PRODUCTION OF OIL FROM FRESHWATER AND MARINE WATER MICROALGAE FOR BIODIESEL PRODUCTION

by

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DEDICATION

TO:

My Parents:

Tayser and Manal Al hattab

My Brothers:

Mohammed and Ahmed Al hattab

My Friends:

Halah and Diana Shahin, Rasha Aludhaib, Niki and Tasha Moideen, Rabiya and Rabeyl Aslam, Amena and Hannan Fayad, Deepika Dave, Ian MacDonald, Vegneshwaran Ramakrishnan and Rishi Ananthashankar

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ABSTRACT

The growth and oil content of *Chlorella saccharophila* (freshwater) and *Tetraselmis suecica* (marine) microalgae were investigated using various nutrient types (ammonium nitrate, ammonium phosphate, ammonium sulfate and combination of nutrients), light durations (9 h, 16 h and 24 h) and carbon sources ($NaHCO_3$ and CO_2). The results indicated that *T. suecica* produced higher cell yields compared to the *C. saccharophila* under all parameters tested. Light exposure of 24 h illustrated the highest biomass yields. The combination of nutrients resulted in the highest growth for both species of microalgae. However, high growth did not necessarily result in high lipid yields, which need to be considered when oil is used as feedstock for biodiesel production.

Oil yield using $NaHCO_3$ were much higher for *C. saccharophila* as opposed to *T. suecica,* but biomass yields were much higher for *T. suecica* as opposed to *C. saccharophila.* Varying light duration had no direct effect on oil yields. The nutrient type significantly influenced the production of lipids. *C. saccharophila* produced the highest lipid yield using ammonium phosphate, but resulted in lower biomass yield. The marine microalgae achieved the highest oil yields using ammonium nitrate system, but the cell yield was the lowest. Consequently, the high lipid yield does not offset the low biomass achieved for the freshwater microalgae which makes these conditions unsuitable for biodiesel production. However, the combination of nutrients at the 24 h light exposure resulted in the highest biomass yields for *C. saccharophila* which resulted in the highest total lipid yield. Despite the low biomass yield obtained using ammonium nitrate at the 24 h light exposure for *T. suecica*, it achieved the highest lipid yield and total lipid yield, which make up for the shortage in biomass yield. Thus, these conditions are more suitable for biodiesel production since the microalgae are producing high lipids as opposed to biomass.

When CO_2 was used as a carbon source at varying CO_2:air percentage ratios (3:97, 6:94 and 9:91), at the best growth and oil promoting conditions achieved using $NaHCO_3$, the biomass yields achieved was higher than those obtained using $NaHCO_3$ for both microalgae species. However, the lipid yields obtained using the freshwater microalgae were significantly lower when CO_2 was used. The marine microalgae showed a slight decrease in lipid yield when using CO_2. Statistical analysis revealed that varying the concentration of CO_2 did not affect the biomass or lipid yield using both microalgae species.

Thus, growing *C. saccharophila* using the combination of nutrients with 24 h light exposure under $NaHCO_3$ and *T. suecica* using ammonium nitrate at 24 h light exposure under $NaHCO_3$ were the optimal growth conditions found for use as biodiesel production feedstock.

LIST OF ABBREVIATIONS AND SYMBOLS USED

AN	Ammonium nitrate
ANOVA	Analysis of variance
AP	Ammonium phosphate
AS	Ammonium sulfate
ATP	Adenosine Triphosphate
C	Carbon Source
CS	*Chlorella saccharophila*
CH_2Cl_2	Dichloromethane
CO_2	Carbon Dioxide
CO_3^{2-}	Carbonate
Comb.	Combination of nutrients
CV	Coefficient of Variation
DCW	Dry Cell Weight
DF	Degrees of Freedom
DHA	Docosahexaenoic Acid
EPA	Eicosapentaenoic Acid
F	F-test, using F-distribution
HCO_3^-	Bicarbonate
L	Light
LED	Light Emitting Diodes
MS	Mean of Squares
N	Nutrient Type
NaCl	Sodium Chloride
NADPH	Nicotinamide Adenine Dinucleotide Phosphate-Oxidase
$NaHCO_3$	Sodium Bicarbonate
NOx	Mono-nitrogen oxide
P	P-value
PBR	Photobioreacters
PUFA	Polyunsaturated Fatty Acids
RNA	Ribonucleic Acid
S	Species
SFE	Supercritical Fluid Extraction
SOx	Sulfur Oxide
SS	Sum of Squares
TAG	Triacylglycerol
TS	*Tetraselmis suecica*

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CHAPTER 1. INTRODUCTION

The increase in the annual global energy consumption over the past century has relied heavily on fossil fuels (oil, coal and natural gas) for powering up cars, farms, factories and for production of electricity (Areva, 2011). The world consumption of crude oil, coal and natural gas in 2011 was 87.4 million barrels/day, 8 144 million short tons/day (approximately 4.64 billion barrels of oil/day) and 3 368 billion m^3 (2 118 barrels of oil/day), respectively (Index Mundi, 2011). However, most of the electricity globally is generated from coal burning (Canadian Center for Energy Information, 2010).

Fossil fuel burning has accelerated carbon dioxide (CO_2) emissions on a global scale from 1.1% per year in 1990 to more than 2.6% per year in 2010 (Adams, 2013). Humans contribute 63% of the greenhouse gas, CO_2 that is emitted into the atmosphere (Mendelsohn et al., 1994; Hofmann et al., 2006). Global warming would increase the atmospheric temperature and impact all living organisms (Root et al. 2003). An increase in the Earth's temperature has been attributed to a decline in the Adelilie penguins species, melting of glaciers, increased precipitation and increased sea level, resulting in floods (Forcada et al. 2006). In Canada, global warming effects are already felt across the nation. Forest fires, floods, insect infestations and drought have all been attributed to global warming (Epstein, 2000).

The environmental concerns associated with greenhouse gas emissions emphasise the need for alternative energy sources that are more environmentally friendly. Various types of biomass can be used as renewable energy sources that offer immediate prospects of producing liquid fuels such as biodiesel and bioethanol which can be used as substitutes for petroleum products (Singh and Gu, 2010). Biofuel that is produced from biomass is a renewable energy source, which can be used as a substitute for petroleum fuels. Using biofuels, as substitutes for traditionally used fuels, offers the benefits of greater energy security, foreign exchange savings and reduced environmental effects (Balat, 2009; Kan, 2009; Yenikaya et al. 2009). Biomass feedstocks for energy production include food waste, municipal waste, agricultural waste, edible and nonedible oilseeds, aquatic plants and algae. Oilseeds are currently the best crops for biofuel production, but are considered

a food source for millions of people around the world (Demirbas, 2005; Singh and Gu, 2010).

Microalgae, which are abundant in nature, can be used as an alternate fuel source (Chisti, 2007; Hu et al., 2008). Using microalgae as an alternative feedstock is ideal because of their high growth rate and their ability to produce lipids that can be used for the production of biodiesel (Song et al., 2008). The majority of lipids produced by microalgae have a low degree of unsaturation, making them a good energy source for replacement of the current fossil fuels (Singh and Gu, 2010). Microalgae are photosynthetic microorganisms capable of surviving in marine and fresh water environments. They tend to have a much higher oil yield than vegetable plants and can produce and store large amounts of oil without the production and release of harmful wastes into the environment (Demirbas, 2011; Wahlen et al., 2011). They are extremely resilient and often unaffected by fluctuations in the environment (Demirbas, 2010; Singh et al., 2010). These microorganisms are beneficial to the environment as well because they utilize the carbon dioxide for their growth, and thus help reducing greenhouse gas emissions (Pokoo-Akins et al. 2010; Demirbas, 2011).

Biodiesel, as a liquid fuel, can be produced by the transesterification of oil extracted from microalgae (Leung et al., 2010; Demirbas and Demirbas, 2011; Wahlen et al., 2011). Microalgae generate oil in the form of triacylglycerols which can be converted into biodiesel by the addition of methanol and the use of a catalyst such as acid, alkali or enzyme (Demirbas, 2005; Chen et al., 2009). The waste generated from the microalgal biomass can be further utilized to produce other biofuels such as methane and ethanol via fermentation or used as animal feed or organic fertilizer (Chen et al., 2009; Demirbas, 2010; Demirbas, 2011). Various microalgae strains contain high percentages by weight of oils, capable of yielding biodiesel fuels while other species possess high sugar content which is suitable for production of bioethanol (Demirbas, 2010). Microalgae appear to offer a viable alternative for biofuel production due to their abundance and cellular structure as well as other high value bioproducts such as protein supplement in animal feed, organic fertilizers, cosmetics and pharmaceutics (Banerjee et al., 2009; Chen et al., 2009; Demirbas, 2010; Demirbas, 2011).

Biodiesel from microalgae generates the same amount of energy (calorific value does not differ significantly) as that generated from petroleum diesel without the release of harsh compounds (NOx, SOx and hydrocarbons) into the atmosphere, it is biodegradable and nontoxic and a much cleaner energy source (Ulusoy et al. 2004; Demirbas, 2005; Kalam and Masjuki, 2005). The biggest advantage of biodiesel, compared with other alternative transportation fuels, is that it can be utilized in existing diesel engines without any modifications (Singh and Gu, 2010). The overall aim of this study was to investigate the possibility of increasing the microalgae growth rate and oil yield of freshwater (*Chlorella saccharophila)* and marine (*Tetraselmis suecica)* microalgae species.

CHAPTER 2. OBJECTIVES

The aim of this study was to investigate the possibility of increasing the microalgae cell growth rate and oil yield by manipulating the environmental parameters (light duration, nutrients, temperature and pH) in a specially designed pilot scale hydroponic system. The specific objectives were:

1. To screen freshwater and marine microalgae and select two strains of microalgae, one freshwater and one marine microalgae for biodiesel production.
2. To evaluate the effect of the following environmental parameters on the microalga biomass yield and oil content of these species in order to determine their potential as a renewable feedstock for production of biodiesel while using $NaHCO_3$ as a carbon source:
    (a) Light duration at three levels (9,16 and 24 h)
    (b) Nutrients type at four levels (ammonium nitrate, ammonium phosphate, ammonium sulfate and a mix of all three) while maintaining the nitrogen concentration at 70 mg/L
3. To study the effect of CO_2 concentration in the air on biomass yield and oil content under optimum light and nutrient conditions.

CHAPTER 3. LITERATURE REVIEW

3.1. Types of algae

Algae are photosynthetic oxygen-producing organisms. They are referred to as thallophytes because they lack roots, stems, leaves and vascular tissues, making them different from other plants (Barsanti and Gualtieri, 2006). Their size can range from 0.2 μm to 60 m in length. They can be divided into macroalgae and microalgae. Some algae are prokaryotic while others are eukaryotic (Barsanti and Gualtieri, 2006; Carlsson et al., 2007; Demirbas, 2010). Both macroalgae and microalgae are found in marine and freshwater environments, forming the base food chains of these environments (Barsanti and Gualtieri, 2006; Brodie and Lewis, 2007). Algae can either be subaerial or aquatic, but the main bulk of algae are microscopic aquatic organisms, that are visible to the eye when colonies are formed (Diaz-Pulido and McCook, 2008).

3.1.1. Macroalgae

Macroalgae are multicellular organisms that are referred to as "seaweeds". They are fast growing plants and can reach up to 60 m in length, growing in salt or fresh water environments (Carlsson et al., 2007). They lack roots, stems and leaves and are instead composed of thallus with a stem and foot (Carlsson et al., 2007). They are divided based on pigmentation into three main groups: brown seaweed (*Phaeophyceae*), red seaweed (*Rhodophyceae*) and green seaweed (*Chlorophyceae*) which are mainly used for food production and hydrocolloids (Carlsson et al., 2007; Demirbas, 2010). In order to provide buoyancy, some of these species have gas filled structures (Barsanti and Gualtieri, 2006; Carlsson et al., 2007). Macroalgae are found to produce both carbohydrates and lipids. The carbohydrates are used as the main energy storage compound and the small amount of lipids produced are used to make up the cell membrane structure (Sheehan et al., 1998).

3.1.1.1. Phaeophyta: Phaeophyta (brown algae) are almost exclusively of marine occurrence. These organisms are found in temperate to subpolar regions and dominate in sublittoral and littoral zones. Brown algae range in size from microscopic to 60 m or greater in length. The cell walls consist of two layers: the inner cellulosic and the slimy

or gummy outer layer. These organisms possess chloroplasts that may be present as a single, a few or many per cell. The number of chloroplasts present is dependent on the taxonomic criterion (Bold and Wynne, 1978).

3.1.1.2. Rhodophyta: Rhodophyta (red algae) are one of the oldest groups of the eukaryotic algae. These organisms possess chlorophyll a (phycobiliproteins), starch as a storage product and singly occurring thylakoids in the chloroplast, but lack flagellated cells. The marine red algae are the predominate species in tropical and temperate regions. These species are much larger in size in cool-temperate areas than those in tropical seas. They are capable of surviving in sea depths down to 200 m. The red algae species found in freshwater are incapable of reaching the large sizes those achieved in marine environments (Lee, 2008).

3.1.1.3. Chlorophyta: Chlorophyta (green algae) belong to the phylum *Chlorophyta* which belongs to the Protista kingdom. These organisms are photosynthetic and are largely marine organisms but are also found in freshwater. They can be unicellular, multicellular, colonial or coenocytic. Coenocytic refers to the species that possess more than one nucleus in a cell. Motile organisms have two apical or subapical flagella (Bold and Wynne, 1985). The green algae predominate in polar and subpolar regions (Lee, 2008).

3.1.2. Microalgae

Microalgae are photosynthetic microscopic organisms capable of tolerating extreme temperature and pH conditions as well as being able to live in different environments such as freshwater, marine water, and wastewater (Barsanti and Gualtieri, 2006; Chen et al., 2009; Demirbas, 2010). Microalgae are capable of producing more neutral oils under unfavourable conditions in comparison to those produced under favourable conditions, and are able to grow rapidly under optimal conditions (Chen et al., 2009). They are single celled organisms and are divided into groups based on pigmentation, basic cellular structure and life cycle (Sheehan et al. 1998: Demirbas 2010). They can also be divided into prokaryotic cyanobacteria and eukaryotic algae. Table 3.1 lists a few of the divisions found in prokaryotic and eukaryotic algae. Eukaryotic algae are classified into 12 divisions (Chen et al. 2009). In terms of abundance, the four most important groups of

Table 3.1. Microalgae group classification (Barsanti and Gualtieri, 2006).

Kingdom	**Division**
Prokaryota eubacteria	Cyanophyta
	Prochlorophtya
Eukaryota	Glucophyta
	Rhodophyta
	Heterokontophtya
	Haptophyta
	Cryptophyta
	Dinophyta
	Euglenophyta
	Chlorarachniophyta
	Chlorophyta
	Bacillariophyta
	Xanthophyta
	Phaeophyta

microalgae are: diatoms (*Bacillariophyceae*), green algae (*chlorophyceae*), golden algae (*chrysophyceae*) and the blue green algae (*cyanophyceae*) (Sheehan et al. 1998; Chen et al. 2009).

3.1.2.1. Eukaryotic Algae: Eukaryotic microalgae are capable of producing oxygen through photosynthesis. Although these organisms possess specific organelles in which photosynthesis occurs, it is believed that these organisms obtained the ability to photosynthesize via primary endosymbiosis with a cyanobacteria (Barsanti and Gualtieri, 2006; Cardol and Franck, 2010). Many eukaryotic algae are able to carry photosynthesis by a secondary endosymbiosis, where a non-photosynthesizing organism engulfs a eukaryotic organism that has acquired photosynthesis via primary endosymbiosis, which gave rise to diatoms (Barsanti and Gualtieri, 2006; Cardol and Franck, 2010). The diatoms formed are known to be the primary base of productivity in the oceans, as opposed to the cyanobacteria (Cardol and Franck, 2010). Diatoms dominate the phytoplankton of the ocean. Their cell walls contain polymerized silica (Si). The carbon in the diatoms is stored in the form of natural oils or as a chysolaminarin, a polymer of carbohydrates (Sheehan et al., 1998).

In freshwater, the green algae are the most abundant species, forming as single cells or as colonies. Their main storage compound is starch, but under certain conditions oil can be produced (Sheehan et al., 1998). The blue green algae have an important role in fixing the atmospheric nitrogen. These algae are much closer to bacteria (based on their structure and organization) than they are to algae (Sheehan et al., 1998).

The golden algae are most similar to the diatoms, but with much more complex pigments. These species tend to exist mainly in freshwater and produce carbohydrates as well as natural oils as part of their storage compounds (Sheehan et al., 1998).

3.1.2.2. Prokaryotic Cyanobacteria: Prokaryotes are single celled organisms that lack membrane bound organelles. There are two divisions in the prokaryotic kingdom: *Cyanophyta* and *Prochlorophya*. *Cyanophyta* are more commonly referred to as cyanobacteria. The name cyanobacteria stresses that these species are more related to bacteria than they are to algae (Barsanti and Gualtieri, 2006). They possess similar

structure as prokaryotes, but they also have the photosynthetic chlorophyll a pigment. Cyanobacteria grow as unicellular cells or colonial and filamentous.

3.2. Microalgae Environment

Microalgae have the ability to withstand various environments. Their main requirements for biomass production include water, light and carbon dioxide. Algae can grow in freshwater, salty water and wastewater (Demirbas, 2011).

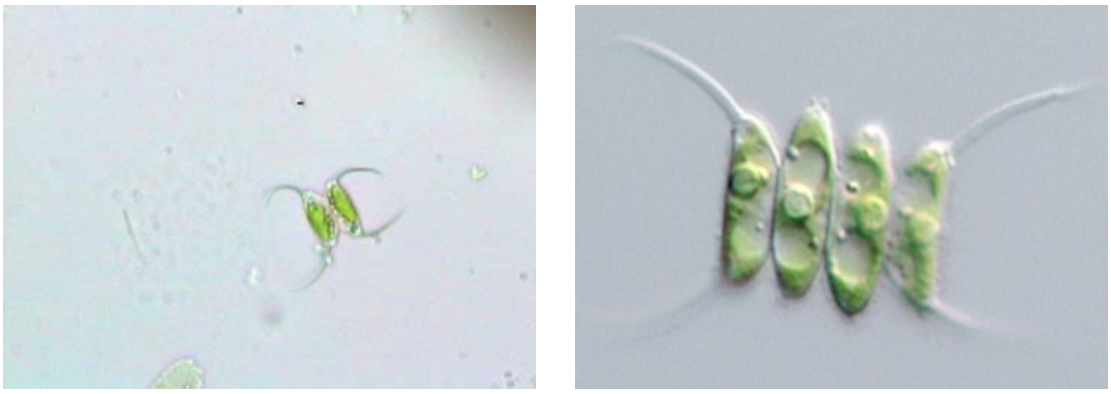
3.2.1. Freshwater Microalgae

As opposed to marine water, freshwater contains a few or no salts, with a freezing point of 0°C and a boiling point of 100°C. In order for algae to be grown in such environments the elements required for algal growth must be supplied into the medium (Aquarius, 2011). Since it has been observed that triacylglycerols (TAGs) content in lipid increases with N-deprivation, growing algae in freshwater allows for limiting nitrogen conditions which result in a higher algal quality and productivity (Chen et al., 2009). There are several species of freshwater microalgae such as: *Scenedesmus obliquus, Chlorella saccharophila* and *Chlorella protothecoides*.

3.2.1.1. Scenedesmus obliquus: *Scenedesmus obliquus* (Figure 3.1) is a freshwater unicellular microalgae belonging to the *Chlorophyceae* genus. The cells are small (6-8 μm in width and 9-12 μm in length) nonmotile and are aligned in a flat plate formation. Inside the cell is a single parietal plate-like chloroplast that has a single pyrenoid as shown in Figure 3.1b (Celekli et al., 2008). Bumps or reticulations cover the cell walls, and can be viewed under a scanning electron microscope (Oilage, 2012).

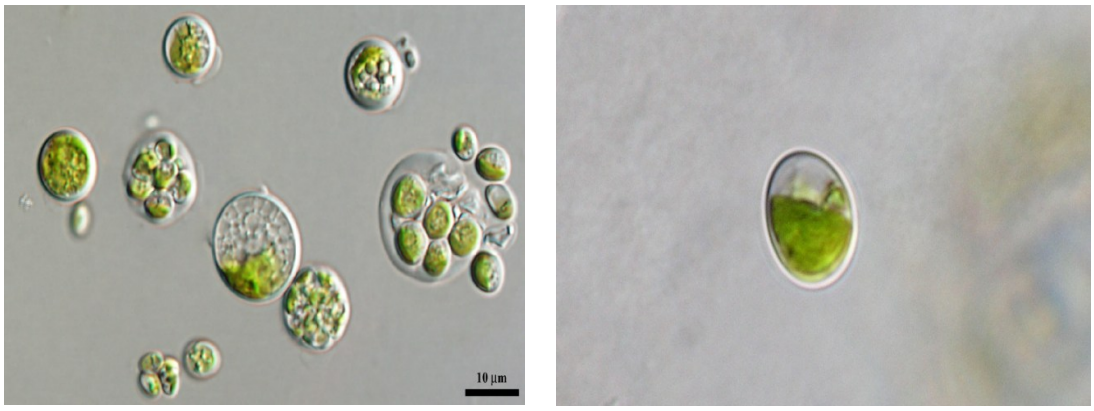
3.2.1.2. Chlorella saccharophila: *Chlorella saccharophila* is a green freshwater unicellular microalgae belonging to the *Chlorella* genus (Lewis, 1997). The cells have an average size of 7.3 μm (Bock et al., 2011). The cells contain a single chloroplast enclosed in a spherical or subspherical form as shown in Figure 3.2. These cells reproduce asexually through production of non-motile autospores (John, 2002).

3.2.1.3. Chlorella protothecoide: *Chlorella protothecoides* is a green freshwater unicellular microalga belonging to the *Chlorella* genus. It is a heterotrophic organism that needs organic carbon in order to thrive which can be obtained through the



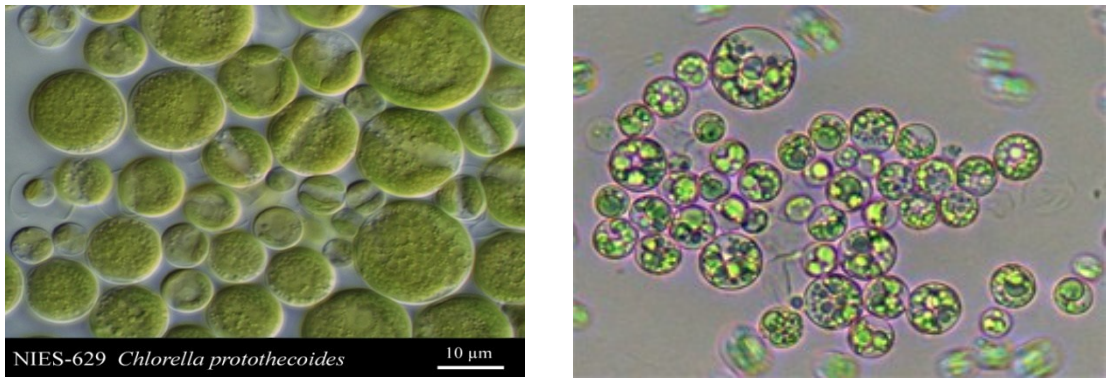
(a) Flat plate aligned cells (b) Chloroplast in cell (640x)

Figure 3.1. *Scenedesmus obliquus* (Picsearch, 2012).



(a) Subspherical form (b) Chloroplast in cell

Figure 3.2. *Chlorella saccharophila* (Skaloud, 2012).



(a) Spherical cells (b) Chloroplast in Cells

Figure 3.3. *Chlorella protothecoides* (Aquaportail, 2012; Ho, 2011).

consumption of organic materials (EOL, 2012). Cells are spherical with dimensions of 10 μm as shown in Figure 3.3 (Aquaportail, 2012).

