Effects of Stress conditions on Lipid Production By Botryococcus braunii, Tetraselmis suecica, and Coccomyxa

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Declaration

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

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Abstract

The effects of stress conditions on neutral lipid accumulation by the microalgae *Botryococcus braunii, Tetraselmis suecica* and *Coccomyxa* were investigated to assess their suitability for biodiesel production. The growth media (3N-BBM+V for *B. braunii,* and *Coccomyxa* and F/2 for *T. suecica*) were altered to impose salt stress (0.1 and 0.2 M NaCl for BBM medium) and up to 1.2 M NaCl for F/2 medium. *Tetraselmis suecica* and *Coccomyxa* were also cultivated under nitrogen depletion conditions (50%, 25% nitrogen and nitrogen free) to monitor growth and investigate neutral lipid accumulation.

It was found that *B. braunii* grew very slowly despite using the relatively nutrient rich BBM medium. It was difficult to reach sufficient biomass to run experiments for *B. braunii,* but faster growth was achieved using a 2 litre fermenter. *B. braunii* had a total lipid content of 29% but only 3.64% were neutral (storage) lipids. This is much less than the 30% neutral lipid normally quoted as the minimum requirement for biodiesel production (Chisti, 2007). The *B. braunii* strain (CCAP 807/1) used failed to grow in the presence of 0.1 or 0.2 M NaCl.

Tetraselmis suecica grew in the higher salinity media, but salt stress did not induce higher lipid accumulation. Nitrogen free medium did induce more neutral lipid accumulation in *T. suecica* even though the growth rate and final biomass level reached were decreased. *Tetraselmis suecica* had less than the 30% neutral lipid normally quoted as the minimum requirement for biodiesel production, but it is an excellent candidate for production of biodiesel based on the fatty acids produced which are principally C16:0 and C18:1.

Neutral lipid accumulation increased rapidly with increasing nitrogen starvation from 25% nitrogen to nitrogen-free medium as *Coccomyxa* grew to reach stationary phase over four weeks. Nitrogen free medium induced more neutral lipid accumulation (31%) in *Coccomyxa,* which was the highest percentage found in the current work and meets the 30% neutral lipid figure suggested by Chisti et al. (2007).

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1.0 Introduction

1.1 Context of Work

As a student funded by my country Qatar, my project is relevant to the needs of Qatar, which is one of the richest countries in fossil fuels such as Oil and Natural Gas. Qatar produces around 1.6 million barrels per day of crude oil and other liquid fuels such as condensates, converting natural gas to liquid fuels by catalytic reactors (GTL) and natural gas plant liquids. Moreover, the production of Natural Gas is around 5,500 billion cubic feet per year with 23% of the consumption used to generate electricity in Qatar (EIA, 2014).

However, carbon dioxide (CO_2) emissions in Qatar are the highest in the world according to the World Bank Group (2014), which states the amount of the CO_2 production in Qatar is approximately 40.3 metric tons per capita. To reduce the CO_2 emissions, Qatar's Government has cited this issue in its vision for 2030 and plans to minimize the dependence on fossil fuels to be around 80% by 2030.

One of the plans to reduce consumption of fossil fuels is developing the production of biofuels which are renewable and environmentally friendly, starting the programme with a collaboration between Qatar Airways, Airbus, Qatar Petroleum and Qatar Science and Technology Park to develop production of biofuels to be used in air transportation.

This PhD project investigates the use of microalgae as a potential source of biofuels; they can be grown in saline water or wastewater without the need of arable land and freshwater (Gilmour and Zimmerman, 2012). Additionally, microalgae use CO_2 and convert it into organic compounds by photosynthesis, which makes them a suitable choice in countries which produce a high amount of CO_2 such as Qatar, but which also have a high level of sunshine for photosynthesis.

Microalgae containing 30% oil by weight of dry biomass could produce almost 600,000 litres per hectare, which is more than the yield from rapeseed or soybean grown in the same area. Moreover, the total oil content could reach up to 70% in some microalgae

species such as *Botryococcus braunii* and *Schizochytrium* spp (Chisti, 2007). The production of biodiesel could reach 121,104 Kg/ha per year by using 70% oil content algae as feedstock. Therefore, it was thought possible to use microalgae for producing oil to replace fossil diesel according to the high yield of oil and the ability of growing rapidly (Deng et al., 2009).

1.2 Microalgae as a source for biodiesel

Microalgae are photosynthetic microorganisms which can grow rapidly in harsh conditions because of their simple structure. They are divided into the following four main categories (Li et al., 2008):

  i.   Diatoms
  ii. Green algae
  iii. Blue-green algae (cyanobacteria – prokaryotes) and
  iv. Brown or golden algae

Studies have found that algae cultivation has the following four major requirements (Campbell, 2008):

- CO_2
- Water (containing nutrients)
- Light
- Space

These requirements will be discussed in the proceeding subsections.

1.2.1 Carbon Dioxide (CO_2)

CO_2 must be provided in significant amounts, much higher than natural conditions (0.03%) and the flue gases from industrial plants, and in particular from power plants, are rich in CO_2 (15-25%) that would be released directly into the atmosphere, harming the environment and causing global warming. Studies suggested that by diverting the CO_2 fraction of the flue gas into an algal cultivation facility, the gas could be diverted back through the energy stream and the rate of algal production will increase (Sarin,

2012). Another recent study carried out by staff at the University of Sheffield in collaboration with Tata Steel (Zimmerman et al., 2011) successfully demonstrated growth of the green microalga *Dunaliella salina* on flue gas pumped through a 2000 litre bioreactor situated outdoors. The CO_2 concentration of the flue gas was up to 23% and a significant amount of CO_2 was sequestered by the *Dunaliella* cells during 10 days of growth in April and September 2010. This was a good proof of concept that CO_2 from flue gas can be sequestered in an industrial setting in the UK (Zimmerman et al., 2010).

1.2.2 Water

Water is the second requirement and treated wastewater from domestic or industrial sources, which already contains nitrogen and phosphate salts, could be used for algal cultivation media directly and provides a cheap way to grow algae. Sea water or saline aquifers can be used as well as a source of salt water (Sarin, 2012). Fresh water is rare in most parts of the world, 70% of the total fresh water in the world is for agricultural uses. The halotolerant/halophilic characteristics of some algae allow a much greater area to be utilized for algaculture. Other sources of salt water, besides seawater, are saline groundwater/aquifers and certain wastewaters. One disadvantage of using saline water is that evaporation leads to an increase in the total salinity over the growth period in an outdoor pond and thus freshwater (or much less saline water) is required to dilute the medium (Borowitzka, 2013).

1.2.3 Light

Abundant light is often accomplished by placing the facility in an area with abundant, uninterrupted sunlight and when working with bioreactors or open ponds. Sunlight quality and quantity can be further enhanced by using solar collectors, solar concentrators and fibre optics in a system called a photobioreactor (Sarin, 2012). The algae absorb light, so the higher algae concentration, the less light enters into the algal broth. For that reason, the algaculture systems are shallow and designed to allow light to penetrate as much as possible. Light quantity depends on the location, only about 45% of the total light spectrum is photosynthetically active radiation (PAR, ~400-700 nm), hence with the fixation of CO_2, a process with 27% efficiency, multiplying these two

factors results in the maximum theoretical conversion of light energy to chemical energy by photosynthesis: about 12% (Gao et al., 2007).

1.2.4 Land

Biodiesel production from algae has a significantly higher yield per hectare over conventional oil crops because algae can be grown in open ponds or bioreactors (Janaun & Ellis, 2010). The cultivation systems have lower land quality then agriculture, because algae are grown in water. There is no need for soil fertility at all. Land needs to be relatively level and sufficiently solid to place the cultivation systems on, which allows a significant area of land to be used, such as deserts, saline soils, polluted land and other land with low economic value. Glenn et al. (1998) indicate that 43% of global land is arid or semi-arid and 15% of undeveloped land has plenty access to seawater, which reaches around 130 million ha. Moreover, Bai et al. (2008) estimated that 24% of the world total land has been degrading over the last 25 years. Some of the desert lands which are away from the sea may have saline ground water that could be useful, or access to wastewater or fresh water (Borowitzka, 2013).

1.3 Microalgae Strains

1.3.1 Botryococcus braunii

Botryococcus braunii is a unicellular photosynthetic microalga which belongs to the class of green algae the Chlorophyceae (phylum Chlorophyta) and is closely related to *Characium vaculatum* and *Dunaliella parva* based on the sequence analysis of the small subunit ribosomal-RNA (18S RNA) and comparison to 18S sequences for other algae (Sawayama et al., 1995b). This colonial alga is common in fresh and brackish waters all around the globe (Metzger and Largeau, 2005).

It is known that *B. braunii* is a potential source of renewable fuel with regard to its ability to produce a significant number of hydrocarbons. Up to 75% of *B. braunii* dry mass can be hydrocarbons, depending on the strain and growth conditions (Banerjee et al., 2002). This alga demonstrates an obvious capability to synthesize a lot of hydrocarbons, i.e. highly reduced compounds comprising only carbon and hydrogen as elements (Brown and Knights, 1969; Knights et al., 1970). Based on their hydrocarbon production, *B. braunii* species can be divided into three "races" – A race which produce odd-numbered C23 to C33 trine hydrocarbons, B race which produce triterpenoid hydrocarbons and the C race which produce a single tetraterpenoid hydrocarbon (Metzger and Largeau, 2005). A number of studies have attempted to alter the chemical composition of *B. braunii* strains by altering the salinity of the medium (Zhila et al., 2011; Ruangsomboon, 2012), nutrient concentration, cultivation time and irradiance (Ruangsomboon, 2012; Yoshimura et al., 2013). One key advantage of using *B. braunii* is that the hydrocarbons are normally found associated with the cell walls (Metzger and Largeau, 2005), and various non-destructive harvesting methods ("milking") have been proposed (Moheimani et al., 2014). This type of harvesting could greatly reduce the cost of downstream processing of the algal biomass (Borowitzka, 2013).

1.3.2 Tetraselmis suecica

Tetraselmis suecica CCAP 66/4 is also a member of the Chlorophyta (green algae), which is found within the class of Prasinophyceae in the Chlorodendrales group. The

strain CCAP 66/4 was received from the Culture Collection of Algae and Protozoa, Oban, Scotland. It is normally grown in large scale as a feedstock in aquaculture using various kinds of photo-bioreactor arrangements (Day and Fenwick, 1993; Naumann et al., 2013). It is normally considered as one of the best sources of long-chain PUFAs that are essentially used for strainer feeders grown in aquaculture systems (Fabregas et al., 2001). It was established also that the conditions to which it is exposed during cultivation play significant roles impacting on its total lipid production. As a result of this discovery, it is seen as an excellent feedstock for commercial production of biofuel as it can be 'stressed' to produce more of the suitable lipids for biodiesel production (Sanchez-Garcia et al., 2013). The fatty acids produced by *T. suecica* are principally C16:0 and C18:1 which are excellent for production of biodiesel (Mendoza Guzman et al., 2010). Stansell et al. (2012) reviewed the requirements for biodiesel feedstock and showed that a mixture of saturated and mono-unsaturated fatty acids gave the best fuel stability without compromising the cold flow characteristics of the biodiesel. The lipid content of *T. suecica* is modest per percentage dry weight biomass but the lipid productivity is very high due to high biomass levels (Griffiths and Harrison, 2009; Mata et al., 2010). In contrast to *B. braunii,* in order to extract the lipids from *T. suecica,* the cell wall needs to be broken to release the lipids into the medium. However, as a result of the proven track record of *T. suecica* in large scale production by utilizing photo-bioreactors and its high lipid potential, it has been included in this work to assess its lipid production in relation to the normal and stress conditions.

1.3.3 Coccomyxa

Another member of the Chlorophyta (green algae) genus is *Coccomyxa* (Trebouxiophyceae, Chlorococcales, Coccomyxaceae), it comprises free-living planktonic freshwater and marine species (Guiry et al., 2005; Hoshina & Imamura, 2008), epiphytes (Lamenti et al., 2000) and symbiotic species including lichens (Lohtander et al., 2003) or even trees (e.g. in Ginkgo biloba; Trémouillaux-Guiller et al., 2002). Very recently, species of *Coccomyxa* have been utilized as oleaginous microalgae e.g. *C. subellipsoidea* and *C. onubensis* (Bermejo et al., 2017; Wang et al., 2017). Several species of *Coccomyxa* are extremophiles. *C. onubensis* is an acidophile

isolated from the Rio Tinto in Spain (Bermejo et al., 2017) and *C. subellipsoidea* was isolated from the Antarctic and is pyschrotolerant (Wang et al., 2017). Brewery effluent plus the addition of a phytohormone was shown to support good growth of *C. subellipsoidea* and led to highly efficient contaminant removal from the effluent (Liu et al., 2018).

In the current study, a strain of *Coccomyxa* appeared in a *B. braunii* culture flask and outgrew the *B. braunii*. The *Coccomyxa* was isolated and identified and therefore it has been included in this work to study and investigate its ability to synthesize neutral lipids.

1.4 Molecular Identification of Algal Strains and DNA Sequencing

1.4.1 Polymerase Chain Reaction (PCR)

PCR utilizes enzymes known as DNA polymerases, these enzymes make a replica of a carefully chosen section of DNA that is to be amplified. Therefore, the selected DNA sequence pairs over and over with each cycle ranging up to millions of times the initial amount and till it is sufficient enough to be perceived by gel electrophoresis (Weaver 2005). There are two encoded sites in a section of a DNA which are amplified with the aid of primers (oligonucleotides) which corresponds to these sites. One of which serves as a primer for the synthetic copies of the DNA sequence. Each cycle of PCR doubles the number of DNA molecules synthesized until a huge amount is produced (Gibbs, 1990). PCR can provide molecular biologists and other researchers with almost unlimited amounts of the exact genetic material that they want to study. The PCR reaction consists of three steps: denaturation, synthesis and reannealing (Weaver, 2005). These three steps are repeated around 30 times and each cycle only takes 3 to 5 minutes to complete using an automated thermal cycler. In addition, amplification of PCR is both simple and elegant. Oligonucleotide primers are used to complement the ends of a DNA sequence to be amplified. Deoxyribonucleotides are used with an appropriate buffer. Healing and cooling are used to denature the original DNA strands and allow annealing of the primer. This process is continued repeatedly and extended in order to create new copies of the original DNA fragment (Arnheim and Erlich, 1992). The worth of PCR lies in its capability to amplify DNA coming from very small amounts of the target DNA or from the DNA of a single cell. Due to this distinguishing characteristic, PCR has become an intrinsic part of molecular biology laboratories. In the field of microalgal identification, the 18S ribosomal (r)RNA gene is used and specific primers are produced for this gene. After PCR, the 18S rDNA can be sequenced and then put into the BLAST database to identify the algal species.

1.5 Lipids in Microalgae

The lipid energy store in microalgae are known to be the Triacylglycerides (TAGs), which could easily be transformed into biodiesel through transesterification reactions once extracted from the microalgae (Fukuda et al., 2001). These neutral lipids bear a common structure of triple esters where usually three long-chain fatty acids (FAs) are coupled to a glycerol molecule. Transesterification displaces glycerol with small alcohols (e.g. methanol). Lipids produced by microalgae generally include neutral lipids, polar lipids, wax esters, sterols and hydrocarbons, as well as prenyl derivatives such as tocopherols, carotenoids, terpenes, quinines and pyrrole derivatives such as the chlorophylls. These lipids produced can be grouped into two categories, storage or neutral lipids (non-polar lipids) and structural lipids (polar lipids). Storage lipids are mainly in the form of TAG made of predominately saturated FAs and some mono-unsaturated FAs which can be transesterified to produce biodiesel. Structural lipids typically have a high content of polyunsaturated fatty acids (PUFAs), which are also essential nutrients for aquatic animals and humans. Of the non-polar lipids, TAGs are abundant storage products, which can be easily catabolized to provide metabolic energy (Gurr et al., 2002). The accumulation of TAG in green microalgae and storage into chloroplastic lipids shows that TAGs play an additional role beyond being an energy storage product in algal cells (Bigogno et al., 2002).

1.6 Methods of Lipid Induction

The ability of microalgae to survive in diverse and extreme conditions is reflected in the tremendous diversity and sometimes unusual pattern of cellular lipids obtained from these microalgae (Sato et al., 2000). Moreover, some of these microalgae can also modify lipid metabolism efficiently in response to changes in environmental conditions (Gushing and Harwood, 2006). Under optimal growth conditions, large amounts of algal biomass are produced but with relatively low neutral lipid contents, and under unfavorable environmental or stress conditions many microalgae alter their lipid biosynthetic pathways towards the formation and accumulation of neutral lipids mainly in the form of TAG, enabling microalgae to endure these adverse conditions. On average oleaginous green algae have a total lipid content of 25.5%, which can double or triple when the cells are put under 'stress' conditions (Hu et al., 2008). Lipid accumulation also occurs when cells come to the end of their life cycle. In either case, the lipid increase comes primarily in the form of the neutral acylglycerols. This is due to a shift in lipid metabolism from membrane lipid synthesis to the storage of neutral lipids as cell division is no longer a priority (Hu et al., 2008). Synthesis and accumulation of large amounts of TAGs accompanied by considerable alterations in lipid and FA composition can occur in microalgae when placed under stress conditions imposed by chemical or physical environmental stimuli, either acting individually or in combination. Many different TAG induction techniques exist such as nutrient starvation, which is described below.

1.6.1 Nutrient Starvation

Nutrient availability has a significant impact on growth and propagation of microalgae and broad effects on their lipid and FA composition. Environmental stress condition when nutrients are limited, invariably cause a steadily declining cell division rate. Surprisingly, active biosynthesis of fatty acids is maintained in some algae species under such conditions, provided there is enough light and CO_2 available for photosynthesis (Thompson, 1996). When algal growth (as measured by cell divisions) slows down and there is no requirement for the synthesis of new membrane

compounds, the cells instead divert and deposit fatty acids into TAG. Under these conditions, TAG production might serve as a protective mechanism. Nutrient starvation is one of the most widely used and applied lipid induction techniques in microalgal TAG production and Nitrogen is the single most critical nutrient affecting lipid metabolism in algae. A general trend towards accumulation of lipids, particularly TAG, in response to nitrogen deficiency has been observed in numerous species or strains of various microalgae (Yeh and Chang, 2011). Nitrogen starvation is most widely applied and studied in almost all the microalgae species that can be considered for the commercial production of biodiesel. The exact combination of induction stresses that provides optimum lipid productivity in a large-scale commercial cultivation system for biodiesel production, will differ for every microalgae strain and depends on nutrient supply, environmental and climatic conditions.

1.6.1.1 Salinity

It is well known that biomass productivity and lipid composition are influenced by conditions like salinity (Renaud and Parry, 1994). Microalgae are also known for their tolerance to changes in salinity, increased salinity affects the rate of respiration, ion toxicity, minerals distribution, rate of photosynthesis and cell membrane permeability (Sudhir, 2004). In some cases, algal growth is retarded with salinity stress as a result of the accrual of simple compatible solutes (i.e. glycine and proline) so as to balance to an equilibrium the external concentrations of the salt in terms of osmotic potential (Ahmed et al., 1989). Formation of proline within cells occurs during osmotic stress, to stabilize the enzymes, it acts as an osmoprotectant (Fatma et al., 2007). Consequently, the amount of saturated fatty acid in the microalgae may be decreased as the salt concentration increases, and on the other hand, the amount of highly unsaturated fatty acid may be increased (Kirrolia et al., 2011).

1.6.1.2 Nitrogen starvation

Nitrogen sources (normally ammonium and nitrate) are essential inorganic salts for cell growth and metabolism. Nevertheless, the most favourable conditions for nitrogen concentration vary from species to species (Huang et al., 2013). A similar conclusion

was drawn by Li et al. (2008) who suggested that nitrogen reduction in microalgae culture led to rapid accumulation of oil.

1.7 Harvesting and Lipid Extraction of Algal Cells

Once a selected algal culture has been grown to a sufficient biomass yield and has the required lipids, it needs to be harvested and the lipids extracted. This downstream processing is of great significance to the overall energy balance and financial feasibility of the algal biofuel production endeavour (Chisti, 2013). As such, there is a great deal of research and funding going into this area both academically and commercially, the review by Kim et al. (2013) gives an excellent summary of the current state of the art. The most widely adopted forms of harvest are based on technologies that were developed for water purification and treatment; namely centrifugation, filtration and flotation (Kim et al., 2013). Lipid extraction methodologies have traditionally been based around mechanical disruption means coupled with solvent fractionation, Newer methods such as electroporation, which have higher throughputs and are energy efficient are showing great promise (Halima et al., 2012). A foam fractionation method has been developed at the University of Newcastle which involves the use of the surfactant CTAB and microtubules to generate a foam of cells which is harvested from the top of the bioreactor (Coward et al., 2013). A beneficial side effect of foam fractionation is that cells that are more buoyant are more likely to be harvested in the foam and high neutral lipid containing cells are more buoyant (Coward et al., 2013).

1.8 Algal Lipid Detection and Quantification

Since there are strict tolerances for biodiesel production, the variability of the algal lipid feedstock depends on the culture conditions, and age is a potentially serious problem (Stansell et al., 2012). Therefore, it is essential that the lipid testing techniques employed (at whatever scale) be as accurate and reliable as possible. To that end, there are many different methodologies available for determining the concentration and composition of lipids in fluids. This differentiation has been driven by advances in biomedical technology for use in the diagnostic process (Lam and Shui, 2013). However, in the last few decades, lipid analysis at the same scale (mass screening) has started to be directed toward non-medical applications such as the production of fuel crops like microalgae (Mutanda et al., 2011). As mentioned in section 1.8, one of the most prevalent methods for calculating lipid yield in microalgae (and many other species) are the gravimetric solvent extraction techniques developed by Bligh and Dyer (1959), Smedes and Askland (1999) and Folch, Lees and Stanley (1957), due to their simple methodologies and effective results. However, as simple as these processes are, they are very time consuming to perform and can be inaccurate due to the number of transfer and estimation steps involved. They also cannot differentiate between the neutral and polar lipid fractions in the biomass (just giving total or crude lipid values), which means additional processing steps are needed, such as liquid or gas chromatography to screen if the correct lipids are present to produce high quality biodiesel (Ichihara and Fukubayashi, 2010). It is worth mentioning there are also other larger scale/industrial combined methods for the extraction and quantification of lipids based around supercritical fluids and hydrothermal liquefaction, but they are out of the scope of this project (Biller and Ross, 2011; Halima et al., 2012).

1.9 Biodiesel Production

Succeeding through the lipid extraction processes, the crude total lipids (typically containing a mix of acylgycerols, polar lipids, free FAs, pigments etc, mentioned previously) are typically fractionated (or separated using another method), as only acylgycerols are suitable for biodiesel production (Medina et al., 1998). Once the lipids are purified, they can be converted into biodiesel via the process of transesterification. The transesterifiction (or alcoholysis) reaction occurs when the acylgycerols (tri, di and mono varieties) are reacted with an alcohol (such as methanol) in the presence of a catalyst to produce fatty acid methyl esters (FAME) or biodiesel and glycerol as a by-product. The reaction can be catalysed by many different acids, alkalis (such as KOH above) or lipase enzymes, each providing their own advantages and disadvantages to the overall process (Fukuda et al., 2001; Kim et al., 2013; Xiao et al., 2009). Alkali 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). 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). The composition of the FAMEs is then checked using a gas chromatography (GC) system equipped with a lipid specific column (Laurens et al., 2012). A number of factors affect the overall efficiency of the FAME conversion process, including; the molar ratio of acylglycerol to catalyst and acylglycerol to methanol, the reaction temperature and exposure time and the water content of the crude lipid extract. To ensure quality of the biodiesel end product, given the inherent variability in the lipid production of the algal feedstock (lipid structure profile and volume), standardization is required to guarantee satisfactory engine performance (Balat and Balat, 2010; Lin et al., 2011). The current properties and qualities that biodiesel must adhere to are the American Standards for Testing Materials (ASTM 6751-3) or the European Union (EN 14214) Standards for biodiesel fuel (Stansell et al., 2012).

1.10 Aims and Objectives

The aims and objectives of the work are stated below:

1. Confirm the identity of *Botryococcus braunii* and *Tetraselmis suecica* strains received from the Culture Collection of Algae and Protozoa using 18S rDNA sequencing.

2. Optimise growth media (3N-BBM+V) to cultivate *B. braunii* and (F/2) to cultivate *T. suecica* and monitor any improvement in growth.

3. Set up a fermenter to grow *B. braunii* to be a continuous source of algae for experiments.

4. Quantify the lipid content of *B. braunii* and *T. suecica* using dry weight measurements, lyophilisation, a gravimetric method and Nile Red fluorescent dye staining.

5. Isolate and identify the new strain of *Coccomyxa* using 18S rDNA sequencing.

6. Quantify the lipid content in *Coccomyxa* using dry weight measurements, lyophilisation, a gravimetric method and Nile Red staining.

7. Quantify the Fatty Acid Methyl Esters (FAMEs) in *T. suecica* and *Coccomyxa* using GC-MS.

8. Quantify the compatible solutes in *T. suecica* and *Coccomyxa* using NMR.

Chapter Two Materials and Methods

2.1 Cleaning and Sterilization Techniques

To keep all experiments from any form of contamination, all the culturing equipment utilized in conducting experiments was autoclaved. Likewise, inoculation and sub-culturing were conducted using a flame after cleaning the bench with 70% ethanol. Furthermore, the glassware was soaked for 2 hours in concentrated sulphuric acid to ensure that all the remaining material from preceding cultures was completely removed.

2.2 Algae Strains

Two (2) algae strains were received from the culture collectuion (Table 2.1), but during the culturing of the strains (*Botryococcus braunii)*, another algal strain (*Coccomyxa)* developed in some of the flasks and outcompeted *B. braunii*. It can be seen from Table 2.1 that three (3) algae strains were investigated even though only two (2) of these were received from the Culture Collection.

Table 2.1: Algae Strains.

Algae strain	Provider
Botryococcus braunii Kützing (1849) (CCAP 807/1)	Culture Collection of Algae and Protozoa, Oban, UK
Tetraselmis suecica (Kylin) Butcher (1959) (CCAP 66/4)	Culture Collection of Algae and Protozoa, Oban, UK
Coccomyxa (new isolate)	Developed in the flask and took over from *Botryococcus braunii*

2.2 Collection of Samples

Botryococcus braunii Kützing (1849) (CCAP 807/1) strain isolated by Droop (1950), was received from the Culture Collection of Algae and Protozoa, Oban, Scotland and cultured in 3N-BBM+V medium as recommended. *Tetraselmis suecica* (CCAP 66/4) was also received from the Culture Collection of Algae and Protozoa, Oban, Scotland and cultured in F/2 medium. While *Coccomyxa,* which is also a genus of green algae in the family Coccomyxaceae, was isolated as a contaminant of *Botryococcus braunii*.

2.3 Medium Preparation

2.3.1 3N-BBM+V Medium for *Botryococcus braunii* and *Coccomyxa*

The medium used to culture *Botryococcus braunii* and *Coccomyxa* is 3N-BBM+V which is Bold's basal medium with 3-fold Nitrogen and vitamins modification. It was prepared as shown in Table 2.2.

Table 2.2: 3N-BBM+V medium Main Recipe.

	Stock solutions in g/100 ml water	1 litre final medium
1	2.5g $NaNO_3$	30.0ml
2	0.25g $CaCl_2.2H_2O$	10.0ml
3	0.75g $MgSO_4.7H_2O$	10.0ml
4	0.75g $K_2HPO_4.3H_2O$	10.0ml
5	1.75g KH_2PO_4	10.0ml
6	0.25g NaCl	10.0ml

7	Trace element solution (see below)	6.0ml
8	Vitamin B1 (see below)	1.0ml
9	Vitamin B12 (see below)	1.0ml

One (1) litre of the medium was made up by adding the stock solutions listed in Table 2.2 to distilled water and then making the volume to 1 litre. For solid medium (agar plates) 15g/1000ml bacterial agar No 1 was also added. In both cases the medium was autoclaved for 15 minutes at 103.42kPa.

To prepare the trace element solution (7) mentioned in Table 2.2, 0.075 g Na_2EDTA was added to 100ml of distilled water and the following minerals were added in the exact sequence shown in Table 2.3 below:

Table 2.3: Addition Sequence Table for Trace Elements.

	Trace elements	g/100 ml water
1	$FeCl_3.6H_2O$	0.0097
2	$MnCl_2.4H_2O$	0.0041
3	$ZnCl_2.6H_2O$	0.005
4	$CoCl_2.6H_2O$	0.002
5	$Na_2MoO_4.2H_2O$	0.004

A measured amount of 0.12g of Thiamin hydrochloride was added to 100ml distilled water as Vitamin B1 (solution 8 as shown in Table 2.2 above) and it was filtered sterile as required.

As well, 0.1 g Cyanocobalamin was added to 100 ml distilled water as Vitamin B12 (solution 9 as shown on Table 2.2 above), but only 1ml of this solution was taken and 99ml distilled water was added to make up the 100ml, it was filtered sterile as required.

2.3.2 Modified 3N-BBM+V Medium

One of the advantages of using algae for biofuel production is the ability to control their accumulation and secretion of biofuels by changing their growth conditions or by metabolic engineering. Therefore different modifications were made to the medium in such a way that the quantity of the chemicals added to the media such as nitrogen source ($NaNO_3$) and NaCl were adjusted to grow the algal samples under stress conditions to determine the best way to increase the neutral lipid accumulation. Some of the conditions considered below include high salinity and nitrogen starvation.

2.3.2.1 High Salinity 3N-BBM+V Medium

The 3N-BBM+V medium was modified to higher salinities by adding NaCl solution, 0.1M and 0.2M NaCl solutions were prepared by adding 5.84g and 11.7g of NaCl to 1 litre of 3N-BBM+V media, respectively, to grow *B. braunii* and *Coccomyxa* in higher salinities. The amount of NaCl present in normal BBM medium (solution 6 in Table 2.2) can safely be ignored since its concentration is only 0.43 mM.

2.3.2.2 3N-BBM+V Medium under Nitrogen Starvation

The 3N-BBM+V medium was also made to contain 50%, 25% and 0% concentrations of nitrogen by adding different concentrations of $NaNO_3$ stock solution (i.e. 1.25g, 0.625g and 0g of $NaNO_3$ in 100ml distilled water) to the 3N-BBM+V medium to provide for different nitrogen starvation stress in the media to determine the best way to increase the neutral lipid accumulation for *B. braunii* and *Coccomyxa*.

2.3.3 F/2 Medium for *Tetraselmis suecica*

F/2 Medium was prepared as prescribed in Guillard and Ryther (1962). It was prepared using the composition of stock and medium as shown in Tables 2.4 below.

Table 2.4: Stocks and Medium Composition For F/2 Medium.

	(1) Trace elements (chelated)	per litre
Stocks	$Na_2EDTA.2H_2O$	4.16g
	$FeCl_3.6H_2O$	3.15g
	$CuSO_4.5H_2O$	0.01g
	$ZnSO_4.7H_2O$	0.022g
	$CoCl_2.6H_2O$	0.01g
	$MnCl_2.4H_2O$	0.18g
	$Na_2MoO_4.2H_2O$	0.006g
	(2) Vitamin mix	
	Cyanocobalamin (vitamin B12)	0.0005g
	Thiamine HCl (vitamin B1)	0.1g
	Biotin (vitamin H)	0.0005g
Medium		
	$NaNO_3$	0.075g
	$NaH_2PO_4.H_2O$	0.00565g
	Trace elements stock solution (1)	1.0ml
	Vitamin mix stock solution (2)	1.0ml

One (1) litre of the medium was made up by adding the stock solutions listed in Table 2.4 to artificial seawater prepared using Ultramarine Synthetica (33.6 g l^{-1}). One M NaOH or HCl was used to adjust the pH to 8.0; and the whole medium was autoclaved at 103.42 kPa for 15 minutes to keep it sterilized.

2.3.4 Modification of F/2 medium

As earlier stated, the advantages of using algae for biofuel production is being able to take control of their biofuel accumulation and secretion by varying their growing conditions. Modifications were made in such a way that the quantity of the chemicals added to the media such as $NaNO_3$, NaH_2PO_4, and NaCl was adjusted. In addition, the F/2 medium which was prepared by utilizing the Ultramarine Synthetica salt mixture precipitates after autoclaving, hence it is unsuitable for use at higher salinities than seawater. Therefore, a fully defined F/2 medium was most suitable for modification with stress conditions to grow *T. suecica* in order to determine an optimum condition to increase the neutral lipid accumulation.

2.3.4.1 High Salinity Defined F/2 Medium

Table 2.5 below shows the composition of the defined F/2 medium including key notes on the preparation procedures.

Table 2.5: Composition of defined F/2 medium stock solutions.

1	Artificial Seawater (Hydrated Salts)			
	Salts	Quantity (g)	Hydrated form	Quantity (g)
	$MgSO_4$	2.92	$MgSO_4.7H_2O$	5.98
	$MgCl_2$	4.66	$MgCl_2.6H_2O$	9.95
	$CaCl_2$	1.17	$CaCl_2.2 H_2O$	

				1.55
	SrCl$_2$	0.02	SrCl$_2$ .6H$_2$O	0.034

2	Articial Seawater (Anhydrous Salts)	
	Salts	Quantity (g)
	NaCl	23.4
	KCl	0.62
	NaHCO$_3$	0.18
	KBr	0.024
	H$_2$BO$_3$	0.020
	Na$_2$S$_2$O$_3$	0.004

3	Nitrate	
	NaNO$_3$	7.5

4	Phosphate	
	NaH$_2$PO$_4$.2H$_2$O	0.5

5	Trace Elements:
	The same composition of trace elements as in F/2 medium (Table 2.4 above)
6	Vitamin Mix:
	The same composition of Vitamin Mix as in F/2 medium (Table 2.4 above)
7	Tris Buffer:
	1M Tris buffer pH 7.8

To prepare the defined F/2 medium, the hydrated salts (1) as shown in Table 2.5 above were added in sequential order to 400ml of distilled water while making sure each dissolved completely before adding the next salt. The volume was made up to 500ml using distilled water (stock solution 1) and the solution was autoclaved to keep it sterilized. A solution of the anhydrous salts (2) was also prepared by adding the anhydrous salts in sequential order to 400ml of distilled water while making sure each of these salts dissolved completely before adding the next salt. The volume was made up to 470ml with distilled water (stock solution 2) and it was autoclaved to keep it sterilized. Additionally, 7.5g of Sodium Nitrate ($NaNO_3$) were dissolved in a 100ml of distilled water to prepare a nitrate stock solution (3) as shown on Table 2.5. A stock solution of Sodium Dihydrogen Phosphate ($NaH_2PO_4.2H_2O$) was also prepared (4) by dissolving 0.5g in 100ml of distilled water (phosphate stock). The composition of trace elements is the same as in F/2 medium (Table 2.4 above), likewise the composition of Vitamin Mix is the same as in F/2 medium (Table 2.4 above). One (1) M Tris buffer was prepared with a pH of 7.8.

