrient, cultivation time and salinity on lipid production of newly isolated strain of the green microalga, *Botryococcus braunii* KMITL 2. *Bioresource Technology*, 109: 261- 265.

Rumin. J., Bonnefond, H., Saint-Jean, B., Rouxel, C., Sciandr, A., Bernard, O., Cadoret J. P., Bougaran, G. (2015). The use of fluorescent Nile red and BODIPY for lipid measurement in microalgae. *Biotechnology for Biofuels*, 8 (42): 1- 16.

Ryckebosch, E., Bruneel, C., Termote-Verhalle, R., Goiris, K., Muylaert, K., Foubert, I. (2014). Nutritional evaluation of microalgae oils rich in omega-3 long chain polyunsaturated fatty acids as an alternative for fish oil. *Food Chemistry*, 160: 393- 400.

Sackett, D. L. and Wolff, J. (1987). Nile red as a polarity-sensitive fluorescent probe of hydrophobic protein surfaces. *Analytical Biochemistry*, 167 (2): 228 - 234.

Saifullah, A. Z. A., Karim, M. A. and Ahmad-Yazid, A. (2014). Microalgae: An Alternative Source of Renewable Energy. *American Journal of Engineering Research*, 3: 330 - 338.

Sakarika, M. and Kornaros, M. (2016). Effect of pH on growth and lipid accumulation kinetics of the microalga *Chlorella vulgaris* grown heterotrophically under sulfur limitation. *Bioresource Technology*, 219: 694 -701.

Sanchez, F. and Vasudevan, P. T. (2006). Enzyme catalyzed production of biodiesel from olive oil. *Applied Biochemistry and Biotechnology*,135: 1- 14.

Santos, A. M., Lamers, P. P., Janssen, M. and Wijffels, R. H. (2013). Biomass and lipid productivity of *Neochloris oleoabundans* under alkaline-saline conditions. *Algal Research*, 2: 204-211.

Sarpal, A. S., Teixeira, L. L., Silva, P. R., Lima, G. M, Silva, S. R., Monteiro, T. V., Cunha, V. S. and Daroda, R. J. (2015). Determination of lipid content of oleaginous microalgal biomass by NMR spectroscopic and GC-MS techniques. *Analytical and Bioanalytical Chemistry*, 407 (13): 3799 - 3816.

Sarpal, A. S., Teixeira, C. M., Silva, P. R., Monteiro, T. V., Silva, J. I. Cunha, V. S. and Daroda, R. J. (2016). NMR techniques for determination of lipid content in microalgal biomass and their use in monitoring the cultivation with biodiesel potential. *Applied Microbiology and Biotechnology*, 100: 2471- 2485.

Satpati, G. G. and Pal, R. (2015). Rapid detection of neutral lipid in green microalgae by flow cytometry in combination with Nile red staining-an improved technique. *Annals of Microbiology*, 65 (2): 937- 949.

Sato, N. and Murata, N. (1980). Temperature shift - induced responses in lipids in the blue-green alga, *Anabaena variabilis*: the central role of diacylmonogalactosyl glycerol in thermo - adaptation. *Biochimica at Biophysica Acta*, 619 (2): 353 - 66.

Sato, N., Hagio, M., Wada, H. and Tsuzuki, A. M. (2000). Environmental effects on acidic lipids of thylakoid membranes. *Biochemical Society,*28: 912- 914.

Schwartz, R. E., Hirsch, C. F., Sesin D. F., Flor J. E., Chartrain M., Fromtling R. E., Harris G. H., Salvatore, M. J., Liesch J. M. and Yudin, K. (1990). Pharmaceuticals from cultured algae. *Journal of Industrial Microbiology*, 5:113-I24.

Schreiber, U. (2004). Pulse amplitude modulation (PAM) fluorometry and saturation pulse method: an overview. Papageorgiou, G. C. (ed). In Chlorophyll a fluorescence: a signature of photosynthesis Dordrecht: *Kluwer Academic*, 279-319.

Scott, S. A, Davey, M. P., Dennis, J. S, Horst, I., Howe, C. J., Smith, D. L. and Smith, A. G. (2010). Biodiesel from algae: challenges and prospects. *Current Opinion in Biotechnology*, 21 (3): 277- 286.