3.2.2. Marine Microalgae

Marine water environments consist of seawaters with varying salt contents. Variation in the salt content is a result of varying water evaporation and precipitation in different parts of the ocean. Numerous elements make up seawater, however only a small number make up more than 99% of the dissolved salts (Table 3.2). All of these dissolved salts are in the form of electrically charged atoms or groups of atoms (Badea et al., 2007). Utilizing marine water for microalgae growth would minimize the need for additional nutrients into the production system. There are several species of marine microalgae such as: *Nannochloropsis oculata*, *Tetraselmis suecica* and *Chaetoceros muelleri.*

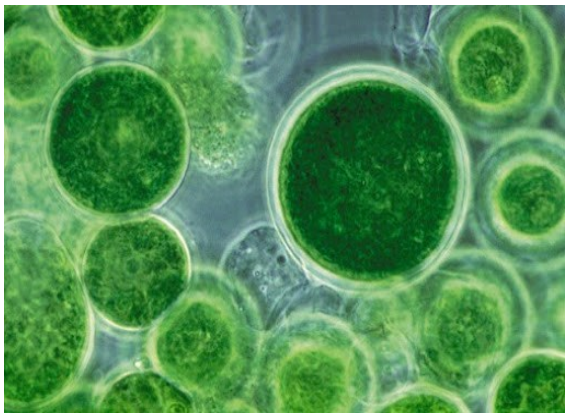
3.2.2.1. Nannochloropsis oculata: *Nannochloropsis oculata* is of the class *Eustigmatophyceae* which consists of small planktonic microalgae or macroalgae that grow attached to various substrates. This class of microalgae is unique because they only possess chlorophyll *a* pigments and lack all others (Fisher et al., 1998). *Nannochloropsis oculata* are characterized by their small cells (1-2 μm length and 1-2 μm width), unicellular shape (Figure 3.4) and salt-water habitats. This microorganism is widely used as a food source in aquaculture (Sukenik et al., 1988).

3.2.2.2. Tetraselmis suecica: *Tetraselmis suecica* is a type of marine microalga that grows as single cells. They are motile and can be compressed or curved, but they are never twisted. Cells are spherical or elliptic with a length of 35 μm and a width of 14 μm, as shown in Figure 3.5 (Acuna and Kiefer, 2000).

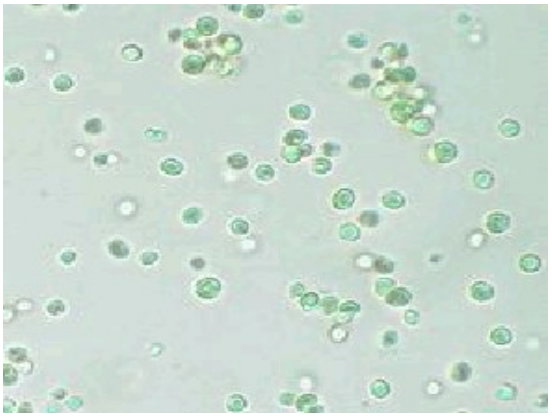
3.2.2.3. Chaetoceros muelleri: *Chaetoceros muelleri* is a marine microalgae that exhibits 8 costae/μm on the valve and valve mantle that are arranged in a radiating pattern. The valves are oval to round shaped and the cells are cylindrical (Figure 3.6). Cell occurrence is singular or on occasion in groups of 2-3 filaments (Johansen and Rushforth, 1985). The valves in *Chaetoceros muelleri* range in length from 3 to 20 μm.

Table 3.2. Composition of marine water (Badea et al., 2007).

Element	Weight Percentage
Chloride (Cl^-)	55.04
Sodium (Na^+)	30.61
Sulphate (SO_4^{2-})	7.68
Magnesium (Mg^{2+})	3.69
Calcium (Ca^{2+})	1.16
Potassium (K^+)	1.10



(a) Unicellular cell type



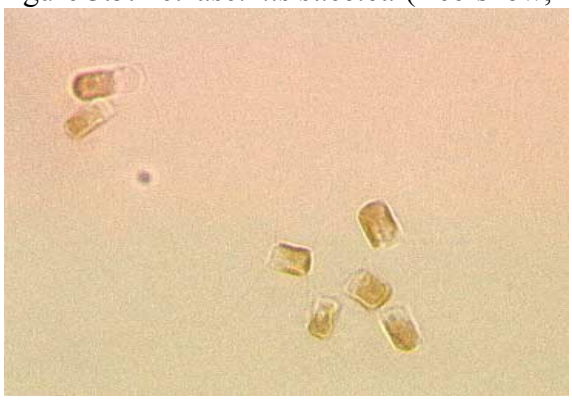
(b) Length and width (1-2 µm)

Figure 3.4. *Nannochloropsis oculata* (Nichols, 2011; Algaelink, 2009).

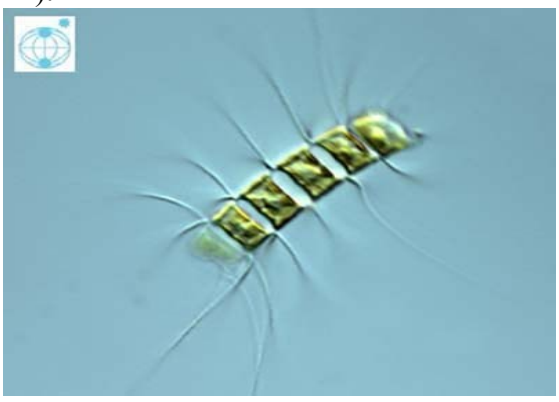
(a) Elliptic cell type

(b) Unicellular cell type

Figure 3.5. *Tetraselmis suecica* (Reefsnow, 2012).



(a) Cylindrical cell type



(b) Cells form groups (8 costae)

Figure 3.6. *Chaetoceros muelleri* (Engel, 2000; Microscope, 2006).

3.2.3. Wastewater Microalgae

Growing algae in wastewater environments has multiple benefits (Chen et al., 2009). The costs for additional nutrient removal from wastewaters will be offset and the costs for algae nutrients, which are needed for algal growth, would be largely reduced (Chen et al., 2009). Algae on municipal wastewaters has the advantage of removing certain elements such as phosphorous and nitrogen from wastewater as well as CO_2 from the atmosphere which if left undealt with, are harmful to the environment (Chen et al., 2009). Both freshwater and marine microalgae can be grown in wastewaters such as: *Chlorella vulgaris, Scenedesmus obliquus, Ourococcus multisporus* (Ji et al., 2013), *Chaetoceros mulleri* (Juan, 2006) and *Tetraselmis suecica* (Perez-Rama et al., 2002).

3.3. Biological Composition of Algae

Algae use light energy in a process called photosynthesis, where water and carbon dioxide from their surrounding environment are converted into lipids, carbohydrates, proteins and nucleic acids in different proportions, depending on the algal type (Demirbas and Demirbas 2011; Singh and Gu, 2010). Carbohydrates are the main constituents of algal cell walls, with small quantities of protein and lipids present as well (Fu et al., 2010). Table 3.3 shows the composition of proteins, carbohydrates, lipids, and nucleic acid in different algae species.

3.3.1. Proteins

Some proteins in algae function as the photoreceptive molecules. A small number of these molecules should be present in algae because they only function to detect light. There are two main types of photoreceptors in algae that are responsible for algal vision, which are rhodopsin-like proteins and flavoproteins. It is important to note that these receptive molecules do not play any role in the color of the organism, because the color plays an important role in the classification of the organism. The protein synthesis of the organism *Stichococcus bacillaris* are strongly inhibited by salt shock, but once returned to lower salinity environments the organism recovers quickly (Ahmed and Hellebust, 1993).

3.3.1.1. Rhodopsin-like Proteins: The rhodopsin protein receptor consists of seven transmembrane α-helices protic parts called the opsin and the retinal which is the group of proteins that absorb the light. Inside the center of the opsin is the location of the retinal.

Table 3.3. Microalgae composition based on dry matter percentage (Demirbas, 2011).

Species	Proteins	Carbohydrates	Lipids	Nucleic Acid
Scenedesmus obliquus	50–56	10–17	12–14	3–6
Chlorella pyrenoidosa	57	26	2	-
Tetraselmis maculata	52	15	3	-
Spirulina maxima	60–71	13–16	6–7	3–4.5
Scenedesmus dimorphus	8–18	21–52	16–40	-
Spirulina maxima	60–71	13–16	6–7	3–4.5

The absorption band of the retinal-opsin complex can be shifted into the visible region of the spectrum. The light reached into the retinal can then isomerize very quickly and efficiently. The reaction that initiates the vision cascade, known as isomerization, can only be initiated by light. Isomerization is important because it is responsible for the movement of the single α-helix. Movement of these structures are initiated by the conversion of light into atomic motion. Upon excitation via light, the process by which the photoreceptor protein undergoes various conformational changes, may return to the original state in a rapid way allowing the photoreceptive machinery to be reset and ready for the next response (Barsanti and Gualtieri, 2006).

3.3.1.2. Flavoproteins: Flavoproteins, known as yellow enzymes, absorb light at 280, 380 and 460 nm. Flavopreoteins are believed to function as blue-light photoreceptors. This protein is also thought to function as a detector in the near-UV-visible light spectrum. Not much is known about this protein to date (Barsanti and Gualtieri, 2006).

3.3.2. Carbohydrates

Carbohydrates are a form of storage product for algae, whereby the end products of photosynthesis are carbohydrates. Carbohydrates are formed by CO_2 fixation. These products can be in the form of sucrose, paramylon and starch. Some algae produce carbohydrates as their primary energy storage. In eukaryotic algae, the fixation process of CO_2 takes place in the stoma, while that of prokaryotic algae takes place in the cytoplasm (Barsanti and Gualtieri, 2006).

3.3.3. Lipids

Another form of storage energy is TAGs. The main building blocks of TAGs and all other cellular lipids are fatty acids. The enzyme acetyl CoA carboxylase is responsible for regulating the rate of fatty acid synthesis which takes place in the chloroplasts (Hu et al., 2008). Small amounts of spherical lipid droplets are contained in the chloroplasts between the thylakoids. These droplets serve for growth and synthesis of lipoprotein membranes within the chloroplasts (Lee, 1999). Algal lipids function as both a structural support for the cell and as metabolic organelles in photosynthesis (Chen et al., 2009). Algae typically have between 5-20% lipid oil when grown under optimal conditions,

while growth in unfavourable conditions increases the lipid content up to 20-50 % (Hu et al., 2008; Pokoo-Aikins et al., 2010).

3.3.4. Nucleic Acid

Production of nucleic acid in algae depends on the stage of growth. Algae growth under batch conditions consists of three stages: lag, exponential and stationary (Fogg and Thake, 1987). During the exponential growth, the accumulation of nucleic acid and other cellular constituents occur in constant proportions (Lavens and Surgeloos, 1996). In this phase, the Ribonucleic acid (RNA) and protein synthesis are formed in a fixed ratio. In contrast, the ratio of proteins to RNA varies in the lag phase with the limitation of certain nutrients. The biomass containing nucleic acid can be used as fertilizer (Demirbas, 2011; Chen et al., 2009).

3.4. Lipid Production

On average, oleaginous green algae consist of 25.5% lipid of their dry cell weight (DCW). However, once they are grown under stress conditions, the lipid content is found to double or even triple (Hu et al., 2008). The average lipid content of oleaginous green algae grown under unfavourable conditions was found to increase to 45.7% DCW (Hu et al., 2008). The average lipid content of oleaginous diatoms was found to be 22.7% DCW under normal conditions, and the lipid content which increased to 44.6% DCW under unfavourable conditions (Hu et al., 2008). However, lipid content and biomass productivity are inversely related as shown in Table 3.4 (Rodolfi et al., 2009).

Aging algal cells maintained under unfavourable conditions consist of TAG as the main neutral lipid (Hu et al., 2008; Chen et al., 2009). The unfavourable conditions altered the synthesis of membrane lipids to the storage of neutral lipids (Hu et al., 2008). An increase in TAG content may also be achieved via De novo biosynthesis and the conversion of the existing polar lipids in the membrane into TAG (Hu et al., 2008). Table 3.5 shows a few species that alter their lipid composition (less PUFA) as the culture is allowed to age (past exponential growth phase). The lipid content of the marine species vary from 8.5% to 39.8%, while those of freshwater yield a lipid content of approximately 20%.

Table 3.4. Microalgae species screened for biomass productivity, lipid content and lipid productivity (Rodolfi et al., 2009).

Microalgae	Biomass Productivity (g/L/day)	Lipid Productivity (mg/L/day)	Lipid Content (% biomass)
Marine strains			
Prophyridium cruentum	0.37	34.8	9.5
Tetraselmis suecica	0.32	27.0	8.5
Nannochloropsis	0.21	61.0	29.6
Isochrysis	0.17	37.7	22.4
Chaetoceros calcitrans	0.04	17.6	39.8
Freshwater strains			
Chlorococcum	0.28	53.7	19.3
Scenedesmus	0.26	53.9	21.1
Chlorella	0.23	42.1	18.7
Scenedesmus quadricauda	0.19	35.1	18.4
Chlorella vulgaris	0.17	32.6	19.2

Table 3.5. Species known to yield higher lipid content with culture aging.

Species	**Reference**
P. Tricornutum	Alonso et al. (2000)
Chlorococcum	Rodolfi et al. (2009)
Scenedesmus	Rodolfi et al. (2009)
Dunaliella	Takagi et al. (2006)

Alonso et al. (2000) noted that the *P. Tricornutum* diatom was not affected by culture age and its fatty acid content remained the same. Hu et al. (2008) reported that the TAGs did accumulate with a reduction in polar lipid content. They also noted that an increase in the total lipid content in the cells, due to unfavorable conditions, resulted in a biomass reduction. Rodolfi et al. (2009) reported that *Chlorococcum* and *Scenedesmus* strains were found to be the highest biomass generating species of freshwater and also produced the highest amount of lipids of 53.7 and 53.9 mg/L/day, respectively. The author also noted that the marine species *Chaetoceros* was found to produce the highest lipid content of 40%, but also resulted in the lowest biomass productivity. Takagi et al. (2006) noted a lipid content in *Dunaliella* cells of 67% and 60% at salt concentrations of 1 and 0.5 M sodium chloride (NaCl), respectively.

3.4.1. Fatty acids

Algal fatty acids are the building blocks for the synthesis of various types of lipids (Hu et al., 2008). The fatty acid chains are made up of even numbers of carbon atoms (in the range of 14 - 24) with a carboxyl group at one end of the chain (Figure 3.7) (Woertz, 2007). Unsaturated fatty acids form when double bonds are present in the carbon chain, in the *cis* configuration. Fatty acids present in microalgae have in total zero to five *cis* double bonds. Fatty acids that are linked by an ester bond to the trihydric alcohol, glycerol or to other alcohols or by an amide bond or amines are the most typical lipid classes of plant and animal (Woertz, 2007). The type of lipid is important because it determines the pre-treatment required for the quality of the final fuel (biodiesel) product. Poor quality lipids are those consisting of a high free fatty acid content and a high degree of unsaturation. Such lipids may need to be processed via thermal depolymerisation in order to transform them into transportation fuels. Pure algae cultures contain a high low degree of unsaturated fatty acids with chain lengths of 16 - 18 carbon atoms. Green algae fatty acids consist of mainly C16 - C18 with a low degree of unsaturation (Halim et al., 2012).

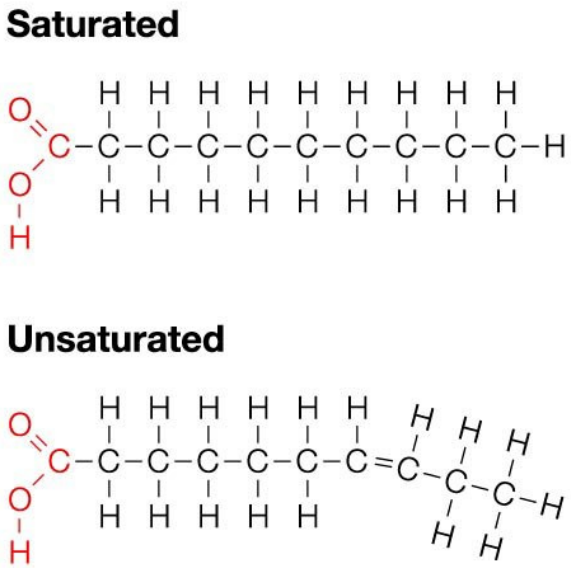


Figure 3.7. Fatty acid chain, both saturated and unsaturated (Education-Portal, 2014).

3.4.2. Membrane Lipids

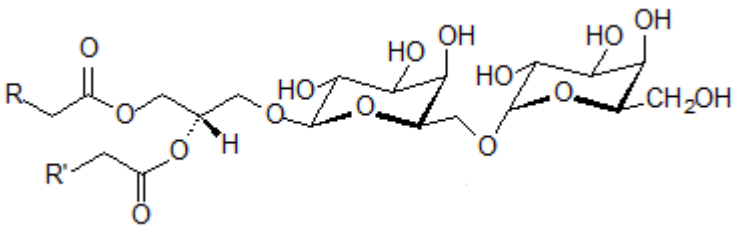
Membrane lipids (Figure 3.8) function as a structural support for the cell (Hu et al., 2008; Rodolfi et al., 2009). About 40% of these lipids are comprised of polyunsaturated fatty acids (PUFA). They can be solubilised by solvent extraction of the wet biomass transesterified into biodiesel (Singh and Gu, 2010). Hu et al. (2008) noted that algae synthesize membrane lipids that make up 5-20% of their DCW under optimal growth conditions. Selstam and Campbell (1996) noted that the membrane lipid digalactosyl diacylglycerol in *Gloeobacter violaceus* makes up 24% of the total lipids. Guschina and Harwood (2006) noted that eukaryotic species contain up to 35% of the membrane lipid monogalactosyldiaclglycerol. Sato et al. (2003) observed that *Chlorella kessleri* had lower membrane lipids under low CO_2 levels compared to high CO_2 levels.

3.4.3. Triacylglycerols (TAGs)

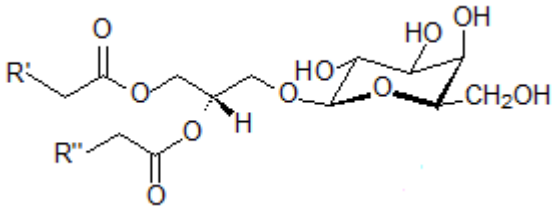
TAGs (Figure 3.9) function as storage compounds (storing carbon and energy) and are the respondents to stress, as opposed to the other lipids that play a role in the structural support (Hu et al., 2008; Rodolfi et al., 2009; Singh and Gu, 2010). TAGs are synthesized in the endoplasmic reticulum (Chen et al., 2009). TAGs (consisting of saturated and monounsaturated fatty acids) tend to generate more energy upon oxidation than carbohydrates and are efficiently packed in the cell (Roessler, 1990).

Algae alter their lipid biosynthetic pathways when exposed to environmental stresses (unfavourable conditions). Such stresses result in the formation of neutral lipids in the form of TAG (Scott et al., 2010). TAGs are used for the production of biodiesel via transesterification (Chen et al., 2009). Therefore, by exposing the algae to unfavourable conditions, more TAGs can be produced (Chen et al., 2009). These stresses include nutrient deprivation including nitrogen, phosphorous and silicon (Chen et al., 2009). However, it should be noted that higher TAG production does not necessarily indicate an increase in biodiesel production, because an increase in TAG is obtained at the expense of the total algal biomass productivity. In addition, species that are known to accumulate high amounts of TAGs are found to have a slow biomass growth (Chen et al., 2009).

Rodolfi et al. (2009) noted an increase in lipid content in *Chlorella vulgaris* from 14-30% of dry weight to 70% under nutrient deficiency. Cohen (1990) reported an increase



(a) Digalactosyldiacylglycerol



(b) Monogalactosyldiacylglycerol

Figure 3.8. Membrane lipid structures (Christie, 2014).

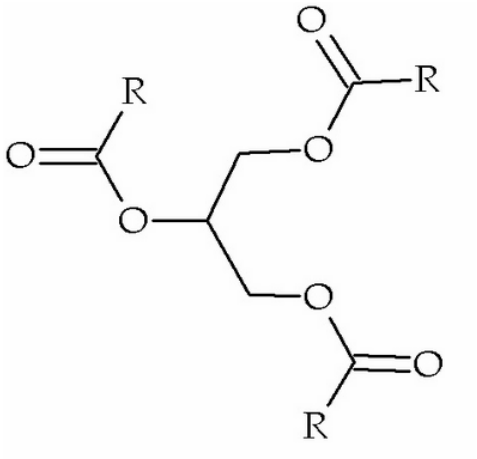


Figure 3.9. Triacylglycerol structure (Feltes et al. 2011).

in TAG production in *Porphyridium cruentum* under nitrogen deficiency. Borowitzka (1988), Hu (2004) and Roessler (1990) noted increases in lipid content (mainly TAGs) of up to 70% in several species under nitrogen starvation. Chen et al. (2009) noted that the species *Botryococcus braunii* can accumulate up to 70% TAGs under nitrogen deficiency. Takagi et al. (2006) noted that an increase in concentration of NaCl from 0.4 M to 4.0 M increased the saturated and monosaturated fatty acid contents and decreased the PUFA content in the marine *Dunaliella* species.

Hu et al. (2008) reported that in the stationary phase of growth, there is an increase in TAG production because as the transition progresses from logarithmic to stationary phase, the lipids that function in membrane structure decrease and mono-unsaturated and saturated fatty acids contents increase, which are the main components of natural lipids. Bigogno et al. (2002) observed an increase in TAG from 43% (total fatty acid) in the logarithmic phase to 77% in the stationary phase in chlorophyte *Parietochloris incise*. Mansour et al. (2003) noted an increase in TAG production of *Gymnodinium* sp. to 30% in the stationary phase in comparison to the 8% in the logarithmic phase.

3.4.3.1. Cytoplasm Lipid: Once the TAGs are synthesized they are then deposited in the cytoplasm lipid bodies. In some green algae (example *Dunaliella bardawil*), the inter-thylakoid space in the chloroplast is responsible for the formation and accumulation of lipid bodies (Hu et al., 2008). In order to obtain the stored TAG, the cell walls as well as other cellular structures must first be broken down prior to the chemical and physical extraction of TAGs in some species (Rodolfi et al., 2009).

3.4.3.2. De Novo Lipids: De novo is a process which eventually leads to the production of TAGs (Rodolfi et al., 2009; Chen et al., 2009; Scott et al., 2010). This process consists of the synthesis of fatty acids, which are added to the glycerol backbone and the acyl transfer from the acyl CoA enzyme which reside in the endoplasmic reticulum (Chen et al., 2009). The synthesis of fatty acids via de novo takes place in the chloroplasts or the stoma of plastids (Hu et al., 2008; Chen et al., 2009). The initial step, of the de novo fatty acid synthesis, is the admission of acetyl CoA, as a substrate for acetyl CoA carboxylase and the initial condensation reaction (Hu et al., 2008). Amongst other enzymes, the final product of the de novo fatty acid synthesis is the production of a fatty acid chain

consisting of 16 or 18 carbons (Hu et al., 2008; Chen et al., 2009). The fatty acid chains produced function as the precursors for the synthesis of lipid storage products and cellular membranes (Hu et al., 2008; Chen et al., 2009). The lipid synthesis capacity via de novo is limited by the characterization of the oleaginous microalgae. When microalgae are grown under nitrogen stress, they store the excess carbon and energy in the form of TAGs (Rodolfi et al., 2009).

Suen et al. (1987) noted that *Nannochloropsis* sp. accumulated 80% lipids in the form of TAGs as a result of de novo fixation of carbon dioxide, under nitrogen deficient environments. Sukenik et al. (1989) reported that under photoinhibitory irradiance the *Nannocholoropsis* species exhibited an increase in TAGs due to de novo lipid synthesis.