 One (1) litre of the defined F/2 medium was prepared by aseptically adding stock solution 1 and stock solution 2, then filter sterilization was used to add 10ml of nitrate

stock to have a 100% nitrogen in the medium followed by 10ml of the already prepared phosphate stock to the resulting solution, 1ml of trace elements and 1ml of vitamin mix were also added by filter sterilization to the medium to provide for the required trace elements and vitamin mix in the medium. Lastly, filter sterilization was also used to add 10ml of 1M Tris earlier prepared. The resulting medium was mixed thoroughly, and a small amount was taken to check the pH which was recorded on the bottle.

2.3.4.2 High salinity F/2 defined medium at different salinities

The F/2 defined medium as prepared above was modified to higher salinity by adding NaCl. Salinities of 0.4M, 0.6M, 0.8M, 1.0M and 1.2M NaCl solution were prepared by adding 23.4g, 35.1g, 46.8g, 58.5g and 70.2g of NaCl to 1 litre of F/2 defined medium respectively to grow *T. suecica* in higher salinity stress to determine the best way to increase the neutral lipid accumulation in *T. suecica.*

2.3.4.3 F/2 defined medium under Nitrogen Starvation

The F/2 defined medium was also made to contain 50%, 25%; and 0% concentrations of nitrogen by adding different dilutions of $NaNO_3$ (i.e. 0.0375g, 0.01875g and 0g of $NaNO_3$ respectively in 1000ml distilled water) to the F/2 defined medium. This provides different concentrations of nitrogen in the medium to determine the optimum condition to increase the neutral lipid accumulation in *T. suecica.*

2.4 Growth of algal strains

2.4.1 *Botryococcus braunii* culture methods

The alga (*B. braunii)* were grown as unialgal cultures in sterilized 3N-BBM+V medium as recommended by the Culture Collection of Algae and Protozoa (CCAP), with continuous irradiance (50 – 70 µmol m^{-2} s^{-1}) at 25°C in shaken (80 rpm) flasks (typically 100 ml medium in 250 ml flask). The medium was renewed every 3 to 4 weeks to reach sufficient biomass to carry out experiments. Flasks *of B.* braunii were also cultured under higher salinities and nitrogen starvation stress media as prepared in sections 2.3.2.1 and 2.3.2.2.

2.4.2 *Botryococcus braunii* Fermenter Growth

Botryococcus braunii microalgae were also cultured in a two litre fermenter for two weeks. The pre-cultured microalgae cells were cultivated in a 250 ml flask for one week before being transferred to the fermenter. The algal cells were cultivated at 25°C using 3N-BBM+V medium. The fermenter was placed on a magnetic stirrer to prevent microalgal cells from accumulating at the bottom of the fermenter as shown in Figure 2.1 below. Cultures were gassed with air through a single nozzle, and illuminated with continuous irradiance (50 – 70 µmol m^{-2} s^{-1}). Samples were taken after one week and again after two weeks to measure the biomass as dry weight. The dry weight of algal cells was measured by filtering a known amount of algal cell suspension through preweighed fiberglass filters (Whatman GF/A), and drying overnight at 110°C.

Figure 2.1: Growing Botryococcus braunii in a 2 litre Fermenter.

2.4.3 *Tetraselmis seucica* **culture methods**

Tetraselmis suecica (CCAP 66/4) was obtained from the Culture Collection of Algae and Protozoa, Oban, UK (Butcher 1959). A primary stock culture (in a 100ml flask) was prepared using the liquid *T. suecica* samples received from the CCAP and allowed to grow in the culture room at $25 \pm 1°C$ with continuous light ($50 - 70$ µmol m^{-2} s^{-1}) supplied by daylight fluorescent lights. The culture was allowed to reach the stationary phase after around 2 weeks of incubation, F/2 defined medium was used to cultivate it and optimize growth by monitoring any improvement in growth using growth curves.

2.4.4 *Coccomyxa* **Culture methods**

Coccomyxa, which developed in a 250 ml flask culture and took over from *B. braunii,* was also grown as unialgal cultures in sterilized 3N-BBM+V medium as recommended by the Culture Collection of Algae and Protozoa (CCAP), with continuous irradiance ($50 - 70$ µmol m^{-2} s^{-1}) at $25°C$ in shaken (80 rpm) flasks (typically 100 ml medium in a 250 ml flask). The medium was renewed every 3 to 4 weeks to reach sufficient biomass to carry out experiments. These experiments were also repeated at higher salinities and under nitrogen starvation stress using media as prepared in sections 2.3.2.1 and 2.3.2.2.

2.5 Molecular Identification of the Algal Strains

Coccomyxa strain studied was identified using 18S rDNA primers. The procedure used to identify the strain included the extraction of the genomic DNA using ZR Soil Microbe DNA Kit, gel electrophoresis to detect the presence of genomic DNA and PCR amplification, and sequencing of the PCR products.

2.5.1 Extraction of DNA

The genomic DNA extraction of the samples studied was performed using ZR Soil Microbe DNA Microprep Kit in accordance with the stated procedure as required.

2.5.1.1 DNA Extraction using ZR Soil Microbe DNA Kit

ZR Soil Microbe DNA Microprep Kit was used for the DNA extraction but in this case, Benchmark tubes which fit the Bead Bug machine were used instead of the kit tubes (Bashingbead^{TM}), the procedure was in accordance with the manufacturer's protocols as follows:

- 20ml of each sample was centrifuged for 10 minutes at 3000g and the supernatant discarded.
- 750µl of Lysis solution was then added to the pellet and transferred into a Benchmark Prefilled tubes (0.1,0.5 or 1mm zirconium beads).
- The tubes were then bead beaten in the Bead bug at 3,000g for 2 minutes as it gives the best breakage without damaging the DNA.
- The benchmark Bead bug tubes were then centrifuged for 1 minute at 10,000 g.
- Approximately 400µl of the supernatant was transferred to a Zymo-spin^{TM} IV filter in a collection tube and centrifuged at 7000g for 1 minute.
- 1,200µl of Soil DNA binding buffer was added to the filtrate in the collection tube from the last step.
- 800µl of the mixture from the last step was transferred to a Zymo–spin^{TM} IC Column in a collection tube and centrifuged at 10,000g for 1 minute.
- The flow through was discarded from the collection tube and the last step was repeated.

- 200µl of DNA Pre-wash Buffer was added to the Zymo–spin™ IC Column in a new collection tube and centrifuged at 10,000g for 1 minute.

- 500 µl of Soil DNA Wash Buffer was added to the Zymo–spin™ IC Column and centrifuged at 10,000g for 1 min.

- The Zymo–spin™ IC Column was transferred to a clean 1.5ml microcentrifuge tube and 50µl of DNA Elution Buffer was added directly to the column matrix. To elute the DNA, the column matrix was centrifuged at 10,000g for 30 seconds.

Figure 2.2: Beadbug Microtube homogenizer for disruption of algal cells.
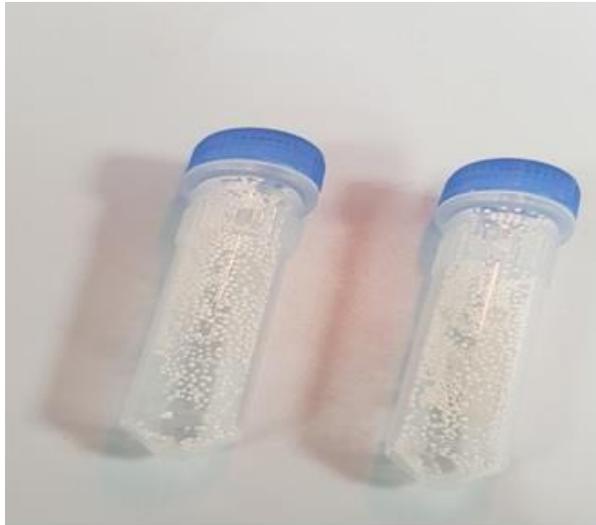
Benchmark prefilled tubes with zirconium beads before adding algal samples

Tubes with algal samples after being bead beaten

Figure 2.3: Benchmark Beadbug Prefilled tubes before and after bead beaten.

2.5.2 Gel Electrophoresis

The existence of genomic DNA in the extracted algal samples considered in this study was explored by preparing a 1% agarose gel as follows. 0.6g agarose powder was added to 60ml of dH_2O and 1.2ml of 50x TAE buffer using a specially designed flask containing a bar for stirring. The agarose gel was dissolved by heating the gel mixture in a microwave oven for 90 seconds at maximum power making sure it all dissolves by reheating if necessary for 30 seconds. While cooling, it was placed on a magnetic stirrer and stirred for a minute or two, 6 μl of Red Gel™ was added while stirring continually. Initially, the gel tank was emptied from any running buffer down the sink, the sink was also rinsed, and end plates and comb were put in place. The liquid gel material was poured into the tank between the end plates and allowed to cool for 15-20 minutes. Now, the comb and end plates were removed, and the tank was filled with 1X TAE running buffer (~20ml 50X TAE made up to 1litre dH_2O in the TAE measuring cylinder) and subsequently covered. Ten (10)μl of the DNA sample was mixed with 2μl of 6x DNA loading dye and loaded onto the agarose gel. Also, 6 μl of 1 kb DNA ladder was

loaded onto the agarose gel and the electrophoresis was run at 80V for 45 minutes. The bands were visualized using a UV lamp (Uvidoc instrument across the corridor) and photographs taken to capture the images.
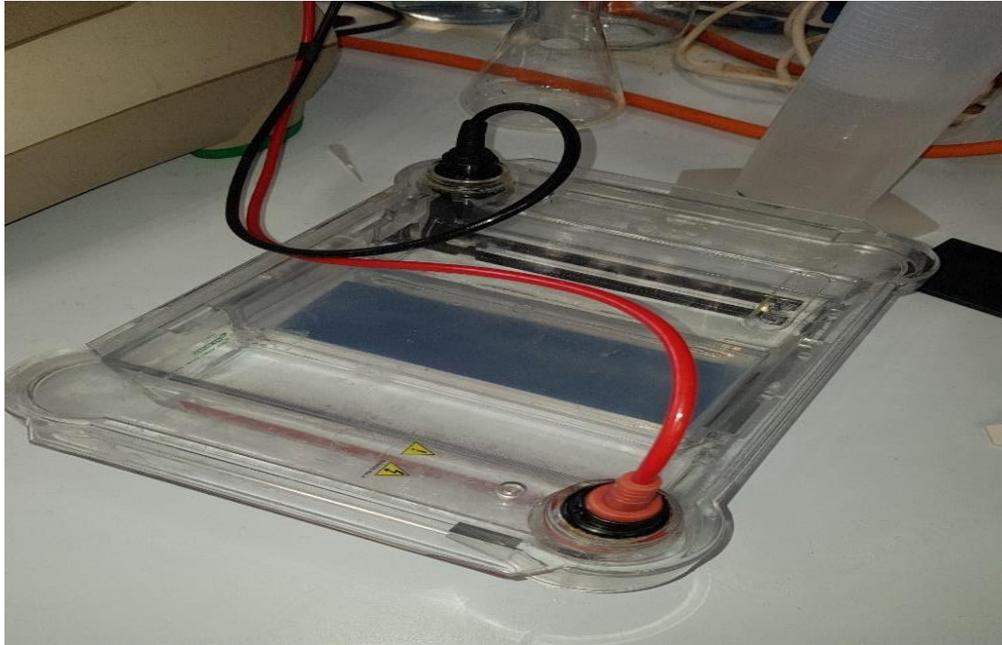
Figure 2.4: Gel Tank for Electrophoresis.

2.5.3 Polymerase Chain Reaction Amplification

The Polymerase Chain Reaction (PCR) amplifications were carried out for the identification and confirmation of the standard algal strain samples under study using 18S primers in a MyCycler thermal cycler (Bio-Rad) (Figure 2.5). The sequences of universal 18S primers are shown in Table 2.6 and the PCR mixtures are shown in Table 2.7. The conditions for the PCR reaction which was a pre-set program in the MyCycler memory as shown in Table 2.8.

Figure 2.5: MyCycler thermal cycler (Bio-Rad).

Table 2.6: Sequence of Universal 18S rRNA Primers.

Primer	Sequence
18S rRNA For Lim	5'-gcg gta att cca gct cca ata gc-3'
18S rRNA Rev Lim	5'-gac cat act ccc ccc gca acc -3'
18S For Sheehan	5'-aat tgg ttg atc ctg cca gc-3'
18S Rev Sheehan	5'-tga ttc tgt gca ggt tca cc-3'

Table 2.7: Contents of Tubes for PCR Amplification.

Sample	18S Lim	Control
Master Mix	20	20
For Primer	4	4
Rev Primer	4	4
Distilled water	17	23
Genomic DNA	5	0
	Total volume is 50 µl for all samples	

Table 2.8: PCR Cycling using 18S rRNA primers.

Initial Denature	94 °C	5 min
Denature	95 °C	30 sec
Anneal	58 °C	30 sec 30 cycles
Elongation	72 °C	1 min
Final Elongation	72 °C	10 min

The PCR products were detected using 1% agarose gel (as described in section 2.5.3), and the positive results were purified using a KeyPrep PCR clean-up kit.

2.5.4 PCR Purification

The PCR products were purified using QIAquick PCR Purification kit (Qiagen) as follows:

- The volume of the samples was determined and adjusted to 100 µl with sterile distilled water. Five volumes of buffer PCR were added, and the samples were mixed thoroughly by vortexing or inverting several times.
- The samples were transferred to a column (max. 1ml) assembled in a clean collection tube, centrifuged at 10,000g for 1 minute. The flow through was discarded.
- The columns were washed with 750µl Wash Buffer and centrifuged at 10,000g for 1 minute and the flow through was discarded.
- The columns were centrifuged at 10,000g for 1 minute to remove residual ethanol.
- The columns were placed into clean microcentrifuge tube. About 80µl 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 10,000g for 1 minute.
- The eluted DNA was confirmed by electrophoresis of the purified DNA and the rest of DNA was stored at 4°C.

2.5.5 DNA Sequencing

The DNA samples were sent out for sequencing to Eurofins/MWG using a sample submission guide for sequences between 300 – 1000bp. The Eurofins requirements were for 5ng/µl in a total volume of 15µl of the purified plasmid DNA and primer concentration of 2pmol/µl in a minimum volume of 15µl. The sequences obtained were

then compared against sequences in the GenBank nucleotide collection using the Basic Local Alignment Search Tool (BLAST).

2.6 Extraction and Determination of Neutral Lipids in Algal Cells

2.6.1 Gravimetric Measurement of Lipid content for Algal Strains

To measure the total lipid content of the algal strains used for this study, a technique known as gravimetric measurement (adapted from Chiu et al., 2009) was used. Four 20ml samples of each well grown culture were centrifuged for 10 minutes at $3000g$ in a bench centrifuge, the centrifuge is shown in Figure 2.6 below. Each pellet was re-suspended in 5ml of distilled water and transferred to 15ml Falcon tubes. The tubes were centrifuged again for 5 minutes at $3000g$, supernatant was discarded, and each pellet was re-suspended in 1ml of distilled water this time. Four Eppendorf tubes were labelled appropriately and weighed on the fine balance, the 1ml samples of each strain were transferred to the four labelled pre-weighed Eppendorf tubes. The lids of another set of four Eppendorf tubes were cut off and holes were made by dissecting needle, and the lids were then placed on each of the four Eppendorf tubes containing the samples. The four samples for each of the strains were frozen at $- 80°C$ overnight and then freeze dried (lyophilized) for 48 hours. The Eppendorf tubes were then re-weighed to estimate the weight of biomass. 500µl of methanol/chloroform (2:1 v/v) was added to each of tubes and then transferred to tubes containing 1mm glass beads and placed on the bead-beater (Figure 2.2) for 3 minutes at 3000 g. The tubes were then centrifuged in the microfuge at full speed for 5 minutes and transferred to fresh Eppendorf tubes, and the supernatant was 2:1 methanol:chloroform, but 1% NaCl and chloroform were then added to give 2:2:1 methanol:chloroform:water as required. The samples were centrifuged again for 2 minutes at full speed in the microfuge. The chloroform phase was then transferred to fresh pre-weighed Eppendorf tubes, the tubes were left in the Fume Cupboard with their lids open to evaporate overnight, and these tubes were re-weighed to obtain the weight of lipids recovered by calculation. This technique was carried out for each of the well grown cultures of the algal strains used for this study.
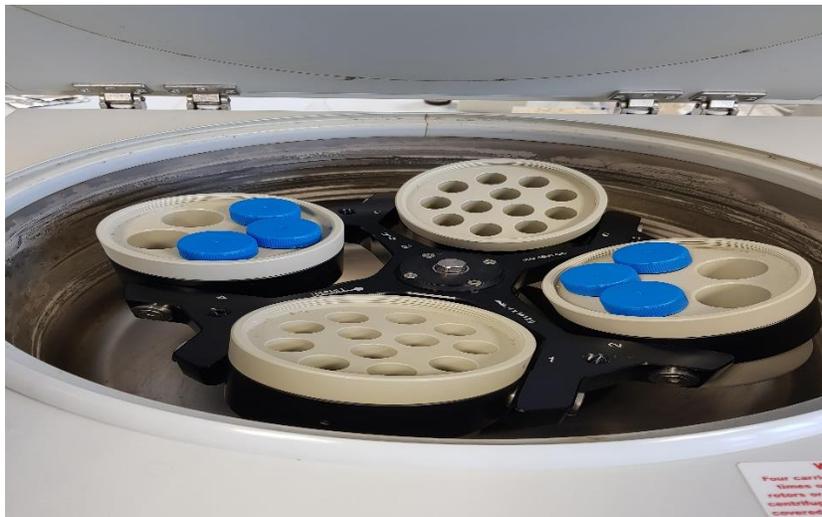
Figure 2.6: Bench Centrifuge (temperature controlled).

2.6.2 Quantification of Neutral Lipid using Nile Red

The method of determining neutral lipids in the algal strains in this study was adapted from Bertozzini et al. (2011). Experiments were carried out to determine optimum concentration of algal cells, peak time and optimum NR dye concentration to plot a Triolein standard curve.

2.6.2.1 Determining optimum cell concentration and peak time for 96 well plate Nile-red method

The algal culture was adjusted to an Optical Density of 1.0 at 595 nm against a dH_2O blank in a volume of 5ml. Eight dilutions of algal cultures were prepared in 1 ml from the adjusted algal culture using dH_2O, at 100, 87.5, 75, 62.5, 50, 37.5, 25 and 12.5% in 1.5 ml Eppendorf tubes as shown in Table 2.9 below.

Table 2.9: Dilution scheme of algal culture concentrations for Nile-Red fluorescence test method.

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
dH$_2$O (µl)	0	125	250	375	500	625	750	875	3.5

The 8 Eppendorf tubes were then centrifuged at 5000g for 10 minutes in a bench top centrifuge, the supernatant was discarded; and the pellets resuspended in 20 µl of dH$_2$O. Two x 2 ml screw top microfuge tubes were prepared for each of the 8 culture concentrations, one for stained and the other for the unstained cells. Ten (10)µl of the resuspended algal pellet was then transferred to each of the stained and unstained labelled tubes. 50µl of dimethyl sulfoxide (DMSO) was then added to each screw capped microfuge tube. All the screw capped microfuge tubes were heated for 50 seconds at full power in a Matsui microwave. The tubes were then removed from the microwave, and 930µl dH$_2$O was added to tubes labelled stained and 940µl of dH$_2$O to the remaining unstained labelled tubes. 10µl of 100µg ml^{-1} NR fluorescent dye dissolved in DMSO was then added to each tube, labelled, and the stop watch was started. All the tubes labelled stained were microwaved again at full power for 60 seconds. The entire 1ml content of each stained tube was then transferred to a multi-pipette reservoir. 200µl aliquots of each cellular concentration were then transferred to rows A to D in a 96 well plate which now provided four technical replicates at each concentration. The same step was also done for the unstained tubes but in this case, rows E to H were used as shown in Table 2.10 (96 well plate layout for optimum concentration of algal cells, R1-R4 are technical replicates from the same concentration). The lid was removed from the plate and the plate was placed in a 96 well plate reader (Biotek flx800).

Table 2.10: 96 well plate layout for determining optimum concentration of algal cells, R1-R4 are technical replicates from the same concentration.

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	staine1	staine5	staine9	staine13	staine17	staine21	staine25	staine29	BLK	BLK	BLK	BLK
B	R2	staine2	staine6	staine10	staine14	staine18	staine22	staine26	staine30	BLK	BLK	BLK	BLK
C	R3	staine3	staine7	staine11	staine15	staine19	staine23	staine27	staine31	BLK	BLK	BLK	BLK
D	R4	staine4	staine8	staine12	staine16	staine20	staine24	staine28	staine32	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	unsta24	unsta28	unsta32	BLK	BLK	BLK	BLK

In order to track the fluorescence, Gen5 2.05 software was used at 5 minute intervals over a 30 minute period. The settings on the plate reader used are shown in Table 2.11 below. The results of the plate readings were exported to an Excel spreadsheet. An average fluorescence value for the four technical replicates (stained and unstained) for each concentration was calculated; and the standard deviation of each average calculated was obtained. The unstained values were subtracted from the stained fluorescence values in order to remove any cellular background fluorescence. Fluorescence values were normalized to select the highest values with lowest standard deviation for both the stained and unstained values allowing for optimum cell concentration, thus optical density with the best (peak) time of running.

Table 2.11: Plate reader setting for algal cells optimum concentration.

Procedure details	
Plate Type	96 well plate
Read	Fluorescence Endpoint Full plate Filter set 1 Excitation: 485/20, Emission: 580/50 Optics: Top, Gain: 60 Read speed: Normal

2.6.2.2 Determining optimum concentration of Nile-Red

Nile-Red stocks were prepared in serial concentrations (0.05, 0.1, 0.2, 0.3, 0.4 and 0.6 µmol ml^{-1}) by dissolving in DMSO 1mg ml^{-1} stock of NR as shown in Table 2.12 below.

Table 2.12: Nile-Red concentrations prepared from 1mg/ml NR Stock.

Nile-Red (µg ml^{-1})	Primary Stock from 1 mg ml^{-1} (µl)	DMSO (µl)
16	16	984
32	32	968
64	64	936
100	100	900
128	128	872
191	192	808

Six Eppendorf tubes were prepared by adding 1ml of each culture with the optimum optical density obtained from the previous section (section 2.6.2.1 above) at 595 nm. These tubes were then centrifuged at 5000g for 10 minutes in a bench top centrifuge, the supernatant was discarded; and the pellets resuspended in 20 μl of dH$_2$O. Two x 2 ml screw top microfuge tubes were prepared for each of the 6 algal culture concentrations, one for stained and the other for the unstained cells. Ten (10)μl of the resuspended algal pellet was then transferred to each of the stained and unstained, labelled tubes. 50μl of dimethyl sulfoxide (DMSO) was then added to each screw capped microfuge tube. All the screw capped microfuge tubes were heated for 50 seconds at full power in a Matsui microwave. The tubes were then removed from the microwave, and 930 μl dH$_2$O was added to tubes labelled stained and 940 μl of dH$_2$O to the remaining unstained, labelled tubes. Ten (10) μl of each primary stock of Nile-red concentration (as shown in Table 2.12) was then added to each tube labelled, stained and the stopwatch was started. The tubes labelled and stained were microwaved again at full power for 60 seconds. The whole 1 ml content of each stained tube was transferred to a multi-pipette reservoir. Aliquots (200μl) of each cellular concentration were then transferred to rows A to D in a 96 well plate which now provided four technical replicates at each concentration. The same step was carried out for the unstained tubes but in this case, rows E to H were used as shown in Table 2.13 96 well plate layout for optimum NR concentration with optimum concentration of algal cells, (R1-R4 are technical replicates from the same concentration). The lid was removed from the plate and the plate was placed in a 96 well plate reader (Biotek flx800).

Table 2.13: 96 well plate layout for optimum Nile Red concentration with optimum concentration of algal cells, R1-R4 are technical replicates from the same concentration).

NR Conc. (µmo/ml)		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
A	R1	staine1	staine5	staine9	staine13	staine17	staine21	BLK	BLK	BLK	BLK	BLK	BLK
B	R2	staine2	staine6	staine10	staine14	staine18	staine22	BLK	BLK	BLK	BLK	BLK	BLK
C	R3	staine3	staine7	staine11	staine15	staine19	staine23	BLK	BLK	BLK	BLK	BLK	BLK
D	R4	staine4	staine8	staine12	staine16	staine20	staine24	BLK	BLK	BLK	BLK	BLK	BLK
E	R1	unsta1	unsta5	unsta9	unsta13	unsta17	unsta21	BLK	BLK	BLK	BLK	BLK	BLK
F	R2	unsta2	unsta6	unsta10	unsta14	unsta18	unsta22	BLK	BLK	BLK	BLK	BLK	BLK
G	R3	unsta3	unsta7	unsta11	unsta15	unsta19	unsta23	BLK	BLK	BLK	BLK	BLK	BLK
H	R4	unsta4	unsta8	unsta12	unsta16	unsta20	unsta24	BLK	BLK	BLK	BLK	BLK	BLK

In order to track the fluorescence, Gen5 2.05 software was used at 5 minute intervals over a 30 minute period. The results of the plate readings were exported to an Excel spreadsheet. An average fluorescence value for the four technical replicates (stained and unstained) for each concentration was calculated. The unstained values were subtracted from the stained fluorescence values to obtain the highest value which is the value for optimum NR concentration selected. The settings on the plate reader used are shown in Table 2.11 as stated earlier.

2.6.2.3 Determination of Neutral Lipids in Botryococcus braunii, Coccomyxa and Tetraselmis suecica

The neutral lipids in well grown cells of *B. braunii*, *Coccomyxa* and *T. suecica* were determined under normal and various stress conditions (i.e. Nitrogen starvation and

high salinity). This occurred on a weekly basis for four weeks as described in section 2.6.2.1 after optimizing NR and cell concentrations.

2.6.2.4 Triolein Calibration Curve for Botryococcus braunii, Coccomyxa and Tetraselmis suecica

To allow for the quantification of neutral lipid within the algal cells under study, a concentration standard curve for NR fluorescence versus concentration of Triolein was set up to measure the NR fluorescence due to Triolein in the presence of algal cells. This is the standard addition method used by Bertozzini et al. (2011). It was set up following the procedures stated below.

50mg of Triolein was completely dissolved in 5ml isopropanol to prepare a 10mg ml^{-1} Triolein lipid standard stock. Eight Eppendorf tubes were prepared by adding 1ml of each culture with the optimum optical density obtained from section 2.6.2.1 at 595 nm. These tubes were then centrifuged at 5000g for 10 minutes in a bench top centrifuge, the supernatant was discarded; and the pellets resuspended in 20 µl of dH$_2$O. Two x 2ml screw top microfuge tubes were prepared for each of the eight Triolein concentrations, one for stained and the other for the unstained cells. Ten (10)µl of the resuspended algal pellet was then transferred to each of the stained and unstained, labelled tubes. 50µl of dimethyl sulfoxide (DMSO) was then added to each screw capped microfuge tube. All the screw capped microfuge tubes were heated for 50 seconds at full power in a Matsui microwave. The tubes were then removed from the microwave, and 910 µl dH$_2$O was added to tubes labelled stained and 920 µl of dH$_2$O to the remaining unstained labelled tubes. Triolein and isopropanol mixed in different concentrations, with a total volume of 20 µl, were added to each stained and unstained tube to provide different concentrations of Triolein as shown in Table 2.14 below. Ten (10)µl of the optimum concentration of NR obtained from section 2.6.2.2 was then added to each tube labelled stained. All the tubes both stained and unstained were microwaved at full power for 60 seconds, and the whole 1 ml content of each stained and unstained tube was transferred to a multi-pipette reservoir.

Table 2.14: Triolein mixture concentrations for Nile-Red fluorescence standard curve.

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

Aliquots (200µl) of each cellular concentration (both stained and unstained) were then transferred to rows A to D for stained and rows E to H were used for the unstained in a 96 well plate which now provided four technical replicates at each concentration for both stained and unstained as shown in Table 2.15 below. The lid was removed from the plate and the plate was placed in a 96 well plate reader (Biotek flx800).

Table 2.15: 96 well plate layout for different concentrations of Triolein mixture for standardization curve, (R1-R4 are technical replicates from the same concentration).

Triolein Conc. (mg/ml)		0.2	0.16	0.12	0.08	0.06	0.04	0.02	0	Empty Wells			
		1	2	3	4	5	6	7	8	9	10	11	12
A	R1	staine1	staine5	staine9	staine13	staine17	staine21	staine25	staine29	BLK	BLK	BLK	BLK
B	R2	staine2	staine6	staine10	staine14	staine18	staine22	staine26	staine30	BLK	BLK	BLK	BLK
C	R3	staine3	staine7	staine11	staine15	staine19	staine23	staine27	staine31	BLK	BLK	BLK	BLK
D	R4	staine4	staine8	staine12	staine16	staine20	staine24	staine28	staine32	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	unsta24	unsta28	unsta32	BLK	BLK	BLK	BLK

In order to track the fluorescence, Gen5 2.05 software was used at 5 minute intervals over a 30 minute period using the same procedure and setting earlier stated in Table 2.11. The results of the plate readings were exported to an Excel spreadsheet. The unstained values were subtracted from the stained fluorescence values and average fluorescence values for the four technical replicates (stained and unstained) for each concentration were calculated in order to plot a graph of Triolein concentration on X-axis and the NR fluorescence intensity in Y-axis to illustrate the Triolein Calibration standard curve for each of the well grown algal strains under this study.

Therefore, each fluorescence intensity value could be converted directly to concentration of lipid based on the following equations derived from the Excel spreadsheet for each of the well grown algal strains under study (*B. braunii, Coccomyxa* and *T. suecica)* in both normal and stress conditions (higher salinities and nitrogen starvation).

For *Botryococcus braunii,* X = Y/69482

For *Coccomyxa,* X = Y/37880

For *Tetraselmis seucica,* X = Y/46231

Where Y = Nile Red Fluorescence Intensity and X = concentration of Triolein (mg/ml).

Therefore, equation (1) below can be used to calculate the neutral lipids content

Neutral lipid (mg/ml) = $\dfrac{\text{Average (Stain-Unstained)}}{\text{Slope of Triolein Standard Curve}}$ ……………..………(1)

2.7 Dry Weight versus Optical Density Relationship

The relationship between the algal cells dry weight and OD was measured based on a study conducted by Storms *et al.* (2014) with a little modification made by preparing a set of well grown cultures of the algal strains under study (OD_{595} = 1). A final volume of 30ml was used for each dilution in a 50ml Falcon tube using fresh medium for each of the algal strains in their corresponding medium as shown in Table 2.16 below. One (1) ml of each dilution was taken and the Optical Density measured at 595nm, and after measurement, the sample was returned to the tube.

Table 2.16: Concentrations of Algal cells for Preparation of Dry Weight versus Optical Density.

Total number	Conc. (%)	Culture(ml)	Medium (ml)
12	0.0	0.0	30.0
11	8.3	2.5	27.5
10	16.6	5.0	25.0
9	33.3	10.0	20.0
8	41.6	12.5	17.5
7	50.0	15.0	15.0
6	58.3	17.5	12.5
5	66.6	20.0	10.0
4	75.0	22.5	7.5

3	83.3	25.0	5.0
2	91.6	27.5	2.5
1	100.0	30.0	0.0

The tubes were all centrifuged for 5 minutes at 3000g, the supernatants discarded, and 5 ml dH$_2$O was used to resuspend the pellets before transferring it into 15ml Falcon tubes. These tubes were then centrifuged, the supernatants discarded again, and 1 ml dH$_2$O was used to resuspend the pellets. The suspended cells were then transferred to pre-weighed Eppendorf tubes, and the tops of the Eppendorf tubes were removed, then holes were made in extra tops to seal the Eppendorf tubes containing the samples. The Eppendorf tubes were then frozen at – 80°C overnight and then freeze dried (lyophilized) for 24 - 48 hours until the samples were completely dried. The tops with the holes were discarded and the Eppendorf tubes were resealed with their own and re-weighed to calculate the dry weight of each sample. This was used to prepare the concentration curve by the used of Excel spreadsheet and the Optical density value with the optimum cell concentration was converted to dry weight in mg/ml based upon the Excel spreadsheet equations for the algal strains as follows:

For *Botryococcus braunii,* Y = 0.1145x

For *Coccomyxa,* Y = 0.1941x

For *Tetraselmis seucica,* Y = 0.3769x

Where Y = Dry weight (mg/ml) and x = OD$_{595}$

Therefore, the dry weight in the optimum concentration can calculated using equation (2) below;

Dry weight biomass in optimum concentration (mg/ml) = Slope of dry weight verses OD

X OD of Optimum

Concentration………………..... (2)

Hence the percentage of neutral lipid could be calculated by dividing the concentration of neutral lipid by the value of the cell dry weight of the algal strain and then multiplying by a 100 using equation (3) below:

Neutral Lipid Content % = $\dfrac{\text{Neutral Lipid Content (mg/ml)} \times 100}{\text{Biomass dry weight in optimum concentration (mg/ml)}}$ ………………… (3)

2.8 Analysis of Compatible Solutes in the Algal Strains using Nuclear Magnetic Resonance

The analysis of compatible solutes from the algal strains utilized in this study was carried out by the use of Nuclear Magnetic Resonance (NMR). The methods and procedures followed were as follows, duplicates of 20 ml samples of well grown samples of algal cells (OD_{595} = 1) were centrifuged for 10 minutes at 3000g using a bench centrifuge and the supernatants were discarded, each of the pellets were re-suspended in 1 ml of distilled water, the tubes were then bead beaten in the Bead bug at 350rpm for 3 minutes using a 0.5mm beating tube as it gives the best conditions for breakage of the cells. It was then transferred to Eppendorf tubes, these tubes were centrifuged for 5 minutes at 3000g, the supernatants were transferred into Eppendorf tubes with two lids freshly prepared by cutting off the lids, making holes in the lids with a needle and putting the lids with a hole on to a complete Eppendorf with its own lid. These samples were frozen at – 80ºC overnight and then transferred to a freeze dryer and frozen without allowing them to thaw, it was left to freeze dry (lyophilize) for 24 – 48 hours until samples were completely dry. The lids with the holes were discarded and the tubes were resealed with their own lid, and the samples were kept on the bench at room temperature until NMR machine was available in the NMR laboratory.