Sforzaa, E., Bertuccoa, A., Morosinottob, T., Giacometti, G. M. (2012). Photobioreactors for microalgal growth and oil production with *Nannochloropsis salina*: from lab-scale experiments to large-scale design. *Chemical Engineering Research and Design*, 90: 1151-1158.

Sharma, K. K., Schuhmann, H. and Schenk, P. M. (2012). High lipid induction in microalgae for biodiesel production. *Energies*, 5: 1532 - 1553.

Sharma, N. K., Rai, A. K., Singh, S. and Brown, R. M. (2007). Airborne algae: their present status and relevance. *Journal of Phycology*, 615- 627.

Sharma R, Chisti, Y., Banerjee, U. C. (2001). Production, purification, characterization, and applications of lipases. *Biotechnology Advances*,19 (8): 627-662.

Shields, R. J. and Lupatsch, I. (2012). Algae for Aquaculture and Animal Feeds. Technikfolgenabschatzung -Theorie und Praxis, 21: 23 -37.

Shia, X., Zhangb, X.and Chenb F. (2000). Heterotrophic production of biomass and lutein by *Chlorellaprotothecoides* on various nitrogen sources. *Enzyme and Microbial Technology*, 27: 312 - 318.

Shimizu, Y. (2003). Microalgal metabolites. *Current Opinion in Microbiology*, 6: 236-243.

Si, L., Kehou, P., Baohua, Z., Xiaolei, M. A., Xin, L. and Guanpin, Y. (2011). Molecular identification of a species in genus *Nannochloropsis*. *Journal of Ocean University of China*, 10 (4): 391- 396.

Siegler, H. D., Ayidzoe, W., Zvi, A.,Burrell, R. E. and McCaffrey, W.C. (2012). Improving the reliability of fluorescence-based neutral lipid content measurements in microalgal cultures. *Algal Research,*1(2): 176 - 184.

Simionato, D., Sforza, E., Carpinelli, E. C., Bertucco, A., Giacometti, G. M. and Morosinotto, T. (2011). Acclimation of *Nannochloropsis gaditana* to different illumination regimes: Effects on lipids accumulation. *Bioresource Technology*, 102: 6026- 6032.

Simionato, D., Block, M. A., Rocca, N. L., Jouhet, J., Maréchal, E., Finazzi, G. and Morosinottoa, T. (2013). The response of *Nannochloropsis gaditana* to nitrogen starvation Includes *de novo* biosynthesis of triacylglycerols, a decrease of chloroplast galactolipids, and reorganization of the photosynthetic apparatus. *Eukaryotic Cell Journal*, 12: 665 - 676.

Simpson, A. G. B. and Roger, A. J. (2014). The real 'kingdoms' of eukaryotes. *Current Biology*, 14 (17): R 693 - R696.

Singh, A., Nigam, P. S. and Murphy, J. D. (2011). Mechanism and challenges in commercialisation of algal biofuels. *Bioresource Technology*, 102: 26 - 34.

Sitepu,I. R., Ignatia,L., Franz, A. K., Wong, D. M., Faulina,S. A., Sui, M. T., Kanti,A., Boundy-Mills, K. (2012). An improved high-throughput Nile red fluorescence assay for estimating intracellular lipids in a variety of yeast species. *Journal of Microbiological Methods*, 91(2): 321 - 328.

Skoog, D. A., Crouch, S. R. And Holler, F. J. (2007). Principles of Instrumental Analysis. 6th Edition, Belmont, C. A., Thomson/Brooks - Cole.

Smith, R. T., Bangert, K., Wilkinson, S. J. and Gilmour, D. J. (2015). Synergistic carbon metabolism in a fast growing mixotrophic freshwater microalgal species *Micractinium inermum*, *Biomass and Bioenergy*, 82: 23- 86

Solovchenko, A., Khozin-Goldberg, I., Didi-Cohen, S., Cohen, Z. and Merzlyak, M. (2008a). Effects of light intensity and nitrogen starvation on growth, total fatty acids and arachidonic acid in the green microalga *Parietochloris incisa*. *Journal of Applied Phycology*, 20: 245- 251.

Solovchenko, A., Khozin-Goldberg, I., Didi-Cohen, S., Cohen, Z. and Merzlyak, M. (2008b). Effects of light and nitrogen starvation on the content and composition of carotenoids of the green microalga *Parietochloris incisa*. *Russian Journal of Plant Physiology*, 55 (4) : 455 - 462.