3.4.3.3. Chloroplasts Lipids: The compartment responsible for absorbing light for photosynthesis is the chloroplast (Barsanti and Gualtieri, 2006). It consists of pigments which function to absorb the light and direct its energy for various photochemical and enzymatic reactions. The pigments are comprised of thylakoids, which are flat-like vesicles compressed onto one another. The thylakoid vesicles vary from prokaryotic to eukaryotic organisms. In prokaryotes the thylakoids are found in the cytoplasm free from a bounding membrane while the eukaryotes possess their thylakoids in an enclosed membrane known as the chloroplast (Barsanti and Gualtieri, 2006). Most of the fatty acid chains synthesised in the chloroplast, function in the assembly of the chloroplast membrane (Chen et al., 2009; Liu et al., 2011). Fatty acids may also aid in the construction of other membranes that are not related to the chloroplast. The free lipid droplets that exist under certain conditions, maybe the result of fatty acids synthesised in the chloroplast (Chen et al., 2009).

3.5. Environmental Factors Affecting the Production of TAGs

The amount of TAG produced by algae is specific to the species/strain (Hu et al., 2008). Oleaginous algae are known to produce only small amounts of TAG when grown under optimal conditions, but once the conditions become unfavourable, it was found that TAG accumulation is increased (Hu, 2004). When algae are exposed to unfavourable environmental conditions, the compositions of the fatty acids and lipids become altered and are found to increase in algae (Hu et al., 2008). Lipids (non-polar TAGs) are the best

substrate for biodiesel production which can be formed by alternating some of the algal growth conditions (Rodolfi et al., 2009). The conditions which if altered can play a major role in TAG synthesis are chemical or physical environmental stimuli (Guschina and Harwood, 2006; Hu, 2004; Hu et al., 2008; Roessler, 1990). The chemical stimuli are deprivation of nutrients and pH variation, while the physical stimuli include light intensity and temperature (Hu et al., 2008). Certain species like *Chlorella* (Illman et al., 2000; Orus et al., 1991; Hsieh and Wu, 2009), *Dunaliella* (Gordillo et al., 1998; Takagi et al., 2006), *Nannochloropsis* (Rodolfi et al., 2009) and *Neochloris* (Li et al., 2008) have been noted to have altered their metabolic pathways from the production of proteins to lipids (TAGs) by diverting the fixed carbon under stress conditions (limitation of phosphorous or nitrogen).

3.5.1. Temperature

Temperature plays a major role in the composition of fatty acids and lipids in microalgae (Hu et al., 2008). The optimal temperature for some microalgal growth is in the range of 18-20°C. Temperatures that do not fall within this range are found to affect the rate of growth and may kill the microalgae (Chen et al., 2009; Demirbas and Demirbas, 2011). It has been observed that with increasing temperatures the saturated fatty acids increase in microalgae and cyanobacteria while a decrease in temperature increases the unsaturated fatty acids (Raison, 1986; Renaud et al., 2002). Lipids on the other hand are found to increase with increasing temperatures (Chen et al., 2009; Hu et al., 2008; Sayegh and Montagnes, 2011; Greenwell et al., 2010) in chrysophytan *Ochromonas danica* (Aaronson, 1973) and in eustigmatophyte *Nannochloropsis salina* (Boussiba et al., 1987). Lipid content increases as a result of increased growth rate which in turn increases the carbon storage of the cells (Chen et al., 2009).

Renaud et al. (2002) tested the temperature effect on growth rate and lipid content in three different species: *Isochrysis* sp., *Nitzschia closterium* and *Nitzschia paleacea*. They noted that over the temperature range of 10-35°C *Isochrysis* sp. had a maximum growth rate and lipid content of 25.6% at 20°C with significantly lower growth rates at temperatures of 10, 15, 25 and 30°C. *Nitzchia closterium* did not grow at temperatures higher than 30°C or lower than 20°C with no significant difference in growth rate at

temperatures of 30, 25 and 20°C, but the maximum lipid production of 20.1% was noted at 20°C. *Nitzschia paleacea* was tolerant to low temperatures, although growing rates at 10°C were very slow. The maximum growth rate for this organism was noted at 15°C while the maximum lipid content of 21.2% was noted at 10°C.

Patterson (1970) noted no significant lipid change upon exposure of *Chlorella sorokiniana* to various temperatures. Tedesco and Duerr (1989) observed an increase in lipid generation and growth in *S. platensis* upon increasing the temperature from 25 to 38°C. Renaud et al. (2002) noted that the *Chaetoceros* sp. grew well at 33-35°C with the highest lipid content of 16.8% noted at 25°C.

3.5.2. pH

Only a few algal organisms are capable of growing in extreme pH environments because the extent of ionization of metabolites is affected by the pH, which in turn affects the reactivity of the organisms and their ability to uptake nutrient. High levels of photosynthesis may result in pH fluctuation, as a result of carbon dioxide removal from an already alkaline culture environment (Rai and Guar, 2001).

The pH of a medium affects the toxicity of the surrounding metals present in the algae and in turn the ability of algae to uptake nutrients from the environment is affected by the toxicity of the metal (Peterson et al., 1984). This might be attributed to the fact that with increasing pH there is a decrease in competition between the metal ion and the H^+ at the cell surface (Franklin et al., 2000; De Schamphelaere et al., 2003). Therefore, the pH of the medium must be maintained at the optimum level for sufficient nutrient uptake.

Rodolfi et al. (2009) reported that the pH of the culture was in the range of 7.5-8.1 while introducing air/CO_2 into the system at a ratio of 97/3% (v/v). Peterson et al. (1984) noted that cadmium toxicity on *Scenedesmus quadricauda* species over the pH range of 5.5-8.5 affected their ability to uptake phosphorous. De Schamphelaere et al. (2005) noted the toxicity of Cu^{2+} to cell growth and found cell growth rate to increase at pH levels over the range of 5.9-8.5 for *Chlorella* sp. and *Pseudokirchneriella subcapitata.* Schenck et al. (1988) noted that the fluxes of metals into the cells decreased over the pH range of 7-5 for *Chlamydomonas variabilis*. Wilde et al. (2006) noted that the growth

rate of *Chlorella* sp. was affected by the copper and zinc toxicity which increased over the pH range of 5.5-8, but the sensitivity to copper was greater than that of the zinc.

3.5.3. Nutrients

Various nutrients, in the surrounding medium of the microalgae play a role in lipid production. Altering the amount of these nutrients, changes the organism's ability to synthesize lipids and affect the rate of growth. Such nutrients include carbon, nitrogen, phosphorous and sulfur.

3.5.3.1. Carbon: A carbon source is required for algal cell production. The carbon can be obtained in the form of sugars, lignocellulose-based substances and/or carbon dioxide. Sugars such as glucose can provide the carbon source required by algal cells for growth (Table 3.6). However, they are not found to be as productive as the lignocellulose based materials such as rice straw, corn powder and sweet sorghum in their hydrolysate forms (Li et al., 2011). Table 3.7 shows that despite the large biomass concentration achieved by cassava starch, the lipid content is much lower in the algal cells than those obtained by glucose. Amongst the various hydrolysate materials, rice straw is found to yield the highest lipid content of 56.3% while running at the shortest time of 48 hours (Li et al., 2011).

Chen (1996) reported that microalgae oil production can be achieved by culturing the algae in heterotrophic conditions where the carbon source can be sugar or organic acids. This method would eliminate the need for light, bringing forward the possibility of increased productivity and cell density. However, growing microalgae in heterotrophic conditions is difficult in large scale systems because bacteria thrive on the sugars (Stephenson et al., 2010).

Liu et al. (2011) noted that *Chlorella zofingiensis* grown under heterotrophic conditions and fed with glucose resulted in a lipid yield of 79.5% of which 88.7% were made of TAGs. Qiao and Wang (2009) noted that *Chlorella sorokiniana* exhibited an increase in lipid content (from 0.053 g/L to 0.272 g/L) when glucose was used as the carbon source in comparison to fructose under heterotrophic conditions.

Table 3.6. Oil content of algae species and optimal conditions (Sobczuk et al. 2000; Sukenik et al. 2009; Demirbas, 2011; Wagenan et al. 2012; Moheimani et al. 2013; Pagnanelli et al. 2013).

Species	Oil Content (% dry basis)	Carbon Sources	pH	Temperature (°C)
Marine				
Nannochloropsis oculata	22-30	CO_2/$NaHCO_3$/glucose	8.4	20-30
Tetraselmis suecica	15-23	CO_2/$NaHCO_3$/glucose	7.6-8.4	20-30
Chaetoceros muelleri	33.6	CO_2/$NaHCO_3$	8	20-30
Freshwater				
Chlorella protothecoides	15-55	CO_2/$NaHCO_3$/glucose	6.0-6.5	20-30
Chlorella saccharophila	36-47	CO_2/$NaHCO_3$/glucose	7.5-8.1	20-24
Scenedesmus obliquus	11-55	CO_2/$NaHCO_3$/glucose	7.5-8.1	20-24

Table 3.7. Impact of various carbon sources on the biomass growth and lipid content of algal cells (Li et al., 2011).

Hydrolysate materials	**Maximum biomass concentration (g/L)**	**Biomass productivity (g/L/day)**	**Lipid content (%, w/w)**
Glucose	0.92	0.37	50.3
Rice straw	2.83	1.10	56.3
Corn powder	3.92	0.65	55.3
Cassava starch	7.20	0.72	28.9
Cassava	4.26	0.82	50.2
Sweet sorghum	5.10	1.02	53.3

Liang et al. (2009) reported that *Chlorella vulgaris* produced the highest amount of lipid when grown with glucose and light. The fastest growth rate was achieved at 1% glucose concentration. However, the highest lipid content was noted at 2% glycerol concentrations.

Liu et al. (2011) noted a higher production of lipids in the microorganism *Chlorella zofingiensis* under heterotrophic conditions in comparison to photoautotrophic. Under heterotrophic conditions, the organism was grown in the dark and exposed to glucose as a source of energy, while under photoautotrophic conditions light was illuminated continuously. Light alone was not capable of lipid synthesis since 51.1% was observed under heterotrophic and only 25.8% under photoautotrophic conditions. The cell biomass rate under photoautotrophic conditions was 1.9 g/L while that under heterotrophic was 9.7 g/L. It was also noted that the cells grown under heterotrophic conditions consumed nitrate very rapidly. The algae cells grown under heterotrophic conditions produced natural lipids, oleic acid and TAGs which are better for biodiesel production than the oils produced by cells grown under photoautotrophic conditions. Thus, illustrating the importance of sugar for high production of biodiesel.

Microalgae are capable of tolerating high levels of CO_2 as shown in Table 3.8 (Demirbas et al., 2011). One ton of algal biomass can fix 1 ton of CO_2 by either autotrophic or heterotrophic metabolism (Chen et al., 2009). Chisti (2007) reported that 1 kg of dry algal biomass uses 1.83 kg of CO_2 which results in air quality improvement. Algae utilize CO_2 and convert it into biomass. Half of the biomass dry weight is made up of carbon obtained from carbon dioxide. During daylight, algae require a continuous carbon dioxide supply (Demirbas and Demirbas, 2011) which can naturally be obtained from the atmospheric CO_2 excreted by power plants (Demirbas, 2011).

Rai and Gaur (2001) reported that during photosynthesis, algae use CO_2 as the carbon source for synthesis of algal biomass and the oxygen released into the atmosphere is more than that released by forests. In seawater, low CO_2 concentrations may be the result because: (a) seawater equilibrated air contains 180 times more inorganic carbon in the forms of bicarbonate and carbonate than CO_2, (b) the algae are capable of changing the way in which the carbon forms are utilized based on the surroundings, (c) the CO_2

Table 3.8. Consumption rate of CO_2 by microalgae.

Species	**pH**	**Rate of CO_2 uptake (mg/g/day)**	**Reference**
B. braunii	7.2	160.7	Sydney et al. (2010)
S. platensis	9	146.3	Sydney et al. (2010)
D. tertiolecta	7.2	126.5	Sydney et al. (2010)
C. vulgaris	7.2	128.6	Sydney et al. (2010)
S. obliquus	6.5	156.5	Tang et al. (2011)

concentrations differ in certain habitats, and (d) the degree of the presence of inorganic carbon depends on other factors (Rai and Gaur, 2001).

Low levels of CO_2 result in: (a) lower levels of growth, (b) over excitation of photosynthesis apparatus which in turn results in a decrease of photosynthetic activity, and (c) damage to the photosynthetic apparatus which is irreversible (Rai and Gaur, 2001; Demmig-Adams and Adams, 1992 and 2000). However, some photosynthetic organisms have developed various different pathways in order to avoid damage to the photosynthetic apparatus by controlling the amount of light allowed to be trapped (Demmig-Adams and Adams, 1992 and 2000). These findings stress the importance of avoiding low CO_2 concentrations (Rai and Gaur, 2001).

Bidwell (1957) noted that in an experiment in which carbon dioxide was labeled (C^{14}), 50% of the labeled carbon was converted to proteins within 4 hours. Wahlen et al. (2011) reported that CO_2 is introduced into the culture by aeration with air including 1% (v/v) of CO_2. Rodolfi et al. (2009) introduced CO_2 into the system by flushing it with air and CO_2 at 95/5% (v/v). Brown et al. (1996) reported that the *Thalassiosira pseudonana* culture was aerated using a mixture of air/CO_2 that contained 1% CO_2.

Round (1973) reported that different forms of carbon (bicarbonate, carbonate and carbon dioxide) exist at various pH levels. At acidic pH levels (pH below 5) carbon is in the form of dissolved CO_2, bicarbonate (HCO_3^-) is the carbon form that exists in the pH range of 6.5-10.5, and at high pH levels (above 10.5) carbonate (CO_3^{2-}) is the main carbon source present. Algae are capable of using bicarbonate as a carbon source (Table 3.9).

Blake et al. (2006) reported on the *C. vulgaris* and *S. obliquus* uptaking sodium bicarbonate at a pH of 7 at the rates of 125 and 126 mg/L/day, respectively. Merrett et al. (1996) reported on *N. oculata* uptaking sodium bicarbonate at the rate of 45.8 mg/L/day at a pH of 8. Huertas et al. (2000) noted that bicarbonate uptake by *Nannochloropsis gaditana* was activated by light.

3.5.3.2. Nitrogen: Nitrogen is the most critical nutrient which plays a large role in algal lipid accumulation (mainly TAGs) (Hu et al., 2008; Huesemann and Benemann, 2009). Large scale production of microalgae for algal oil requires 8-16 tons/ha/year of nitrogen fertilizer (Demirbas, 2011). However, lipid accumulation in diatoms is largely

Table 3.9. Consumption rate of $NaHCO_3$ by microalgae.

Species	**pH**	**Rate of uptake (mg/L/day)**	**Reference**
C. vulgaris	7	125	Blake et al. (2006)
	9	70	Blake et al. (2006)
S. obliquus	7	126	Blake et al. (2006)
	9	50	Blake et al. (2006)
N. oculata	8	45.8	Merrett et al. (1996)

affected by silicon (Hu et al., 2008). Under nitrogen starvation, diatoms with a high lag-phase, do not respond by increasing their lipid content (Benemann and Oswald, 1996; Shifrin and Chisholm, 1981). Roessler (1988) noted that *Cyclotella cryptica* contain higher amounts of natural lipids under silicon deficient environments in comparison to nitrogen deficiency. Some species of green microalgae such as *C. pyrenoidosa* show a multiple fold increase in lipid content upon nitrogen deprivation while the species *Dunaliella* and *Tetraselmis suecica* show almost no change/slight reduction (Borowitzka, 1988). However, within the same genus *Chlorella* was found to produce starch under nitrogen stress (Hu, 2004).

In nitrogen deficient environments, the photosynthesis products shift from protein to carbohydrate and then to lipid (Rodolfi et al., 2009). However, the accumulation of lipids results at the expense of biomass production (Rodolfi et al., 2009; Scott et al., 2010). Cell division in cultures under nitrogen deficiency is halted as a result of the accumulation of inhibitory products that are formed with nitrogen starvation (Rai and Gaur, 2001). Fogg and Thake (1987) reported that young cells grown in nitrogen deficient environments are able to grow and divide, generating a second generation of cells with an alteration in their metabolic pattern. These cells favour the production of carbohydrates.

Shifrin and Chisholm (1981) noted that in some microalgae (mostly the green microalgae), nitrogen deficiency results in lipid accumulation that is higher than the amount of total biomass. Rodolfi et al. (2009) noted that growing the microalgae species *Chlorella* and *Scenedesmucs* under nitrogen deprivation in freshwater resulted in a reduction in biomass productivity and the growth was ceased after 7 days. However, despite the reduction in biomass productivity, the lipid production was not affected until the growth ceased.

Rodolfi et al. (2009) noted that 90% of the fatty acid content in *Nannochlorpsis* sp. consisted of saturated and monounsaturated fatty acids, which are the main makeup of storage TAGs during nitrogen deficiency. Arnold (1971) reported that *Chlorella* species showed an increase in fat production from 28 to 70% during nitrogen deprivation at the expense of proteins which decreased from 30 to 8%. Rai and Gaur (2001) noted that *Synechococcus* in nitrogen limited environments showed signs of protein destruction.

However, Saha et al. (2003) reported that nitrogen deprivation reduced the formation of lipids and fatty acids.

The nitrogen source present affects the pH, if ammonium salt is present and absorbed by the algal cells then the pH decreases (Round, 1973). In Blue-green algae, the element molybdenum is required for nitrogen fixation and assimilation of nitrogen, but when the nitrogen source is ammonium salt, no molybdenum is required (Glass et al., 2012). In cultures where nitrogen starvation is in effect, the algal cells assimilate ammonium much more rapidly than normal cells (Ress, 1989) while the nitrate and nitrite assimilation in starved conditions is slower than with ammonium and occurs at the same rate as for the normal cells (Fuggi, 1993). In the dark, the nitrogen starved cells continue to assimilate ammonium until they have exhausted their carbohydrate reserves (Rigano et al., 1990). The cells recovering from nitrogen deficiency experience an increase in chlorophyll content to restore the original rate of photosynthesis (Solovchenko et al., 2008).

It has been reported that under nitrogen sufficient conditions, 13.7% of the DCW of the species *C. vulgaris* consisted of lipids, the major portion being phospholipids with only 3% TAGs. The nitrogen deficiency increased the lipid content to 20% (dry cell weight) and the phospholipids and glycolipid content decreased, with TAGs making up 50%. In an attempt to increase the lipid levels in *C. vulgaris*, the algae culture was made up in 100 ml Erlenmeyer flasks and the light intensity was increased to illustrate similar conditions to those found in natural ponds. It was noted that under nitrogen sufficient environments, the culture had reached its maximum cell density of 4.2 g/L after 9 days with biomass, lipid and biodiesel lipid productivities of 480, 66 and 2 mg/L/day, respectively. In limited nitrogen conditions, it was noted that the cultures with low initial cell densities resulted in the highest cell content. However, the highest TAG contents were achieved in most dense cultures. The composition of the fatty acids produced was altered with oleic acid being the major fraction and the highly unsaturated fatty acids were reduced (in comparison to those in nitrogen sufficient environments) (Stephenson et al., 2010).

Several researchers noted that algal cells do not uptake nitrate as the nitrogen source in the presence of ammonium (Florencio and Vega, 1983; Fernandez and Cardenas, 1982;

and Tischner and Lorenzen, 1979). Scherholz and Curtis (2013) reported significant growth in *Chlorella vulgaris* cultures provided with 0 and 0.0135 g L^{-1} nitrogen in the form of ammonium and that higher levels of ammonium halted the growth of the culture. Rodolfi et al. (2009) noted that nitrogen deprived cultures showed a higher lipid content, and a lower biomass production compared to those with sufficient nitrogen. Costa et al. (2001) noted *Spirulina platensis* biomass yields of 1.559, 0.993 and 0.081 g L^{-1} using sodium nitrate, ammonium nitrate and ammonium sulfate, respectively. They suggest that ammonium is the preferential source of nitrogen for lipid production, but not the best for high biomass yields.

Lin and Lin (2011) reported that unicellular green algae *Chlorella* and *Chlamydomonas* can obtain their nitrogen source from urea. They also noted that the microalgae *Scenedesmus rubescens* achieved a biomass productivity of 0.54 g/L/d and lipid content of 42.9% using the mixture of urea and sodium nitrate as the nitrogen source.

Pai and Lai (2011) noted an increase in algae biomass from 28.3 mg/L to 254 mg/L using ammonium as the nitrogen source. Arumugam et al. (2013) noted that the species *Scenedesmus bijugatus* resulted in equal biomass using urea and nitrates as the nitrogen sources. Danesi et al. (2002) reported on urea having a positive effect on the growth of *Spirulina platensis* when fed continuously with exponentially increasing amounts.

3.5.3.3. Phosphorous: Phosphorous plays an important role in the building blocks of nucleic acids, phospholipids, complex carbohydrates (Rai and Gaur, 2001) normal algal growth (Xin et al., 2010), catabolic and anabolic pathways and the conversion of energy through the energy rich phosphoanhydride bonds (Rai and Gaur, 2001).

Xin et al. (2010) reported on increasing the lipid accumulation to 53% of *Scenedesmus sp*. by limiting phosphorous concentrations to 0.1 mg/L. Khozin-Goldberg and Cohen (2006) noted that *Monodus subterraneus* had higher levels of lipid accumulation (mainly TAG) as a result of phosphorus limitation.

Rodolfi et al. (2009) reported that during phosphorous starvation *Nannochloropsis* survived a week before a decrease in productivity was noted and the growth was halted after 20 days. The same fatty acids observed to increase in nitrogen-starved environments

were increased from 13.2% to 50.1% upon phosphorous deprivation. Once phosphate was reintroduced back into the system the growth was immediately resumed suggesting that the stored fatty acids were broken down and used for growth continuation. The lipid content of the phosphorous starved environment consisted of 67% TAGs.

Reitan et al. (1994) observed a higher lipid accumulation in *P. tricornutum* and *Chaetoceros* (Bacillariophyceae) and a decrease in lipid content in *Nannochloris atomus* and *Tetraselmis* during phosphorous deficiency. Reitan et al. (1994) also noted an increase in lipid content in phosphorous deprived cultures and a decrease in biomass as a result of the stress imposed on the system.

3.5.3.4. Sulfur: The lipid content was found to be enhanced under sulfur deprivation in *Chlorella* sp. by Otsuka (1961) and in *C. reinhardtii* by Sato et al. (2000). The chlorophyll content of *Chlorella* was noted to decrease by Mandels (1943) as a result of sulfur deficiency. Matthew et al. (2009) achieved an increase in lipid content with sulfur limitation in *Chlamydomonas reinhardtii*.

Hu et al. (2008) stated that sulfate limitation in microalgae promotes lipid accumulation. Sato et al. (2000) achieved an increase in lipid content with limitation of sulphur in *Chlamydomonas reinhardtii*. Cakmak et al. (2012) noted an increase of 258% in sulfur deprived *Chalamydomonas reinhardtii*, which was higher than the 240% in nitrogen deprived medium. Held and Raymond (2011) noted a 5 fold increase in cellular lipid content of *Chlorella vulgaris* in sulfur deprived media.

3.5.4. Light Requirements

Light regulates and stimulates algal growth (Barsanti and Gualtieri, 2006). Light is an electromagnetic radiator characterized by its quality and possesses different wavelengths and intensities. In order for algae to be able to detect light and react to it they must possess a photocycling protein and have sensitivity to light. Photosynthetic algae obtain their energy and nutrients via photosynthesis which is driven by solar energy that the algae converts it to chemical energy. The chemical energy is stored in organic matter (Chen et al., 2009).

Photosynthesis consists of two reactions, the light and dark reactions. In the light reaction, the photons emitted from the light, are absorbed in order to excite the water

electrons into a higher energy state by the chlorophylls and accessory pigments (Chen et al., 2009). This is converted to nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) and adenosine triphosphate (ATP) with the release of oxygen resulting from the water splitting process. The dark reaction consists of the conversion of CO_2 into sugars, using the energy forms of ATP and NADPH which are generated from the light reaction. The two carboxylation pathways that convert CO_2 into organic carbons in algae are the C3 and C4 carbon fixation pathways. Most algae follow the C3 pathway in order to fix the inorganic carbons. The C3 pathway consists of the enzyme Rubisco that catalyzes the reaction to form a 3-carbon compound, known as phosphoglyceric acid which is converted into sugar after entering the Calvin cycle. Some algae have evolved and follow a C4 pathway. This pathway consists of the initial conversion of CO_2 into a four carbon compound and then releasing the CO_2 in order to undergo fixation by Rubisco (Chen et al., 2009). Rai and Gaur (2001) noted that the photosynthesis in culture environments undergoing nutrient deprivation is utilized for the formation of reduced storage products (fats).