In the NMR Laboratory, the freeze-dried biomass samples were solubilized by adding 500 µl 1:6 of deuterated chloroform ($CDCl_3$): deuterated (CD_3Cl_3) mix, 5 µl of chloroform ($CHCl_3$) was added to serve as an internal standard and then transferred to a 5 mm NMR tube. NMR spectral lines were obtained by utilizing a Bruker Avence 600 which was equipped with a cryoprobe having 16k complex data points on a simple pulse-acquiring programme with a 3 second recycle time operating with 4 dummy background scans prior to the eight scans of each sample used in this study. Fourier transformation was applied to transform the given function by using a 1Hz line that broadens and then followed by the manual baseline correction. Bruker Topspin Version 1.3 software was used for processing and integration of the final spectra.

2.9 Determination of Total Lipids as Fatty Acid Methyl Esters (FAME) through Transesterification and GC-MS

Total algal lipids could be expressed as biofuel relevant fatty acid methyl esters (FAME) and expressed as percent FAME content based on the dry weight of the algal biomass. The method for doing this will be explained in this section.

2.9.1 Apparatus, Reagents and Materials

2.9.1.1 Apparatus

The following set of apparatus were used in order to determine the biofuel relevant fatty acid methyl esters (FAME) in the algal samples under this study include

- Analytical balance with accuracy to 1mg or 0.1mg

- Digital dry block, capable of maintaining 85°C ± 3°C

- Gas chromatograph (GC) equipped with a variable split-flow injector or equivalent device: Agilent 7890A GC system equipped with a split/splitless inlet or equivalent

- Automated sampler compatible with the chosen GC system: Agilent 7693A Automatic Liquid Sampler or equivalent

- Detector compatible with the chosen GC system and type of analysis: Agilent 7890A equipped with a flame ionization detector (FID) or equivalent

- Capillary column with polyethylene glycol stationary phase: Agilent J&W GC Column DB-Wax length 30 m, internal diameter 0.25 mm, film thickness 0.25 μm or equivalent.

2.9.1.2 Reagents

The reagents used in order to determine the biofuel relevant fatty acid methyl esters (FAME) in the algal samples under this study included the following:

- Chloroform, high-performance liquid chromatograph (HPLC) grade (Acros Organics 404635000)

- Methanol, HPLC grade (Fisher Chemical A4525K-1)

- Hexane, HPLC grade (Sigma Aldrich 34859-1L)

- Hydrochloric acid (HCl), concentrated (36.5%–38%) (~12M) (J.T. Baker 9535-03)

- Chloroform: methanol solution (2:1, v/v)

- HCl:methanol solution (0.6M [2.1% v/v] HCl in methanol), prepared by adding 5 mL of concentrated HCl (12M) to 95mL of methanol—may be prepared ahead of time and stored for up to 2 months

- Tridecanoic acid methyl ester (C13:0 ME) standard (Sigma Aldrich #91558- 5ML)

- FAME Standard Calibration Mix C8:0–C24:0 (NuChek-Prep GLC 461C)

2.9.1.3 Materials

- Volumetric flask (class A), 10 mL

- Gas-tight syringes, covering ranges from 5–1000 µL

- Adjustable pipette, covering ranges from 100–300 µL

- Vials, clear, crimp tops, 1.5 ml fill volume (Agilent #5182-0543 or equivalent)

- Vials with inserts, clear, crimp tops, 300 µl fill volume (Agilent #9301- 1388 or 5188-6572 [amber])

- Crimp caps, polytetrafluoroethylene (PTFE)/silicone/PTFE septa (Agilent #5181-1211)

2.9.2 Transesterification of Algal Cells

2.9.2.1 Preparation of Algal Samples

Samples of the algal cells were prepared for direct transesterification together with gas chromatography (GC) *in-situ* as described by Van Wychen and Laurens (2013) and Laurens et al. (2012). Using an acid catalyzed reaction, all the lipids in the algal cells were transesterified to FAME (including phospholipid and galactoglycerolipid), the following procedure was conducted to prepare the algal samples for transesterification.

A permanent marker was used to label the 1.5-ml GC vials for each of the samples to be analyzed and the label was allowed to completely dry before weighing and recording the weights of the vials to an accuracy of 0.1mg. Three replicate cultures of 30 ml each from late stationary, well grown samples in each of the culture media were centrifuged at full speed for 5 minutes, the supernatant discarded while the pellets were resuspended using 1 ml of distilled water for each sample. The cell suspensions were each transferred to a new pre-weighed Eppendorf tube and sealed with a lid from another Eppendorf tube containing a hole, these samples were frozen overnight and then transferred to a freeze dryer and left to freeze dry (lyophilize) until samples were completely dry and 5-10 mg of these samples each were then transferred into the pre - weighed GC vials.

2.9.2.2 Preparation of the Recovery standard

Internal standard was also prepared to make 10 mg/ml by weighing100 mg methyl tridecanoate (C13:0ME) into a 10-mL, class A, volumetric flask; weighing the C13:0ME to an accuracy of 0.1 mg and then adding hexane of HPLC grade to bring to volume and mixing thoroughly. This mixture was transferred and sealed in a 1.5-ml GC vials using PTFE/silicone/PTFE crimp caps. The standard prepared was stored in an upright position at -20°C in the laboratory freezer until samples were ready for running GC-MS.

2.9.2.3 Transesterification of the Samples

In order to enable the transesterification reaction to occur, the hot plate was pre-heated at 85°C. While preheating, the following were added to each of the sample vials: 300 μL

of 0.6M HCl:methanol using a 200 µL pipette and 100 µL digital pipette, 200 µL of chloroform:methanol (2:1, v/v) with the aid of a gas-tight syringe; and 25 µL of the earlier prepared C13:0 ME internal standard (10 mg/mL) with the aid of a gas-tight syringe. The crimps vials were promptly sealed with PTFE/silicone/PFTE crimp caps and mixed thoroughly, the sealed vials were immediately transferred to the pre-heated hot plate at 85°C for 60 minutes to allow the transesterification reaction to occur. Afterwards, the samples were removed from the hot plate and cooled for at least 15 minutes and not longer than 60 minutes at room temperature.

2.9.2.4 FAME Extraction and Preparation for GC Analysis

To extract the FAME from the mixture after the samples have been cooled as required,1 ml of HPLC grade hexane was added to each vial with the aid of a gas tight syringe (1 ml polypropylene syringe, BD Plastipak) and hollow core needles without removing the cap, the vials were vortexed for about 15 seconds to mix well and then allowed to stand for at least 60 minutes and not longer than 4 hours at room temperature in order to allow isolation into phases. The samples were additionally diluted as required depending on estimated FAME concentration to fall within the calibration curve, the diluted samples in the 1.5-ml GC vials were then sealed using PTFE/silicone/PTFE crimp caps and stored in an upright position at -20°C in the laboratory freezer until samples were ready for running on the GC-MS. Also prepared were well labelled new 300-µL insert vials for each level of FAME standard.

2.9.2.5 Preparation and Addition of Internal standard

In order to correct for any instrumental variability and evaporation of solvent while running the FAME analysis, an internal standard was prepared by weighing about 10 mg pentadecane (C15) standard into a 1.5-ml vials and weighing to an accuracy of 0.1 mg then adding hexane of HPLC grade to the vials, diluted to 1:10 and sealed using PTFE/silicone/PTFE crimp caps. To each of the new 300-µL insert vials for each level of FAME standard, 5 µL of the diluted 1:10 pentadecane and 200-µL of diluted FAME extracted samples (prepared in section 2.9.2.4 above) were added.

2.9.2.6 Preparation of FAME standard

A series of FAME standard solutions was prepared from 10 mg/ml C4:0-C24:0 FAME neat mix (Sigma, 18919-1AMP) and HPLC grade hexane by transferring the neat mix to a clean 10 ml class A volumetric flask and using the HPLC grade hexane to bring to volume following the scheme outlined in Table 2.17 below. Furthermore, a calibration verification standard (CVS) was created by mixing 90 µl FAME mix and 910 µl HPLC grade hexane. The standards were transferred into labelled 1.5 ml GC vials and sealed immediately using PTFE/silicone/PTFE crimp caps. They were stored upright at -20°C in a freezer until the samples were ready for running on the GC-MS.

Table 2.17: Standards Prepared using C4:0 - C24:0 working Solution.

Standard Level	C4 - C24 Working Solution	HPLC Grade Hexane
	µl	µl
5	500	500
4	250	750
3	100	900
2	30	970
1	10	990
CVS	90	910

2.9.2.7 Gas Chromatography-Mass Spectrometric Analysis of FAME

Gas Chromatography Mass Spectrometry (GC-MS) was used to analyze the FAMEs of each sample prepared using an AutoSystem XL Gas Chromatograph (CHM-100-790, Perkin Elmer), which was coupled with a TurboMass Mass Spectrometer (13657, Perkin Elmer). The GC had a close-fitting with DB-WAX 30m x 0.25 mm Internal Diameter x

0.25 μm Film Thickness GC capillary column. About 1μl of samples were injected at 10:1 split ratio and a helium constant carrier flow at 1 ml/min at an injection temperature of 250°C via an auto-sampler onto the column. To optimize for the peak separation fatty acid isomers, a temperature tailored programme (FAME_FINAL) was utilized appropriately. The temperature optimized programme was set to 100°C to hold for 1 minute, then ramped to 200°C at 25°C/min and hold for 1 minute, followed by a final ramp up to 250°C at 5°C/min and hold for 7 minutes. Consequently, the mass spectrometer was operated in electron ionization (EI+) mode, and the time for scanning was 90 minutes while the start mass was set to 50 and the end at 600. Turbomass software (Ver 5.2.4, Perkin Elmer) in conjunction with the National Institute of Standard Technology (NIST) spectral database was used to identify the sample peaks with the relative quantification. Therefore, all the responses were calculated for each dilution point of each FAME component of interest based on the FAME standard dilution series and all these responses were calculated by the software utilized. Consequently, base line separations, manual and automatic peak integration were all carried out in the quantification tool of the software.

Chapter 3: *Botryococcus braunii* Strain Identity Confirmation and Lipid Content Determination under normal and stress conditions

3.0 Introduction

There is recent interest in the possibility of utilising microalgae as a source of renewable fuels, in particular to replace fossil diesel, as a result of high yield of oil and the ability to grow rapidly (Christi, 2007). It is also known that *Botryococcus braunii* is a potential source of renewable fuel with regards to its ability to produce a significant number of hydrocarbon compounds (Metzger and Largeau, 2005). Up to 75% of *B. braunii* dry mass can be hydrocarbons, depending on the strain and growth conditions (Banerjee et al., 2002). This alga is categorized by an obvious capability to synthesize a lot of hydrocarbons, i.e. highly reduced compounds comprising only carbon and hydrogen as elements (Brown and Knights, 1969; Knights et al., 1970). Based on this, it seems to be an excellent candidate to be exploited for biodiesel production. In this chapter, *B. braunii* was evaluated by modifying its growth conditions in order to study its neutral lipid production under normal and stress growth conditions.

Botryococcus braunii Kützing (1849) (CCAP 807/1) strain isolated by Droop (1950) was received from the Culture Collection of Algae and Protozoa, Oban, Scotland. It was checked for any bacteriological adulteration and the identity of the strain was confirmed with the aid of a microscope. It was grown as unialgal cultures in sterilised 3N-BBM+V medium as recommended by the Culture Collection of Algae and Protozoa (CCAP), with continuous irradiance ($50 – 70$ µmol m^{-2} s^{-1}) at 25ºC in shaken (80 rpm) flasks (typically 100 ml medium in 250 ml flask). The medium was renewed every 3 to 4 weeks to reach sufficient biomass to perform experiments (i.e. the growth medium was optimized for culture of this *B. braunii* strain).

The total lipid content of *B. braunii* was measured using the gravimetric method while Nile red fluorescence was used to quantify the cellular neutral lipid content of *B. braunii*.

Accurate determination of neutral lipid in *B. braunii* using this method mainly depends on three factors: the best concentration of algal cells, the best concentration of Nile Red to be used and, finally, the best incubation time that showed the highest (peak) fluorescence intensity of Nile Red using a Varian 96-well plate spectrophotometer. Therefore, the optimum concentration of Nile Red dye and optimum concentration of algal cells were determined and a Triolein calibration curve was prepared using a procedure adapted from (Bertozzini et al., 2011), in order to allow for the quantification of neutral lipid within algal cells (a concentration curve for Nile-Red fluorescence versus concentration of Triolein, a common lipid standard used in the food industry).

As stated earlier, one of the advantages of using algae for biofuel production is the ability to control their accumulation and secretion of biofuels by changing their growth conditions or by metabolic engineering. Therefore, different modifications were made to the 3N-BBM+V medium in such a way that the quantity of the chemicals added to the media such as NaCl was adjusted to grow the algal samples under stress conditions to determine the best way to increase the neutral lipid accumulation. The stress condition considered in this study of *B. braunii* was high salinity. Therefore, the algal cells were also cultivated using high salinity 3N-BBM+V medium in order to evaluate the effect of salinity on *B. braunii* growth. However, no promising growth was observed after one-month incubation in higher salinities as is described in section 3.2.1 below. And even under normal conditions, the growth rate was too slow to reach sufficient biomass to accurately measure neutral lipid production. Nevertheless, neutral lipid accumulated by *B. braunii* grown in standard (non-saline) medium was assessed in this chapter.

3.1 Aims and Objectives

The aims and objectives of this chapter are stated below:

1. Confirm the identity of *Botryococcus braunii* strain received from the Culture Collection of Algae and Protozoa.

2. Optimise growth media (3N-BBM+V) to cultivate *Botryococcus braunii* and monitor any improvement in growth.

3. Set up a fermenter to grow *Botryococcus braunii* to be a continuous source of algae for experiments.

4. Quantify the lipid content of *Botryococcus braunii* using dry weight measurements, lyophilisation, a gravimetric method and Nile Red fluorescent dye staining.

3.2 *Botryococcus braunii* Strain Identity Confirmation using Microscope

As soon as the *Botryococcus braunii* Kützing (1849) (CCAP 807/1) strain isolated by Droop (1950), was received from Culture Collection of Algae and Protozoa, Oban, Scotland, the cells were checked for any bacterial contamination and the identity of the strain was checked using a microscope to ascertain the strain. Figure 3.1 below shows the microscopic image of *Botryococcus braunii* Kützing (1849) (CCAP 807/1) strain.
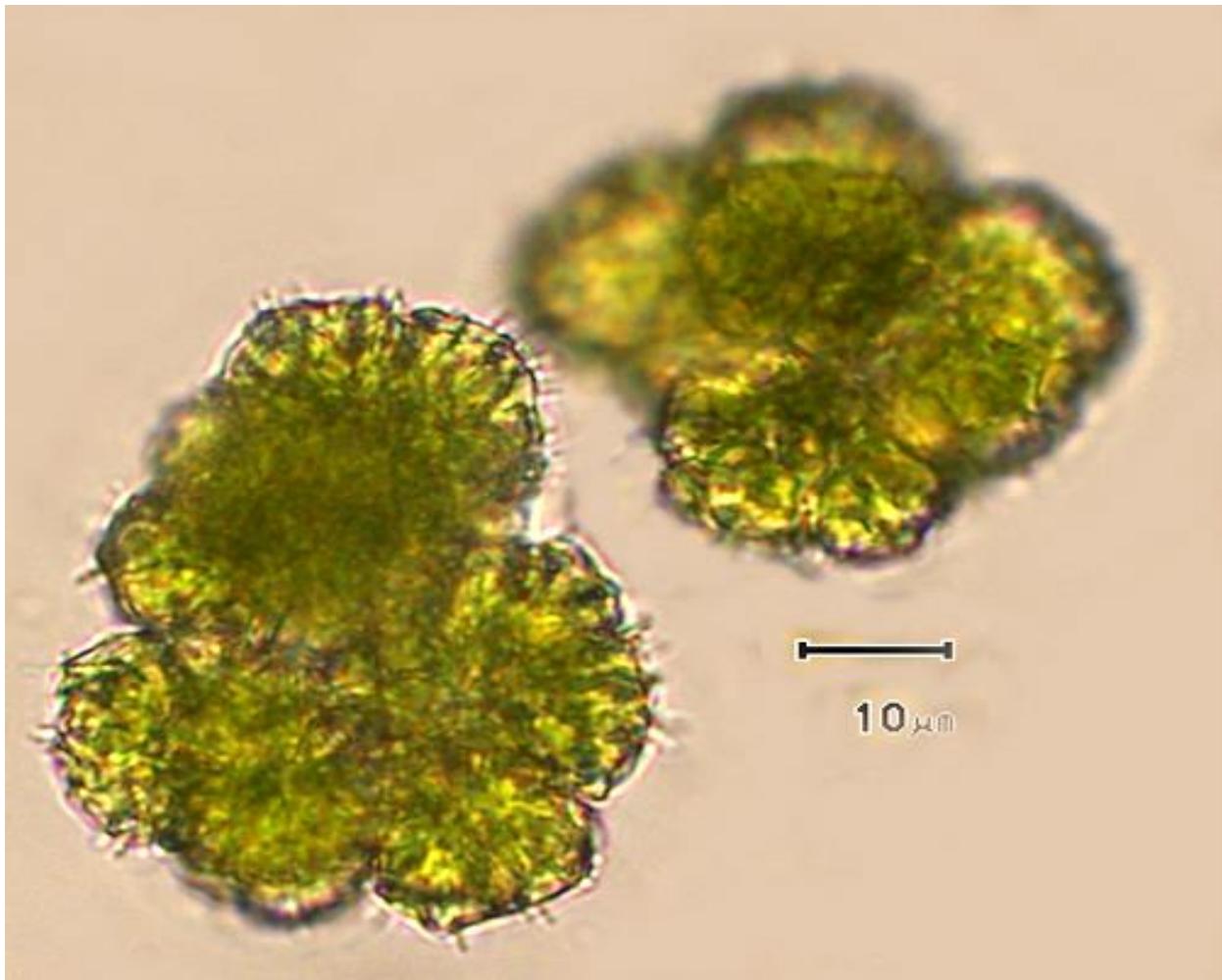
Figure 3.1: Microscopic image of Botryococcus braunii Kützing (1849) (CCAP 807/1) strain.

3.3 Algal Growth under Normal and Stress Conditions

3.3.1 Effects of Salinity on growth

Botryococcus braunii was grown in 3N-BBM+V medium as prepared in accordance to section 2.3.1, It was then grown under stress conditions of higher salinities by adding NaCl (0.1M and 0.2M NaCl) to give a higher salinity 3N-BBM+V medium as prepared in section 2.3.2. These media were used to investigate the effect on the growth and lipid accumulation in *B. braunii*, and growth curves were obtained from the readings of optical density at 595 nm over a 30 days period. Figure 3.2 below shows the growth curve of *B. braunii* grown under normal conditions and in different salinities, and it can be seen from the OD readings obtained that it takes 14 days to obtain 0.5 OD at 595 nm and then 30days to reach 0.9 OD reading clearly indicating that *Botryococcus braunii* is very slow growing alga. This means that *B. braunii* takes a very long time to reach sufficient biomass to be used to perform experiments even when the medium is optimized under normal conditions. It can also be seen from the growth curve that no promising growth was observed even after one-month incubation period in higher salinities (Figure 3.2).
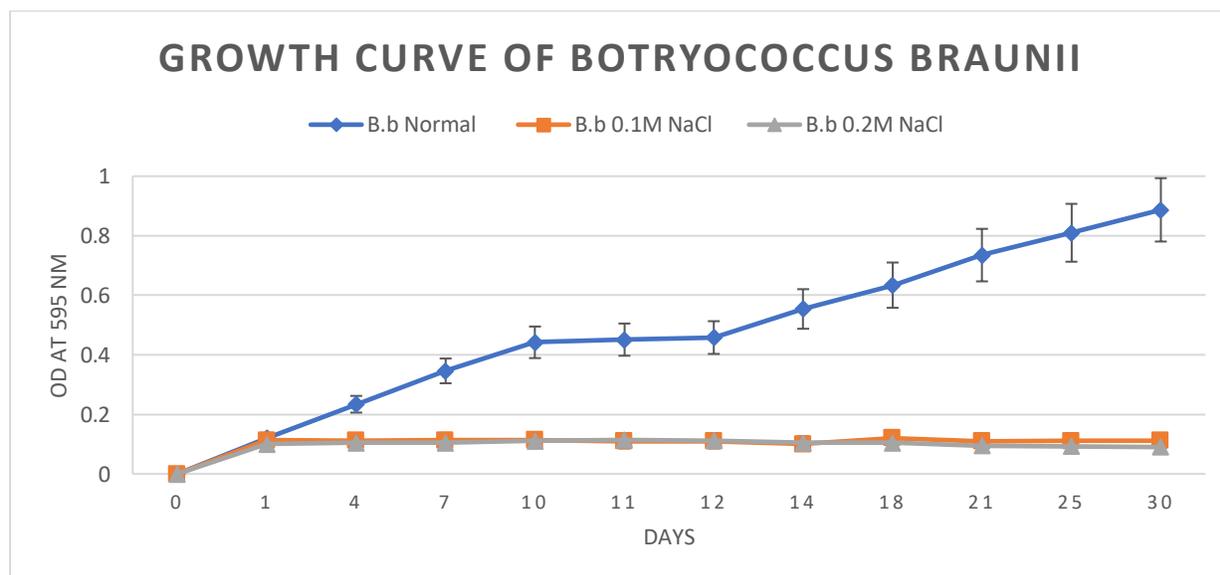
Figure 3.2: Botryococcus braunii Optical Density readings to show the growth curve for normal medium and at different salinities. Error bars represent means ± standard error for three replicates.

3.4 Determination of Lipids in *Botryococcus braunii*

There are diverse methods that have been used for the determination and quantification of algal lipids such as the gravimetric method which is the most conventional method for algal lipid evaluation adapted from Chiu et al. (2009). This technique mainly uses cell disruption and solvent extraction of algal biomass lipids, and the total algal lipids can be quantified by drying and weighing the lipid extract (Kumari et al., 2011). It was clearly shown in Figure 3.2 of section 3.2.1 that *B. braunii* is a very slow growing alga and that it takes a very long time even under optimized, normal conditions to reach sufficient biomass to perform experiments. It also shows no promising growth in higher salinities even after incubation for a one-month period. Therefore, the total algal lipids and the percentage neutral lipids for only full-grown cultures under normal conditions were considered in this work.

3.4.1 Total lipid content by the Gravimetric Method

The cells were grown under normal conditions until sufficient biomass was generated to conduct assessment because a large quantity of biomass is required to determine the total lipids by this method. The well-grown samples were taken, and the lipid content measured in accordance with the procedure discussed in section 2.6.1. It was found that the percentage of lipid content of the dry weight biomass was around 29% which is in close agreement with Lee et al. (1998) who measured the lipid content and found it equal to around 30% of dry weight using the same method.

3.4.2 Nile red method for measuring neutral lipid content

Fluorescence spectroscopy has been utilized as the easiest, quick and an economical tool for studying the neutral lipid content of microalgae based on a lipid soluble glowing dye called Nile Red (9-diethylamino-5H-Benzo[α]phenoxazine-5-one). The vital feature of employing Nile Red as a fluorescent dye was to accomplish a quick screening of oleaginous microalgae to allow the selection of promising strains for economically feasible production of biofuel (Chisti, 2008). Nile Red has the ability to pass through the cell barriers including the cell wall, cell membrane and dissolve in the intracellular

neutral lipid. In combination with organic solvents like dimethyl sulfoxide (DMSO) which have been used with different microalgal species in terms of facilitating the staining of microalgae with Nile Red (Pancha et al., 2014; Wu et al., 2014).

The effectiveness of Nile Red to pass through the algal cell wall and consequently of the spectroscopy filter and these wavelengths depend mainly on the hydrophobicity of the carrier solvent utilized. In general, the excitation wavelength for determination of neutral lipid by Nile Red varies between different algal species and sometimes within the same species. The variations in the emission and excitation wavelength are based mainly on the individual microalgal isolates and neutral lipid composition of fatty acids (Chen et al., 2009). The excitation and emission filters of the spectroscopy are used to read the fluorescence of the dye which is related indirectly to the lipid content (Cirulis et al., 2012).

As the fluorescent intensity of Nile Red is variable, the precise evaluation of neutral lipid in algal cells required an optimization of two factors: the algal cell concentration and Nile Red concentration. Therefore, optimum cell concentration at peak time (optimum time is that which gives the highest fluorescence intensity) was determined, and optimum concentration of Nile-Red was also found using the 96 well plate method described in sections 3.3.2.1 and 3.3.2.2, respectively.

3.4.2.1 Optimum cell concentration and peak time for 96 well plate Nile-red method

The concentrations of the *B. braunii* cells were optimized as described in section 2.6.2. Figure 3.3 shows the fluorescence intensities of *B. braunii* for eight different percentage concentrations of the cells (i.e. 100, 87.5, 75, 62.5, 50, 37.5, 25 and 12.5% where 100% was OD_{595} = 1) over 30 minute at 5 minute intervals starting from 15 minutes after staining with Nile red. It can be seen from the chart that the highest fluorescence intensity is at 75% with 15 minutes staining time. Hence, the optimal cell concentration were at 75% which is equivalent to an OD_{595} = 0.743. This shows that the optimum conditions were a 75% cell concentration exposed to Nile Red for 15 minutes staining.
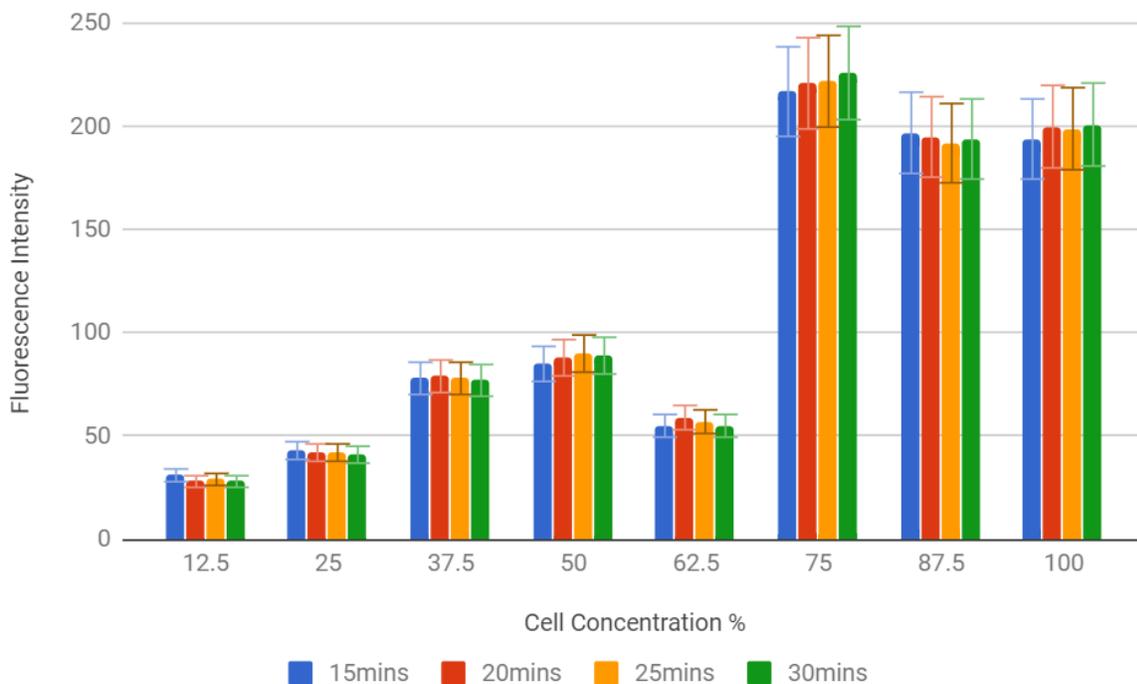
Figure 3.3: Nile Red staining time and cell concentration optimization on fluorescence intensity of B. braunii. Each column represents the mean of four reading plus/minus standard deviation.

Tables 3.1 and 3.2 below show the 96 well plate readings for the fluorescence intensity of *B. braunii* for different cell concentrations ranging from 12.5% to 100 at the peak time (15 minutes) after staining with Nile red including the calculation of averages, standard deviation and normalization. It can be seen from Table 3.2 that the optimum concentration of *Botryococcus braunii* cells was found to be 75% based on the equilibrium with the standard deviation after normalization (lowest standard deviation, highest normalized and highest number when stained was subtracted from unstained).

Table 3.1: Plate readings of different algal cell concentrations at 15 minutes.

T° Read 1:485/20,580/50	Dilution%		100	87.5	75	62.5	50	37.5	25	12.5
Stained Cells	R1	A	332	331	340	187	212	206	167	151
	R2	B	337	320	353	181	215	196	158	145
	R3	C	332	323	343	182	211	206	164	151
	R4	D	346	321	332	178	199	198	166	146
Unstained Cells	R1	E	141	126	123	125	123	125	119	118
	R2	F	149	126	128	128	125	122	121	116
	R3	G	142	127	124	129	124	121	120	116
	R4	H	140	129	126	128	126	125	124	119

Table 3.2: Averages, standard deviation and normalised calculations of different algal cell concentrations at 15 minutes.

Concentration	100	87.5	75	62.5	50	37.5	25	12.5
Average Stained	337	324	342	182	209	202	164	148
STDEV Stained	7	5	4	4	7	5	4	3
Average Unstained	143	127	125	128	125	123	121	117
STDEV Unstained	4	1	2	2	1	2	2	2
Stain-Unstained	194	197	217	55	85	78	43	31
Normalised	2	2	3	1	2	2	2	2

3.4.2.2 Optimum Concentration of Nile-Red

Investigation of optimum concentration of Nile-Red was also conducted at the appropriate time interval for peak fluorescence i.e. at 15 minutes using 75% algal culture with OD_{595} = 0.743 by utilizing the 96 well plate method as described in section 3.3.2.1. Tables 3.3 and 3.4 below show the plate readings for the fluorescence intensity for the six different concentrations of the Nile-Red at the peak time of 15 minutes including the calculation of averages, standard deviation and subtraction of unstained cells from the stained. It can be seen from Table 3.4 that the optimum Nile-Red concentration was 0.4 μmol ml^{-1} which is equal to 128 μg ml^{-1} based on the balance with the standard deviation.

Table 3.3: Plate readings of different Nile-Red concentrations at 15 minutes.

T° Read 1:485/20,580/50	Concentration		0.05	0.1	0.2	0.3	0.4	0.6
Stained Cells	R1	A	182	225	247	301	341	401
	R2	B	154	221	247	305	347	425
	R3	C	177	221	252	308	351	417
	R4	D	177	228	232	311	354	418
Unstained Cells	R1	E	137	119	122	117	117	119
	R2	F	134	121	123	118	119	119
	R3	G	139	121	122	119	115	120
	R4	H	137	129	128	137	159	128

Table 3.4: Averages and standard deviations for fluorescence of different Nile-Red concentrations at 15 minutes.

Concentration	0.05	0.1	0.2	0.3	0.4	0.6
Average Stained	173	224	245	306	348	415
STDEV Stained	13	3	9	4	6	10
Average Unstained	137	123	124	123	128	122
STDEV Unstained	2	4	3	10	21	4
Stain-Unstained	36	101	121	184	221	294

3.4.2.3 Triolein Calibration Curve

To quantify the neutral lipid in *B. braunii*, a concentration standard curve was prepared as described earlier in section 2.6.2.4. Triolein is a standard neutral lipid derived from glycerol and three molecules of unsaturated oleic fatty acids. Various concentrations of Triolein with isopropanol were added to the well grown *B. braunii* cells and the mixture of different concentrations was stained with Nile Red dye. The reason for adding algae to the concentration curve is to try to take into account the potential absorption of Nile Red fluorescence by algal pigments. The Triolein calibration curve was plotted with cell concentration of 75% OD_{595} = 0.743, and 0.4 µmol ml^{-1} (128 µg ml^{-1}) as the concentration of Nile-Red dye found in the previous section (section 3.3.2.2). The resulting Triolein standard curve for *B. braunii* is shown in Figure 3.4 below, and this is to allow for quantification of the neutral lipid within the *B. braunii* cells.
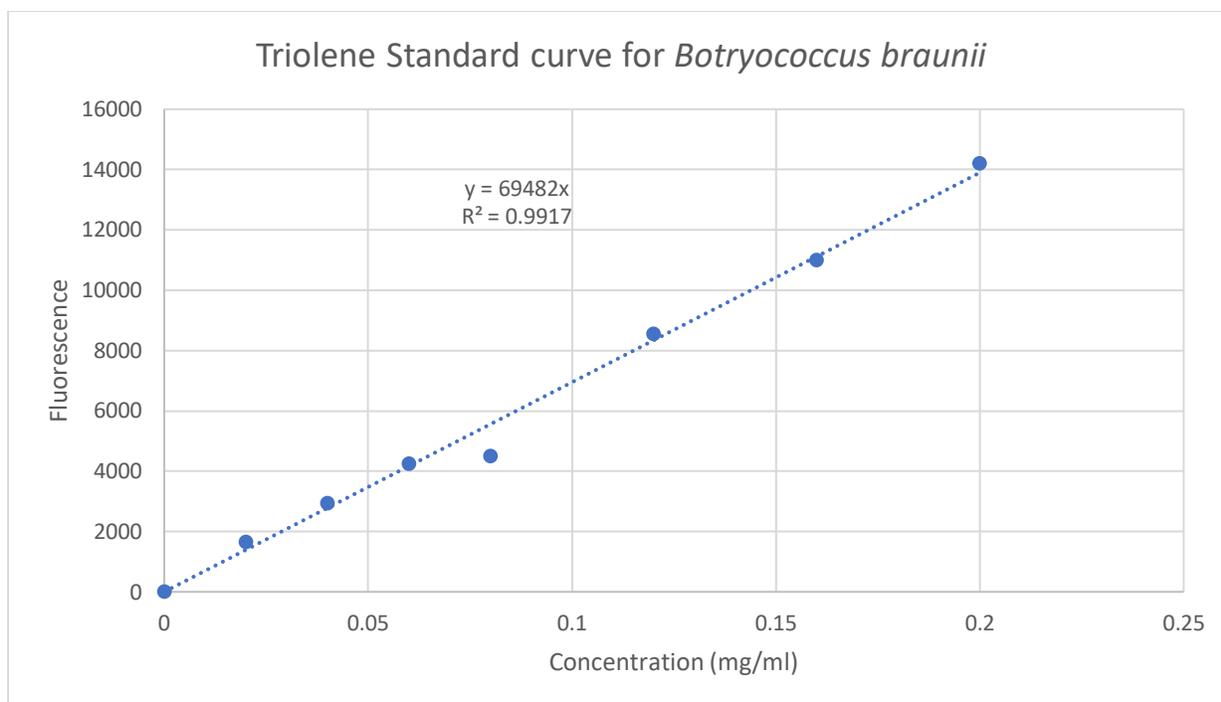
Figure 3.4: Triolein Standard curve for Botryococcus braunii.