Solovchenko, A., Lukyanov, A., Solovchenko, O., Shoshana, D. C., Boussiba, S. and Goldberg, I. K. (2014). Interactive effects of salinity, high light, and nitrogen

starvation on fatty acid and carotenoid profiles in *Nannochloropsis oceanic* CCALA804. *European Journal of Lipid Science and Technology,* 1- 62.

Somenrille, C. (1995). Direct tests of the role of membrane lipid composition in low-temperature-induced photoinhibition and chilling sensitivity in plants and cyanobacteria.*Proceedings of the National Academy of Sciences of the United States of America*, 92: 6215- 6218.

Spratt, D. A. (2004). Significance of bacterial identification by molecular biology methods. *Endodontic Topics*, 9: 5-14.

Spolaore, P., Cassan, C. J., Duran, E. and Isambert, A. (2006). Commercial Applications of Microalgae, *review*. *Journal of Bioscience and Bioengineering*, 101: 87- 96.

Srinivas, R. and Ochs, C. (2012). Effect of UV-A irradiance on lipid accumulation in *Nannochloropsis oculata*. *Photochemistry and Photobiology*, 88 (3): 684 - 689.

Stansell, G. R., Gray, V. M. and Sym, S. D. (2012). Microalgal fatty acid composition: implications for biodiesel quality. *Journal of Applied Phycology,*24: 791- 801.

Starkenburg, S. R., Kwon, K. J., Jha, R. K., McKay, C., Jacpbs, M., Chertkov, O., Twary, S., Rocap, G. and Cattolico, R. A. (2014). A pangenomic analysis of the *Nannochloropsis* organellar genomes reveals novel genetic variation in key metabolic genes. *BMC Genomic,* 15: 212 - 233.

Storms, Z. J., Cameron, E., Siegler, H. H. and McCaffrey,W. C. (2014). A Simple and rapid protocol for measuring neutral lipids in algal cells using fluorescence. *Journal of Visualized Experiments*, May 30 (87): pp. 1-7.

Suali, E, Sarbatly, R. (2012). Conversion of microalgae to biofuel. *Renewable and Sustainable Energy Reviews*, 16 (6): 4316 - 4342.

Suda, S., Atsumi, M. and Miyashita, H. (2002). Taxonomic characterization of a marine *Nannochloropsis* species, *N. oceanica* sp. nov. (Eustigmatophyceae). *Phycologia,* 41: 273- 279.

Sun, X., Cao, Y., Xu, H., Liu, Y., Sun, J., Qiao, D. and Cao, Y. (2014). Effect of nitrogen starvation, light intensity and iron on triacylglyceride / carbohydrate production and fatty acid profile of *Neochloris oleoabundans* HK-129 by a two stage process. *Bioresource Technology*, 155: 204 - 212.

Sunda, W. G., Price, N. M. and Morel, F. M. M. (2005). Trace metal ion buffers and their use in culture studies. *Algal culturing techniques*, pp. 35- 63.

Suresh, B. and Ravishankar, G. A. (2004). Phytoremediation-A novel and promising approach for environmental clean-up. *Critical Reviews in Biotechnology,* 24: 97-124.

Sushchik, N. N., Kalacheva, G. S., Zhila, N. O., Gladyshev, M. I. and Volova, T. G. (2003). A temperature dependence of the intra and extracellular fatty-acid composition of green algae and cyanobacterium. *Russian Journal of Plant Physiology,* 50 (3): 374 - 380.

Tababa, H. G, Hirabayashi, S, and Inubushi, K. (2012). Media optimization of *Parietochloris incisa* for arachidonic acid accumulation in an outdoor vertical tubular photobioreactor. *Journal of Applied Phycology*, 24: 887 - 95.

Taggar, M. S., Singh, I. and Sooch, S. S. (2014). Lipid accumulation in microalgae and its induction under different stress conditions for biodiesel. *Production. Impending Power Demand and Innovative Energy Paths* -ISBN: 978-93-83083-84-8, 222 - 229.

Takagi, M., Karseno and Yoshida, T. (2006). Effect of salt concentration on intracellular accumulation of lipids and triacylglyceride in marine microalgae *Dunaliella* Cells. Journal of Bioscience and Bioengineering, 101 :223 -226.

