# The Effect of Salinity Stress and Nitrogen Depletion on Neutral Lipid Production by the Green Microalgae *Auxenochlorella* and *Chlorella*

By

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## Declaration

***I hereby declare that this thesis and the work presented in it are my own and they have been generated by me as the result of my own original research.***

***Furthermore, I have acknowledged all sources used and have cited them in the reference section***.

***Signature: Fauzeya M.Albalwe***

# Dedication

***I dedicate my thesis work to my family and friends. A special expression of gratitude goes to my loving parents, Fatima and Mataq, who continually provided me with their moral, spiritual, emotional, and financial support.***

# Abstract

During the past decade, microalgae have been studied as potential sources for biofuel production. In this study, two species of green microalgae were isolated from Weston Park pond in Sheffield, UK and identified using 18S rDNA sequencing as *Auxenochlorella* and *Chlorella*. The neutral lipid (triacylglycerol, TAG) content of *Auxenochlorella* and *Chlorella* was measured using the fluorescent dye Nile Red. Neutral lipid, in the form of TAG, can easily be converted to biodiesel using transesterification. Therefore, the effects of stress conditions on neutral lipid accumulation by both microorganisms were tested to evaluate their potential for the production of biodesel. For salinity stress, the highest percentage TAG content (24%) was found in *Auxenochlorella* cells grown at 0.8 M NaCl for three weeks. For *Chlorella,* the highest TAG was 26% in cells grown at 1 M NaCl for 4 weeks. Neutral lipid accumulation also increased significantly with nitrogen starvation for 4 weeks reaching 44% in *Auxenochlorella*, while in *Chlorella,* it was 70%. Therefore, the percentage of neutral lipid accumulated was much higher under nitrogen stress than under salinity stress.

Further work concentrated on the Fatty Acid Methyl Esters (FAME) conversion yield, which was examined using a direct transesterification method; besides, the composition of fatty acids was investigated using GC-MS. *Auxenochlorella* and *Chlorella* grown in N-free BBM medium showed the highest yield and content of C16 and C18 fatty acids, which are suitable for biodiesel production. Further analysis of these strains for lipid content was based on $^{1}$H-NMR and the results confirmed that the fatty acid content is much higher in the N free grown cells.

The final sets of experiments focused on the effect of mixotrophic growth of the *Chlorella* strain on lipid production and on examining high lipid containing cells using Nile red staining and automated fluorescence assisted cell sorting (FACS) flow cytometry technique. The results showed that *Chlorella vulgaris* cells subjected to nitrogen limitation exhibited higher lipid content.

# Abbreviations

| | |
|---|---|
| **Acetyl CoA** | **Acetyl coenzyme A** |
| **ADP** | **Adenosine diphosphate** |
| **ATP** | **Adenosine triphosphate** |
| **BBM** | **Bold Basal Medium** |
| **B12** | **Cyanocobalamin** |
| **DMSO** | **Dimethyl sulfoxide** |
| **FACS** | **Fluorescence Activated cell sorting** |
| **FAMEs** | **Fatty acid methyl esters** |
| **FAs** | **Fatty acids** |
| **FAS** | **Farming Advisory System** |
| **FC** | **Flow Cytometry** |
| **FSC** | **Forward Scatter** |
| **GC-MASS** | **Gas Chromatography- Mass Spectrophotometry** |
| **NMR** | **Nuclear Magnetic Resonance** |
| **NR** | **Nile Red dye** |
| **PBR** | **Photobioreactor** |
| **SCS** | **Side scatters** |
| **TAG** | **Triacylglycerol** |

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# Literature Review and Aims

Chapter One

### 1.1 Global Carbon Cycle

Global climate change occurs as a result of the increase of carbon dioxide ($CO_2$) in the Earth's atmosphere leading to a corresponding increase in greenhouse gas emission. It is widely accepted that the increase in $CO_2$ is due to human activity. Furthermore, the conservation of conventional sources of fuel resulting from fossil fuel depletion became a matter of concern for the world. **Figure 1.1** illustrates the annual increases in the level of $CO_2$ between 1965 and 2011. It indicates that $CO_2$ emissions have risen steadily in recent years (Solomon *et al*., 2007).

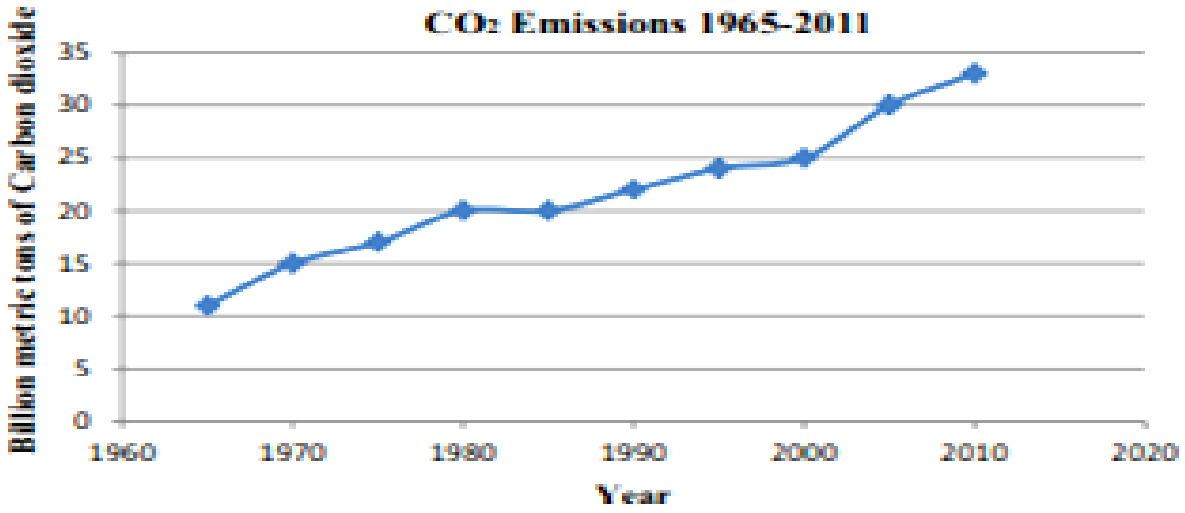


*Figure 1. 1: Average increase $CO_2$ between 1965 and 2011 showing a faster rate growth in recent years (Modified from Solomon* et al., *2007).*

Kyoto protocol, promoted in 1997 by The United Nations, targets decreasing greenhouse gases like $CO_2$, $CH_4$, $SO_2$, $NO_2$ which made up 5.2% of the emissions in 1990, and this protocol was approved by more than one hundred and seventy countries (Gutierrez *et al*., 2008). The Intergovernmental Panel on Climate Change (IPPC) provided data on the $CO_2$ concentration between 1960 and 2007 displaying an increase from 315ppm to 380ppm, with a 35% increase since 1990 (Solomon *et al*., 2007). At present, this gradual but persistent rise in $CO_2$ in the atmosphere requires research into finding the appropriate capture process to mitigate the effects of $CO_2$ on global warming. In (**Figure 1.2),** various techniques for $CO_2$ mitigation strategy are revealed. In that regard, the reduction of $CO_2$ will be a strong motivation for researchers to produce biomass energy using photosynthetic microorganisms a process which enables the fixation of $CO_2$ in the atmosphere (Kondili and Kaldellis, 2007).

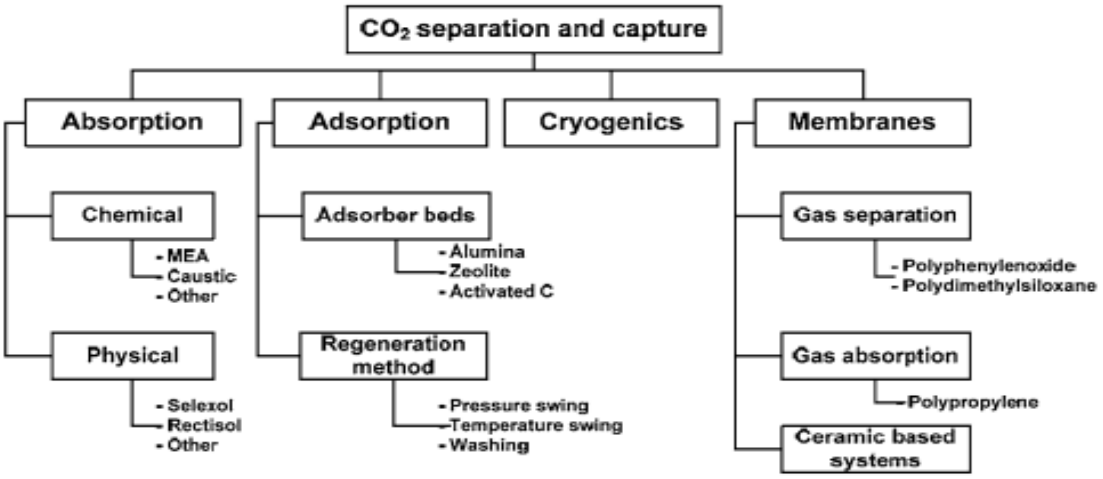


*Figure 1.2: Chart shows various techniques for capturing $CO_2$ (Adapted from Rao and Rubin, 2002).*

Green plants absorb a small percentage of $CO_2$, consisting of 3-6 % of fossil fuel emissions, as a result of the relatively slow growth of plants (Skjanes *et al*., 2007). On the other hand, microalgae have attracted interest, as they can grow faster than terrestrial plants. Microalgae have an immense capability to fix $CO_2$ and to convert sunlight into energy. In comparison to plants, microalgae can be more efficient by 10-50 times (Li *and Liu*, 2008). Many studies have been carried out using several microalgae species to uptake $CO_2$ such as *Chlorella kessleri*, *Scenedesmus obliquus* (Morais and Costa, 2007), Dunaliella *tertiolecta*, *Botryococcus braunii*, *Spirulina platensis* (Sydney *et al*., 2010), *Nannochloropsis oculata* (Chiu *et al*., 2009) and *Chlorocuccum littorale* (Ota *et al*., 2009). Researchers have concentrated on the impact of different concentrations of $CO_2$ on biomass production and have indicated that the rate of $CO_2$ capture is a consequence of light intensity, pH and temperature (e.g. Yue and Chen, 2005).

The use of fossil fuel causes problems that negatively impacts on society, such as the global energy crisis; besides, the worldwide energy demand is rising in the world, especially in the developing countries such as China and India. Biomass derived from agriculture; trees, grass plants and crops is considered the raw material for the first generation of biofuels. Providing sufficient quantities from fat or oil, such as flower oil, soybean oil, canola oil, and palm oil, would be unsuccessful for biofuel production on a large scale (Ghosh *et al*., 2016). Additionally, biofuels derived from agriculture compete with food crops in a way potentially detrimental to the world food supply (Chisti, 2007; Nepstad *et al*., 2008) whereas, biofuels derived from microalgae are involved in $CO_2$ uptake and the process is considered one of the methods that helps decrease $CO_2$ production (Madhumanti-Mondal *et al*., 2017).

The most frequent method utilized for capturing $CO_2$ in a biological system is exhibited in photobioreactors (PBRs) that mitigate $CO_2$ in the atmosphere and reduce global warming (Salih 2011). From all the procedures applied to utilize agriculture produce or waste for biofuels production, microalgae are the best alternative resource. Microalgae have attracted the attention of scientists due to their properties in terms of the high amount of lipid production and their applications in many aspects of biotechnology (Rawat *et al*., 2011). Algae have existed on Earth for at least a billion years taking up $CO_2$ significantly and contributing to the development of life as we now know it today (Demirbas, 2010).

### 1.2. Microalgae

Algae are thallophytes and photosynthetic non-vascular plants that lack roots, stems and leaves. Their primary photosynthetic pigment is chlorophyll (Vonshak and Maske, 1982). All existing Earth ecosystems have algae, and a wide variety of species is found in a wide range of environmental conditions (Mata *et al*., 2010). Microalgae include prokaryotic (cyanobacteria) and eukaryotic microorganisms that as noted above lack roots, leaves and stems. They can multiply in a variety of areas such as aquatic and terrestrial environments. Microalgae can be unicellular, filamentous or colonial (Lee, 2008). However, in recent times, based on molecular phylogenetic systematics, algae have not been classified under the Kingdom Plantae, although some types of algae (e.g. Chlorophyta) do resemble higher plants (Graham and Wilcox, 2000).

Algae can be divided into two major categories of organisms depending on their sizes; macroalgae which are known as seaweeds and microalgae; a single microscopic cell that can be filamentous or colonial ranging in size from a few micrometres (µm) to a few millimetres (Sheehan *et al*., 1998). Researchers have pointed out that airborne algae can cause unfavourable impacts on human health, as they can produce allergic reactions in humans, but eukaryotic algae rarely cause human health problems (Chu, 2012). Microalgae are photosynthetic organisms that only need simple inorganic nutrients to grow; plus water, light and $CO_2$.

A number of microalgal species are more suitable to produce lipids (oil) when compared to other sources such as plants due to their ease of cultivation, purification method and extraction (Hu *et al*., 2008 and Borowitzka and Moheimani., 2013).Microalgae can produce around 80% oil by dry weight of biomass (Khan *et al*., 2009 and Abou-shanab *et al*., 2014). Green microalgae are a promising alternative resource for biodiesel (Borowitzka and

Moheimani 2013; Zhang *et al.*, 2014). Algae can be used for the production of sugars and carbohydrates that could be converted to ethanol and utilized for hydrogen output (Benemann, 2000). On the other hand, algae can be autotrophic, heterotrophic or mixotrophic organisms cultivated from a variety of feedstocks (Liang *et al.*, 2009; Drexler and Yeh, 2014). Furthermore, as noted above algae can be divided into two major categories of organisms; microscopic (unicellular or filamentous) microalgae and multicellular macroalgae; more commonly known as seaweed (Lundquist *et al.*, 2010). Algae species can be found in a vast range of sizes, for example, from picoplankton of only 0.2 - 2.0 μm in diameter to giant kelps with up to 60 m long fronds (Chu, 2012).

Furthermore, algae are potentially important organisms for biotechnological usage especially for products, processes and services, with a substantial effect on the production of food, pharmaceutical industry and public health (Chisti, 2007; Ahmad *et al*., 2011; Chu, 2012; Drexler and Yeh, 2014). Algae are considered as a possible source of biofuel (biodiesel) in their ability to produce neutral lipids (triacylglycerides) by photosynthesis (Meng *et al*., 2009; Vicente *et al*., 2009; Devi and Mohan, 2012; Bellou and Aggelis, 2012; Campos *et al*., 2014; Drexler and Yeh, 2014). The most commercially appealing microalgae belong to *Chlorella*, *Spirulina*, *Dunaliella* and *Haematococcus* genera (Bruton *et al.*, 2009). They can grow in different environments such as freshwater, marine water and soil (Pei *et al*., 2010).

### 1.3. Microalgae Cultivation

There are several types of algae cultivation (photoautotrophic, photoheterotrophic, mixotrophic and heterotrophic, see Table 1.1) and each type affects the composition of the biomass produced and the characteristics of microalgae growth (Ahmad *et al*., 2011; Amaro *et al*., 2011). Photoautotrophic cultivation uses light to obtain energy like sunlight and utilizes inorganic carbon like $CO_2$ by photosynthesis to release chemical energy and to fix $CO_2$ (Huang *et al*., 2010). Some species of microalgae use organic carbon under dark conditions, which is referred to as heterotrophic cultivation. Organic carbon is used as a source of energy and carbon (Huerlimann *et al*., 2010; Xiong *et al*., 2008). The problems related to light becoming a limiting factor for growth at high biomass levels can be solved with heterotrophic cultivation, the output of biomass being much higher than that under phototrophic culturing (Huang *et al*., 2010). *Chlorella protothecoides* lipid production was reported to be enhanced by 40% when cultivation was switched from phototrophic to heterotrophic (Xu *et al*., 2006).

Microalgae in mixotrophic cultivation can grow under both heterotrophic and phototrophic conditions, using organic compounds and inorganic ($CO_2$) as a source of carbon., $CO_2$ produced by heterotrophic respiration can be used to drive photoautotrophic cultivation (Mata, *et al*., 2010). Photoheterotrophic cultivation is rarely mentioned in the literature. Usually, no distinction is made between mixotrophic and photoheterotrophic growth (Griffiths and Harrison, 2009; Mata *et al*., 2010). Photoheterotrophic cultivation of microalgae demands light as a source of energy while using organic compounds as their carbon source (Chojnacka and Marquez-Rocha, 2004).

*Table 1.1: Outline of the characteristics for the different types of microalgae cultivation methods (Adapted from Gruz et* al.*, 2018).*

| | Phototrophic growth | Heterotrophic growth | Mixotrophic growth | Photoheterotrophic growth |
|---|---|---|---|---|
| Energy source | Light | Organic | Luz e orgânica Light and Organic | Light |
| Carbon source | Inorganic - Carbon dioxide | Organic - External carbon source | Organic and inorganic carbon source simultaneously | Organic External carbon source |
| Cell density | Low | High | Medium | Medium |
| Growing systems | Open pond or Photobioreactor | Conventional fermenter | Photobioreactor | Photobioreactor |
| Cost | Low | Medium | High | High |
| Main disadvantages | Low cell density. Contamination of open pond algae cultivation systems | High cost of substrate. Risk of contamination | High cost of substrate and Photobioreactor | High cost of substrate and Photobioreactor |

### 1.3.1 Comparison of microalgae cultivation methods

The most common systems of microalgae cultivation are open ponds, and enclosed cultivation system photobioreactors (PBRs). Many studies focus on open pond cultivation. Some of its features are cheaper land, reduced operation cost and less energy for culture mixing. Open systems demand a large land area and are exposed to contamination and bad climate conditions. Scientists experienced in growing algae in open ponds have come to terms with a lot of bacteria being present in the open pond cultures. In addition, scientists have noted the harmful effects of the appearance of rotifers in cultures of *Tetraselmis*, *Chlorella*, *Nannochloropsis* and *Scenedesmus*. Amoebas continually prey on diatoms. Moreover, open pond systems are difficult to control, in terms of growth parameters such as temperature, pH, etc. (Narala *et al*., 2016).

In contrast to open ponds, PBRs do not require much land area for culturing, solving the problem of limited land areas available in some countries such as the UK. Essentially, PBRs are designed to provide sufficient light and to avoid contamination issues. However, PBRs also have some drawbacks such as algae growth on the walls of the PBR; increased regional temperature, issues related to cleaning and to the increase in the cost of operation (Chisti, 2008). The design of an open pond in wastewater treatment can be shaped as circular or gravity flow watering. The design of PBRs in recent years has been tubular, providing sufficient light, and mixed culture with pharmaceutical products to enhance nutritional yield (Otero and Fabregas, 1997; Mata *et al*., 2010).

### 1.4. Metabolic Pathways for Lipid Accumulation

Research on microalgae for biodiesel production and its enhancement has drawn a lot of attention lately. Algae normally store energy in the form of lipids and polyglycans (polysaccharides, carbohydrates), but lipids appear to be more concentrated stores of energy than polyglycans (Vitova *et al*., 2015). Lipids and polyglycans have benefits represented by the microalgae sustainability under dark conditions and variable light intensities, providing further energy for biological processes in microalgae cells such as DNA replication, nuclear division and microalgal reproduction to form daughter cells. Lipids found in algae can be divided into two major types: neutral lipids, which are energy reserves and polar lipids which are key components of the cell and organelle membranes. Neutral lipids (NLs) are accumulated in microalgae in the form of triacylglycerols (Zhu, *et al*., 2016).

The cell cycle of microalgae passes through several stages sequentially: the growth of cells, the replication of DNA and division of cells. Carbohydrate and lipid metabolism begins with three-carbon molecules like 3-phosphoglycerate (3PG) and glyceraldehyde 3-phosphate (GAP) (de Jaeger *et al*. 2014). **Figure 1.3** presents the metabolic effects of C3 precursor pathways on lipid accumulation. The biological processes in a cell cycle as mentioned above, utilizes the conserved energy as a result of photosynthesis to complete these processes and to face the demand on carbon and energy (Bisova and Zachleder, 2014). Competition for carbon to form TAG and starch by C3 precursors leads to a division in the use of carbon. Until now, the mechanism of carbon partition, with the change from starch production to TAG production, is still not clear in the literature. However, the formation of TAG increases with the inhibition of starch formation (Wang *et al*., 2014). Li *et al*. (2010a) recommends the use of a starch-less mutant of *C. reinhardtii* for enhanced production of TAG based on a comparison of TAG production between the starch-less mutant and wild type strains. The

authors also reported that the growth of the starch-less mutant *C. reinhardtii* was inhibited and that there was an increase in the degradation of TAG offsetting production.

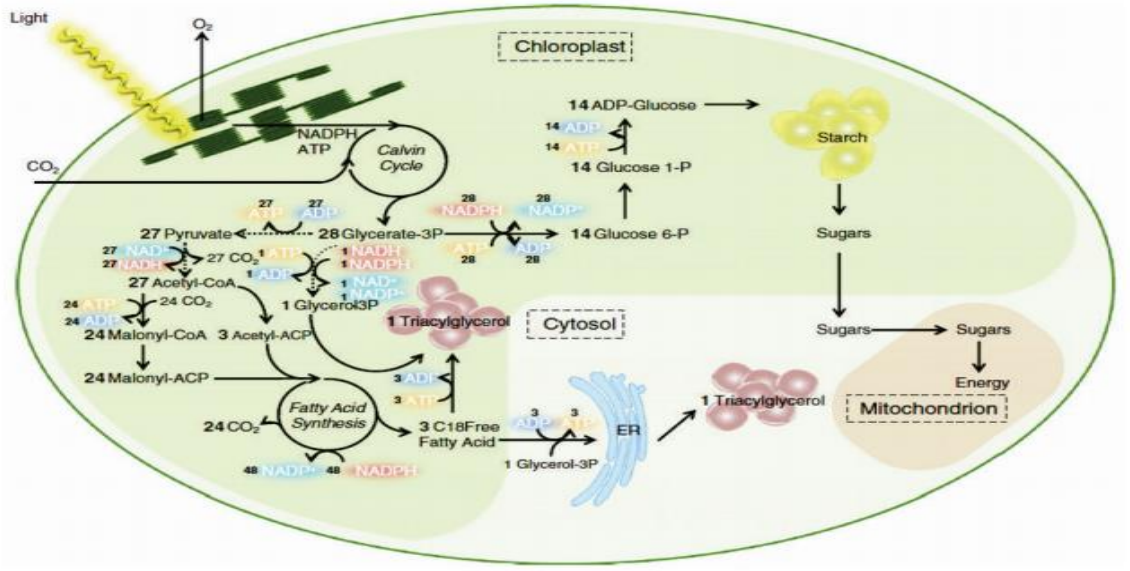


*Figure 1.3: A simplified drawing of the metabolism of triacylglycerol (TAG) in microalgae (Adapted from de Jaeger et al., 2014). The dashed lines indicated the reactions that happened in the cytosol. The figure shows the pathways of TAG formation following the way in chloroplasts or endoplasmic reticulum membrane.*

### 1.5. Production of Biofuels from Microalgae

There has been a hitherto unparalleled surge in economic growth across the entire world-supported by the ready and inexpensive availability of energy in the past decades (Smil, 2000). This ongoing and massive need for power tends to be met by reserves of combustible fossil fuels. Indeed, it was noted that 81 percent of energy requirements have come from non-renewable sources and this persists to be the case as to the present day. In the long term, this situation is not sustainable, and it is clear that society will not be able to depend on fossil fuels indefinitely. New reserves of fossil fuels are not being exploited at a rate which keeps abreast with the high levels of consumption of this finite reserve.

It has been asserted that, if there is no reduction in consumption of electricity by the end of the twenty-first century, the world's oil and gas reserve will be used up. Secondly, climate change has been brought about by widespread and never-ending fossil fuel combustion (Frame and Myles, 2007). Scientists maintained that carbon dioxide has aggravated the greenhouse effect for more than a century. However, the atmospheric $CO_2$ concentration has

been rising since the industrial revolution. The combustion of fossil fuel has significantly increased and the rate of the increase is not compatible with natural climate cycles (Keeling, 1961).

To reduce dependence on fossil fuel and to reverse global warming; bioenergy is a possible option. Biomass from the sun's energy could be converted to accessible power useful for the masses (Rittman, et al., 2008). Biofuel production has now entered into its third generation, with the feedstocks used for fuel production evolving over time (**Figure 1.4**).

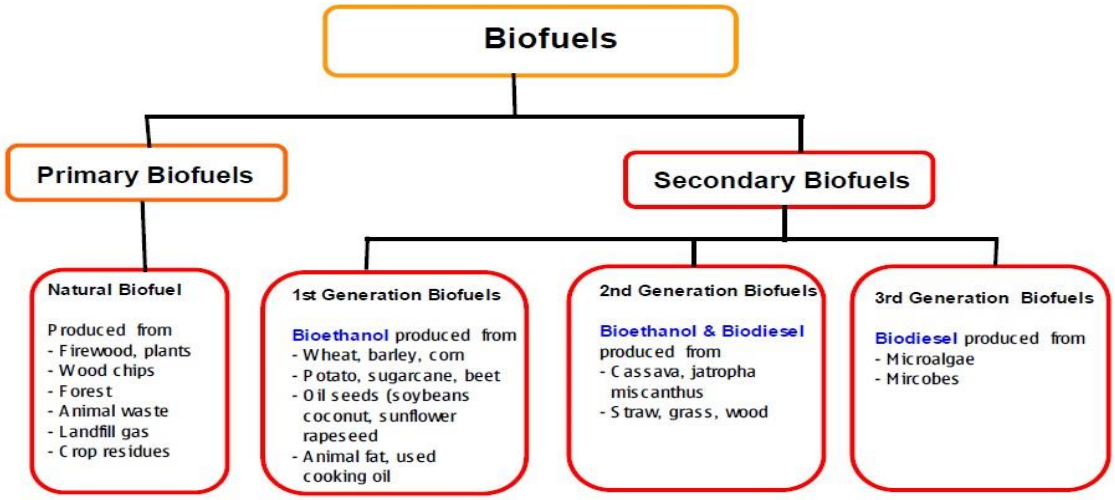


*Figure 1.4: The classification of biofuels (Adapted from Dragone et al., 2010; Alam et al., 2012).*

First-generation biofuels were derived from edible sources; such as seed, grain, sugars, oil crops and animal fats. This resulted in competition for the use of arable land between food producers and fuel producers and a subsequent hike in price for both parties (Scharlemann and Laurance, 2008). It became clear that biofuels would need to be sourced differently if the pressure on land and prices was to be avoided. The second-generation began to focus on using lignocellulosic biomass residues generated agriculturally and industrially. These were seen as a viable source of renewable energy, avoiding the problems of the first generation. However, these types of raw materials are composed of mixtures of polymers that are cross-linked, making the processes for breaking down these feedstocks into simple sugars utilized by microorganisms complex and environmentally unfriendly due to use of strong acids and high temperatures (Sanderson, 2011).

The precursors of biofuel production need to be accumulated over an extended period without being degraded. Endorsed as a valuable third-generation biofuel feedstock; microalgae can be developed on traditionally uncultivated land and saltwater, and they do not interfere with the growth of conventional food crops. They make excellent biofuel producers due to their high synthesising capacity of fats and lipids, exhibiting fast-growth as well as a net-zero $CO_2$ emission balance as they help counteract greenhouse gases (Nigma and Singh, 2011). Among all these renewable energy systems, biofuels can be recognised as renewable energy produced by biological systems that can be used for heating, electricity generation and transport. The biofuels are seen as an appealing form of renewable energy due to their worldwide availability and straightforward processing and use, leading to low-costs of manufacturing. Biofuels play a crucial role in diminishing $CO_2$ emissions (Griffiths and Harrison, 2009).

### 1.6. Biodiesel

Oil industries face the challenge of environmental protection and exhaustion of feedstock oil supplies. Biodiesel can be derived from triglycerides (triacylglycerols, TAG) or free fatty acids by transesterification with short-chain alcohols (**Figure 1.5**) and it has attracted great attention for many decades as a renewable, biodegradable, and non-toxic fuel (Li, *et al*., 2008).

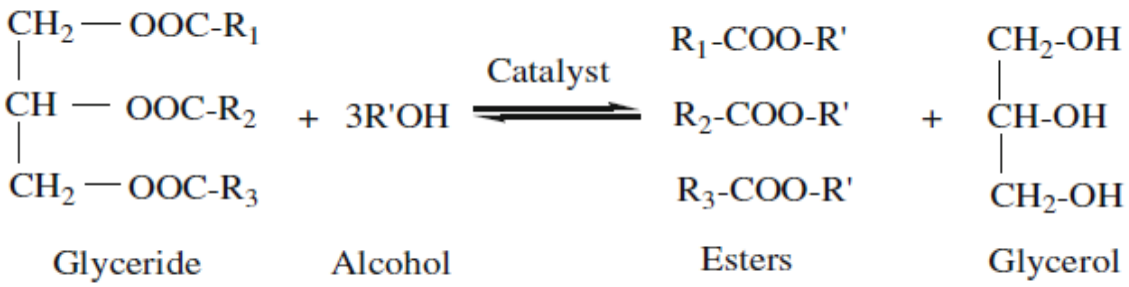


*Figure 1.5: Transesterification reaction for the production of biodiesel. Esters are the biodiesel and glycerol is a waste product (Adapted from* Li, *et al*., 2008.

The long-chain alkyl esters (methyl, propyl, ethyl) produced during various chemical processes involving lipids comprise a mixture, which creates biodiesel. Free fatty acids are the primary requirement for biodiesel, which is a renewable, carbon-neutral alternative fuel that burns cleanly and that can be utilised in combination with fossil fuels, or on its own in internal combustion engines. Biodiesel is a good alternative to petro-diesel in conventional diesel engines as it has comparable combustion properties and it does require changes to the

current fuel set-up. It has been estimated that global production of biodiesel reached approximately four billion gallons in 2009, production throughout the world has been growing steadily and the preceding ten years witnessed a sixteen-fold elevation, principally in the USA and the EU States. It has been pointed out that biodiesel has many advantages over petro-diesel, including fewer emissions, more productive combustion, renewability, greater lubricity, improved safety and generally less negative impact on the environment (Demirbas, 2010). The production of biodiesel has increased in many countries, reaching about 82% of all biofuel production. European Biodiesel Board reported that Germany, France and Spain are considered to be the main leading countries in Europe which increased the production of biodiesel to 22.117 million tons in 2011.

The price of biodiesel mainly depends on the type of feedstock used, which represents about 60-70% of the total cost of biodiesel fuel (Canacki and Sanli, 2008). The chemical reaction in which biodiesel is produced is called transesterification (**Figure 1.5**) and is the reaction between triacylglycerol and short-chain monohydric alcohols (mainly methanol) at a high temperature in the presence of a catalyst that synthesises the initial units of biodiesel, which are fatty acid alkyl esters. Regarding several pieces of research on various organisms that may be utilised for biodiesel products, microalgae have been proposed as a beneficial source due to their high levels of lipid; besides, the algal process does not compete for land, food or water (Chisti 2007; Williams, 2007).

## 1.7. Factors Involved in the Growth and Lipid Production of Microalgae

### 1.7.1. Temperature

Temperature is an important factor that enhances the yield of a closed growth system such as bioreactors and open ponds. The daily changes in the temperature can impact on lipid production leading to a reduction of the output (Wehr, 2007). So, the strategy of temperature control has been extensively utilized to increase FAs production and to induce lipid production in several microalgae considered for biodiesel production (Juneja *et al.,* 2013; Taggar *et al.,* 2014). It is reported that when there was a rise in temperature, the cell volume decreases. In general, the optimum temperature for growth falls between 20ºC and 30ºC (Chisti, 2008; Venkata *et al*., 2014). Several microalgae strains can resist down to 15ºC, but a low growth average occurs if the temperature increases by a slight degree; this leads to the death of the algae (Venkata *et al.* 2014).

However, growing microalgae under dark conditions or low climate temperature resulted in a reduced biomass (Griffiths *et al.*, 2011). Low-temperature adjustment for lipid was applied to *Dunaliella salina* and an increase of about 20% in the content of unsaturated lipids (FAs) was observed (Thompson, 1996; Sharma *et al.*, 2012). *Ochromonas danica* was studied and the study revealed that when increasing the temperature for the culture from 15*ºC* to 30*ºC*, the number of cells exponentially multiplied; with a rise in the total content of the sample's lipid (Aaronson, 2006). The temperature should be maintained optimally; otherwise, biochemical pathways in the cells may damage the accumulation of storage lipid; resulting in an unsuitable condition for lipid accumulation (Kurpan, *et al*., 2015). Based on the study carried out by Singh and Singh, (2015), the optimum temperature for growth of some microalgae strains like *Chlorella*, *Spirogyra*, *Botrycoccus* and brown algae varies from 20 ºC to 30 ºC as well as 33–400 mmol/$m^2$/s light intensity (Lee *et al*., 2011).

Temperature can affect all metabolic pathways and productive cultivation (Borowitzka, 2016). Temperature plays a major role in photosynthesis, respiration and growth rate. All these processes decrease when temperature is increased over its optimum appliance leading to a defect in the production of adenosine triphosphate (ATP) a source of energy, and to protein destruction necessary for facilitating photosynthesis (Raven and Geider, 1988; Ras *et al*., 2013). Accordingly,, high-temperature impacts fatty acids as in lipid resulting in decreased unsaturated fatty acids and composition imbalance (Montensen *et al*., 1988; Renaud *et al*. 1995; Jiang and Chen, 2000; Wei *et al*., 2015). In contrast with the green algal species *Chlorella sorokiniana* there is no change in the content of lipid under various temperature regimes (Patterson, 1970).

### 1.7.2 Light

Microalgae essentially prefer light as a source of energy, $CO_2$ as a carbon source, culture medium for growth harbouring nutrients such as nitrogen and phosphorus. Light is a very important factor for the algae growth, as it is known that microalgae are phototrophic, and synthesis nicotinamide adenine dinucleotide phosphate (NADP) and adenosine triphosphate (ATP) in the presence of light (Matthew *et al*., 2014).

Generally, the optimum light intensity is different within the algae community. For example, the light saturation intensity in *Chlorella sp* was found to be 8000 lux, whereas in *Nannochloropsis sp* it was 10000 lux (Cheirslip and Torpee, 2012). The optimum light intensity was 6000 lux in *Scenedesmus sp* (Mandotra *et al*., 2016). In *Scenedesmus sp*, it was

found that lipid production increased gradually from 3000 to 6000 lux under controlled culturing (Mandorta *et al*., 2016). The type of lipid is also affected by light intensity. When the intensity of light was decreased, the algae cells produced a high amount of polar lipid that leads to maximization of the synthesis of chloroplast membranes, whereas increasing light intensity results in the accumulation of neutral lipids with no effect on the growth rate of algae (Breuer *et al*., 2013a).

### 1.7.3 Salinity

A profusion of microbial cultures can colonise a wide range of environments. These natural ecosystems, are often noted for their absence of developed forms; they are classified by humans as extreme environments; meaning that they may be too saline, alkaline, acidic or hot to support meaningful life (Galinski and Truper, 1994). In 1978, Kushner categorised them into: extreme, moderate and slight halophiles: extreme halophiles exhibited peak development at a minimum of twelve per cent w/v salt, moderate halophiles between three and fifteen per cent w/v salt, and slight halophiles at approximately three per cent w/v NaCl. As the methods employed by cells to endure salt could have significant marketable functions, many biotechnologists, physiologists, microbial biochemists and ecologists have expressed interest in the study of salinity as an essential environmental consideration (Galinski and Truper, 1994).

Each of the life domains: Eukarya, Archaea and Bacteria have been noted to support halophilic microorganisms. Their capacity to sustain osmotic balance enables halophilic microorganisms to develop and thrive in hypersaline environments (Margesin and Schinner, 2001; Madern and Zaccai, 2004). Osmotic stress and ion toxicity are the two products of salt stress (Mager and Siderius, 2002). Cation transport system speeds up the elimination of sodium, and osmotic regulation via osmolyte synthesis is a means to reduce the harmful effects of salt stress (Yancey, 2005). Microalgae fit within all three Gilmour's (1990) classifications of microorganisms which rely on salt for development; by employing the method of organic osmolyte (compatible solute) to adapt to saline environments. Gilmour categorised salt-dependent microorganisms into slight halophilic organisms including marine systems such as seawater, comprising approximately 0.5 M NaCl, moderate halophiles organisms which developed best between 0.2 - 2M NaCl, and extreme halophilic-organisms which exhibited peak growth at more than 3M NaCl (Gilmour, 1990).