3.5.4.1. Light Intensity: The amount of light that algae are exposed to plays a role in photosynthesis, pigment content and chemical composition (Post et al., 1985; Sukenik et al., 1987; Rodolfi et al., 2009). Low light intensity prompts the synthesis of polar lipids (phospholipids and glycolipids) which function as membrane lipids of the chloroplast (Hu et al., 2008), while high light intensity increases the amount of lipid synthesis in the form of TAGs (Roessler, 1990) at the expense of polar lipid content (Brown et al., 1996; Khotimchenko and Yakovleva, 2005). The high light intensity alters the fatty acid composition and produces natural lipids in the form of mono-unsaturated and saturated fatty acids.

Hu et al. (1996) reported that there exists a relationship between light intensity and biomass production, in which the biomass production and cell concentration were enhanced by maximal mixing at the highest light intensity. Since biomass production requires energy which is obtained from light for photosynthetic algae, the light intensity and degree in which it reaches the algal cells affects biomass production (Chen et al., 2009).

Algae have light harvesting systems that vary depending on the light intensity. With high light intensity, the harvesting system is small. However with low light intensity, the harvesting system becomes large. This variation aids in the protection of the photosynthetic apparatus from damage that may be caused by lights of high intensities (Rai and Guar, 2001). Demmig-Adams and Adams (1992 and 2000) noted that light energy captured in excess is very harmful to sea plants. Nedbal et al. (1996) reported higher rates of growth using flashing light as opposed to continuous light. Yoshimoto et al. (2005) noted that in *Chaetoceros calcitrans*, flashing light enhanced the photosynthesis process.

Algae have evolved and are capable of storing more of the absorbed light when under competition for it (due to low light), but high levels of light have also been noted to cause photoinhibition, biochemical damage to the photosynthetic machinery. The highest efficiency for photosynthetic reactions has been noted at lower light wavelength (blue light) (Scott et al., 2007).

3.5.4.2. Photocycling Protein: When a photon is absorbed, the photocycling protein undergoes various conformational changes which include the active state that will begin the signal transmission. It is restored to its original formation by a second absorption of a photon of different wavelength or by a thermal process. This protein is important to algae because the cells depend on their photoreceptive system (Barsanti and Gualtieri, 2006).

The photoorganisms should possess a high sensitivity to light in order to be able to fully adapt to the changing light conditions. Being able to detect a low photon of light is important because aquatic organisms tend to compete for light in the water due to shading by other organisms (Barsanti and Gualtieri, 2006). Being able to detect light becomes an advantage because light acquiring organisms with a higher sensitivity to light will move to more lit areas in order to obtain their metabolic energy. Light reactions have two major categories: physical and chemical reactions. The physical reaction consists of the energy absorption and transferring it between the different chloroplast pigments (Lewin, 1962). The chemical reaction involves the mechanisms and intermediates of the light energy conversion into chemical energy via the chlorophyll reactions.

3.6. Light Sources

Microalgae can grow under various light sources including natural light and artificial light (red, blue and fluorescent).

3.6.1. Natural Light

Through the process of photosynthesis, microalgae are capable of taking photons from natural light source and converting them into algal biomass (Chen et al., 2009). The absorption of light photons depends on the algal pigmentation cells, culture density, and the specific position of the cells (Chen et al., 2009; Barsanti and Gualtieri, 2006). The quality and intensity of natural light varies from place to place and it also varies daily. Therefore, in an open pond system the algae cultures must adjust to the light intensity in order to be able to sense the light photons for the different seasons and avoid shading by other cells. Algae cultures growing on water surface or close to it may be able to obtain their photons for photosynthesis, but algae growing at a lower depth may not sense the photons from the natural light, thus lower biomass productivity (Chen et al., 2009).

The pigments (chlorophylls) in charge of light absorption have the best absorption at wavelengths of 440 and 680 nm (Chen et al., 2009; Barsanti and Gualtieri, 2006). Thus the white light, which covers the whole spectrum, is not absorbed fully. The unabsorbed light is reflected off or transmitted as wasted energy. Blanken et al. (2013) noted that using sunlight for algae is advantageous because of its abundance and free cost. Sarisky-Reed (2010) noted that algae require 2800 h of sunlight a year, making outdoor cultivation more economically feasible than using artificial light. Richmond and Cheng-Wu (2001) noted a biomass productivity of 0.35 g/L/d using natural sunlight in an outdoor raceway pond.

3.6.2. Artificial Light

Using artificial light will provide algal growth during the day, as opposed to sunlight which is only available for a certain number of hours each day. Light emitting diodes (LEDs) or fluorescent light may be used for algal growth. The LEDs are more energy efficient than fluorescent lights but cost more. LEDs are capable of converting 80% of electrical energy into radiation energy thus making them more efficient light sources (Chen et al., 2009).

Rodolfi et al. (2009) reported increased biomass productivity from 0.61 to 0.85 g/L/day of the marine species *Nannochloropsis* grown with one sided illumination when the light intensity was increased from 115 to 230 μmol photons/m^2/s. They also noted that the fatty acid content also increased from 14.7 to 19.6% with the increase in the light intensity. The authors also reported that when the light intensity was increased from 115 to 230 μmol photons/m^2/s for the same species under a two-sided illumination, both the biomass production and fatty acid content increased from 0.97 to 1.45 g/L/day and from 24.3% to 32.5%, respectively. The increase in fatty acid content is the result of the increases in saturated and monounsaturated fatty acids which mainly function as storage lipids.

3.6.2.1. Red LED: The red LED is the most attractive for photosynthesis because its emission spectrum corresponds with the energy required for a photon to reach the first excited state of chlorophyll *a* and *b*. In the conversion of photons to biomass, it was concluded that the red LED resulted in the highest growth rate for the species *Spirulina platensis* (Chen et al., 2009). Round (1973) reported that the red light has the ability to increase carbohydrate formation in algae.

Chen et al. (2009) and Rai and Gaur (2001) noted that light consisting of red wavelength was found to be the most efficient in biomass production. Algae are in competition for the red photons because they are strongly absorbed by water molecules and as such is not available for algal photosynthesis in most habitats (Rai and Gaur, 2001). Light of shorter wavelength may also not reach algae because the photons may be absorbed by the solute and other organic materials that are capable of retaining the light. It may also be scattered from the surface by suspended materials (Rai and Guar, 2001). Only 10-30% of the light intensity reaches the molecules under the water surface forming a gradient in the quality and quantity of light going from the surface of the water to deeper layers (Rai and Guar, 2001).

3.6.2.2. Blue LED: The blue light photons consist of 40% more energy than those of the red light, making them suitable for chlorophyll absorption. However, in the conversion of photons to biomass, it was concluded that the blue LED showed the least efficiency (Chen

et al., 2009). Kowalik and Bartling (1984) reported that blue light increases the production of RNA and protein content in the algal cells.

3.6.2.3. Fluorescent light: A study done on the growth of *Chlorella vulgaris* under artificial fluorescent light illustrated no difference in the overall total biomass, but are comparable to LED (Chen et al., 2009). Lee and Palsson (1996) noted that the *Chlorella vulgaris* species grown resulted in double the cell volume of that cultivated under red LED.

3.7. Algae Production Systems

Algal cultivation can be done in either open or closed system as long as the light and right nutrients needed for growth are present. In open systems, it is much harder to control the amount of nutrients entering the system. On the other hand, closed systems are much easier for algal cultivation but cost more than open systems. Sunlight energy for algal cultivation can be utilized in open pond systems and closed pond systems (Demirbas, 2011). Microalgae production systems include open pond (circular and raceway), enclosed photobioreactors (tubular, plastic bag and plate types,) and hybrid systems.

3.7.1. Open ponds

For mass cultivation, open ponds are the simplest and oldest methods known for microalgae cultivation. Open pond systems for algal cultivation are shallow, with nutrients entering the systems via runoff water. Nutrients may also be obtained from sewage water (Demirbas, 2011; Demirbas, 2010). Although open ponds require low operation and construction costs, they have many limitations including low productivity, temperature fluctuation, water loss via evaporation, high harvesting costs, low dissolved carbon dioxide and contamination by predators (Chen et al., 2009). The productivity of open ponds is assessed based on the biomass production per day per unit of surface area available (Demirbas, 2010). There are various types of open pond systems including circular pond and raceway ponds. Tafreshi and Shariati (2006) reported that in an open pond system *Dunaliella salina* was successful at producing carotenoids which protect it from harsh climate conditions.

3.7.1.1. Circular Pond: Agitation in circular ponds is provided by the rotation arm as shown in Figure 3.7. The pivoted agitator arm can extend 45 m in diameter. Circular ponds are less popular than raceway ponds because of their high construction expenses. They are made up of concrete and consume a lot of energy for stirring. They are also inefficient in land use and face high complexity when it comes to supplying CO_2 (Chen et al. 2009; Demirbas, 2010).

Borowitzka (1999) reported on a large scale (15,000 L) production facility in Taiwan utilizing *Chlorella* spp. using circular ponds with a rotating arm. Henrikson (2011) reported on a mass cultivation of *Spirulina* and *Chlorella* in Taiwan using circular ponds producing hundreds of tons of algae per year. Ranga Rao et al. (2012) noted a biomass productivity in *Botryococcus braunii* of only 1.25 g/L/d in a circular pond in comparison to 1.75 g/L/d in a raceway pond.

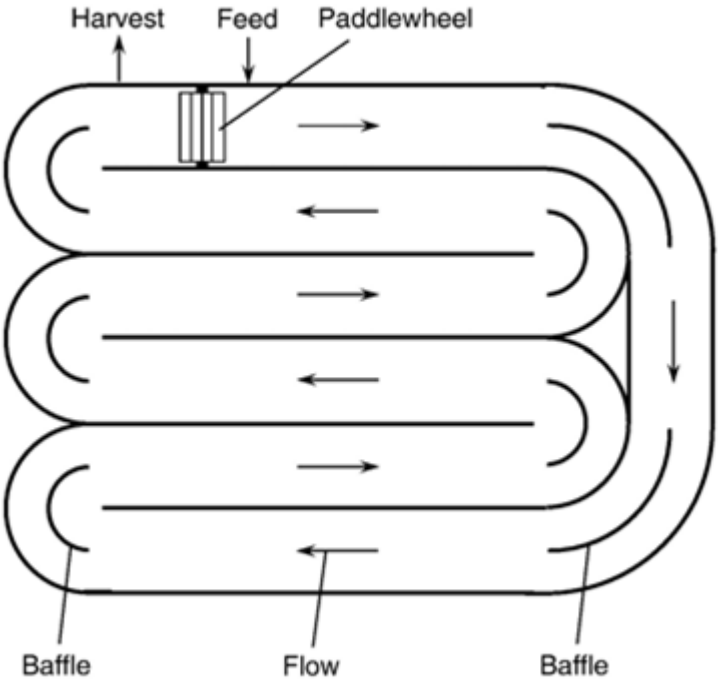
3.7.1.2. Raceway Pond: Raceway pond designs consist of a paddle wheel, propeller or air lift pumps which function to circulate and mix the water, algae and nutrients around the pond as shown in Figure 3.8 (Chen et al., 2009). Agitation and circulation is produced by the paddle wheel which operates continuously in order to bring the algae to the surface of the water and to prevent sedimentation. Shallow ponds are necessary for algal exposure to sunlight due to its limited penetration (typically 15-25 cm deep). Raceway ponds can be made from concrete or more simply earth dug and lined with a plastic liner. The plastic liner prevents the water from seeping into the ground. In this system, the water and nutrients are fed into the pond continuously while the water containing the algae is removed on the other end (Demirbas, 2010; Demirbas, 2011). Jorquera et al. (2010) calculated the positive net energy ratio for raceway ponds as follows:

$$\text{Net Energy Ratio} = \frac{\Sigma\, Energy\ Produced\ (lipid\ or\ biomass)}{\Sigma\, Energy\ requirments} \quad (1)$$

Moheimani and Borowitzka (2006) achieved a lipid content of 33% and a productivity of 0.19 g/L/day for *Pleurochrysis carterae* cultivated in a raceway pond. Blanco et al. (2007) reported on lutein rich cells of *Muriellopis* sp. cultivated in a raceway pond operated with a wheel paddle. Hase et al. (2000) cultivated *Chlorella* sp. and *Chlorophta* in a raceway system and achieved a stable photosynthetic efficiency. Jimenez et al.

Figure 3.7. Circular pond (Henrikson, 2011).

(a) Paddle wheel



(b) Raceway pond schematic

Figure 3.8. Raceway pond for algal cultivation (Chisti, 2007; Henrikson, 2011).

(2003) reported on *Spirulina* cultivated in raceway ponds with a productivity of 10.3 $g/m^2/d$ over the duration of 9 months. Borowitzka (1999) reported on large scale production facilities using raceway ponds capable of holding 3 000 L of algae cultures.

3.7.2. Enclosed Photobioreacters

Although the cost of enclosed production systems is much higher than open ponds, they require less light and land area. Cultivation of algae in photobioreacters (PBRs) does not only function to grow the algae for oil, but also removes nutrients from wastewaters and adsorb CO_2 released into the atmosphere by power plants (Demirbas, 2011). PBRs are closed bioreactors consisting of a light source. Enclosed PBRs can achieve high cell densities and are easily maintained (Chen et al., 2009). Tubular and plate are the two major types of PBRs. Their advantages over open pond systems include their narrow light path (1.2-1.3 cm) which allows for more cell concentration, large illuminating area and less contamination. Disadvantages of enclosed PBRs include wall growth, fouling, formation of dissolved oxygen and CO_2 along the tube, pH gradients, hydrodynamic stress and high cost (Chen et al., 2009). Singh and Gu (2010) stated that although enclosed photobioreacters produce higher fuel per hectare in comparison to open ponds, the start-up cost is much greater.

3.7.2.1. Tubular Photoreactor: Tubular PBRs consist of several horizontal, vertical or inclined tubes made up of transparent glass or plastic and run parallel to one another as shown in Figure 3.9 (Demirbas, 2011; Chen et al. 2009). Due to the amount of sunlight capable of penetrating into the tubes, the diameter of these tubes is typically 0.1 m or less, in order to achieve high biomass productivity (Demirbas, 2011). Mixing can be accomplished in the system by an airlift or a pump system (Chen et al., 2009). The advantages of using a tubular photobioreactor include its large illumination surface area, good biomass productivity, and suitability for outdoor cultures.

Kong et al. (2009) achieved a productivity of 2.0 g/L/day of *Chlamydomonas reinhardtii* grown in a vertical coil reactor. Ugwu et al. (2008) reported on *Porphyridum cruentum* grown in an airlift tubular photobioreactor achieving a productivity of 1.50 g/L/d. Lee et al. (1995) reported on a tubular PBR that achieved a biomass density of

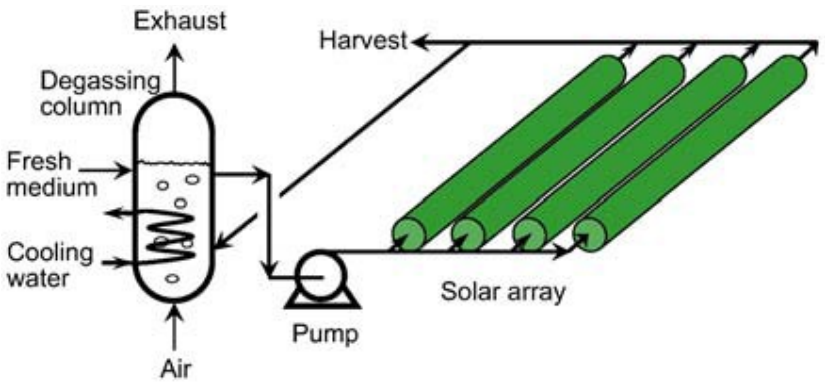


Figure 3.9. Tubular photobioreactor (Chisti, 2007).

more than 72 g/m^2/d. Alias et al. (2004) achieved a productivity of 0.25 g/L/d from *S. Platensis* grown in a horizontal tubular reactor. Richmond et al. (1993) reported a biomass productivity of 1.60 g/L/d of *Spirulina platensis* cultivated in a tubular horizontal PBR. Grima et al. (1994) reported a 0.32 g/L/d biomass productivity of *Isochrysis galbana* using a horizontal tubular PBR.

3.7.3.2. Plate Type Photoreactors: Plated PBRs (Figure 3.10) are made up of a transparent material. The large surface area allows for more illumination and thus increased photosynthesis (Demirbas, 2010; Demirbas, 2011). Plate PBRs also possess low concentrations of accumulated dissolved oxygen. The plate PBRs can be horizontal, vertical and inclined (Chen et al., 2009). Jorquera et al. (2010) reported a positive net energy ratio for flat-plate PBRs, the resulting energy is higher than that required for operating.

Cuaresma et al. (2009) cultivated *Chlorella sorokiniana* in a flat panel photobioreactor and achieved a productivity of 12.2 g/L/d. Ugwu et al. (2008) reported a productivity of 0.27 g/L/d from *Nannochloropsis sp*. grown in a flat plate reactor. Hu et al. (1996) noted of *Spirulina platensis* grown in an inclined flat plate PBR achieved a 4.30 g/L/d biomass productivity. Hu et al. (1998) achieved growth rate of 380 mg/L/h for the marine species *Chlorococcum littorale* using a flat-plate PBR. Zhang et al. (1999) reported a 32.2 g/m^2/d biomass productivity of *Synechocystis aquatilis* cultivated in a vertical flat-plate PBR. Tredici and Zittelli (1998) reported a 24.2 g/m^2/d biomass productivity for *Spirulina platensis* grown in a horizontal flat-plate PBR.

3.7.3. Hybrid Systems

A hybrid system is a combination of both open pond and closed bioreactor (Chen et al. 2009; Demirbas, 2011). A mix of both open and closed systems is most likely the most cost effective algae cultivation method (Demirbas, 2011). Benemann and Oswald (1996) reported on a hybrid system that resulted in an algal lipid yield of 35% at an aerial productivity of 70.4 mg/m2/d. Huntley and Redalje (2007) reported on Haematococcus pluvialis cultivated in a two stage system for the production of oil and astaxanthin (salmon feed) which achieved an oil production rate of 10 ton/ha annually. Rodolfi et al. (2009) described a two stage production plantthat was dedicated to 22% algae biomass

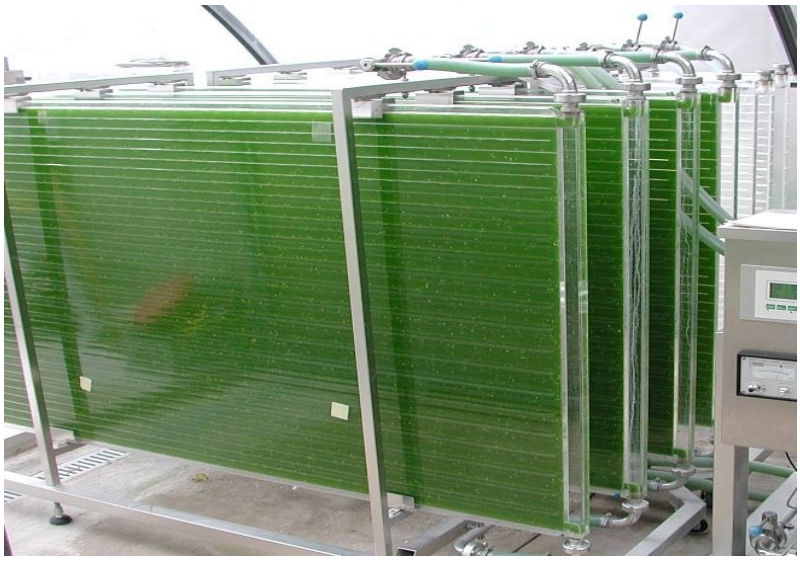


Figure 3.10. Plate type photobioreactor (IGV Biotech, 2003).

production and 78% oil production, first stage is to grow algae biomass in a photobioreactor and the second stage is for oil accumulation in an open pond. Huntley and Redalje (2007) reported on *Haematococcus pluvialis*, cultivated in a two stage system for the production of oil and astaxanthin (salmon feed), achieving an oil production rate of 10 tons/ha annually.

3.8. Utilization of Algae

Algal biomass can be used to produce non-fuel products such as food supplements and livestock feed. They may also be used to produce biofuels including biodiesel, biomethane and bioethanol.

3.8.1. Non-Fuel Products

Algal biomass possesses high protein content and other health beneficial ingredients. The algal biomass can be used as feed for human consumption or animals (fish, pets and farm animals) (Chen et al., 2009).

3.8.1.1. Food Supplements and Other Compounds: Microalgae are a source of Omega 3 fatty acids, eicosapentaenoic acid (EPA), chlorophyll, and docosahexaenoic acid (DHA) (Luiten et al., 2003). Harun et al. (2010) reported that microalgae naturally contain Omega 3 fatty acids, which can be extracted via purification to provide a high value food supplement. These are a major group of high value chemicals that contain PUFA. These chemicals are important source of nutrients for humans and animals.

The C*rypthecodinium* and *Schizochytrium* species are capable of producing DHA, which is helpful in the development of infant brain and eyes as well as improving the cardiovascular health of adults (Chen et al., 2009). DHA is also in clinical use for a cure and prevention of cancer, AIDS, heart disease to control or lower cholesterol, boost immune system as well as a body detoxification (Singh and Gu, 2010). Pyle et al. (2008) reported of DHA production form *Schizochytrium* fermentation of crude glycerol. Burgess et al. (1993) noted that *Isochrysis galbana* produced 5.4 mg/g DHA in a closed photobioreactor. Jiang et al. (1999) reported on *Crypthecodinium cohnii* consisting of 51.12% DHA and a biomass concentration of 2.04 g/L. Kyle and Gladue (1991) reported on *Crypthecodinium cohnii* biomass consisting of 15-30% oil of which DHA is 20-35%.

EPA is a compound known to play a role in the prevention and treatment of various human diseases and disorders. EPA is used in the medical industry as a source of treatment for inflammatory and heart diseases such as asthma, arthritis, migraine headache and psoriasis (Singh et al., 2005). This compound is found in the microalgae species *Nannochloropsis*, *Phaeodactylum,* and *Nitzschia* (Chen et al., 2009). Ohta et al. (1993) noted a high EPA portion in *Porphyridium propureum*. Renaud et al. (1999) noted an EPA content of 8.7 and 12.0% in *Rhodomonas* sp. and *Chromonas* sp., respectively. Blanchemin and Grizeau (1999) noted a 29.2% EPA content in the diatom *Skeletonema costatum*. Wen and Chen (2000) reported an EPA content of 19.1% in *Nitzschia laevis*. Yongmanitchai and Ward (1991) reported that over the pH range of 6.0-8.8, the maximum EPA was achieved at a pH of 7.6 in *P. tricornutum*.

Chlorophyll is a product that can be obtained from microalgae (Singh and Gu, 2010). Chlorophyll is of great importance in the pharmaceutical industry and can be used as ointment treatment, liver recovery and ulcer treatment. It is capable of repairing cells, increasing haemoglobin in the blood stream and increases cell growth (Puotinen, 1999). Ferruzi and Blakeslee (2007) noted that algae chlorophyll can be used in food products and pharamaceuticals because of their anti-inflammatory properties and that it makes up 0.5-1.5% of dry matter. Danesi et al. (2002) reported that the chlorophyll amount present in *Spirulina platensis* was not affected by the addition of urea as the nitrogen source. Henriques et al. (2007) noted that chlorophyll content achieved from *Nannochloropsis gaditana* was three times less than the concentration achieved using methanol solvent.