Tang. Y., Zhang, Y., Rosenberg, J. N., Betenbaugh, M. J. and Wang, F. (2016). Optimization of one-Step in situ transesterification method for accurate Quantification of EPA in *Nannochloropsis gaditana*. *Applied Sciences,* 6 (343): 1- 11.

Tatsuzawa, H., Takizawa, E., Wada, M. and Yamamoto, Y. (1996). Fatty acid and lipid composition of the acidophilic green alga *Chlamydomonas* sp. *Journal of Phycology,* 32: 598 - 601.

Terry, K. L. and Raymond, L. P. (1985). System design for the autotrophic production of microalgae. *Enzyme and Microbial Technology*, 7(10): 474- 487.

Terry, N. and Abadia, J. (1986). Function of iron in chloroplasts. *Journal of Plant Nutrition* ,9: 609 - 646.

Thompson, G.A. (1996). Lipids and membrane function in green algae. *Biochimica et Biophysica Acta*, 1302: 17- 45.

Tsukahara, K. and Sawayama, S. (2005). Liquid fuel production using microalgae. *Journal of the Japan Petroleum Institute,* 48(5): 251- 259.

Ugwu, C. U. and Aoyagi, H. **(2012).** Microalgal Culture Systems: An Insight into their Designs, Operation and Applications, *Biotechnology,* 11 (3): 127- 132.

Umdu, E.S., Tuncer, M. and Seker, E. (2009). Transesterification of *Nannochloropsis oculata* microalga's lipid to biodiesel on Al_2O_3 supported CaO and MgO catalysts. *Bioresource Technology*, 100 (11): 2828 - 2831.

Unnithan, V. V., Unc, A. and Smith, G. B. (2014). Role of *Nannochloropsis salina* for the recovery and persistence of MS2 virus in wastewater. *Algal Research*, 4: 70-75.

Van den Hoek, C., Mann, D. G. and Jahns, H. M. (1995). Algae: An Introduction to Phycology, *Cambridge University Press*, Cambridge, UK.

Van Wychen, S. V. and Laurens, L. M. L. (2013). Determination of Total Lipids as Fatty Acid Methyl Esters (FAME) by *in situ* Transesterfication. Laboratory Analytical Procedure (LAP) NREL/TP-5100 - 60958. Golden, Colorado (United States): *National Renewable Energy Laboratory*.

Vaishampayan, A., Sinha, R. P., Hader, D. P., Dey, T., Gupta, A. K., Bhan, U. and Rao, A. L. (2001). Cyanobacterial Biofertilizers in Rice Agriculture. *The Botanical Review*, 67: 453-516.

Vazquez-Duhalt, R. and Arredondo-Vega, B. O. (1991). Haloadaptation of the green alga *Botryococcus braunii* (RACE A). *Phytochemistry*, 30: 2919 - 2925.

Velmurugan, N. Sung, M., Yim, S. S., Park, M. S. Min S.. Yang, J. W. and Jeong, K. J. (2013). Evaluation of intracellular lipid bodies in *Chlamydomonas reinhardtii* strains by flow cytometry. *Bioresource Technology*, 138 **:** 30 - 37.

Vicente, G., Bautista, L. F., Rodriguez, R., Gutierrez, F. J., Sadabaa, I., Vazquez R. M. R., Martinez, S. T., Garre, V. (2009). Biodiesel production from biomass of an oleaginous fungus. *Biochemical Engineering Journal*, 48: 22 - 27.

Vieler, A., Wu, G., Tsai, C. H., Bullard, B., Cornich, A. J., Harvey, C., Reca, I. B., Thornburg, C., Achawanantakun, R., Buehl, C. J., Campbell, M. S., Cavalier, D., Childs, K. L., Clarck, T. J., Deshpande, R., Erickson, E., Ferguson, A. A., Handee, W., Kong, Q. Li, X., Lui, B., Lundback, S., Peng, C., Roston, R. L., Simpson, J. P., Terbuch, A., Warakanont, J., Zäuner, S., Farre, E. M., Hegg, E. L., Jiang, N., Kuo, M. H., Lu, Y., Niyogi, K. K., Ohlrogge, J., Osteryoung, K. W., Shachar, H. Y., Sear, B. B., Sun, Y., Takahashi, H., Yandell, M., Shiu, S. H. and Benning, C. (2012). Genome, functional and gen annotation and nuclear transformation of the heterokont oleaginous alga *Nannochloropsis oceanica* CCMP1779. *PLOS Genetics*, 8: 1- 25.