3.4.2.4 Dry Weight versus Optical Density

There exists a relation between OD and cell dry weight and it was determined for *B. braunii* as described in section 2.7. This is aimed at converting the OD value of the optimized concentration of cells to dry weight (mg/ml). Figure 3.5 shows the relationship between OD and dry weight for *B. braunii.* It can be seen that for *B. braunii,* Y = 0.1145x, where Y = Dry weight (mg/ml) and x = OD_{595}. Hence the percentage of neutral lipid could be calculated by dividing the neutral lipid content by the value of the cell dry weight of the algae in the optimum concentration and then multiplying by 100.

Relation between OD and Dry Weight

$y = 0.1145x$

$R^2 = 0.8483$
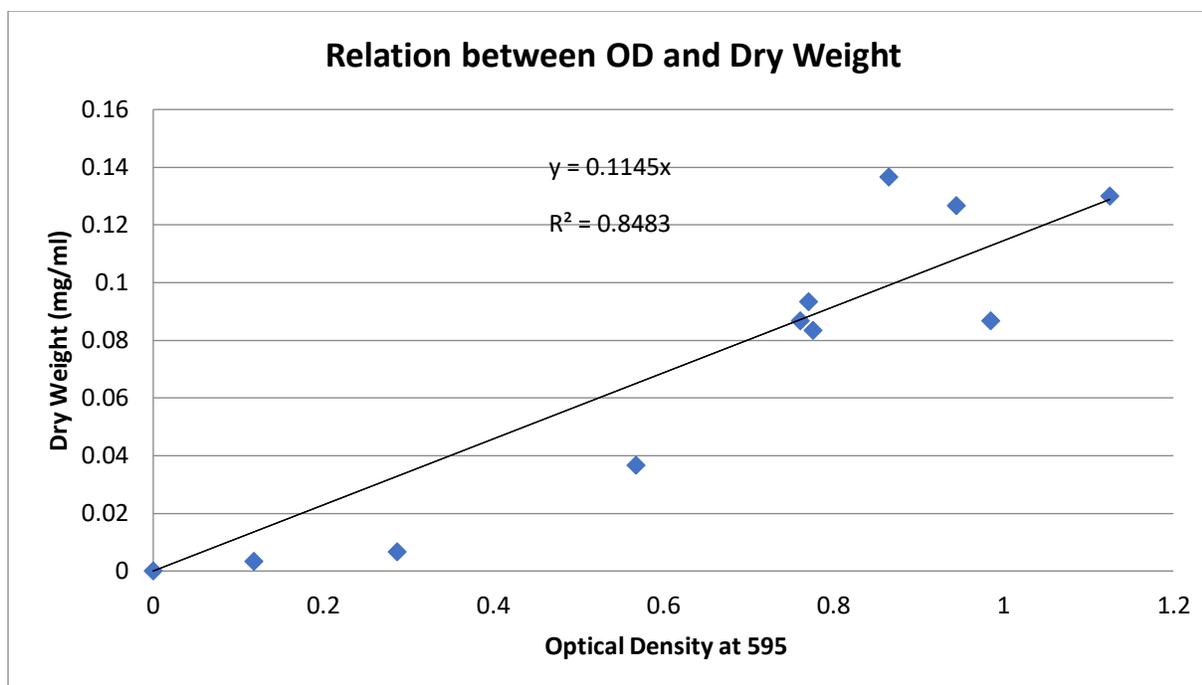
Figure 3.5: Relation between OD and dry weight of Botryococcus braunii.

3.4.2.5 Neutral Lipids in *Botryococcus braunii*

The concentration of neutral lipid 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 (i.e OD_{595}=75%= 85.1 µg/ml dry weight) for *Botryococcus braunii* cells as described in section 3.2.2.1 using the formula below:

Neutral lipid (mg/ml) = Average (Stain-Unstained)
Slope of Triolein Standard Curve

Biomass dry weight in optimum concentration (mg/ml) = Slope of dry weight verses OD

X OD of Optimum Concentration

Neutral Lipid Content % = Neutral Lipid Content (mg/ml)   X 100
Biomass dry weight in optimum concentration (mg/ml)

Therefore, neutral lipid (mg/ml) =   217
69482

= 0.003123 (mg/ml)

= 3.123 (µg/ml)

Biomass dry weight in optimum concentration (mg/ml) = 0.1145 X 0.743 (mg/ml)

= 85.1 (µg/ml)

Neutral Lipid Content % = $\dfrac{3.123 \times 100}{85.1}$

= 3.64%

Therefore, the highest lipid content and percentage of lipid accumulated by *Botryococcus braunii* cells under normal conditions of well grown cells were 3.1 µg/ml and 3.64% respectively after 30 days.

3.5 Discussion

Recent interests in the possibility of utilizing microalgae as a source of renewable fuels to replace fossil diesel emanated in the search for microalgae with high yield of oil which can grow rapidly. *Botryococcus braunii* dry mass has up to 75% hydrocarbons depending on the strain and its incubation conditions.

In the work described in this chapter, the identity of *B. braunii* strain was confirmed using microscope and it was first checked for any bacteriological contamination. It was grown as unialgal cultures in BBM medium which was optimized for culture of green algae strains similar to *B. braunii.* Despite using a relatively nutrient rich medium, it was difficult to reach sufficient biomass to run experiments. The growth rate was observed by the Optical Density readings obtained which showed that it took 14 days to get 0.5 OD at 595 nm and then 30 days to reach a 0.9 OD reading. In comparison, cultures of the unicellular green alga *Dunaliella* can reach an OD_{595} of around 2 after 14 days growth (D.J. Gilmour, personal communication). This clearly indicates that *B. braunii* grows very slowly in 250 ml flask cultures. Therefore, an attempt was made to grow *B. braunii* in a 2 litre fermenter. *B. braunii* then were cultured in a 2 litre fermenter for 2 weeks, the fermenter was placed on magnetic stirrer to prevent algae accumulating at the bottom. Cultures were gassed with air through a single nozzle. The fermenter was illuminated with continues irradiance (50-70 µmol m-2 s-1) at 25°C. Biomass dry weight of 2 weeks was found to be equal to 3 weeks of flask cultivation.

The total lipid content of *Botryococcus braunii* was measured using the gravimetric method adapted from Chiu et al. (2009). The total lipid content for the full-grown culture of *B. braunii* was found to be 2.98 mg/ml in 10.2 mg/ml dry weight biomass. Hence the percentage of total lipid content in dry weight biomass was around 29% (section 3.3.1) which is in agreement with Lee et al. (1998) who measured the lipid content and found it equal to around 30% of dry weight using the same method.

Nile red fluorescence was used to quantify the cellular neutral lipid content of *B. braunii*. The time to peak fluorescence, optimum cell concentration and optimum concentration of Nile Red were all determined for *B. braunii* – it is essential to optimise these

parameters for each algal species tested (Pick and Rachutin-Zalogin, 2012). The optimum Nile red parameters for *B. braunii* were cell concentration OD_{595} = 0.743, Nile red concentration of 0.4 µmol ml^{-1} (128 µg ml^{-1}) and peak fluorescence staining time of 15 minutes. A Triolein calibration curve (Bertozzini et al., 2011) and a dry weight versus OD calibration curve were set up to allow the quantification of neutral lipid in *B. braunii*.

Hence the percentage of neutral lipid was calculated by dividing the concentration of neutral lipid by the value of the cell dry weight of the algae in the optimum concentration and then multiplying by 100. The neutral lipid content and percentage of neutral lipid were found to be 3.1 µg/ml and 3.64%, respectively, after 30 days growth under normal conditions. Thus, *B. braunii* has a total lipid content of 29% but only 3.64% are neutral (storage) lipids. This is much less than the 30% neutral lipid normally quoted as the minimum requirement for biodiesel production (Chisti, 2007).

A great advantage of using algae for biofuel production is the ability to control their accumulation and secretion of biofuels by changing their growth conditions. Therefore, the next step of the project was to modify the medium (3N-BBM+V) by adding NaCl in two different concentrations (0.1M and 0.2M) to cultivate *B. braunii* under stress conditions to investigate the lipid production. However, no growth was observed after one-month incubation in higher salinities.

In summary, *B. braunii* might have a significant total lipid content compared to other algal species but accessing it for biodiesel product has a lot of disadvantages, one of which is its very slow growth rate as it takes a long time to reach stationary phase. Even in normal conditions, the growth rate is very slow to reach sufficient biomass and the percentage of neutral lipid produced is very low. It could not grow in higher salinities which shows that it is intolerant to a diverse medium for growth. In conclusion, *Botryococcus braunii* CCAP807/1 is a very slow growing alga that takes a very long time to reach high levels of biomass. Also, it could not grow under the stress of higher salinities to improve its lipid accumulation. For these reasons, the next part of the study focused on an alternative species *Tetraselmis suecica* (CCAP 66/4) and this strain is assessed in the subsequent chapter (Chapter 4).

Chapter 4: *Tetraselmis suecica* Strain Identity Confirmation and Lipid Content Determination under normal and stress conditions

4.0 Introduction

As stated in the previous chapter, about 75% of *Botryococcus braunii* dry mass can be hydrocarbons, depending on the strain and growth conditions (Banerjee et al., 2002). This should provide a better basis for biodiesel production than the neutral lipids normally stored by the other algae. However, exploitation of *B. braunii* was very difficult as it was found to be slow growing and the *B. braunii* CCAP 807/1 strain could not grow under the stress of higher salinities. Therefore, to continue the work to find a strain that will accumulate high levels of intracellular lipid, an alternative species (*Tetraselmis suecica* CCAP 66/4) was obtained, since it is known to be more salt tolerant than *B. braunii* and to be capable of producing high levels of lipids (Montero et al., 2011).

A number of other microalgae were considered, before *T. suecica* was chosen. For example *Schizochytrium*, which is an unusual alga found within the *Heterokonta* group. *Schizochytrium* has been mainly studied by research groups in Japan and China, and produces the polyunsaturated fatty acid (PUFA) docosahexanoic acid (DHA) in large amounts, which is used as a human dietary supplement (Qu et al., 2013). *Nannochloropsis*, which like *Schizochytrium*, is also a heterokont alga belonging to the class *Eustigmatophyceae* (Van den Hoek, 1995) has also been well studied and high levels of neutral lipids (in excess of 25% of biomass) have been measured in this alga on a regular basis (e.g. Pal et al., 2011). However, *Nannochloropsis* cells are small (around 1 to 2 μm) and have thick cell walls, which makes the task of harvesting the neutral lipids more difficult (Rios et al., 2013). The *Dunaliella* genus (Ying et al., 2015) and species related to the *Chlorella* genus (Smith et al., 2015) are being actively studied in the Gilmour laboratory at the University of Sheffield, UK, so it was decided to use *T. suecica*.

Tetraselmis suecica CCAP 66/4 is one of the members of the Chlorophyta (green algae), which is found within the class of Prasinophyceae in the Chlorodendrales group. The strain CCAP 66/4 was received from the Culture Collection of Algae and Protozoa, Oban, Scotland. It is normally grown on a large scale as a feedstock in aquaculture using numerous types of photo-bioreactor arrangements (Day and Fenwick, 1993; Naumann et al., 2013). It is normally considered as one of the best sources of long-chain PUFAs that are essentially used for strainer feeders grown in aquaculture systems (Fabregas et al., 2001). It was established also that the conditions by which it is exposed during cultivation play significant roles impacting on its total lipid production. As a result of this discovery, it is seen as an excellent feedstock for commercial production of biofuel as it can be 'stressed' to produce more of the suitable lipids for biodiesel production (Sanchez-Garcia et al., 2013). The fatty acids produced by *T. suecica* are principally C16:0 and C18:1, which are excellent for the production of biodiesel (Mendoza Guzman et al., 2010; Stansell et al., 2012). The lipid content of *T. suecica* is modest per percentage dry weight biomass but lipid productivity is very high (Griffiths and Harrison, 2009; Mata et al., 2010). In this work, *T. suecica* was re-evaluated by modifying its growth conditions in order to study its neutral lipid production under normal and stressed (higher salinity and nitrogen starvation) growth conditions. The CCAP 66/4 strain was chosen since it was not previously studied for neutral lipid production under stress conditions. The main aim of this chapter was to determine the best way to increase the neutral lipid accumulation of *T. suecica* CCAP 66/4.

Immediately *T. suecica* (CCAP 66/4) was received from the CCAP, it was cultivated in primary stock culture (in a 100ml flask) prepared using the liquid *Tetraselmis suecica* samples received from the CCAP. Flask cultures were grown in the culture room at 25 ± 1°C with continuous light (50 – 70 µmol m^{-2} s^{-1}) supplied by daylight fluorescent lights until the culture reached stationary phase. It was earlier stated that one of the advantages of using algae for biofuel production is the ability to control their accumulation and secretion of biofuels by changing their growth conditions (Montero et al., 2011). 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 to optimize growth by monitoring any improvement in growth using growth curves. It was also

cultivated in different salinities (0.2M, 0.4M, 0.6M, 0.8M, 1M and 1.2M NaCl) to understand its tolerance to salinity and nitrogen starvation was also used to find out if there is any improvement in the lipid content of *T. suecica* as a result of these stress conditions. The development of a defined F/2 medium was a very important step in this work, because normal F/2 uses seawater as its base and thus nutrients are not fully quantified.

In both normal and stress conditions of the well grown algal cells, the total lipid content of *T. suecica* was measured using the gravimetric method while Nile red fluorescence was used to quantify the cellular neutral lipid content of *T. suecica*. To determine the neutral lipids accurately by the use of this method, the best concentration of algal cells, the best concentration of Nile Red to be used and finally the best incubation time that showed the highest fluorescence intensity of Nile Red were determined using a Varian 96-well plate reader. Although these parameters were determined for *B. braunii* in Chapter 3, they must be re-determined for *T. suecica* cells. Therefore, the optimum concentration of Nile Red dye and optimum concentration of algal cells were determined at peak time and a Triolein calibration curve was prepared using a procedure adapted from Bertozzini et al. (2011). In order to allow the calculation of the percentage neutral lipid within the algal cells, the Nile red fluorescence readings were converted to Triolein equivalents and the linear relationship between the OD_{595} and dry weight of *T. suecica* cells was determined.

The total algal lipids expressed as biofuel relevant fatty acid methyl esters (FAMEs) could be expressed as percent FAME content based on the dry weight of the algal biomass. Therefore, the FAME profiles from the neutral lipids of *Tetraselmis suecica* were determined using GC-MS for cells cultured in both higher salinities and under nitrogen starvation. The compatible solutes production was also analysed using NMR in cells exposed to increasing salinity and under nitrogen starvation.

4.1 Aims and Objectives

The aims and objectives of this chapter are stated below:

1. Confirm the identity of *Tetraselmis suecica* strain received from the Culture Collection of Algae and Protozoa using 18S rDNA sequencing.

2. Optimise growth media (F/2) to cultivate *Tetraselmis suecica* and monitor any improvement in growth.

3. Quantify the lipid content of *Tetraselmis suecica* using dry weight measurements, lyophilisation, a gravimetric method and Nile Red fluorescent dye staining

4. Quantify the Fatty Acid Methyl Esters (FAMEs) in *Tetraselmis suecica* using GC-MS.

5. Quantify the compatible solutes in *Tetraselmis suecica* using NMR.

4.2 *Tetraselmis suecica* Strain Identity Confirmation using Microscope

As soon as the *Tetraselmis suecica* CCAP 66/4 strain was received from the Culture Collection of Algae and Protozoa, Oban, Scotland, the cells were checked for any bacterial contamination and the identity of the strain was checked using a microscope to ascertain the strain. Figure 4.1 below shows the microscopic image of *Tetraselmis suecica* CCAP 66/4 strain

Figure 4.1: Microscopic image of Tetraselmis suecica CCAP 66/4 strain.

4.3 Algal Growth under Normal and Stress Conditions

Tetraselmis suecica was grown in defined F/2 medium as prepared in accordance with section 2.3.3, It was then grown under stress conditions of higher salinities by adding NaCl to give concentrations of 0.2M, 0.4M, 0.6M, 0.8M, 1.0M and 1.2M NaCl. The higher salinity defined F/2 medium was prepared as described in section 2.3.4.2 to investigate the effect of salinity on the growth and lipid accumulation in *T. suecica*. The growth curves were obtained from the readings of optical density at 595 nm over a 30 days period. Three replicate OD_{595} measurements for each salinity were performed each day.

4.3.1 Effects of Salinity on growth

Figure 4.2 shows the growth curves of *T. suecica* in different salinities in order to investigate the effects of salinity on neutral lipids. It shows that it could be grown in higher salinities and neutral lipid production could be studied under stress conditions to determine the optimum salinity to increase the neutral lipid accumulation. Increasing the salinity can increase the lipid content of microalgae, but often lowers the growth rate of a species. Just as expected, growth rate and final biomass level reached after 30 days were decreased as salinity increased. It can also be seen from the level of growth that there was a significant difference at the highest salinity tested (1.2M NaCl) from that at normal condition (0.4M NaCl, equivalent to seawater salinity). The normal condition showed a higher growth rate and final biomass level as indicated in Figure 4.2 below.
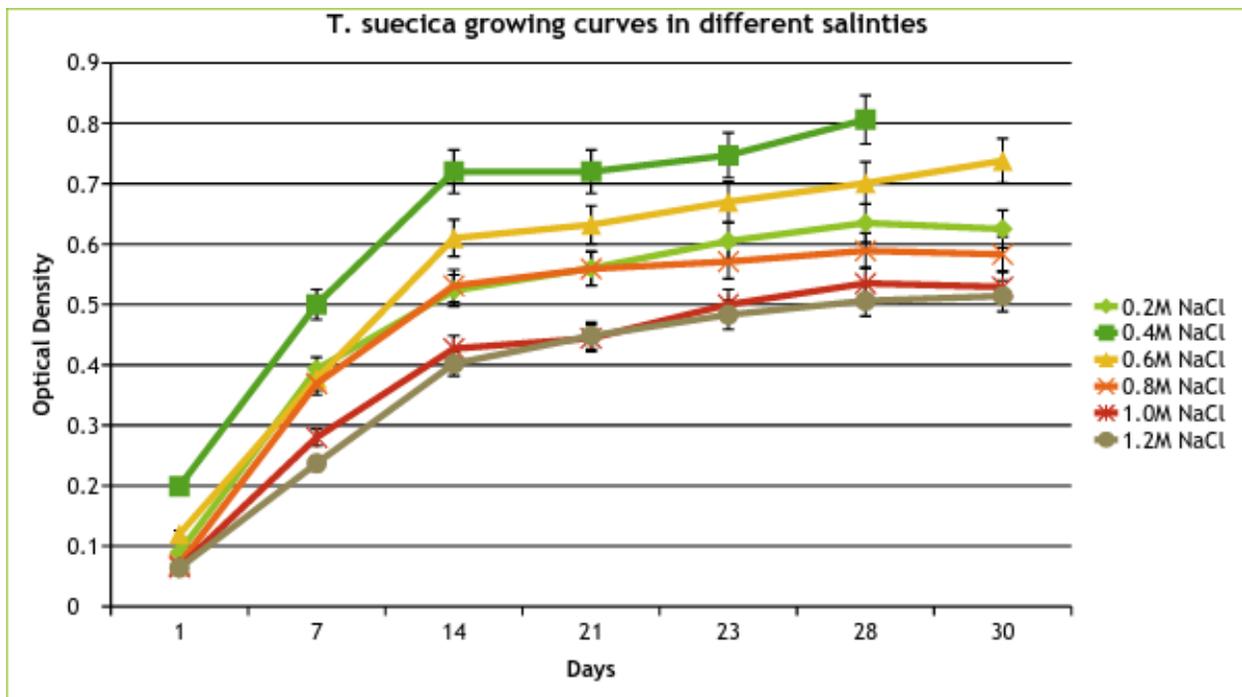
Figure 4.2: Tetraselmis suecica Optical Density readings to show the growth rate at different salinities. Bars represent means ± standard error for three replicates.

4.3.2 Effects of Nitrogen Starvation

Figure 4.3 shows the growth curves for *T. suecica* cells under nitrogen starvation in order to subsequently investigate the effects of nitrogen starvation on neutral lipids accumulation. It can be seen that the growth rate and final biomass level reached after 30 days were decreased with nitrogen starvation. It can also be seen from the level of growth that there were significant differences between the nitrogen free, 25% and 50% environments from that found under the normal conditions. But there was no significant difference in the level of growth for the 25% and 50% nitrogen grown cells. The normal condition showed a higher growth rate and final biomass level as indicated from Figure 4.3 below.
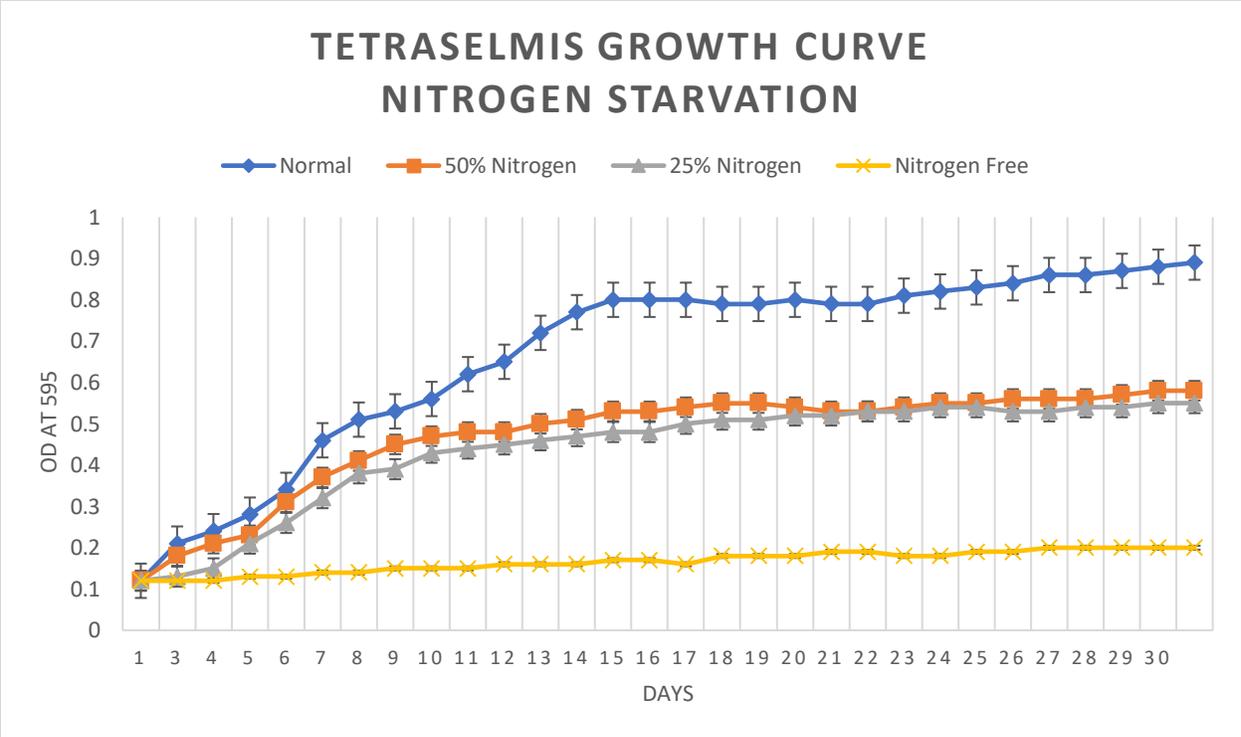
Figure 4.3: Tetraselmis suecica Optical Density readings to show the growth rate under nitrogen starvation. Bars represent means ± standard error for three replicates.

4.4   Determination of Neutral Lipids in *Tetraselmis suecica*

As discussed in the previous chapter, diverse methods have been used for the determination and quantification of algal lipids such as gravimetric method which is the most conventional method for algal total lipid evaluation adapted from Chiu et al. (2009). This is a technique that mainly uses cell disruption and solvent extraction of the algal biomass lipids, and the total algal lipids can be quantified by drying and weighing the lipid extract (Kumari et al., 2011). *Tetraselmis suecica* was grown under normal conditions and under stress to investigate the effects on neutral lipids accumulation, therefore, the total algal lipids and the percentage neutral lipids for full-grown cultures under normal and stress conditions were considered in this part of the work.

4.4.1 Total Lipid Content by the Gravimetric Method

The *T. suecica* cells were grown under normal and 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. Full-grown samples were taken from the normal, 0.6M NaCl and Nitrogen free conditions; and the total lipid was measured in accordance with the procedure described in section 2.6.1. As shown in Figure 4.4, the percentage of total lipid content of the dry weight biomass was found to be around 31%, 29% and 56% for normal, 0.6M NaCl and Nitrogen free conditions, respectively.
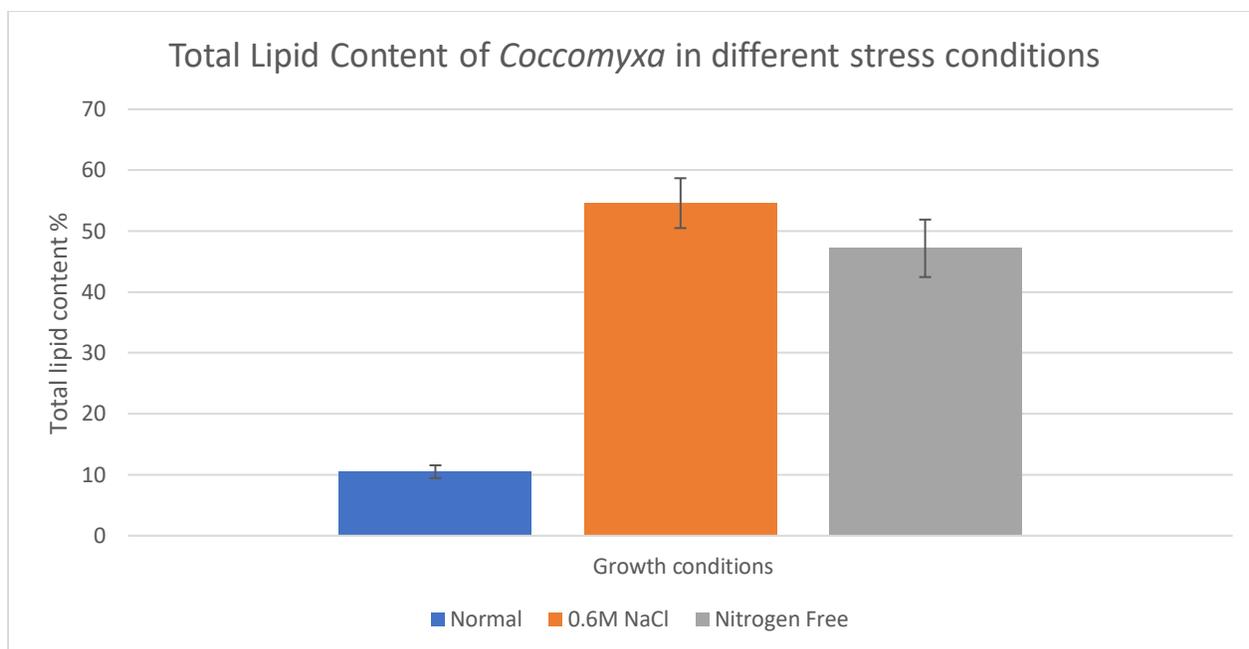
Figure 4.4: Tetraselmis suecica grown in different Stress Conditions Total Lipid Content percentage by the Gravimetric Method. Bars represent mean ± standard error for three replicates.

This shows clearly that Nitrogen starvation stress increased total lipid levels in *T. suecica* cells, whereas salinity stress had no effect on total lipid production.

4.4.2 Nile Red Method for Measuring Neutral Lipid Content

It was earlier discussed that fluorescence spectroscopy has been utilized as the easiest, quick and an economical tool for studying the neutral lipid content of microalgae based on a lipid soluble glowing dye called Nile red (9-diethylamino-5H-Benzo[α]phenoxazine-5-one). The vital feature of employing Nile Red as a fluorescent dye was to accomplish a quick screening of oleaginous microalgae to allow the selection of promising strains for economically feasible production of biofuel (Chisti, 2008). Nile red has the ability to pass through the cell barriers including the cell wall, cell membrane and dissolve in the intracellular neutral lipid. In combination with organic solvents like dimethyl sulfoxide (DMSO) which have been used with different microalgal species to facilitate the staining of microalgae with Nile red (Pancha et al., 2014; Wu et al., 2014).

However, the fluorescent intensity of Nile red is variable, thus the precise evaluation of neutral lipid in algal cells requires an optimization of two factors: the algal cell concentration and Nile red concentration. Therefore, optimum cell concentration at peak time (optimum time which gives the highest fluorescence intensity) was determined, and optimum concentration of Nile-Red was also found using the 96 well plate reader as described in sections 4.3.2.1 and 4.3.2.2, respectively.

4.4.2.1 Optimum cell concentration and peak time for 96 well plate Nile red method

The concentrations of the *T. suecica* cells were optimized as described in section 2.6.2. Figure 4.5 below shows the fluorescence intensities of *T. suecica* cells for eight different percentage concentrations of the cells (i.e. 100, 87.5, 75, 62.5, 50, 37.5, 25 and 12.5% where 100% was OD_{595} = 1) measured over 30 minutes at 5 minutes intervals starting from 15 minutes after staining with Nile red. The highest fluorescence intensity was at 87.5%, but 75% was taken as best since the 87.5% sample had higher standard deviations and lower normalized values. The peak time of 15 minutes after staining had the lowest standard deviation while 20 minutes, 25 minutes and 30 minutes had higher standard deviations after normalization. Hence, the optimal cell concentration was 75% which is equivalent to an OD_{595} = 0.7.
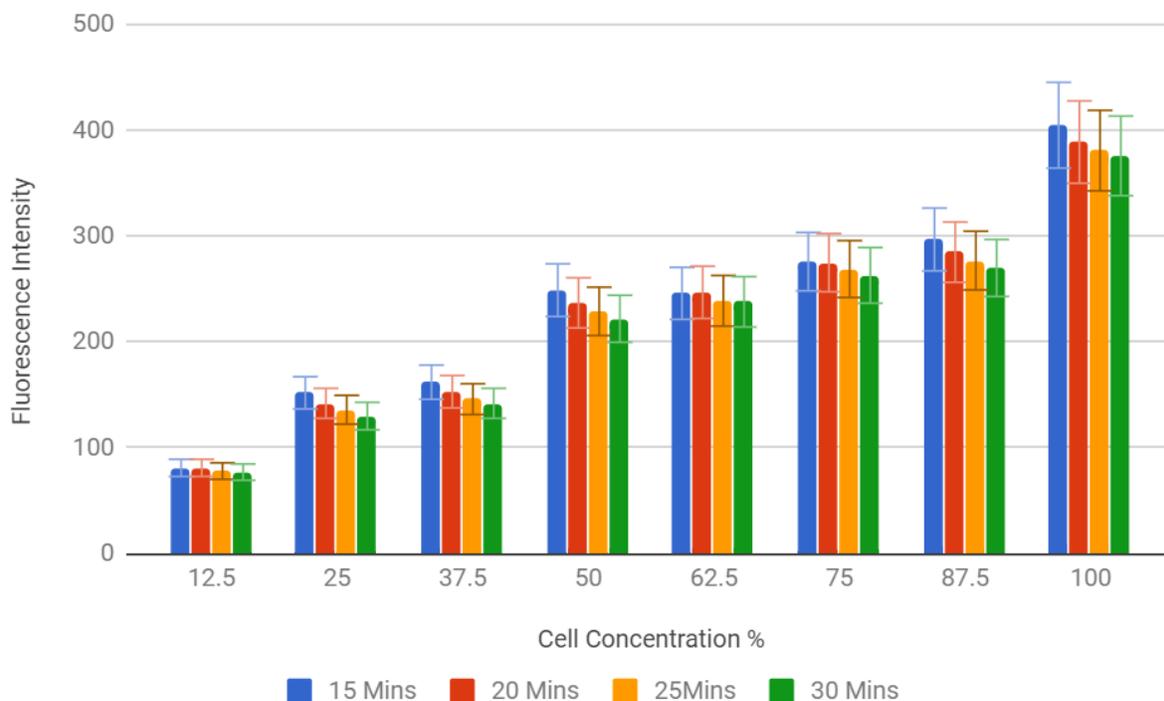
Figure 4.5: Nile Red staining time and cell concentration optimization for fluorescence intensity of Tetraselmis suecica. Each column represents the mean of four readings plus/minus standard deviation.

Tables 4.1 and 4.2 below show the plate readings for the fluorescence intensity of *T. suecica* for different cell concentrations ranging from 12.5% to 100 at the peak time (15 minutes) after staining with Nile red including the calculation of averages, standard deviation and normalization respectively. It can be seen from Table 4.2 that the optimum concentration of *T. suecica* cells was found to be 75% based on the equilibrium with the standard deviation after normalization (lowest standard deviation, highest normalized and highest number when unstained was subtracted from stained).