3.8.1.2. Livestock Feed: Halama (1990) and Phang (1992) reported that algae are an important source of nutrients for animals, fish, and humans. After the extraction of oil from the algae, the remaining biomass, which is high in protein, can be utilized for livestock feed, thus reducing the amount of waste generated by algal biomass (Demirbas and Demirbas, 2011). Da Silva and Barbosa (2008) reported an increase in shrimp growth rates when their feed diets consisted of algae because algae are rich in protein. Algae present in chicken feed reduced the cholesterol level of egg yolk by 10% and the color of the egg yolk became darker, indicating a high carotenoid content (Ginzberg et al., 2000). Schlichting (1971) reported that the brown marine algae (*Ascophyllum*) has been used as livestock feed in Great Britain and Ireland.

3.8.2. Biofuel Products

Microalgae strains that are selected for biodiesel production must yield a high lipid content (Rodolfi et al., 2009). Microalgae are capable of producing 20,000-80,000 L oil per acre per year, making algae oil production 7-31 times greater than palm oil crops, which are the next best producing crops (Demirbas, 2010). Various types of algae contain high percentages (by weight) of oils which is suitable for production of biodiesel, while other species possess high sugar content which is suitable for production of bioethanol (Demirbas, 2010). Algae appear to offer a viable alternative for biofuel production due to their abundance and cellular structure. The waste generated from the algal biomass can be further utilized to produce biofuels such as methane and ethanol via fermentation or used as animal feed or organic fertilizer (Chen et al. 2009; Demirbas, 2010; Demirbas, 2011).

3.8.2.1. Biodiesel: Biodiesel is a fuel form that generates the same calorific value as that generated from petroleum diesel without the release of harsh compounds (NOx, SOx and HC) into the atmosphere, it is biodegradable and nontoxic and is a much cleaner energy source and environmentally friendly (Ulusoy et al., 2004; Demirbas, 2005; Kalam and Masjuki, 2005). The biggest advantage of biodiesel, compared with other alternative transport fuels, is that it can be utilized in existing diesel engines without any modification (Singh and Gu, 2010). Table 3.10 lists the advantages and disadvantages associated with utilizing algae for biodiesel production.

Biodiesel as a liquid fuel can be produced by transesterification of oil found in algae (Demirbas and Demirbas, 2011; Wahlen et al. 2011). Algae generate oil in the form of triacylglycerols which can be converted into biodiesel by the addition of methanol and the use of a catalyst (Chen et al., 2009).

Mata Teressa et al. (2010) reported that microalgae have a much higher oil content than vegetable crops. Table 3.11 lists the oil content achieved by various microalgae species (Chisti, 2007).

Sheehan et al. (1998) noted that for biodiesel production, the algae strain should be capable of producing high lipid content in nutrient sufficient or deficient environments,

Table 3.10. Advantages and disadvantages of using algae for biodiesel production (Hu et al. 2008; Johnson and Wen, 2009; Rodolfi et al. 2009; Pokoo-Aikins, 2010; Singh and Gu, 2010; Demirbas, 2011; Demirbas and Demirbas, 2011; Liu et al. 2011;).

Advantages	**Disadvantages**
• No land space competition with other crops • Capable of growing in water with very high salt levels, no need for freshwater use • Require less water than oilseeds • Require less land for growth than terrestrial plants • Utilize CO_2 for growth, removing the common industrial pollutant from the atmosphere • Rapid growth rate • High intracellular lipid content • High volumes of algal biomass can be achieved yielding higher oil content than other sources • Algal oil has limited competition in the market • Consume resources that are otherwise considered waste • Algal biofuel contains no sulfur, is non-toxic, and highly biodegradable • Algal biodiesel consisting of high levels of polyunsaturates is suitable for cold weather • Algal fertilizers can be obtained from wastewaters • Reduce nitrous oxide release • Can be used in diesel engines without much modification	• Open pond culture systems are prone to contamination • Many polyunsaturates make biodiesel unstable • In comparison with its mainstream alternative, biodiesel has poor performance • Difficult to maintain certain outdoor cultures • High energy inputs for mixing the culture, CO_2 transfer, harvesting/dewatering • Concentration of biomass is low due to poor light penetration • Require more nitrogen fertilizer (8-16 tons/ha/year) than plants which could damage the environment if commercial fertilizers used.

Table 3.11. Various microalgae oil content (Chisti, 2007).

Microalgae	**Oil Content (% Dry Weight)**
Botryococcus braunii	25-75
Chlorella sp.	28-32
Crypthecodinium cohnii	20
Cylindrotheca sp.	16-37
Dunaliella primolecta	23
Isochrysis sp.	25-33
Monallanthus salina	>20
Nannochloris sp.	20-35
Nannochloropsis sp.	31-68
Neochloris oleoabundans	35-54
Nitzschia sp.	45-47
Phaeodactylum tricornutum	20-30
Schizochytrium sp.	50-77
Tetraselmis suecica	15-23

although these two are mutually exclusive to one another. The cell wall and the amount of PUFA present in algae strains must also be considered when they are desired for biodiesel production. The main substrate desired for biodiesel production is saturated and monounsaturated fatty acids that are stored in the organism in the form of TAGs (Rodolfi et al., 2009; Pokoo-Aikins et al., 2010; Johnson and Wen, 2009). The extracted algal oils (TAGs) are converted into fatty acid methyl esters by mixing them with an alcohol (usually methanol) and an acid or a base as the catalyst for the reaction (Demirbas and Demirbas, 2011).

Biodiesel from microalgae must meet the international standards which include a concentration of phosphorous and sulfur no more than 10 ppm. Thus, the removal of phospholipids must be achieved before conversion of oil to biodiesel because it promotes water accumulation, increases the consumption of the catalyst during alkaline-catalyzed transesterification and increases the phosphorous content in the oil. The glycolipids are noted to increase the sulfur content of the biofuel (Stephenson et al., 2010).

Miao and Wu (2006) reported a biodiesel yield of 70% from *Chlorella protothecoides* at 50°C using acid as the catalyst. Li et al. (2011) reported 95% biodiesel yield using *Chlorella pyrenoidosa* with rice straw hydrolysate as the lignocellulose-based carbon source. Johnson and Wen (2009) achieved a biodiesel conversion efficiency of 95.9% from the oil extracted (57%) from *Schizochytrium limacinum* using an extraction-transesterificaiton method. Stephenson et al. (2010) reported that *Chlorella vulgaris* are capable of producing 8200 L/ha/year of biodiesel. Rodolifi et al. (2009) reported that *Nannochloropsis* can produce 23,000-34,000 L/ha/year of biodiesel.

3.8.2.2. Biomethane: Anaerobic digestion of algal waste results in the production of methane, carbon dioxide and ammonia. The residues produced in the anaerobic digestion process maybe further used to produce fertilizers (Singh and Gu, 2010). Nitrogen and phosphorous compounds are rich in algal fertilizer (Demirbas, 2010). Methane production from algae can be enhanced by pre-treating the cells in order to break down the cell wall order to make organic matter more accessible to microbes (Demirbas, 2010). The methane produced from the anaerobic digestion can be converted into electricity or used as a fuel gas (Hon-Nami, 2006).

Vergara-Fernandez et al. (2008) stated that algae exhibit a good process stability and high conversion efficiency for anaerobic digestion because of the absence of lignin and lower cellulose. The biogas production by the anaerobic process is affected by the organic loading, pH, temperature and the retention time. High methane yields are achieved with long solid retention times and high organic loading rates (Chynoweth, 2005).

Chynoweth (2005) reported that the *Macrocyotics* and *Laminaria* sp. had a methane yield of 0.39-0.41 and 0.26-0.28 m^3/kg, respectively. Bird et al. (1990) noted a biomethane yield of 0.28-0.4 m^3/kg from *Gracilaria* sp. Morand and Briand (1999) reported on the algae *L. digitata* producing a methane yield of 0.5 m^3/kg. Briand and Morand (1997) noted that *Ulva* sp. produced a methane yield of 0.2 m^3/kg solids.

3.8.3.3. Bioethanol: Ethanol is produced from crops via fermentation, in which the sugars and starch are converted into ethanol (Chen et al. 2009; Demirbas, 2011). Algae have high degrees of carbohydrates and proteins which can be utilized as carbon sources for fermentation (Singh and Gu, 2010). In algal biomass, ethanol production can be achieved by firstly releasing starch from the cells using mechanical equipment or enzymes. The fermentation begins by the addition of yeast (*Saccharomycess cerevisiae*) into the biomass, resulting in ethanol, water and CO_2 as the by-products as follows (Chen et al. 2009; Demirbas, 2010; Demirbas, 2011).

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \quad (2)$$

Harun et al. (2010) reported a 38% (by weight) ethanol using the yeast species *Chlorococum* sp. Harun and Danquah (2011) reported an ethanol yield of 53% (by weight) from *Chlorococcum humicola*.

3.9. Algae Harvesting Methods

The algal biomass must be harvested and treated to release TAGs which can then be utilized to produce biodiesel. It is best to release the oil from the algal biomass while avoiding contamination of DNA and chlorophyll which are also present in the cells (Scott

et al., 2010). There are a number of harvesting techniques including membrane filtration, chemical flocculation, air flotation, centrifugation and ultrasound wave.

3.9.1. Membrane filtration

Membrane filtration is a simple method used for removal and collection of algal cells. The easiest way to use this method is using a vacuum pump. Modified fibers or cellulose are used in filters. The problem associated with this method is membrane clogging or fouling due to the ability of algal cells to penetrate into the membrane. In order to avoid cell penetration into the filter, a reverse-flow vacuum filter method is utilized to move the liquid upward across the membrane (Chen et al., 2009). A drum filter and disc filter can also be used.

Tsukahara and Sawayama (2005) used membrane filtration for *B. braunii* and achieved high cell density. Sawayama et al. (1992) filtered *Botryococcus braunii* through filter paper of 0.2 μm pore size to recover the algal cells grown in secondarily treated sewage from domestic wastewaters. Grima et al. (2003) noted that microfiltration is a cost effective method for small volumes of algae (less than 2 m^3/d) compared to centrifugation.

3.9.2. Centrifugation

This method is more commonly used either on its own or as a second step for further water removal. Cream separator centrifuges are used to separate large volumes of algal cultures. In this method, the algal cells form a paste on the walls of the centrifuge tubes (Chen et al., 2009).

Heasman et al. (2000) reported a harvesting efficiency of 95-100% and a cell viability of 88-100% using centrifugation at 13000xg. Sim et al. (1988) compared air flotation, drum filtration and centrifugation and noted that the most efficient method for biomass recovery was centrifugation. Chen et al. (2011) reported a 80-90% microalgae recovery efficiency from the liquid media using centrifugation at 500-1000x g. Grima et al. (2003) noted that the preferred method for microalgae biomass recovery is centrifugation.

3.9.3. Chemical Flocculation

Algal cells possess a negatively charged surface, which allows the separation from one another upon suspension. The surface charge of the cells may be disrupted by the addition of iron, alum, lime, cellulose, salts, polyacrylamide polymers, surfactants and chitosan, which would result in cell flocculation and settling. Filtration through this method has been reported to result in 95% recovery of the algal cells from the culture media (Chen et al., 2009).

Bilanovic and Shelef (1988) reported that microalgae flocculation was effective at salinity levels less than 5 g/L. Sukenik et al. (1988) reported that marine microalgae *Isochrysis galbana* and *Chlorella stigmatophora* require 5-10 times more flocculent dosages than those required by freshwater microalgae. Morales et al. (1985) noted a 100% flocculation efficiency using chitosan concentration of 40 mg/L in 20 L batch cultures of *Chlorella* sp. and *Thalassiosira nordenskoldii*.

3.9.4. Air Flotation

Flotation method for filtration of algal cells is performed by the generation of fine air bubbles, through the injection of air. Air is then adhered to the cells, causing them to float to the surface of the column as a form of foam. The foam formed on the surface top may be removed or the water may be drained from below. Different types of flotation methods may be performed including air flotation, dissolved air flotation and suspended air flotation. This method is expensive and impractical because it requires a lot of energy for air compression (Chen et al., 2009).

Wiley et al. (2009) reported that suspended air flotation and dissolved air flotation capture efficiencies of microalgae of 76.6% and 84.9%, respectively. Golueke and Oswald (1965) compared flotation, filtration and centrifugation for algal removal and concluded that centrifugation was the only economically feasible method. Boussiba et al. (1988) reported a 100% biomass removal efficiency by means of flocculation with 180 mg/L of $FeCl_3$ and dissolved air flotation using *Isochrysis galbana*. Chen et al. (1998) noted that removal of microalgae is more efficient by flotation in comparison to sedimentation.

3.9.5. Ultrasound Wave

In this method the algal cells agglomerate to the low pressure nodes of ultrasound waves, generated by low energy ultrasound waves. The particle-particle interaction and acoustic interaction forces aid the mass collection of the cells. When the ultrasonic field is turned off, the cells settle by gravity. This technique for dewatering of algal biomass is non-fouling, free of mechanical failures (no movement of parts involved) and offers continuous operation. However, this method requires high consumption of power and is only used for low concentrations of biomass (Chen et al., 2009).

Bosma et al. (2003) reported efficiencies higher than 90% using ultrasound separation of *Mondus subterraneus*. Zhang et al. (2006) noted that ultrasonic irradiation improved algae settle ability, but also change the structure of the algal cells.

3.10. Oil Extraction

Prior to conversion of algal biomass to biodiesel oil extraction must firstly be performed (Li et al., 2011). There are four common methods for oil extraction: oil press, solvent extraction, supercritical fluids and ultrasound (Demirbas and Demirbas, 2011). Table 3.12 summarizes some of the advantages and limitations of oil extraction methods.

3.10.1. Oil Press

This method works by applying pressure to break the cells in order to compress the oil out. The oil press method is the simplest way known for oil extractions and is capable of extracting 70-75% of the algal oil (Singh and Gu, 2010; Demirbas and Demirbas, 2011). Popoola and Yangomodou (2006) reported an oil extraction efficiency of 75% using oil press. Demirbas (2009) reported a microalgae oil extraction efficiency of 70-75% using the oil press method. Singh and Gu (2010) stated that for maximum efficiency of this method, the algae should first be dried. Despite the high extraction efficiency and simplicity of this method, it has been noted to be less efficient than other methods because of the long extraction time required (Popoola and Yangomodou, 2006).

Table 3.12. Advantages and limitations associated with various microalgae oil extraction methods (Harun et al., 2010)

Extraction Method	Advantages	Limitations	Reference
Oil Press	No solvent required Easy to use	Time consuming Large amount of sample required	Mata et al. (2010)
Solvent Extraction	Inexpensive solvents Reproducible	Organic solvents are highly flammable or toxic Energy intensive solvent recovery Large volume of solvent required	Herrero et al.(2004) Galloway et al.(2004)
Supercritical Fluid Extraction	Non-toxic Non Flammable Simple operation	Often fails in large extractions of polar analyte Insufficient interaction between supercritical CO2 and the sample	Macias-Sanchez et al. (2005) Pawliszyn, (1993)
Ultrasound	Reduced extraction time Reduced solvent use Higher solvent penetration Improves release of cell content into the medium	High power consumption Scale up difficulty	Luque-Garcia and Castro, (2003) Martin, (1993)

3.10.2. Solvent Extraction

Microalgae oil extraction can also be achieved via solvent extraction. In this method, the wet algae paste (in water) is extracted utilizing solvents (benzene, cyclo-hexane, hexane, acetone, or chloroform) which break the algal cell walls (Singh and Gu, 2010). By breaking the algal cell wall, the oil can then be extracted from the aqueous medium, due to its higher solubility in the organic solvents in comparison to water. The oil can then be separated via distillation from the solvent extract (Singh and Gu, 2010). For maximum extraction efficiency, the solvent used should possess several features; (a) lipid polarity of the organic solvent should match those of the cells and (b) solvent should be cheap, (c) solvent should be easily removed and (d) should be toxic free, water insoluble and ideally recyclable (Chen et al., 2009). However, on a large commercial scale this method would be impractical because these solvents are environmentally destructive and high in costs (Singh and Gu, 2010; Demirbas, 2011).

Demirbas (2009) and Serrato (1981) reported that hexane solvent would be the most efficient for algal oil extraction because it has the highest extraction capability and is low in cost. Fajardo et al. (2007) reported an 80% lipid recovery via two stage extraction, by ethanol extraction first followed by hexane in order to purify the lipids. Xiou and Xu, (2005) reported that butanol is effective in extracting lysophospholipids, but the high boiling point of this solvent makes it difficult to evaporate and its high polarity tends to extract more impurities. Hexane is also an inexpensive chemical known for algal oil extraction (Demirbas and Demirbas, 2011). Chen et al. (2009) noted that the mixture of chloroform-methanol provided the highest extraction efficiency of microalgal lipids. Li et al. (2011) noted that methanol is a poor solvent for oil extraction and since it plays the major role in transesterification for the production of biodiesel from algal biomass, solvents such as n-hexane or chloroform must firstly be used since they have been noted to be good oil extractors.

3.10.3. Supercritical Fluid

Fluids above their critical point are known as supercritical fluids. The diffusivity is enhanced and the viscosity of the fluid is decreased upon its critical point. Such properties allow fluids to diffuse easily through solid materials as opposed to non-super

critical fluids (Chen et al., 2009). The supercritical fluid extraction (SFE) method for oil extraction is the most efficient of the three methods because of its high selectivity, time efficiency, and free of toxicity (Chen et al. 2009; Singh and Gu, 2010; Demirbas and Demirbas, 2011). It results in a product of high purity (Chen et al. 2009; Demirbas and Demirbas, 2011). If carbon dioxide is the chemical used for extraction, it would have to be liquefied under heat and pressure to a point where its properties consist of both liquid and gas, acting as the oil extracting solvent (Demirbas and Demirbas, 2011).

This method utilizes high temperatures and pressures in order to rupture the algal cells (Singh and Gu, 2010). Canela et al. (2002) reported that the pressure and temperature of SFE do not influence the yield of the extracted compounds, but instead influence the extraction rate. Andrich et al. (2005) reported that over the temperature range of 45-55°C and pressure of 400-700 bar, there was no impact on the extraction of bioactive lipids (PUFA) from the species *Nanochloropsis*. Demirbas (2009) and Demirbas and Demirbas (2011) reported that supercritical fluid is capable of extracting 100% of oils. Andrich et al. (2006) reported a higher PUFA extraction yield from the species *Spirulina platensis* using SFE system as opposed to solvent extraction method.

3.10.4. Ultrasound

Ultrasound is another method that can be used for oil extraction. In this method, algae are exposed to high intensity ultrasonic waves, creating tiny cavitation bubbles around the cells. The desired compounds are released into the solution when the bubbles collapse and emit shockwaves that shatter the cell walls (Singh and Gu, 2010).

Wiltshire et al. (2000) reported a 90% extraction efficiency of fatty acids and pigments from the species *Scenedesmus obliquus* using ultrasound extraction. Pernet and Tremblay (2003) concluded that the ultrasonic method for oil extraction from *Chaetoceros gracilis* increased the extraction rate, which affects the recovery of lipid extracts. Hu et al. (2007) reported an oil extraction yield of 93% in adlay seeds using ultrasound assisted supercritical fluid extraction.

3.10.5. Liquefaction

A more practical and effective method for algal oil separation of hydrocarbons to be utilized as liquid flues is liquefaction of the algal cells with high moisture content

(Demirbas, 2011). Hydrothermal liquefaction is done in an aqueous solution consisting of alkali or alkaline earth salts at temperatures of 575 K and pressures of 10 MPa (Demirbas, 2010; Demirbas, 2011). High conditions such as this result in the formation of supercritical water, thus enhancing the reaction rate (Chen et al., 2009). In this method, the algal cells are liquefied and the product is then extracted with dichloromethane (CH_2Cl_2) to separate the oil fraction (Demirbas, 2011). This method is performed utilizing a stainless steel autoclave with mechanical mixing (Demirbas, 2010).

Liquefaction of algal cells is advantageous because it does not require water drying (Chen et al., 2009). Algal cell liquefaction is done using hexane, in order to obtain the primary oil (Demirbas, 2011). Sodium carbonate is used as a catalyst in the liquefaction of wet biomass into liquid fuel. The liquefaction of these cells results in an oil-like product by the reaction of carbon monoxide/hydrogen in the presence of sodium carbonate. The oil product is achieved via a series of changes in the chemical and physical properties in the liquefaction process (Demirbas, 2010). Minowa et al. (1995) reported a 37% oil yield from *Dunaliella tertiolecta* using liquefaction method at 575 K.

CHAPTER 4. MATERIALS AND METHODS

4.1. Selection of Microalgae

Several freshwater and marine microalgae species were selected based on their ability to yield high biomass and store lipids.

4.1.1. Freshwater Microalgae

The selected freshwater microalgae (8 species) deemed suitable for biodiesel production (Table 4.1) included *Botrycoccus brauni, Chlorella protothecides, Chlorella pyrenoidsa, Chlorella saccharophila, Chlorella sorokiniana, Chlorella vulgaris, Chlorococcum sp.* and *Scenedesmus obliqus*.

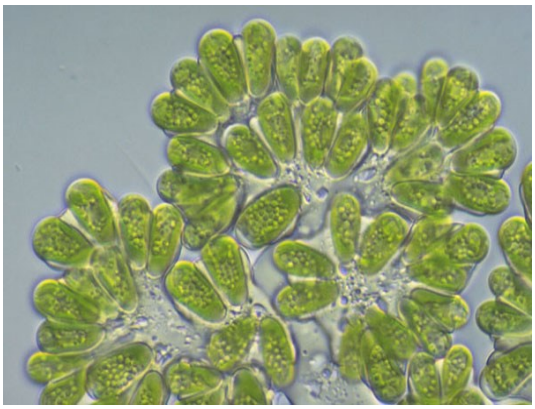
***4.1.1.1. Botrycoccus brauni*:** *Botrycoccus brauni* is a unicellular green freshwater microalga that forms in colonies as shown in Figure 4.1 (Hamana et al., 2013). This species belongs to the Botrycoccus genus. The cells are oval or obovioid in shape and range in size from 7-11 μm. This species can utilize glucose, mannose (Tanoi et al. 2011) and carbon dioxide as the carbon source (Ranga et al. 2007). The optimal temperature and pH for growth of this species are 20°C and 7.6, respectively (Li and Qin, 2005). *Botrycoccus brauni* can yield a biomass of 1.84 g/L and a lipid content of 25.2% (Velichkova et al., 2012).

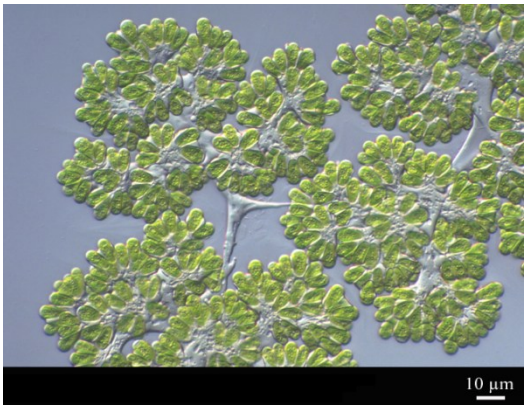
***4.1.1.2. Chlorella protothecides*:** *Chlorella protothecoides* is a green freshwater unicellular microalga belonging to the Chlorella genus. It is a heterotrophic organism that obtains carbon for growth through the consumption of organic or inorganic compounds (EOL, 2012). Cells are spherical with dimensions of 10 μm and possess a chloroplast as shown in Figure 4.2b (Aquaportail, 2012). The optimal temperate and pH for growth of this microalga are 25°C and 6.0, respectively (Shi et al., 2006). The biomass and oil content yields for *Chlorella protothecoides* are 1.32 g/L and 31.2%, respectively (Liu et al., 2011).

***4.1.1.3. Chlorella pyrenoidsa*:** *Chlorella pyrenoidsa* is a green freshwater unicellular microalga belonging to the Chlorella genus. The cells are spherical in shape and are 4 μm in diameter as shown in Figure 4.3 (Czeczuga et al., 2003). This species can utilize glucose, galactose, acetate (Samejima and Myers, 1958) and carbon dioxide (Tanget al.