Visviki, I. and Palladino, J. (2001). Growth and cytology of *Chlamydomonas acidophila* under acidic stress. *Bulletin of Environmental Contamination and Toxicology*, 66 (5) : 623 - 630.

Visviki, I. and Santikul, D. (2000). The pH tolerance of *Chlamydomonas applanata* (*Volvocales, Chlorophyta*). *Archives of Environmental Contamination and Toxicology*, 38 (2): 147 - 151.

Vooren, V. G., Grand, G. L., Legrand, J., Cuine, S., Peltier, G and Pruvost, J. (2012). Investigation of fatty acids accumulation in *Nannochloropsis oculata* for biodiesel application. *Bioresource Technology*, 124: 421- 432.

Wagenen, J. V., Miller, T. W., Hobbs, S., Hook, P., Crowe, B. and Huesemann, M. (2012). Effects of Light and Temperature on Fatty Acid Production in *Nannochloropsis Salina*. *Energies*, 5:731-740.

Wahidin, S., Idris, A. and Shaleh, S. R. M. (2013). The influence of light intensity and photoperiod on the growth and lipid content of microalgae *Nannochloropsis sp. Bioresource Technol*ogy, 129: 7 - 11.

Walter, T. L., Purton, S., Becker, D. K. and Collet, C. (2005). Microalgae as bioreactors. *Plant Cell Reports*, 24: 629 - 641.

Wan, C., Bai, F. and Zhao, X. (2013). Effects of nitrogen concentration and media replacement on cell growth and lipid production of oleaginous marine microalga *Nannochloropsis oceanica* DUT01. *Biochemical Engineering Journal*, 78: 32- 38.

Wang, B., Li, Y., Wu, N. and Lan, C. Q. (2008). CO_2 bio-mitigation using microalgae. Applied Microbiology and Biotechnology,79: 707 - 718.

Wang, D., Ning, K., Li, J., Hu, J., Han, D., Wang, H., Zeng, X., Jing, X., Zhou, Q., Su, X., Chang, X., Wang, A., Wang, W., Jia, J., Wei, L., Xin, Y., Qiao, Y., H. R., Chen, J., Ha, B., Yoon, K., Hill, R. T., Zohar, Y., Chen, F., Hu, Q. and Xu, J. (2014a). *Nannochloropsis* Genomes Reveal Evolution of Microalgal Oleaginous Traits. *PLOS Genetics*,10: 1-13.

Wang, J., Sommerfeld, M. R., Lu, C. and Hu, Q. (2013). Combined effect of initial biomass density and nitrogen concentration on growth and astaxanthin production of *Haematococcus pluvialis* (Chlorophyta) in outdoor cultivation. *Algae*, 28: 193 - 202.

Wang, J., Yang, H. and Wang, F. (2014b). Mixotrophic Cultivation of Microalgae for Biodiesel Production: Status and Prospects. *Applied Biochemistry and Biotechnology*, 172 : 3307- 3329.

Wang, X., Hao, C., Zhang, F., Feng, C., Yang, Y. (2011). Inhibition of the growth of two blue-green algae species (*Microsystis aruginosa* and *Anabaena spiroides*) by acidification treatments using carbon dioxide. *Bioresource Technology*, 102: 5742 - 5748.

Wang, Z. T., Ullrich, N., Joo, S., Waffenschmidt, S. and Goodenough, U. (2009). Algal lipid bodies: stress induction, purification, and biochemical characterization in wild-type and starchless *Chlamydomonas reinhardtii*. *Eukaryot Cell*, 8 (12): 1856 - 1868.

Wei, L., Xin, Y., Wang, D., Jing, X., Zhou, Q., Su, X., Jia, J., Ning, K., Cheng, F., Hu, Q. and Xu, J. (2013). *Nannochloropsis* plastid and mitochondrial phylogenomes reveal organelle diversification mechanism and intragenus phylotyping strategy in microalgae. *BMC Genomics*, 14: 534 - 551.

Wei, N., Quarterman, J., and Jin, Y. (2013). Marine macroalgae: an untapped source for producing fuels and chemicals. Trends in biotechnology, 31:70 -77.