### 1.7.4 Nitrogen starvation

Nitrogen is an essential requirement for the growth of all organisms. It is critical for cell structure, functional proteins and genetic material, including nucleotides and chlorophyll (Cai *et al.*, 2013). Nitrogen sources are primarily inorganic salts needed for the growth of all organisms. However, the compatible conditions for nitrogen source and concentration differ according to the species concerned, e.g. use of $NO_3$ or $NH_4$ (Huang *et al.*, 2013a). Nitrogen limitation decreases cell division rate and thus induces a shift in the biosynthetic pathway of lipid to build up neutral lipid (TAG) instead of membrane lipids to form cell membranes (Hu, 2004). Furthermore, nitrogen limitation led to a ten times increase in the lipid content of *Micractinium pusillum* during a six-day growth experiment (Li *and Deng* 2012).

### 1.7.5 Heavy metal stress

There are some important micronutrient metal ions such as Fe, Mn, Cu, Zn and Ni (Janssen *et al*., 2010, Kar *et al.*, 2008). Several studies reported that lipid accumulation was enhanced under heavy metal stress (Einicker-Lamas *et al*., 2002). Ren *et al*. (2014) estimated the impact of $Fe^{3+}$, $Mg^{2+}$ and $Ca^{2+}$ on lipid content of *Scenedesmus sp.* He suggested that the amount of total lipid increased up to 28 and 29 % under culturing with EDTA, respectively. This indicates that heavy metals like $Fe^{3+}$and $Ca^{2+}$ can modify the pathways for biosynthesis and breakdown of lipids (Liu *et al*. 2008). Battah *et al*. (2015) studied the effects of two heavy metals $Mn^{2+}$ and $Co^{2+}$ on the accumulation of lipid in *C. vulgaris*.

Also, the lipid content in *Botryococcus* increased under cultivation with high iron concentration (Yeesang and Cheirsilp, 2011). A similar instance occurred in another examination conducted for *Monoraphidium sp.* after cultivation with magnesium (Huang *et al*., 2013b). Other metals, such as zinc and copper, being enzyme cofactors, displayed corresponding impacts on growth and lipid production in algae (Zhao *et al*. 2013).

## 1.8. Industrial Application of Microalgae

Algae are considered a low-cost product for most people, and they are a source of nutrition available as proteins, pigments, fatty acids and triglycerides. Microalgal biomass has been used as food and it feeds animals in farms (Pignolet *et al*., 2013; Borowitzka, 2013; Lu *et al*., 2016). Moreover, microalgae are medicinal sources treating diseases such as high cholesterol, atherosclerosis and cancer; drugs from microalgae are consumed as powders and capsules (Raja *et al*. 2007). Japan started cultivation of microalgae in the 1960s in large quantities, especially *Chlorella sp;* it is as an additive in food. In the 1970s and 1980s, industrial

applications and commercialisation of microalgae moved to some countries such as China, Taiwan, USA and Australia focusing on *Spirulina sp* and *Chlorella* and helping to address the gap of protein inadequacies.

Many researchers have been studying *Arthrospira* (*Spirulina*) and *Haematococcus* with their paramount focus on cell accumulation and on expanding its yield, trying to stabilise the products derived. There are many reports on astaxanthin and β-carotene produced by Zion Market Global Research in 2016 (Mohammadi-Gouraji *et al*., 2018). There is an increasing world demand to meet needs of the people and to feed animals. Enriched feeds of a small amount of biomass of microalgae contributed to strengthening the immunity of animals giving them resistance against disease, increased antiviral and antibacterial protection, enhancing reproductive performance. They contributed immensely to weight gain (Harel *et al*., 2007; Madeira *et al*., 2017). *Chlorella vulgaris* was used to feed the dairy cattle and to change the fatty acid composition of milk reducing the proportion of saturated fatty-acid residues and increasing the content of DHA (Kouˇrimská *et al*., 2014).

Adding extra microalgae to feeding sheep and horses enhanced the amount of fatty acid in their meats. While pigs and poultry fed with *Arthrospira platensis* increased weight of the domesticates (Simkus *et al*.,2013), Furthermore, microalgae are used for feeding aquaculture; for example, astaxanthin derived by microalgae as an additive in the aquaculture feed in industries improved colour and the outside-look in fishes and shrimps. It was found that adding astaxanthin enhanced the growth rate and life of larvae in aquaculture. Besides, it has proven to improve the immunity and resistance against infectious disease in farmed fish. The application and commercialization of microalgae as feed-in aquaculture are limited due to the high cost of Algae production whether stored or harvested upstream (Franciele *et al*., 2019).

### 1.9. Aims of Project

The following were the aims of the remaining chapters:

The aim of Chapter Three was to isolate and identify species of unicellular non-motile green algae isolated from pond water using a molecular approach.

The aim of Chapter Four was to evaluate the comparative potential of *Auxenochlorella* and *Chlorella* species for the production of biofuels by determining the neutral lipid content under salinity and nitrogen stress.

The aim of Chapter Five was to optimize growth media for enhanced biomass and lipid production by *Auxenochlorella* and *Chlorella* for biodiesel production.

The aim of Chapter Six was to determine the influence of glucose on the mixotrophic growth and lipid production in *Chlorella vulgaris*.

The aim of Chapter Seven was to determine the neutral lipid content in *Auxenochlorella* and *Chlorella* using flow cytometry technique in combination with Nile Red.

# Materials and Methods

Chapter Two

## 2.1. Cleaning and Sterilisation Techniques

To avoid any contamination during the experiments, all the culture equipment used in the experiments was autoclaved. Also, inoculation and sub-culturing were carried out with a flame after cleaning the bench with 70% ethanol.

## 2.2. 3N-BBM+V (Bold Basal Medium with 3- Fold Nitrogen and Vitamins)

Both water samples (see section 2.3) were grown in BBM medium. The BBM medium was prepared according to Bischoff and Bold (1963) using a series of stock solutions that were then combined. The stock solutions were prepared as follows:

*Table 2.1: Stock solutions prepared on the basis of grams per litre*

| Stock solutions | Gram per litre |
|---|---|
| (1)25.0 g $NaNO_3$ | 30.0 ml |
| (2)2.5 g $CaCl_2.2H_2O$ | 10.0 ml |
| (3)7.5 g $MgSO_4.7H_2O$ | 10.0 ml |
| (4)7.5 g $K_2HPO_4.3H_2O$ | 10.0 ml |
| (5)17.5 g $KH_2PO_4$ | 10.0 ml |
| (6)2.5 g NaCl | 10.0 ml |
| (7) Trace element solution (see below) | 6.0 ml |
| (8) Vitamin B1 (see below) | 1.0 ml |
| (9) Vitamin B12 (see below) | 1.0 ml |

1 litre of the medium was made up with distilled water and autoclaved at 15 psi for 15 minutes

To the trace element solution (7) in 1000 ml of distilled water, the following were added: 0.75 g $Na_2EDTA$ and then the minerals in the following sequence: $FeCl_3.6H_2O$ 97.0 mg $MnCl_2.4H_2O$ 41.0 mg $ZnCl_2.6H_2O$ 5.0 mg $CoCl_2.6H_2O$ 2.0 mg $Na_2MoO_4.2H_2O$ 4.0 mg

Vitamin B1 (8) 0.12 g Thiaminhydrochloride in 100 ml distilled water was then filtered and sterilised. Vitamin B12 (9) 0.1 g Cyanocobalamin in 100 ml distilled water 1 ml of this solution was taken and 99 ml distilled water was added. This was then filtered and sterilised.

### 2.2.1. Modified BBM media

Different modifications were made to the BBM media to grow algal samples under stress conditions**.**

#### 2.2.1.1. High-salinity BBM medium

High-salinity BBM media (0.2, 0.4, 0.6, 0.8 M NaCl) were prepared by adding 11.7 g, 23.4 g, 35 g, 47g NaCl litre of BBM media, the media was adjusted to pH 7.2 using 1 M HCl or NaOH.

#### 2.2.1.2 BBM medium containing different concentrations of $NaNO_3$

To provide different concentrations of nitrogen in BBM media, different volumes of stock solution 1 (15 g/ l $NaNO_3$) were added to BBM media to make 50% and 25% concentrations of nitrogen in the BBM media.

#### 2.2.1.3 BBM medium containing different concentrations of glucose

*Auxenochlorella* and *Chlorella* were cultured in BBM media with different glucose concentrations (0.1, 0.5,1 and 2%) and (1, 5, 10 and 20 g glucose) per litre to examine how carbon source influences growth and lipid production under mixotrophic culture.

## 2.3. Collection of Samples

Water samples were collected from different locations around the edge of Weston Park pound (Sheffield, UK) using sterile 50-ml Falcon tubes, each of which was filled with 50 ml of pond water. Each sample was then transferred to a sterile 250-ml flask and 50 ml BBM medium was added.

## 2.4. Microscopic Examination of the Pond Samples and purity of microalgae strains

After the water pond samples showed growth in BBM medium flasks, serial slides from each culture were prepared and examined using a light microscope (Nikon, Japan). Most of the slides contained a mixture of microalgae and bacteria. Therefore, different techniques were

used for purification including serial dilution, streak plating technique and centrifuge washing technique.

### 2.5. Maintenance of the Microalgae Strains

Sub-culturing of the *Chlorella* and *Auxenochlorella* was performed routinely once every two weeks to minimise contamination over time by adding 5 ml of active stationary phase culture of microalgae cells into 250-ml conical flasks plugged with foam bungs containing 100 ml of BBM medium, incubated and incubated at 25˚C with white fluorescent lamps. *Auxenochlorella* cultures were put on a shaker (90 – 100 rpm).

### 2.6. Measurement of Growth

The following procedure was followed: it consists of the three steps:

**1.** Exactly 100 ml of BBM media was added to at least three autoclaved 250-ml flasks then 5ml of the appropriate culture was added as an inoculum.

**2.** Cell growth was monitored by using spectrophotometry which is considered an indirect method whereby the optical density was measured at 595 nm.

**3.** The cultures were then incubated in a 25˚C room under continuous light. The OD was taken every day.

### 2.7. Molecular Identification of the Strains

Both water samples were identified using 16S and 18S rDNA primers respectively. The identification procedure included genomic DNA extraction, PCR amplification, and PCR product sequencing.

#### 2.7.1. Extraction of DNA

Genomic DNA extraction for *Auxenochlorella* and *Chlorella* were performed using the surfactant hexadecyltrimethylammonium bromide (CTAB) based on the protocol described by Li *et al.* (2002):

**1.** 5-10 ml of culture was centrifuged for 10 minutes at 3000 G.

**2.** The supernatant was discarded and the pellet was re-suspended in 500 µl of CTAB* then sonicated for 60 seconds.

**3.** The sample was then incubated at 65˚C for 1 hour.

**4.** 500 µl of phenol chloroform isoamyl alcohol was added then vortexed, the top layer was removed into a fresh Eppendorf tube and 500 µl of chloroform was added.

**5.** The sample was then centrifuged for 5 minutes at full speed then the top layer was measured and transferred into a fresh Eppendorf tube.

**6.** The sample was then incubated on ice for 10 minutes then centrifuged.

**7.** The supernatant was discarded and 1 ml of ethanol was added then centrifuged and the supernatant was discarded. The same procedure was repeated and the remaining ethanol was removed.

**8.** Air drying was performed for 10 minutes and the pellet was re-suspended in 50 µl of MilliQ water.

**9.** The DNA pellet was left on the bench to be re-suspended overnight and incubated for 60 minutes at 50˚C in the morning.

* **CTAB:**

2% CTAB (Cetyltrimethylammonium Bromide)

2% β-mercaptoethanol

0.1 M Tris-HCl pH 8.0

1.4 M NaCl

20 mM EDTA

### 2.7.2. Gel electrophoresis

To detect the presence of DNA in the extracted samples, a 1% agarose gel was prepared by dissolving 0.6 g of agarose powder and 60 ml of distilled water with 1.2 ml of 50x TAE buffer. 0.6 µl aliquot of Gel Red was added to the gel mix after heating the mix in a

microwave oven at power 6 for 90 seconds. 10 µl of the sample was mixed with 2 µl 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 80 V for 45 min. Bands were visualised with a UV lamp and photographs were taken to capture the image.

### 2.7.3. Polymerase chain reaction amplification

PCR amplifications were performed for identification of the pond samples using 18S primer (Lim et al., 2012) for *Chlorella* and 16S primer for *Auxenochlorella* in a Mycycler thermal cycler (Bio-Rad).The sequences of both 16S and 18S primers are illustrated in Table 2.1 and the PCR mixtures are shown in Table 2.2.

*Table 2.2: Sequence of 16S rRNA and 18S rRNA Primers.*

| **Primer** | **Sequence** |
|---|---|
| 27 F 16S | 5-AGA GTT TGA TCC TGG CTCAG -3 |
| 1492 R 16S | 5-TACGGCTACCTTGTTACGACTT -3 |
| 18S rRNA For Lim | 5-GCGGTAATTCCAGCTCCAATAGC-3 |
| 18S rRNA Rev Lim | 5 –GACCATACTCTCCCCCGGAACC-3 |

*Table 2.3: The Contents of Tubes for PCR Amplification.*

| **Sample** | **16S** | **18S Lim** | **Control** |
|---|---|---|---|
| Master mix | 20 | 20 | 20 |
| For primer | 4 | 4 | 4 |
| Rev primer | 4 | 4 | 4 |
| Molecular water | 17 | 17 | 23 |
| Genomic DNA | 5 | 5 | 0 |

| |
|---|
| Total volume is 50µl for all samples |

*Table 2.4: PCR Cycling using 16S rRNA Primers*

| | | |
|---|---|---|
| Initial Denature | 94 °C | 3 min |
| Denature<br>Anneal<br>Elongation | 95 °C<br>58 °C<br>72 °C | 1 min<br>1 min<br>1 min<br>30 cycles |
| Final Elongation | 72 °C | 5 min |

*Table 2 .5.: PCR cycling using 18S rRNA primers*

| | | |
|---|---|---|
| Initial Denature | 94 °C | 5 min |
| Denature<br>Anneal<br>Elongation | 95 °C<br>58 °C<br>72 °C | 30 sec<br>30 sec<br>30 sec<br>30 cycles |
| Final Elongation | 72 °C | 10 min |

The PCR products were detected using 1% agarose gel (as described in Section 2.7.2), and the positive results were purified using a KeyPrep PCR clean-up kit as follows:

- The volume of the samples were determined and adjusted to 100 µl with sterile distilled water. 5 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. 1 ml) assembled in a clean collection tube, centrifuged at 10,000 × *g* for 1 minute. The flow through was discarded.

- The columns were washed with 750 µl Wash Buffer and centrifuged at 10,000 × *g* for 1 min and the flow through was discarded.
- The columns were centrifuged at 10,000 × *g* for 1 minute to remove residual ethanol.
- The columns were placed into clean microcentrifuge tube. About 80 µl of Elution Buffer was added onto the column membrane and allowed to stand for 2 minutes. The eluted DNA was collected by centrifuging the column at 10,000 × g for 1 minute.

The eluted DNA was confirmed by electrophoresis of the purified DNA and the rest of the DNA was stored at 4°C.

### 2.7.4. PCR Purification

PCR products were purified by QIAquick PCR Purification Kit (Qiagen). Furthermore, the purified PCR product was measured in quartz cuvette at 260 nm using a spectrophotometer.

### 2.7.5. DNA sequencing

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

### 2.7.6. Phylogenetic tree constructions

After PCR amplifications, 18S gene sequences of pond water samples were discovered with specific gene sequences through National Center for Biotechnology Information (NCBI) database by using the basic local alignment tool (BLAST).

The highest identity scores of genus sequences were chosen and with the query sequences restored from the NCBI GenBank database, Jalview software (Waterhouse *et al.*, 2009) was utilised to conduct multiple sequence alignment of the query and the retrieved sequences by using the muscle method. The phylogenetic tree was constructed by Molecular Evolutionary Genetics Analysis (Mega, version 7) using the neighbour joining method and the maximum likelihood method (Kumar *et al*., 2016).

## 2.8. Microalgae Dry Weight Determination

To produce the standard curve of dry weight (DW) in biomass of *Auxenochlorella* and *Chlorella*, well-grown algal culture was adjusted with a fresh medium to an OD of 1 at 595 nm. The relationship between OD and the algal cell dry weight was measured, based on Storms *et al*., (2014). A set of dilutions was made according to the Table below; the final volume of each dilution was 30 ml.

*Table 2.6: Dilution for dry weight determination*

| Tube Number | Conc (%) | Culture (ml) | Medium (ml) |
|---|---|---|---|
| 12 | 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 | 0.00 |

12x50 ml Falcon tubes were centrifuged and the supernatants were discarded. The pellets were resuspended in 5 ml distilled water and transferred to 12x15 ml Falcon tubes. These Falcon tubes were centrifuged again and the supernatant was discarded. After that, each pellet was re-suspended in 1 ml of distilled water and it was transferred to pre-weighed (on final balance) Eppendorf tubes. The tops of the Eppendorf tubes were removed and a hole was

made in each in order to to be used as extra tops to the Eppendorfs containing samples. The samples Then the tubes with pellets were frozen overnight at - 80˚C and then freeze-dried for 24 to 48 hours until the samples were completely dried. After that the tops with holes were discarded and weighed on a fine balance. The concentration curve was prepared on Excel using optical density (OD) reading against the dry weight of each sample.

### 2.9. Quantification of Neutral Lipid Using the Nile Red Method

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

#### 2.9.1. Determination of optimum cell concentration and peak time for 96 well plate method

This experiment was performed based on the study carried out by Chen *et al.* (2009) and Simionato *et al.* (2011). To determine the optimum cell concentration to be used, 5 ml samples of well grown algal culture of *Auxenochlorella and Chlorella* (active stationary phase culture) were taken aseptically and adjusted with a fresh medium to an OD of 1 at 595 nm. The following series of 8 dilutions of algal culture were then made in 1 ml from this adjusted culture using a fresh medium at 12.5, 25, 37.5, 50, 62.5, 75, 87.5 and 100% in 8 x autoclaved 1.5 ml Eppendorf tubes as shown in Table 2.7 below.

*Table 2.7: Dilutions scheme to produce a range of cell concentration for a Nile Red peak fluorescence test.*

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

The ODs were then taken at 595nm for each of these dilutions in order to provide ODs at the given culture concentration. These tubes were then centrifuged at 3000 *g* for10 minutes in a bench top centrifuge. The supernatant was discarded immediately and then each pellet was resuspended in 20 µl of distilled water. Two 2-ml screw top microfuge tubes were then prepared for each of the 8 culture concentrations, one for stained and one for unstained cells. Then 10 µl of each resuspended algal pellet was transferred to each of the labelled stained and unstained screw top microfuge tubes. Then 50 µl of dimethyl sulfoxide (DMSO) was transferred to each screw capped microfuge tube. The caps of the tubes were then tightened loosely and all tubes were heated for 50 seconds at full power in a Matsui microwave. The tubes were then removed from the microwave and then 930 µl of distilled water was added to tubes labelled stained and 940 µl of distilled water to all unstained labelled tubes. Nile-red fluorescent dye dissolved in 100% dimethyl sulfoxide (DMSO) was then added to each tube labelled stained. All tubes were microwaved again at full power for 60 seconds and a timer was started as soon as the heating was completed. The entire 1 ml content of each stained tube was transferred to a multi-pipette reservoir. Then 200 µl aliquots of each cellular concentration was transferred to rows A - D in a 96 well plate, providing 4 technical replicates at each concentration. The same procedure was applied on the unstained tubes in rows E - H. The plate was then put in the plate reader after removing the lid of the plate as shown in Figure 2.1. The details of the plate reader settings for the peak fluorescence test are presented in Figure 2.2. To track the fluorescence, Gen5 2.05 software was used for 30 minutes at 5-minute intervals.

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

| Dilution (%) | | 100 | 87.5 | 75 | 62.5 | 50 | 37.5 | 25 | 12.5 | Empty Wells | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | R1 | Stain1 | Stain5 | Stain9 | Stain13 | Stain17 | Stain21 | Stain25 | Stain29 | BLK | BLK | BLK | BLK |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| B | R2 | Stain2 | Stain6 | Stain10 | Stain14 | Stain18 | Stain22 | Stain26 | Stain30 | BLK | BLK | BLK | BLK |
| C | R3 | Stain3 | Stain7 | Stain11 | Stain15 | Stain19 | Stain23 | Stain27 | Stain31 | BLK | BLK | BLK | BLK |
| D | R4 | Stain4 | Stain8 | Stain12 | Stain16 | Stain20 | Stain24 | Stain28 | Stain23 | BLK | BLK | BLK | BLK |
| E | R1 | unsta1 | Unsta5 | Unsta9 | unsta13 | unsta17 | Unsta21 | Unsta25 | Unsta29 | BLK | BLK | BLK | BLK |
| F | R2 | Unsta2 | Unsta6 | unsta10 | unsta14 | unsta18 | Unsta22 | Unsta26 | Unsta30 | BLK | BLK | BLK | BLK |
| G | R3 | Unsta3 | Unsta7 | unsta11 | unsta15 | unsta19 | Unsta23 | Unsta27 | Unsta31 | BLK | BLK | BLK | BLK |
| H | R4 | Unsta4 | Unsta8 | unsta12 | unsta16 | Unsta20 | Unsta42 | Unsta28 | Unsta32 | BLK | BLK | BLK | BLK |

*Figure 2.1: 96 well plate layout for peak fluorescence test (cell concentration experiment).Note: Rows R1to R4 are technical replicates obtained from the same concentration.*

| Procedure details | | | |
|---|---|---|---|
| Plate Type | 96 Well Plate | | |
| | Fluorescence end point | | |
| | Full plate | | |

| | | | | |
|---|---|---|---|---|
| | | | | |
| Read | Filter Set 1 | | | |
| | Excitation 485/20, Emission 580/50 | | | |
| | Optics Top, Gain:60 | | | |
| | Read Speed: Normal | | | |

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

### 2.9.2. Determination of optimum Nile Red concentration method

This experiment was performed based on the work initiated by Chen *et al.* (2009). In this procedure, the Nile Red stock was diluted to give concentrations of 0.05, 0.1, 0.2, 0.3, 0.4 and 0.6 µmol $ml^{-1}$ by being dissolved in DMSO from a 1 mg $ml^{-1}$ stock of Nile Red as shown in Table 2.8 below. NB 0.3 µmol $ml^{-1}$ = 100 µg $ml^{-1}$

*Table 2.8: A series of 6 dilutions of Nile Red stock concentration prepared by being dissolved in 100% dimethyl sulfoxide (DMSO).*

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

The same procedure described in section 2.9.1 was followed to prepare algal suspensions at optimum OD at 595 nm and stained and unstained 2-ml screw top microfuge tubes. The samples were also prepared as mentioned above for each Nile Red concentration using each of the 6 Nile Red concentrations separately in place of the 100 µg $ml^{-1}$ dilution used in section 2.9.1. The 96 well plate was then prepared and run as described in section 2.9.1, ensuring that fluorescence values were read at the optimum time after microwaving as stated in section 2.9.1, (**Figure 2.3**).

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

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

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | R4 | H | 200 | 200 | 200 | 200 | 200 | 200 | 0 | 0 | 0 | 0 | 0 | 0 |

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

### 2.9.3. Lipid quantification of *Auxenochlorella and Chlorella* cells (Nile Red sample measurement test)

Neutral lipids were quantified for cells grown until the stationary phase (14-21 days old) under normal growth conditions. The protocol was followed using the optimum conditions defined including optimum cell concentration, peak time and optimum Nile Red concentration as described in section 2.9.1 and 2.9.2, respectively. As three flasks of culture were set up under normal growth conditions, results from one plate from each flask were averaged. Results of the experiments were then converted to quantify lipid using the calibration curves to produce the concentration as well as the dry weight percentage according to Bertozzini *et al.* (2011).

### 2.9.4. Determination of neutral lipid of *Auxenochlorella and Chlorella* cells grown at different salinities

A common observation reveals that an increase in salinity can increase the lipid content in microalgal cells while lowering the growth rate of cells. Therefore, the effect of increasing external salinity on the growth and lipid content of microalgal species was investigated in *Auxenochlorella and Chlorella* cells grown in BBM culture medium by the Nile Red method. Measurements were made over a significant time period with a range of different salinity levels (0.2, 0.4, 0.6, 0.8 and 1 M NaCl) at 25ºC for a 4-week incubation period in comparison with normal growth conditions.

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

incubation. The lipid accumulation was determined every week using Nile Red dye using optimum conditions of Nile Red method including 75% cell concentration of *Auxenochlorella* which is equivalent to an OD595 = 0.858 and Nile Red concentration of 0.4 μmol $ml^{-1}$ with 15 minutes staining. Whereas for *Chlorella*, 75% cell concentration (OD595 = 0.715), Nile Red concentration of 0.3 μmol $ml^{-1}$ with 25 minutes staining was utilized. The lipid content in *Auxenochlorella and Chlorella* cells was plotted against the concentration of salinity used over the incubation time for 4 weeks.

### 2.9.5. Determination of neutral lipid of *Auxenochlorella and Chlorella* cells grown under nitrogen starvation

The effect of nitrogen limitation on the growth and lipid content of microalgal species was examined in *Auxenochlorella and Chlorella* cells grown in BBM growth medium by the Nile Red method. The culture BBM medium was prepared with different nitrogen concentrations (Nitrogen free, 25%, and 50% of $NaNO_3$) and then growth experiments were carried out for a four-week incubation period at 25°C in comparison with normal growth conditions. The lipid content in Auxenochlorella and Chlorella cells was plotted against nitrogen concentrations used over incubation time.

### 2.9.6. Triolein calibration curve method (Nile Red triolein concentration test)

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

To allow quantification of neutral lipid within algal cells, a concentration curve of Nile Red fluorescence versus concentration of Triolein (a model neutral lipid) was set up. The Triolein was added to an algal sample so that the Nile Red fluorescence due to Triolein could be measured in the presence of algal cells. 10 mg $ml^{-1}$ Triolein lipid standard stock was made by adding 50 mg of Triolein (Sigma T7140) to 5 ml isopropanol and then dissolving it. Then 1 ml aliquots were transferred to five 1.5 ml Eppendorf tubes labelled 1 to 5. Eight algal samples were prepared under the conditions optimised in section 2.9.1; they then were processed by adding 50 μl DMSO and microwaving the samples. At this point 910 μl of distilled water was added to tubes labelled stained with 920 μl of distilled water added to all unstained labelled tubes. Then 20 μl of triolein and isopropanol was added to each of the stained and unstained tubes as shown in Table 2.11 below to produce concentrations of 0.2, 0.16, 0.12, 0.08, 0.06, 0.04, 0.02 and 0.0 mg $ml^{-1}$.

*Table 2.9: Dilutions for Triolein Concentration.*

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

10 µl of 100 µg $ml^{-1}$Nile Red fluorescence dye dissolved in DMSO was then added to each tube labelled stained. All tubes were microwaved again at full power for 60 seconds and a timer was started as soon as heating was completed. The method was then continued as described in section 2.9.1 (**Figure 2.4**).

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

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | R1 | E | 200 | 200 | 200 | 200 | 200 | 200 | 200 | 200 | 0 | 0 | 0 | 0 |
| Unstained | R2 | F | 200 | 200 | 200 | 200 | 200 | 200 | 200 | 200 | 0 | 0 | 0 | 0 |
| Cells | R3 | G | 200 | 200 | 200 | 200 | 200 | 200 | 200 | 200 | 0 | 0 | 0 | 0 |
| | R4 | H | 200 | 200 | 200 | 200 | 200 | 200 | 200 | 200 | 0 | 0 | 0 | 0 |

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

## 2.10. Extraction and Determination of Neutral Lipids in Algal Cells

### 2.10.1. 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. Each pellet was resuspended in 5ml of distilled water and transferred to 15ml Falcon tubes. The tubes were centrifuged again for 5 minutes at 3000 *g*, 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 needles; 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 the freeze was 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 the tubes and then transferred to tubes containing 1 mm glass beads; they were placed on the bead-beater 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; the supernatant was 2:1 methanol:chloroform, but 1% NaCl and chloroform were then added to produce 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 allowing the liquid to evaporate overnight. 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.

### 2.11. Analysis of Compatible Solutes in Microalgae using Nuclear Magnetic Resonance

The analysis of compatible solutes from the algal strains utilised in this study was carried out by the use of Nuclear Magnetic Resonance (NMR) based on the method devised by Derome, (1987). The methods and procedures applied were the following: duplicates of 20 ml samples of well grown samples of algal cells (OD595 = 1) were centrifuged for 10 minutes at 3000*g* using a bench centrifuge and the supernatants were discarded. Then each of the pellets was re- suspended in 1 ml of distilled water; the tubes were then bead beaten in the Bead Bug at 350 rpm for 3 minutes using a 0.5 mm beating tube as it gives the best conditions for the breakage of the cells. They were then transferred to Eppendorf tubes; these tubes were centrifuged for 5 minutes at 3000 *g*. 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 – 80$^{o}$C overnight and then transferred to a freeze dryer; they were frozen without being allowed to thaw. They were then left to freeze dry (lyophilize) for 24 – 48 hours until the samples became completely dry. The lids with the holes were discarded and the tubes were resealed with their own lids. The samples were kept on the bench at room temperature until an NMR machine was made 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 (CDCl3): deuterated (CD3Cl3) mix, 5 µl of chloroform (CHCl3) was added, serving as an internal standard; they were then transferred to a 5mm 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 1 Hz line that is broadened, followed by the manual baseline

correction. Bruker Topspin Version 1.3 software was used for processing and integrating the final spectra.

## 2.12. 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). They could be identified as percent FAME content based on the dry weight of the algal biomass. The method applied will be clarified in the following section.

### 2.12.1. Apparatus, reagents and materials

#### *2.12.1.1. Apparatus*

The following sets of apparatus were used to determine the biofuel relevant fatty acid methyl esters (FAME) in the algal samples of this study; they include:

- 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.

#### *2.12.1.2. Reagents*

The reagents used 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 (0.6 M [2.1% v/v] HCl in methanol), prepared by adding 5 ml of concentrated HCl (12 M) to 95 ml of methanol—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).

#### *2.12.1.3. 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).

### 2.12.2. Transesterification of algal cells

#### *2.12.2.1. 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 analysed and the label was allowed to dry completely before being weighed and recorded; the weights of the vials were recoeded 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 was then 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; they were left to freeze dry (lyophilize) until the samples were completely dry; 5-10 mg of each of these samples was then transferred into the pre-weighed GC vials.

#### *2.12.2.2. Preparation of the recovery standard*

Internal standard was also prepared to make 10 mg/ml by weighing 100 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 bring it to the volume required and to be mixed thoroughly. This mixture was transferred and sealed in 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 the samples were ready for running GC-MS.

#### 2.12.2.3. Transesterification of 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 µlof 0.6 M 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 crimp 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, no longer than 60 minutes at room temperature.

#### 2.12.2.4. FAME extraction and preparation for GC analysis

To extract the FAME from the mixture after the samples were 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 were made 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, no longer than 4 hours at room temperature in order to allow isolation into phases. The samples were additionally diluted as required depending on the 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 well labelled new 300-µl insert vials for each level of FAME standard were prepared.

**2.12.2.5. Preparation and addition of internal standard**

In order to correct any instrumental variability and evaporation of solvents while running the FAME analysis, an internal standard was prepared by weighing about 10 mg pentadecane (C15) standard into 1.5-ml vials. They are weighed to an accuracy of 0.1 mg then hexane of HPLC grade was added to the vials, diluted to 1:10 and sealed using PTFE/silicone/PTFE crimp caps. To each of the new 300-µl insert vials at each level of FAME standard, 5 µl of the diluted 1:10 pentadecane and 200-µl of diluted FAME extracted samples were added (Section 2.12.2.4. above).

***2.12.2.6. 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 The HPLC grade hexane was used to bring to volume following the scheme outlined in Table 2.9 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 2.10: Standards Prepared using C4:0 - C24:0 Working solutions:*

| 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** |

### 2.12.2.7. Gas Chromatography-Mass Spectrometric analysis of FAME

Gas Chromatography Mass Spectrometry (GC-MS) was used to analyse 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 1 µl of samples were injected at a 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 was observed. To optimise the peak separation fatty acid isomers, a temperature tailored programme (FAME_FINAL) was appropriately utilised. The temperature optimised programme was set to 100°C, being held for 1 minute; it was then ramped to 200°C at 25°C/min and held for 1 minute, followed by a final ramp up to 250°C at 5°C/min and held for 7 minutes. Consequently, the mass spectrometer was operated in electron ionization (EI+) mode. 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. All these responses were calculated by the software utilised. Consequently, base line separations, manual and automatic peak integration were all carried out in the quantification tool of the software.

## 2.13. Flow Cytometry Analysis of Neutral Lipid Content

The Flow Cytometry Technique was used for the analysis of neutral lipid in *Auxenochlorella and Chlorella* cells grown under different growth conditions included in a BBM medium containing 0.8 M NaCl and nitrogen starvation BBM medium in comparison with a normal BBM growth medium as a control according to the method described by Satpati and Pal (2015).

### 2.13.1. Determination of DMSO concentration

Different concentrations of DMSO ranging from 10 to 60% were prepared using distilled water; they were used to identify the best concentration of DMSO to be used in the experiment. An aliquot of 500 µl of culture which was grown under normal and stress growth conditions as mentioned above for different time periods (high OD =1.5 after 14-21 days culture) was transferred to a 2 ml Eppendorf tube; 7 of of these tubes were labelled as stained and 7 were labelled as unstained. Then 5 µl of 100 µg $ml^{-1}$ of Nile Red solution was added to the stained tubes. From each concentration of DMSO, 295 µl was added to one of the stained tubes and 300 µl was transferred to unstained tubes; the final concentrations of DMSO were 3%, 7%, 11%, 15%, 18% and 22%.

All the tubes were vortexed and left in the dark at room temperature for 10 minutes; then the stained and unstained tubes were washed 2 to 3 times with 1 ml of phosphate buffer (PBS). The samples were then transferred to a multi-pipette reservoir (Thermo Scientific). Using a multi-channel pipette, 200 µl aliquots of each stained tube were transferred to the 96 well plate rows from A to D, to give 4 technical replicates of each concentration. The same step was undertaken for the unstained tubes in rows E to H as shown in (**Figure 2.5**).

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

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Unstained Cells | R1 | E | 200 | 200 | 200 | 200 | 200 | 200 | 0 | 0 | 0 | 0 | 0 | 0 |
| | R2 | F | 200 | 200 | 200 | 200 | 200 | 200 | 0 | 0 | 0 | 0 | 0 | 0 |
| | R3 | G | 200 | 200 | 200 | 200 | 200 | 200 | 0 | 0 | 0 | 0 | 0 | 0 |
| | R4 | H | 200 | 200 | 200 | 200 | 200 | 200 | 0 | 0 | 0 | 0 | 0 | 0 |

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

### 2.13.2. Optimising of Nile Red concentration

After determining the optimal concentration of DMSO as described in Section 2.13.1., the Nile Red concentration was optimised by preparing serial concentrations of Nile Red with DMSO at 0.05, 0.1, 0.2, 0.3, 0.4 and 0.6 mg $ml^{-1}$ as the final concentration. The same steps were carried out except for adding 5 µl of each concentration of Nile Red to one of the stained tubes. After the cell was washed with a phosphate buffer (PBS), the stained and unstained tubes were transferred to run through the flow cytometer.

### 2.13.3. Determination of neutral lipid under stress conditions

The neutral lipid contents of *Auxenochlorella* and *Chlorella* cells grown in BBM medium with 0.8 M NaCl, BBM growth medium with nitrogen starvation in comparison with normal BBM growth medium as a control were measured using the flow cytometry analysis.

### 2.13.4. Flow cytometry analysis

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

To obtain the fluorescence readings, the Nile Red stained cells at different concentrations were excited with a 475-nm laser; the emission was 568/42 nm while the unstained cells were employed as an auto-fluorescence control. The features of the microalgae cells, including cell size and granularity using a flow cytometer, were obtained by the Forward Scatter (FSC) and Side Scatter (SSC) signals. Flow cytometry data were analysed depending on many parameters including the stained cells percentage and the median of the stained cells using Flow Jo software.