Table 4.1: Plate readings of different algal cell concentrations at 15 minutes.

T° Read 1:485/20,580/50	Dilution%		100	87.5	75	62.5	50	37.5	25	12.5
Stained Cells	R1	A	536	419	404	384	357	272	264	196
	R2	B	530	393	388	354	366	259	263	187
	R3	C	531	421	399	361	351	261	249	181
	R4	D	502	392	383	356	364	273	261	205
Unstained Cells	R1	E	130	113	115	109	109	106	109	106
	R2	F	127	112	112	108	109	103	108	107
	R3	G	122	111	110	111	112	104	109	102
	R4	H	129	119	121	118	123	112	115	112

Table 4.2: Averages, standard deviation and normalised calculations of different algal cell concentrations at 15 minutes.

Concentration	100	87.5	75	62.5	50	37.5	25	12.5
Average Stained	532	411	390	357	362	269	263	188
STDEV Stained	3	16	4	4	6	7	6	8
Average Unstained	127	114	115	112	113	106	110	107
STDEV Unstained	4	4	5	5	7	4	3	4
Stain-Unstained	405	297	276	246	249	162	152	81
Normalised	4	3	4	4	3	4	6	7

4.4.2.2 Optimum concentration of Nile red

The optimum concentration of Nile red was also determined at the time interval for peak fluorescence i.e. 15 minutes using 75% algal culture with OD_{595} = 0.7 by utilizing the 96 well plate method as described in section 4.3.2.1. Tables 4.3 and 4.4 below show the plate readings for the fluorescence intensity for the five different concentrations of the Nile red at the peak time of 15 minutes including the calculation of averages, standard deviation and subtraction of unstained cells from the stained. It can be seen from Table 4.4 that the optimum Nile-Red concentration was 0.4 µmol ml^{-1} based on the balance with the standard deviation.

Table 4.3: Plate readings of different Nile-Red concentrations at 15 minutes.

T° Read 1:485/20,580/50	Concentration		0.05	0.1	0.2	0.4	0.6
Stained Cells	R1	A	195	186	180	200	208
	R2	B	192	189	191	201	208
	R3	C	196	184	187	208	215
	R4	D	200	200	191	210	271
Unstained Cells	R1	E	169	142	150	150	141
	R2	F	153	136	138	136	134
	R3	G	158	137	135	134	135
	R4	H	167	140	135	145	140

Table 4.4: Averages, standard deviation and subtracting Calculations of different Nile-Red concentrations at 15 minutes.

Concentration	0.05	0.1	0.2	0.4	0.6
Average Stained	196	190	187	205	226
STDEV Stained	3	7	5	5	31
Average Unstained	162	139	140	141	138
STDEV Unstained	8	3	7	8	4
Stain-Unstained	34	51	48	64	88

4.4.2.3 *Tetraselmis suecica* **Triolein calibration curve**

In order to accurately quantify the neutral lipid in *T. suecica*, a concentration standard curve was prepared as described earlier in section 2.6.2.4. Triolein is a standard neutral lipid derived from glycerol and three molecules of unsaturated oleic fatty acids. Various concentrations of Triolein with isopropanol were added to the well grown *T. suecica* cells and the different concentrations were stained with Nile red dye. The reason for adding algae to the concentration curve is to try to take into account the potential absorption of Nile red fluorescence by algal pigments (Bertozzini et al., 2011). The Triolein calibration curve was plotted with cell concentration of 75% OD_{595} = 0.7, and 0.4 μmol ml^{-1} as the concentration of Nile red dye found in the previous section (section 4.3.2.2). The resulting Triolein standard curve for *T. suecica* is shown in Figure 4.6 below, and this is to allow for quantification of the neutral lipid within the *T. suecica* cells.
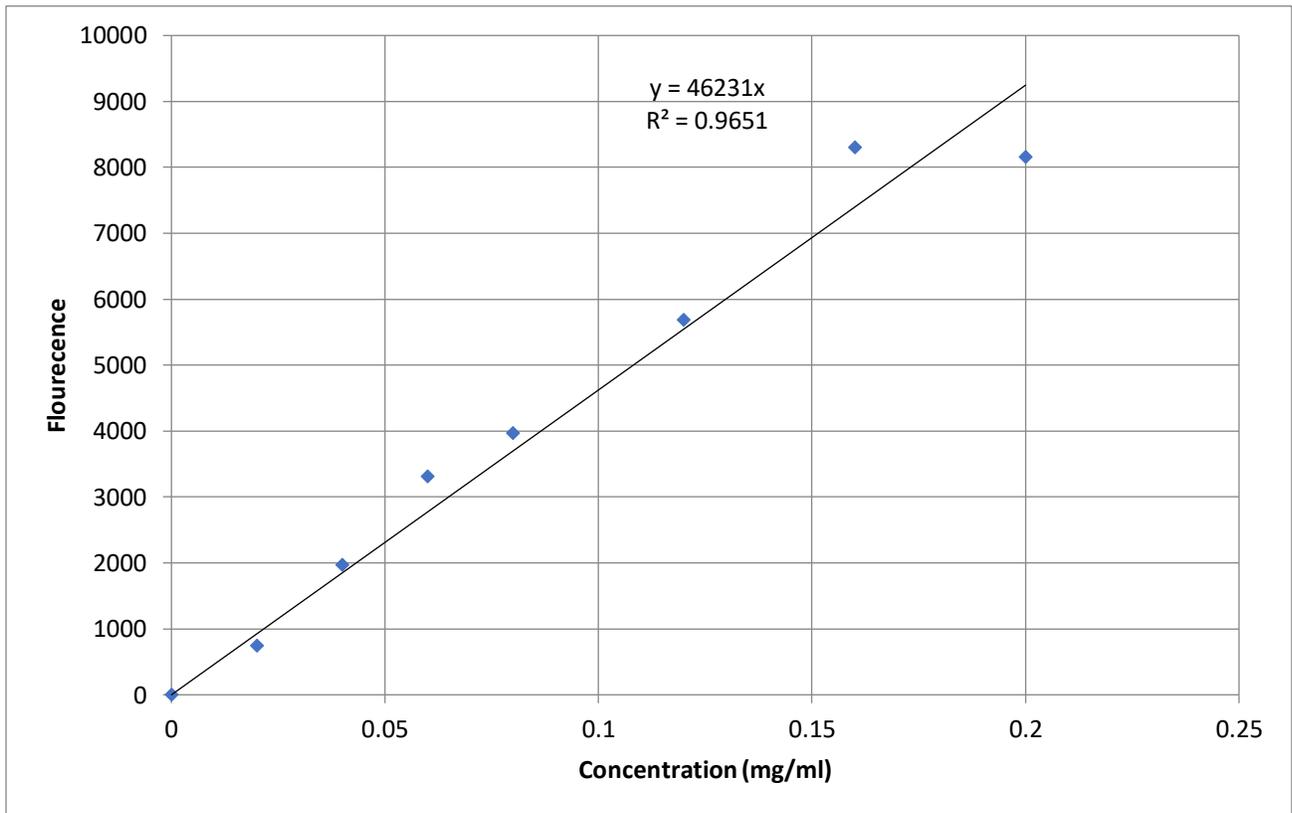
Figure 4.6: Triolein Standard curve for Tetraselmis suecica.

4.4.2.4 Dry weight versus Optical Density

The relationship between OD_{595} and cell dry weight was determined for *T. suecica* as described in section 2.7. The aim was to convert the OD_{595} value of the optimized concentration of cells to dry weight (mg/ml). Figure 4.7 shows the relationship between OD and dry weight for *T. suecica.* It can be seen that for *T. suecica,* Y = 0. 0.3769x, where Y = Dry weight (mg/ml) and x = OD_{595}. Hence the percentage of neutral lipid could be calculated by dividing the neutral lipid content by the value of the cell dry weight of the algae in the optimum concentration and then multiplying by 100.
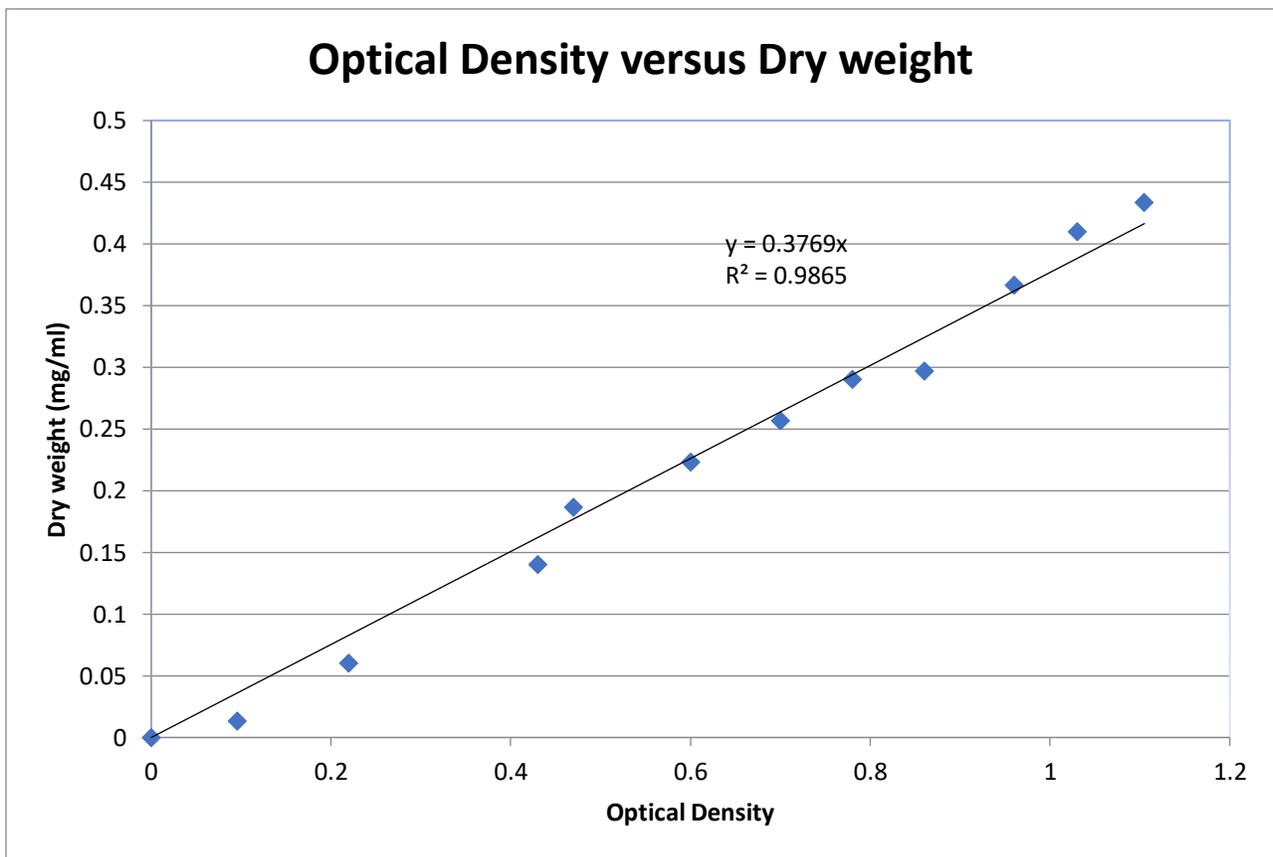
Figure 4.7: Relationship between Optical Density and dry weight of Tetraselmis suecica.

4.5 Neutral Lipids in *Tetraselmis suecica* under Normal and Stress Conditions

The relation between Optical Density and Dry weight was used to convert the values of Nile Red fluorescence obtained from the Triolein calibration curve into percentage of lipids for *T. suecica* cells under normal and stress conditions of salinity and nitrogen starvation.

4.5.1 Neutral Lipids in *Tetraselmis suecica* with Increasing Salinity

Figure 4.8 below shows the percentage of lipid content accumulated over a period of four weeks (week 1 - week 4) which is measured using the Nile red fluorescence method. It can be seen that the percentage of neutral lipid content decreases with increasing salinity stress for *T. suecica* cells. However, it should be noted that the trend over time is for neutral lipid to increase at all salinities tested i.e. as the cells enter stationary phase, more neutral lipid is produced. It can be seen also that the highest percentage of lipid content of *T. suecica* cells after four weeks of incubation was found in cells grown under normal conditions, followed by cells grown in 0.6M NaCl (Figure 4.8). The percentage of neutral lipid contents for the normal, 0.6M, 0.8M, 1.0M and 1.2M were 2.7%, 2.1%, 1.6% and 1.3%, respectively, as the cells enter stationary phase in the fourth week.
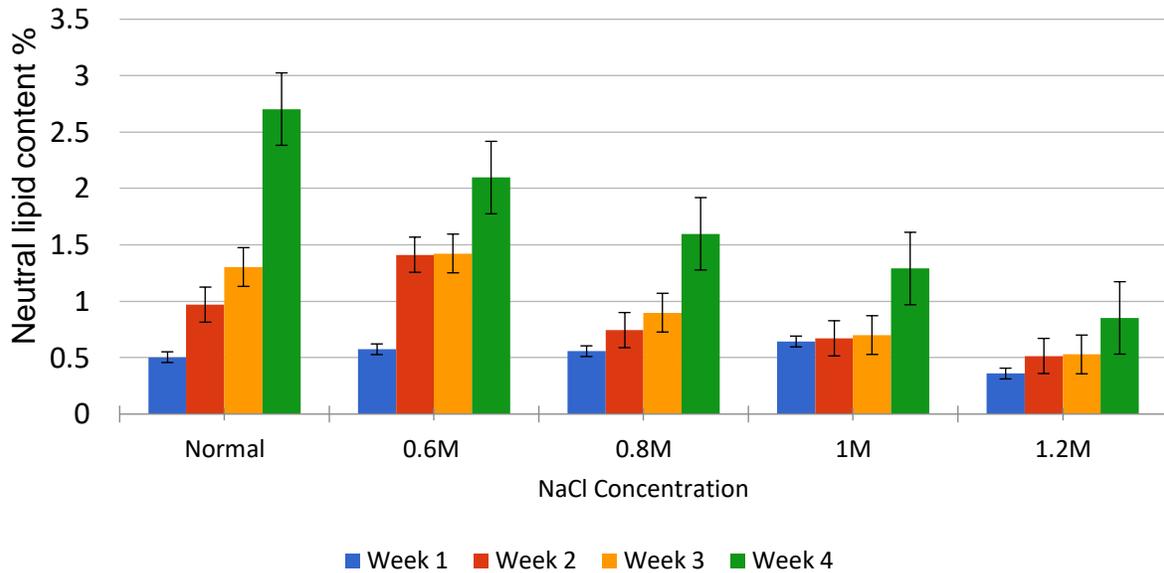
Figure 4.8: Percent neutral lipid content measured by Nile red fluorescence for Tetraselmis suecica under increased salinity conditions. Measurements were made weekly for four weeks. Bars are standard deviation for three replicates.

4.5.1.1 Statistical Analysis for *Tetraselmis suecica* with Increasing Salinity

The t-Test analysis for *Tetraselmis suecica* with increasing salinity is shown below for Two-Sample Assuming Unequal Variances (normal and 0.6M NaCl conditions).

H_0: $\mu_1 - \mu_2 = 0$: Null hypothesis: There is no significant difference in the sample mean

H_1: $\mu_1 - \mu_2 \neq 0$: Alternative hypothesis: There is significant difference in the sample mean

t-Test for *Tetraselmis suecica* with increasing salinity

	Variable 1 (Normal)	Variable 2 (0.6M NaCl)
Mean	1.918653532	1.927845061
Variance	1.758396989	0.761437684
Observations	4	4
Hypothesized Mean Difference	0	
Df	5	
t Stat	-0.011580618	
P(T<=t) one-tail	0.495604038	
t Critical one-tail	2.015048373	
P(T<=t) two-tail	0.991208075	
t Critical two-tail	2.570581836	

If t Stat< -t Critical two-tail or t Stat >t Critical two-tail

Reject null hypothesis

If -t Critical two-tail < t Stat value < t Critical two-tail

Accept null hypothesis

In this case, -2.57 < -0.012 < 2.57

There is no significant difference

The observed difference between the sample means (1.91 - 1.92) is not convincing enough to say that the average percentage of lipid accumulated between 0.6M NaCl and normal condition differs significantly based statistical analysis using t-Test

4.5.2 Neutral Lipids in *Tetraselmis suecica* under Nitrogen Starvation

Figure 4.9 below shows the percentage of lipid content accumulation over four weeks (week1 - week 4) for *T. suecica* cells subjected to nitrogen limitation and starvation. It shows that the percentage of neutral lipid content increased with increasing nitrogen starvation from 50% nitrogen to nitrogen free for *T. suecica.* It can be seen also that the highest percentage of lipid content of *Tetraselmis suecica* was in the nitrogen free environment after four weeks of incubation (Figure 4.9). The percentage neutral lipids accumulation with 100%, 50%, 25% nitrogen and nitrogen free environment were 2.4%, 6.1%, 8% and 10.2%, respectively, as the cells entered stationary phase in week four.
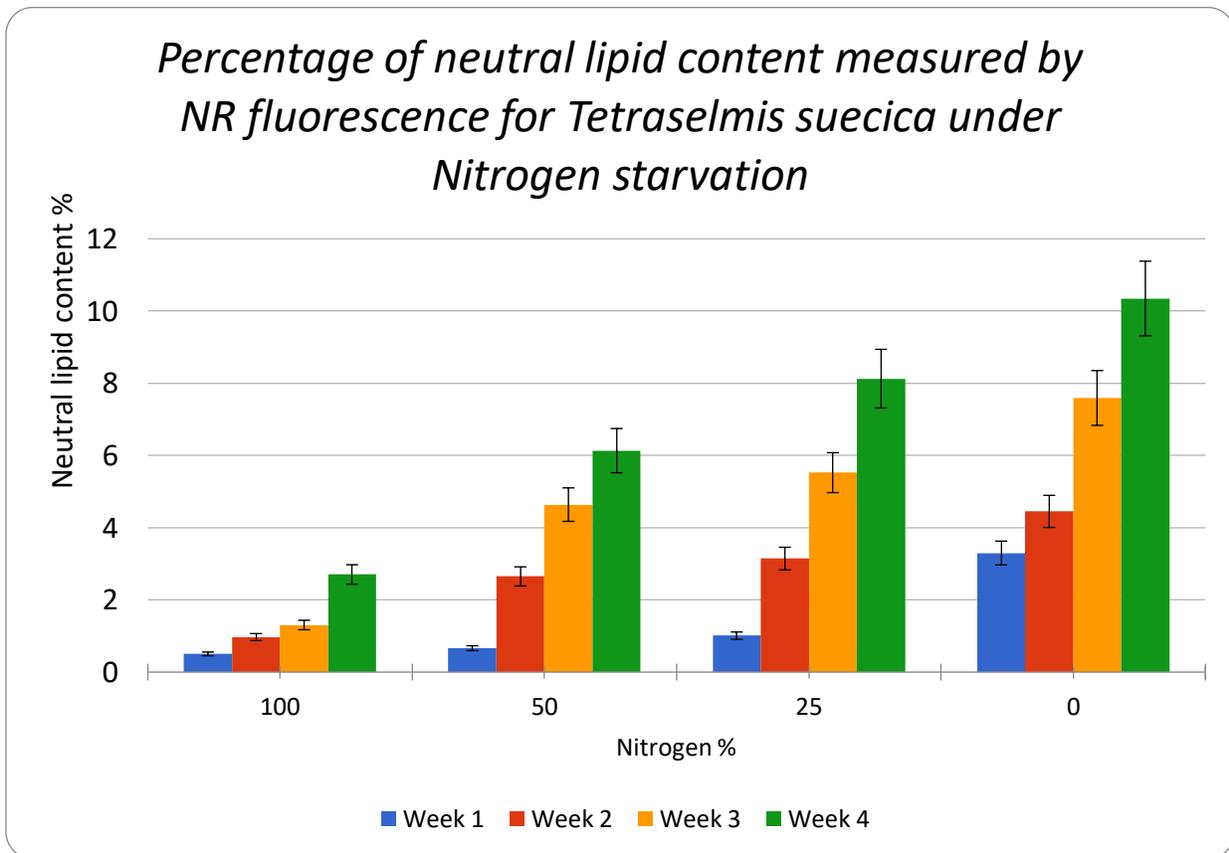
Figure 4.9: Percent neutral lipid content accumulation over four weeks measured by Nile red fluorescence for Tetraselmis suecica under nitrogen starvation. Bars are standard deviations for three replicates.

4.5.2.1 Statistical Analysis for *Tetraselmis suecica* under Nitrogen Starvation

The t-Test analysis for *Tetraselmis suecica* under Nitrogen Starvation is shown below for Two-Sample Assuming Unequal Variances (normal and nitrogen-free conditions).

H_0: $\mu_1 - \mu_2 = 0$:	Null hypothesis: There is no significant difference in the sample mean
H_1: $\mu_1 - \mu_2 \neq 0$:	Alternative hypothesis: There is significant difference in the sample mean

t-Test for *Tetraselmis suecica* under Nitrogen Starvation

	Variable 1 (Nitrogen-free)	Variable 2 (Normal)
Mean	8.989352923	1.918653532
Variance	19.86729759	1.758396989
Observations	4	4
Hypothesized Mean Difference	0	
Df	4	
t Stat	3.040936398	
P(T<=t) one-tail	0.019183514	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.038367029	
t Critical two-tail	2.776445105	

If t Stat< -t Critical two-tail or t Stat >t Critical two-tail	Reject null hypothesis
If -t Citical two tail < value < t Critical two-tail	Accept null hypothesis
In this case, 3.04 > 2.78	There is a significant difference
	The observed difference between the sample means (8.99 - 1.91) is convincing enough to say that the average percentage of lipid accumulated between Nitrogen free and normal condition differs significantly based statistical analysis using t-Test

4.6 Fatty Acid Methyl Esters (FAMEs) from the neutral lipid using GC-MS

To identify fatty acid methyl esters (FAMEs) that occur in *T. suecica* neutral lipid, gas chromatography mass spectrometry (GC-MS) was used and samples were prepared based on the procedure described in section 2.9.2. Figure 4.10 shows the FAMEs found in cells grown under normal conditions.
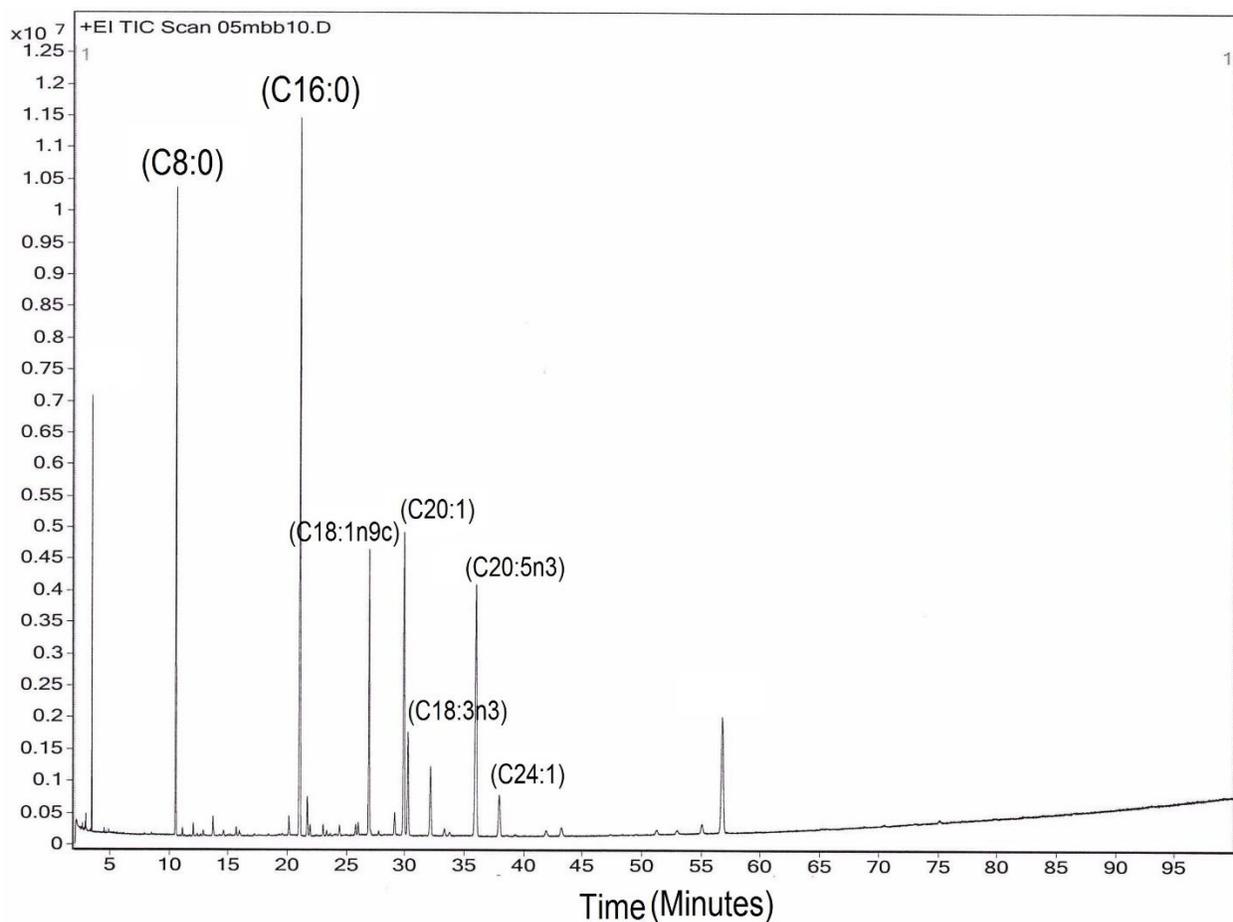
Figure 4.10: Fatty Acid Methyl Esters (FAMEs) found in Tetraselmis suecica grown under normal conditions.

As seen in Figure 4.10 above, the lipid of *T. suecica* cells grown under normal conditions contained several FAMEs such as (C8:0) caprylic acid methyl ester, (C16:0) palmitic acid methyl ester, (C18:1n9c) oleic acid methyl ester, (C20:1) cis-11,14-eicosenoic acid methyl ester, (C18:3n3) linolenic acid methyl ester, (C20:5n3) cis-

5,8,11,14,17-eicosapentaenoic acid methyl ester, and (C24:1) nervonic acid methyl ester.

4.6.1 Fatty Acid Profiles under higher Salinities
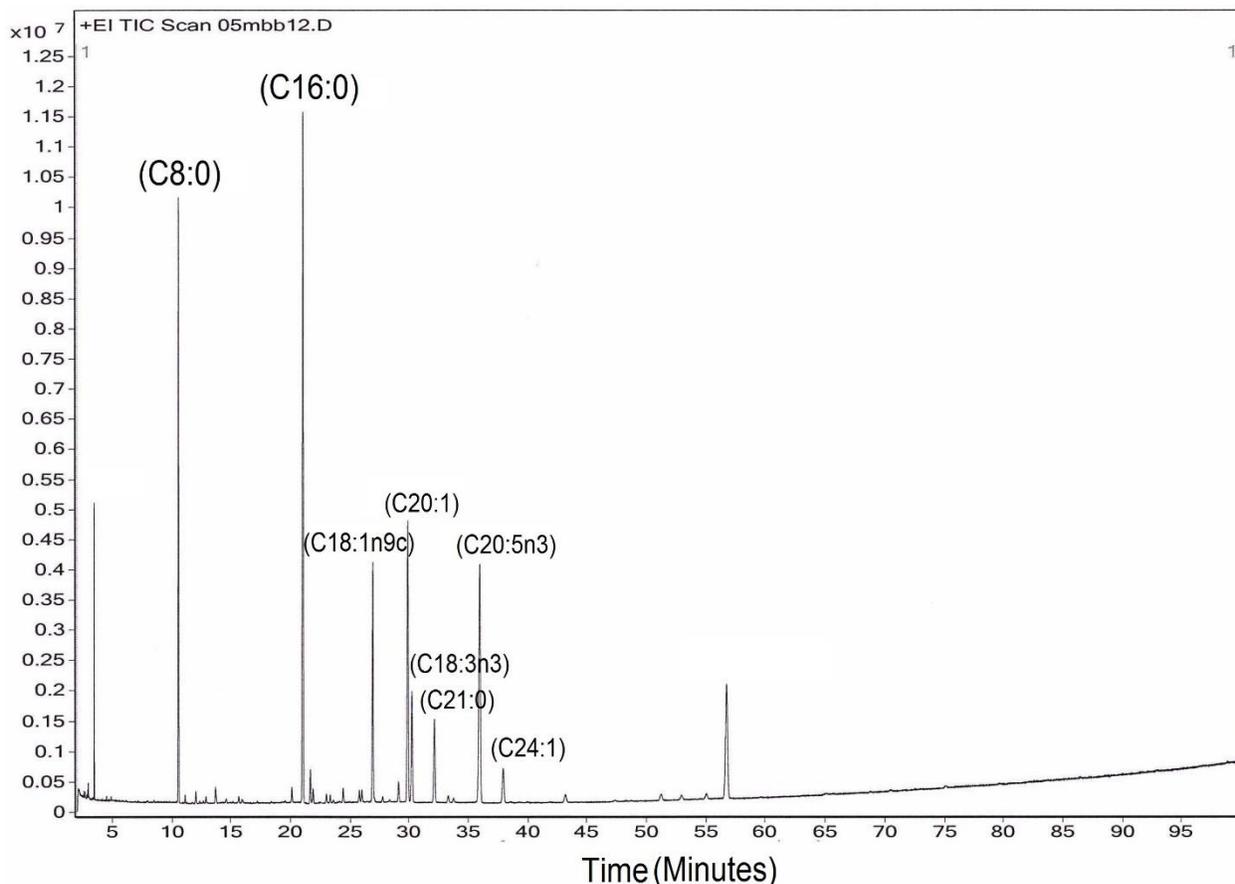
Figure 4.11: Fatty Acid Methyl Esters (FAMEs) found in Tetraselmis suecica grown in 0.6M NaCl Medium.

As under normal conditions, in higher salinity (0.6M NaCl) the lipid contained several FAMEs such as (C8:0) caprylic acid methyl ester, (C16:0) palmitic acid methyl ester, (C18:1n9c) oleic acid methyl ester, (C20:1) cis-11,14-eicosenoic acid methyl ester, (C18:3n3) linolenic acid methyl ester, (C20:5n3) cis-5,8,11,14,17-eicosapentaenoic acid methyl ester, and (C24:1) nervonic acid methyl ester as shown in Figure 4.11. This means that there was no change in the FAME composition with increased salinity to 0.6M NaCl.

4.6.2 Fatty Acid Profiles under Nitrogen starvation
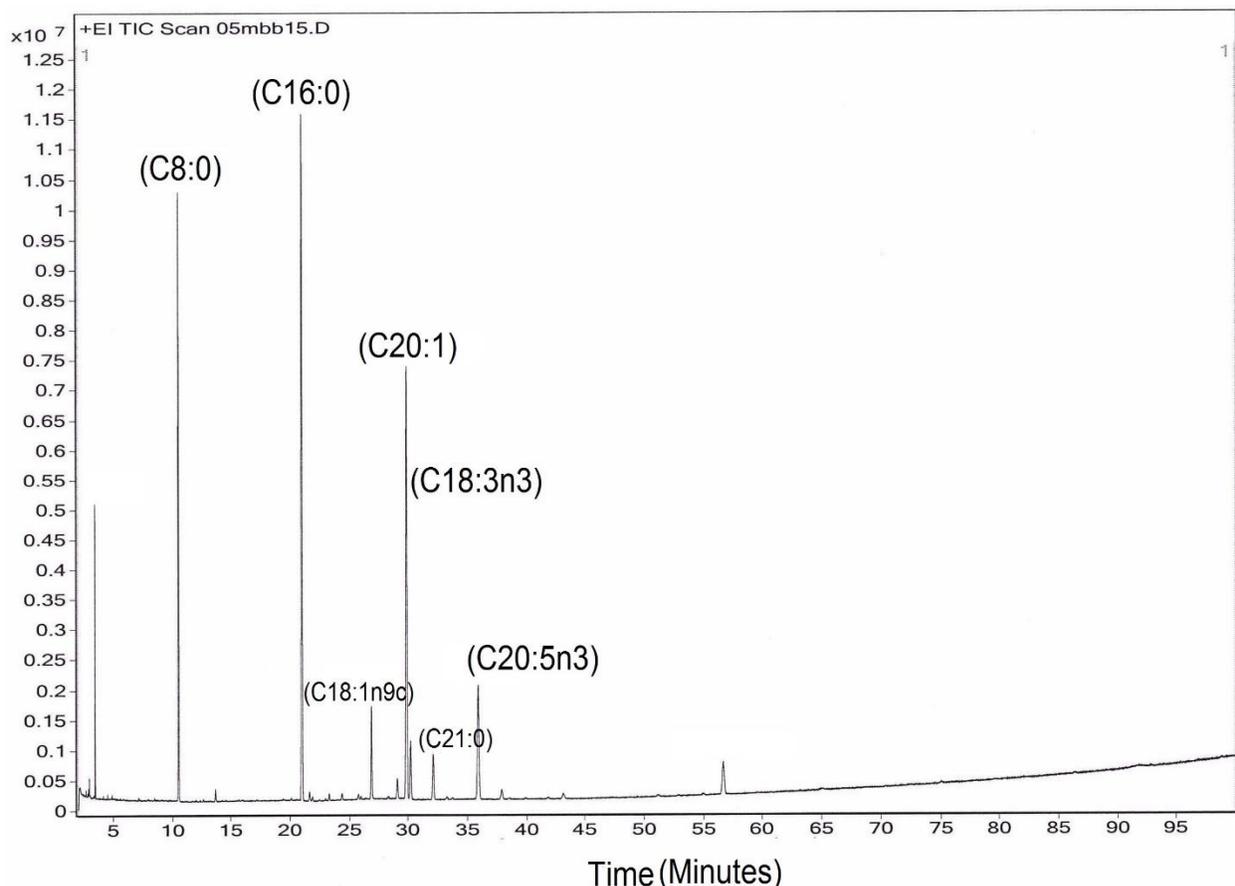
Figure 4.12: Fatty Acid Methyl Esters (FAMEs) found in Tetraselmis suecica grown in Nitrogen-Free Medium.