Table 4.1. Oil content of freshwater microalgae species.

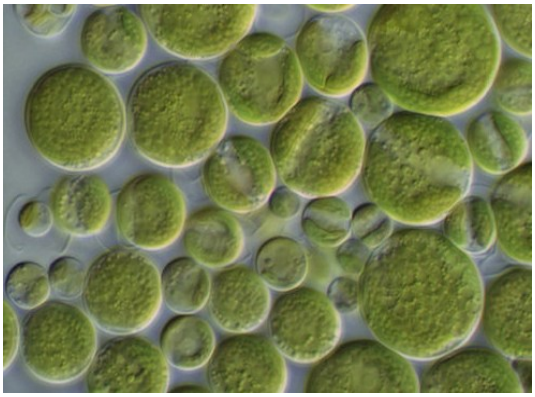
Species	Biomass (g/L)	Lipid Content (%)	Lipid Productivity (mg/L/d)	Temperature (°C)	pH	Reference
Botryococcus brauni	1.84	25.2	5.51	20	7.6	Velichkova et al. (2012)
Chlorella protothecides	1.32	31.23	39.6	25	6.0	Liu et al. (2011) ; Shi et al. (2006)
Chlorella pyrenoidosa	2.84	38.95	107.9	25-30	7.4	Liu et al. (2011)
Chlorella saccharophila	**3.88**	**45.46**	**153.38**	**20-24**	**7.5-9**	Liu et al. (2011)
Chlorella sorokiniana	3.22	19.3	44.7	30	7-8	Rodolfi et al. (2009) ; Moronta et al. (2006)
Chlorella vulgaris	1.005	27.66	27.61	25-30	7	Liu et al. (2011)
Chlorococcum sp.	3.92	19.3	53.7	25-30	8-8.5	Rodolfi et al. (2009)
Scenedesmus obliqus	4.355	38.98	117	20-30	8	Liu et al. (2011)

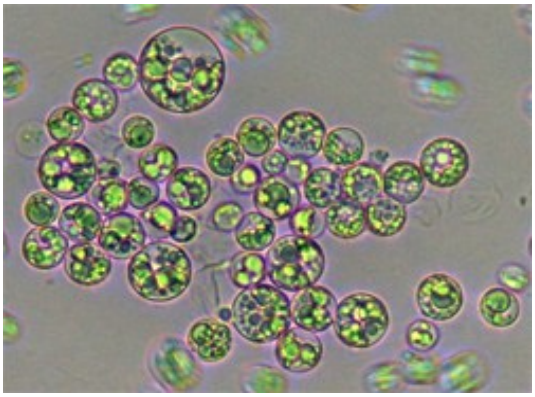




(a) Unicellular (b) Colony forming

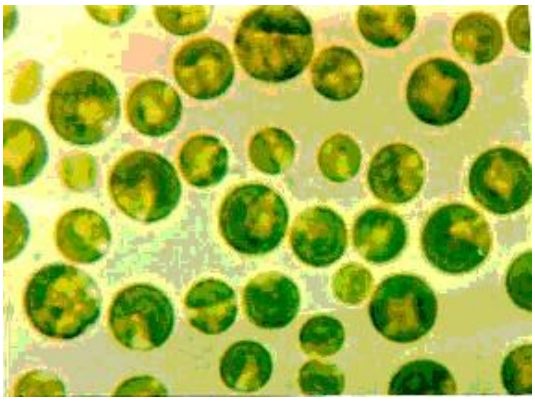
Figure 4.1. *Botrycoccus brauni* (NIES, 2001).

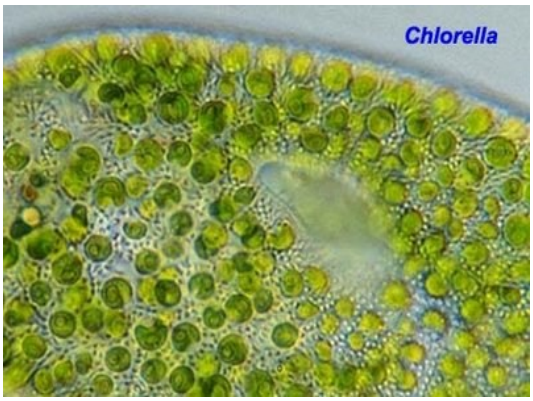




(a) Spherical cells (b) Chloroplast in cells

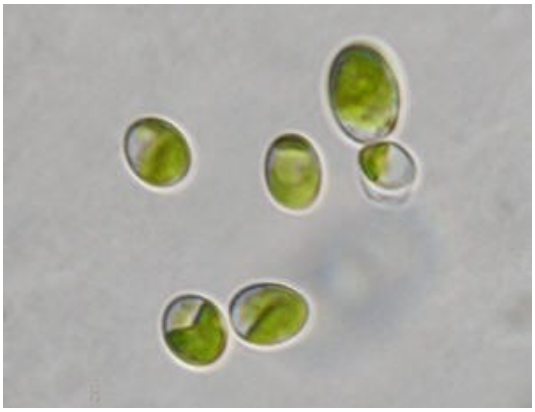
Figure 4.2. *Chlorella protothecoides* (Ho, 2011; Aquaportail, 2012).





(a) Spherical cells (b) Form individually

Figure 4.3. *Chlorella pyrenoidsa* (NPB, 2007; Bioinformatics Centre, 2013).



(a) Subspherical form (b) Chloroplast in cell

Figure 4.4. *Chlorella saccharophila* (Skaloud, 2012).

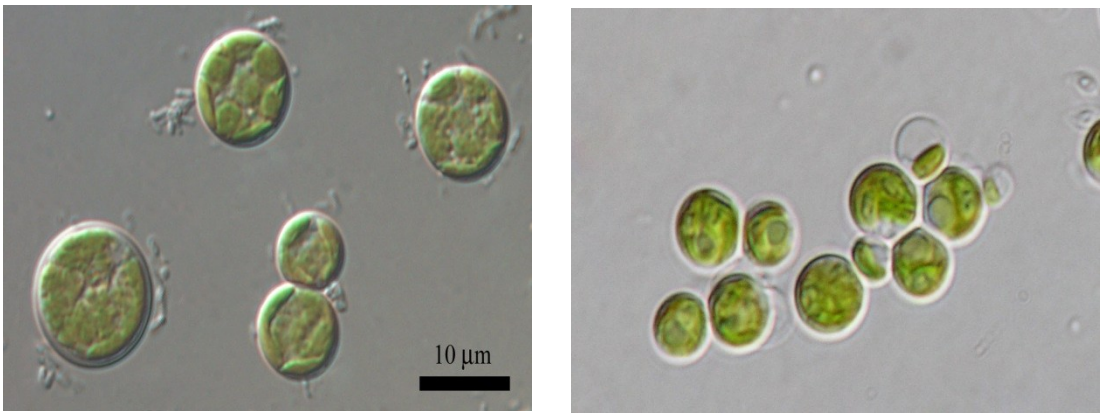
2011) as the carbon source for growth. The optimal temperature and pH for growth of this species are 25-30°C and 7.4, respectively (Sharma et al., 2012). *Chlorella pyrenoidsa* can yield a biomass of 2.84 g/L and a lipid content of 39% (Liu et al., 2011).

***4.1.1.4. Chlorella saccharophila*:** *Chlorella saccharophila* is a green freshwater unicellular microalga belonging to the Chlorella genus (Lewis, 1997). The cells have an average size of 7.3 μm (Bock et al. 2011). The cells contain a single chloroplast enclosed in a spherical or subspherical form as shown in Figure 4.4. These cells reproduce asexually through production of non-motile autospores (John et al., 2002). This microalgae species is able to use glucose (Singh et al. 2013), bicarbonate, and carbon dioxide as the carbon source for growth (Matsuda and Colmen, 1996). The optimal temperature and pH for growth are 20-24°C and 7.5-8, respectively. *Chlorella saccharophila* can yield a biomass of 3.88 g/L and a lipid content of 45%, respectively (Liu et al. 2011).

***4.1.1.5. Chlorella sorokiniana*:** *Chlorella sorokiniana* is a green freshwater unicellular microalga belonging to the Chlorella genus (Lewis, 1997). The cells are spherical in shape as shown in Figure 4.5 (Czeczuga et al., 2003). The cells can range in size from 2 to 10 μm (Lee and Hur, 2012). This microalga species is able to use glucose, fructose, sodium acetate (Qiao and Wang, 2009) and carbon dioxide (Kumar et al. 2014) as the carbon source. The optimal growth temperature and pH for this species are 25-30°C and 7-8, respectively (Moronta et al. 2006). *Chlorella* sorokiniana can yield a biomass of 3.22 g/L and a lipid content of 19%, respectively (Rodolfie et al., 2009).

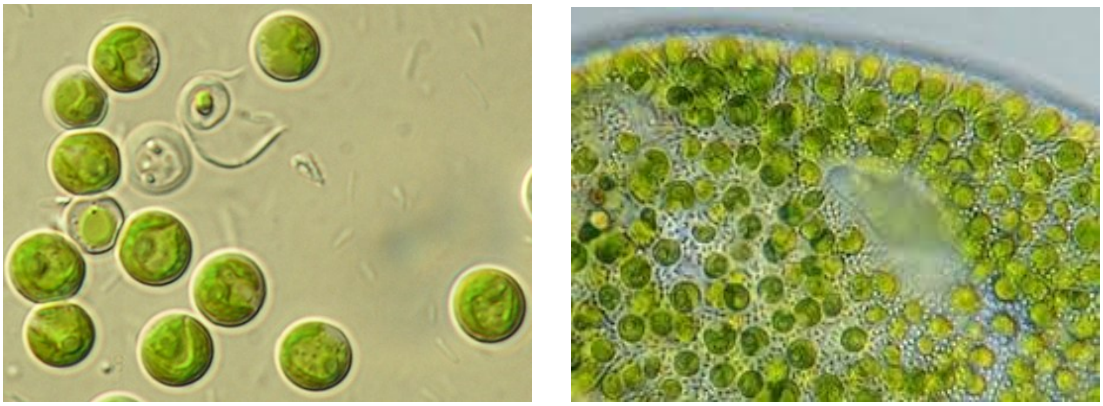
***4.1.1.6. Chlorella vulgaris*:** *Chlorella vulgaris* is a green freshwater unicellular microalga belonging to the Chlorella genus (Lewis, 1997). The cells are spherical in shape as shown in Figure 4.6. The cells can range in size from 5 to 10 μm (Myers, 1953). This species is able to use sodium bicarbonate and carbon dioxide as the carbon source for growth (Zheng et al. 2011). The optimal growth temperature and pH for this species are 25-30°C and 7-8, respectively (Cassidy, 2011; Wang et al., 2010). *Chlorella* vulgaris can yield a biomass of 1.00 g/L and a lipid content of 27%, respectively (Liu et al. 2011).

***4.1.1.7. Chlorococcum sp*:** *Chlorococcum sp.* is a green freshwater unicellular microalga, belonging to the Chlorococcum genus (Harwati et al., 2012). The cells are spherical or



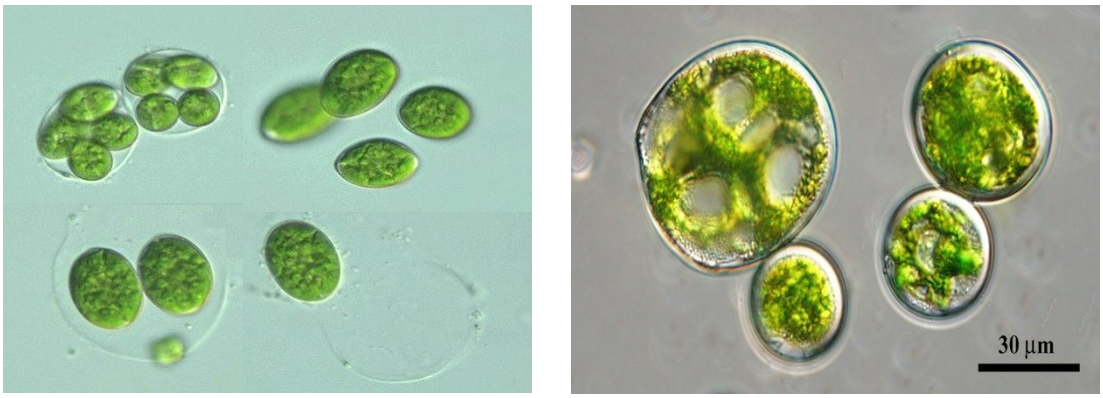
(a) Spherical cells (b) Unicellular

Figure 4.5. *Chlorella protothecoides* (Skaloud, 2007).



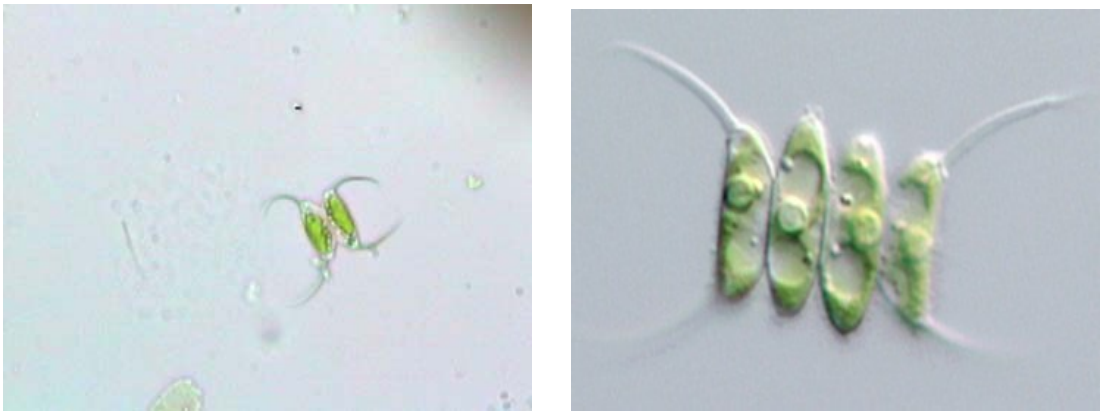
(a) Spherical cells (b) Form in colonies

Figure 4.6. *Chlorella vulgaris* (Matsos, 2013).



(a) Spherical cells (b) 24 µm length

Figure 4.7. *Chlorococcum sp.* (PIS, 2013; CCALA, 2013).



(a) Flat plate alignment (b) Chloroplast in cell

Figure 4.8. *Scenedesmus obliquus* (Picsearch, 2012).

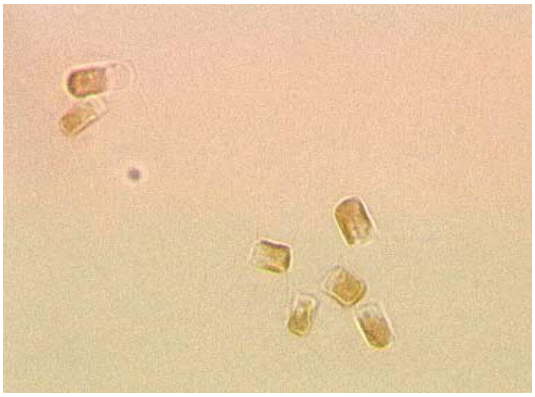
slightly ellipsoidal in shape as shown in Figure 4.7 (PIS, 2013). Cells are typically 24 μm long and 18 μm wide (PIS, 2013). This microalgae species is able to use both bicarbonate and carbon dioxide as the carbon source for growth (Mukund et al. 2013). The optimal growth temperature and pH for this species are 25-30°C and 8-8.5, respectively (Harwati et al., 2012; Zhang et al., 1997). Chlorococcum sp. can yield a biomass of 3.92 g/L and a lipid content of 19%, respectively (Rodolfi et al., 2009).

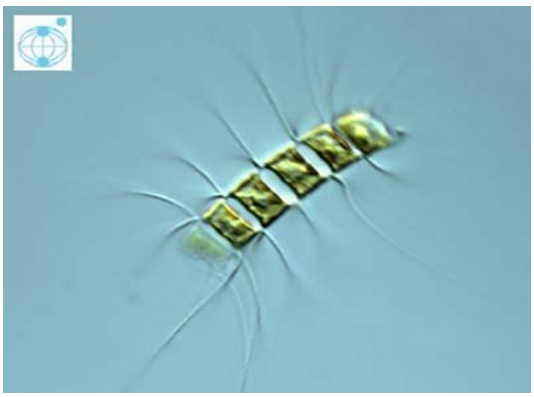
4.1.1.8. Scenedesmus obliquus: *Scenedesmus obliquus* is a freshwater unicellular microalgae belonging to the *Chlorophyceae* genus. Its cells are small (6-8 μm in width and 9-12 μm in length) and nonmotile, which are aligned in a flat plate as shown in Figure 4.8. This microalga species is able to use carbon dioxide and bicarbonate as the carbon source for growth (Morais and Costa, 2007; Thielmann et al. 1990). The optimal growth temperature and pH for this species are 25-30°C and 7-8, respectively (Moronta et al. 2006). Inside the cell is a single parietal plate-like chloroplast that has a single pyrenoid (Celekli et al., 2008). Bumps or reticulations cover the cell walls, which can be viewed under a scanning electron microscope (Oilgae, 2012). *Scenedesmus obliquus* can yield a biomass of 4.34 g/L and a lipid content of 39%, respectively, Table 4.1 (Liu et al., 2011).

4.1.2. Marine Microalgae

The selected marine microalgae (6 species) deemed suitable for biodiesel production include *Chaetoceros muelleri, Isochrysis sp., Nannochloropsis sp., Pavlova salina, Phaeodactylum tricornutum, Skeletonema costatum* and *Tetraselmis suecica* (Table 4.2).

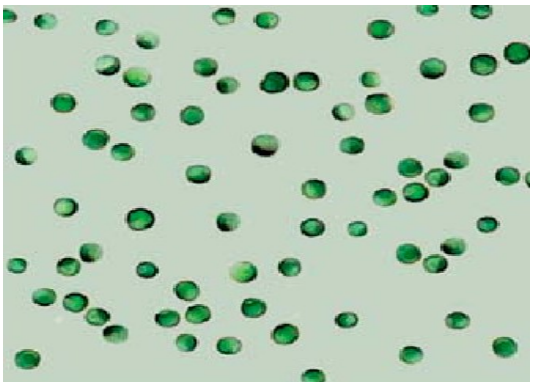
4.1.2.1. Chaetoceros muelleri: *Chaetoceros muelleri* is a marine microalga that exhibits 8 costae/μm on the valve and valve mantle that are arranged in a radiating pattern (Figure 4.9). The valves are oval to round shaped and the cells are cylindrical. Cell occurrence is singular or on occasion in groups of 2-3 filaments (Johansen and Rushforth, 1985). The valves in *Chaetoceros muelleri* range in a length from 3 to 20 μm. This microalga species is able to use carbon dioxide (Thornton, 2009) and glucose (Gomez-Gil et al. 2002) as the carbon source for growth. The optimal growth temperature and pH for this species are 20-30°C (McGinnis et al. 1997) and 7-8, respectively (Moronta et al. 2006). *Chaetoceros*

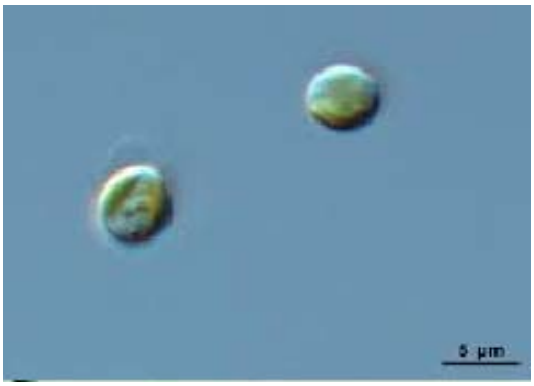




(a) Cylindrical cell type (b) Cells form groups

Figure 4.9. *Chaetoceros muelleri* (Engel, 2000; Microscope, 2006).





(a) Spherical cell shape (b) Cell Size ~5µm

Figure 4.10. *Isochrysis sp*. (NCMA, 2013).

Table 4.2. Oil content of marine water microalgae species.

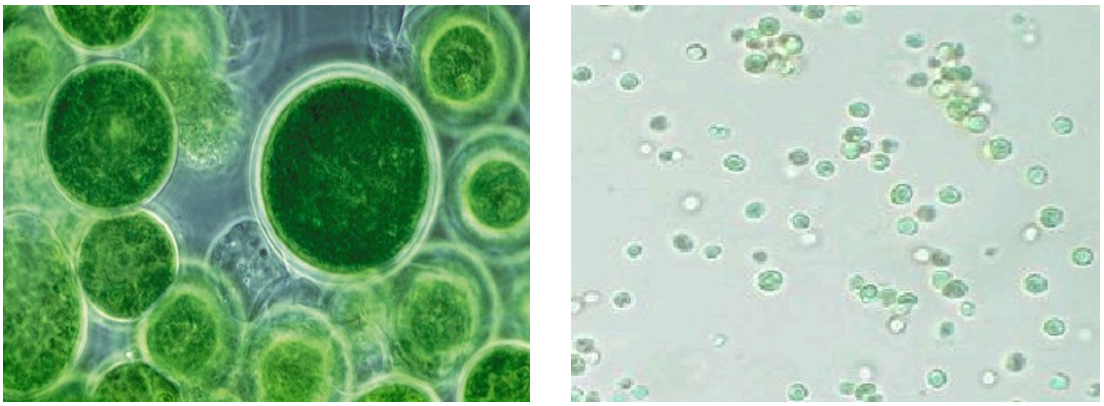
Species	Biomass (g/L)	Lipid Content (%)	Lipid Productivity (mg/L/d)	Temperature (°C)	pH	Reference
Chaetoceros muelleri	0.98	33.6	21.8	20-30	8	Rodolfi et al. (2009)
Isochrysis sp.	2.38	22.4	37.7	25	8	Rodolfi et al. (2009); Liu and Lin, (2001)
Nannochloropsis sp.	2.8	24.4	48.2	20-25	8.4	Rodolfi et al. (2009); Spolaore et al. (2006)
Phaeodactylum tricornutum	3.36	18.7	44.8	25-30	8	Rodolfi et al. (2009); Okauchi & Tokuda (2003)
Skeletonema costatum	1.12	21.1	17.4	25	7.4	Rodolfi et al. (2009); Yan et al. (2002)
Tetraselmis suecica	**4.48**	**23**	**36.4**	**18-24**	**7-9**	Rodolfi et al. (2009)

muelleri can yield a biomass of 0.56 g/L and a lipid content of 39%, respectively (Rodolfi et al., 2009).

4.1.2.2. Isochrysis sp.: *Isochrysis sp.* is a marine microalga that is spherical in shape (Figure 4.10). The cells are non-motile and can range from 3.5 to 6 μm in size (Bendif et al., 2013). *Isochrysis sp.* can yield a biomass of 2.38 g/L and a lipid content of 22.4%, respectively (Rodolfi et al., 2009). This microalga species is able to use both carbon dioxide (Roncarati et al. 2004) and bicarbonate as the carbon source for growth (Camiro-Vargas et al. 2005). The optimal temperature and pH for this species are 25°C and 8, respectively (Liu and Lin, 2001).

4.1.2.3. Nannochloropsis oculata: *Nannochloropsis oculata* is of the class Eustigmatophyceae. This class consists of small planktonic microalgae or macroalgae that grow attached to various substrates. This class of microalgae is unique because they lack chlorophyll pigments different from chlorophyll a (Fisher et al. 1998). *Nannochloropsis oculata* are characterized by their small cells (1-2 μm length and 1-2 μm width), unicellular shape and salt-water habitats (Figure 4.11). This microorganism is widely used as a food source in aquaculture (Sukenik et al., 1989). *Nannochloropsis oculata* can yield a biomass of 2.8 g/L and a lipid content of 24%, respectively (Rodolfi et al., 2009). This microalga species is able to use glucose, sodium bicarbonate (Dou et al. 2013) and carbon dioxide (Chiu et al. 2009) as the carbon source for growth. The optimal temperature and pH for this species are 20-25°C and 8.4, respectively (Spolaore et al., 2006).