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

# Isolation and Identification of Microalgae from Pond Water

## Chapter Three

### 3.1. Introduction

The study of form and structure of species is not sufficient for the characterization of microalgae. There are several ways of investigating characteristics of microalgae and in recent times, molecular techniques have been used in numerous fields and are helping to strengthen scientific findings in diverse areas of research. Since the 1970s, microalgae have been identified as feedstock for alternative biodiesel production owing to the higher oil yields compared to the conventional plants (Chisti, 2007; Miao and Wu, 2006; Rodolfi *et al*., 2009). Due to the rapid growth and ease of cultivation, *Chlorella* strains were seen as favourable candidates for viable lipid production. Early research demonstrated that *Chlorella* spp are rarely contaminated by other species of algae and that they are able to grow in open ponds (Huntley and Redalje, 2006). Many microalgae accumulate lipid in the cells under environmental stress such as nitrogen deprivation, phosphate limitation (Rodolfi *et al*., 2009), and silicon deprivation (Griffiths and Harrison, 2009). When microalgae cells were exposed to stress conditions the lipid productivity values increased to over 70% more than in normal conditions (El Arroussi *et al*., 2015).

The marketing of biodiesel production processes from microalgae is still hard to implement due to the huge initial cultivation cost. This makes developing an effective process for lipid production and improvement factors for growth critical (Chisti, 2007). Environmental variables cause biosynthesis of lipid in most algae (Flynn and Bulter, 1986; Roessler 1990; Guschina and Harwood, 2006). Freshwater microalgae are considered to be one of the most favourable sources for the production of a wide range of bio-products which can be used in industry to produce lipids, recognized as the most promising feedstock for biodiesel production (Chisti, 2007; Sharma *et al*., 2014). Antioxidants and emulsifiers that are utilized in the nutrition sector are also potential products (Chisti, 2007; Ahmed *et al*., 2014).

Weather conditions and forecasts influence the rate of growth rate of algae in outdoor ponds and the weather is considered as a key environmental factor that affects the selection of potential algae candidates for outdoor growth. In recent years, many studies have investigated some key factors such as temperature, nutrients, pH and light period that affect the growth and improved lipid storage by microalgae. The results were promising with improved growth and increased storage lipid (Lv *et al*., 2010; Sforza *et al*., 2012a; Adams *et al*., 2013). However, the success of algal growth has been shown to be fundamentally controlled by the algal environment (Wang *et al*., 2016) and as such the optimization of their growth conditions is important for strains of algae used for lipid production. Furthermore, choosing a microalgal

strain in order to produce biodiesel should depend on the lipid composition, i.e. degree of unsaturation, and chain length rather than the percentage of lipid alone (Hong, 2013).

The precise identification of the strains of algae plays an important role in the choice of oleaginous microalgae for biodiesel production. In phylogenetic terms, exciting oleaginous algae associated with closely related strains have the capacity to produce high lipid (Duong *et al*., 2012). The traditional identification of microalgae has been performed by several methods such as morphological characteristics of algal cells (Boyer *et al*., 2001). The estimation and identification based morphological methods are very time-consuming and complicated (Huss *et al*., 1993).

*Chlorella vulgaris* was described around 120 years ago, the structure and form of over 100 species have been defined since the characterization of the genus (John *et al*., 2003). Depending on the molecular and biochemical data, Huss *et al*. (1999) indicated that just five *Chlorella* "true" types are available. However, 16S rRNA and 18S rRNA gene analysis has helped to identify the genus *Chlorella* excluding symbiotic relationship between species (Nakahara *et al.,* 2004). For the identification of species, rDNA and ITS genes have been utilized in diverse studies because the sequence is known to be conserved across species (Rogers *et al*., 2006).

### 3.2. Aim and Objectives

The aim of this chapter was to isolate and identify two species of unicellular non-motile green algae isolated from pond water.

The specific objectives of the research were:

1. To isolate species of green microalgae from pond water using Bold's Basal Medium (BBM),
2. To identify the green algal species isolated from pond water using a molecular biology approach,
3. To determine the evolutionary relationships between the newly isolated green algal obtained from pond water and the known species deposited in NCBI using phylogenetic trees,
4. To produce the initial growth data on the green algal strains of *Auxenochlorella* and *Chlorella* isolated from the pond water.

## 3.3. Results

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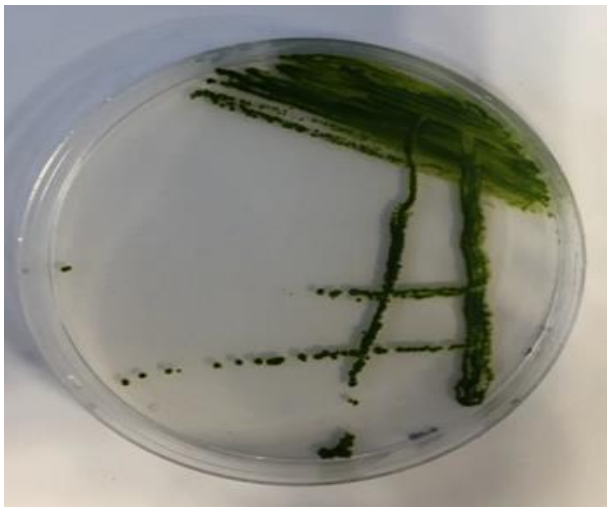


*Figure 3.1: Chlorella-like green microalga1 strain obtained from pond water in the garden; subsequently identified as Auxenochlorella protothecoides (F1).*

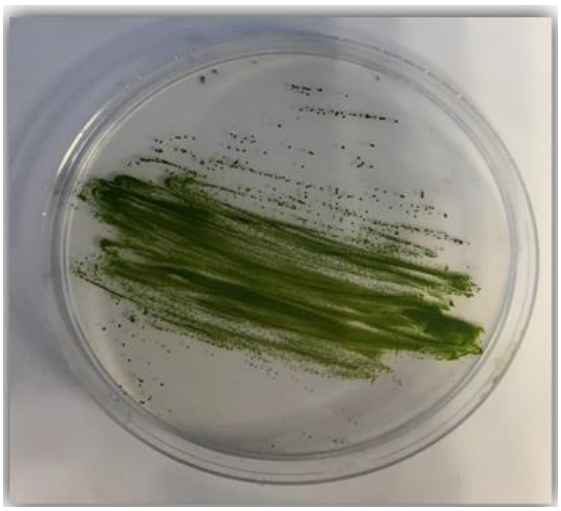


*Figure 3.2: Chlorella-like green microalga1 strain obtained from pond water in the garden; subsequently identified as Chlorella vulgaris (F2).*

### 3.3.1. Identification of two Chlorella-like species

The results of the microscopic examination of the pond water collected from different places revealed mixed cultures of algae with bacteria. Therefore, different purification techniques were performed including serial dilution, streak plating and centrifuge washing to obtain axenic strains **(Figures 3.1 and 3.2)**. At the end of these processes two species of non-motile green microalgae were isolated; they that looked to be possible species of *Chlorella*. The microscopic examination revealed that both organisms have a thick cell wall and that their cell morphology is unicellular and spherical. Both strains were grown in liquid culture without any apparent contamination with bacteria.

#### 3.3.1.1. Genomic DNA extraction of pond water isolates

For precise identification of the two *Chlorella*-like strains, molecular identification was performed as described in **section 2.7**. The genomic DNA of both strains was extracted using one protocol (CTAB). The protocol resulted in good quality DNA which was verified using agarose gel electrophoresis. It was further validated by polymerase chain reaction amplification and sequencing. The results presented in (**Figures 3.3** & **3.4)** showed clear bands of genomic DNA in both strains.

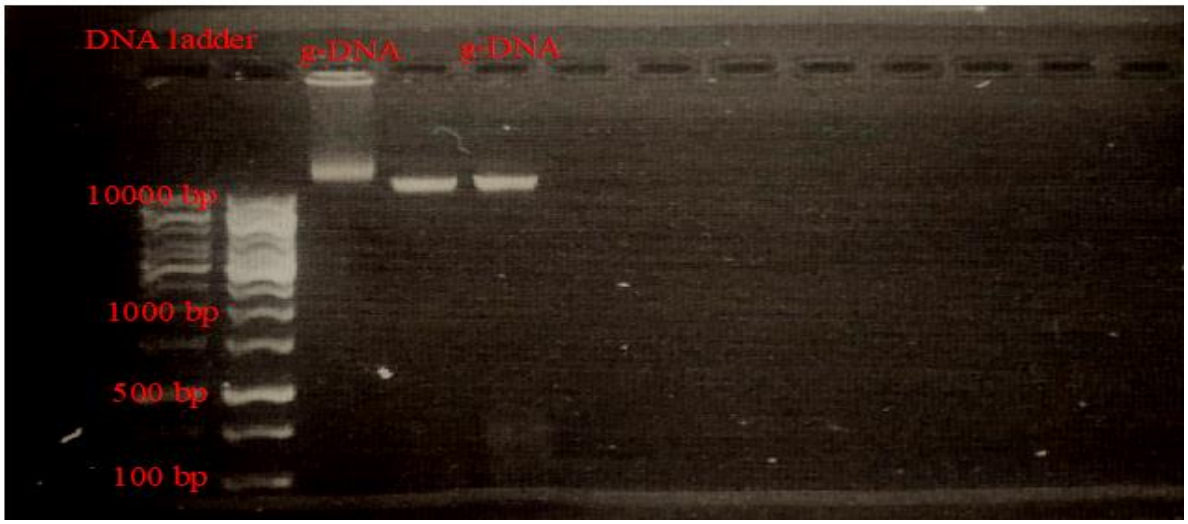



*Figure 3.3: Extraction of DNA from F1 strain using CTAB protocol and run on 1% Agarose gel. Lane 2: 1Kb DNA ladder, lane: 3 genomic DNA extracted from 20 ml culture (OD 595 = 1).*

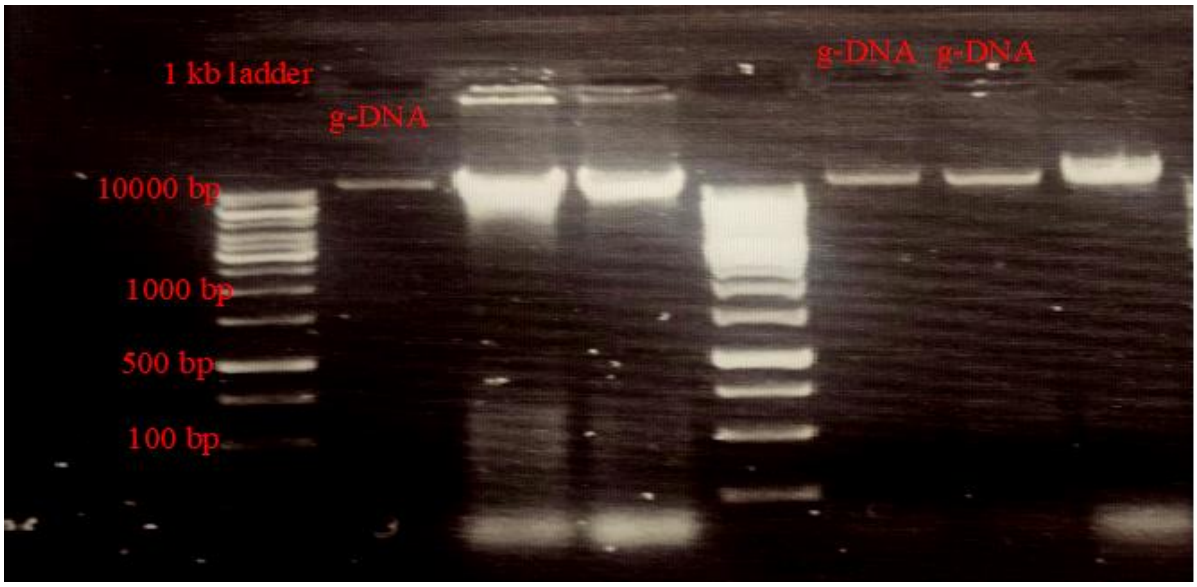



*Figure 3.4: Extraction of DNA from F2 strain using CTAB protocol; 1% agarose gel was run. Lane 1, 5 and 9: 1Kb DNA ladder, lane 3: genomic DNA extracted from 20 ml culture (OD 595 = 1).*

### 3.3.1.2. PCR amplification

Genomic DNA from F1 strain was amplified using 16S rRNA gene primers as described in section 2.7.3. The results showed successful amplification of the 16S rDNA genes (**Figure 3.5**) with clear band (approximately 1500 bp). For F2 isolate, the 18S rRNA gene was amplified using two different 18S rRNA primers Lim and Sheehan (Lim, et al., 2012) as described in section 2.7.3. Figure 3.6 showed the successful amplification of 18S rRNA gene

by the appearance of clear bands (approximately 500 bp), while, Sheehan primers did not yield any PCR product.

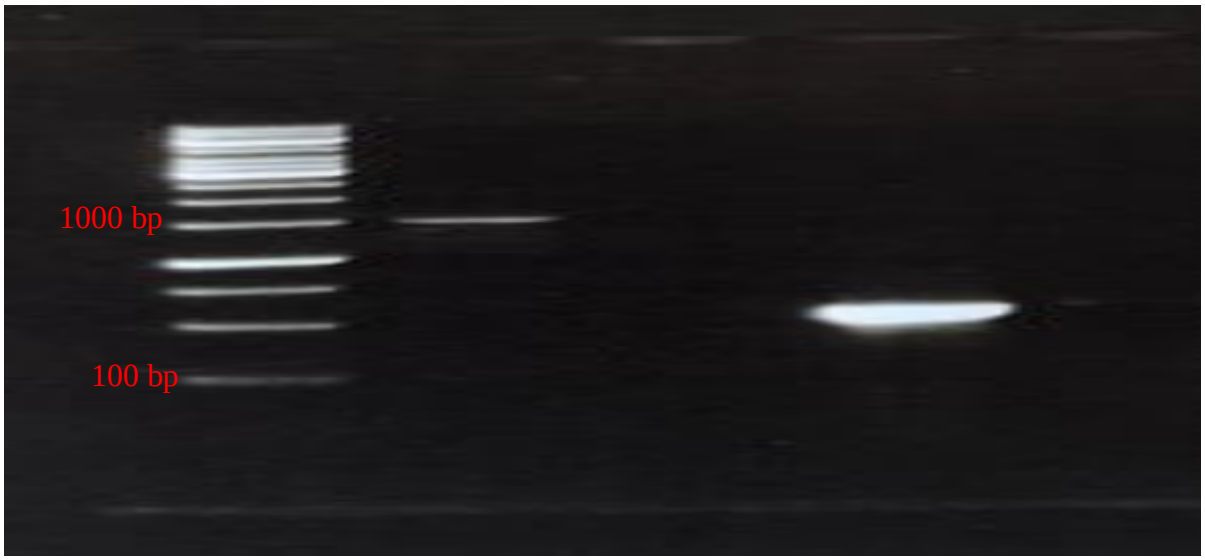



*Figure 3.5: PCR amplification of F1 and F2 genomic DNA using 16S rRNA and 18S rRNA primers respectively. Lane1: 1Kb ladder, Lane 2:16S rRNA band at around 1500 bp and Lane 4:18S rDNA band at around 500bp.*

### 3.3.1.3. Sequencing and Identification of the strains using Blast

The purified PCR product was sent to Eurofins/MWG for Sanger sequencing using both forward and reverse primers. The number of bases received for forward primer was 1093 bases and 1093 bases of reverse 16S rRNA, which corresponds to the expected size indicating that the gene was sequenced successfully (**Appendix A**). The sequences were compared against the NCBI database using Blastn command and the blast hits confirmed that both forward and reverse 16S primer sequences were well matched with *Auxenochlorella protothecoides* with 99% % identity (**Table 3.1**). The other microalgae isolate had the same sequences of 1093 bases of forward and reverse 18S rRNA Lim primers when sequenced (**Appendix B**). The blast hit against the NCBI database confirmed that the forward and reverse 18S rRNA primer sequences highly matched *Chlorella vulgaris* with 99% identity (**Table 3.1**).

*Table 3.1a: Closest matches to F1 isolated from pond based on the Blast of the 16S Forward sequences.*

| Species Matched | Accession number | Species Matched (% Identity) |
|---|---|---|
| *Auxenochlorella protothecoids* | AY553213.1 | 99.4 |
| *Chlorella sorokiniana* 1230 | KJ742376.1 | 97.5 |
| *Chlorella vulgaris* strain S708 | KF981995.1 | 99.2 |
| *Peudochlorella pringsheimii* | MF683077.1 | 96.5 |

*Table 3.1b: Closest matches to the F2 isolated from pond based on the Blast of the 16S Forward sequences.*

| **Species Matched** | **Accession number** | **Species Matched (% Identity)** |
|---|---|---|
| *Chlorella vulgaris* C-27 | AB001684.1 | 99.4 |
| *Chlorella vulgaris* (strain 211-1e) | D11347.1 | 99.1 |
| Chlorellaceae SP.QLD | KJ612008.1 | 99.1 |

### 3.3.2. Phylogenetic tree construction

The evolutionary distance of the identified microalgae from pond water against similar species of microalgae whose sequences have been deposited in Genbank was determined by the inference from phylogenetic tree construction. As shown in **Figure 3.6,** there were two evolutionary groups: the first group consisting of the *Chlorella vulgaris, Auxenochlorella protothecoides*, the isolates, which matched closely *Chlorella vulgaris and the Auxenochlorella protothecoides* with high percentage; the the second group was related to other *Chlorella* species.

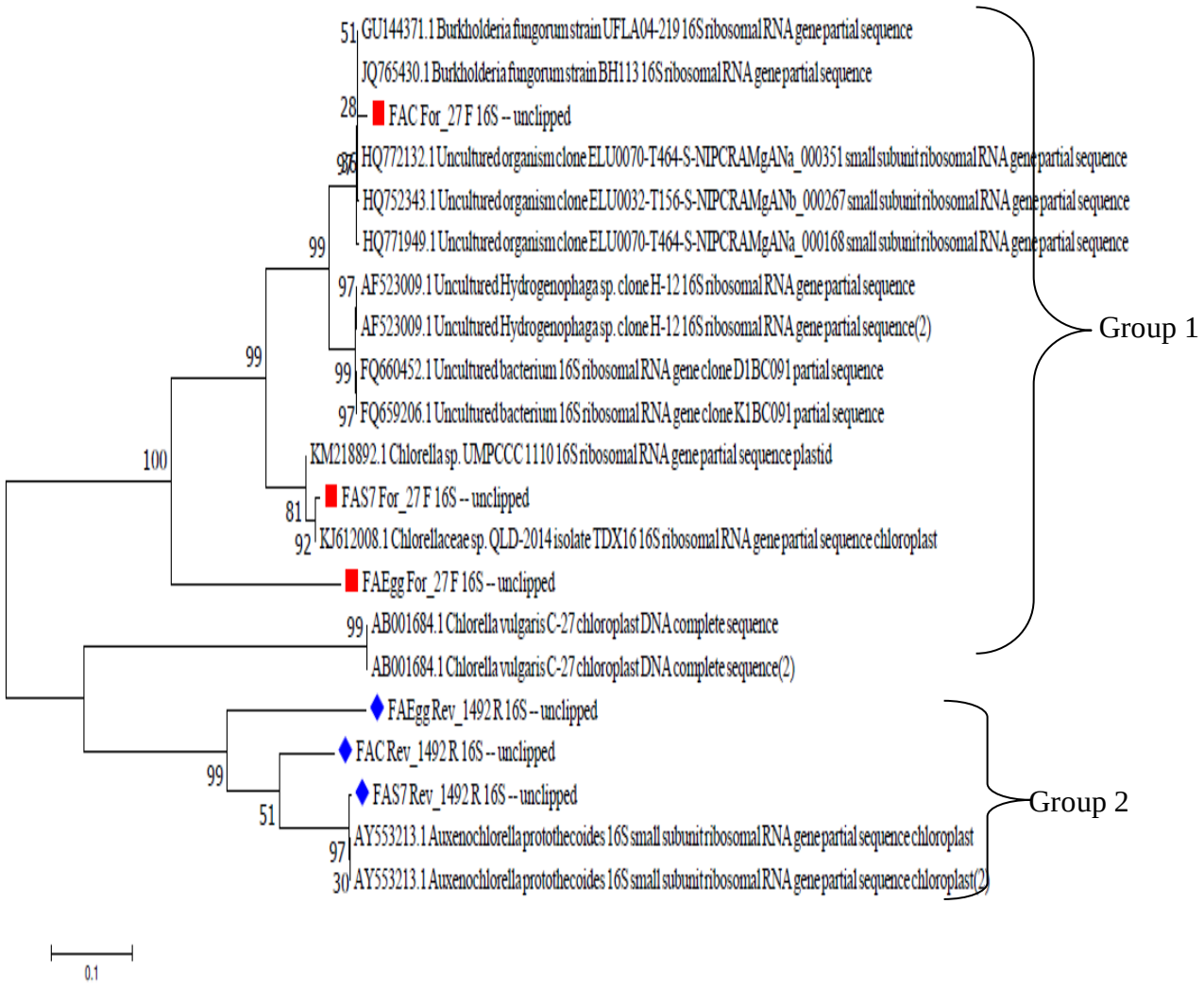



*Figure 3.6: Phylogenetic tree analysis of 16S sequences of pond water samples. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The tree is drawn*

*to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method); they are in the units of the number of base substitutions per site. The analysis involved 6 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 976 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).*

### 3.3.3. Growth of *Auxenochlorella protothecoides* (F1) and *Chlorella vulgaris* (F2)

Growth curves for *Auxenochlorella* and *Chlorella sp*. strains were performed as described in **section 2.6**, by monitoring the OD at 595 nm every day. **Figure 3.7** shows the growth curve of both strain cells over 24 days period of cultivation. The results showed clearly that they reached the exponential phase after 5 days until the 12th day of cultivation while the stationary phase was about 20 days of cultivation.

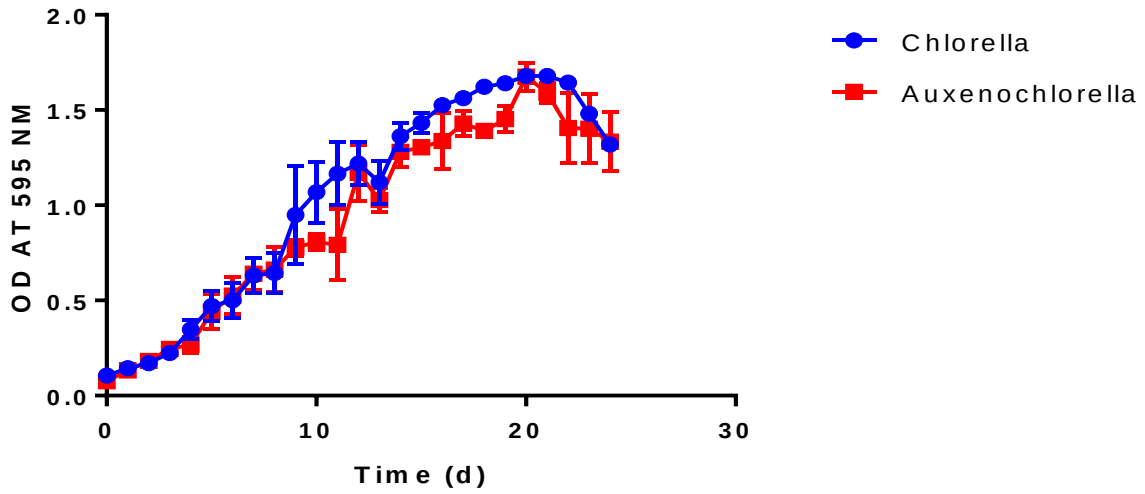



*Figure 3.7: Growth curve of Auxenochlorella and Chlorella cells grown over 26 days of cultivation.*

## 3.4. Discussion

### 3.4.1. Identification and Characterization of Pond Water Isolates

The cultures of the isolated samples (F1 and F2) were axenic after processing of serial dilution techniques and streaking cells across agar plates. The initial characterisation based on onmorphological featires showed similarity with genus *Chlorella*. The cells were dark green, spherical and unicellular; they formed discrete colonies on plates (Figures 3.1 and 3.2). These characteristics are not sufficient to identify the strains, although they can aid

identification. Alternatively, Songdong (2008) used DNA primers suitable for use in green algae and PCR amplification for the purpose of identification. The genomic DNA extracted from *Chlorella vulgaris* and 18S rDNA primers allowed the amplification of the desired PCR products. The result simply means that the DNA isolation method used in the study allowed the extraction of clean and suitable DNA for downstream application (PCR amplification). Previous studies used both 18S rRNA and 16S rRNA for the identification of microalgae (Nakahara *et al*., 2004).

This molecular biology approach has been found to be robust enough for elucidating the environmental diversity of green algae (**Chlorophyta) (**Haddad *et al*., 2014**).** The **18S** rDNA sequence analysis proved to be useful for the classification of chlorophycean and trebouxiophycean algae. Abundant data allowed the researchers to determine the phylogenetic positions of closely related taxa (Hoshina *et al*., 2004). In the present work, a molecular approach allowed the identification of the two microalgae: *Auxenochlorella* and *Chlorella* isolated from pond water with above 97% identity (**Table 3.1**). It also permitted the separation of the two strains into different genera (Huss *et al*., 2002). The comparative analysis of the phylogenetic relationship of the *Auxenochlorella* and *Chlorella* strains isolated from the pond water exhibited two major clusters with group 2 clustering together with *Auxenochlorella* (**Figure 3.6**). Most of the sequences in this research fall with bootstrap values above 70%, which correspond to 95% confidence statistically, although the statistical confidence is still debated by some researchers (Xiong, 2006).

The growth curves of *Auxenochlorella* and *Chlorella* reached exponential phase in about 5 days. The exponential growth continued until the $12^{th}$ day of cultivation under the experimental growth conditions (**Figure 3.7**). At the stationary phase, the biomass yield has been found to decrease irrespective of the microalgae culture (Thangavel *et al*., 2018). The exponential phases of the isolated microalgae in this work were found to be shorter than those reported for oleaginous microalgal isolates from Nilgiri biosphere reserve of India, which was 3 – 5 days. The stationary phase of the algal strains studied in this work indicated that cultivating the two strains beyond 20 days would not yield more biomass. Additionally, the short exponential phase suggests that the algal strains in our study could be exploited for the rapid production of algal biomass and lipid within 3 days, and is comparable with other strains isolated from previous studies (Thangavel *et al*., 2018).

# Determination and Quantification of Netural Lipid content and fatty Acid composition

## Chapter Four

## 4.1. Introduction

In recent times, microalgae have become the most common feedstocks for third generation biofuel. Due to their minimum impact on agricultural land, microalgae can be cultivated on saline land or waste water. Compared to the first generation biofuel feedstocks, they have a higher rate of neutral lipid production (Chisti, 2007; Wijffels and Barbosa 2010). Microalgae produce neutral lipid in addition to polar lipids and these neutral lipids consist mainly of triacylglycerol esters (TAG). Under favourable growth conditions microalgae can produce basically polar lipids such as glycolipids and phospholipids that contain chloroplast and cellular membranes. However, under environmental stress conditions that are unfavourable to growth, neutral lipids are stored in bodies in the cytoplasm (Deng *et al.*, 2011).

The discovery of new microalgae species has been intensified in recent years due to their high popularity in biofuel production. Algae contribute to reduced economic costs of biofuel production requirements. The main problems identifying the full chain process are known (Cadoret and Bernard, 2008; Rios, et al., 2013). However, lipid production needs to be performed accurately, reliably and easily. Additionally, the quantity and quality of lipid matters greatly. In biodiesel production, TAG is the target which can be transformed by transesterification (Rumin, *et al*., 2015). Several methods exist for the quantification of natural lipids, and often these methods required heavy and expensive equipment and in some cases highly trained technicians to manage and run such equipment (Han, *et al.*, 2011). The analysis and characterization of the lipid content in Algal species have been performed using several tools and techniques such as Nile Red staining, gravimetric and spectrometric techniques (Barupal, *et al*., 2010). Table 4.1 summarises the various methods used for microalgae lipid determination, their advantages and disadvantages.

*Table 4.1: Features and drawbacks of methods used for microalgae lipid determination (Gong and Jiang, 2011)*

| Methods | Advantages | Disadvantages |
|---|---|---|
| 1. Solvent extraction and gravimetric method | Accurate and reproducibility | Time consuming, labour intensive, large amount of biomass (15 mg) |
| 2. Chromatographic method | Good reproducibility, single analysis generates data of both quantity and profile of fatty acids, small sample required | Requirement of cell disruption, requirement of expensive analytical equipment |
| 3. Nile Red staining (NR) | A high throughput for rapid quantification, simple, and efficient | Efficiency variance in some microalgae, accuracy can be affected by many factors |
| 4. Time domain nuclear magnetic resonance (TD-NMR) | In situ measurement, rapid and Cheap | Precise is dependent on high lipid content |
| 5. Colorimetric quantification | Quick, simple, less expensive. | Not applicable to detect fatty acids with length chain of less than 12C atoms. |

However, many studies have shown that these methods are not always suitable for detection of lipid in Algae due to the loss of some lipid after applying additional steps required to differentiate various types of lipid. The additional steps include chromatographic separation and transesterification. Furthermore, methods such as gravimetric have other disadvantages of time consumption and labour-intensive manipulation (Bertozzini *et al*., 2011). Moreover, other drawbacks comprise lipid assessment, which includes even non-fatty acids such as pigments, and sterols, not only total lipid; it is connected with the choice of the method for the determination of the lipid. These non-fatty acids are shown to contribute about 50%

increase in the total lipid content (Mata *et al*., 2010; Chen *et al*., 2018). Therefore, to specifically target conversion of natural lipid to biodiesel it is advised to utilize the gravimetric method, which estimates the total lipid content over (Breuer *et al*., 2013b).

In recent time, spectrophotometry has become a favourite protocol for detecting algal lipids in-Situ screening Algal lipids content. Fluorescence spectroscopy has been recommended for estimating the Algal lipid content due to its ease, fastness and inexpensive nature as it depends on a fluorescent dye (Nile Red). Also, the fluorescence of the dye is involved directly in the quantification of the lipid content using the stimulation and emission filters of the spectroscopy (Cirulis *et al*., 2012).

Nile red (NR) is a stain that has been utilised to detect lipid droplets within cells and the existent fluorogenic dye (Greenspan and Fowler, 1985). It has also been used to stain fat droplets in different organism like zooplankton (Alonzo and Mayzaud, 1999), mammalian cells (Genicot *et al*., 2005), bacteria (Izard and Limbergere, 2003) and Yeast (Sitepu *et al*., 2012). NR it is very soluble in lipids; it has many features to be used for detecting Natural lipids. It has strong fluorescence in the presence of a hydrophobic environment, non-polar in contrast with aqueous solutions with very weak fluorescence (Fowler *et al*., 1979). When dissolved, this stain in aqueous/ DMSO solutions penetrated the cell wall of diverse Algal species.

The dye fluorescent lipid droplet inside the cytoplasm (Chen *et al*., 2009), and in *Chlorella vulgaris*, lipid reaches 20% weight of the dry biomass under normal growth conditions (IIIman *et al*., 2000) while under environment stress condition, lipid can be more than 80% by weight of dry biomass (Khan *et al*., 2009). There are a number of other techniques applied to measure lipids; they include gravimetric technique, Gas Chromatography–Mass Spectrometry (GC-MS) technique and Nuclear Magnetic Resonance (NMR) technique. Lipid-soluble fluorescent dye, the Nile Red staining is a simple, rapid, sensitive, accurate and specific method for lipid determination and it includes the solvent extraction and gravimetric method, which is time-consuming; moreover, it only allows for low level throughput of neutral lipid measurement (Govender *et al*., 2012).

### 4.2. Aim and Objectives

The aim of this chapter was to evaluate the comparative potential of *Auxenochlorella* and *Chlorella* species for the production of biofuels.

The specific objectives were:

1. To compare *Auxenochlorella* and *Chlorella* as model organisms for biodiesel production,
2. To quantify the concentration of lipid (fatty acid) produced by both microalgae,
3. To determine the relationship between stress factors and lipid production,
4. To establish the fatty acid profile of the lipid produced by *Auxenochlorella* and *Chlorella* using GC-MS analysis.

## 4.3. Results

### 4.3.1. Fluorescence microscopy for visualisation of lipid droplets

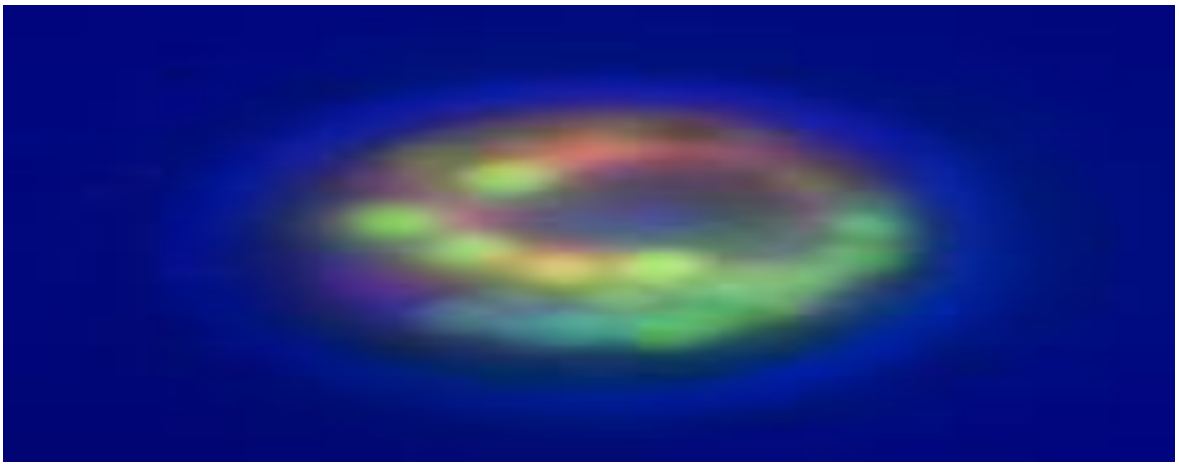


*Figure 4.1: Nile red evaluation of the natural lipid in Chlorella.*

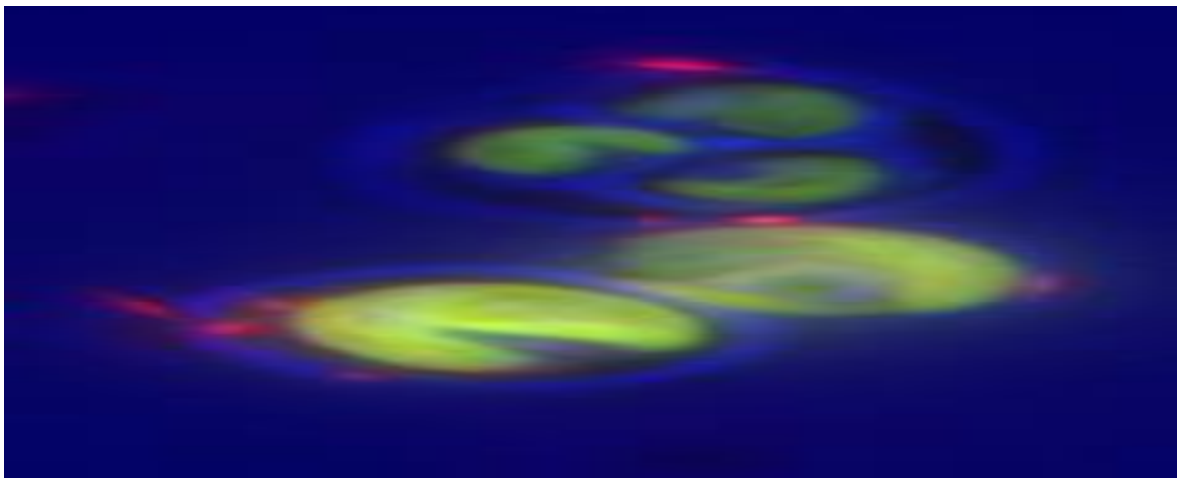


*Figure 4.2: Nile red evaluation of the natural lipid in Auxenochlorella.*

### 4.3.2 Determination of neutral lipid using Nile Red

The estimation of neutral lipid in algal cells accurately with fluorescent intensity of Nile Red requires the optimization of algal cell concentration and Nile red concentration.