In the case of growing *T. suecica* in nitrogen free medium, the same FAMEs that occurred in normal and 0.6M NaCl conditions also occur in the nitrogen free sample which are (C8:0) caprylic acid methyl ester, (C16:0) palmitic acid methyl ester, (C18:1n9c) oleic acid methyl ester, (C20:1) cis-11,14-eicosenoic acid methyl ester, (C18:3n3) linolenic acid methyl ester, (C20:5n3) cis-5,8,11,14,17-eicosapentaenoic acid methyl ester, and (C24:1) nervonic acid methyl ester ass shown in Figure 4.12 above.

4.7 *Tetraselmis suecica* Compatible Solutes Analysis using Nuclear Magnetic Resonance (NMR) Analysis

For microalgae to survive extreme saline conditions, microorganisms need to synthesize compatible solutes to balance the increase in osmotic pressure in the external medium. The NMR results shown in Figure 4.13 identified betaine at 3.78ppm, acetate at 1.95ppm and lactate at 1.3ppm in the normal, 0.6M NaCl and Nitrogen free conditions as the compatible solutes for *T. suecica*. Betaine performs an important function in microalgae subjected to environmental stresses. It plays an adaptive role in mediating osmotic adjustment and protecting the sub-cellular structures in stressed algal and bacterial cells (Cummings et al., 1995).
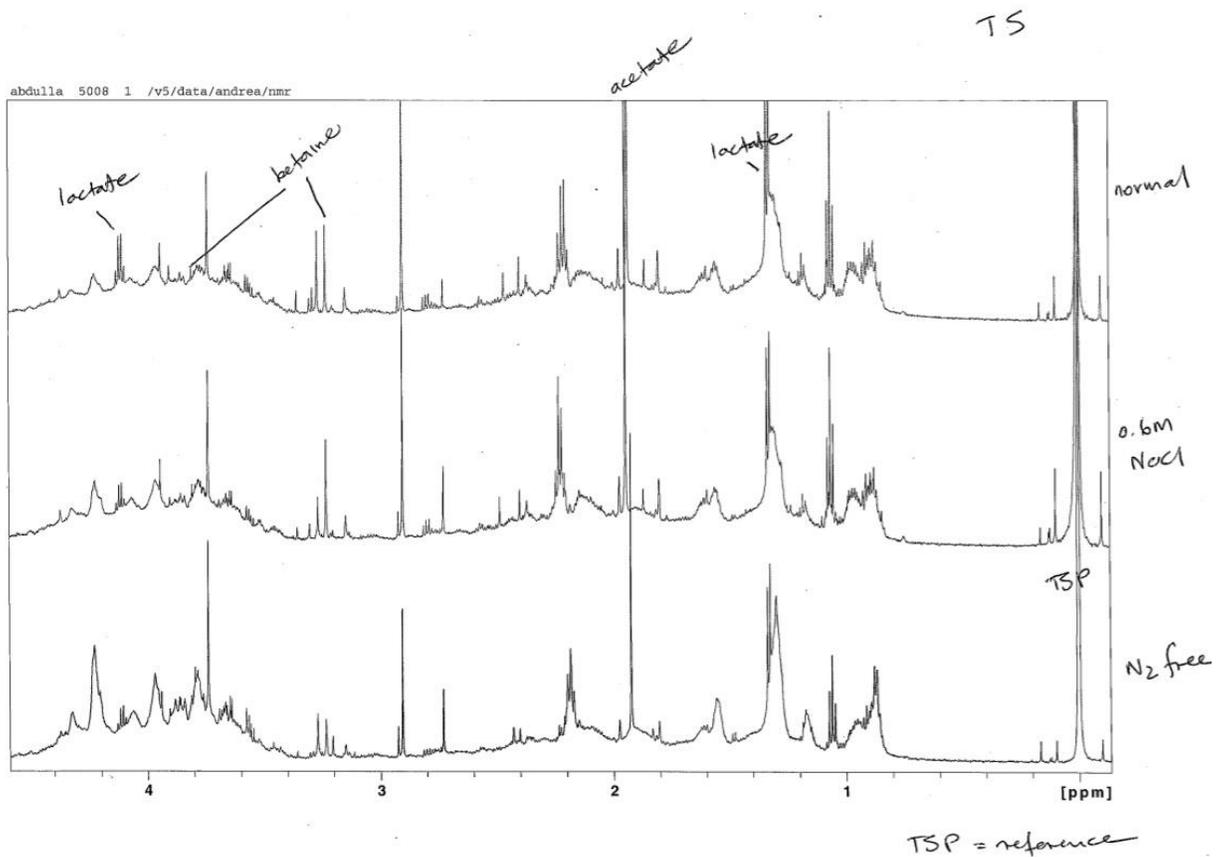
Figure 4.13: NMR Spectra for Tetraselmis suecica cell-free extracts grown under normal, high salinity and N-free conditions.

4.8 Discussion

In Chapter 3, *Botryococcus braunii* was studied as it was thought to give a better basis of stored neutral lipids for biodiesel production than the neutral lipids normally stored by the other algae. However, exploitation of *Botryococcus braunii* was very difficult as it was found to be slow growing and it could not grow under the stress of higher salinities to improve the accumulation of lipid content. Hence, an alternative strain of *Tetraselmis suecica* (CCAP 66/4) was obtained and through the course of this chapter evaluated by optimizing and modifying its growth conditions to study its neutral lipid accumulation under normal and stress growth conditions.

It was also cultivated under stress conditions of higher salinities by adding NaCl, 0.2M, 0.4M, 0.6M, 0.8M, 1.0M and 1.2M NaCl solution to give a higher salinity defined F/2 medium. It was seen that the growth rate and final biomass level reached after 30 days were decreased as salinity increased. It was also found from the level of growth that there was a significant difference at the highest salinity tested (1.2M NaCl) from that at normal condition; and that the normal condition showed a higher growth rate and final biomass level as indicated in Figure 4.2

Tetraselmis suecica was also grown under nitrogen starvation with 50%, 25% nitrogen and nitrogen free environment. It was found that the growth rate and final biomass level reached after 30 days were decreased with nitrogen starvation. It was also seen from the level of growth that there was a significant difference at the nitrogen free, 25% and 50% environment from that at the normal condition. However, the normal condition showed a higher growth rate and final biomass level as indicated from Figure 4.3.

The total lipid content of full-grown samples taken from the normal, 0.6M NaCl and Nitrogen free conditions were measured using the gravimetric method adapted from Chiu et al. (2009). The total lipid content for the well-grown culture of *T. suecica* under normal conditions was found to be 3.7 mg/ml in 12 mg/ml dry weight biomass, 2.9 mg/ml in 10 mg/ml dry weight biomass for 0.6M NaCl, and 1.5 mg/ml in 2.7 mg/ml dry weight biomass for Free Nitrogen. Hence the percentage of lipid content of the dry weight biomass was found to be around 31%, 29% and 56% for normal, 0.6M NaCl and

Nitrogen free conditions, respectively. However, the concentration per ml of neutral lipid was highest under normal conditions and actually decreased under the stress conditions. This makes the point that decreased biomass produced under stress conditions can reduce the overall lipid production, even though the amount of lipid per cell may have increased.

Nile red fluorescence was used to quantify the cellular neutral lipid content of *T. suecica*. Accurate determination of neutral lipid using this method mainly depends on the best concentration of algal cells, the best concentration of Nile red to be used and finally the best peak time that showed the highest fluorescence intensity of Nile Red using a Varian 96-well plate reader (Biotek flx800). Hence, the best *T. suecica* cell concentration was found to be 75% after normalization. Likewise, the ideal Nile red concentration was found to be 128 µg/ml at a peak time of 15 minutes. Furthermore, these optimum results (cell concentration of 75% OD_{595} = 0.7, and 0.4 µmol ml^{-1}) were used to prepare Triolein calibration curve for *Tetraselmis suecica* as shown in Figure 4.6.

The relationship between Optical Density and cell dry weight was shown in Figure 4.7. It was found that for *Tetraselmis suecica,* Y = 0. 0.3769x, where Y = Dry weight (mg/ml) and x = OD_{595}. Along with the Triolein concentration curve, this allowed the calculation of percentage lipid content accumulation over a four weeks period. The highest percentage of lipid content accumulated by *T. suecica* after the normal condition was in higher salinity (0.6M NaCl) after a four weeks incubation period. The percentage of neutral lipid content for the normal, 0.6M, 0.8M, 1.0M and 1.2M were found to be 2.7%, 2.1%, 1.6% and 1.3%, respectively, as the cells entered stationary phase in the fourth week. It was also clear that the percentage of neutral lipid content increased with increasing nitrogen starvation from 50% nitrogen to nitrogen free for *T. suecica.* The highest percentage of lipid content of *Tetraselmis suecica* was in the nitrogen-free environment after four weeks of incubation (10.2%). The percentage neutral lipids accumulation with 100%, 50%, 25% nitrogen and nitrogen-free environment were found to be 2.4%, 6.1%, 8% and 10.2%, respectively, as the cells entered stationary phase in week four. The overall conclusion from the stress experiments is that nitrogen stress is

much more effective at inducing lipid accumulation in *T. suecica* cells than salinity stress and this agrees with previous work on *Tetraselmis* (Alsull and Omar, 2012).

For microalgae to survive extreme saline conditions, microorganisms need to synthesize compatible solutes to balance the increase in osmotic pressure in the external medium. The NMR results identified betaine at 3.78ppm, acetate at 1.95ppm and lactate at 1.3ppm in the normal, 0.6M NaCl and Nitrogen free conditions as the compatible solutes for *T. suecica*. Betaine performs an important function in microalgae subjected to environmental stresses. It plays an adaptive role in mediating osmotic adjustment and protecting the sub-cellular structures in stressed algal and bacterial cells (daCosta et al., 1998). Betaine is also found in human diet and sometimes as dietary supplements, and because it occurs entirely as a simple solute, which is highly water soluble and is not protein bound, it has two important roles in mammalian physiology. One is as a major osmolyte, accumulated in most tissues to assist cell volume regulation and the other role is as a methyl donor for the remethylation of homocysteine to methionine (Feng et al., 2001; Schliess and Haussinger, 2002).

Total algal lipids could be expressed as biofuel relevant fatty acid methyl esters (FAME) and could be identified as percent FAME content. Therefore, the FAME profiles from the neutral lipids of *T. suecica* were determined using GC-MS for cells cultured in both higher salinities and under nitrogen starvation. The results of GC-MS showed that there are several fatty acid methyl esters found in the neutral lipid of *T. suecica* such as (C16:0) palmitic acid methyl ester, (C18:1n9c) oleic acid methyl ester, and (C18:3n3) linolenic acid methyl ester. The first two are suitable for biofuel production since they are saturated or monounsaturated fatty acids, but polyunsaturated fatty acids such as linolenic acid are important dietary supplements, but are not suitable for biofuel production due to their inherent instability (Stansell et al., 2012).

In this chapter, *T. suecica* has been shown to be a better candidate for biofuel production than *B. braunii*, but the levels of neutral lipid accumulation still fall well below the 30% required for commercial exploitation (Chisti, 2007). Therefore, in the next results chapter, the contaminant species of *B. braunii* cultures, *Coccomyxa* was evaluated for its ability to act as a feed stock for biofuel production.

Chapter 5: Molecular Identification and Lipid Content of *Coccomyxa* under Normal and Stress Conditions

5.0 Introduction

In the process of culturing *Botryococcus braunii* in a 250 ml flask culture, *Coccomyxa* appeared and outgrew *B. braunii.* Therefore, *Coccomyxa* was also incorporated into this study to investigate its growth and neutral lipids accumulation as a member of the Chlorophyta (green algae), the genius *Coccomyxa* (Trebouxiophyceae, Chlorococcales, Coccomyxaceae), comprises together free-living planktonic freshwater and marine species (Guiry et al., 2005), protozoans (Hoshina & Imamura, 2008), epiphytic (Lamenti et al., 2000) and symbiotic species including lichens (Lohtander et al., 2003) or even trees (e.g. in Ginkgo biloba; Trémouillaux-Guiller et al., 2002).

The identity of *Coccomyxa* was confirmed using 18S rDNA sequencing and then it was grown in purified 3N-BBM+V medium with continuous irradiance (50 – 70 µmol m^{-2} s^{-1}) at 25°C in shaken (80 rpm) flasks. The medium was renewed every 3 to 4 weeks to reach sufficient biomass to carry out experiments. It was grown in higher salinities and under nitrogen starvation stress using media as prepared in sections 2.3.2.1 and 2.3.2.2, respectively.

In both normal and stress conditions of the well grown *Coccomyxa* cells, the total lipid content was measured using the gravimetric method, while Nile red fluorescence was used to quantify the cellular neutral lipid content. As required for accurate determination of neutral lipid in *Coccomyxa* using this method, the best concentration of algal cells, the best concentration of Nile Red to be used and finally the best peak time that showed the highest fluorescence intensity of Nile Red using a Varian 96-well plate reader was determined in order to prepare the Triolein calibration curve using a procedure adapted from Bertozzini et al. (2011). This will then allow for the quantification of neutral lipid within algal cells (a concentration curve for Nile-Red fluorescence versus concentration

of Triolein) by converting the OD readings to dry weight which can be utilized to estimate the percentage of neutral lipids within the algal cells.

For microalgae to survive extreme stress conditions, microorganisms need to synthesize compatible solutes to balance the increase in osmotic pressure in the external medium. Hence the compatible solute production in *Coccomyxa* was also analysed using NMR with increasing salinity and under nitrogen starvation. To the best of my knowledge, this is the first time that compatible solute production has been determined in *Coccomyxa.*

Total algal lipids can be expressed as biofuel relevant fatty acid methyl esters (FAME) and could be expressed as percent FAME content based on the dry weight of the algal biomass. Therefore, the FAME profiles from the neutral lipids in *Coccomyxa* were determined using GC-MS in cultures exposed to both higher salinities and nitrogen starvation.

5.1 Aims and Objectives

The aims and objectives of this chapter are stated below:

1. Isolate and identify the new strain of *Coccomyxa* using 18S rDNA sequencing.

2. Optimise growth media (3N-BBM+V) to cultivate *Coccomyxa* and monitor any improvement in growth.

3. Quantify the lipid content of *Coccomyxa* using dry weight measurements, lyophilisation, a gravimetric method and Nile Red fluorescent dye staining.

4. Quantify the Fatty Acid Methyl Esters (FAMEs) in *Coccomyxa* using GC-MS.

5. Quantify the compatible solutes in *Coccomyxa* using NMR.

5.2 Molecular Identification of *Coccomyxa* using 18S rDNA Sequencing

It was observed that one of the flask of *Botryococcus braunii* culture transformed its colour from light green (*Botryococcus braunii* colour) to dark green colouration, hence it was viewed under the microscope and found that it was another strain appeared and outgrew *Botryococcus braunii* in the culture and this strain was found to be *Coccomyxa*, but the identity of the strain had to be confirmed using 18S rDNA sequencing. Figure 5.1 below shows the microscopic image of *Coccomyxa* strain. The DNA was first extracted then amplified using PCR and purified for sequencing using 18S rDNA and identification with BLAST.
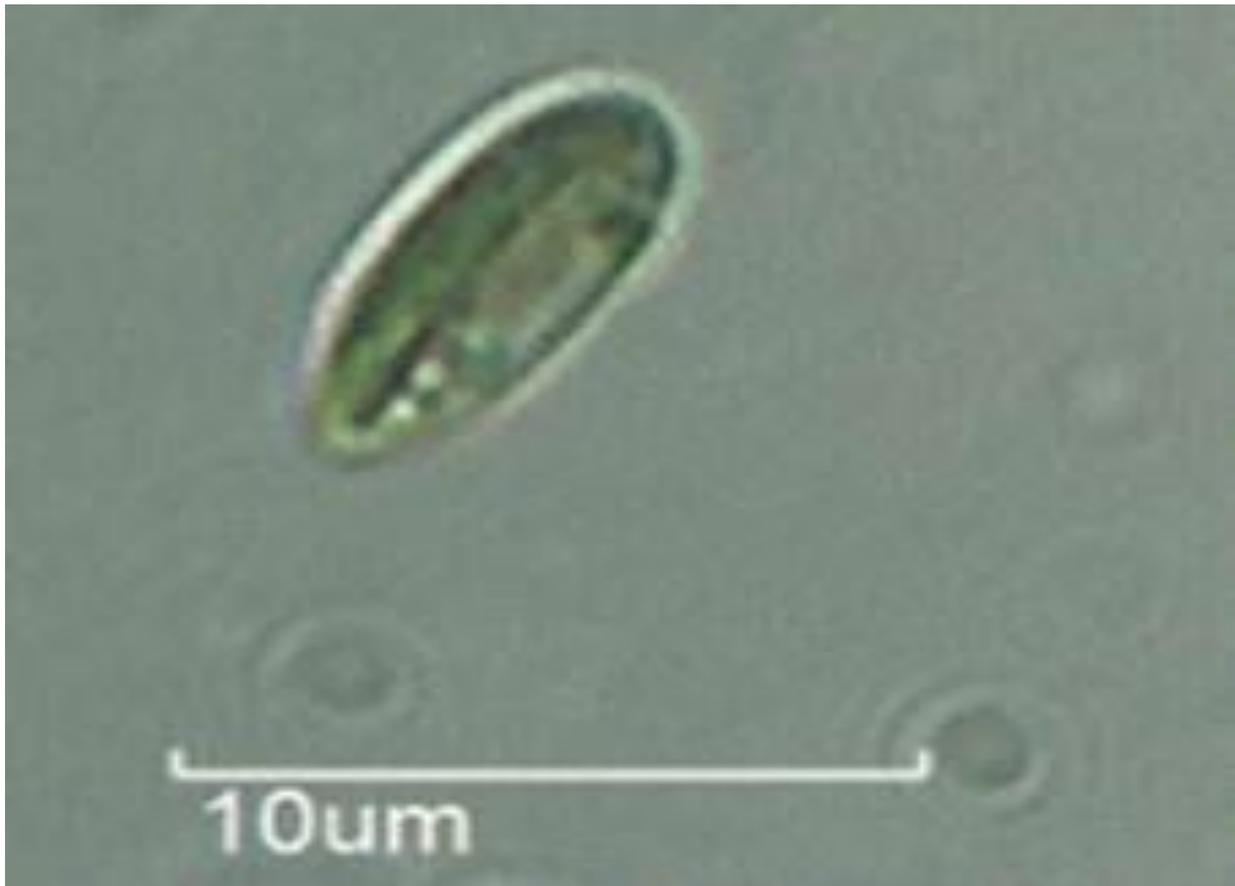
Figure 5.1: Microscopic image of Coccomyxa strain.

5.2.1 DNA extraction and PCR

Coccomyxa cells were harvested through centrifugation and the genomic DNA was extracted using ZR Soil Microbe DNA Microprep Kit as described in section 2.5.1. The method successfully extracted the genomic DNA showing a clear bright DNA band on the gel (Figure 5.2), the band was amplified using PCR.
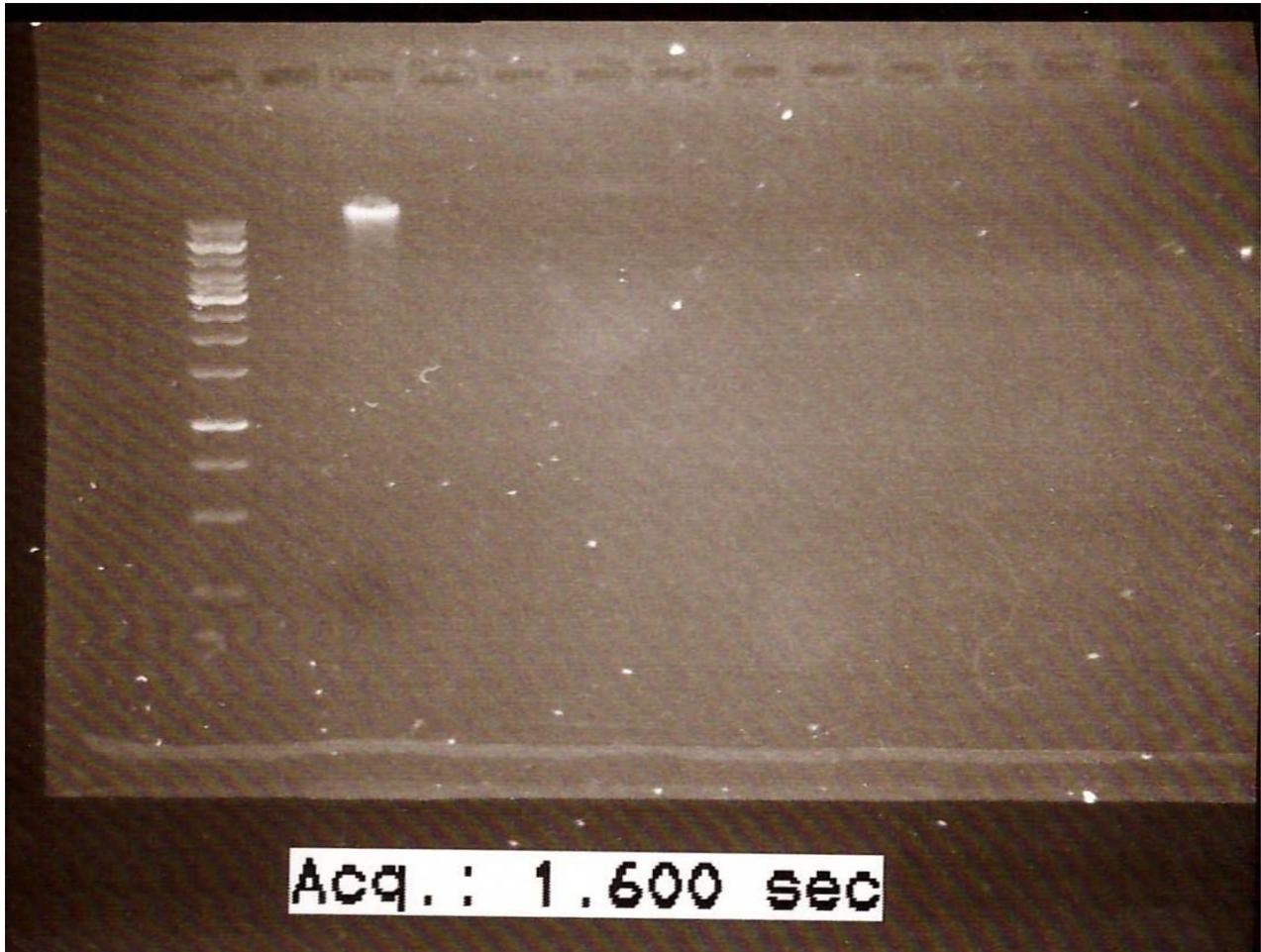
Figure 5.2: Samples of extracted DNA run in a 1% agarose gel. Lane 1 is the ladder.

5.2.2 PCR Amplification

18S rRNA gene was amplified using the Lim primers and the correct sized band (approximately 1800 bp) was amplified (Figure 5.3). The DNA was cleaned up using a PCR purification kit.

*Figure 5.3: PCR amplification results **u**sing 18S Lim primers, Lane 1 = ladder and lane 2 shows the 18S rDNA band at around 550 bp.*

5.2.3 PCR Purification

The amplified PCR products were purified using QIAquick PCR Purification kit (Qiagen) (Figure 5.4), the purified PCR products were of the correct size (approximately 550 bp). It clearly shows that 550bp bands remained after the purification process (Lanes 2 and 5).
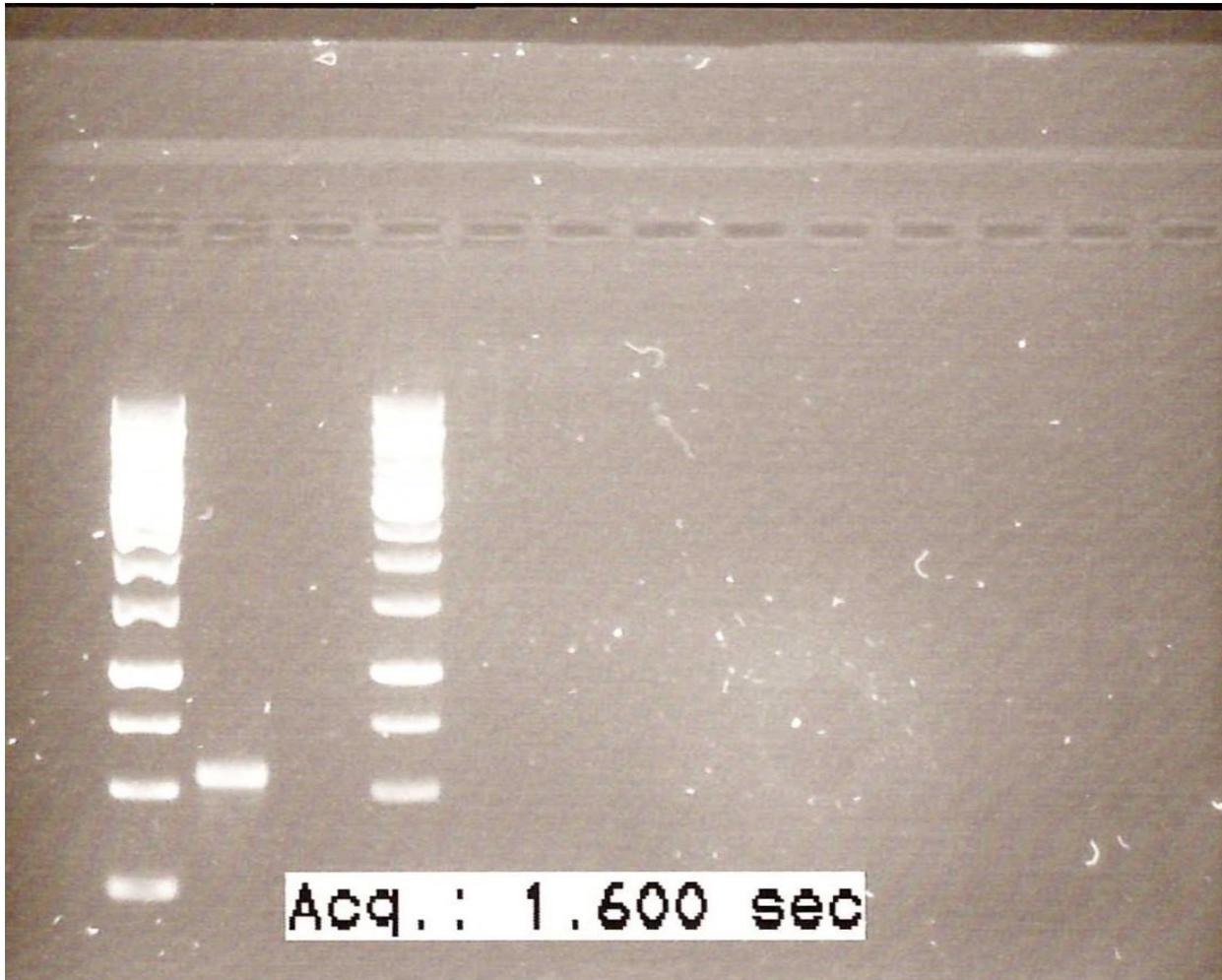
Figure 5.4: 18S rDNA after PCR Purification.

5.2.4 Sequencing of 18S rDNA and Identification using BLAST

After cleaned up using PCR purification kit, PCR products were sent for sequencing to Eurofins/MWG using a sample submission guide for sequences between 300 – 1000bp and the results showed that 500 bases of forward and 499 bases of reverse Lim 18S rDNA primer were successfully sequenced as shown below. Using BLAST on the NCBI website indicated that both forward and reverse Lim 18S primer sequences were highly matched with *Coccomyxa* (with 99% identity).

Forward 18S-Lim sequence (500 letters):

AGTTAAAGCTCGTAGTTGGATTTCGGGCGGGCTCGGCCGGTCCGCCTATGGGTGTGCACT

GACCGAGCCCGTCTTGTTGCCGGGGACGGGCTCCTGGGCTTAACTGTCCGGGACTCGGAG

TCGGCGAGGTTACTTTGAGTAAATTAGAGTGTTCAAAGCAGGCCTACGCTCTGAATACAT

TAGCATGGAATAACACGATAGGACTCTGGCCTATCTTGTTGGTCTGTGGGACCGGAGTAA

TGATTAAGAGGGACAGTCGGGGGGCATTCGTATTTCATTGTCAGAGGTGAAATTCTTGGAT

TTATGAAAGACGAACTACTGCGAAAGCATTTGCCAAGGATGTTTTCATTAATCAAGAACG

AAAGTTGGGGGGCTCGAAGACGATTAGATACCGTCCTAGTCTCAACCATAAACGATGCCGA

CTAGGGATTGGCGGGCGTTCTTTTGATGACCTCGCCAGCACCTTATGAGAAATCAAAGTT

TTTGGGTTGCGGGGGGGGGGTG

Reverse 18S-Lim sequence (499 letters):

CTAGGTGCTGGCGAGGTCATCAAAAGAACGCCCGCCAATCCCTAGTCGGCATCGTTTATG

GTTGAGACTAGGACGGTATCTAATCGTCTTCGAGCCCCCAACTTTCGTTCTTGATTAATG

AAAACATCCTTGGCAAATGCTTTCGCAGTAGTTCGTCTTTCATAAATCCAAGAATTTCAC

CTCTGACAATGAAATACGAATGCCCCCGACTGTCCCTCTTAATCATTACTCCGGTCCCAC

AGACCAACAAGATAGGCCAGAGTCCTATCGTGTTATTCCATGCTAATGTATTCAGAGCGT

AGGCCTGCTTTGAACACTCTAATTTACTCAAAGTAACCTCGCCGACTCCGAGTCCCGGAC

AGTTAAGCCCAGGAGCCCGTCCCCGGCAACAAGACGGGCTCGGTCAGTGCACACCCATAG

GCGGACCGGCCGAGCCCGCCCGAAATCCAACTACGAGCTTTTTAACTGCAACAACTTAAA

TATACGCTATTGGAGTAGAC

5.3 Algal Growth under Normal and Stress Conditions

5.3.1 Effects of Salinity on Growth

As *Coccomyxa* was observed to outgrow *Botryococcus braunii* in the same medium (3N-BBM+V), the medium was adopted to culture and study *Coccomyxa* as prepared in accordance with section 2.3.1. It was then grown under stress conditions of higher salinities by adding NaCl (0.1M and 0.2M NaCl) to give higher salinity 3N-BBM+V medium as prepared in section 2.3.2. These media were used to investigate the effect of salinity on the growth and lipid accumulation in *Coccomyxa*. Growth curves were obtained from the readings of optical density at 595 nm over a 30 days period. Figure 5.5 shows the growth curves for *Coccomyxa* grown under normal conditions and in different salinities. It can be seen from the Optical Density readings obtained that the growth rate and final biomass level reached after 30 days were decreased as salinity increased. It can also be seen from the level of growth that there was a significant difference at the other salinities from that under normal conditions. The *Coccomyxa* cells grown under normal conditions (i.e. 3N-BBM+V medium) showed a higher growth rate and final biomass level as seen below in Figure 5.2.
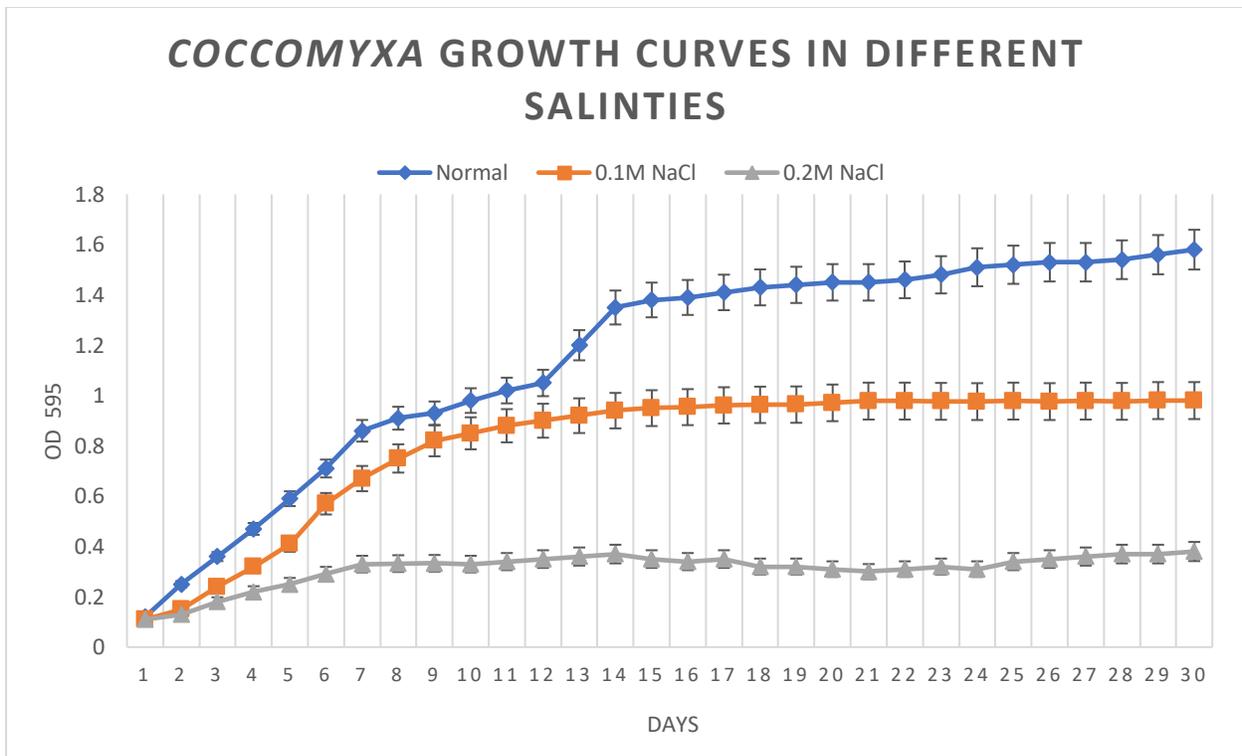
Figure 5.5: Coccomyxa Optical Density readings to show the growth rate at different salinities. Bars represent means ± standard error for three replicates.