Weselake, R. J., Shah, S., Tang, M., Quant, P. A., Snyder, C. L,. Furukawa-Stoffer, T. L., Zhu, W.,. Taylor, D. C., Zou, J., Kumar, A., Hall, L., Laroche, A., Rakow, G., Raney, P., Moloney, M. M. and Harwood, J. L. (2008). Metabolic control analysis is helpful for informed genetic manipulation of oilseed rape (*Brassica napus*) to increase seed oil content. *Journal of Experimental Botany*, 59 (13): 3543 - 3549.

Weyer, K. M., Bush, D. R., Darzins, A. And Wilso, B. D. (2010). Theoretical Maximum Algal Oil Production. *Bioenergy Reearch*, 3: 204 - 213.

Widjaja, A., Chien, C. C. and Ju, Y. H. (2009). Study of increasing lipid production from fresh water microalgae *Chlorella vulgaris*. *Journal of the Taiwan Institute of Chemical Engineers*, 40: 13 -20.

Wijffels, R. H. and Barbosa, M. J. (2010). An Outlook on Microalgal Biofuels. **Science**, 329: 796-799.

Williams, P. J. L. and Laurens, L. M. L. (2010). Microalgae as biodiesel and biomass feedstocks: Review and analysis of the biochemistry, energetics and economics. *Energy and Environmental Science*, 3: 554-590.

Wong, D. M., Nguyen, T. T. N. and Franz, A. K. (2014). Ethylenediamin etetraaceticacid (EDTA) enhances intracellular lipid staining with Nile red in microalgae *Tetraselmis suecica*. *Algal Research*, 5: 158 - 163.

Woo, P.C., Fung, A. M, Wong, S. S., Tsoi, H. W. and Yuen, K. Y. (2001). Isolation and characterization of a *Salmonella enterica* serotype *typhi* variant: its clinical and public health implications. *Journalof Clinical Microbiology*, 39: 1190-1194.

Woo, P.C., Ng, K. H., Lau, S. K., Yip, K.T., Fung, A. M., Leung, K. W., Tam, D. M., Que, T. L. and Yuen, K. Y. (2003). Usefulness of the MicroSeq 500 16S ribosomal DNA-based bacterial identification system for identification of clinically

significant bacterial isolates with ambiguous biochemical profiles. *Journal of Clinical Microbiology*,41: 1996 - 2001.

Wu, S., Baoyu, H., Aiyou, H., Li, H., Lin, A., Niu, J. and Wang, G.(2014). Detection of intracellular neutral lipid content in the marine microalgae *Prorocentrum micans* and *Phaeodactylum tricornutum* using Nile red and BODIPY 505/515. *Journal of Applied Phycology*, 26 (4): 1659 - 1668.

Xia, L., Song, S. and Hu, C. (2016). High temperature enhances lipid accumulation in nitrogen-deprived *Scenedesmus obtusus* XJ-15. *Journal of Applied Phycology*, 28 (2): 831- 837.

Xin, L., Hong-ying, H., Ke, G. and Ying-xue, S. (2010). Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga *Scenedesmus* sp. *Bioresource Technology*, 101 (14) : 5494 - 5500.

Xu, X. O. and Beardall, J. (1997). Effect of salinity on fatty acid composition of a green microalga from an antarctic hypersaline lake. *Phytochemistry*,655 - 658.

Xu, D. Gao, Z., Li, F. Fan, X., Zhang, X., Ye, N., Mou, S., Liang, C. and Li, D. (2013). Detection and quantitation of lipid in the microalga *Tetraselmis subcordiformis* (Wille) Butcher with BODIPY 505/515 staining. *Bioresource Technology*, 127: 386 - 390.

Xu, Y. and Boeing, W. J. (2014). Modeling maximum lipid productivity of microalgae: Review and next step. *Renewable and Sustainable Energy Reviews*, 32: 29 - 39.

Xue, L., Zhang, Y., Zhang, T., An, L., Wang, X. (2005). Effects of enhanced ultraviolet-B radiation on algae and cyanobacteria. *Critical Reviews in Microbiology*, 31 (2): 79 - 89.

Xue, S., Su, Z. and Cong, W., (2011). Growth of *Spirulina platensis* enhanced under intermittent illumination. *Journal of Biotechnology*, 151 (3), 271 - 277.

Yaakob, Z., Ali, E, Zainal, A., Mohamad, M. and Takriff, M. S. (2014). An overview: biomolecules from microalgae for animal feed and aquaculture. *Journal of Biological Research-Thessaloniki*, 21(6): 1-10.