#### 4.3.2.1 Determination of optimum cell concentration and peak time for Auxenochlorella 96 well plate method

Table 4.2 presents the fluorescence intensities for the different cell concentrations of *Auxenochlorella* strain ranging from 12.5% to 100% (where 100% was $OD_{595}$ = 1) at different staining times (10, 15, 20, 25 and 30 minutes). It was found that the highest fluorescence intensities at cell concentrations were 100%, 87.5% and 75% respectively. The other lower cell concentrations gave low fluorescence intensities, which decreased as the cell concentration decreased. However, based on the smallest standard error value of stain average (2) and the higher stained average (379), the cell concentration of 75% (which is equivalent to an $OD_{595}$ = 0.858) and 15 minutes staining time was selected as optimum cell concentration for use in the determination of optimum Nile red concentration.

*Table 4.2: Optimization of cell concentration on the fluorescence intensity of Auxenochlorella; the optimum conditions were at 75% of cell concentration after 15 min staining; Data points are the mean of four replicates plus or minus standard error*

| Concentration of cells (%) | 100.0 | 87.5 | 75.0 | 62.5 | 50.0 | 37.5 | 25.0 | 12.5 |
|---|---|---|---|---|---|---|---|---|
| Stained Average | 339 | 322 | 379 | 231 | 301 | 232 | 277 | 137 |
| StDev Stain | 4 | 4 | 2 | 9 | 19 | 10 | 7 | 6 |
| Unstained Average | 134 | 124 | 116 | 112 | 114 | 113 | 113 | 110 |
| St.Dev. unstain | 6.1 | 2.0 | 1.5 | 1.0 | 1.2 | 2.0 | 1.2 | 2.5 |
| Stained-unstained | 205 | 198 | 262 | 119 | 188 | 119 | 164 | 28 |
| Normalised per % dilution | 2.0 | 2.3 | 3.5 | 1.9 | 3.8 | 3.2 | 6.6 | 2.2 |

#### 4.3.2.2. Determination of optimum cell concentration and peak time for Chlorella for 96 well plate methods.

The fluorescence intensities for different cell concentrations of *Chlorella* strain ranging from 12.5% to 100% (where 100% was OD595 = 1) at different staining times (10, 15, 20, 25 and 30 minutes) were evaluated (**Table 4.3**). It was found that the highest fluorescence intensities were recorded for cell concentrations of 100%, 87.5% and 75% respectively. The other lower cell concentrations gave low fluorescence intensities simply suggesting a decrease as the cell concentration decreased. However, based on the higher stained average (329) and the smallest standard error value of stain average (13.87) by avoiding first and last concentration, the cell concentration of 75% (equivalent to an $OD_{595}$ = 0.715) at 25 minutes staining time was found to be the optimum cell concentration to use for determining the optimum Nile red concentration to be used.

*Table 4.3: Optimization of cell concentration on fluorescence intensity of Chlorella; the optimum conditions were at 75% of cell concentration after 25 min staining; data points are the mean of four replicates plus or minus standard error*

| Concentration of cells (%) | 100.0 | 87.5 | 75.0 | 62.5 | 50.0 | 37.5 | 25.0 | 12.5 |
|---|---|---|---|---|---|---|---|---|
| Stained Average | 312 | 305 | 329 | 208 | 260 | 220 | 248 | 140 |
| StDev Stain | 4.93 | 24.54 | 13.87 | 15.10 | 31.01 | 20.84 | 26.15 | 5.29 |
| Unstained Average | 135 | 123 | 115 | 112 | 112 | 112 | 112 | 109 |
| StDev unstain | 6.00 | 2.52 | 1.53 | 0.58 | 1.73 | 2.00 | 3.00 | 2.08 |
| Stained-unstained | 177 | 182 | 214 | 96 | 148 | 108 | 136 | 31 |
| Normalised per % dilution | 1.8 | 2.1 | 2.9 | 1.5 | 3.0 | 2.9 | 5.4 | 2.5 |

#### 4.3.2.3 Determination of optimum Nile Red concentration method for Auxenochlorella

**Table 4.4** shows the effect of using different concentrations of Nile Red ranging from 0.05 to 0.6 µmol $ml^{-1}$ dissolved in 100% dimethyl sulfoxide (DMSO) with the 75% cell concentration

($OD_{595}$ = 0.858) of *Auxenochlorella* strain at different staining times (10, 15, 20, 25 and 30 minutes). It was found that the highest fluorescence intensities were observed when Nile Red concentration was 0.05, 0.4 and 0.6 µmol $ml^{-1}$ respectively (Table 4.4). Based on the higher stained average and stain minus stain, the Nile Red concentration of 0.4 µmol $ml^{-1}$ at 15 minutes staining was chosen as optimum Nile Red concentration to be used for neutral lipid determination experiments at cell concentration of 75%.

*Table 4.4: Optimization of Nile Red staining concentration on fluorescence intensity of the green alga Auxenochlorella; the optimum conditions were at 75% of cell concentration after 15 min staining; data points are the mean of four replicates plus or minus standard error*

| Concentration of Nile Red µmol $ml^{-1}$ | 0.05 | 0.1 | 0.2 | 0.3 | 0.4 | 0.6 |
|---|---|---|---|---|---|---|
| Stained Average | 178 | 159 | 156 | 158 | 174 | 171 |
| StDev stain | 7.2 | 7.0 | 3.2 | 7.8 | 2.3 | 6.5 |
| Unstained Average | 131 | 120 | 117 | 115 | 112 | 112 |
| StDev unstain | 2.6 | 1.2 | 2.5 | 2.1 | 1.0 | 1.5 |
| Stained-unstained | 47 | 39 | 40 | 43 | 62 | 60 |

#### 4.3.2.4 Determination of optimum Nile Red concentration method for Chlorella

**Table 4.5** shows the effect of using different concentrations of Nile Red ranging from 0.05 to 0.6 µmol $ml^{-1}$ dissolved in 100% dimethyl sulfoxide (DMSO) with the 75% cell concentration ($OD_{595}$ = 0.715) of *Chlorella* strain at different staining times (10, 15, 20, 25 and 30 minutes). It was found that the highest fluorescence intensities were observed when Nile Red concentration was 0.2, 0.3 and 0.6 µmol $ml^{-1}$ respectively (**Table 4.5**). Based on the higher stained average and higher stained minus unstained, the Nile Red concentration of 0.3 µmol $ml^{-1}$ with 25 minutes staining has been chosen as optimum Nile Red concentration to use for neutral lipid determination experiments with a cell concentration of 75%.

*Table 4.5: Optimization of Nile Red staining concentration on fluorescence intensity of the green alga Chlorella; the optimum conditions were at 75% of cell concentration after 25 min staining; data points are the mean of four replicates plus or minus standard error*

| Concentration of Nile Red µmol $ml^{-1}$ | 0.05 | 0.1 | 0.2 | 0.3 | 0.4 | 0.6 |
|---|---|---|---|---|---|---|

| | | | | | | |
|---|---|---|---|---|---|---|
| Stained Average | 202 | 380 | 415 | 474 | 324 | 455 |
| StDev stain | 3.1 | 17.8 | 20.1 | 26.4 | 15.6 | 11.7 |
| Unstained Average | 143 | 145 | 144 | 127 | 131 | 125 |
| StDev unstain | 3.5 | 13.3 | 15.6 | 12.1 | 2.6 | 8.5 |
| Stained-unstained | 60 | 235 | 271 | 347 | 193 | 330 |

**4.3.2.5: Triolein calibration curve method for *Auxenochlorella* (Nile Red Triolein concentration test).**

The final part of the Nile Red set-up experiments was to produce the Triolein calibration curve (Triolien concentration curve) in the range of 0.2, 0.16, 0.12, 0.08, 0.06, 0.04, 0.02 and 0.0 mg $ml^{-1}$ using Triolien as neutral lipid standard with algal cell concentration of 75% and 0.4 µmol $ml^{-1}$ Nile Red dye concentration for 15 minutes staining time. This will allow the conversion of fluorescence in arbitrary units to Triolein equivalents. The Triolein calibration curve was plotted from Triolein concentration as neutral lipid for each dilution against the Nile Red fluorescent intensity for each dilution. The resulting concentration curve is shown in (**Figure 4.3).**

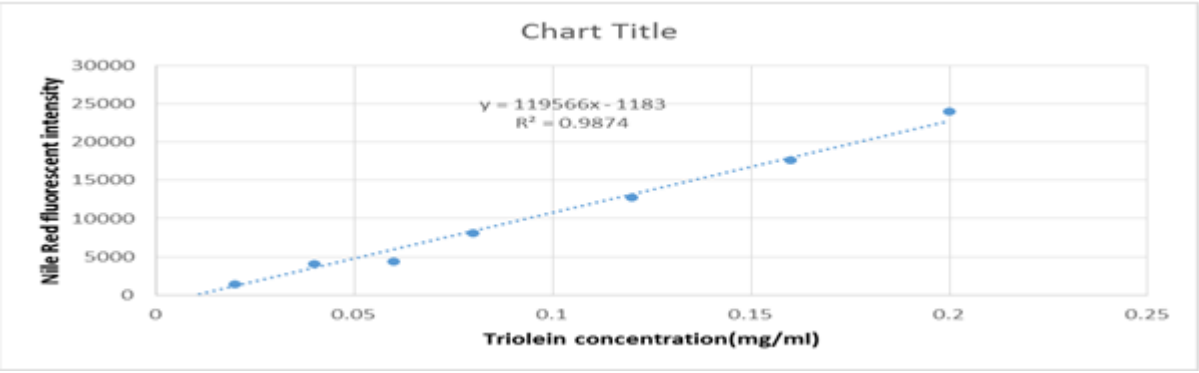



*Figure 4.3: Linear correlation for Auxenochlorella cells between fluorescence intensity and Triolein concentration, allowing the conversion of fluorescence readings to Triolein equivalents. Data points are the mean of four replicates plus or minus standard error.*

### 4.3.2.6. Triolein calibration curve method for *Chlorella* (Nile Red Triolein concentration test)

The final part of the Nile Red set-up experiments for *Chlorella* aimed also to produce the Triolien calibration curve (Triolein concentration curve) in the range of 0.2, 0.16, 0.12, 0.08, 0.06, 0.04, 0.02 and 0.0 mg $ml^{-1}$. Triolein was used as neutral lipid standard with algal cell concentration of 75% and 0.3 µmol $ml^{-1}$ Nile Red dye concentration for 25 minutes staining. This will allow the conversion of fluorescence in arbitrary units to Triolein equivalents. The Triolein calibration curve was plotted from Triolein concentration as neutral lipid for each dilution against the Nile Red fluorescent intensity. The resulting concentration curve is shown in (**Figure 4.4).**

### 4.3.2.7 Effect of Salinity on the neutral lipid of Auxenochlorella and Chlorella cells

Neutral lipid contents of cells of *Auxenochlorella* and *Chlorella* are grown under normal conditions (BBM medium) and under stress conditions (different salinities i.e. 0.2, 0.4, 0.6, 0.8, and 1 M NaCl) over a period of four weeks to investigate the effect of high salinity as stress condition on the natural lipid content were measured using Nile red fluorescence. The cell concentrations, staining times and Nile red concentrations were described in Tables 4.4 and 4.5. Using both dry weight calibration curve at $OD_{595}$ and triolein calibration curve as neutral lipid standard, the % neutral lipid contents were calculated (**Figures 4. 3 and 4.4**).

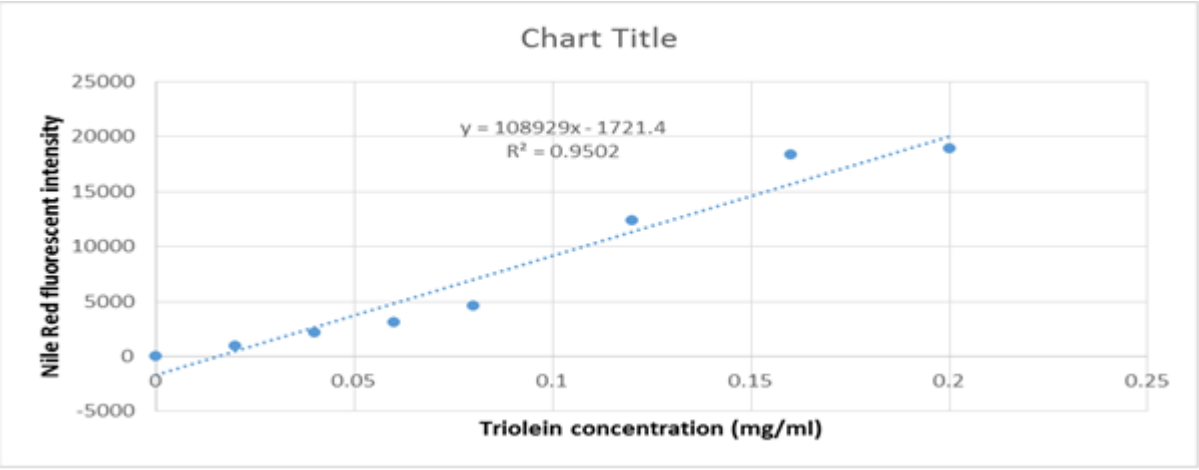



*Figure 4.4: Linear correlation for Chlorella cells between fluorescence intensity and Triolein concentration, allowing the conversion of fluorescence readings to Triolein equivalents. Data points are the mean of four replicates plus or minus standard error.*

For *Auxenochlorella* (**Figure 4. 5**), no clear trend is shown in relation to % lipid and salinity. The highest % lipid is found in cells grown in 0.8 M NaCl, but this is only slightly higher than that found in cells grown in normal BBM medium. The highest % neutral lipid for *Auxenochlorella* cells is 24% at 0.8 M NaCl. For *Chlorella* (**Figure 4.6**), there is an increase % lipid with increasing salinity levels which level off after 0.8 M NaCl. The highest % neutral lipid for *Chlorella* cells is 26%:

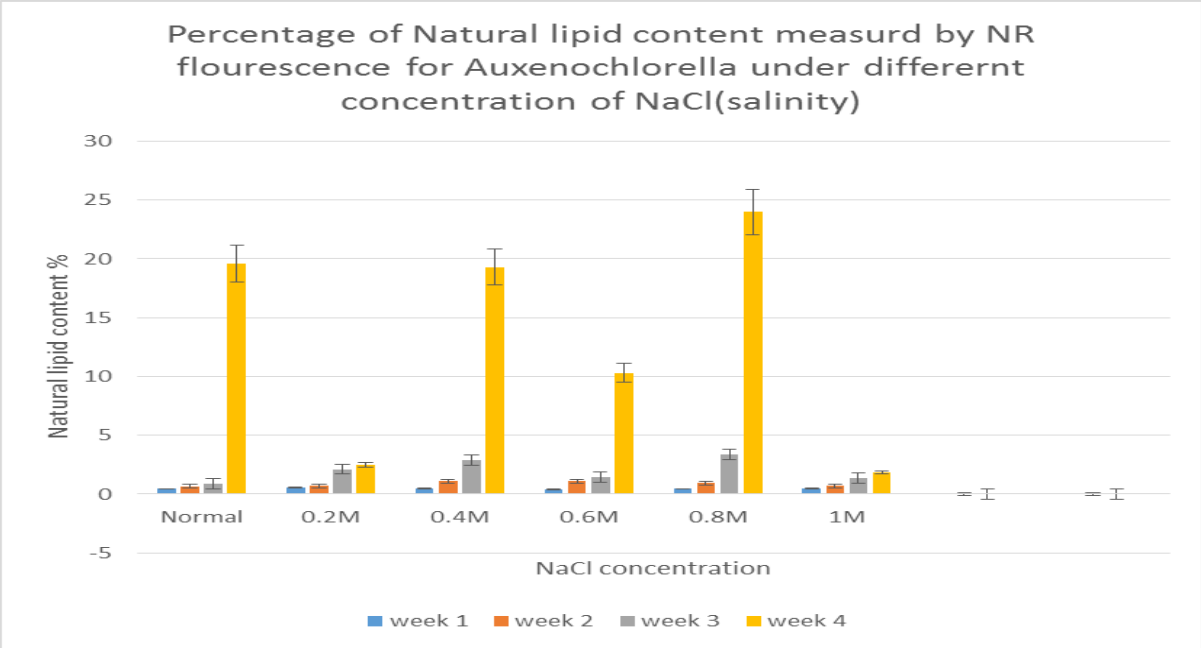



*Figure 4.5: Nile Red Fluorescence Intensity measurements of Auxenochlorella cells grown in BBM media with Normal concentration of NaCl, 0.2 and 0.4, 0.6, 0.8 and 1 NaCl M respectively. Each measurement represents the difference between the average of four stained and four unstained readings. Error bars represent technical repeats (n=3).*

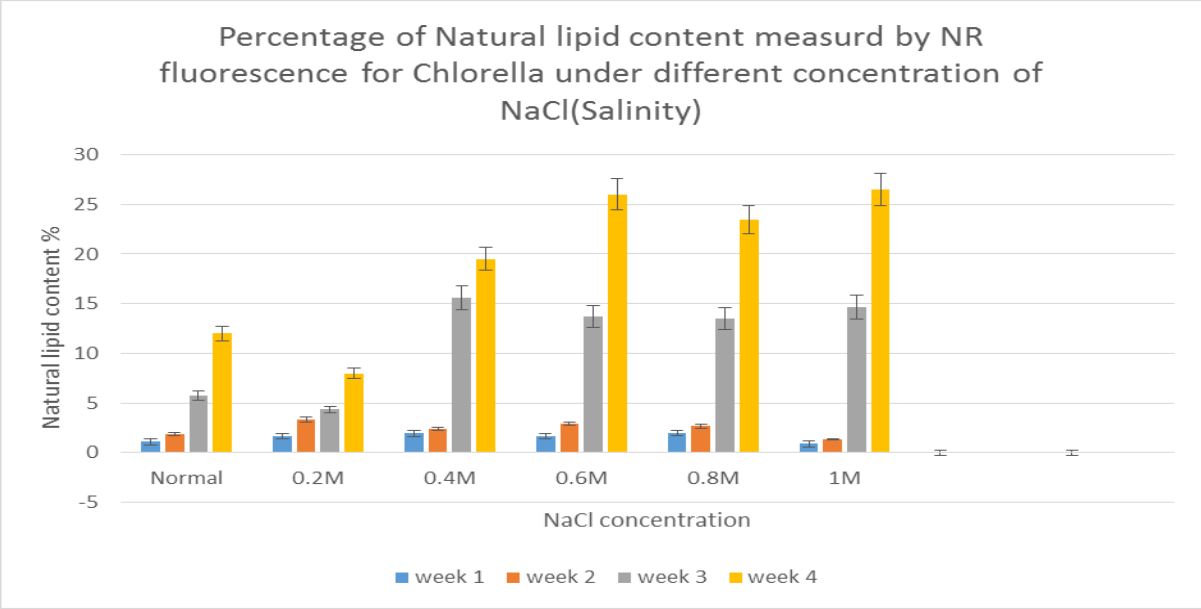



*Figure 4.6: Nile Red Fluorescence Intensity measurements of Chlorella cells grown in BBM media with Normal concentration of NaCl, 0.2 and 0.4, 0.6, 0.8 and 1 NaCl M respectively. Each measurement represents the difference between the average of four stained and four unstained readings. Error bars represent technical repeats (n=3).*

### 4.3.2.8 Determination of the neutral lipid content of *Auxenochlorella* and *Chlorella* under nitrogen starvation

**Figures 4.7** and **4.8** reveal the percentage of neutral lipid content accumulation over the course of 4 weeks when *Auxenochlorella* and *Chorella* were exposed to nitrogen limitation. It can be seen that the percentage of natural lipid content in *Auxenochlorella* and *Chlorella* increased with increasing nitrogen starvation. The highest percentage of neutral lipid content was in the nitrogen-free stress condition. The percentage of the neutral lipid was much higher under nitrogen stress than under salinity stress (**Figures 4.5 to 4.6**).

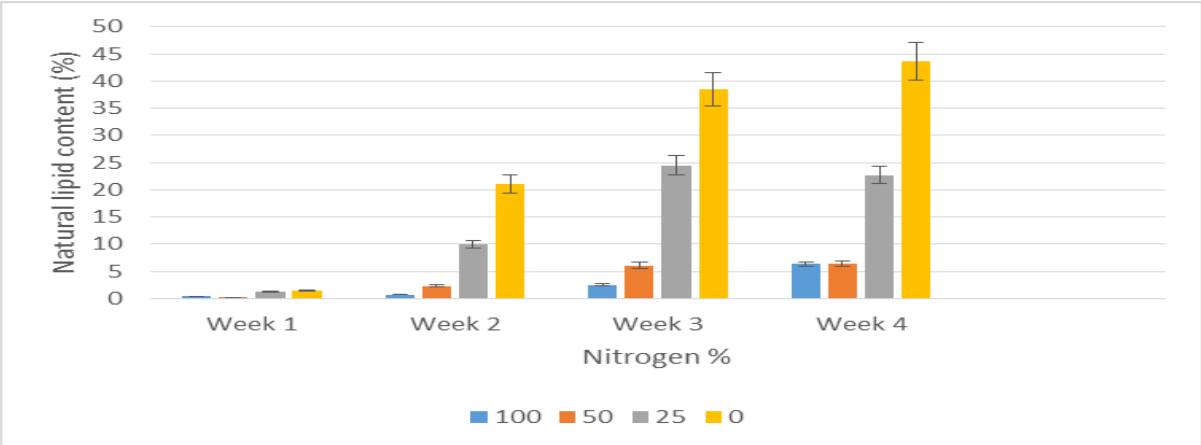


*Figure 4.7: Percentage of the neutral lipid accumulation over the course of 4 weeks measured using Nile red fluorescence for Auxenochlorella under nitrogen starvation. The bars represent the standard deviation for three replications.*

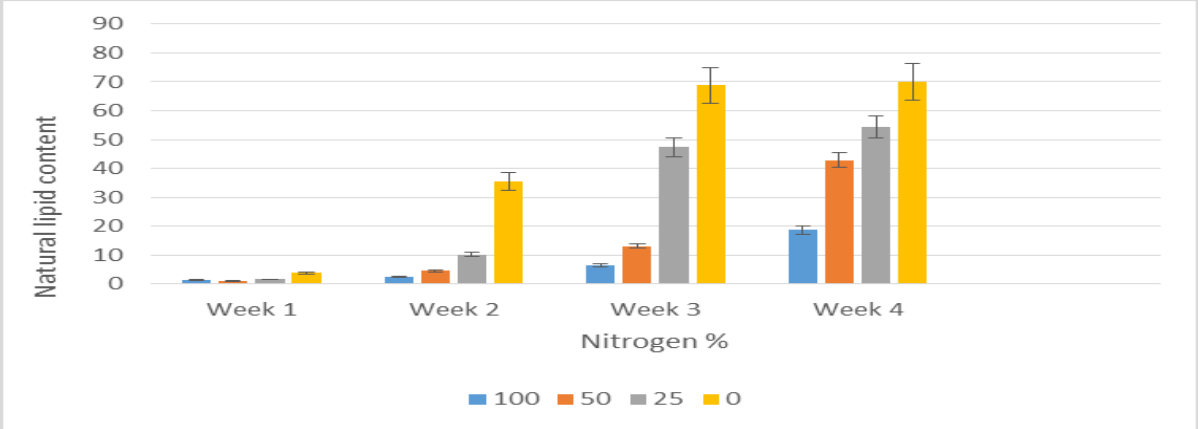



*Figure 4.8: Percentage of neutral lipid accumulation over the course of 4 weeks measured using Nile red fluorescence for Chlorella under nitrogen starvation. The bars represent the standard deviation for three replications.*

### 4.3.3. Quantification of total lipid content using the gravimetric method

The percentage lipid content of the dry weight biomass of *Auxenochlorella* and *Chlorella* grown in normal, 0.8 M NaCl, and nitrogen-free conditions was evaluated (**Figures 4.9** and **4.10**). The lipid contents were 29%, 28% and 43% respectively for *Auxenochlorella*, and 21%, 30% and 55%, respectively for *Chlorella*.

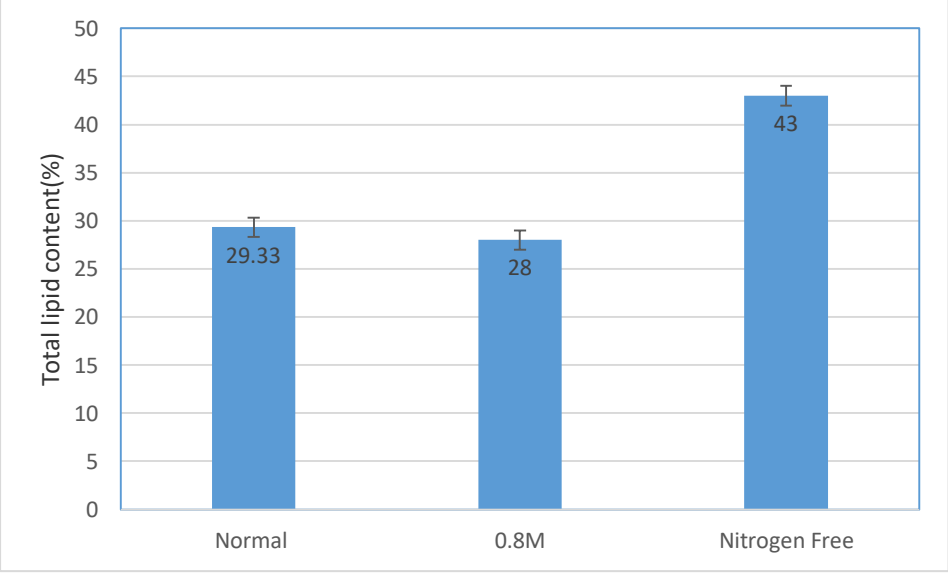


*Figure 4.9: Gravimetric measurement of total lipid content (Total Lipid Content percentage) in Auxenochlorella cultures grown in different growth conditions, including 0.8 M NaCl and nitrogen starvation, compared to normal growth conditions. Each column represents the mean of four readings plus or minus standard deviation.*

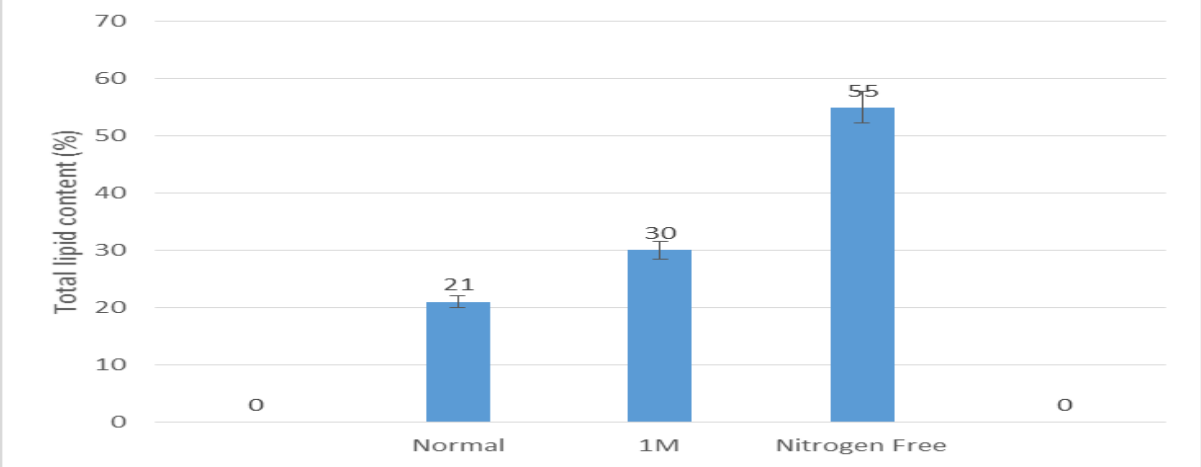



*Figure 4.10: Gravimetric measurement of total lipid content (Total Lipid Content percentage) in Chlorella cultures grown in different growth conditions, including 1M NaCl and nitrogen starvation, compared to normal growth conditions. Each column represents the mean of four readings plus or minus standard deviation.*

### 4.3.4: Determination of the fatty acid methyl ester (FAME) content in the neutral lipids using GC-MS

The fatty acid methyl esters (FAMEs) profile of *Auxenochlorella and Chlorella* neutral lipid was determined by GC-MS (**Figure 4.9**), following the sample preparation step as described in section 2.12. The result shows that FAMEs were found in cells grown under normal conditions. As seen from Figures 3.1 and 3.2, the lipids of *Auxenochlorella* and *Chlorella* grown under normal conditions consisted of FAMEs such as palmitic acid (C16:0), oleic acid (C18:1) and linolenic acid (C18:3).

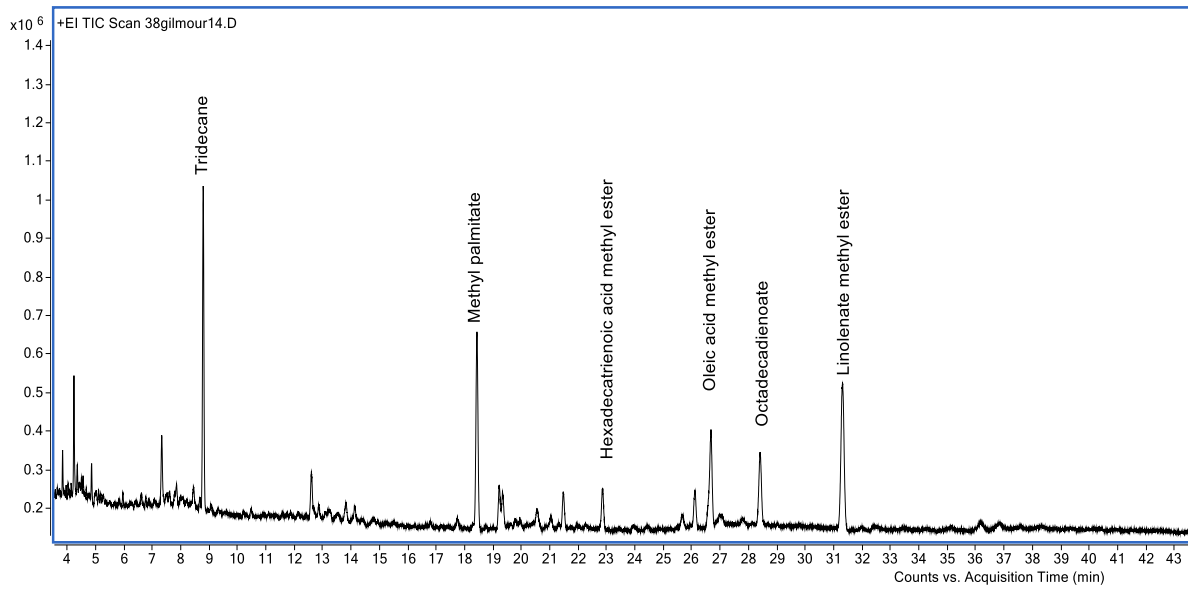



*Figure 4.11: Fatty acid methyl esters (FAMEs) found in Auxenochlorella grown under normal conditions.*

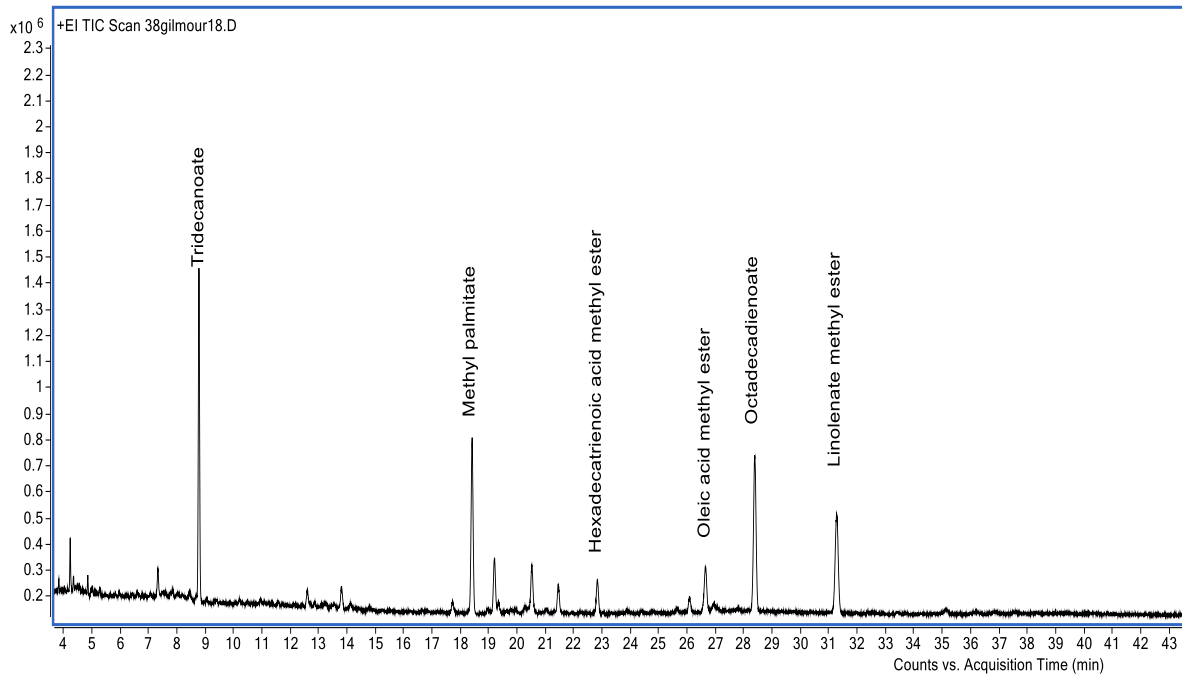



*Figure 4.12: Fatty acid methyl esters (FAMEs) found in Chlorella grown under normal conditions.*

### 4.3.4.1. Fatty acid profiles under higher salinities

The fatty acid profile of the lipid produced by *Auxenochlorella* and *Chlorella* cultivated under high salt concentration was determined (**Figures 4.13** and **4.14**). The FAME composition did not change except for the addition of stearic acid (C18:0) in *Auxenochlorella* produced lipid.

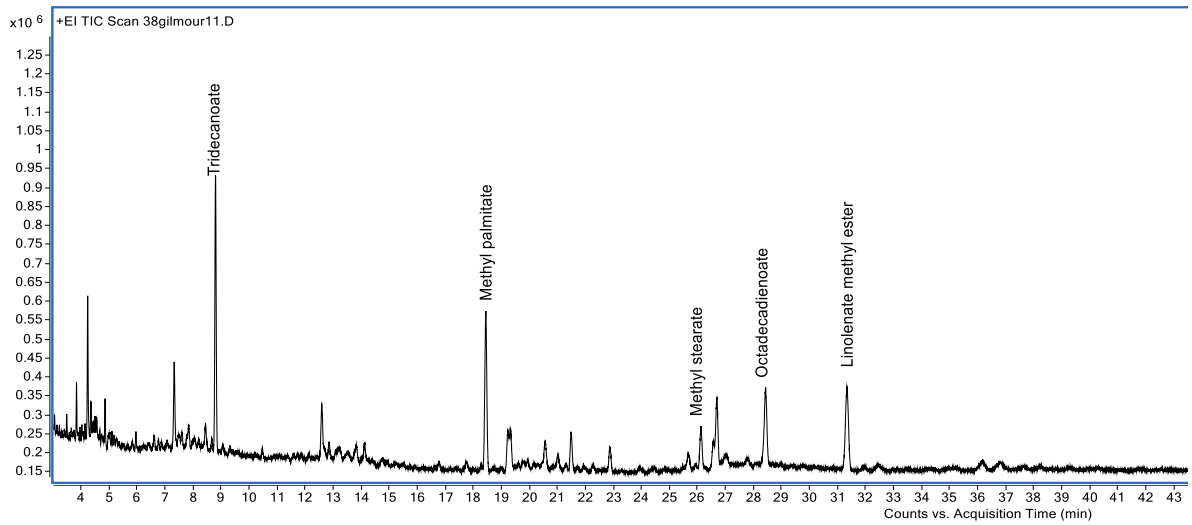



*Figure 4.13: Fatty acid methyl esters (FAMEs) found in Auxenochlorella grown in 0.8M NaCl medium.*

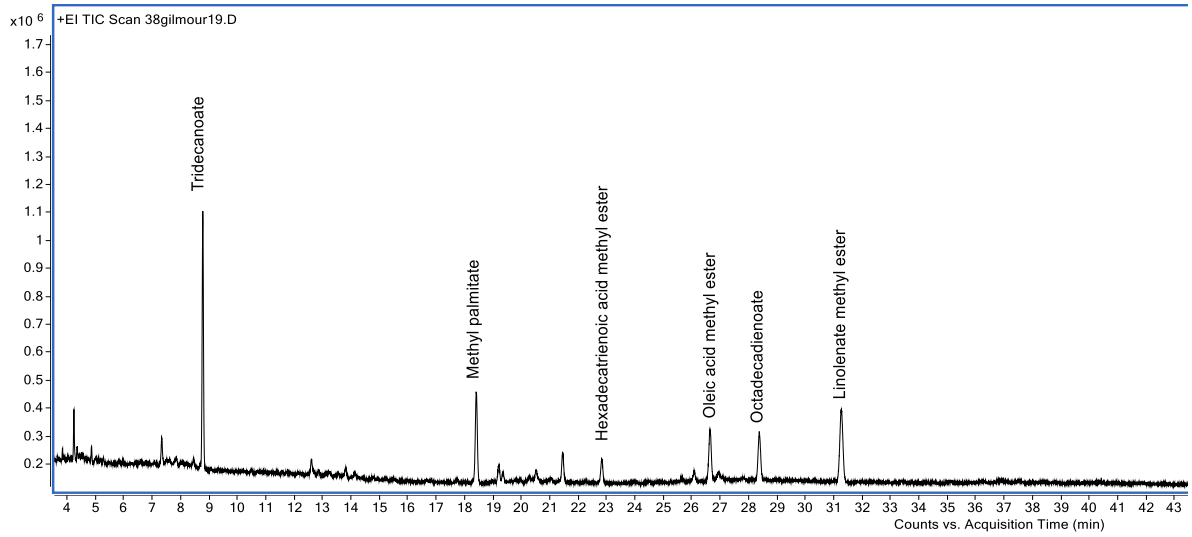



*Figure 4.14: Fatty acid methyl esters (FAMEs) found in Chlorella grown in 1M NaCl medium.*

### 4.3.4.2 Fatty acid profiles under nitrogen starvation

The fatty acid profile of lipid produced nitrogen-stressed cells of *Auxenochlorella* and *Chlorella* showed oleic acid as the major FAME for both organisms (**Figure 4.15** and **4.16**).