***4.1.2.4. Phaeodactylum tricormutum*:** *Phaeodactylum tricormutum* is a marine microalga of the genus Phaeodactylum (Guiry, 2013). Cells can be fusiform, triadate and ovoid in form as shown in Figure 4.12 (Francius et al., 2008). Average biovolume of the cells is 74.4 μm^3 (Ruivo et al., 2011). This microalga is able to use glucose, acetate (Ceron Garcia et al. 2005) and carbon dioxide (Valenzuela et al. 2012) as the carbon source for growth. The optimal growth temperature and pH for this species are 25 - 30°C and 8, respectively (Okauchi and Tokuda, 2003; Dixon and Merrett, 1988). *Phaeodactylum tricormutum* achieves a biomass yield of 3.36 g/L and a lipid content of 19% (Rodolfi et al. 2009).



(a) Unicellular cell type (b) Length and width (~1-2 μm)

Figure 4.11. *Nannochloropsis oculata* (Nichols, 2011; Algaelink, 2009).

(a) Fusiform Shape (b) Colony

Figure 4.12. *Phaeodactylum tricormutum* (Martino, 2013; NCMA, 2013).

4.1.2.5. Skeletonema costum: *Skeletonema costum* is a marine microalgae of the genus Skeletonema (Hernandez-Becerrill et al., 2013). The cells of this microalga are yellow-brown in color, cylindrical in shape and connected in long straight chains as shown in Figure 4.13 (Horner, 2002). These chains range from 2 to 61 μm in length and from 2 to 21 μm in diameter (Hasle and Syvertsen, 1997). *Skeletonema costum* can yield biomass values of 1.12 g/L and a lipid content of 21%, respectively (Rodolfi et al., 2009). This microalga species is able to use both glucose (Kogure et al. 1979) and carbon dioxide (Xu et al. 2010) as the carbon source for growth. The optimal temperature and pH for this species are 25°C and 7.4, respectively (Yan et al., 2002; Gao et al., 1993).

4.1.2.6. Tetraselmis suecica: *Tetraselmis suecica* is a marine microalga that grows as single cells. They are motile, as shown in Figure 4.14a, and can be compressed or curved, but they are never twisted (Acuna and Kiefer, 2000). Cells are spherical or elliptic with a length of 35 μm and a width of 14 μm (Figure 4.14b). This microalga species is able to use both sodium bicarbonate (White et al. 2012) and carbon dioxide (de Castro Araujo et al. 2005) as the carbon source for growth. The optimal temperature and pH for growth are 18-24°C and 7-9, respectively (Lavens and Sorgeloos, 1996). *Tetraselmis suecica* achieves biomass yields of 4.48 g/L and a lipid content of 36% (Rodolfi et al., 2009).

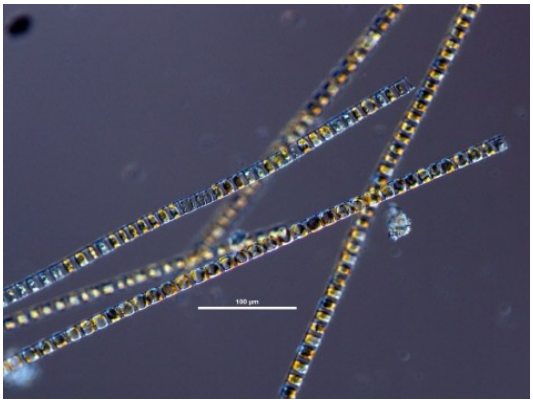
4.2. Experimental Materials and Equipment

4.2.1. Glassware

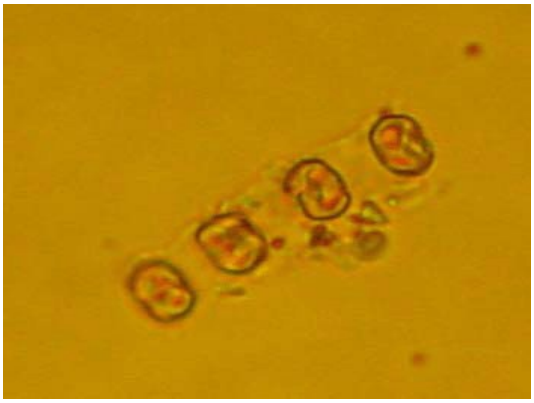
The glassware used included test tubes, volumetric flasks, graduated cylinder, Erlenmeyer flasks, funnel, beakers, reagent bottles, pyrex bottles, centrifuge tubes and pipettes. All glassware were washed with acetone and left to dry overnight in an oven set at 105°C (HeraTherm Oven, OMS100, Thermo Fisher Scientific, Ontario, Canada).

4.2.2. Chemicals

The chemicals used included: Proteose peptone, sodium nitrate, calcium chloride, magnesium sulfate, dipotassium phosphate, monopotassium phosphate, sodium chloride and iron (III) chloride, ethylenediaminetetraacetic acid disodium salt, cupper sulfate, zinc sulfate, cobalt chloride, manganese chloride, sodium molybdate, thiamine HCl, biotin,



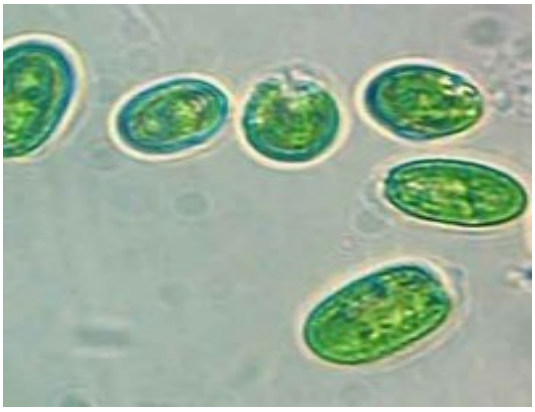
(a) Cells are connected in chains



(b) Yellow, brown color

Figure 4.13. *Skeletonema costatum* (UBC, 2012).

(a) Elliptic cell type



(b) Unicellular cell type

Figure 4.14. *Tetraselmis suecica* (Reefsnow, 2012).

iron (II) sulfate, boric acid, ammonium molybdate tetrahydrate, cadmium chloride, aluminium ammonium bis(sulfate) dodecahydrate and sulfuric acid, were used as nutrients to make up the growth media for the microalgae species. Chloroform, methanol, potassium chloride and sodium sulfate were used for oil extraction process. All the chemicals were obtained from Sigma-Aldrich, Oakville, Ontario, Canada and Thermo Fisher Scientific, Ontario, Canada.

4.2.3. Equipment

Several pieces of equipment's were used in the experiments: Analytical Balance (PI-314, Denver Instruments, New York, USA) was used to weigh out the samples and the resulting oil extracted. A UV-Vis spectrophotometer (Genesys 10S UV-Vis, Thermo Fisher Scientific, Ontario, Canada) was used to measure the optical density of the microalgae. Yamato RE-51 (HiTEC RE-51, Yamato Scientific America, California, USA) was used in this experiment to remove the solvent in the hexane extraction. HeraTherm Oven (OMS100, Thermo Fisher Scientific, Ontario, Canada) was used to dry all glassware after it was washed. The biomass was harvested from the liquid media using a Sorvall T1 Centrifuge (Thermo Scientific, Ohio, USA). Disruption of microalgae cell walls was accomplished using a Branson 2510 Sonicator (2510R-DTH, Branson Ultrasonics Corporation, Danbury, USA). Orion 5 Star pH meter (Thermo Scientific, Massachusetts, USA) was used to measure the pH of the growth media. Hund Wetzlar Microscope (H 500, Hund Wetzlar, Wilhelm Will Str. 7, Germany) was used to determine the cell count of the marine microalga. A Colony Counter (Model No. 7-910, Fisher Scientific, Ottawa, Ontario) was used to determine the cell count for the freshwater microalga.

CHAPTER 5. EXPERIMENTAL APPARATUS

A fully automated multiple open pond system consisted of a frame, 18 open pond units, a cooling unit, a lighting unit, a supernatant collection unit and control unit.

5.1. Frame

The frame was used to house the open pond, light, cooling, water collection and control units (Figure 5.1). The frame was constructed of angle iron and measured approximately 244 cm (width) x 41 cm (depth) x 283 cm (height). It consisted of three shelves, 76 cm apart. Each shelf was divided vertically into two cells by a 1.2 cm thick plywood sheet to provide a better control of light and feed. The two sides, back and top were covered with 0.6 cm thick plywood sheets.

5.2. Open Pond Unit

The open pond unit consisted of six growth troughs, each was made of galvanized steel and was divided into three compartments (Figure 5.2). The dimensions of each compartment were 38 cm (length) x 38 cm (width) x 12.5 cm (height). Each compartment can hold up to 18 L.

5.3. Lighting Unit

The lighting unit provided approximately 430 hectolux of illumination per shelf (Figure 5.1). This was achieved by a mixture of fluorescent and incandescent lamps. Six 40 W cool white fluorescent lamps 122 cm in length were fastened above each trough. Four 100 W incandescent bulbs were also mounted amongst the fluorescent tubes on each shelf, that sit 100 cm away from the pond units. This gave a total of twelve fluorescent lamps per shelf and four incandescent ones. The light intensity achieved for each pond is 480 μmol m^{-2} s^{-1}.

5.4. Cooling Unit

A cooling unit was designed to continuously remove the heat produced by the lamps to avoid heating of the algae on the upper and middle shelves. For each of these two shelves, a 5 cm diameter PVC pipe (having 6 mm diameter holes spaced 6 cm apart and facing out) was placed under the backside of the troughs. Two metal blocks placed under

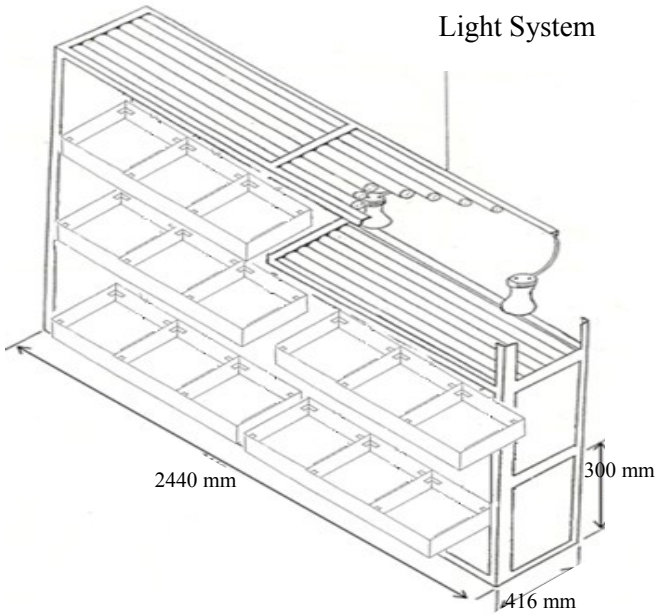


Figure 5.1. Schematic view of the open pond system.

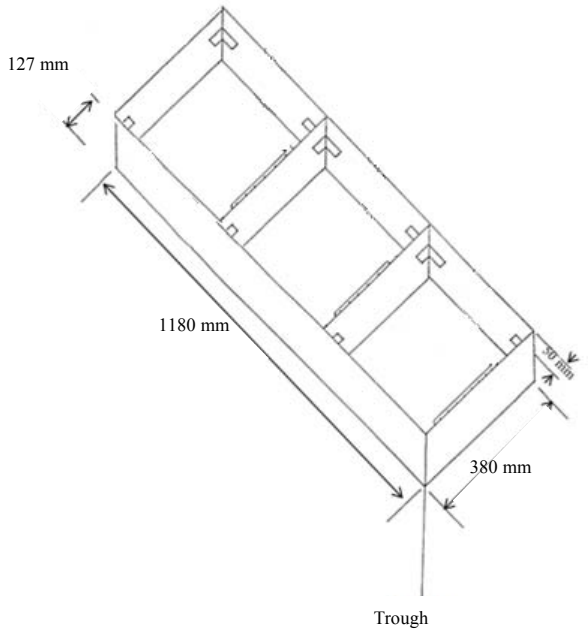


Figure 5.2. Top view of the open pond unit.

each trough provided a 5 cm space between the trough and the lighting system of the shelf below it. A 5 cm diameter PVC pipe was attached vertically to the left side of the frame and acted as a manifold through which air was blown by means of a motor driven fan (Model AK4L143A Type 821, Franklin Electric, Bluffton, IN).

5.5 Collection System

The supernatant from each tray was collected in a separate container (2.7 L each) located at the bottom of the system (Figure 5.3). The outlets were connected to plastic tubes of 1 cm outside diameter, which were passed through a solenoid valve.

5.6. Control System

A computer was used to operate and control the various components of the open pond system and record the various measurements. The light intensity was measured using a Quantum Sensor, SQ-316 Series (Apogee, Logan, Utah). The pH was measured using pH electrodes (EW-59001-65, Cole Parmer, Montreal, Quebec). The temperature was measured using thermocouples (WD-08541-12, Nova-Tech International, Houston, Texas).

A basic computer program (BASIC Stamp Editor v2.5) allowed the configuration of the operating frequency and duration of the light, aeration unit and collection system. The computer was connected to a data coordinator (cDAQ-9178, National Instruments) which had 24 digital output ports and 24 digital input ports. The digital output ports were connected to electronic circuits which were responsible for the lighting, cooling and collection systems.

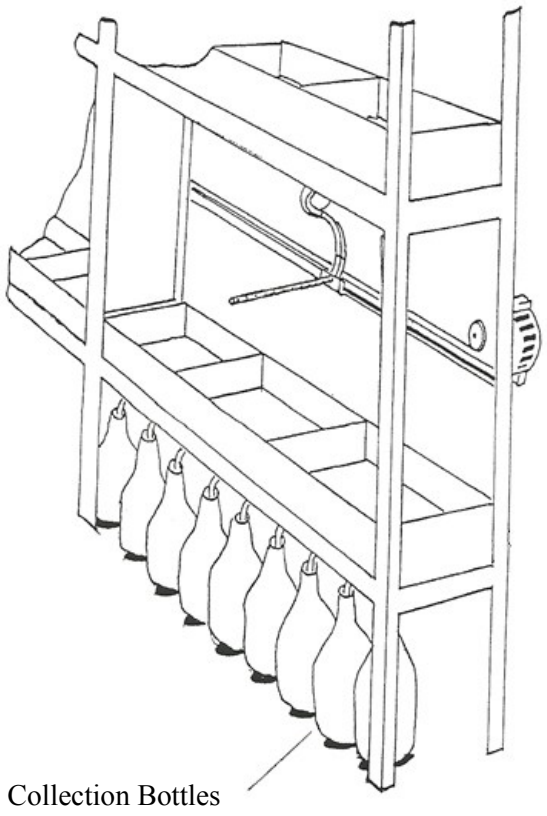


Figure 5.3. Schematic view of the collection unit.

CHAPTER 6. EXPERIMENTAL PROCEDURE

6.1 Experimental Design

The selected freshwater (*Chlorella saccharophila*) and marine (*Tetraselmis suecica*) microalgae species were grown under various environmental conditions in the open pond system in order to determine the optimal conditions for achieving the fastest growth and the highest oil yield. The study was carried out in two stages. In the first, the effects of light duration and nutrient on the algae biomass and oil yield were evaluated using $NaHCO_3$ as a carbon source while maintaining the pH and temperature constant. In the second stage, the effect of CO_2 as a carbon source on the biomass and oil yield was evaluated at the optimum light duration and nutrient levels.

The optimal ranges for pH and temperature for these species are shown in Table 6.1. The average pH for the freshwater (*Chlorella saccharophila*) and marine (*Tetraselmis suecica*) microalgae was 8.9 and 8.3, respectively. The temperature was kept constant by controlling the air flow of the cooling unit. The average temperature for the freshwater and marine microalgae was 22.0°C (range of 21.8-22.3°C) and 21.8°C (range of 21.7-21.9°C), respectively.

Sodium bicarbonate ($NaHCO_3$) was used as the carbon source for the microalgae and was administered at a concentration of 1300 mg/L, which is higher than the reported requirement for consumption (Table 6.2) for various freshwater and marine microalgae species. This concentration was used to ensure that enough carbon was present in the system so that carbon limitation is not a factor that needs to be considered when assessing the biomass yield using the various nitrogen sources.

The light intensity was kept at 480 μmol m^{-2} s^{-1} while the optimal light duration was determined by exposing the algae to three light periods throughout the cultivation process: (a) the shortest day light in the winter of ~9 h, (b) the longest day light in the summer of ~16 h and (c) full light exposure (24 h) using the automated lighting and control units in the open pond system.

The effect of nutrient dosing of ammonium nitrate, ammonium phosphate, ammonium sulfate, and a mix of all three was investigated to evaluate their potential as source of

Table 6.1. Optimal pH, temperature and carbon source for microalgae species.

Species	Oil Content (% dry basis)	Carbon Sources	pH	Temperature (°C)
Marine				
Tetraselmis suecica	15 - 23	CO_2/$NaHCO_3$/ glucose	7.0 - 9	20 - 30
Freshwater				
Chlorella saccharophila	36 - 47	CO_2/$NaHCO_3$/ glucose	7.5 - 9	20 - 24

Table 6.2. Consumption rate of $NaHCO_3$ by microalgae.

Species	Rate of uptake (mg/L/day)	Reference
Freshwater		
C. vulgaris	125	Blake et al. (2006)
S. obliquus	126	Blake et al. (2006)
Scenedesmus sp.	119.9	Nayak et al. (2013)
Marine Water		
N. oculata	45.8	Merrett et al. (1996)
Haematococcus pulvialis	72.9	Devgoswami et al. (2011)

Table 6.3. Concentration of micronutrient components.

Compound	Molecular Weight (g/mol)	Amount (mg/L)	Concentration (mg/L)		
			Nitrogen	Phosphorus	Sulfur
Ammonium nitrate (NH_4NO_3)	80	200	70		
Diammonium phosphate $(NH_4)_2HPO_4$	132	330	70	77.5	
Ammonium sulfate (NH_4SO_4)	132	330	70		80
Combination					
Ammonium nitrate (NH_4NO_3)	80	85.7	30		
Diammonium phosphate $(NH_4)_2HPO_4$	132	194.3	20	22.1	
Ammonium sulfate (NH_4SO_4)	132	94.3	20		22.8

nitrogen, phosphorus and sulphate and their impact on the growth of algae and oil yield. The nitrogen concentration was maintained at 70 mg/L (Table 6.3).

Tables 6.4 and 6.5 provide a detailed description of the investigation parameters for microalgae growth conditions for the freshwater and marine water algae. Each experiment was done in three replicates for each species, giving a grand total of 72 runs for both species. Each experiment was carried out for 10 days. The optimal conditions were determined based on the algae rate of growth and oil yield and quality.

Once the best light duration and nutrient were determined, CO_2 was used as carbon source for the freshwater and marine algae and the experiments were carried out for 10 days at the same pH and temperature.

6.2. Preparation of Liquid Medium for the Growth of Inoculum

The freshwater microalgae medium was prepared on algal proteose medium (ATCC Catalog Medium No. 847, American Type Culture Collection, Manassas, VA, USA). Table 6.6 provides the freshwater liquid medium components. The liquid medium was made up by adding 1 g of proteose peptone (Difco 0120) to 1 L of Bristols solution. Bristols solution (Table 6.7) was prepared by adding the following amounts from the prepared stock solution: 10 mL of $NaNO_3$, 10 mL of $CaCl_2$, 10 mL of $MgSO_4$ $7H_2O$, 10 mL of K_2HPO_4, 10 mL of KH_2PO_4, 10 mL of NaCl, 0.05 mL of $FeCl_3$, and 940 mL of distilled water. The stock solutions were prepared as follows: 10 g of $NaNO_3$ in 400 mL of distilled water, 1g of $CaCl_2$ in 400 mL of distilled water, 3 g of $MgSO_4$ $7H_2O$ in 400 mL of distilled water, 3 g of K_2HPO_4 in 400 mL of distilled water, 7 g of KH_2PO_4 in 400 mL of distilled water and finally 1 g of NaCl in 400 mL of distilled water.

The marine microalgae medium was prepared in F/2 medium (Guillard and Ryther, 1962). Table 6.8 provides the marine water liquid medium stock solution components. The trace element liquid medium stock solution was prepared by the addition of 4.16 g of Na_2 EDTA, 3.15 g of $FeCl_3 \cdot 6H_2O$, 0.01 g of $CuSO_4 \cdot 5H_2O$, 0.022 g of $ZnSO_4 \cdot 7H_2O$, 0.01 g of $CoCl_2 \cdot 6H_2O$, 0.18 g of $MnCl_2 \cdot 4H_2O$ and 0.006 g of $Na_2MoO_4 \cdot 2H_2O$ into 1 L of autoclaved seawater (Halifax Waterfront, Halifax, Nova Scotia). The vitamin mix stock solution was prepared by the addition of 0.1 g of Thiamine HCl and 0.0005 g of biotin

Table 6.4. Experimental design for the freshwater microalga *Chlorella saccharophila*.

Light Duration (h)	**Nutrient source (70 mg/L Nitrogen)**
9	Ammonium nitrate
	Ammonium phosphate
	Ammonium sulfate
	Ammonium nitrate Ammonium phosphate Ammonium sulfate
16	Ammonium nitrate
	Ammonium phosphate
	Ammonium sulfate
	Ammonium nitrate Ammonium phosphate Ammonium sulfate
24	Ammonium nitrate
	Ammonium phosphate
	Ammonium sulfate
	Ammonium nitrate Ammonium phosphate Ammonium sulfate

Carbon Source: $NaHCO_3$

Table 6.5. Experimental design for the marine microalga *Tetraselmis suecica*.

Light Duration (h)	**Nutrient source (70 mg/L Nitrogen)**
9	Ammonium nitrate
	Ammonium phosphate
	Ammonium sulfate
	Ammonium nitrate Ammonium phosphate Ammonium sulfate
16	Ammonium nitrate
	Ammonium phosphate
	Ammonium sulfate
	Ammonium nitrate Ammonium phosphate Ammonium sulfate
24	Ammonium nitrate
	Ammonium phosphate
	Ammonium sulfate
	Ammonium nitrate Ammonium phosphate Ammonium sulfate

Carbon Source: $NaHCO_3$

Table 6.6. Components of the freshwater liquid and solid media.

Component	Quantity	
	Proteose Agar Medium	**Proteose Broth Medium**
Agar	15 g	
Proteose peptone	1 g	1 g
Bristols solution	1 L	1 L

Table 6.7. Components of the Bristols solution.

Component	**Amount (mL)**
$NaNO_3$ Solution	10
$CaCl_2$ Solution	10
$MgSO_4 \cdot 7H_2O$ Solution	10
K_2HPO_4 Solution	10
KH_2PO_4 Solution	10
NaCl Solution	10
$FeCl_3$ Solution	0.05
Distilled Water	940

Table 6.8. Components of the F/2 marine liquid media (Guillard and Ryther, 1962).

Component	**Quantity (g/L*)**
Trace Element Stock Solution	
Na_2 EDTA	4.16
$FeCl_3 \cdot 6H_2O$	3.15
$CuSO_4 \cdot 5H_2O$	0.01
$ZnSO_4 \cdot 7H_2O$	0.022
$CoCl_2 \cdot 6H_2O$	0.01
$MnCl_2 \cdot 4H_2O$	0.18
$Na_2MoO_4 \cdot 2H_2O$	0.006
Vitamin Mix Stock Solution	
Thiamine HCl	0.1
Biotin	0.0005

***per liter of autoclaved seawater**

into 1 L of autoclaved seawater. The liquid medium was prepared by the addition of 0.075 g of $NaNO_3$, 0.00565 g of $NaH_2PO_4 \cdot 2H_2O$, 1.0 ml of trace element stock solution and 1 ml of vitamin mix stock solution.