5.3.2 Effects of Nitrogen Starvation

Growth curves of *Coccomyxa* under nitrogen depletion were set up in order to investigate the effects of nitrogen starvation on neutral lipid accumulation (Figure 5.6). It can be seen that the growth rate and final biomass level reached after 30 days were the same for 50%, 25% and normal nitrogen environment. Thus, means that for *Coccomyxa*, there was no significant difference in the growth rate and final biomass level for 50%, 25% and normal nitrogen environment. It can also be deduced from the level of growth that there was a little difference at nitrogen-free from that at 25%, 50% and normal nitrogen environment. The latter result is surprising, since cells should not be able to continue growing in the absence of nitrogen and this requires further work to find an explanation for the Nitrogen-free growth shown in Figure 5.6.

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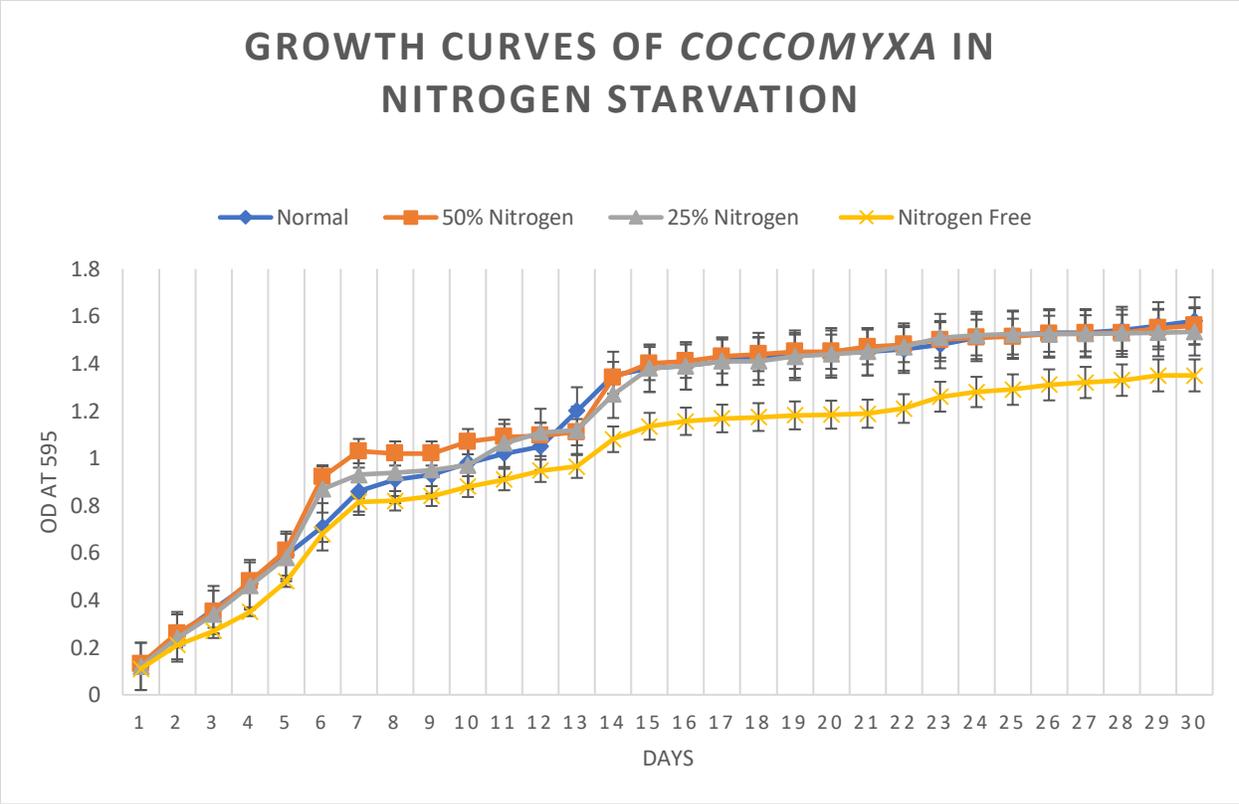
Figure 5.6: Coccomyxa Optical Density readings to show the growth rate under nitrogen starvation. Bars represent means ± standard error for three replicates.

5.4   Determination of Neutral Lipids in *Coccomyxa*

It has been discussed earlier that diverse methods have been utilized to determine and quantify algal lipids such as the gravimetric technique which is the most conservative method for algal lipid evaluation as adapted from Chiu et al. (2009). It is a procedure that principally uses cell disruption and solvent extraction of the algal lipids, and by drying and weighing the lipid extract, the total algal lipids can be quantified (Kumari et al., 2011). *Coccomyxa* was grown under normal conditions and stress to evaluate the effects on neutral lipids accrual. Hence, the total algal lipids and the percentage neutral lipids for full-grown *Coccomyxa* cultures in normal and stress conditions were studied in this part of the work.

5.4.1 Total Lipid Content by the Gravimetric Method

Full-grown samples of *Coccomyxa* were taken from the normal, 0.1M NaCl and nitrogen-free condition, and the lipid content was measured in accordance with the procedure discussed in section 2.6.1.
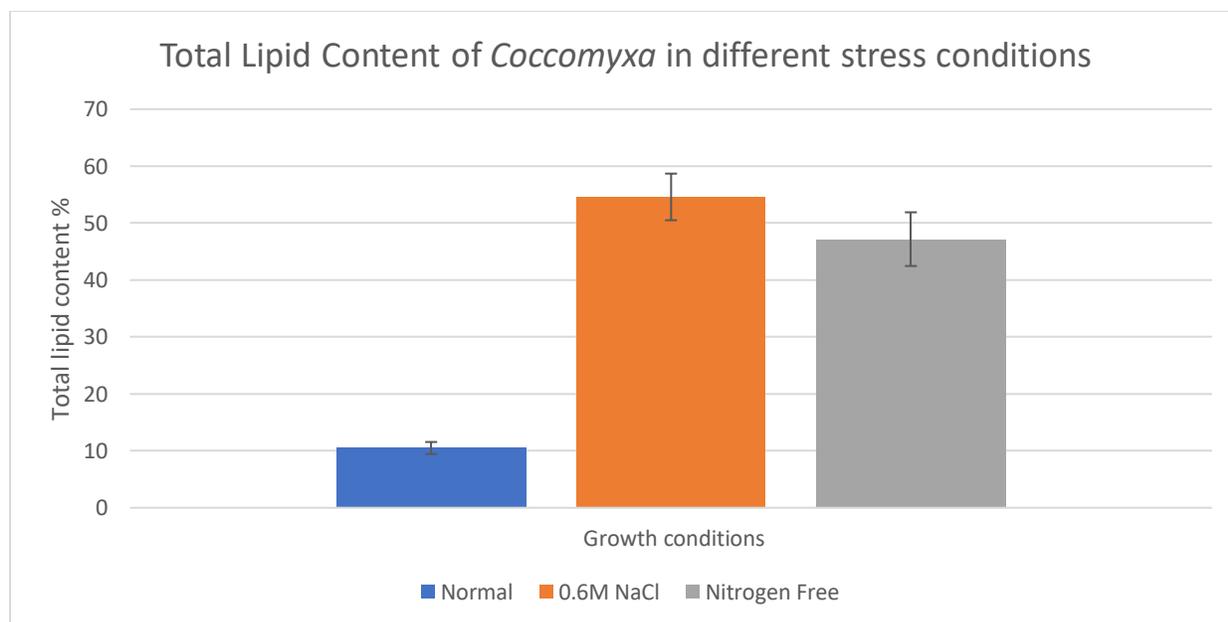
Figure 5.7: Coccomyxa grown in different Stress Conditions Total Lipid Content percentage by the Gravimetric Method. Bars represent means± standard error for three replicates.

As shown in Figure 5.7, the percentage lipid content of the dry weight biomass was found to be around 21%, 55% and 47% for normal, 0.1M NaCl and Nitrogen-free conditions, respectively. Therefore, in contrast to *Tetraselmis* (Chapter 4), increased salinity does increase total lipid production in *Coccomyxa*.

5.4.2 Nile red Method for Measuring Neutral Lipid Content

In Chapters 3 and 4, it was discussed that fluorescence spectroscopy has been utilized as the easiest, rapid and most cost-effective tool for studying the neutral lipid content of microalgae based on a lipid soluble glowing dye called Nile red (9-diethylamino-5H-Benzo[α]phenoxazine-5-one). The vital feature of employing Nile red as a fluorescent dye was to accomplish a quick screening of oleaginous microalgae to allow the selection of promising strains for economically feasible production of biofuel (Chisti, 2008). This is because it has the ability to pass through the cell barriers including the cell wall, cell membrane and dissolve in the intracellular neutral lipid, in combination with organic solvents like dimethyl sulfoxide (DMSO) to facilitate the staining of the microalgae with Nile red (Pancha et al., 2014; Wu et al., 2014).

Nonetheless, since the fluorescent intensity of Nile Red is variable, precise evaluation of neutral lipid in algal cells required an optimization of the algal cell concentration and Nile Red concentration and optimum time after staining. These parameters were again optimised for *Coccomyxa* by running the same set-up experiments as described in Chapters 3 and 4.

5.4.2.1 Optimum cell concentration and peak time for 96 well plate Nile-red method

The concentrations of the *Coccomyxa* cells were optimized as described in section 2.6.2. Figure 5.8 shows the fluorescence intensities of *Coccomyxa* for eight different percentage concentrations of the cells (i.e. 100, 87.5, 75, 62.5, 50, 37.5, 25 and 12.5% where 100% was $OD_{595} = 1$) over 30 minutes at 5 minute intervals starting from 15 minutes after staining with Nile red. It can be seen from the chart that the highest fluorescence intensity was at 62.5% and 20 mins peak time after staining, it had the

lowest standard deviation after normalization. Hence, the optimal cell concentration was 62.5% which is equivalent to an OD_{595} = 0.7.
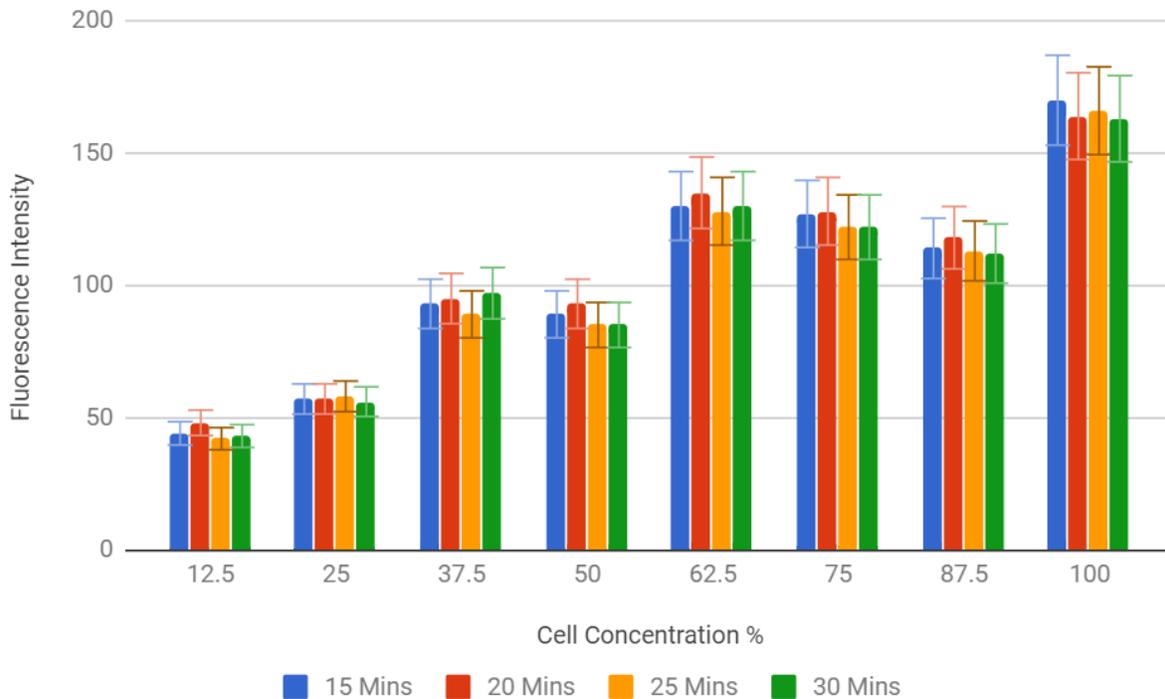
Figure 5.8: Nile Red staining time and cell concentration optimization for fluorescence intensity of Coccomyxa. Each column represents the mean of four readings plus/minus standard deviation.

The plate readings for the fluorescence intensity of *Coccomyxa* are shown in Tables 5.1 and 5.2 below for different cell concentrations ranging from 12.5% to 100 at the peak time (20 minutes) after staining with Nile red and the calculation of averages, standard deviation and normalization respectively. It can be deduced from Table 5.2 that the optimum concentration of *Coccomyxa* cells was found to be 62.5% based on the consistency with the standard deviation after normalization (lowest standard deviation, highest normalized and highest number when unstained was subtracted from stained).

Table 5.1: Plate readings of different algal cell concentrations at 20 minutes.

T° Read 1:485/20,580/50	Dilution%		100	87.5	75	62.5	50	37.5	25	12.5
Stained Cells	R1	A	369	303	305	320	271	274	235	226
	R2	B	360	279	290	296	248	259	222	210
	R3	C	369	281	290	299	251	258	221	212
	R4	D	370	281	292	296	251	269	242	217
Unstained Cells	R1	E	199	164	162	164	161	171	180	169
	R2	F	209	171	169	169	160	170	170	170
	R3	G	200	169	168	169	165	169	168	167
	R4	H	207	192	183	191	187	188	178	189

Table 5.2: Averages, standard deviation and normalised calculations of different algal cell concentrations at 20 minutes.

Concentration	100	87.5	75	62.5	50	37.5	25	12.5
Average Stained	366	286	294	303	255	265	230	216
STDEV Stained	6	1	1	2	2	6	12	4
Average Unstained	203	168	166	167	162	170	173	169
STDEV Unstained	6	4	4	3	3	1	6	2
Stain-Unstained	164	118	128	135	93	95	57	48
Normalised	2	1	2	2	2	3	2	4

5.4.2.2 Optimum concentration of Nile-Red

Optimum concentration of Nile-Red was also determined at appropriate time intervals for peak fluorescence (20 minutes) using the 62.5% algal culture with OD_{595} = 0.7 using the 96 well plate reader. Tables 5.3 and 5.4 below show the plate readings for the fluorescence intensity for the six different concentrations of the Nile red at the peak time of 20 minutes including the calculation of averages, standard deviation and subtraction of unstained cells from the stained. It can be deduced from Table 5.4 that the optimum Nile-Red concentration was 0.3 µmol ml^{-1} which is equal to 100 µg ml^{-1} based on the balance with the standard deviation.

Table 5.3: Plate readings of different Nile-Red concentrations at 20 minutes.

T° Read 1:485/20,580/50	Concentration		0.05	0.1	0.2	0.3	0.4	0.6
Stained Cells	R1	A	281	274	273	324	302	219
	R2	B	275	281	265	303	298	251
	R3	C	262	238	244	279	258	271
	R4	D	298	283	307	336	342	214
Unstained Cells	R1	E	181	161	157	159	157	157
	R2	F	184	163	161	159	161	161
	R3	G	178	165	161	160	164	164
	R4	H	195	176	177	181	180	180

Table 5.4: Averages, standard deviations and subtracting calculations of different Nile red concentrations at 20 minutes.

Concentration	0.05	0.1	0.2	0.3	0.4	0.6
Average Stained	285	279	282	321	314	228
STDEV Stained	12	5	22	17	24	20
Average Unstained	181	163	160	159	161	161
STDEV Unstained	3	2	2	1	4	5
Stain-Unstained	104	116	122	162	153	67

5.4.2.3 *Coccomyxa* Triolein Calibration Curve

Triolein concentration calibration curve was prepared as described earlier in section 2.6.2.4 in order to quantify the neutral lipid in *Coccomyxa*. Triolein is a standard neutral lipid derived from glycerol and three molecules of unsaturated oleic fatty acids. Various concentrations of Triolein with isopropanol were added to the well grown *Coccomyxa* cells and the mixture of different concentrations were stained with Nile Red dye. The reason for adding algae to the concentration curve has been discussed earlier in Chapters 3 and 4 (i.e. to take into account the potential absorption of Nile Red fluorescence by algal pigments). The Triolein calibration curve was plotted with the cell concentration of 62.5% (OD_{595} = 0.7), and 0.3 µmol ml^{-1} (100 µg ml^{-1}) as the concentration of Nile red dye found in the previous section (section 5.3.2.2). The resulting Triolein standard curve for *Coccomyxa* is shown in Figure 5.9. Hence, the neutral lipid in *Coccomyxa* cells can be quantified.
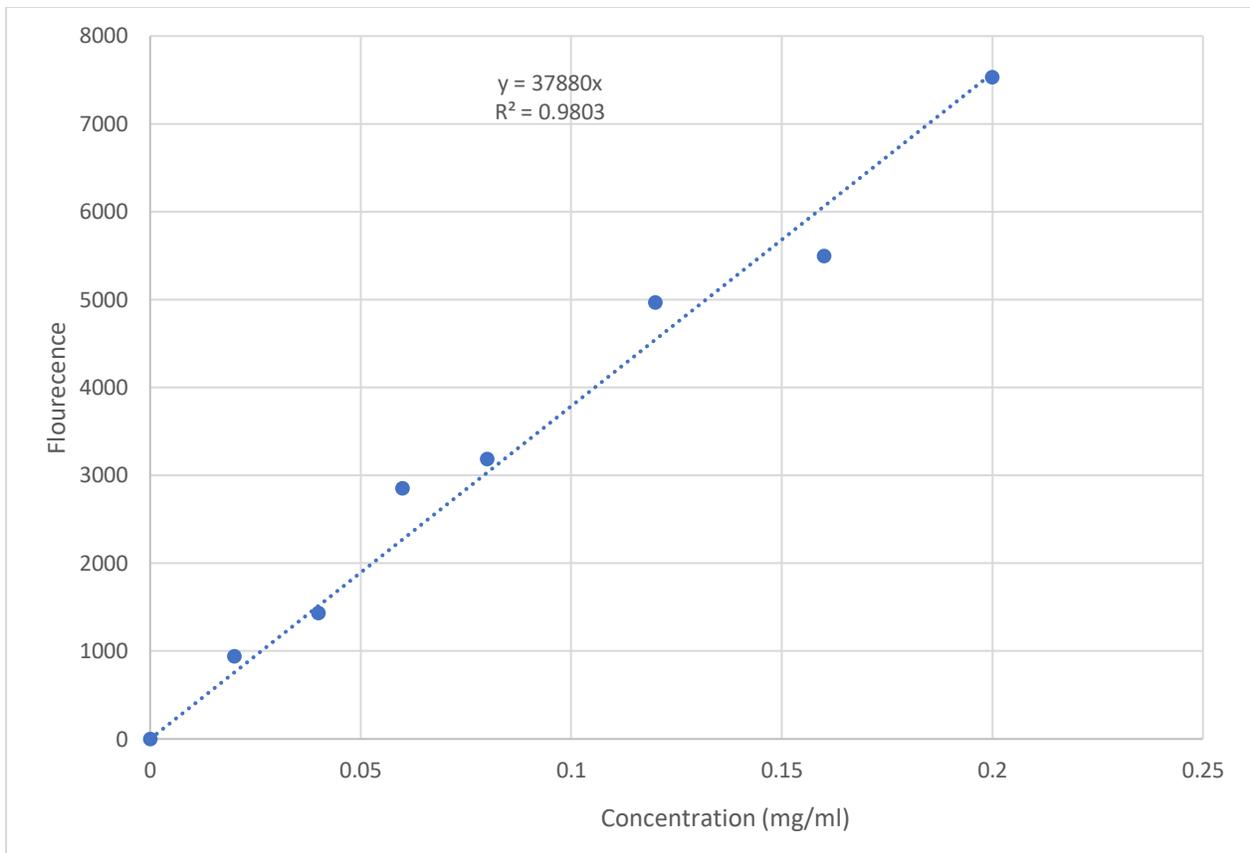
Figure 5.9: Triolein Standard Curve for Coccomyxa.

5.4.2.4 Dry Weight versus Optical Density

The relationship between Optical Density and cell dry weight was determined for *Coccomyxa* as described in section 2.7. It is aimed at converting the Optical Density value of the optimized cell concentrations to dry weight (mg/ml). Figure 5.10 shows the relationship between Optical Density and dry weight for *Coccomyxa.* It can be seen that, Y = 0. 0.1941x, where Y = Dry weight (mg/ml) and x = OD_{595}. Therefore, the percentage of neutral lipid could be calculated by dividing the neutral lipid content by the value of the cell dry weight of the algae at the optimum concentration and then multiplying by 100.
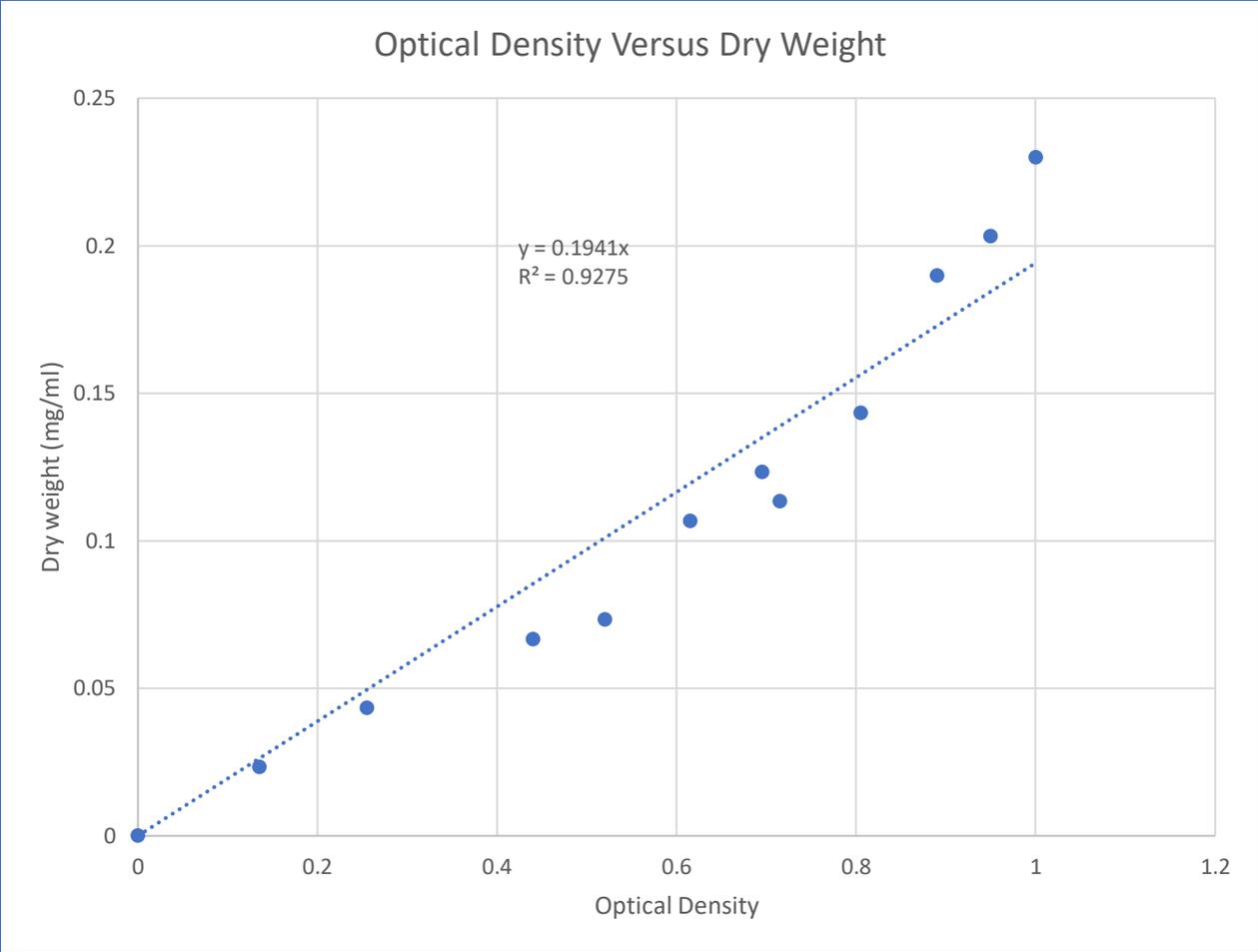
Figure 5.10: Relationship between Optical Density and Dry Weight of Coccomyxa.

5.5 Neutral Lipids in *Coccomyxa* under Normal and Stress Conditions

The relationship between Optical Density and Dry weight was used to convert the values of Nile red fluorescence obtained from the Triolein calibration curve into percentage of lipids for *Coccomyxa* under normal conditions and stress conditions of salinity and nitrogen starvation.

5.5.1 Neutral lipid content in higher salinities

The percentage of lipid content accumulation over a four weeks period (week 1 - week 4) was measured using the Nile red fluorescence method (see Figure 5.11 below). It can be deduced that the percentage of neutral lipid content peaked at 0.1M NaCl and then decreased remarkably with increasing salinity stress from 0.1M to 0.2M NaCl. As *Coccomyxa* grows to reach stationary phase in weeks 3 and 4, it should be noted that the trend over time is that neutral lipid increased at all salinities tested as the cells entered stationary phase (i.e. more neutral lipid is produced). This is particularly dramatic for 0.1M NaCl grown cells, but it is also true under normal conditions and for cells grown at 0.2M NaCl (Figure 5.11). It can be seen also that the highest percentage of lipid content of *Coccomyxa* cells was found at 0.1M NaCl salinity after a period of four weeks incubation. The percentages of neutral lipid content for the normal, 0.1M and 0.2M were found to be 3.4%, 17% and 6.8%, respectively, as the cells entered stationary phase in week four. The figure of 17% was the highest lipid percentage found for salt stressed cells in the work described in this thesis.
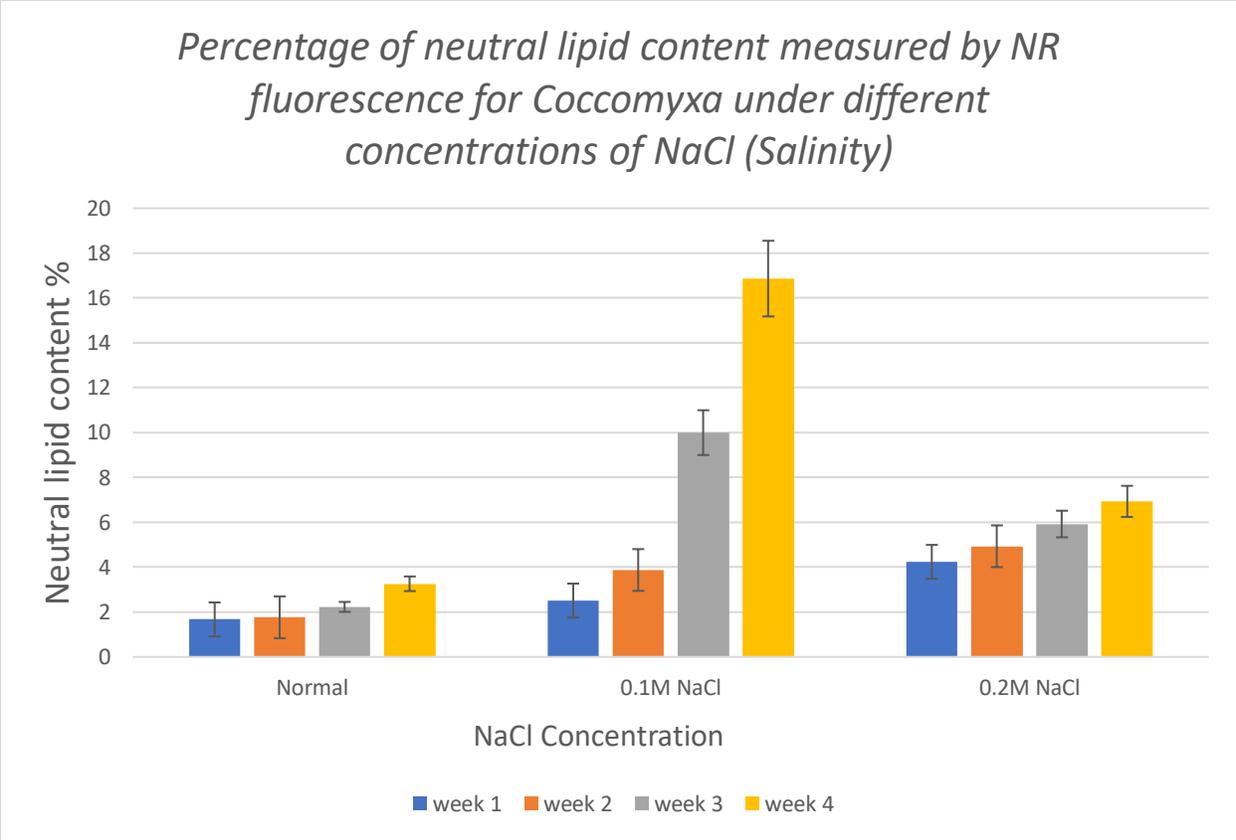
Figure 5.11: Percent neutral lipid content measured by Nile red fluorescence for Coccomyxa under increased salinity conditions. Measurements were made weekly for four weeks. Bars are standard deviations for three replicates.

5.5.1.1 Statistical Analysis for *Coccomyxa* with Increasing Salinity

The t-Test analysis for *Coccomyxa* with increasing salinity is shown below for Two-Sample Assuming Unequal Variances (normal and 0.1M NaCl conditions).

H_0: μ_1 - μ_2 = 0:		Null hypothesis: There is no significant difference in the sample mean
H_1: μ_1 - μ_2 ≠ 0:		Alternative hypothesis: There is significant difference in the sample mean

t-Test for *Coccomyxa* with increasing salinity

	Variable 1 (0.1M NaCl)	Variable 2 (Normal)
Mean	8.312327303	2.23168418
Variance	43.10985088	0.528668702
Observations	4	4
Hypothesized Mean Difference	0	
Df	3	
t Stat	1.840960653	
P(T<=t) one-tail	0.081441047	
t Critical one-tail	2.353363435	
P(T<=t) two-tail	0.162882094	
t Critical two-tail	3.182446305	

If t Stat< -t Critical two-tail or t Stat >t Critical two-tail	Reject null hypothesis
If -t Citical two-tail < value < t Critical two-tail	Accept null hypothesis
In this case, -3.18 < 1.84 < 3.18	No significant difference

The observed difference between the sample means (8.31 - 2.23) is not convincing enough to say that the average percentage of lipid accumulated between 0.1M NaCl and normal condition differs significantly based statistical analysis using t-Test

5.5.2 Neutral lipids content under Nitrogen starvation

Figure 5.12 shows the percentage of lipid content accumulation over four weeks (week1 - week 4) for *Coccomyxa* which has been measured using Nile red fluorescence. It shows that *Coccomyxa* grown in nitrogen-free environment had the highest percentage of lipid content after four weeks of incubation. It can also be seen that the percentage of neutral lipid content increased rapidly with increasing nitrogen starvation from 25% nitrogen to nitrogen-free for *Coccomyxa.* But there were much less significant differences in the percentage neutral lipid produced in the normal, 50% and 25% nitrogen environments as *Coccomyxa* grew to reach stationary phase over four weeks.
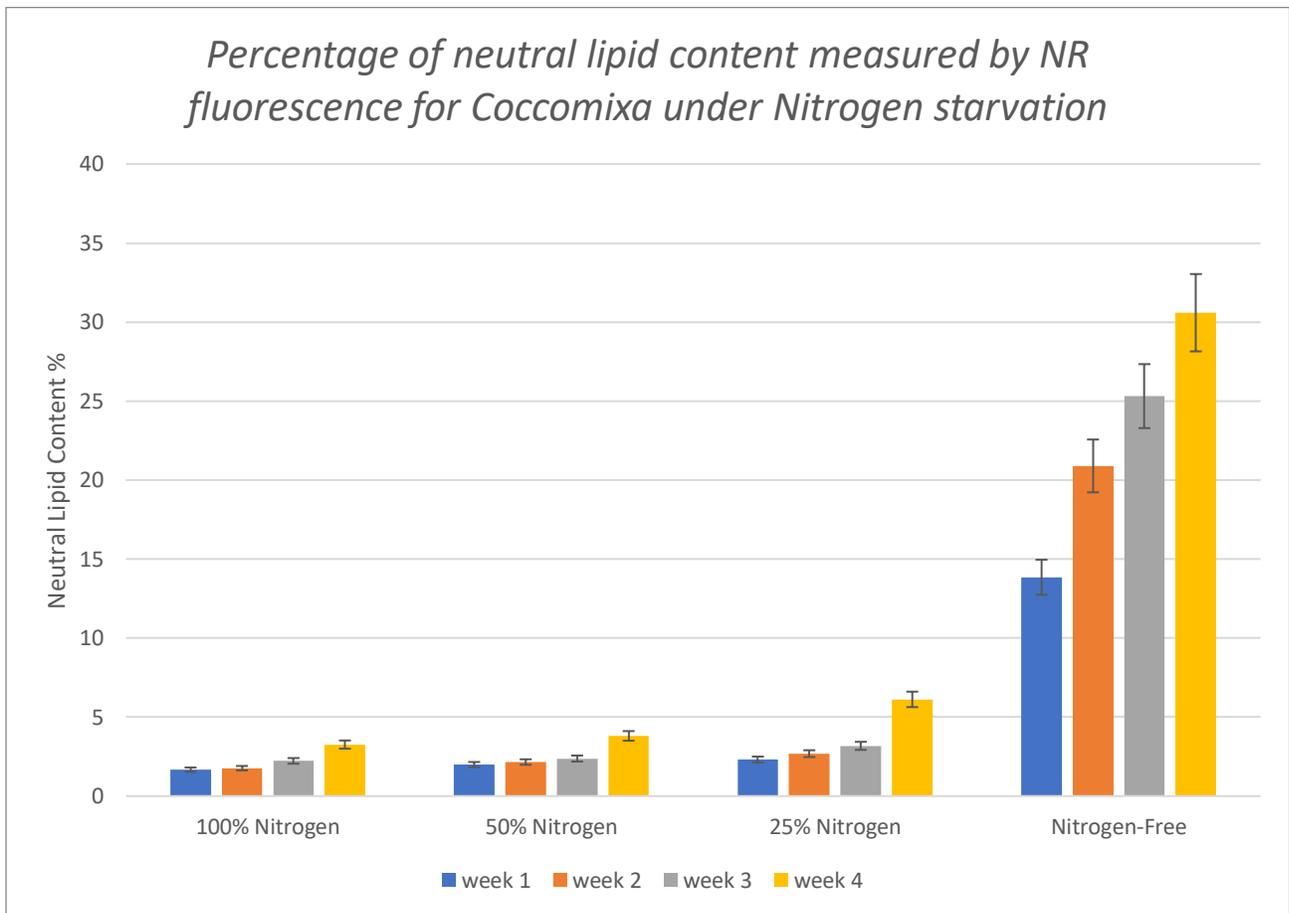
Figure 5.12: Percent neutral Lipid content accumulation over four weeks measured by NR fluorescence for Coccomyxa under nitrogen starvation. Bars are standard deviations for three replicates.