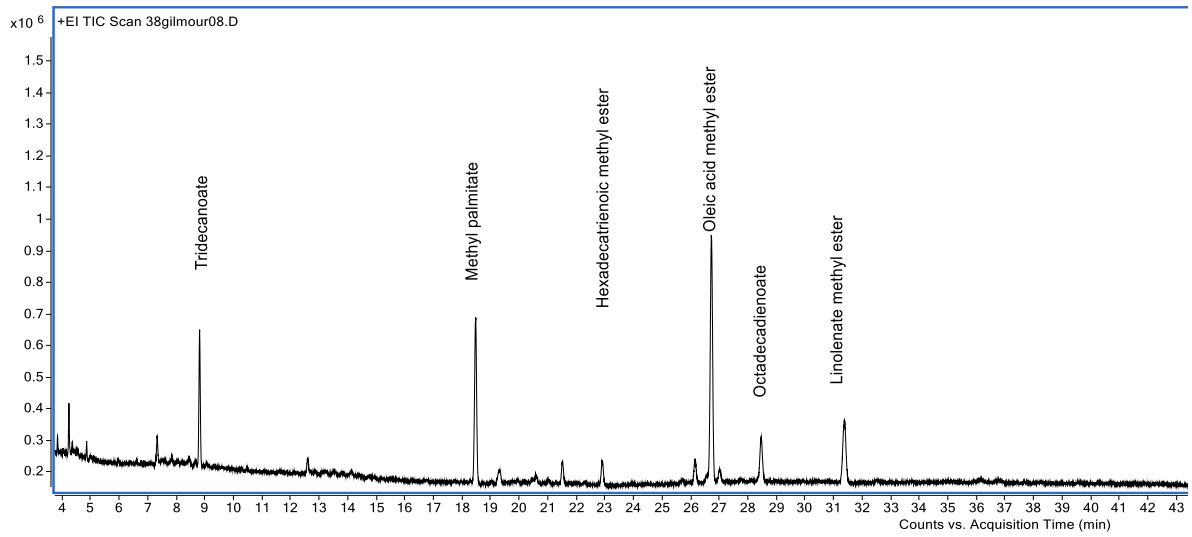



*Figure 4.15: Fatty acid methyl esters (FAMEs) found in Auxenochlorella grown in nitrogen-free medium.*

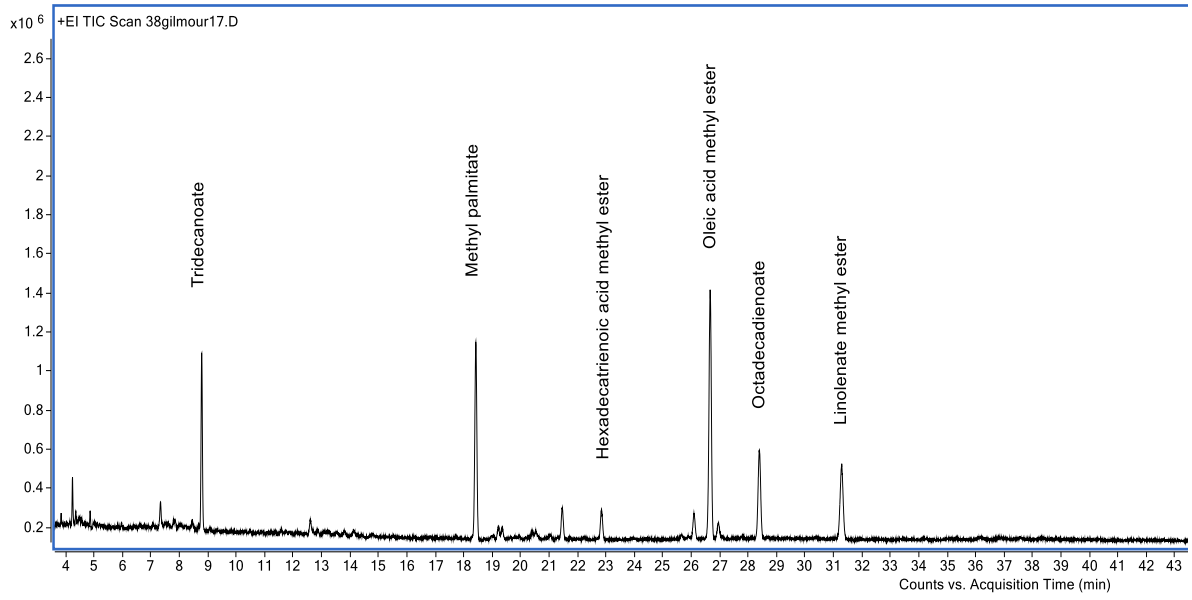



*Figure 4.16: Fatty acid methyl esters (FAMEs) found in Chlorella grown in nitrogen-free medium.*

## 4.5. Compatible solutes analysis using Nuclear Magnetic Resonance (NMR) analysis in *Auxenochlorella* and *Chlorella*

Nuclear magnetic resonance (NMR), at the beginning, was suggested by Rabi *et al*. (1939). It depends on the part of specific nuclei that absorb and diffuse energy within the magnetic field. NMR is an effective and powerful tool for the lipid analysis and it can be applied to liquid and solid samples even in combination with cells (Chauton *et al*., 2004). NMR

energetic nuclei in lipid properties contain ($^{1}$H), carbon ($^{13}$C), phosphorus ($^{31}$P), oxygen ($^{17}$O) and H in very large quantity in Algal cells (Todt *et al*., 2001); therefore, it has to be taken into consideration (Danielewicz *et al*., 2011). In the present study, both *Auxenochlorella* and *Chlorella* show a large amount of fatty acid (the broad signals) under nitrogen starvation whereas the other four are very similar with nothing standing out in larger quantities within the other experimental samples (**Figure 4.17**).

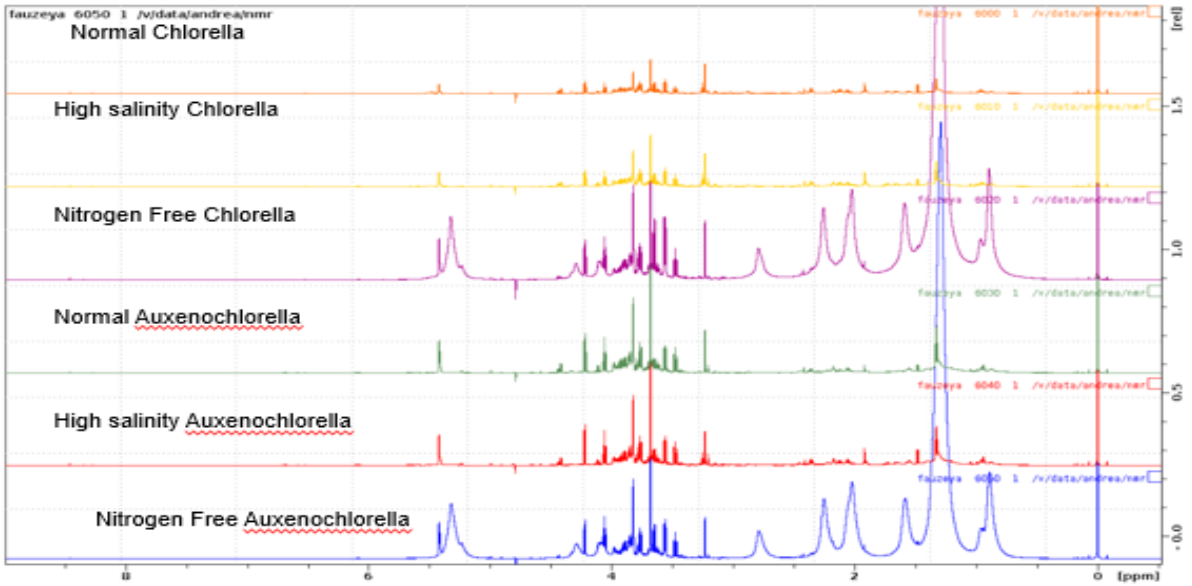



*Figure 4.17: NMR Spectra for Auxenochlorella and Chlorella cell-free extracts grown under normal, high salinity and N-free conditions.*

## 4.4. Discussion

The approaches for microalgae lipid extraction and quantification include the valuable NLs and fatty acid from water and matrix of the cells. Several methods have been used for the quantification of algae lipids. These methods are within the range of the conventional gravimetric method using Nile red lipid visualization extraction solvents, SPV and TLC (Cheng *et al*., 2011).

### 4.4.1. Microwave-assisted staining

Microwave-assisted staining methods follow the extra treatments such as the combination with DMSO processing and microwave radiation (e.g., on *Pseudochlorococcum* sp. ASU strain 1, *Scenedesmus dimorphus* ASU strain 1). The radiation by microwave affects algal cells and the dye properties by increasing the molecular clash and motion between them allowing the dye to enter the dye into the cells (Chen *et al*., 2011b). In the current study, two

procedures were used for natural lipid quantification: they relied on the use of acetone as strain, utilizing Nile Red directly. Acetone was not a good solvent carrier and showed little impact.

The Nile Red was unable of penetrate the cells of Algae because the cell wall is thick and cytoplasmic membrane might affect the penetration of dye making it a main disadvantage of Nile Red. Consequently, it will not reach the target, the intracellular lipid in this case (Chen *et al.*, 2011a). Several studies found that DMSO was good to be used as a strain carrier with Nile Red because it increases the fluorescent intensity as many as 10-fold (Chen *et al*., 2009; Frick *et al*., 2014). This makes it a better solvent compared to other solvents such as acetone, ethanol, ethylene glycol, and diaminotetraacetic acid (Cooksey *et al*., 1987; Castell and Mann, 1994; Doan and Obbard, 2011a; Siegler *et al*., 2012; Wong *et al*., 2014).

Two microwave steps with organic solvent have allowed for the determination of natural lipid content of the algal cells because it increases the fluorescent intensity. Previous study showed that organic solvent alone leads to low fluorescent intensity of algal cells because of the fluorescent refrigerate of Nile Red under high DMSO concentration (Chen *et al*., 2009). *Chlorella pyrenoidosa* and *Scenedesmus obliquus* are difficult to stain due to rigidity of their cell making NR unable to pass through (Ibid, 2009). Chen *et al*. (2009) checked the effect of DMSO on NR applied it to many *Chlorella* species. The researchers found that it could improve quantification; besides, the DMSO increases the fluorescence of NR of green Algae species of this study as well.

### 4.4.2 Lipid production in algae based on Nile Red concentration and cell concentration

*Chlorella* is considered a promising source of biofuel with a high rate of lipid production, cultivated under heterotrophic condition; it also accumulates lipid with a high calorific value (Xu *et al.*, 2006). The quantification of lipid from bio-organisms is shown to be fast and accurate compared with traditional method of gravimetric that needs around 3-4 days with at least 10-15 mg wet weight of cells (Bligh and Dyer 1959; AKoto, *et al.*, 2005). The Nile red fluorescence is fast, uncomplicated and sensitive (Cooksey, *et al.*, 1987; Priscu, 1990). Presently, the determination of lipid content in *Chlorella vulgaris* from the fluorescence intensity of Nile red relies on calibration curve to stain cells (Liu *et al*., 2008). Quantification of Natural lipid in microalgae was determined by the Nile red method in 96-well plate (Chen *et al*., 2009). However, now it is found out that it depends on different factors such as Algae

species, the polarity of solvent to dissolve Nile red and the various measurable conditions that probably affect the Nile red combination to lipid content in algal cells. In the end, it highlights the differences in the fluorescence intensity which is not reproducible (Cooksey *et al*., 1987; Chen *et al*., 2009; Greenspan and Fowler 1985; Elsey *et al*., 2007).

The selection of the optimum concentration of *Auxenochlorella* and *Synechocystis* cells was based on the balance among the cell-normalised value, the ratio of NR/concentration of algal cells, and the time of staining. The normalization technique was used because it is an accurate method applied to compare the fluorescent intensity readings of the same sample or different samples, prepared at the same cell density or the same amount of biomass (Chen *et al*., 2009). Furthermore, the highest fluorescence intensity of algal cells with Nile red depends on the time of staining. It is considered the main factor that significantly depends on the structure of cell wall of the chosen algal strain (Balduyck *et al*., 2015). These results showed that the maximum fluorescence intensity was achieved after 15 minutes for *Auxenochlorella*, slightly decreased after 20 minutes incubation. On the other hand, 25 minutes was the optimal staining time for *Chlorella* because of the fluorescence intensity.

The influence of Nile Red concentrations on the quantification of fluorescent intensity was investigated in both *Auxenochlorella* and *Chlorella*. The optimum Nile red concentration was selected based on the balance between high and low Nile Red concentrations due to the drawbacks of relying on the lowest or highest concentration. The optimum concentration of Nile Red for *Auxenochlorella* was (0.4µg $ml^{-1}$), and for *Chlorella* was (0.6µg $ml^{-1}$). The importance of dye concentration was related to the fact that the fluorescence intensity varies based on the concentration with an increase in the dye concentration above the optimal level. The fluorescence then decreased with the further increase in the dye concentration probably because of excess intense collisional quenching of the dye molecules at high concentration (Lakowicz, 2006). Furthermore, the quenching of the dye was decreased when compared with the access of Nile Red. However, Pick and Rachutin-zalogin, (2012) observed that low concentration of Nile Red affected the interaction between the dye and lipid droplets and consequently decreased the possibility of the dye to access the hydrophilic quenchers while the selection of a high Nile Red 135 concentration would probably lead it to react not only to the neutral lipids but also to the phospholipidic coat and hydrophobic surface of proteins (Sackett and Wolff, 1987).

### 4.4.3. Influence of salinity on the neutral lipid accumulation

Salinity affects the natural lipid accumulation in *Auxenochlorella* and *Chlorella* in high NaCl concentration in BBM medium. In *Auxenochlorella*, the highest percentage of TAG content found in cells was around 24% in a period of three weeks at 0.8 M NaCl; additionally, the control was 19% while *Chlorella* showed an increase of total lipid about 26% after 4 weeks at 1M NaCl and the control was 12%. This agrees with the previous study which shows an increase of lipid content in *Chlorella vulgaris* from 11.5% to 16.1% (Jared *et al*., 2017). Similar results were recorded with the *Chlorella*, which shows a high accumulation of lipid of around 21.4% (Rai *et al*., 2015). Also, increase in the lipid production with increase the level of salinity has previously been shown (Battah *et al.*, 2014). The maximum percent of lipid was recorded; it was about 43.15 % compared with control with 29.47% following the treatment of *Chlorella vulgaris* with 0.45 mM NaCl at 12 days old culture. The study showed that salinity is a tool suitable to get over production of lipid; furthermors, inverse relation was found between lipid production and growth. It has been discovered that exposing plants to salt lead to decreasing the growth and biomass as compared with control (Riguez *et al*., 2006).

### 4.4.4. Influence of nitrogen depletion

The percentage of lipid accumulation over four weeks for *Auxenochlorella* and *Chlorella* cells subjected to nitrogen limitation and starvation showed high percentage of lipid in *Auxenochlorella* in the nitrogen free stress condition after 4 weeks incubation (44%) of total dry weight followed by cells grown in normal growth BBM (5% of total dry weight). While in *Chlorella*, the percentage of lipid reached 70% under nitrogen starvation of total dry weight while normal growth was 20 % of total dry weight. This result agrees with that of Negi, *et al.*, (2016) which showed that in *Chlorella sorokiniana* under nitrogen starvation conditions, there was an increase in lipid accumulation ~20 times relative to culture supplied well with N leading a 1.6-fold higher total energy productivity in N depleted than in culture with N. Several researchers documented high lipid content in microalgae by achieving N-limitation or under other unfavourable growth conditions (Cakmak *et al*., 2012; Li *et al.*, 2013; Siaut *et al*., 2011). The results are in agreement with the observation on *Chlamydomonas* cells when grown under stress conditions such as nitrogen starvation, where their growth nearly stops and the cells accumulate large amounts of TAGs (Grossman, 2000; Zhang *et al.*, 2004). Also, this study agrees with that of Iwai *et al*. (2014), who reported that

natural lipid content was the highest in *C. reinhardtii* cells grown with nitrogen or phosphate deprivation at logarithmic growth phase.

The algal species *Chlorella* sp. illustrated the storage granules of starch as well as lipid droplet after three-day incubation in N-starvation, resulting in a great amount of total biomass and total energy yield (Wang *et al*., 2011). Many studies have shown that nutrient stress conditions induce lipid accumulation; they also decrease growth rates, probably due to huge fat remodelling or stress enhancing poor growth (Sharma *et al*., 2012; Spolaore *et al*. 2006). The results of this study contrast with the work of Araujo (2019) who observed higher growth rate and total production of biomass in *Chlorella vulgaris* and *Tetraselmis tetrathele* with no limitation of sodium nitrate (1.1 to 3.4 g/L dry weight) so these organisms did not get any support in the production of lipid under limited sodium nitrate. Oleic acid was the major FAME for *Auxenochlorella* and *Chlorella* under nitrogen stress. For biodiesel production, saturated or mono-unsaturated fatty acids are favoured (Knothe, 2008), so the composition of FAME in *Auxenochlorella* and *Chlorella* is reasonably well suited to biodiesel production.

### 4.4.5. Fatty acid profile of cells

The results of GC-MS for cells under high salinity stress and nitrogen starvation showed that there are several fatty acid methyl esters found in the natural lipid of both organism such as (C16:0) palmitic acid methyl ester and (C18:1) oleic acid methyl ester which are suitable or monounsaturated fatty acids, in addition to polyunsaturated fatty acids. Polyunsaturated fatty acids like linolenic are important dietary supplements; they are convenient for biofuel production as well due to their underlying instability (Stansell *et al*., 2012). The results of fatty acid profile of *Auxenochlorella* and *Chlorella* total lipid after 21 days incubation under nitrogen depletion (N Free BBM media) revealed the presence of Oleic acid (C18:1) as the main fatty acid. Our results agree with with those of the previous studies (Olmstead, 2013) which documented that the fatty acid, oleic acid and palmitic acid under nitrogen depletion. The detection of oleic methyl ester in biodiesel develops the properties of biodiesel (Knothe, 2008; Prabakaran and Ravindran, 2012).

### 4.4.6. NMR determination of lipid content

NMR spectra are able to examine the interior of intact cells so they can quantify the lipids and other biological metabolites within the entire cells (Beal *et al*., 2010; Merkley and

Syvitski, 2012). In addition, one of the features of using NMR in microalgal lipid content determination is their ability to estimate the composition of biological mixture with the smallest amount of the extract that significantly minimizes the complexity of chromatographic separation or chemical deduction (Bearden, 2012). The H-NMR spectrum of *Chlorella vulgaris* extract displays all signals of biodiesel such as saturated fatty esters as C18:N = 0-3 and C22:6 accordingly. The algal extract shows signals at 1.4, 2, 3.7 and 5.2 ppm, which are related to the proton signals of different triglyceride moieties. Signal at 1.4 ppm is related to the terminal methyl groups of saturated C14, C18 or n-6/n-9 of unsaturated fatty acids C18:1, C18:2. At 2 ppm that referred to a long alkyl chain (CH2) n. At 5.3 ppm indicates Oleic acid. The results of 1H-NMR showed an increase in the content of all lipids in stressed *Auxenochlorella* and *Chlorella* cells compared with the same microalga grown under normal conditions. When the results of NMR and GC-MS are compared, it becomes apparent that *Auxenochlorella* and *Chlorella* grown under N free BMM medium accumulated a high percentage of TAG in the form of saturated and unsaturated fatty acids, which makes them potentially suitable. Many other factors determine suitability for biodiesel production.

# Optimization of Growth Medium for Enhanced Biomass and Lipid Production of *Auxenochlorella* and *Chlorella*

## Chapter Five

## 5.1. Introduction

Microalgae are currently being considered as an alternative source of lipid (oil) due to fast growth and their ability to convert solar energy into chemical energy via photosynthesis and also their ability to fix $CO_2$ (Chen *et al.*, 2011b; Mata *et al*., 2010; Morales-Sánchez *et al*., 2017). Algal biomass can be produced in various environments including polluted water; high lipid yields are achieved and as such algae cultivation does not compete with agriculture; thus, they are a good source of biodiesel production (Chisti 2007; Lu *et al.*, 2015; Luque, 2010; Yang *et al*., 2016; Zhao *et al*., 2013). Many researchers have developed approaches to increase biomass and oil production by the cultivation of microalgae under different environmental conditions (e.g. Markou and Nerantzis, 2013; Minhas *et al*., 2016).

In order to enhance biomass and other bioactive compounds, microalgae are subjected to environmental stresses, which is considered to be one of the best methods for biomass production with the desired quantity of lipids or other compounds. These stresses include starvation of nitrogen or phosphorus, pH, light and salinity (Cheng and He, 2014). Optimization of stress conditions particularly the type and magnitude should be studied well to minimize the negative effect on the growth of microalgae and to optimise the desired commercial production of microalgae (Nagarajan *et al*., 2013).

Many companies have been optimizing methods for algal growth and lipid production. For example, the optimization of the medium component namely a type of a carbon source, salts, vitamins, and other nutrients that have been investigated. Also, the enhancement of environmental parameters namely temperature, light intensity and pH is carried out along with amendments to the metabolism in microalgae e.g. phototrophic, heterotrophic, mixotrophic growth (Dibenedetto *et al*., 2016; Ho *et al*., 2010; Jiménez *et al*., 2003; Langley *et al*., 2012; Negi *et al*., 2016; Yeh and Chang, 2012; You and Barnett, 2004). There are several other factors that affect growth and lipid content in many microalgae species. These include energy sources (organic or light), type of cultivation procedure, and the carbon sources (Chen *et al*., 2010; Přibyl *et al*., 2012; Talukder *et al*., 2012; Wang *et al*., 2007). Furthermore, algae have the ability to adapt and manipulate lipid metabolism pathways under various physiochemical parameters (Mohammed *et al*., 2013).

Nitrogen depletion is known to be an effective method that enhances lipid accumulation; however, it relies on the type of microalgae species involved. As N starvation increases, the medium composition sometimes limits total biomass yield. Numerous strategies have been

proposed like growth in two stages: first under enrichment media with nitrogen to stimulate biomass growth and then to transfer to free nitrogen medium to increase lipid production (Minhas *et al*., 2016; Singh *et al*., 2016). Constant use of nitrogen limitation was suggested to increase lipid accumulation in oleaginous *Chlorella vulgaris*, exceeding 1.35 times higher than the storage of lipid and similar biomass production when compared to conventional batch nitrogen limitation strategy; therefore, it points to the possibility of continuous large-scale production (Liu *et al*., 2016).

In order to get more value and sustainability of the microalgae as a source of biofuel and chemicals, after the extraction of lipids and protein; what remains of biomass needs valorisation using effective methods (Chisti 2007; Luque 2010). It can be utilized for animal feed or for biogas production by digestion and compost processes (Park *et al*., 2012). Culture conditions of microalgae have an impact on lipid and biomass productivity. The composition of production media is important, namely carbon, nitrogen, phosphorus and trace metals that promote and significantly support growth and biochemical characteristics of microalgae (Lam and Lee, 2012; Li *and Deng*, 2012; Prathima *et al*., 2012; Wang *et al*., 2014; Lin and Wu, 2015). Hence, it is necessary to look for and select an appropriate medium and to determine the right composition of nutrients for the cultivation of microalgae to induce fast growth and high amount of lipid production (Huerlimann *et al*., 2010).

## 5.2. Aim and Objectives

The aim of this chapter is to optimize growth media for enhanced biomass and lipid production by *Auxenochlorella* and *Chlorella* for biodiesel production.

The specific objectives of the research are:

1. To determine a suitable medium for *Auxenochlorella* and *Chlorella* using some well-known media BBM, JM and BG11,
2. To optimize the various growth parameters for high production of biomass.

## 5.3. Results and Discussion

### 5.3.1. Effect of BG11 medium on the growth of *Auxenochlorella* and *Chlorella* cells

Both organisms were grown in BG11 medium and the standard BBM medium (Amit *et al*., 2016). Algae growth rate is based on algal density in the culture media. Three replicate measurements at $OD_{595}$ were performed for each medium at two days interval for both cultures for a period of 25 days at 25°C in order to reach a growth plateau (**Figure 5.1**). The

result showed that the growth of *Auxenochlorella* cells in BBM medium was slightly higher than its growth in BG11 medium. In the BBM medium, the optical density of *Auxenochlorella* cells at $OD_{595}$ nm reached 5.54 and 9 after 12- and 18-days incubation respectively. For the BG11 medium, the optical density of *Auxenochlorella* cells at 595 nm reached 7.14 and 9.4 after 14- and 20-days respectively; therefore, the BG11 medium supported the growth of *Auxenochlorella* cells, but at a slightly slower growth rate.

With *Chlorella* cells in BBM medium, growth reached 4.46 and 5.80 after 14- and 20-days incubation, respectively (**Figure 5.2**). For BG11 medium OD reached 4 and 4.37 at the same time points. It is clear that both media are appropriate for the growth of *Chlorella*, but growth in the BBM medium was better. BBM has been found to be a most suitable medium for many species of green microalgae; nevertheless, there are exceptions e.g. *Chlorococcum aquaticum* (Kulvinder, *et al*., 2017). As the main goal in this part of the study was microalgae cultivation, BG11 and BBM were both preferred media for growth. Growth in BBM medium can lead to high rates of lipid production and yield due to lower nitrogen and phosphate concentrations, while the BG11 medium is a rich medium with high levels of nitrogen. However, nitrogen deficiency is a good way to increase lipid production (Li *and Deng*, 2012).

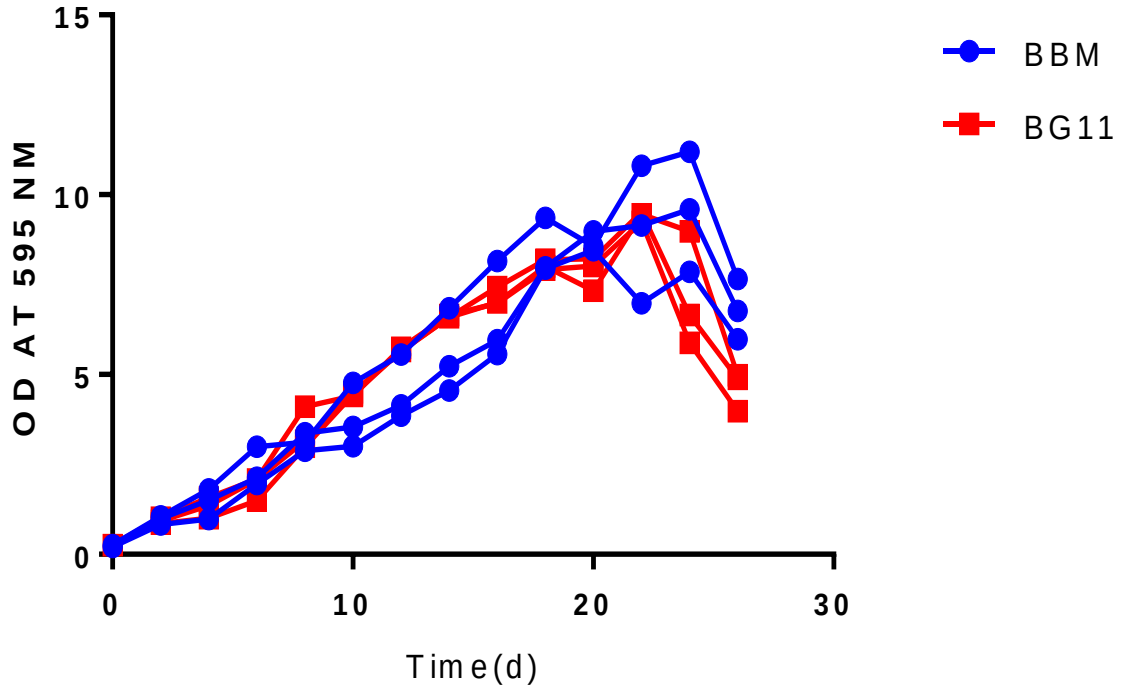



*Figure 5.1: Growth curves for Auxenochlorella grown in BBM and BG11 medium incubated in a 25ºC constant temperature room for 26 days.*

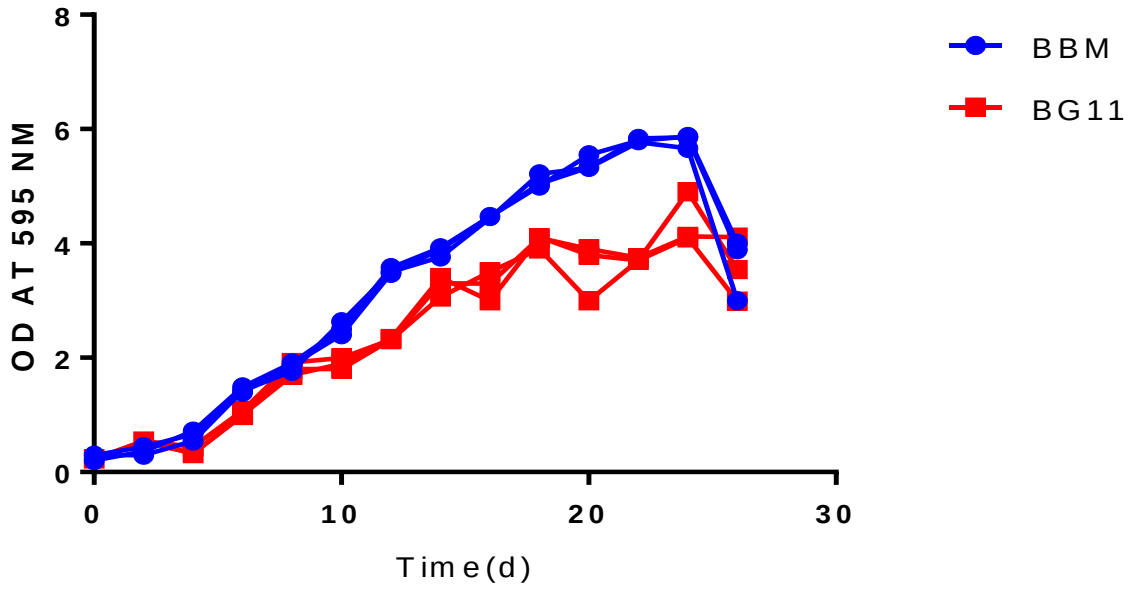



*Figure 5.2: Growth curves for Chlorella grown in BBM and BG11 medium incubated in a 25ºC constant temperature room for 26 days.*

### 5.3.2. Comparison of BBM and JM media

In order to determine the best growing conditions for *Auxenochlorella* and *Chlorella* to produce lipid (that can be converted to biodiesel fuel) various nutritional conditions were evaluated. Jaworski medium (JM) and BBM were prepared for biomass and lipid production. The JM medium was selected because it favoured the growth of green algae cultures (Sostaric *et al*., 2009). A comparison with the growth rate of *Auxenochlorella* cells in BBM was carried out to observe which medium is the most appropriate based on the growth yield (**Figure 5.3**). The growth curves of *Auxenochlorella* cells grown in BBM and JM at 25 ºC over26 days of incubation show that an $OD_{595}$ of 9.55 and 5.75 were reached after 22 days respectively for BBM and JM media while *Chlorella* cultures after 22 days incubation reached 5.79 and 3.46 respectively as shown in **Figure 5.4**. It is clear that the best growth resulted in BBM for both organisms. Therefore, BBM was selected as growth medium for further study in this work.

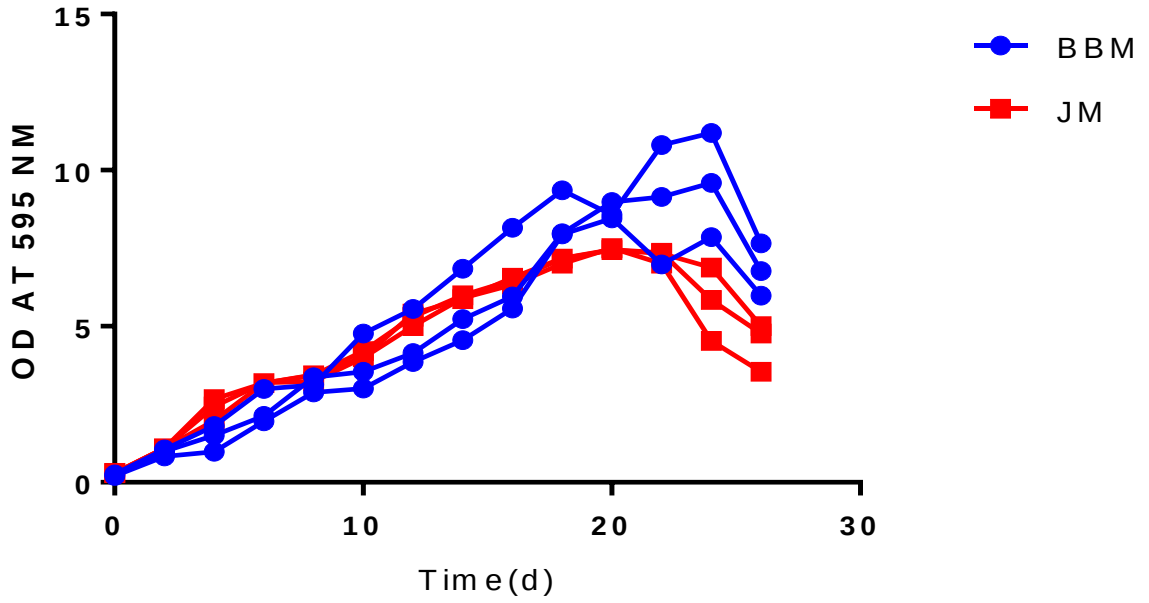



*Figure 5.3: Comparison of growth of Auxenochlorella cells in BBM and JM. Cells were incubated in a 25ºC constant temperature room for 26 days. The OD for each medium was measured at 595 nm.*

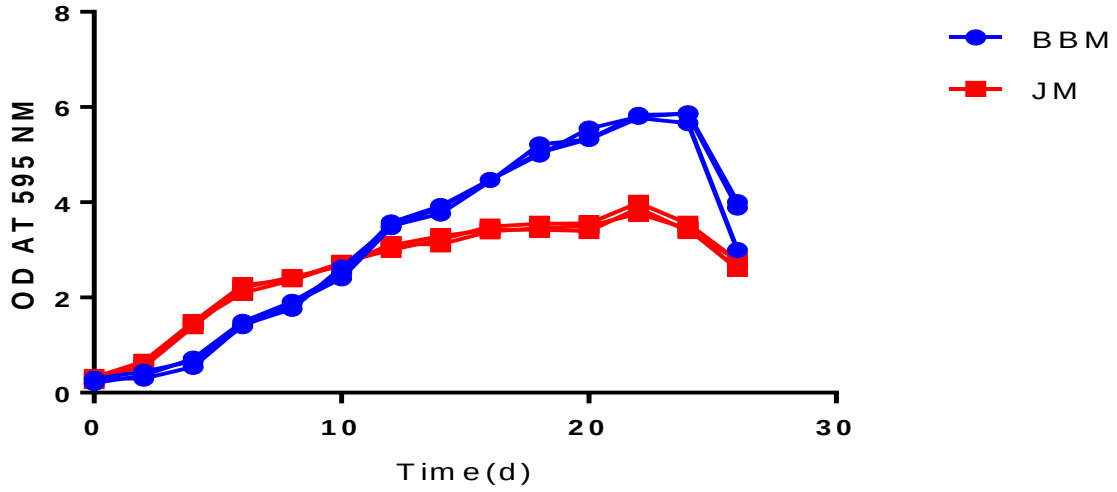



*Figure 5.4: Comparison of growth of Chlorella cells in BBM and JM. Cells were incubated in a 25 ºC constant temperature room for 26 days. The OD for each medium was measured at 595 nm.*

### 5.3.3. Effect of vitamin B12 concentration on the growth of *Auxenochlorella* and *Chlorella*

Several microalgae species require an exogenous supply of vitamin B12 for their growth as an essential factor in vitamin B12-dependent enzymes, which are gained during symbiosis with bacteria (Croft *et al.*, 2005). Vitamin B12 is an important supplement for many organisms and human cells as a cofactor for methionine synthase and methylmalonyl-CoA

mutase enzymes (Helliwell *et al*., 2011). In order to investigate the demand for vitamin B12 for the growth of *Auxenochlorella* and *Chlorella* cells, both strains were grown in BBM medium with or without vitamin B12 under the same growth conditions at a temperature of 25 ºC for 26 days incubation with the same initial $OD_{595}$ for both cultures. It was observed that *Auxenochlorella* showed only very slightly higher growth in the presence of B12, while *Chlorella* showed significantly higher growth with vitamin B12. After 12 days of incubation, the optical density for *Auxenochlorella* was 6.38 without B12 and 7.16 with B12, while in *Chlorella* the OD values were 2.15 and 5 in normal medium and vitamin B12 medium respectively. Over 18 days period *Auxenochlorella* cells reached optical density of 10.55 and 11.3 at $OD_{595}$ while *Chlorella* optical density of 5 and 6.3 was measured in normal and B12 media (**Figures 5.5 and 5.6**).