Yao, C, Ai, J., Cao, P. and Xue, S. (2013). Salinity manipulation as an effective method for enhanced starch production in the marine microalga *Tetraselmis subcordiformis*. *Bioresource Technology*, 146: 663 - 671.

Yeesang, C. and Cheirsilp, B. (2011). Effect of nitrogen, salt, and iron content in the growth medium and light intensity on lipid production by microalgae isolated from freshwater sources in Thailand. *Bioresource Technology,* 102: 3034 - 3040.

Yeh, K. L. and Chang, J. S. (2011). Nitrogen starvation strategies and photobioreactor design for enhancing lipid production of a newly isolated microalga *Chlorella vulgaris* esp-31: Implications for biofuels. *Biotechnology Journal*, 6:1358 - 1366.

Yen, H. W., Hu, I. C., Chen, C. Y., Ho, S. H., Lee, D. J. and Chang, J. (2013). Microalgae-based biorefinery-From biofuels to natural products. *Bioresource Technology*, 135: 166 -174.

Yen, H. W., Hu, I. C., Chen, C. Y. and Chang, J. S. (2014). Chapter 2 – Design of photobioreactors for algal cultivation. *Biofuels from Algae*, 23 - 45.

Ying, K., Gilmour, D. J. and Zimmerman, W. B. (2015). Periodic CO_2 dosing strategy for *Dunaliella salina* batch culture. *International Journal of Molecular Sciences,*16 : 11509 -11521.

Yoshida, N., Ikeda, R. and Okuno, T. (2006). Identification and characterization of heavy metal-resistant unicellular alga isolated from soil and its potential for phytoremediation. *Bioresource Technology*, 97 (15): 1843 - 1849.

Yu, W., Ansari, W., Schoepp, N. G., Hannon, M. J, Mayfield, S. P. and Burkart M. D. (2011). Modifications of the metabolic pathways of lipid and triacylglycerol production in microalgae. *Microbial Cell Factories,* 10 (91): 1-11.

Yun, H., Smith, V. H., Pate, R, C. (2015). Managing nutrients and system operations for biofuel production from freshwater macroalgae. *Algal Research,* 11: 13 - 21.

Zhang, Z., Shrager, J., Jain, M., Chang, C. W., Vallon, O. and Grossman, A. R. (2004). Insights into the survival of *Chlamydomonas reinhardtii* during sulfur starvation based on microarray analysis of gene expression. *Eukaryot Cell*, 3 (5): 1331-1348.

Zhekisheva M, Boussiba S, Khozin-Goldberg I, Zarka A, Cohen Z (2002). Accumulation of oleic acid in *Haematococcus pluvialis* (Chlorophyceae) under nitrogen starvation or high light is correlated with that of astaxanthin esters. *Journal of Applied Phycology,* 38: 325 - 331.

Zhila, N. O., Kalacheva, G. S. and Volova, T. G. (2011). Effect of salinity on the biochemical composition of the alga *Botryococcus braunii* Kutz IPPAS H-252. *Journal of Applied Phycology*, 23:47 - 52.

Zhu, L. D., Li, Z. H. and Hiltunen, E. (2016). Review article strategies for lipid production improvement in microalgae as a biodiesel feedstock. *BioMed Research International*, ID 8792548, 1- 8.

Zhu, M., Zhang, X., Mao Y., *et al.* (2003). Effects of temperature, salinity and illumination on the growth of *Thalassiosira sp. Marine Sciences,* 27 (12) : 58 - 61.

Zimmerman, W. B., Zandi, M., Bandulasena, H.C. H., Tesar, V., Gilmour, D. J. and Ying, K. (2011). Design of an airlift loop bioreactor and pilot scales studies with fluidic oscillator induced microbubbles for growth of a microalgae *Dunaliella salina*. *Applied Energy*, 88: 3357- 3369.

Zosel, J., OelBner, W., Decker, M., Gerlach, G. and Guth, U. (2011). The measurement of dissolved and gaseous carbon dioxide concentration. Measurement Science and Technology, *Review* 22: 1- 45.

Appendixs

Appendix A

50 X TAE:

242 g Trizma base, 57.1 ml glacial acetic acid and 18.6 g EDTA are added to 900 ml dH_2O before adjusting the final volume to 1 litre with additional dH_2O. This solution was diluted 1 in 50 to produce 1X TAE suitable for use as an electrophoresis buffer.