The percentage neutral lipid accumulation with 100%, 50%, 25% nitrogen and nitrogen-free environment were found to be 3%, 4%, 6% and 31%, respectively, as *Coccomyxa* grew to reach stationary phase over four weeks. The figure of 31% was the highest percentage neutral lipid accumulation found in the work described in this thesis and meets the 30% neutral lipid figure suggested by Chisti (2007) as being of potential commercial value.

5.5.2.1 Statistical Analysis for *Coccomyxa* under Nitrogen starvation

The t-Test analysis for *Coccomyxa* under Nitrogen starvation is shown below for Two-Sample Assuming Unequal Variances (normal and nitrogen-free conditions).

H_0: μ_1 - μ_2 = 0:   Null hypothesis: There is no significant difference in the sample mean

H_1: μ_1 - μ_2 ≠ 0:   Alternative hypothesis: There is significant difference in the sample mean

t-Test for *Coccomyxa* under Nitrogen starvation.

	Variable 1 (Nitrogen - free)	Variable 2(Normal)
Mean	22.66235123	2.23168418
Variance	50.23169067	0.528668702
Observations	4	4
Hypothesized Mean Difference	0	
df	3	
t Stat	5.735221533	
P(T<=t) one-tail	0.005262458	
t Critical one-tail	2.353363435	
P(T<=t) two-tail	0.010524917	
t Critical two-tail	3.182446305	

If t Stat< -t Critical-two tail or t Stat >t Critical two-tail	Reject null hypothesis
If -t Citical two tail < value < t Critical two-tail	Accept null hypothesis
In this case, 5.74 > 3.18	There is a significant difference
	The observed difference between the sample means (22.66 - 2.23) is convincing enough to say that the average percentage of lipid accumulated between Nitrogen free and normal condition differs significantly based statistical analysis using t-Test

5.6 Fatty Acid Methyl Esters (FAMEs) from the neutral lipid using GC-MS

To identify fatty acid methyl esters (FAMEs) that occur in *coccomyxa* neutral lipid, gas chromatography mass spectrometry (GC-MS) was used and samples were prepared based on the procedure described in section 2.9.2. Figure 5.13 shows the FAMEs found in cells grown under normal conditions.
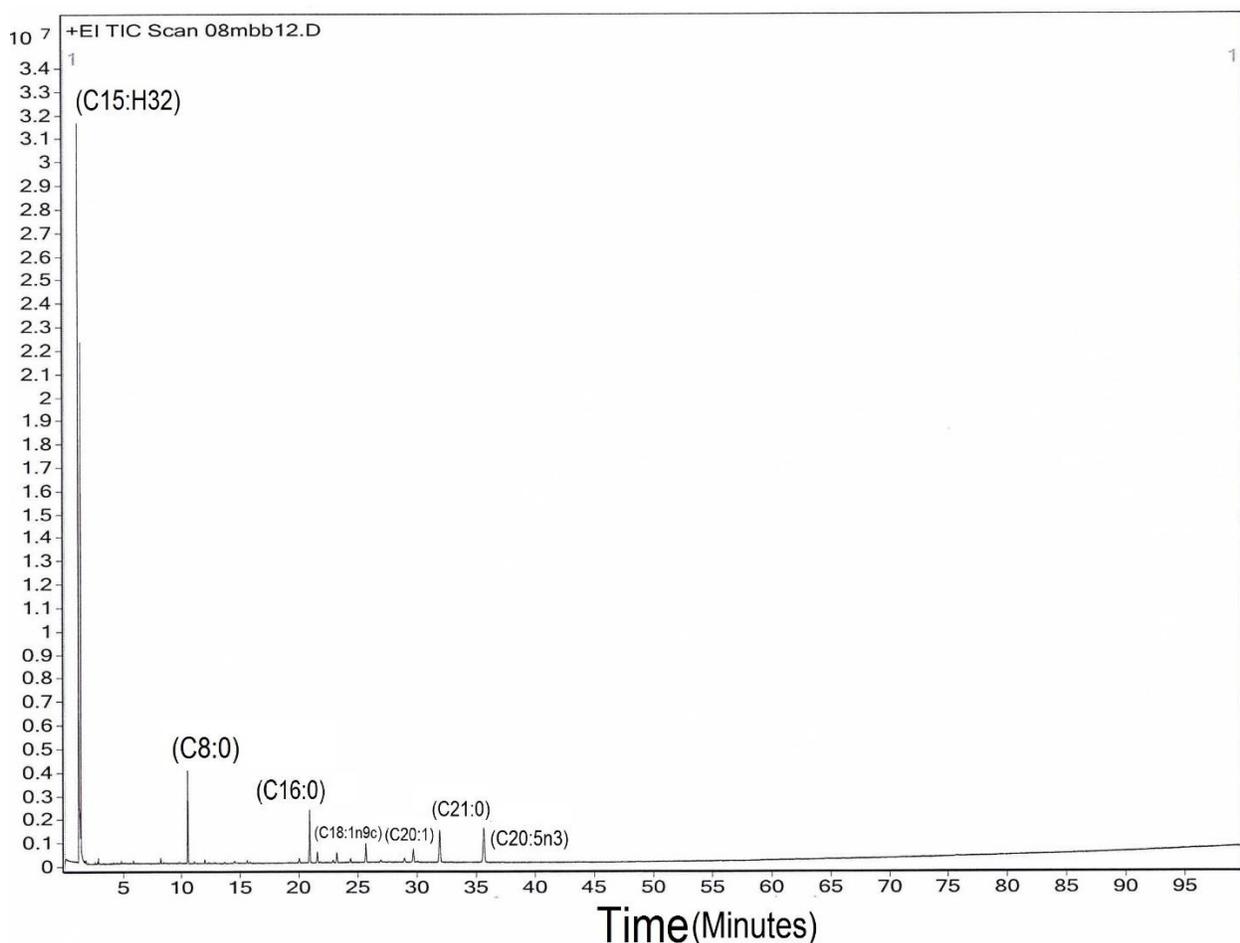
Figure 5.13: Fatty Acid Methyl Esters (FAMEs) found in Coccomyxa grown under normal conditions.

As seen in Figure 5.13 above, the lipid of *coccomyxa* cells grown under normal conditions contains several FAMEs such as (C8:0) caprylic acid methyl ester, (C16:0) palmitic acid methyl ester, (C18:1n9c) oleic acid methyl ester, (C20:1) cis-11,14-

eicosenoic acid methyl ester, (C21:0) heneicosanoic acid methyl ester, and (C20:5n3) cis-5,8,11,14,17-eicosapentaenoic acid methyl ester.

5.6.1 Fatty Acid Profiles under higher Salinities
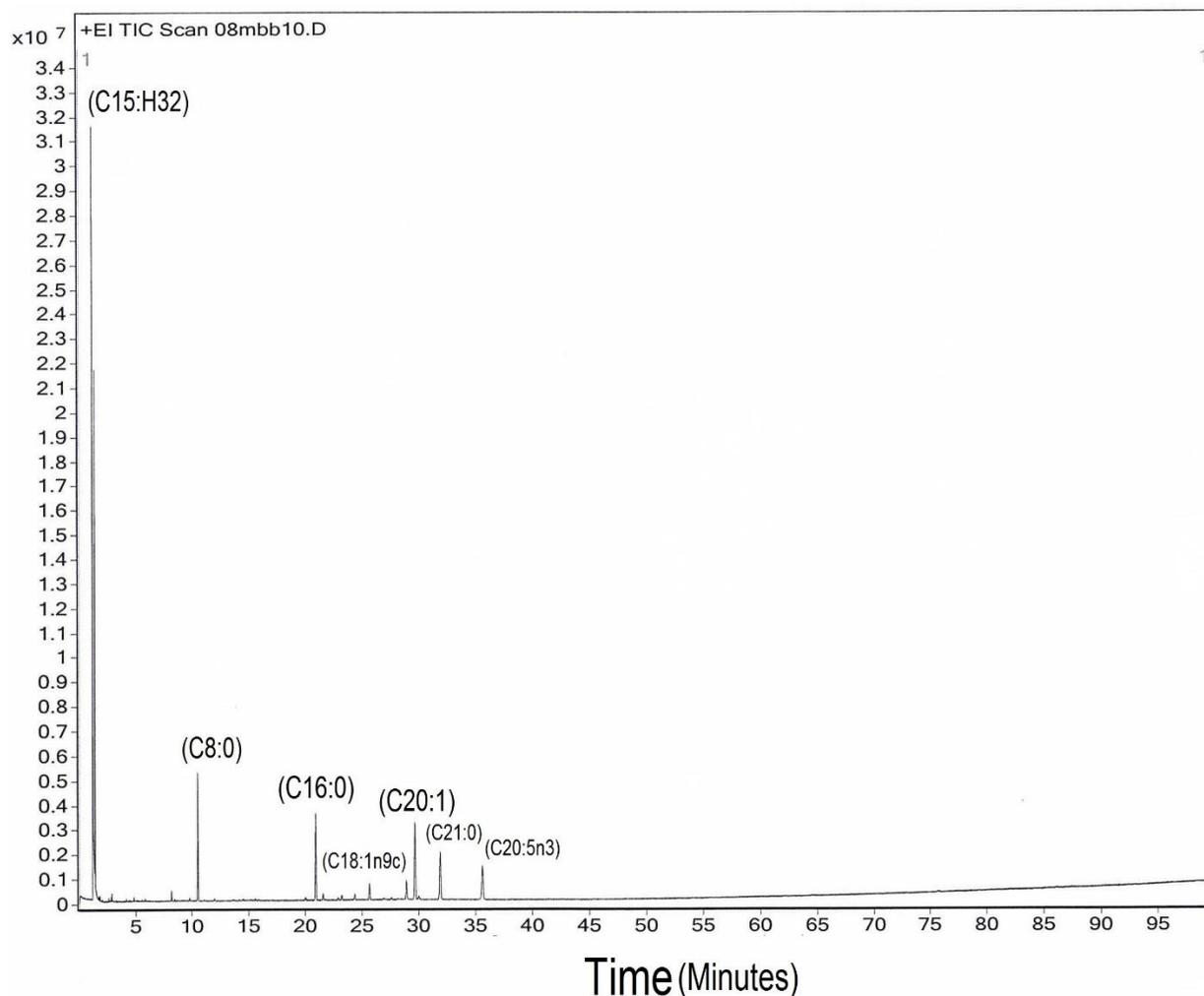
Figure 5.14: Fatty Acid Methyl Esters (FAMEs) found in Coccomyxa grown in 0.1M NaCl Medium.

As in normal conditions, in higher salinity (0.1M NaCl) the lipid contains several FAMEs such as (C8:0) caprylic acid methyl ester, (C16:0) palmitic acid methyl ester, (C18:1n9c) oleic acid methyl ester, (C20:1) cis-11,14-eicosenoic acid methyl ester, (C21:0) heneicosanoic acid methyl ester and (C20:5n3) cis-5,8,11,14,17-eicosapentaenoic acid methyl ester as shown in Figure 5.14. This means that there was no change in the FAME composition with increased salinity to 0.1M NaCl

5.6.2 Fatty Acid Profiles under Nitrogen starvation
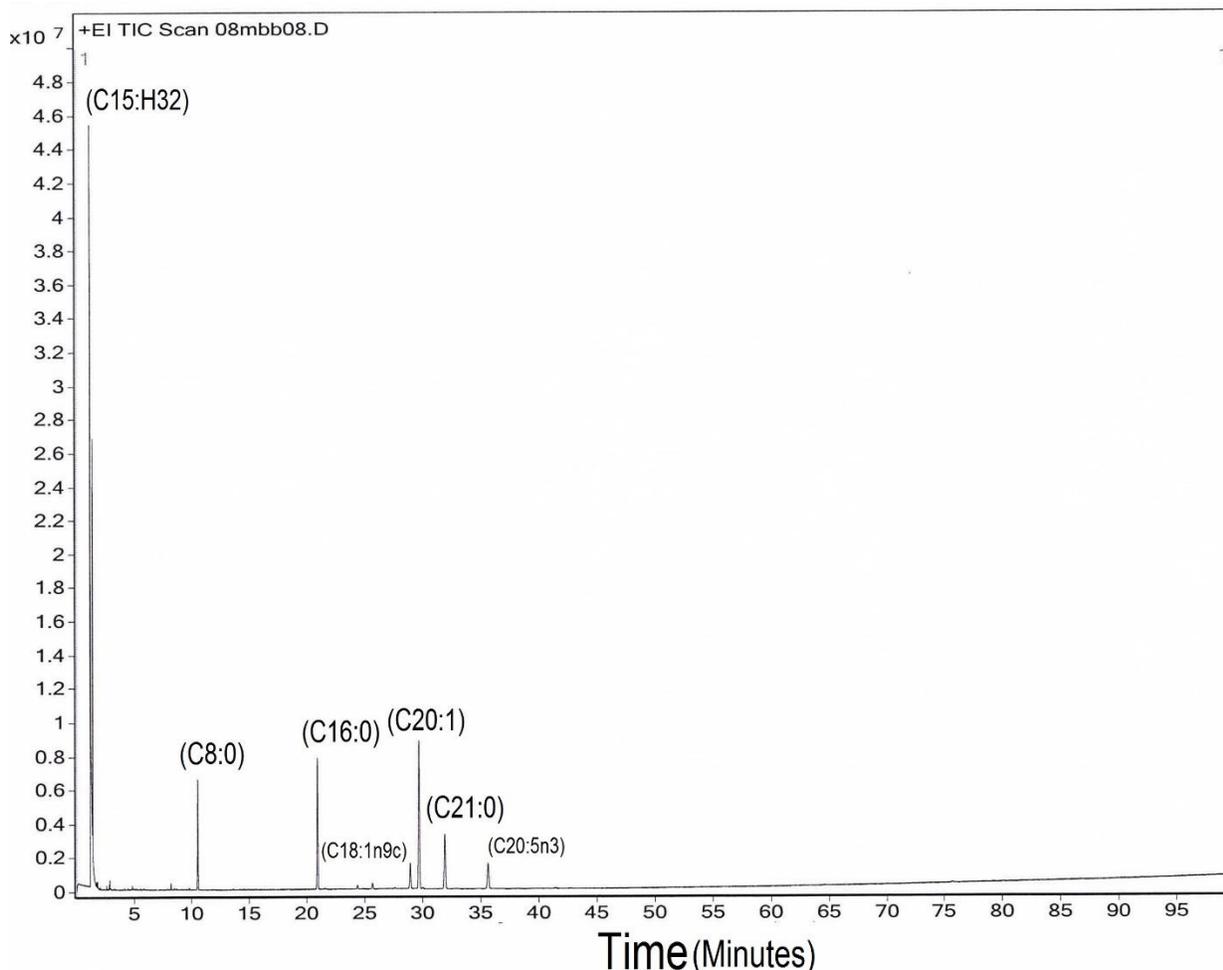
Figure 5.15: Fatty Acid Methyl Esters (FAMEs) found in Coccomyxa grown in Nitrogen-free Medium.

As in the case of growing *T. suecica* in nitrogen free medium, the same FAMEs that occurred in normal and 0.1M NaCl conditions also occur in the nitrogen free sample which are (C8:0) caprylic acid methyl ester, (C16:0) palmitic acid methyl ester, (C18:1n9c) oleic acid methyl ester, (C20:1) cis-11,14-eicosenoic acid methyl ester, (C21:0) heneicosanoic acid methyl ester, and (C20:5n3) cis-5,8,11,14,17-eicosapentaenoic acid methyl ester as shown in Figure 5.15 above.

5.7 *Coccomyxa* Compatible Solutes Analysis using Nuclear Magnetic Resonance (NMR) Analysis

To survive extreme saline conditions, microalgal cells need to synthesize compatible solutes to balance the increased osmotic pressure related to the external medium. Therefore, the NMR results shown in Figure 5.16 below identified sucrose at 5.4ppm, lactate at 4.1ppm and 1.37ppm and acetate at 2.0ppm in the nitrogen free environment grown cells. For the 0.1M NaCl, trehalose at 5.2ppm in addition to lactate at 4.1ppm and 1.37ppm, and acetate at 2.0ppm were seen. Under normal conditions, only lactate at 4.1ppm and 1.37ppm, and acetate at 2ppm were found. These results suggest that trehalose is the main salt induced compatible solute in *Coccomyxa.*
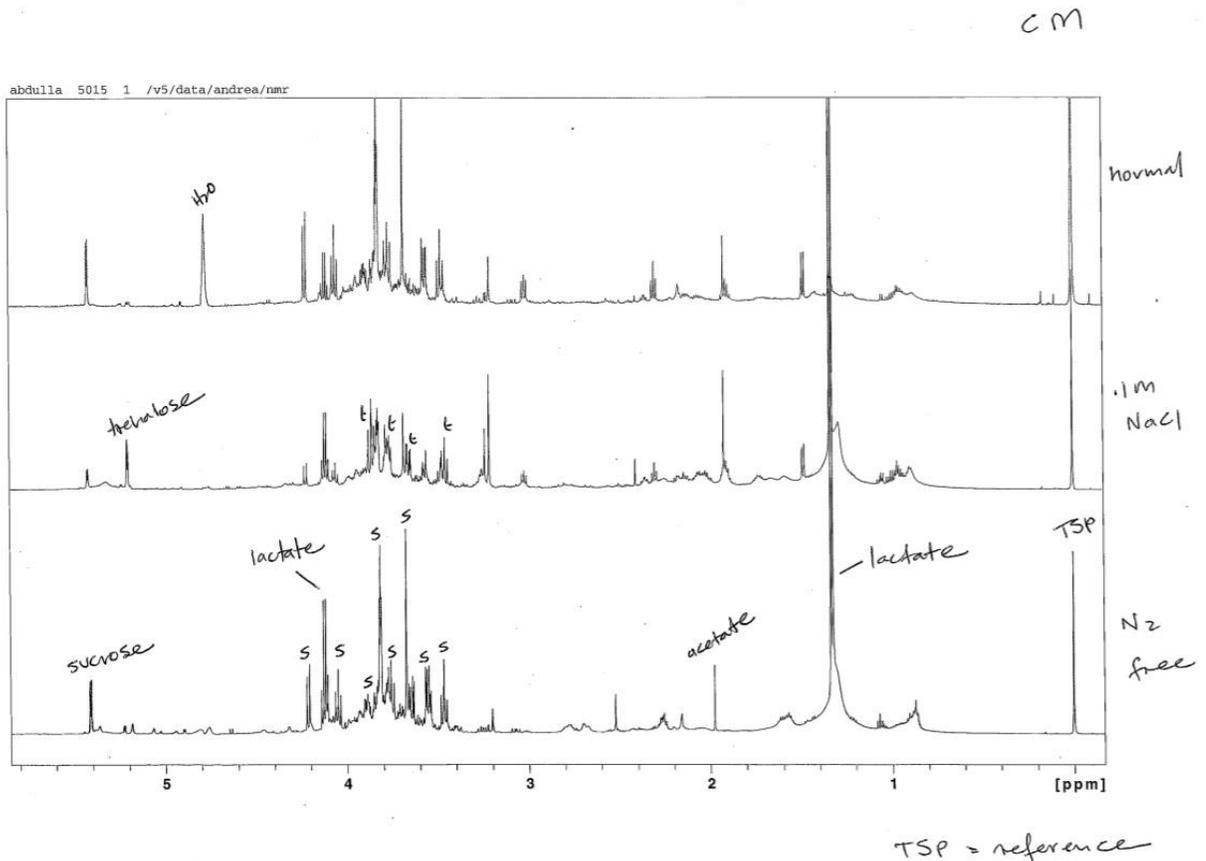
Figure 5.16: NMR spectra of Cell-Free Extracts from Coccomyxa grown under normal, N-free or Higher Salinity (0.1M NaCl) Conditions.

5.8 Discussion

Coccomyxa appeared and outgrew *B. braunii* in the process of culturing *B. braunii* in a 250 ml flask. As a consequence, *Coccomyxa* was studied in the work described in this Chapter to investigate its growth and neutral lipids accumulation. It is a member of the Chlorophyta (green algae) genus is *Coccomyxa* (Trebouxiophyceae, Chlorococcales, Coccomyxaceae). Its identity was confirmed using 18S rDNA sequencing and 3N-BBM+V medium was adapted to grow *Coccomyxa* with continuous irradiance (50 – 70 µmol m^{-2} s^{-1}) at 25°C in shaken (80 rpm) flasks. The medium was renewed every 3 to 4 weeks to reach sufficient biomass to carry out experiments.

It was also grown under stress conditions of higher salinities (0.1M and 0.2M NaCl) to investigate the effect on the growth and lipid accumulation in *Coccomyxa*. Growth curves over a 30 days period showed that the growth rate and final biomass levels decreased as salinity increased i.e. the normal condition gave a higher growth rate and final biomass level as shown in Figure 5.5.

Coccomyxa was also cultivated under nitrogen depletion in order to investigate the effects of nitrogen starvation on neutral lipid accumulation. It was found that the growth rate and final biomass level reached after 30 days were the same for 50%, 25% and normal nitrogen environment, meaning that for *Coccomyxa,* there was no significant difference in the growth rate and final biomass level for 50%, 25% and normal nitrogen environment. It was also shown that there was a little difference at nitrogen-free from that at 25%, 50% and normal nitrogen environment (Figure 5.6). The latter observation of good growth in nitrogen-free conditions needs further work to explain this phenomenon.

The total lipid content of well grown *Coccomyxa* cells taken from the normal, 0.1M NaCl and Nitrogen free conditions was found to be around 11%, 55% and 47%, respectively (section 5.3.1). Nile red fluorescence was used to quantify the cellular neutral lipid content of *Coccomyxa*. Based on the relationship between OD and cell dry weight and on the Triolein calibration curve, the percentage of lipids for *Coccomyxa* under normal and stress conditions of salinity and nitrogen starvation was determined.

The percentage of lipid content accumulation in weeks 1 - 4 is shown in Figure 5.11 and the percentage of neutral lipid content decreased remarkably with increasing salinity stress from 0.1M to 0.2M NaCl as *Coccomyxa* grew to reach stationary phase in weeks 3 and 4. However, there were no significant differences in the percentage neutral lipid produced in the normal and 0.2M NaCl as *Coccomyxa* grew to reach stationary phase. Therefore, the highest percentage of lipid content of *Coccomyxa* cells was for 0.1M NaCl salinity after a period of four weeks incubation. The percentage of neutral lipid content for the normal, 0.1M and 0.2M were found to be 3.4%, 17% and 6.8%, respectively, as the cells entered stationary phase in week four. In terms of nitrogen depletion, it was seen that the percentage of neutral lipid content increased rapidly with increasing nitrogen starvation from 25% nitrogen to nitrogen-free for *Coccomyxa.* The percentage neutral lipid accumulation with 100%, 50%, 25% nitrogen and nitrogen-free environment were found to be 3%, 4%, 6% and 31%, respectively, as *Coccomyxa* grew to reach stationary phase over four weeks.

To survive extreme stress conditions, microalgal cells need to synthesize compatible solutes to balance the increased osmotic pressure related to the external medium. The NMR results in Figure 5.16 showed that the main compatible solute in *Coccomyxa* was trehalose.

Total algal lipids could be expressed as biofuel relevant fatty acid methyl esters (FAME) and could be expressed as percent FAME content based on the dry weight of the algal biomass. Therefore, the FAME profiles from the neutral lipids of *Coccomyxa* were determined using GC-MS cultured in both higher salinities and nitrogen starvation. The results of GC-MS showed that there are several fatty acid methyl esters found in the neutral lipid for *coccomyxa* such as (C8:0) caprylic acid methyl ester, (C16:0) palmitic acid methyl ester, (C18:1n9c) oleic acid methyl ester, (C20:1) cis-11,14-eicosenoic acid methyl ester, (C21:0) heneicosanoic acid methyl ester, and (C20:5n3) cis-5,8,11,14,17-eicosapentaenoic acid methyl ester. However, only (C16:0) palmitic acid methyl ester and (C18:1n9c) oleic acid methyl ester are suitable for biofuel production since they are saturated or monounsaturated fatty acids.

Chapter 6: General Discussions and Future Work

6.0 General Discussion

Qatar's Government has cited the issue of reducing CO_2 emissions in its vision for 2030 and plans to minimize the dependence on fossil fuels to be around 80% by 2030. One of the plans to reduce consumption of fossil fuels is developing the production of biofuels which are renewable and environmentally friendly, starting the programme with a collaboration between Qatar Airways, Airbus, Qatar Petroleum and Qatar Science and Technology Park to develop production of biofuels to be used in air transportation.

Therefore, as a student funded by Qatar, this PhD project investigated the use of microalgae as a potential source of biofuels; they can be grown in saline water or wastewater without the need for arable land and freshwater (Gilmour and Zimmerman, 2012). Additionally, microalgae use CO_2 and convert it into organic compounds by photosynthesis, which makes them a suitable choice in countries which produce high amounts of CO_2 such as Qatar which also have a high level of sunshine for photosynthesis.

It was thought possible to use microalgae for producing oil to replace fossil diesel according to the high yield of oil and the ability of growing rapidly (Deng et al., 2009) and it is known that *Botryococcus braunii* in particular is a potential source of renewable fuel in regard to its ability to produce a significant number of hydrocarbon compounds (Metzger and Largeau, 2005). Up to 75% of *B. braunii* dry mass can be hydrocarbons, depending on the strain and growth conditions (Banerjee et al., 2002).

6.1 *Botryococcus braunii*

In Chapter 3, *B. braunii* was evaluated for neutral lipid production under normal growth conditions (i.e. growing in 3N-BBM+V medium) and by modifying its growth conditions in order to study its neutral lipid production under salt stress. Despite using a relatively nutrient rich medium, it was difficult to reach sufficient biomass to run experiments. The growth rate in 3N-BBM+V medium was slow, even compared to other green microalgae and the results shown in Chapter 3 conclusively demonstrate that *B. braunii* was unable to grow at higher salinities. This means that salt stress cannot be used to induce neutral lipid production in at least this strain (CCAP 807/1) of *B. braunii.*

The total lipid content for the well-grown culture of *B. braunii* measured using the gravimetric method under normal conditions was found to be 2.98 mg/ml in 10.2 mg/ml dry weight biomass. This indicated that the percentage of total lipid content in dry weight biomass was around 29% (section 3.3.1) which agrees with Lee et al. (1998) who found it be to around 30% of dry weight by the same method. The neutral lipid content and percentage of neutral lipid was also found to be 3.1 μg/ml and 3.64%, respectively, after 30 days growth under normal conditions using Nile red fluorescence method. Thus, *B. braunii* had a total lipid content of 29% but only 3.64% were neutral (storage) lipids when grown in normal 3N-BBM+V medium, which is not sufficient for commercial production of biodiesel (Chisti, 2007).

Interestingly, another strain of *B. braunii* (strain LB 572) did respond to salt stress by increasing neutral lipid production and carotenoid production (Ranga Rao et al., 2007). However, in this work the highest salinity used was 0.085M NaCl and the medium was Chu 13, not BBM (Ranga Rao et al., 2007). Therefore, the salt concentrations used in the current study may have been too high.

6.2 *Tetraselmis suecica*

Tetraselmis suecica was considered as an alternative species to *Botryococcus braunii* and was grown in defined F/2 medium and under stress conditions of higher salinities of 0.2M, 0.4M, 0.6M, 0.8M, 1.0M and 1.2M NaCl solution to investigate the effect on the growth and lipid accumulation in *Tetraselmis suecica*. Increasing the salinity decreased the growth rate, but it did not increase the total lipid production. Under normal conditions the percentage of lipid content of the dry weight biomass was found to be around 31%, and for cells grown in 0.6M NaCl it was found to be 29%. For Nitrogen starved *Tetracelmis suecica* cells, the total lipid production was increased to 56%.

The percentage neutral lipid content accumulated after four weeks growth was found to decrease with increasing salinity. The percentage of neutral lipid content for the normal, 0.6M, 0.8M, 1.0M and 1.2M were found to be 2.7%, 2.1%, 1.6% and 1.3%, respectively. It was also found that the percentage of neutral lipid increased with increasing nitrogen depletion, peaking at 10.2% for nitrogen-free growth conditions. This is still less than the 30% neutral lipid normally quoted as the minimum requirement for biodiesel production (Chisti, 2007). The important point is that nitrogen stress is much more effective at inducing neutral lipid accumulation in *Tetraselmis suecica* cells than salinity stress and this agrees with previous work on *Tetraselmis suecica* (Alsull and Omar, 2012).

Very little work has been done to investigate the production of compatible solutes in *Tetraselmis suecica* species. Therefore, NMR was used in this work to identify betaine as a possible compatible solute in *Tetraselmis suecica.* Betaine is an excellent compatible solute, but it has not been identified in microalgae until now (daCosta et al., 1998). One possible explanation is that the betaine comes from bacteria contaminating the *Tetraselmis suecica* culture. In the current work, all cultures were unialgal, but it is likely that some bacteria were also present. To confirm this interesting result, the NMR experiment needs to be repeated with an axenic *Tetraselmis suecica* culture.

Finally, the TAG content of *Tetraselmis suecica* cells was examined using GC-MS to determine which fatty acid methyl esters (FAMEs) were present. A mixture of FAMEs was found including (C16:0) palmitic acid methyl ester, (C18:1n9c) oleic acid methyl

ester, and (C18:3n3) linolenic acid methyl ester. These results agree with Mendoza et al. (2010) who found that the fatty acids produced by *Tetraselmis suecica* are principally C16:0 and C18:1. Saturated or monounsaturated fatty acids, but not polyunsaturated fatty acids such as linolenic acid, are suitable for biofuel production (Stansell et al., 2012).

6.3 *Coccomyxa*

Chapter 5 described work to study *Coccomyxa,* which was isolated as a contaminant in cultures of *B. braunii. Coccomyxa* is much less well studied than either *Botryococcus braunii* or *Tetraselmis suecica* and it was decided to investigate its ability to accumulate neutral lipids. In common with both *Botryococcus braunii* and *Tetraselmis suecica*, salinity decreased growth rate, but in the case of *Coccomyxa* high salinity increased total lipid content from around 11% in normal 3N-BBM+V medium to 55% in 0.1M NaCl medium. This is a promising result, but how much of this lipid is neutral lipid?

To answer this question, the percentage of neutral lipid accumulation was measured over a period of 4 weeks and at the end of the four weeks, the percentage of neutral lipid accumulation for the normal, 0.1M and 0.2M grown cells were found to be 3.4%, 17% and 6.8%.   Therefore, 0.1M NaCl stress was the most effective salinity stress found in the current work and led to 17% TAG production.

For nitrogen limitation/starvation, it was found that the percentage of neutral lipid accumulation increased rapidly with increasing nitrogen starvation from 25% nitrogen to nitrogen-free. The percentage neutral lipids accumulated with 100%, 50%, 25% nitrogen and nitrogen free environment were found to be 3%, 4%, 6% and 31%, respectively, as *Coccomyxa* reached stationary phase over four weeks. This means that the nitrogen free condition induces more neutral lipid accumulation (31%) in *Coccomyxa* and this is in line with the 30% neutral lipid normally quoted as the minimum requirement for biodiesel production (Chisti, 2007).   Based on the results for *Botryococcus braunii, Tetraselmis suecica* and *Coccomyxa*, the latter is clearly the most promising strain for biodiesel production.

Since, the compatible solutes used by *Coccomyxa,* had not been previously studied, NMR was used to look at the compatible solute profile for *Coccomyxa.* The key finding was that trehalose and sucrose were found in the higher salinity grown cells, suggesting that these compounds act as compatible solutes in *Coccomyxa* (daCosta et al .,1998)*.*

Lastly, the TAG content of *Coccomyxa* cells was examined using GC-MS to determine which fatty acid methyl esters (FAMEs) were present. A mixture of FAMEs was found

including (C8:0) caprylic acid methyl ester, (C16:0) palmitic acid methyl ester, (C18:1n9c) oleic acid methyl ester, (C20:1) cis-11,14-eicosenoic acid methyl ester, (C21:0) heneicosanoic acid methyl ester, and (C20:5n3) cis-5,8,11,14,17-eicosapentaenoic acid methyl ester. However, only (C16:0) palmitic acid methyl ester and (C18:1n9c) oleic acid methyl ester are suitable for biofuel production since they are saturated or monounsaturated fatty acids (Stansell et al., 2012).

6.4 Future Work

- Use lower salinity values (less than 0.1M NaCl) to investigate the effect of very low salinity stress on *Botryococcus braunii* growth and lipid accumulation

- Cultivate *Botryococcus braunii* in Nitrogen starvation conditions to investigate the effect on growth and lipid accumulation

- Cultivate *Tetraselmis suecica* in a combination of Nitrogen starvation and higher salinities (double stress) conditions to investigate the effect on growth and lipid accumulation.

- Cultivate *Tetraselmis suecica* in a combination of pH and Nitrogen starvation (double stress) conditions to investigate the effect on growth and lipid accumulation.

- Cultivate *Coccomyxa* in a combination of Nitrogen starvation and higher salinities (double stress) conditions to investigate the effect on growth and lipid accumulation.

- Cultivate *Coccomyxa* in a combination of pH and Nitrogen starvation (double stress) conditions to investigate the effect on growth and lipid accumulation.

- Cultivate *Coccomyxa* in a combination of pH and higher salinities (double stress) conditions to investigate the effect on growth and lipids accumulation.

- The observation of good growth of *Coccomyxa* in nitrogen-free conditions needs further work to explain this phenomenon.

Chapter 7: References

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