The results indicate that BBM successfully supported the growth of both organisms but it was found that the growth of *Chlorella* was better supported with vitamin B12 suggesting that vitamin B12 is an essential compound for *Chlorella* cells growth. The results of *Auxenochlorella* agree with those of Jinkerson *et al*. (2013) who maintained that *Nannochloropsis* contains B12 dependent enzymes and that it did not need vitamin B12 for growth. Generally, many species of microalgae can produce methionine without using vitamin B12- dependent methionine synthase, as the synthesis of methionine can take place without vitamin B12 by the synthesis of B12-independent methionine synthase (Croft *et al.*, 2005). Although *Chlorella* species have some amount of endogenous B12 (Watanabe *et al*., 1997), it was found that the growth of *Chlorella* is higher with the addition of Vitamin B12 to the media. For further studies in this work, BBM medium containing vitamin B12 was considered to be the most suitable medium for growing *Chlorella*.

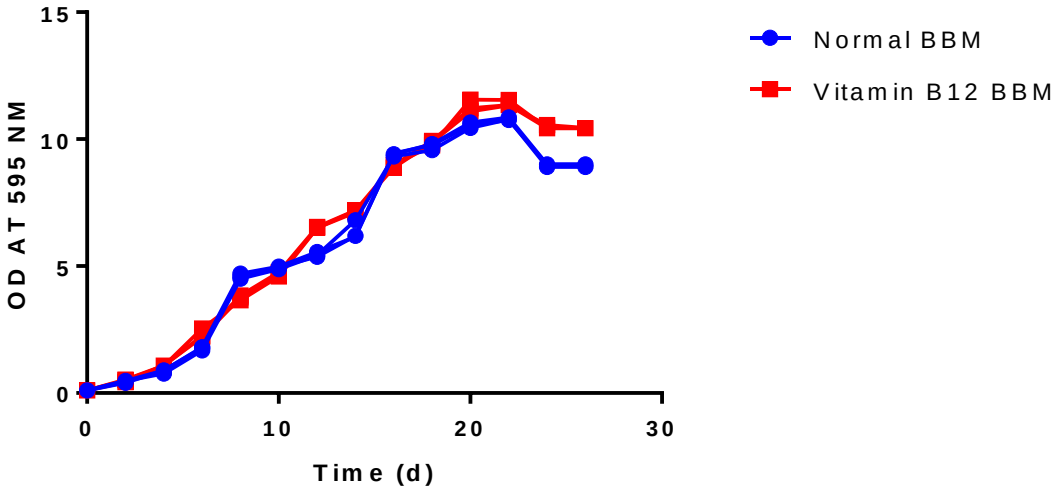



*Figure 5.5: Growth rate of Auxenochlorella cells grown in BBM medium in comparison with the growth rate of Auxenochlorella cells grown in vitamin B12 BBM medium. Cells were incubated in a 25ºC constant temperature room for 26 days. Growth rate was determined by measuring the OD at 595 nm every two days.*

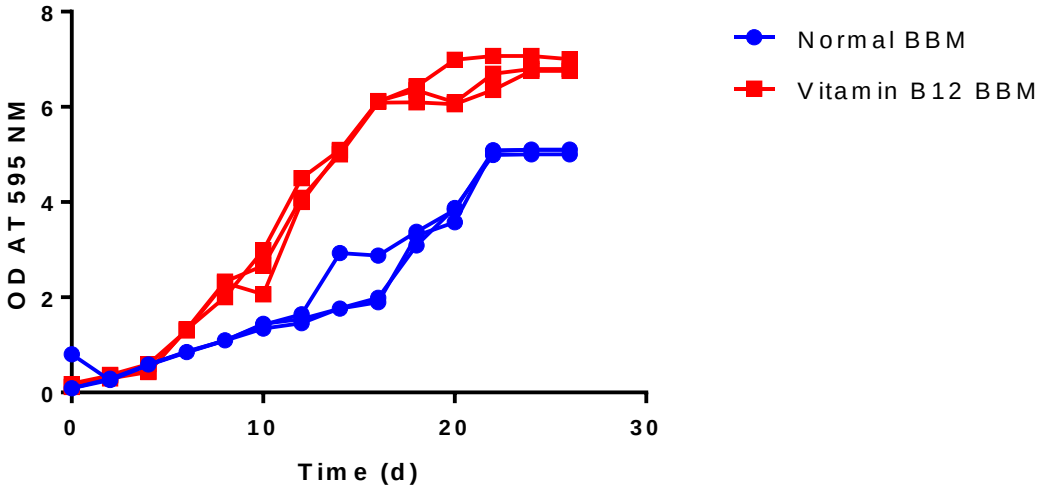



*Figure 5.6: Growth rate of Chlorella cells grown in BBM medium in comparison with the growth rate of Chlorella cells grown in vitamin B12 BBM medium. Cells were incubated in a 25ºC constant temperature room for 26 days. Growth rate was determined by measuring the OD at 595 nm every two days.*

### 5.3.4. Microalgal dry weight determination

The biomass dry weight of microalgae cells grown in the BBM medium was determined at different cell concentrations as described in **section 2.8** using 12 dilutions of algal culture. **Table 5.1** shows the result of the biomass dry weight of *Auxenochlorella* cells and **Table 5.2** shows the result of *Chlorella.* The results allow for the calculation of dry weight (mg $ml^{-1}$) based on the optical density measured at 595 nm for each dilution. It was clear that from findings, the biomass dry weight of the microalgae cells increased with an increasing concentration of samples ($OD_{595}$). However, the main purpose of this experiment is to establish the relationship between optical density at 595 nm and dry weight biomass of *Auxenochlorella* and *Chlorella* cells for use as a standard dry weight curve of cells **(Figures 5.7 and 5.8)**. For example, in order to convert the amount of neutral lipid measurements in Nile Red procedure (fluorescence readings) to percentage lipid per biomass dry weight of algal cells (% dry weight), the equations shown in (**Figures 5.7 and 5.8)** were used.

*Table 5.1: Auxenochlorella culture concentrations (12 culture dilutions), optical density at 595 nm reading and amount of dry weight calculated as mg/30ml and mg/ml of microalgal biomass dry weight respectively.*

| Tube | $OD_{595}$ | Weight before | Weight After | Total weight | Dry Weight (mg/30ml) | Dry Weight (mg/ ml) |
|---|---|---|---|---|---|---|
| 1 | 1.006 | 1.009 | 1.012 | 0.010 | 9.700 | 0.323 |
| 2 | 0.975 | 1.024 | 1.034 | 0.011 | 10.900 | 0.363 |
| 3 | 0.817 | 1.009 | 1.018 | 0.009 | 9.200 | 0.307 |
| 4 | 0.736 | 1.002 | 1.010 | 0.006 | 7.500 | 0.250 |
| 5 | 0.685 | 1.008 | 1.015 | 0.007 | 6.900 | 0.230 |
| 6 | 0.631 | 0.997 | 1.005 | 0.007 | 7.100 | 0.237 |
| 7 | 0.527 | 1.011 | 1.017 | 0.007 | 6.700 | 0.223 |
| 8 | 0.470 | 0.999 | 1.005 | 0.006 | 5.900 | 0.197 |
| 9 | 0.393 | 0.991 | 0.994 | 0.003 | 3.300 | 0.110 |
| 10 | 0.225 | 1.002 | 1.005 | 0.003 | 2.800 | 0.093 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 11 | 0.096 | 1.002 | 1.004 | 0.002 | 2.000 | 0.067 |
| 12 | 0 | 1.007 | 1.007 | 0 | 0 | 0 |

The standard concentration curve of dry weight in *Auxenochlorella* cells was plotted using optical density ($OD_{595}$) reading (X axis) against dry weight biomass measurements of each concentration (Y axis) as shown in (**Figure 5.7)**.

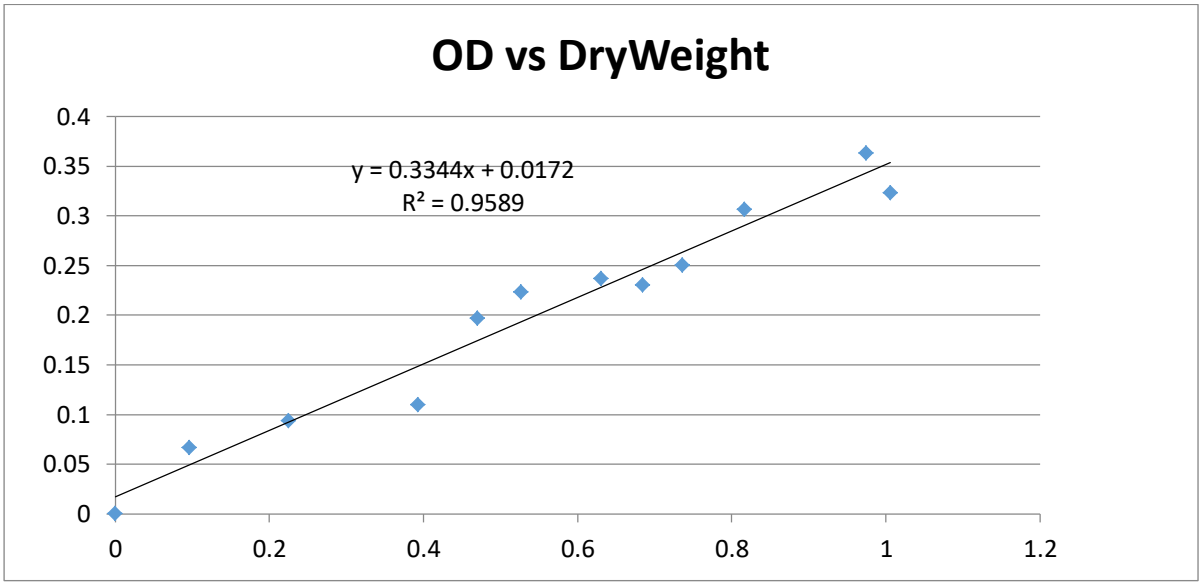



*Figure 5.7: Linear relationship between OD and dry weight of Auxenochlorella cells. The concentration curve was plotted using optical density (OD) reading against dry weight of each sample.*

*Table 5.2: Chlorella culture concentrations (12 culture dilutions), optical density at 595 nm reading and amount of dry weight calculated as mg/30ml and mg/ml of microalgal biomass dry weight, respectively.*

| Tube No. | $OD_{595}$ | Weight before | Weight after | Total weight | Dry Weight (mg/30ml) | Dry Weight (mg/ ml) |
|---|---|---|---|---|---|---|
| 1 | 1.036 | 1.029 | 1.034 | 0.005 | 4.6 | 0.153 |
| 2 | 0.986 | 1.008 | 1.011 | 0.004 | 3.5 | 0.117 |
| 3 | 0.844 | 1.008 | 1.010 | 0.003 | 2.7 | 0.090 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 4 | 0.791 | 1.015 | 1.017 | 0.002 | 2.0 | 0.067 |
| 5 | 0.773 | 1.012 | 1.014 | 0.002 | 1.7 | 0.057 |
| 6 | 0.699 | 1.016 | 1.017 | 0.002 | 1.6 | 0.053 |
| 7 | 0.575 | 1.002 | 1.003 | 0.001 | 1.4 | 0.047 |
| 8 | 0.544 | 1.002 | 1.003 | 0.001 | 1.2 | 0.040 |
| 9 | 0.363 | 1.018 | 1.019 | 0.001 | 1.0 | 0.033 |
| 10 | 0.269 | 1.010 | 1.011 | 0.001 | 0.9 | 0.030 |
| 11 | 0.107 | 1.012 | 1.013 | 0 | 0.4 | 0.013 |
| 12 | 0 | 1.016 | 1.016 | 0 | 0 | 0 |

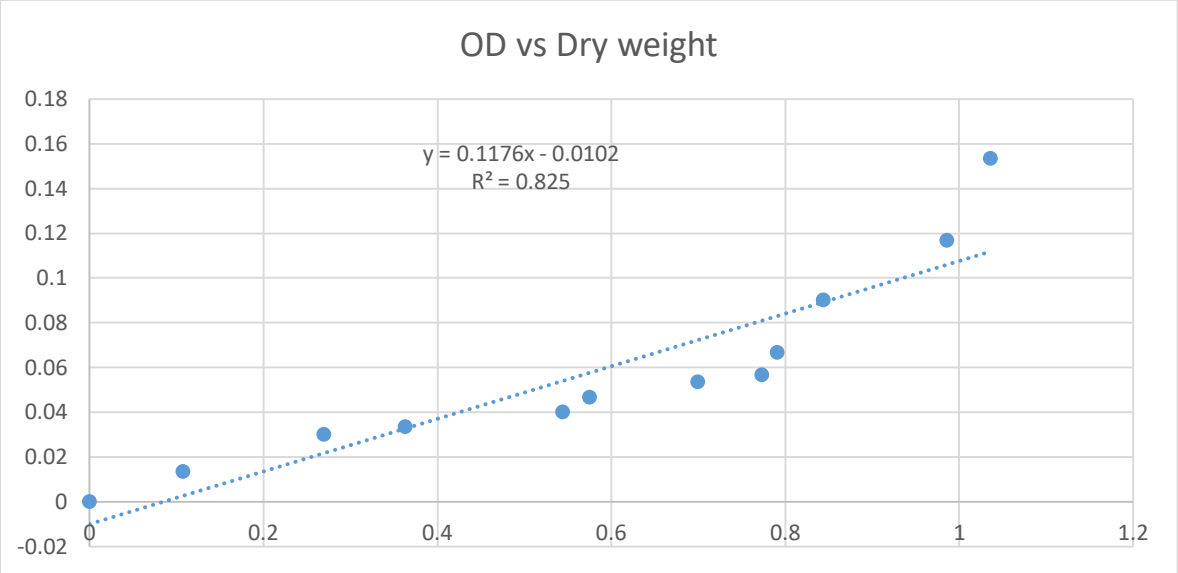



*Figure 5.8: Linear relationship between OD and dry weight of Chlorella cells. The concentration curve was plotted using optical density (OD) reading against dry weight of each sample.*

### 5.3.5. Effect of Adaptation to Different Salinity Concentrations on *Auxenochlorella* and *Chlorella*

In order to determine the salinity tolerance of *Auxenochlorella and Chlorella*, the microalgae were subjected to a range to of salinities. In this experiment, five different salinity

concentrations were used on the microalgae cells and growth was evaluated every day using a spectrophotometer to measure the cell density (OD) at 595 nm during the incubation period. The cell density measurements at 595 nm of *Auxenochlorella* cells adapted to grow at different salt concentration values (0.2 M, 0.4 M, 0.6 M, 0.8 M and 1M NaCl) in BBM medium are shown in (**Figure 5.9)**. It was found that the specific growth rate and cell growth by *Auxenochlorella* cells was higher at 0.2 M, 0.4 M, and 0.6 M salinity. However, the highest specific growth rate of *Auxenochlorella* cells was observed at 0.2 M salinity and it was the lowest at 1 M NaCl. Generally, *Auxenochlorella* cells grew well at 0.2 M and 0.4 M salinity with the cell density reading reaching 1.626 and 1.5 at 595 nm after 18 days incubation at 25°C compared with their growth at 0.8 M and 1 M of NaCl; the cell density reading reached 0.6 and 0.2 after 8 days incubation at 25°C, respectively. *Chlorella* cells showed higher growth at 0.2, 0.4 and 0.6 M NaCl with cell density readings reached after 17 days being 1.621, 1.472 and 1.200 and the lowest growth was at 1 M, which was 0.329 after 8 days (**Figure 5.10**).

This findings agree with those concluded by Rai et al. (2015) who suggested that the microalgae strain *Chlorella* sp showed greatest growth of 0.822 g/l and 1.021 g/l in Fogg's medium and under 0.2 M NaCl respectively. In our study, a reduction in growth and biomass with 0.8 and 1 M NaCl was observed. This finding is also in agreement with that of Kalla and Khan, et al., (2009) observed the response of *Chlorella vulgaris* growth to various concentrations of NaCl. They found that the cell growth was favoured at minimum concentration of NaCl between 0.0 (control) and 0.25M until 21 days when it started to decline. So, to obtain favourable growth of microalgae, the optimum NaCl concentration is deemed to be below the point where there is a reduction in growth value (Ruangsomboon, 2012; Takagi *et al.*, 2006). Rodriguez *et al*. (2006) stated that when plants are exposed to salinity, the growth and biomass is reduced compared with the control. The most likely reason for the inhibition of growth in *Chlorella vulgaris* under salinity is the partial inhibition of cell division (Hagemann *et al*., 1989). Salinity stress can significantly impact the biologically grown systems of microalgae that lead to the improvement and progress of microalgae. Salinity stress is able to bring changes in the metabolic systems of lipid and fatty acid synthesis so it contributes to the acceleration in the increase of lipid substances within the cells (Kalita *et al*., 2011).

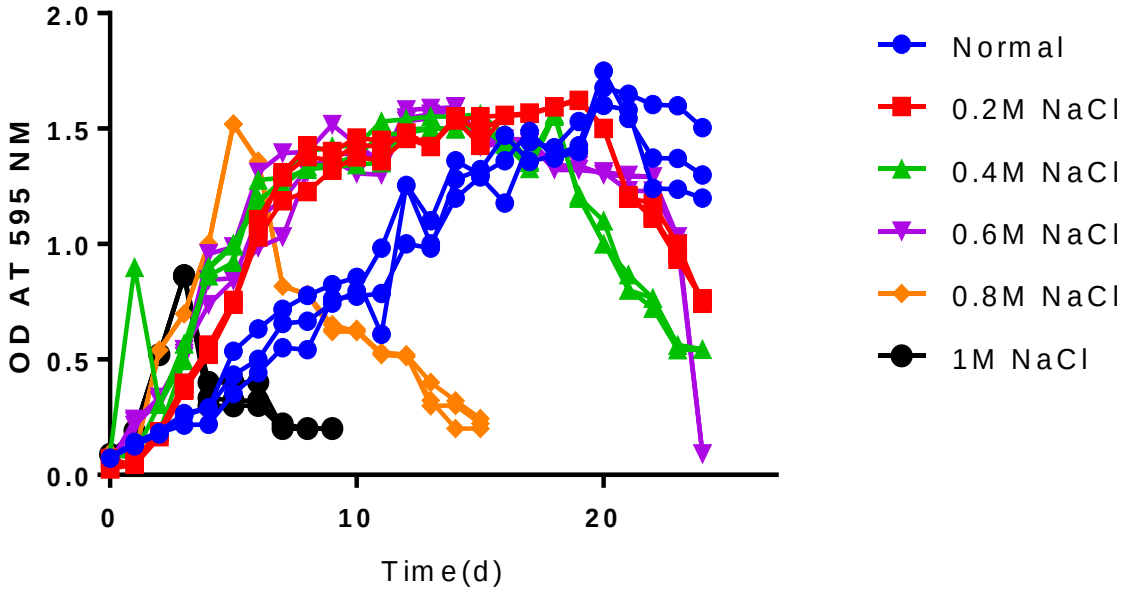



*Figure 5.9: Growth curves for Auxenochlorella cells grown in BBM adapted to grow at different salinity values (0.2, 0.4, 0.6, 0.8 and 1M NaCl) incubated for a period of 26 days; incubation is 25ºC constant temperature room; the OD for each medium was measured at 595 nm.*

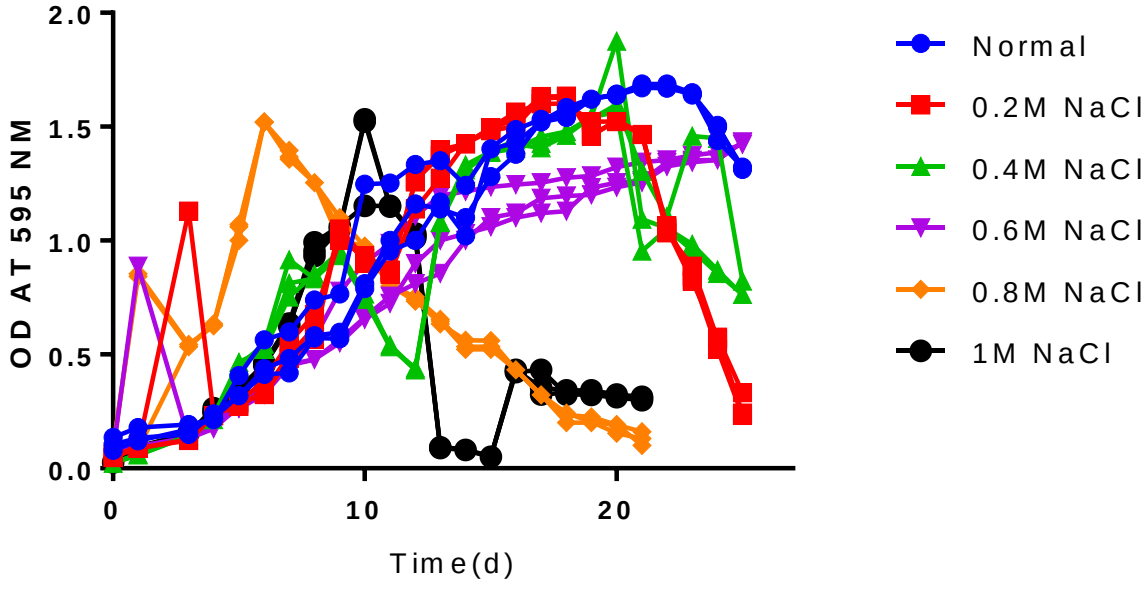



*Figure 5.10: Growth curves for Chlorella cells grown in BBM adapted to grow at different salinity values (0.2, 0.4, 0.6, 0.8 and 1M NaCl) incubated for a period of 26 days; incubation is at 25ºC constant temperature room. The OD for each medium was measured at 595 nm.*

### 5.3.6. Effect of nitrogen concentration on cell growth of *Auxenochlorella* and *Chlorella*

Nitrogen is considered an essential ingredient and component of biological macromolecules such as protein, chlorophylls and DNA, and it plays a primary role in the cultivation of microalgae. Variation in nitrogen concentration could impact the growth rate, as well as protein, lipid and carbohydrate composition in microalgal cells (Pancha *et al*., 2014). Studies have reported that although nitrogen starvation leads to a stimulation of lipid production, it causes reduction in biomass production (El-Kassas, 2013). Therefore, it is most important to establish the right culture medium and cultivation conditions to obtain optimum lipid production. In our study, the effect of different nitrogen concentrations on the cell growth of *Auxenochlorella and Chlorella* was determined using a spectrophotometer at 595 nm for 26 days incubation period to investigate the appropriate culture medium to obtain optimum lipid production. **Figures 5.7** and **5.8** illustrate the growth curves of cells grown at different nitrate concentrations (Nitrogen free, 25%, and 50% of $NaNO_3$) in BBM cultivated for 26 days. The relationship between cell growth and nitrogen limitation in the medium indicated that the microalgae cells grew better at 25%, and 50% of $NaNO_3$. Although the highest biomass was found under the highest nitrate concentration, there was no significant difference. The lowest specific growth rate was found under nitrogen-deficient condition with nitrogen free BBM.

The findings of this study agree with those arrived at by Wong *et al*. (2017) who showed that nitrogen starvation leads to significant decline in the cell division and to the low optical density that we found in nitrogen-free medium. Also, our finding agrees with Rodolfi *et al*. (2009) who reported that N-limitation could enhance lipid accumulation with a decrease in the biomass production. It has been previously observed that loss of biomass of the green alga, *Botrycoccus* sp occurred under nitrogen limitation conditions in culture medium (Yeesang and Cheirsilp, 2011). A similar observation was made for *C. vulgaris*, where the growth rate was lower in low nitrogen medium but the final cell numbers were a little higher (Illman *et al.*, 2000). Also, a reduced growth pattern in *Scenedesmus obliquus* was observed (Mandal and Mallick, 2009; Gouveia and Oliveira, 2009); when exposed to nitrogen deficient conditions and to *Chlorella pyrenoidosa* (Nigam *et al.*, 2011). Microalgae grown under nitrogen deficient condition mainly accumulate a surplus of carbon metabolites that is stored as lipid (Ahlgren and Hyenstrand, 2003).

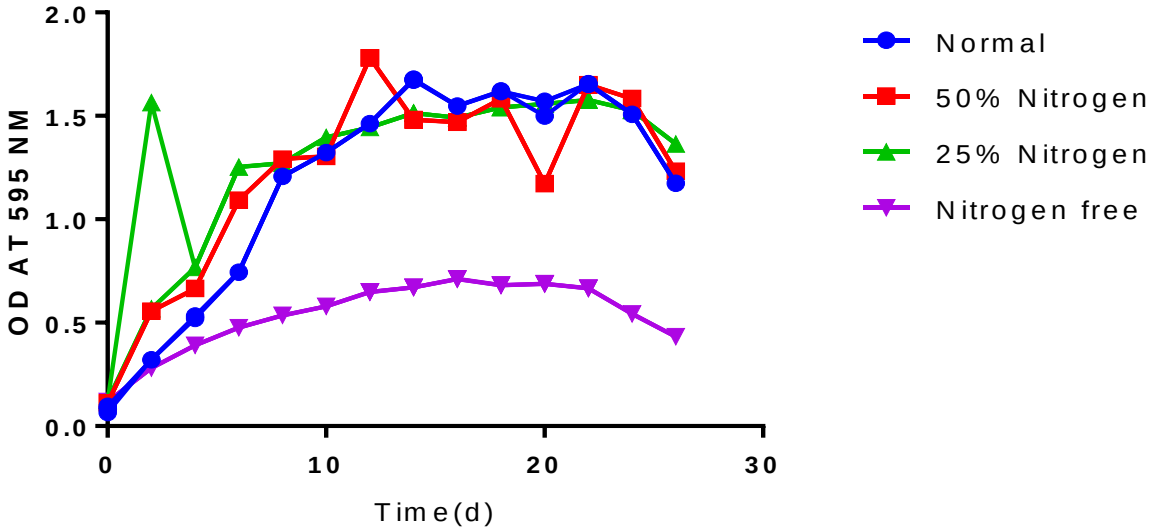



*Figure 5.11: Effect of nitrogen concentrations (Nitrogen free, 25%, and 50% of $NaNO_3$) on cell growth of Auxenochlorella grown in BBM. Cells were incubated in a 25ºC constant temperature room for 26 days. The OD for each medium was measured at 595 nm.*

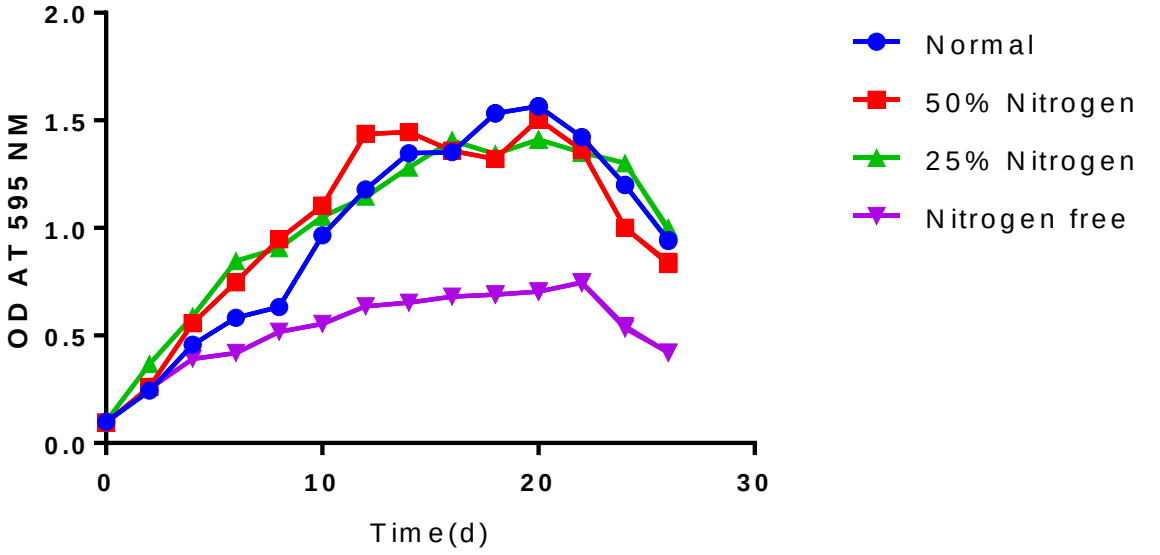



*Figure 5.12: Effect of nitrogen concentrations (Nitrogen free, 25%, and 50% of $NaNO_3$) on cell growth of Chlorella grown in BBM. Cells were incubated in a 25ºC constant temperature room for 26 days. The OD for each medium was measured at 595 nm.*

# Effect of Glucose on Enhancement of Biomass and Lipid Production by *Chlorella vulgaris* in Mixotrophic Culture

Chapter Six

## 6.1. Introduction

Microalgae are a promising resource that produce different types of bioenergy; additionally, they have the ability to produce $CO_2$ (EISA, 2007; Ahmed *et al*., 2017). When compared to land crops, microalgae can grow faster under favourable conditions and produce higher levels of biomass. Moreover, microalgae can be cultivated in a relatively small land area, which is an advantage for biofuel production (Hossein *et al*., 2019). The lipid in microalgae can be converted to biodiesel in the form of fatty acid methyl esters (FAME) through the process of transesterification (Liu *et al*., 2016). Large-scale microalgae cultivation is the best method to generate a higher amount of these lipids. The cultivation of microalgae has several benefits for the world, especially in the medical and cosmetic fields, besides its use for feeding animals (Das *et al*., 2011). Microalgae can be cultivated in the closed culture systems such as bioreactors or open systems such as outdoor ponds and cultured under phototrophic conditions by using $CO_2$ and light as carbon and energy source (Chen *et al*., 2011a).

The production of new energy fuels from microalgae depends on the technique of microalgal cultivation (*Ibid*, 2015). Indeed, several microalgae species are able to grow under autotrophic, mixotrophic and heterotrophic conditions (Chojnacka and Marquez-Rocha, 2004; Bhatnagar *et al*., 2010). Microalgae cultivated under mixotrophic conditions can grow using photosynthesis, but at the same time it can assimilate organic material as sources of energy and carbon. This mechanism decreases dependence on the light source so that the cell density will be higher than autotrophic culture. Mixotrophy also utilizes less organic compound per unit of biomass than dark heterotrophic culture (Boyle and Morgan, 2009).

Mixotrophic culturing has drawn attention to the microalgal ability to grow with autotrophic and heterotrophic cultivation by utilizing sunlight as a source of light energy and both organic and inorganic carbon (Li *et al*., 2014**a).** Throughout the use of different microalgae cultivation methods, two strategies have proved to be successful so far, namely optimum growth conditions to enhance biomass production and the utilization of stress conditions to accumulate lipid (Markou and Nerantzis, 2013; Devi and Mohan, 2012). Many researchers have worked on improving the biomass production in an attempt to decrease the cost of microalgae production. This will enhance lipid yield to increase biodiesel production via approaches designed to optimize metabolic pathways under microalgae cultivation utilizing the nutrients in the culturing media especially carbon element.

The mixotrophic mode has benefits over hetrotrophic growth and mixotrophy avoiding the drawbacks of autotrophic cultivation. In 1997, it was found that when *Chlorella vulgaris* was grown mixotrophically using acetate as the carbon source, the cell growth rate combined autotrophic and heterotrophic metabolism (Endo *et al*., 1977). Mixotrophic culturing can be a good way for cultivating microalgae to produce biofuel. Mixotrophic culturing has many merits: firstly, it minimizes the growth cycles and increases biomass production. Secondly, it lengthens the exponential growth rate. Thirdly, it reduces the loss of biomass during dark conditions. Fourthly, this cultivation method can be changed to either heterotrophic or phototrophic. Fifthly, it avoids the risk of photo-oxidation by providing oxygen in photobioreactors (Octavio and Yoav, 2015).

Even though mixotrophic culturing is considered a good and effective process, it is associated with some issues. For example, not all microalgal species can use organic compounds. Also, it may expose the cultures to bacteria and fungi which cause contamination; besides, excessive unconsumed organic substrate not consumed may result in inhibition of the growth of microalgae (Perez-Garcia *et al*., 2011b; Pires 2015). Mixotrophic cultivation in microalgae involves both photosynthesis and use of organic substrates as well as $CO_2$. Mixotrophic culture is appealing in industrial biotechnology because light is commonly the main limiting factor in photobioreactors; it restricts the maximum biomass obtainable (Woodworth *et al*., 2015).

Microalgae most commonly use phototrophic culture to convert $CO_2$ and water to biomass (Li *et al*., 2014 **b).** Due to many drawbacks of phototrophic culture, such as the limitation of biomass concentration, the longer time of cultivation and the harvesting the cost becomes huge. Mixotrophic cultivation is considered an advantageous approach that could solve issues associated with phototrophic culture (Wang *et al*., 2014). Heterotrophic cultivation uses organic substrates as carbon and energy sources, while mixotrophic cultivation utilizes more sources of organic carbon and inorganic $CO_2$. Thus mixotrophic organisms combine phototrophic and hetrotrophic metabolism to produce a high yield of biomass. In addition, mixotrophic growth costs less energy when compared to phototrophic cultivation due to its demands for lower light intensities.

Heterotrophic and mixotrophic microalgae can utilize many exogenous sugars like glucose, galactose, mannose, fructose and sucrose. The most commonly studied substrate in mixotrophic metabolism is glucose, which is a monosaccharide (Perez-Garcia *et al*., 2011a).

It has been reported that several microalgae such as *Nannochloropsis*, *Scenedesmus*, *Arthrospira* and *Chlorella* can use glucose (Cheirsilp and Torpee, 2012; Chojnacka and Zielińska, 2012). Glucose as a substrate is used by microalgae in mixotrophic culture due the fact that it releases more energy content in contrast with other carbon sources (Tan *et al*., 2018). For example, glucose releases more energy content per mole than acetate, which produces 0.8 kJ $mol^{-1}$ while glucose produces 2.8 kJ $mol^{-1}$ (Boyle and Morgan 2009). Most lipid production growth systems using microalgae have glucose as the carbon source (Perez-Garcia *et al*., 2011 b).

Some strains of microalgae such as *Chlorella* can grow successfully in the presence of glucose and galactose under different light intensities. In addition, several researchers have reported that supply of glucose to a medium enhanced the biomass and biofuel production for biorefinery (Kong *et al*., 2012). Mixotrophic cultivation of microalgae can grow by photosynthesis and at the same time, it can assimilate organic material as source of energy and carbon. This mechanism decreases dependence on light source so the cell density will be higher than autotrophic culture compared to utilizing less organic compound per unit of biomass than dark heterotrophic growth (Boyle and Morgan, 2009).

It has been shown that when *Chlorella vulgaris* is cultivated under mixotrophic conditions. The biomass levels are better than those under hetrotrophic and autotrophic conditions (Heredia-Arroyo *et al*., 2011). The same was found for *Nannochloropsis* sp where the biomass was significantly improved under mixotrophic conditions reaching 1.6 times higher concentration compared to autotrophic conditions (Andrade and Costa 2007; Marquez *et al*., 2017). The biomass of *Nannochloropsis salina* and *Chlorella vulgaris* scored a higher yield under mixotrophic conditions and it grew faster than autotrophic or hetrotrophic growth (Sforza *et al*., 2012b). It was reported that in *Chlorella protothecoides* biomass and lipid increased under mixotrophic conditions (Marquez *et al*., 2017; Wang *et al*., 2013).

### 6.2. Aim and Objectives

The aim of this Chapter was to determine the influence of glucose on the mixotrophic growth and lipid production in *Chlorella vulgaris*.

The objectives were;

1. To determine the effect on *Chlorella vulgaris* growth of different concentrations of glucose,

2. To assess the effect of glucose on lipid accumulation in *Chlorella vulgaris* under mixotrophic conditions.

## 6.3 Results and Discussion

### 6.3.1 Growth of Chlorella vulgaris on different concentrations of glucose

*Chlorella vulgaris* cells were grown in the BBM medium under mixotrophic conditions in which an organic carbon source is provided to algal cells. The growth profile of *C. vulgaris* was measured when it was cultivated in the presence of different concentrations of glucose. It can be seen that the cells grew much faster in mixotrophic culture i.e. in the presence of glucose. After 7 days, $OD_{595}$was 5.7, 7.8, 10.7 and 7 with increasing concentration of glucose (0.1, 0.5, 1 and 2%) while under normal non-mixotrophy conditions, the OD after 7 days was 1.3 (**Figure 6.1**).

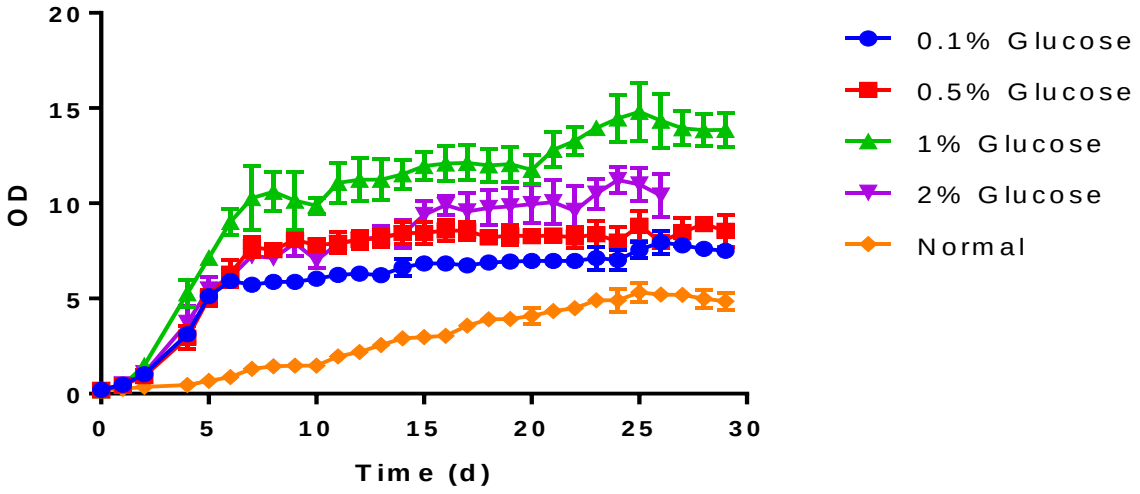



*Figure 6.1: Growth profiles of C. vulgaris cultivated with different concentrations of glucose incubated in a 25ºC constant temperature room for 29 days. Data points are the means of three replicates plus or minus the standard error.*

The most significant increase in growth was observed when the glucose concentration was 1%. Previous studies showed that determining the optimum concentration of glucose is important. Usually the tested concentrations of glucose range between 1 and 25 g/L i.e. 0.1 – 2.5%. Higher concentrations of glucose (≥15 g/ L, 1.5%) lead to inhibition of the growth of microalgae in mixotrophic cultures (Deng *et al*., 2011). Under optimum conditions, *Chlorella*

spp and *Nannochloropsis* spp produce higher biomass levels with glucose under mixotrophic growth compared to hetrotrophic and photoautotrophic cultivation (Cheirsilp and Torpee, 2012). The present results are also in agreement with those inferred by Heredia-Arroyo and Ren (2011) who reported that glucose is taken up and utilized within the first week of growth in *C. vulgaris* and *C. protothecoides* cultures, with a concentration of 5 and 10 g/l glucose respectively (Deng *et al*., 2011).

### 6.3.2 Lipid accumulation on microalgae under mixotrophic conditions

Neutral lipid contents of cells of *Chlorella vulgaris* grown under normal conditions (BBM medium) and under mixotrophic conditions (1% glucose) were serutinized over a period of four weeks to investigate the effect of mixotrophic growth on the neutral lipid content as measured by Nile red fluorescence. As shown in **Figure 6.2**, when 1% concentration of glucose was used as carbon source in mixotrophic culture, the lipid increased to 79% in the last week of the experiment as compared to 11% under normal photoautotrophic conditions.

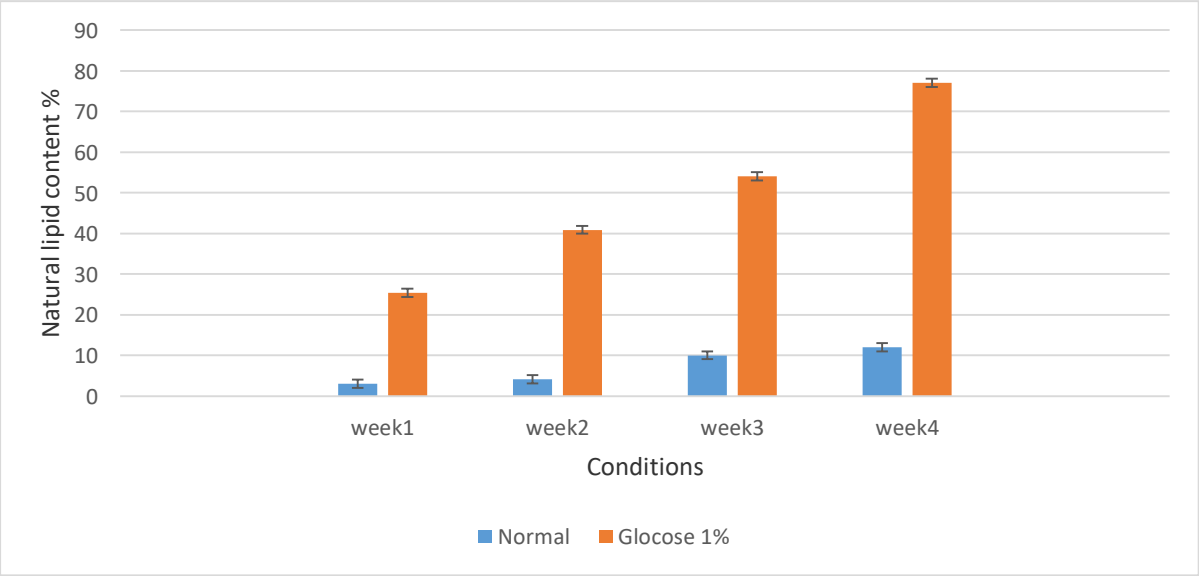



*Figure 6.2: Nile Red fluorescence intensity measurements of Chlorella cells grown in normal BBM medium and in BBM medium with 1% glucose respectively. Each measurement represents the difference between the average of four stained and four unstained readings, which was then converted into % lipid. Error bars represent technical repeats (n=3).*

The results coincide with those drawn by Park *et al*., (2012) who used 14 Chlorophyta strains isolated from north Canada. The authors found that in mixotrophic cultures the lipid increased compared to those under photoautotrophic regimes. The same observation was

found in *Chlorella* spp and *Nannochloropsis* where the lipid production is higher under mixotrophic conditions compared with photoautotrophic and heterotrophic cultivation (Cheirsilp and Torpee, 2012). Also, agree with those arrived at by Cheirsilp and Torpee (2012) who found that lipid content of *Chlorella* sp. and *Nannochloropsis* sp under concentration of glucose from 0 to 4 g/L significantly increased and remained the same above 4 g/L (0.4%) of glucose. In 2009, Cheng *et al*. compared lipid production under the three cultivation conditions of mixotrophic, photoautotrophic and heterotrophic with supplement of glucose about 1% and found higher lipid content under mixotrophic conditions, which was about 1.5 times and 13.5 times respectively higher compared to hetrotrophic and photoautotrophic growth. It was shown that the composition of FAMEs in hetrotrophic and mixotrophic cultures comprised three fatty acid methyl esters, namely palmitic acid, linoleic acid and oleic acid. These fatty acids formed about 70-90 % of the fatty acids transformed to FAMEs from these cultivations (Octavio and Yoav, 2015). The same was found in *Chlorella vulgaris* under mixotrophic growth supplemented with glucose as carbon source leading to the higher amount of lipid (Li *et al*., 2011; Liang *et al*., 2009). In 1996, Day and Tsavolos reported that the lipid content in another green alga (*Tetraselmis*) under mixotrophic and phototrophic cultivation was 5.8 times higher than that in hetrotrophic cultivation.

# Enhancement of Intracellular Lipid Production by *Auxenochlorella* and *chlorella* via Flow Cytometric Cell Sorting

## Chapter Seven

## 7.1 Introduction

The increase of greenhouse gas emissions from the combustion of fossil fuel and the limitation of fossil fuel stocks have led researchers to focus on alternative energy derived from biomass (Scurlock *et al*., 1993; Brune *et al*., 2009). Microalgae are a known promising alternative resource for biofuels as many species have high lipid content and can be cultivated at a large scale (Chisti, 2007; 2008). The selection of optimum strains of microalgae and suitable lipid production conditions are a concern for scientists in the biofuel production process (Stuart *et al*., 2010). Therefore, there is a need to identify the microalgae species and cultivation conditions necessary to allow for the development of suitable techniques for quantification and screening lipid in microalgae (Yuchi *et al*., 2011).

Algal lipid content can be measured using various conventional techniques such as extraction, gravimetric determination, nuclear magnetic resonance, dielectrophoresis and flow cytometry. The latter has become a widespread technique for the analysis and characterization of algal lipid yields with single cell resolution. Flow cytometry is a common and inexpensive tool; moreover, it is a reliable protocol applied for measuring algal lipid content along with other parameters like size of the cell and autofluorescence of chlorophyll (Michael *et al*., 2015).

At present, the applications of strategies to overproduce lipid in microalgae, which can be utilized for biofuel, are based on genetic engineering and biochemical engineering approaches (Courchesne *et al*., 2009). The biochemical engineering strategy relies on nutrient starvation or limitation, high salinity or other environmental stresses that channel the flow of the metabolic pathways to favour lipid accumulation in microalgae. The genetic engineering approach depends on overexpressing the rate limiting enzymes to make metabolic pathways more efficient at lipid biosynthesis in microalgae strains (Courchesne *et al*., 2009).

Flow cytometry is cell sorting based on fluorescence, which is considered a reliable technique. It is used in different organisms such as bacteria, fungi and microalgae determining the features and factors such as the size of the cell and the quality of being granular (Velmurugan *et al*., 2013; Hyka *et al*., 2013). In different microalgae, photosynthetic pigment autofluorescence can be screened by flow cytometry; these pigments include carotenoids, chlorophylls and phycobilins (Hyka *et al*., 2013). Using flow cytometry in combination with an appropriate fluorescent dye is a successful method for measuring lipid stored in microalgae that could be used to produce biodiesel (Cooper *et al*., 2010; Yao *et al*., 2012; Doan and Obbard, 2011b; Velmurugan *et al*., 2013).

In this study, flow cytometry was used with the fluorescent dye Nile red to screen lipid under variable conditions such as high salinity and nitrogen starvation. The staining was modified by incubation time, solvent and dye concentration. These treatments successfully allowed Nile red to interact with intracellular lipid bodies in *Auxenochlorella* and *Chlorella*.

## 7.2 Aim and Objectives

The aim of the chapter is to determine the neutral lipid content in *Auxenochlorella* and *Chlorella* using flow cytometry technique in combination with Nile Red.

## 7.3 Results and Discussion

### 7.3.1 Flow cytometry analysis of neutral lipid content.

#### 7.3.1.1 Determination of the influence of DMSO

The universal organic solvent dimethyl sulfoxide (DMSO) was used to help support Nile Red to stain intracellular neutral lipid. Different concentrations of DMSO ranging from (10-60%) were prepared with distilled water to dissolve Nile Red. As shown in **Figure (7.1)**, it was clearly found that in *Auxenochlorella*, 60% DMSO (final concentration) was the optimum concentration to be used as a stain carrier for the determination of lipid content using flow cytometry in combination with Nile Red while in *Chlorella*, the optimum concentration was 30% (**Figure 7.2**). These results agree with the findings of Satpati and Pal, (2015) who contended that 40% DMSO with 15 min incubation was an optimum concentration for gaining high fluorescent intensity of Nile Red using two green microalgae of *Chlorella ellipsoidea* and *Chlorococcum infusionum*.

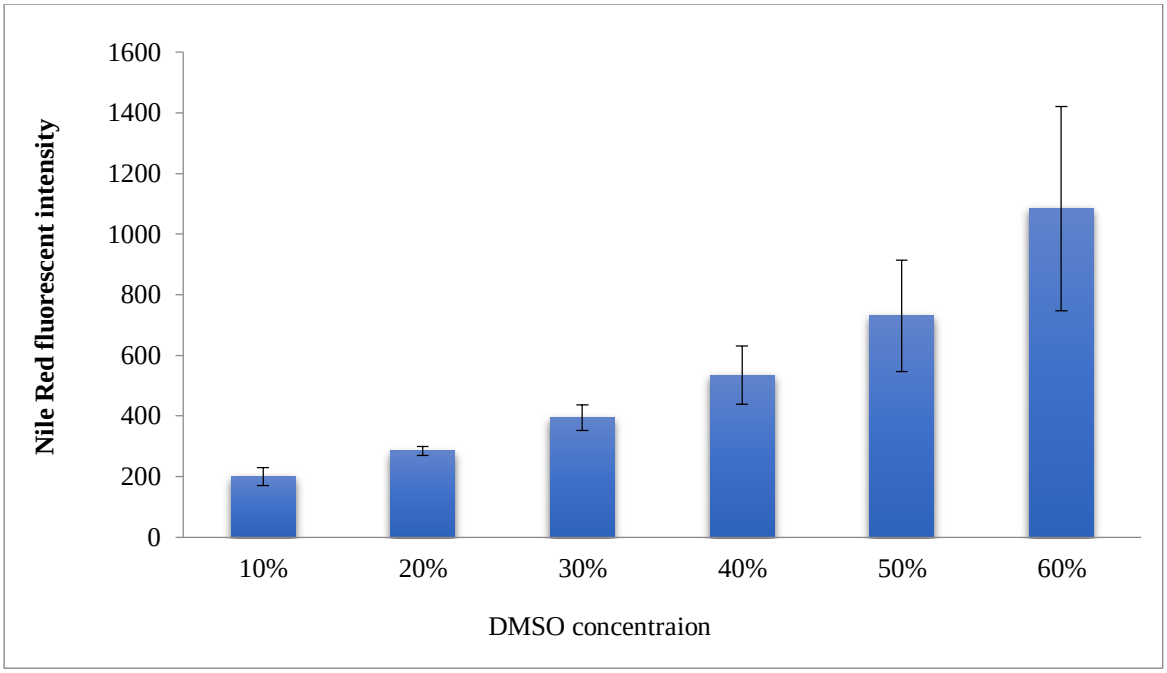



*Figure 7.1: Optimization of DMSO concentration to be used a stain carrier for Auxenochlorella strain. Different concentrations of DMSO ranging from (10-60%) were prepared to find out the best concentration of DMSO to be used in the experiment using flow cytometry. Data points are the means of four replicates plus or minus the standard error.*

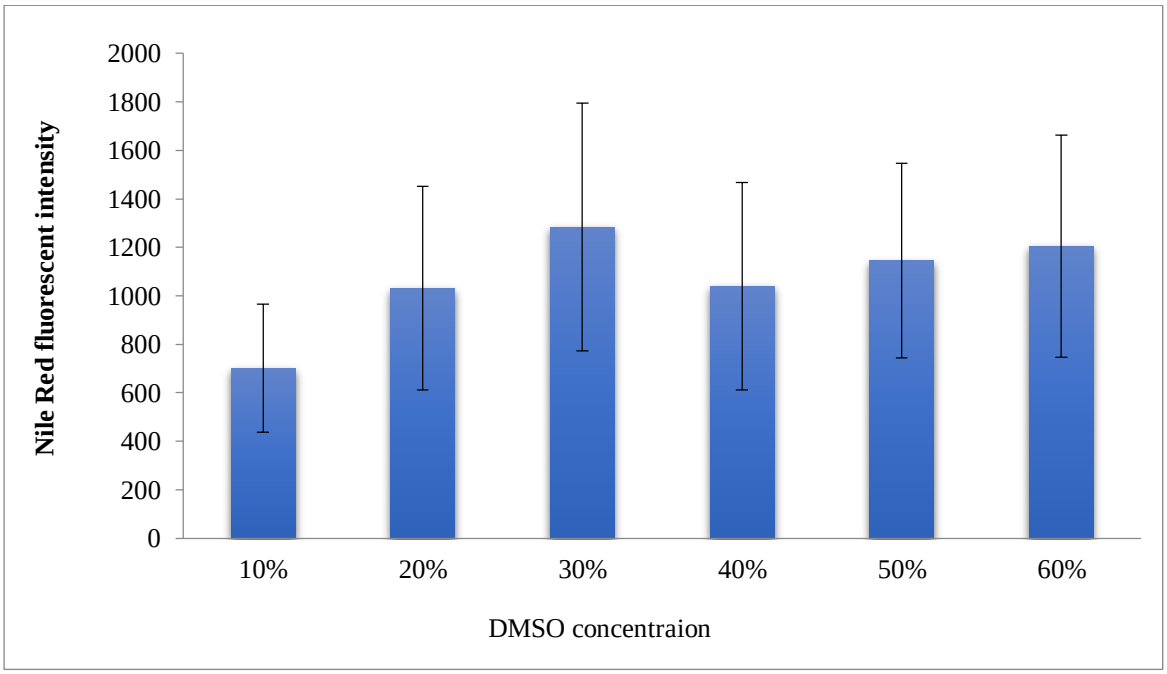



*Figure 7.2: Optimization of DMSO concentration for using as a stain carrier for Chlorella strain. Different concentrations of DMSO ranging from (10-60%) were prepared to find out the best concentration of DMSO to be used in the experiment using flow cytometry. Data points are the means of four replicates plus or minus the standard error.*

### 7.3.1.2 Optimising the concentration of Nile Red

Serial concentrations of Nile Red dissolved in 60% DMSO with *Auxenochlorella* and 30% DMSO for *Chlorella* were prepared to optimise suitable concentrations of the dye to be used in the flow cytometry technique as described in **section 2.13.2**. **Tables 7.1** and **7.2** illustrate the flow cytometry results using flow Jo software; it is evident that the concentration of 0.1 µg $ml^{-1}$ was the optimum concentration of Nile Red in *Auxenochlorella* cells based on the percentage of stained cells under normal conditions. However, the optimum concentration of Nile Red was selected based on the highest fluorescent intensity of *Auxenochlorella* cells (% of stained cells) which was 20% while in *Chlorella* it was 24% with 0.4 µg $ml^{-1}$ optimum Nile Red concentrations. The results arrived at by *Auxenochlorella* and *Chlorella* disagree with those stated by Chen *et al.* (2009) who noted that the optimum Nile Red concentration was 0.5 and 0.8 µg $ml^{-1}$ respectively. In contrast, the result for *Chlorella* is similar to that obtained by Satpati and Pal (2015) who observed that the optimum Nile Red concentration was 0.5 µg $ml^{-1}$ utilized in staining diverse species of microalgae. Due to that fact that each strain of microalgae has a varied response to Nile red stain, the selection of the optimum concentration of Nile red dye will be different because of the rigidity of cell wall effect on the penetration of fluorescent dye into the cells as well as the organic solvent used (Rumin *et al*., 2015).

*Table 7.1: Flow cytometry analysis of Auxenochlorella strain: Cells stained with different concentration of Nile Red using 60% DMSO as best concentration (final concentration) for 10 min staining in comparison with unstained cells as control. Each value is the average of three technical repeats.*

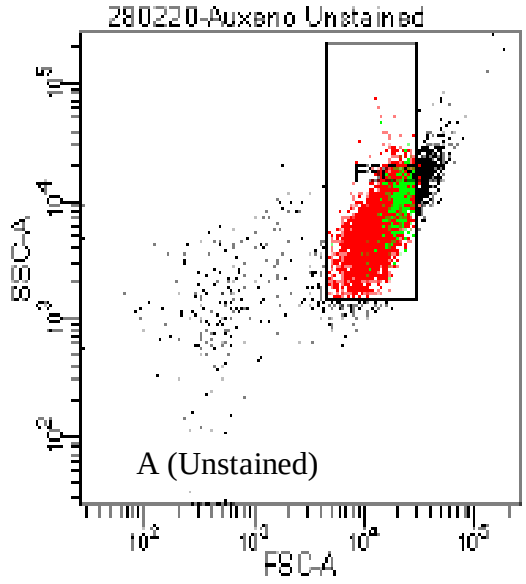
| **Specimen No.** | **Nile Red concentration (µg $ml^{-1}$)** | **Cell population** | **Stained cells population** | **Stained cells (%)** |
|---|---|---|---|---|
| 1 | unstained | 9936 | 391 | 3.9 |
| 2 | 0.05 | 9429 | 1579 | 16.7 |
| 3 | 0.1 | 9505 | 1954 | **20.6** |
| 4 | 0.2 | 9538 | 1523 | 16 |

| | | | | |
|---|---|---|---|---|
| 5 | 0.3 | 9402 | 1629 | 17.3 |
| 6 | 0.4 | 9602 | 1889 | 19.6 |
| 7 | 0.6 | 9736 | 1655 | 17 |

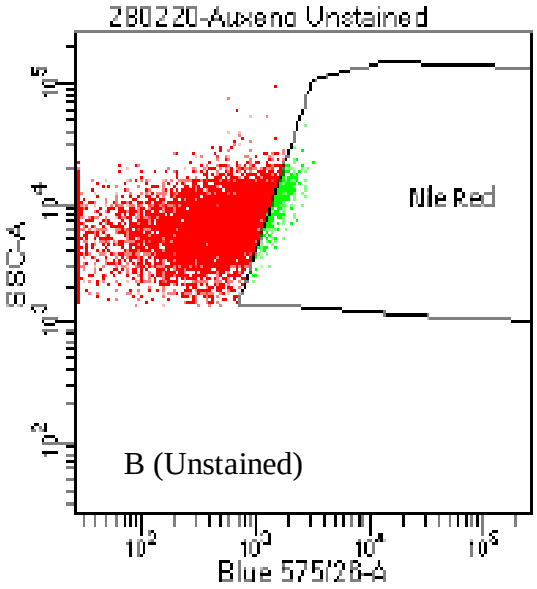
*Table 7.2: Flow cytometry analysis of Chlorella strain; cells stained with different concentration of Nile Red using 30% DMSO as best concentration (final concentration) for 10 min staining in comparison with unstained cells as control. Each value is the average of three technical repeats.*

| **Specimen No.** | **Nile Red concentration (µg ml$^{-1}$)** | **Cell population** | **Stained cells population** | **Stained cells (%)** |
|---|---|---|---|---|
| 1 | Unstained | 10690 | 292 | 2.7 |
| 2 | 0.05 | 10572 | 1980 | 18.7 |
| 3 | 0.1 | 10000 | 2110 | 21.1 |
| 4 | 0.2 | 10000 | 1695 | 17 |
| 5 | 0.3 | 10000 | 1932 | 19.3 |
| 6 | 0.4 | 10000 | 2436 | **24.4** |
| 7 | 0.6 | 10000 | 2431 | 24.3 |

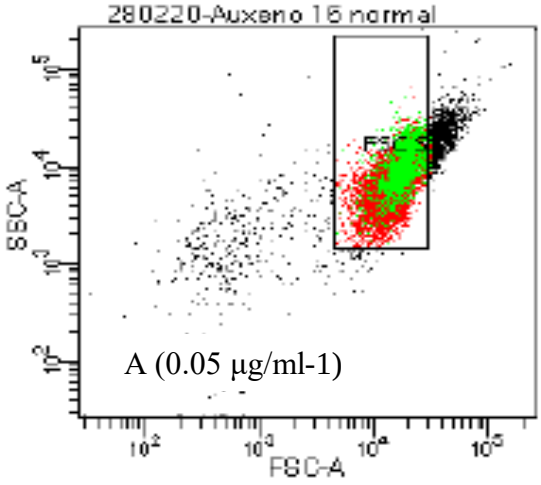
*Figures 7.3 and 7.4 show the FSC vs (A) and SSC (B) area dot plots of scattered unstained and stained Auxenochlorella and Chlorella cells with different concentrations of Nile Red (0.05, 0.1, 0.2, 0.3, 0.4, 0.6 µg ml$^{-1}$). The unstained cells were used to estimate the autofluorescence of the pigments of the microalgae; they therefore, create the gate that distinguishes between the stained and unstained cells. The stained cells showed uniform scatter.*



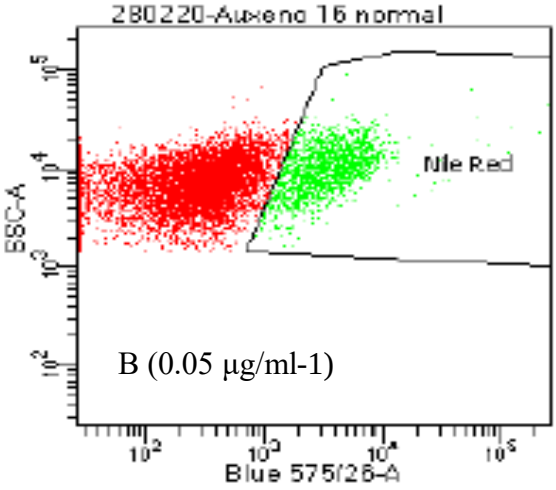

A (Unstained)



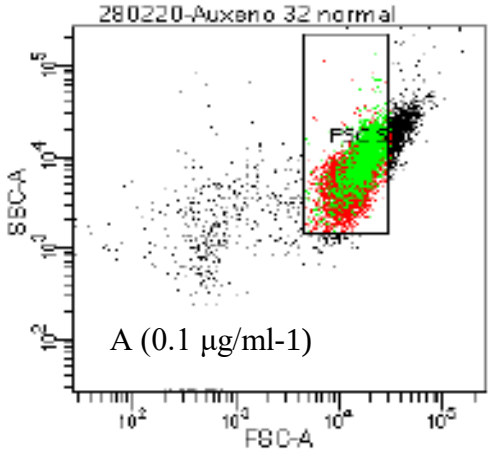

B (Unstained)



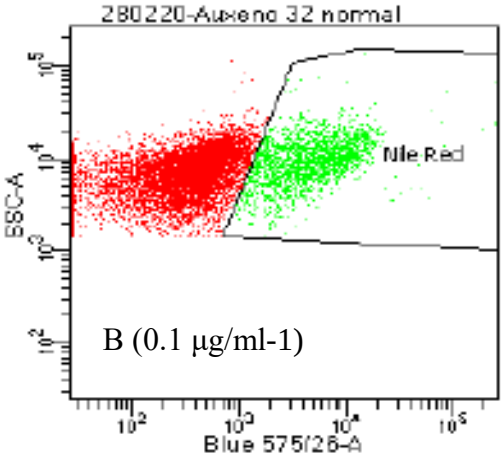

A (0.05 µg/ml-1)



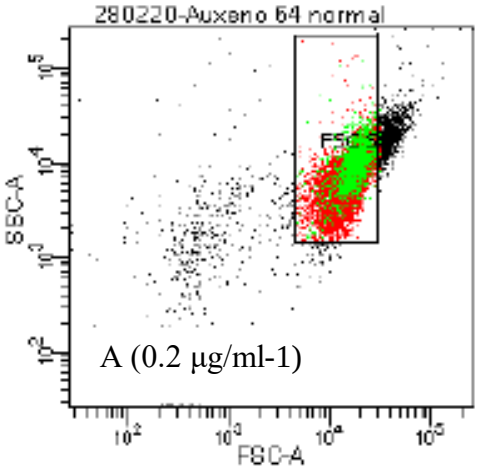

B (0.05 µg/ml-1)



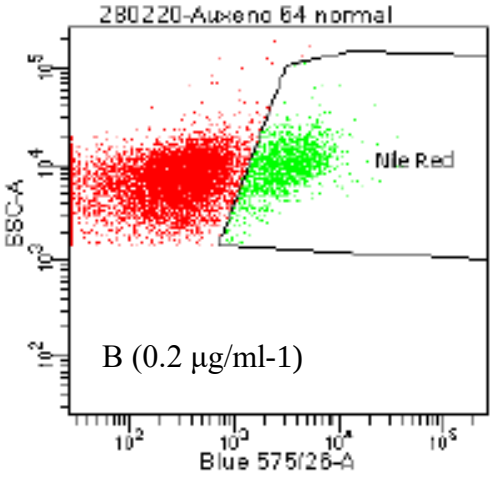

A (0.1 µg/ml-1)



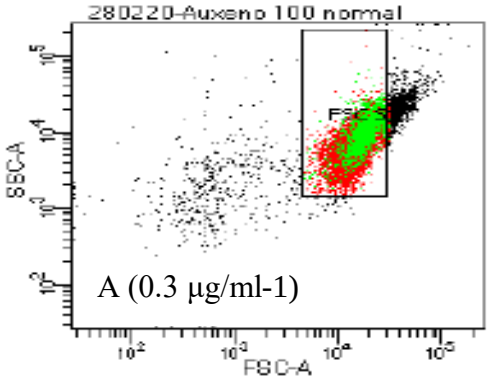

B (0.1 µg/ml-1)



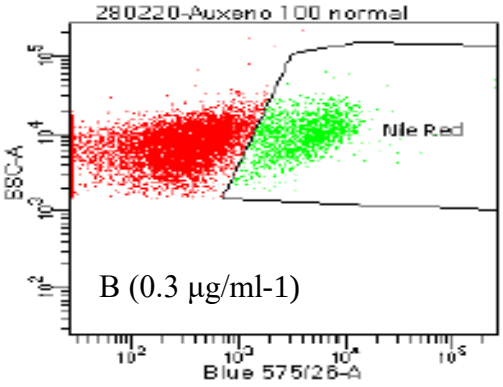

A (0.2 µg/ml-1)



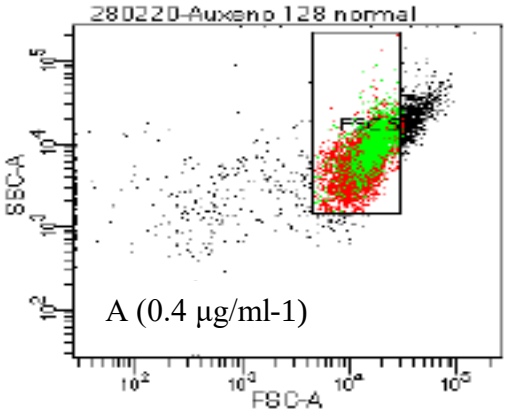

B (0.2 µg/ml-1)



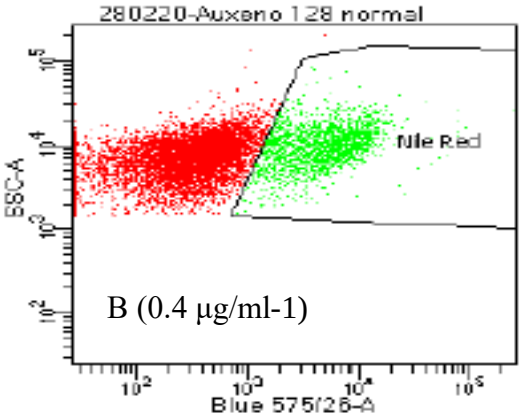

A (0.3 µg/ml-1)




B (0.3 µg/ml-1)




A (0.4 µg/ml-1)




B (0.4 µg/ml-1)

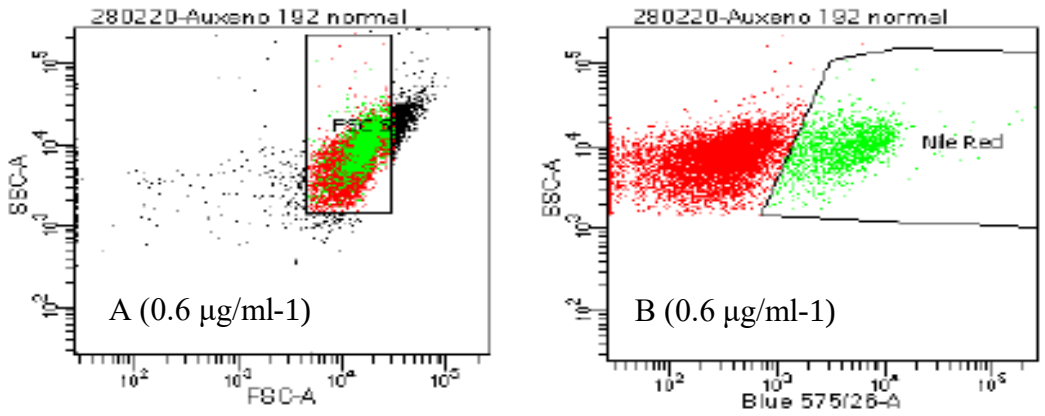
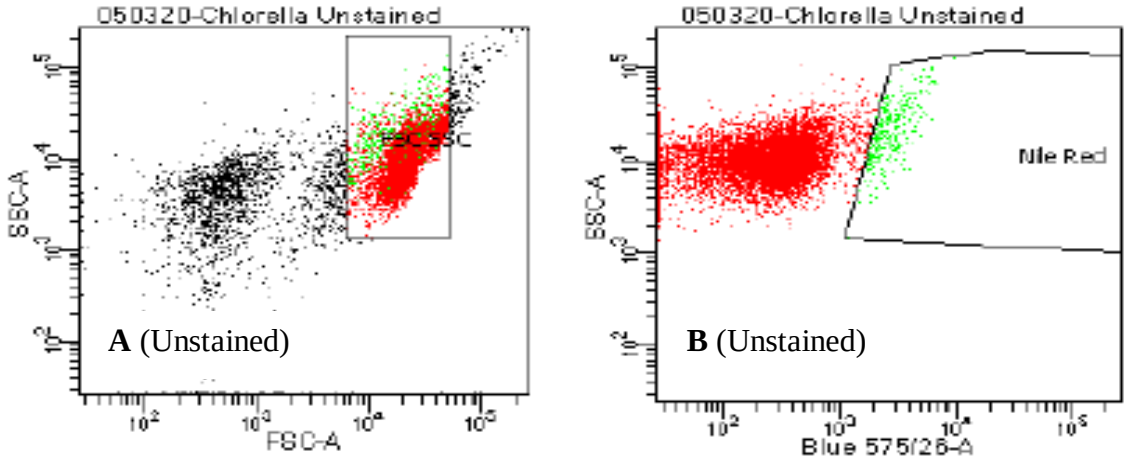
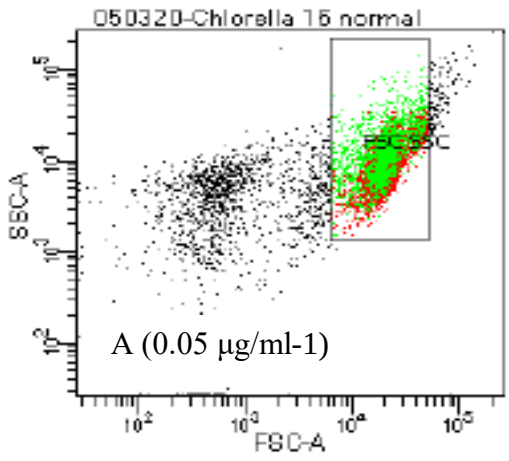
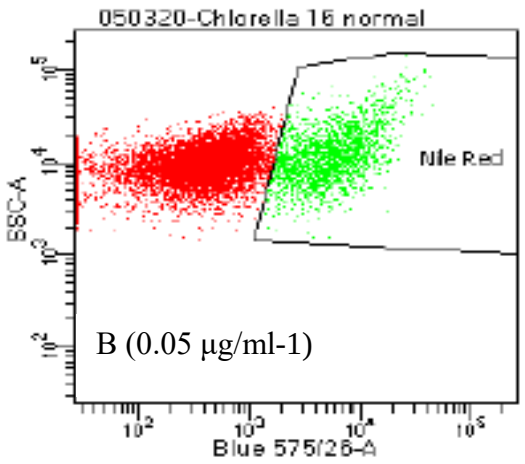
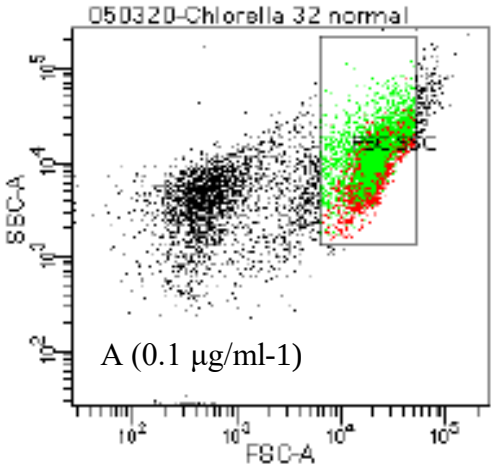


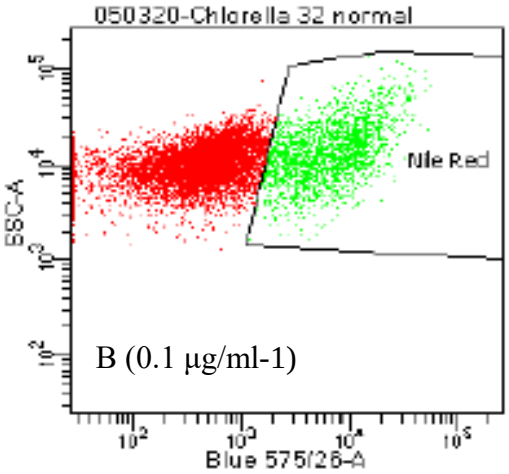

*Figure 7.3: Flow cytometry analysis of Auxenochlorella cells unstained and stained, revealed the FSC vs (A) and SSC (B) area plot of scattered cells with different concentrations of Nile Red (0.05, 0.1, 0.2, 0.3, 0.4, 0.6 µg $ml^{-1}$) with 60% DMSO as the final concentration for 10 min staining to determine the optimum concentration of Nile Red to be used in the experiment using the flow cytometry. Each value is the average of three technical repeats.*

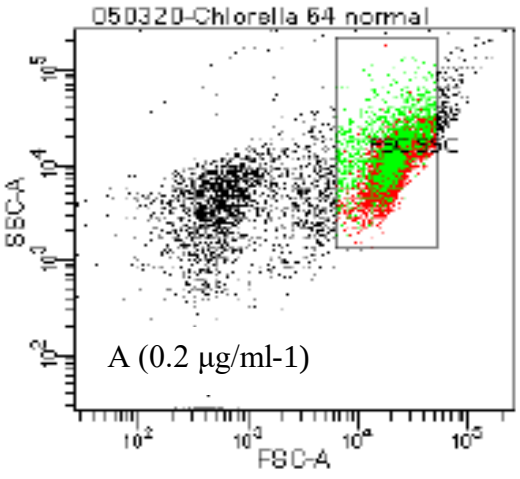


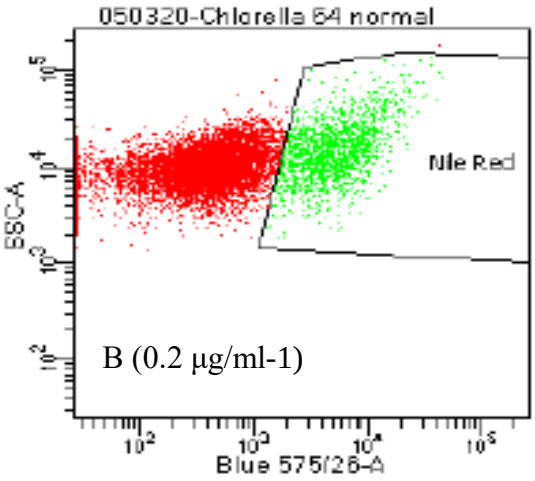

050320-Chlorella 16 normal
SSC-A
FSC-A
A (0.05 µg/ml-1)


050320-Chlorella 16 normal
Nile Red
SSC-A
Blue 575/26-A
B (0.05 µg/ml-1)


050320-Chlorella 32 normal
SSC-A
FSC-A
A (0.1 µg/ml-1)


050320-Chlorella 32 normal
Nile Red
SSC-A
Blue 575/26-A
B (0.1 µg/ml-1)


050320-Chlorella 64 normal
SSC-A
FSC-A
A (0.2 µg/ml-1)


050320-Chlorella 64 normal
Nile Red
SSC-A
Blue 575/26-A
B (0.2 µg/ml-1)

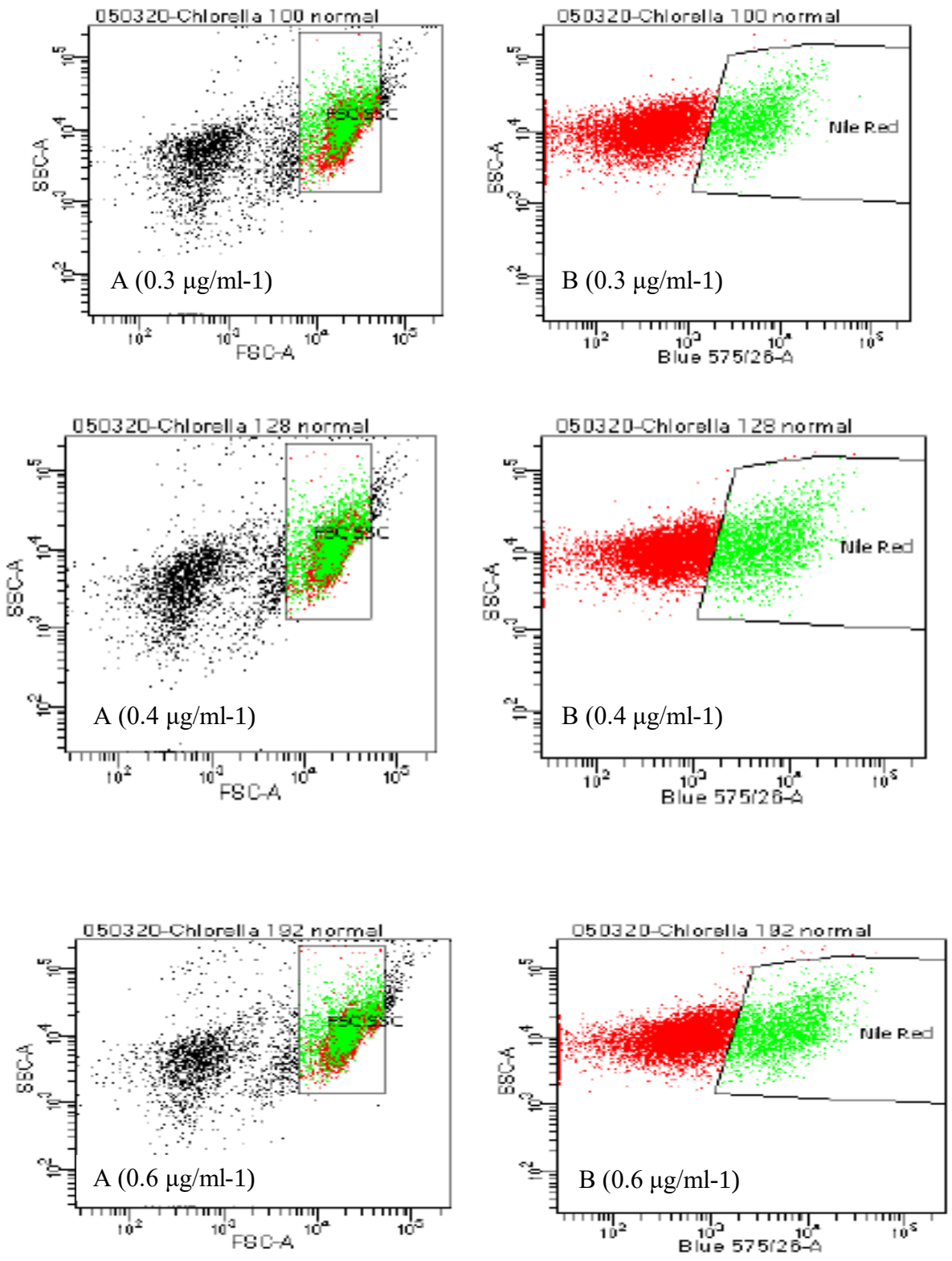



*Figure 7.4: Flow cytometry analysis of Chlorella cells unstained and stained, revealed the FSC vs (A) and SSC (B) area plot of scattered cells with different concentrations of Nile Red (0.05, 0.1, 0.2, 0.3, 0.4, 0.6µg ml$^{-1}$) with 30% DMSO as the final concentration for 10 min staining to determine the optimum concentration of Nile Red to be used in the experiment using the flow cytometry. Each value is the average of three technical repeats.*

### 7.3.1.3 Determination of the neutral lipid in *Auxenochlorella* and *Chlorella* cells grown under stress conditions

The neutral lipid content of *Auxenochlorella* and *Chlorella* was measured under stress conditions i.e. 0.8 M NaCl and nitrogen free in comparison to normal BBM medium. In **Tables 7.3** and **7.4**, it can be seen that the highest percentage of neutral lipid in *Auxenochlorella* (25.3%) was observed with the cells grown in 0.8 M NaCl salinity under 0.3 µg/ml Nile red concentration while in *Chlorella* the highest percentage of lipid under free nitrogen with Nile red concentration 0.05 µg/ml, which was 79.5%. In addition, unstained cells (control) were used to exhibit the level of auto fluorescence of the chlorophyll and other photosynthetic pigment (**Figure 7.5** and **7.6**).

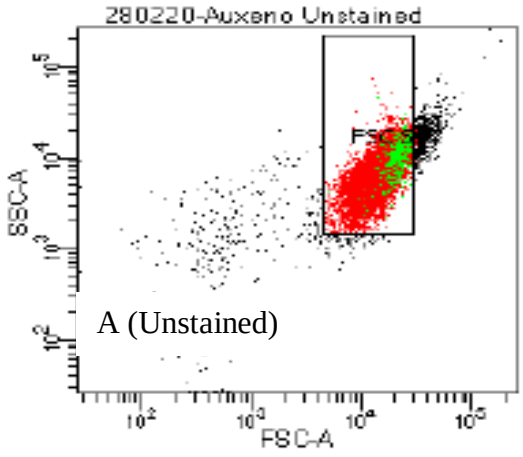
*Table 7.3: Flow cytometry analysis of Auxenochlorella cells grown under different growth conditions including BBM containing 0.8 M NaCl, BBM medium with nitrogen starvation and BBM growth medium with normal BBM growth medium as control, with 60% DMSO and 0.3 µg $ml^{-1}$ of Nile Red as optimum concentration (final concentration). Each value is the average of three replicates for 10 min staining.*

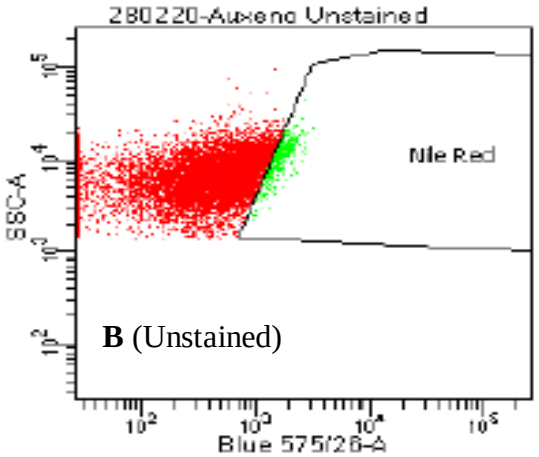
| **Specimen number** | **NileRed concentration (µg/ml-1)** | Stained cells with salinity (%) | Stained cells with free N(%) |
|---|---|---|---|
| 1 | 0.05 | 19.7 | 2.4 |
| 2 | 0.1 | 21.1 | 2.8 |
| 3 | 0.2 | 24.8 | 2.8 |
| 4 | 0.3 | 25.3 | 2.7 |
| 5 | 0.4 | 24.7 | 2.7 |
| 6 | 0.6 | 24.7 | 4.1 |

*Table 7.4: Flow cytometry analysis of Chlorella cells grown under different growth conditions including BBM containing 1M NaCl, BBM medium with nitrogen starvation and BBM growth medium*

*with normal BBM growth medium as control, with 30% DMSO and 0.05 µg/ml-1of Nile Red as optimum concentration (final concentration). Each value is the average of three replicates for 10 min staining.*

| **Specimen number** | **NileRed concentration (µg/ml-1)** | Stained cells with salinity (%) | Stained cells with free N (%) |
|---|---|---|---|
| 1 | 0.05 | 74.2 | 79.5 |
| 2 | 0.1 | 42.1 | 8 |
| 3 | 0.2 | 33.8 | 14.1 |
| 4 | 0.3 | 34.3 | 11.4 |
| 5 | 0.4 | 31.3 | 77.4 |
| 6 | 0.6 | 38.2 | 78.7 |



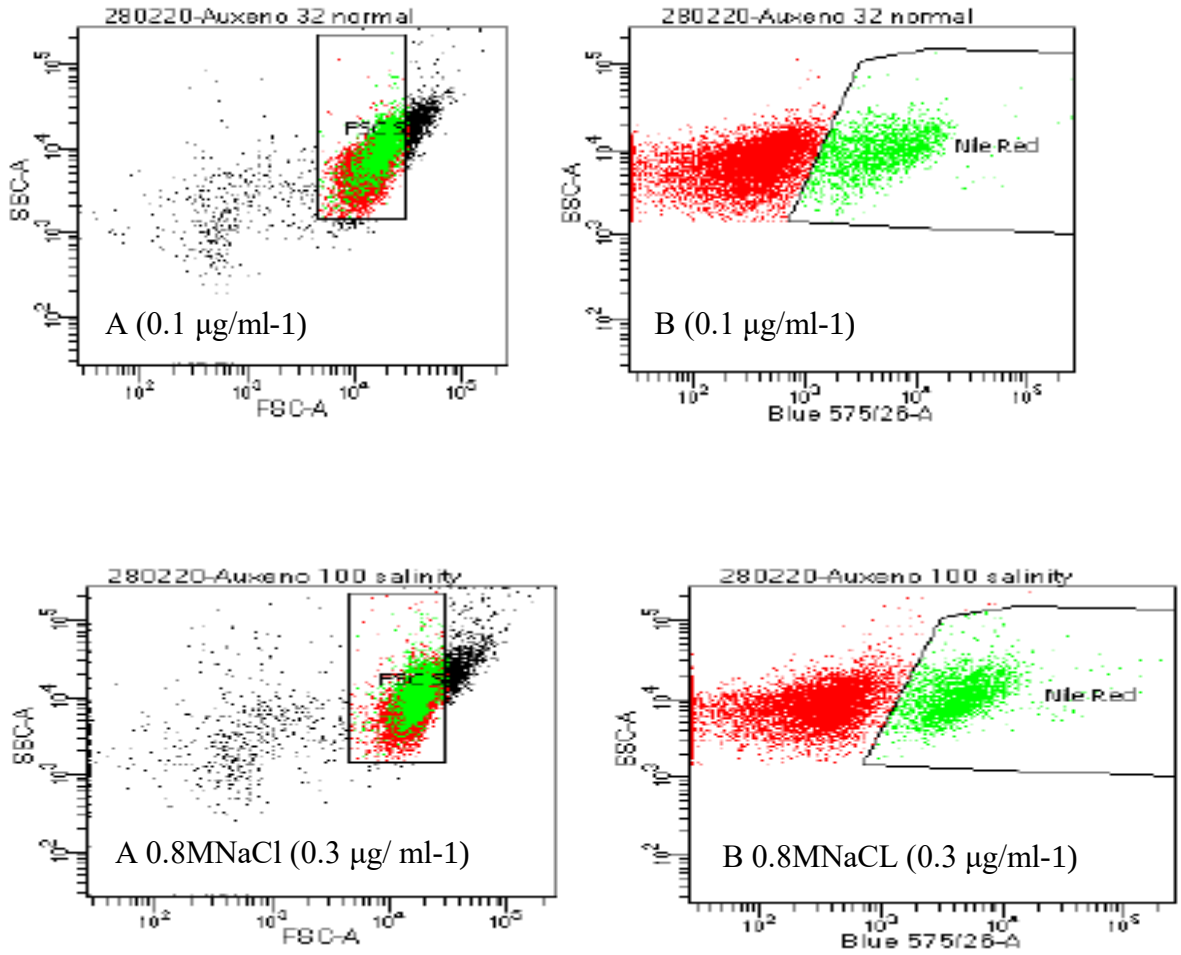
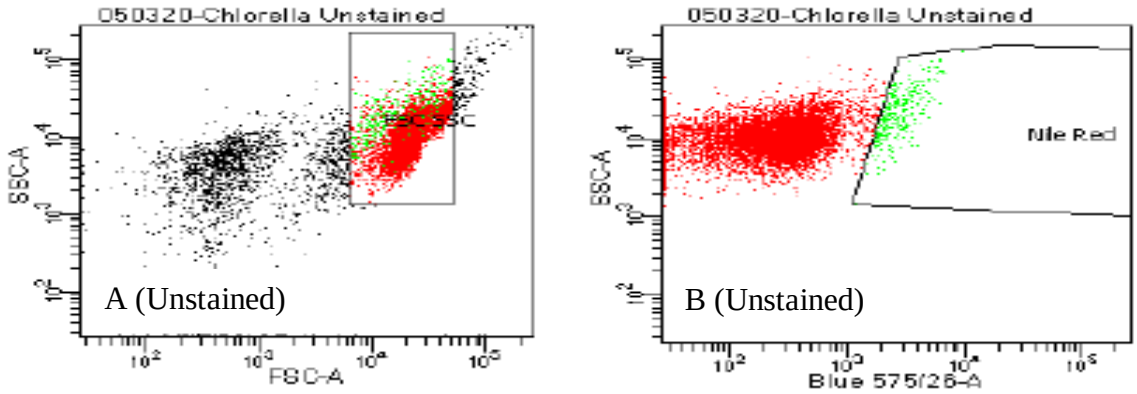



*Figure 7.5: Flow cytometry analysis of neutral lipid content in Auxenochlorella cells grown under different growth conditions including BBM medium supplemented with 0.8 M NaCl, BBM normal growth medium as control, using 60% DMSO and 0.1 µg ml$^{-1}$ of Nile Red as optimum concentration. Cells unstained and stained, revealed the FSC vs (A) and SSC (B) area plot of scattered cells; each value is the average of three replicates.*



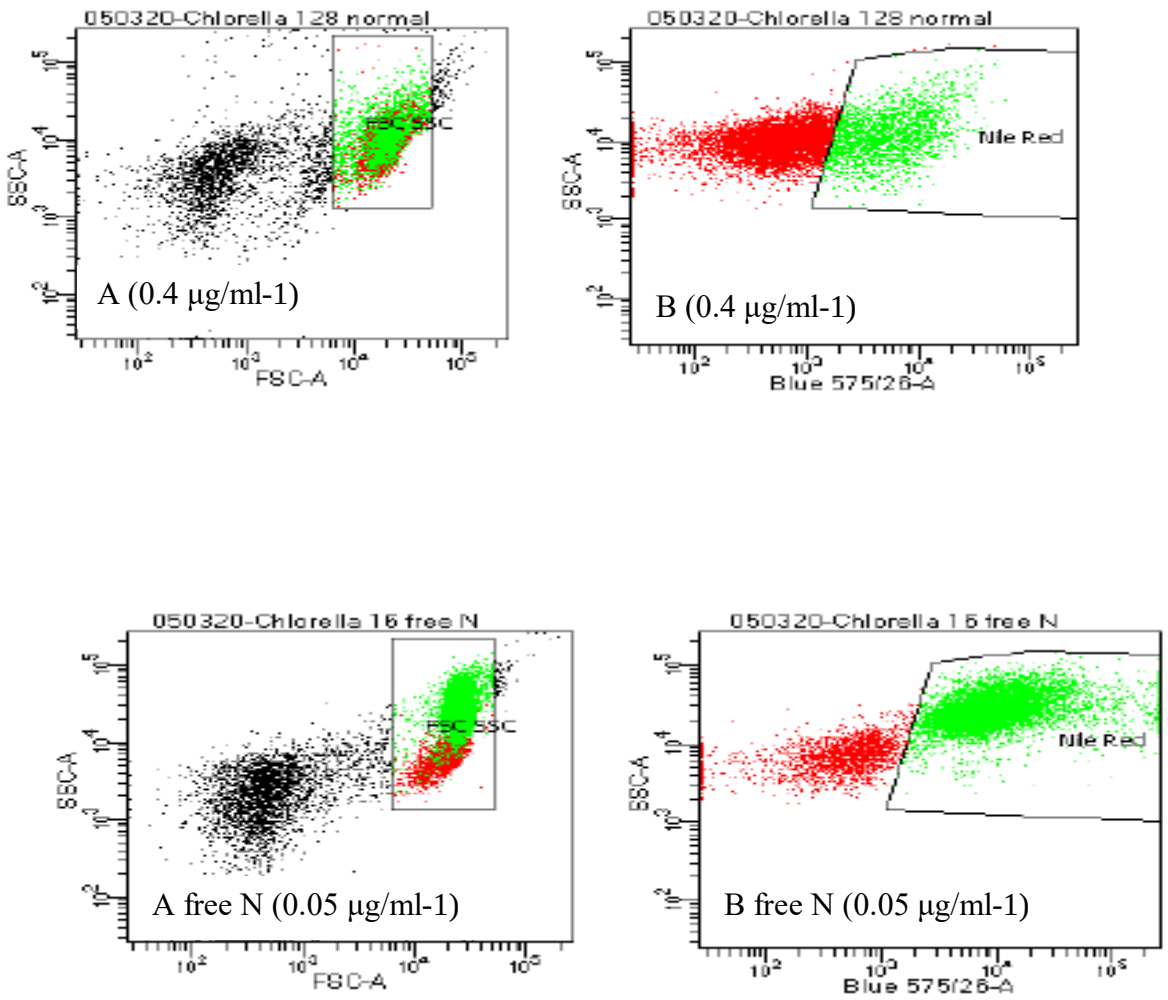



*Figure 7.6: Flow cytometry analysis of neutral lipid content in Chlorella cells grown under different growth conditions including BBM medium supplemented with 0.05 µg/ml free N, BBM normal growth medium as control, using 30% DMSO and 0.4 µg ml$^{-1}$ of Nile Red as optimum concentration. Cells unstained and stained, revealed the FSC vs (A) and SSC (B) area plot of scattered cells each value is the average of three replicates.*

## 7.4 Conclusions

In this chapter, flow cytometry using Nile Red fluorescent dye was optimised to measure neutral lipid in *Auxenochlorella* and *Chlorella*. Nile Red stained cells could be separated from the autofluorescence of chlorophyll and other pigments to allow the effect of salinity stress and nitrogen limitation on neutral lipid production to be measured.

The results are preliminary in nature, but they show that salt stress did induce increased neutral lipid production for both organisms. Their response to nitrogen limitation was different with only *Chlorella* showing an increase in neutral lipid when nitrogen was removed from the medium.

# General Conclusions and Future Work

## Chapter Eight

## 8.1. General Conclusion

Microalgae are believed to be a good source of renewable energy due to their fast growth and their ability to produce neutral lipid; they can be cultivated in waste water and on waste land. What is interesting in the use of these organisms is that they have a higher amount of lipids, nutrients and carbohydrates compared with other feedstocks. In addition, they have a wide range of industrial applications in areas such as cosmetics, health products and biofuel production. The production of biodiesel from microalgae has received attention in recent years due to their high biomass and oil (lipid) yields.

The first step undertaken in this research (as detailed in Chapter Three) involved genomic identification and characterization for the isolation of microalgae strains from Weston Park in Sheffield (UK). Examining the axenic strains of microalgae using conventional microscopic tools was not sufficient to distinguish between strains because of their closely related morphological features. The rDNA sequencing showed that the two main isolated organisms belonged to *Auxenochlorella protothecoides* and *Chlorella vulgaris*. The last step for identification of our locally isolated strains was the phylogenetic tree construction that confirms the precise taxonomy of our strains.

In Chapter Four, the work focused on the investigation of neutral lipid content of the strains with the fluorometric technique of using Nile Red dye which was an efficient tool for the quantification of neutral lipid in microalgae. As part of the process, optimization of algal cell concentration and Nile Red concentration were carried out. DMSO is an organic solvent that was used to help the penetration of Nile Red through the algal cell walls for accurate *in situ* detection of lipid droplets.

Furthermore, the gravimetric technique was used to measure all lipids present, whereas Nile Red measures was only used to measure lipid (triacylglycerol, TAG). The highest percentage of lipid content accumulation over four weeks for *Auxenochlorella* under different concentrations of salinity was 24% at 0.8M NaCl while in *Chlorella* it was 26% at 0.8M NaCl.

On the other hand, the percentage of total lipid under nitrogen starvation increased significantly in both organisms after 4 weeks of incubation. It could be seen that the percentage accumulation of total lipid in *Auxenochlorella* was 44% while in *Chlorella* it was 70%. These results indicated that nitrogen starvation was the optimal stress condition inducing the highest accumulation of neutral lipid in both organisms.

To carry out further investigation, fatty acid methyl esters (FAMEs) profile of *Auxenochlorella* and *Chlorella* neutral lipid was determined by GC-MS. The results demonstrate that FAMEs were found in cells grown under normal conditions. The lipid of *Auxenochlorella* and *Chlorella* grown under normal conditions consisted of FAMEs such as palmitic acid (C16:0), oleic acid (C18:1) and linolenic acid (C18:3). However, the fatty acid profile of lipid produced by nitrogen-stressed cells of *Auxenochlorella* and *Chlorella* showed oleic acid as the major FAME for both organisms.

In this study, NMR spectra were used to identify the compatible solutes produced by the cells to survive extreme conditions under both high salinity and nitrogen stress conditions. It was found that the fatty acids accumulated by cells grown in nitrogen free medium were higher than those for cells grown in normal BBM medium and 0.8 M NaCl BBM medium.

After that, in Chapter Five the investigation focused on determining a suitable medium for *Auxenochlorella* and *Chlorella* using some well-known media (BBM, JM and BG11) and investigating the demand of vitamin B12 for the growth. The results indicated that BBM successfully supported the growth of both organisms. The addition of vitamin B12 for *Chlorella vulgaris,* cultures produced better growth whereas *Auxenochlorella* does not need the addition of vitamin B12 to its media.

Furthermore, by optimising the various growth parameters for better production of biomass such as high salinity and nitrogen starvation, it was found that the cell growth was favoured at a minimum concentration of NaCl (0.2M NaCl) in both organisms. It could be seen that nitrogen limitation leads to a significant decline in the growth rate of the cells, when compared with normal conditions.

Additionally, in Chapter Six the growth and lipid content of the *Chlorella vulgaris* strain was studied during 29 days of mixotrophic cultivation that was carried out in BBM media enriched with different concentrations of glucose as carbon source (0.1, 0.5, 1 and 2%).

Finally, in Chapter Seven, the flow cytometry technique was used to determine the cellular lipid accumulated by *Auxenochlorella* and *Chlorella* cells grown in BBM media supplemented with 0.8 M NaCl and BBM media without nitrogen. The stress media were compared with normal BBM growth media as a control using 60% DMSO with *Auxenochlorella* and 30% DMSO in *Chlorella.* The Nile Red concentration was 0.1 µg $ml^{-1}$ for *Auxenochlorella* and 0.4 µg $ml^{-1}$ for *Chlorella* with 10 min staining. It was found that the maximum percentage of accumulated cellular lipid was found in *Auxenochlorella* cells grown

under salinity stress whereas for *Chlorella*, the highest values were found under nitrogen starvation.

### 8.2. Future Work

Based on the results of this study, the following future research studies are recommended:

1- Fatty acid profile of *Chlorella vulgaris* cells grown in glucose BBM medium under mixotrophic culture.
2- Examining in detail the effect of environmental stress on photosynthesis using Pulse Amplitude Modulated (PAM) fluorometry technique.
3- Quantification of neutral lipid by a direct transesterification method.
4- Measurement of respiration rate and photosynthesis in *Auxenochlorella and Chlorella* under different stresses using oxygen electrode.
5- In flow cytometry, inducing random mutations in *Auxenochlorella* and *Chlorella* using UV-C light as a mutagen and fluorescent activated cell sorting (FACS) technique for screening *Auxenochlorella* and *Chlorella* mutated cells and sorting the mutated cells for high neutral lipid production.

# References and Appendices

## Chapter Nine

## 9.1. Reference List

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## 9.2. Appendices

## Appendix (A)

```
ALIGNMENTS
>AY553213.1 Auxenochlorella protothecoides 16S small subunit ribosomal RNA
gene, partial sequence; chloroplast
Length=1475

 Score = 2019 bits (1093),  Expect = 0.0
 Identities = 1122/1135 (99%), Gaps = 6/1135 (1%)
 Strand=Plus/Minus

Query  6     TGG-GGAATGCTT-AC-C-TGC-AGTCGTACGCATGCAATTTGGCTTGCCAGATTGCGAT  60
             ||| || ||||||| || | ||| ||||||||||||||||||||||||||||||||||||
Sbjct  1444  TGGCGGCATGCTTAACACATGCAAGTCGTACGCATGCAATTTGGCTTGCCAGATTGCGAT  1385

Query  61    GAGTGGCGGACGGGTGAGTAACACGTAAGAACCTACCTTTTGGAGAGGGATAACCATTGG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1384  GAGTGGCGGACGGGTGAGTAACACGTAAGAACCTACCTTTTGGAGAGGGATAACCATTGG  1325

Query  121   AAACGATGGCTAATACCTCGTATTGCTGAGAAGTGAAAGATGAAAATCGCCAATAGATGG  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  241   GGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1204  GGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG  1145

Query  301   CAGCAGTGAGGAATTTTCCGCAATGGGCGACAGCCTGACGGAGCAATGCCGCGTGAAGGA  360
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             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  601   AATTCCCGGTGGAGCGGTGAAATGCGTAGAGATCGGGAGGAACACCAAAGGCGAAAGCAC  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  784   TCTGCTGGGCCATAACTGACACTGAGAGACGAAAGCGAGGGGAGCAAAAGGGATTAGATA  725

Query  721   CCCCTGTAGTCCTCGCCGTAGACGATGGATACTAGATGTTGGGTAGGTTAAATCACTCAG  780
             |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  724   CCCCTGTAGTCCTCGCCGTAAACGATGGATACTAGATGTTGGGTAGGTTAAATCACTCAG  665

Query  781   TATCGTAGCTAACGCGTGAAGTATCCCGCCTGGGGAGTATGCTCGCAAGAGTGAAACTCA  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  664   TATCGTAGCTAACGCGTGAAGTATCCCGCCTGGGGAGTATGCTCGCAAGAGTGAAACTCA  605

Query  841   AAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCG  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  604   AAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCG  545

Query  901   AAGAACCTTACCAGGACTTGACATGCCACTTTTTCCCTGAAAGGGGAAGTTCCAGAGTGG  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  544   AAGAACCTTACCAGGACTTGACATGCCACTTTTTCCCTGAAAGGGGAAGTTCCAGAGTGG  485

Query  961   ACACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCTTGAGATGTTGGGTTAAGTCCCGCA  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  484   ACACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCTTGAGATGTTGGGTTAAGTCCCGCA  425

Query  1021  ACGAGCGCAACCCTTGTTTTGAATTGCCAGTAATGGGAAATTCAAAAAACTGCCGGTGAC  1080
             |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  424   ACGAGCGCAACCCTTGTTTTGAATTGCCAGTAATGGGAAATTCAAAAGACTGCCGGTGAC  365

Query  1081  AAAGCCGAAGGAAGGTGAGGATGACTTCAAGTCAGCCTGCCCCTTAAGTCCTGGG  1135
             || |||| |||||||||||||||| |||||||||| ||||||||| ||||||||
Sbjct  364   AA-GCCGGAGGAAGGTGAGGATGACGTCAAGTCAGCATGCCCCTTACGTCCTGGG  311
```

## Appendix (B)

```
ALIGNMENTS
>MF686452.1 Chlorella vulgaris isolate 18s rRNA small subunit ribosomal RNA
gene, partial sequence
Length=1795

 Score = 937 bits (507),  Expect = 0.0
 Identities = 507/507 (100%), Gaps = 0/507 (0%)
 Strand=Plus/Minus

Query  16    AGGTGCCGGCGGAGTCATCGAAGAAACATCCGCCGATCCCTAGTCGGCATCGTTTATGGT  75
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1079  AGGTGCCGGCGGAGTCATCGAAGAAACATCCGCCGATCCCTAGTCGGCATCGTTTATGGT  1020

Query  76    TGAGACTAGGACGGTATCTAATCGTCTTCGAGCCCCCAACTTTCGTTCTTGATTAATGAA  135
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1019  TGAGACTAGGACGGTATCTAATCGTCTTCGAGCCCCCAACTTTCGTTCTTGATTAATGAA  960

Query  136   AACATCCTTGGCAAATGCTTTCGCAGTAGTTCGTCTTTCATAAATCCAAGAATTTCACCT  195
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  959   AACATCCTTGGCAAATGCTTTCGCAGTAGTTCGTCTTTCATAAATCCAAGAATTTCACCT  900

Query  196   CTGACAATGAAATACGAATGCCCCCGACTGTCCCTCTTAATCATTACTCCGGTCCTACAG  255
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  899   CTGACAATGAAATACGAATGCCCCCGACTGTCCCTCTTAATCATTACTCCGGTCCTACAG  840

Query  256   ACCAACAGGATAGGCCAGAGTCCTATCGTGTTATTCCATGCTAATGTATTCAGAGCGTAG  315
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  839   ACCAACAGGATAGGCCAGAGTCCTATCGTGTTATTCCATGCTAATGTATTCAGAGCGTAG  780

Query  316   GCCTGCTTTGAACACTCTAATTTACTCAAAGTAACAGCGCCGACTCCGAGTCCCGGACAG  375
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  779   GCCTGCTTTGAACACTCTAATTTACTCAAAGTAACAGCGCCGACTCCGAGTCCCGGACAG  720

Query  376   TGAAGCCCAGGAGCCCGTCCCCGGCAACAAGGTGAGCCCTGCCAGTGCACACCGAAACGG  435
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  719   TGAAGCCCAGGAGCCCGTCCCCGGCAACAAGGTGAGCCCTGCCAGTGCACACCGAAACGG  660

Query  436   CGGACCGGCAGGTCCCACCCGAAATCCAACTACGAGCTTTTTAACTGCAGCAACTTAAAT  495
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  659   CGGACCGGCAGGTCCCACCCGAAATCCAACTACGAGCTTTTTAACTGCAGCAACTTAAAT  600

Query  496   ATACGCTATTGGAGCTGGAATTACCGC  522
             |||||||||||||||||||||||||||
Sbjct  599   ATACGCTATTGGAGCTGGAATTACCGC  573
```