6.3. Preparation of Solid Medium for the Growth of Inoculum

The freshwater microalgae medium was prepared on algal proteose agar medium (ATCC Catalog Medium No. 847). Table 6.6 provides the freshwater solid medium components. The solid medium was made up by the addition of 1 g of proteose peptone (Difco 0120) and 15 g of agar to 1 L of Bristols solution. Bristols solution was prepared by adding the following amounts form the prepared stock solution: 10 mL of $NaNO_3$, 10 mL of $CaCl_2$, 10 mL of $MgSO_4$ $7H_2O$, 10 mL of K_2HPO_4, 10 mL of KH_2PO_4, 10 mL of NaCl, 0.05 mL of $FeCl_3$, and 940 mL of distilled water. The stock solutions were prepared as follows: 10 g of $NaNO_3$ in 400 mL of distilled water, 1g of $CaCl_2$ in 400 mL of distilled water, 3 g of $MgSO_4$ $7H_2O$ in 400 mL of distilled water, 3 g of K_2HPO_4 in 400 mL of distilled water, 7 g of KH_2PO_4 in 400 mL of distilled water and finally 1 g of NaCl in 400 mL of distilled water.

6.4. Preparation of Inoculum

Sufficient amounts of inoculum were prepared for all the experimental runs for both freshwater and marine microalgae in order to maintain consistency.

6.4.1. Chlorella Saccharophila Inoculum

The procedure for preparing the freshwater inoculum is depicted in Figure 6.1. Initially, the freeze dried *Chlorella saccharophila* (ATCC® 30408™) sample was revived in 5 mL of Bristols liquid media. Using an inoculating loop, cells were transferred from the liquid media onto 3 petri dishes containing proteose agar medium. The plates were incubated for 3 days at room temperature at 14 h light and 10 h dark periods. The cells were then transferred, by scraping the cells off the solid media using an inoculating loop and submerging them into a 125 mL Erlenmeyer flask containing 25 mL of Bristols liquid medium. These cells were then left to grow for 2 weeks a photocycle of 14 h light and 10 h dark. The sample was then transferred to a 500 mL Erlenmeyer flask containing 250 mL of Bristols liquid media which was left to grow for 2 weeks at a photocycle of 14 h light and 10 h dark. Finally, the media was transferred

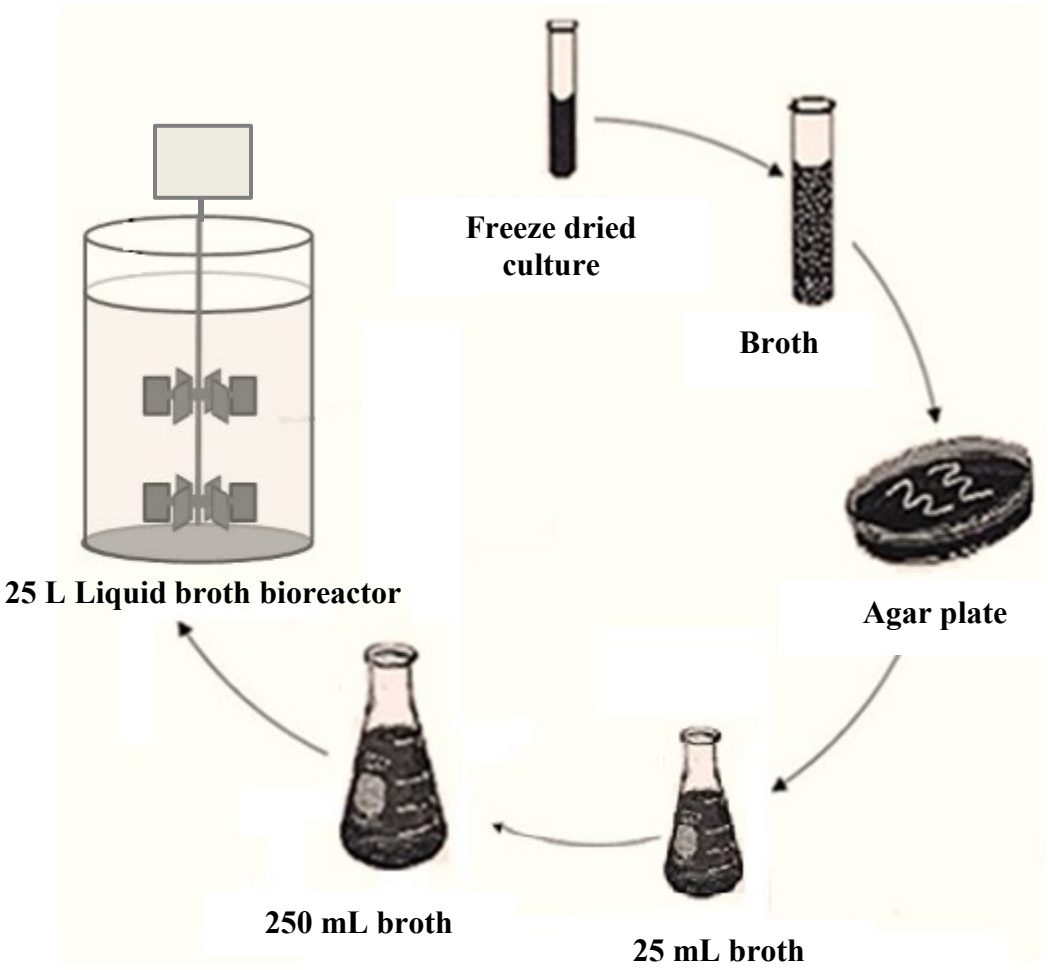


Figure 6.1. Preparation of Inoculum.

from the 500 mL flask into a 30 L bioreactor containing 25 L of Bristols liquid media and left to grow for 2 more weeks at a cycle of 14 h light and 10 hour dark.

6.4.2. Tetraselmis Suecica Inoculum

The inoculum for *Tetraselmis suecica* (UTEX LB 2286, Cedarlane, Burlington, Ontario) microalga was prepared by taking 5 mL of the liquid sample received and adding it to 125 mL Erlenmeyer flask containing 25 mL of F/2 liquid media. The sample was left to grow at room temperature for 2 weeks at a photocycle of 14 h light and 10 h dark. The sample was then transferred to a 500 mL Erlenmeyer flask containing 250 mL of F/2 liquid media which was left to grow for 2 weeks at a photocycle of 14 h light and 10 h dark. Finally, the media was transferred from the 500 mL flask into a 30 L bioreactor containing 25 L of F/2 liquid media and left to grow for 2 additional weeks at a cycle of 14 h light and 10 hour dark.

6.5. Preparation of Algae Production Media

A specially formulated nitrogen deprived medium was used as the production medium for freshwater and marine microalgae.

6.5.1. Chlorella Saccharophila Production Medium

The freshwater production medium is a modification of the Fitzgerlad medium (Hughes et al., 1959). The preparation of the stock solutions for this media is shown in Table 6.9. The freshwater microalgae medium was made up by the addition of 1 mL of each of the stock solutions A, B, C and D per L of distilled water (Table 6.10).

6.5.2. Tetraselmis Suecica Production Medium

A modification of the F/2 media was used as the production media for the marine microalga. The media was modified by eliminating the addition of sodium nitrate. The marine production medium consists primarily of autoclaved ocean water (Halifax Waterfront, Halifax, NS, Canada). Table 6.11 depicts the elemental analysis (Mineral Engineering Center of Dalhousie University, Halifax, Nova Scotia) of the components present in the marine water used.

Table 6.9. Formulation of stock solutions for *Chlorella Saccharophila* production medium.

Stock Solutions (per 200 mL)	**Composition**
A	24.648 g $MgSO_4 \cdot 7H_2O$
B	1.36 g KH_2PO_4 8.70 g K_2HPO_4
C	1.392 g $FeSO_4 \cdot 7H_2O$ 1.864 g EDTA tri Na
D	0.620 g H_3BO_3 0.340 g $MnSO_4 \cdot H_2O$ 0.057 g $ZnSO_4 \cdot 7H_2O$ 0.018 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.027 g $CoCl_2 \cdot 6H_2O$ 0.024 g KBr 0.017 g KI 0.023 g $CdCl_2 \cdot 5/2H_2O$ 0.091 g $Al_2(SO_4)_3(NH_4)_2SO_4 \cdot 24H_2O$ 0.00004 g $CuSO_4 \cdot 5H_2O$ 0.56 mL 97% H_2SO_4

Table 6.10. Components of freshwater production medium.

Component	Amount (mL)
A	1 mL
B	1 mL
C	1 mL
D	1 mL
Distilled Water	996 mL

Table 6.11. Elemental analysis of autoclaved ocean water used for marine production media.

Element	Amount (mg/L)
Na	10,254
Mg	1,078
S	1,010
K	395
Ca	386
Sr	6.79
Si	2.8
P	0.1
Ba	0.05
Al	0.05
Ni	0.04
Zn	0.02
Mo	0.01
Cd	0.01
Co	0.01
Cu	0.01

6.6. Experimental Protocol

6.6.1. Freshwater Microalgae Protocol

To each compartment in the open pond unit a total of 4.75 L of freshwater production media was prepared. The desired nutrient (ammonium nitrate, ammonium phosphate, ammonium sulfate or combination of all three) for investigation was added to the production media (Table 6.3 lists the amounts). This solution was enriched with 1.3 g/L of sodium bicarbonate (total of 6.5 g). Additionally 250 mL of *Chlorella saccharophila* inoculum was added to each compartment. The cells were exposed to either 9, 16 and 24 h or light and left to grow for 10 days. Every other day 100 mL of sample was taken for experimental analyses. The biomass was harvested from the liquid media using a Sorvall T1 Centrifuge (Thermo Scientific, Ohio, USA). The supernatant from the centrifuge tubes was decanted and the cells were collected for analysis.

6.6.2. Marine Microalgae Protocol

To each compartment in the open pond unit a total of 4.75 L of marine production media was prepared. The desired nutrient (ammonium nitrate, ammonium phosphate, ammonium sulfate or combination of all three) for investigation was added to the production media (Table 6.3 lists the amounts). This solution was enriched with 1.3 g/L of sodium bicarbonate (total of 6.5 g). Additionally, 250 mL of *Tetraselmis suecica* inoculum was added to each compartment. The cells were exposed to either 9, 16 and 24 h or light and left to grow for 10 days. Every other day 100 mL of sample was taken for experimental analyses. The biomass was harvested from the liquid media using a Sorvall T1 Centrifuge (Thermo Scientific, Ohio, USA). The supernatant from the centrifuge tubes was decanted and the cells were collected for analysis.

6.7. Experimental Analyses

The samples collected from marine and freshwater algae medium were analyzed for pH, temperature, nitrite concentration, algae yield, oil content and elemental analysis.

6.7.1. Freshwater Microalgae Yield

The freshwater mciroagla yield was determined by counting the colony forming units observed on the petri dishes. A standard curve between the cell count and optical density

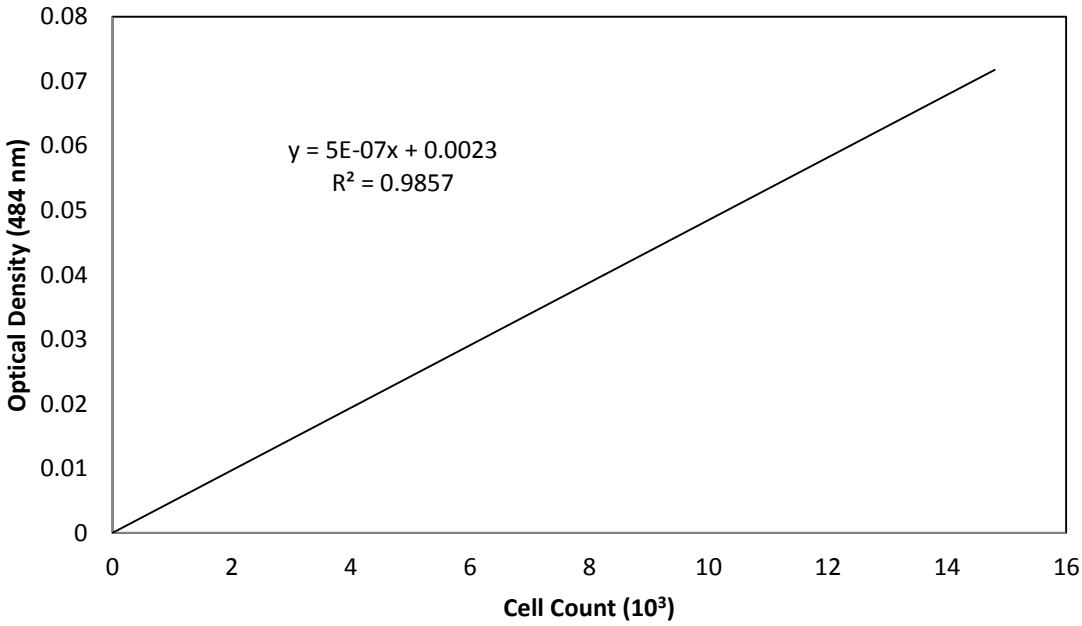


Figure 6.2. Standard curve for *Chlorella saccharophila* at 484 nm.

at 484 nm was determined (Figure 6.2). This allowed for cell counts to be determined by optical density measurements during experiments.

The number of Colony Forming Units (CFU) for *Chlorella saccharophila* was determined using a series of dilutions. A test tube containing 9 mL of distilled water was autoclaved and a 1 mL aliquot sample was added to the tube. The contents of the tube were vortexed (Thermolyne Maxi Mix, Thermolyne Corporation, Hampton, New Hampshire, USA) to distribute the cells. A 1 mL aliquot of this solution was added to another tube that had been autoclaved with 9 mL of distilled water. This tube was again vortexed to distribute the cells. This was carried out seven times to obtain dilutions of 1:1, 1:10, 1:100, 1:1000, 1:10 000, 1:100 000, 1: 1 000 000. For each of the six dilutions made, 0.1 mL of the solution was added to a petri dish containing solid freshwater or marine water medium. The plates were then sealed with parafilm, inverted and incubated at room temperature (~24°C) at a cycle of 14 hours light and 10 hours dark for 3 days. After the incubation period was complete, the plates were removed and the colonies were counted using a colony counter (Model No. 7-910, Fisher Scientific, Ottawa, Ontario). The plates consisting of 30-300 CFU were used for calculating the CFU of the sample.

In a 250 mL Erlenmeyer Flask, 175 mL of liquid Bristols media was autoclaved and inoculated freshwater algae grown. The flask was then incubated at room temperature (~24°C) for 2 weeks with 14 h light and 10 h dark periods. At 2, 5, 8, 11 and 14 days samples were taken to determine plate count using the plate count procedure and Optical Density (OD) content. The optical density readings were taken at 484 nm (Genesys 20, Thermo Scientific, Mississauga, Ontario, Canada). A standard curve was prepared by plotting the optical density against the plate count (Figure 6.2). The correlation of this curve results in the determination of cell count using the optical density measurements. Equation 1 depicts the relationship between cell yield and optical density for *Chlorella saccharophila*. Equation 2 illustrates the rearrangement necessary to solve for cell yield.

$$Optical\ Density = 5x10^{-7}\ (Cell\ Yield) + 0.0023 \quad (1)$$

$$Cell\ Yield = \left(\frac{Optical\ Density - 0.0023}{5x10^{-7}}\right)\ x\ 10^3 \quad (2)$$

6.7.2. Marine Microalgae Yield

The marine microalgae yield was determined by counting the cells using a hemocytometer under a light microscope. A standard curve between the cell count and optical density at 750 nm was determined (Figure 6.3). This allowed for cell counts to be determined by optical density measurements during experiments.

The cell count yields for *Tetraselmis suecica* were determined using a hemocytometer, since the cells do not grow very well on the agar plate. In a 250 mL Erlenmeyer flask, 175 ml of F/2 media was prepared and inoculated with *Tetraselmis suecica.* The flask was then incubated at room temperature (~24°C) for 2 weeks with 14 h light and 10 h dark periods. At 2, 5, 8, 11 and 14 days 0.01 mL of sample was taken to determine cell counts using Hemocytometer slide. The cells were counted under a Hund Wetzlar microscope (H 500, Hund Wetzlar, Wilhelm Will Str. 7, Germany).

The optical density readings were taken at 750 nm (Genesys 20, Thermo Scientific, Mississauga, Ontario, Canada). A standard curve was prepared by plotting the optical density against the plate count (Figure 6.3). The correlation of this curve results in the determination of cell count using the optical density measurements. Equation 3 depicts the relationship between cell yield and optical density for *Chlorella saccharophila*. Equation 4 illustrates the rearrangement necessary to solve for cell yield.

$$Optical\ Density = 0.0023\ (Cell\ Yield) \quad (3)$$

$$Cell\ Yield = \left(\frac{Optical\ Density}{0.0023}\right)\ x\ 10^4 \quad (4)$$

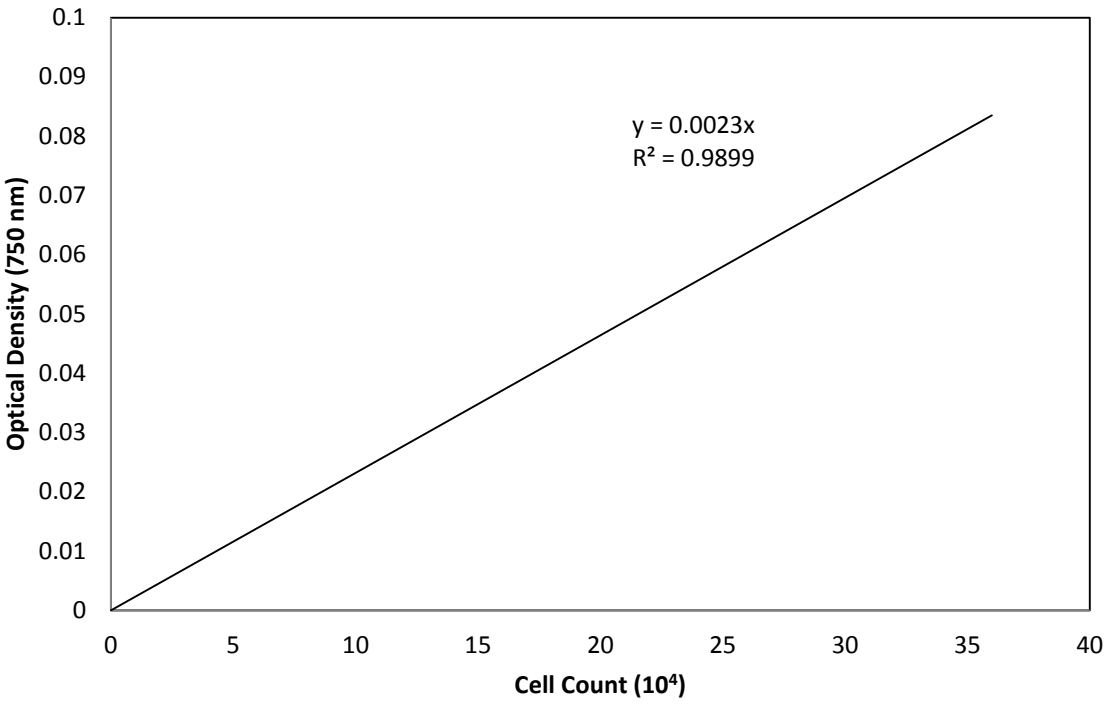


Figure 6.3. Standard curve for *Tetraselmis suecica* at 750 nm.

6.7.3. Oil Content

The oil content in the biomass was determined according to Bligh and Dyer (1959) method as described by Araujo et al. (2013). This is a simple method for oil extraction from microalgae using ultrasound assisted solvent extraction. Firstly, the algae biomass were homogenized and mixed with 25 mL of methanol, 12.5 mL of chloroform and 5 mL of distilled deionized water. This mixture was exposed to ultrasonic energy (Branson 2510R-DTH, Branson Ultrasonics Corporation, Danbury, USA) for 40 min. To this, an additional 12.5 mL of chloroform and 12.5 mL of 1.5% (w/v) sodium sulfate solution were added and sonicated for another 20 min. The solid biomass particles were filtered out of the solution and the liquid fraction was transferred to a separatory funnel with the addition of 75 mL of 0.88% (w/v) KCl. The mixture was vigorously shaken and then left to separate for 24 h. The solubility of oils in the chloroform solvent and the insolubility of solvents in water allowed for a separation to occur into two phases (organic and aqueous). The oil containing phase (on the bottom) was drained out of the separatory funnel and collected into a pre-weighed distill flask. The flask was distilled using rotary evaporator (HiTEC RE-51, Yamato Scientific America, California, USA) set at 45°C. The oil left behind has weighed in the flask and the yield was determined based on Equation 5.

$$\text{Oil Yield (\%)} = \frac{weight\ of\ Oil\ (g)}{weight\ of\ Algae\ Biomass\ (g)} \text{ x } 100 \qquad (5)$$

6.7.4. Elemental Analysis

The elemental analyses were performed at the Mineral Engineering Center of Dalhousie University. The sample was initially filtered to remove any solid particulates. Following which, the filtrate was analyzed using Varian Vista Pro ICP OES (Radical View).

CHAPTER 7. RESULTS

7.1. Selection of Microalgae

Due to time constraints, the freshwater and marine microalgae species identified for biodiesel production (Table 7.1) were evaluated (based on literature information) in order to select one freshwater and one marine microalga for further study. Selection of the suitable microalgae was based on biomass yield and lipid content of each species. The relationship between lipid content (%) and biomass yield (g/L) depicted as lipid yield (g/L of biomass) is illustrated by Figures 7.1 and 7.2 for the freshwater and marine microalgae species, respectively.

The freshwater strain *Chlorella saccharophila* was selected for study because of its high lipid content (45%). This strain is capable of achieving a biomass yield of 3.88 g/L, which is not the highest among the freshwater species, but can however be offset by the fact that it achieves the highest lipid content. This results in a lipid yield of 1.75 g/L. The highest biomass yielding algae *Scenedesmus obliqus* of 4.34 g/L only achieves a lipid content of 38%, which intern results in a lipid yield of 1.69 g/L (Table 7.1).

The marine microalgae strain *Tetraselmis suecica* was selected for this study because of its high biomass yield of 4.48 g/L and comparatively high lipid content. This species achieves a lipid content of 23% which is not the highest among the other species, but can however be offset by the fact that it achieves the highest biomass yield. This results in a lipid yield of 1.03 g/L, while the highest marine lipid yielding algae *Chaetoceros muelleri* of 34% only achieves a biomass yield of 0.98 g/L, which results in a lipid yield of 0.33 g/L (Table 7.1).

7.2. $NaHCO_3$ as a Carbon Source

7.2.1. Microalgae Cell Biomass Yield

Microalgae yield was determined for two types of microalgae *Chlorella saccharophila* (freshwater) and *Tetraselmis suecica* (marine) using four types of nitrogen sources (ammonium nitrate at 70 mg nitrogen L^{-1}, ammonium phosphate 70 mg nitrogen L^{-1} and 77.5 mg phosphorous L^{-1}, ammonium sulfate at 70 mg nitrogen L^{-1} and 80 mg

Table 7.1. Lipid yield of freshwater and marine water microalgae species.

Species	Biomass (g/L)	Lipid Content (%)	Lipid Yield (g/L of biomass)	Lipid Productivity (mg/L/d)
Freshwater				
Botryococcus brauni	1.84	25.2	0.46	5.51
Chlorella protothecides	1.32	31.23	0.41	39.6
Chlorella pyrenoidosa	2.84	38.95	1.10	107.9
Chlorella saccharophila	**3.88**	**45.5**	**1.75**	**153.38**
Chlorella sorokiniana	3.22	19.3	0.62	44.7
Chlorella vulgaris	1.005	27.6	0.28	27.61
Chlorococcum sp.	3.92	19.3	0.76	53.7
Scenedesmus obliqus	4.355	38.9	1.69	117
Marine Water				
Chaetoceros muelleri	0.98	33.6	0.33	21.8
Isochrysis sp.	2.38	22.4	0.53	37.7
Nannochloropsis sp.	2.8	24.4	0.68	48.2
Phaeodactylum tricornutum	3.36	18.7	0.63	44.8
Skeletonema costatum	1.12	21.1	0.24	17.4
Tetraselmis suecica	**4.48**	**23**	**1.03**	**36.4**

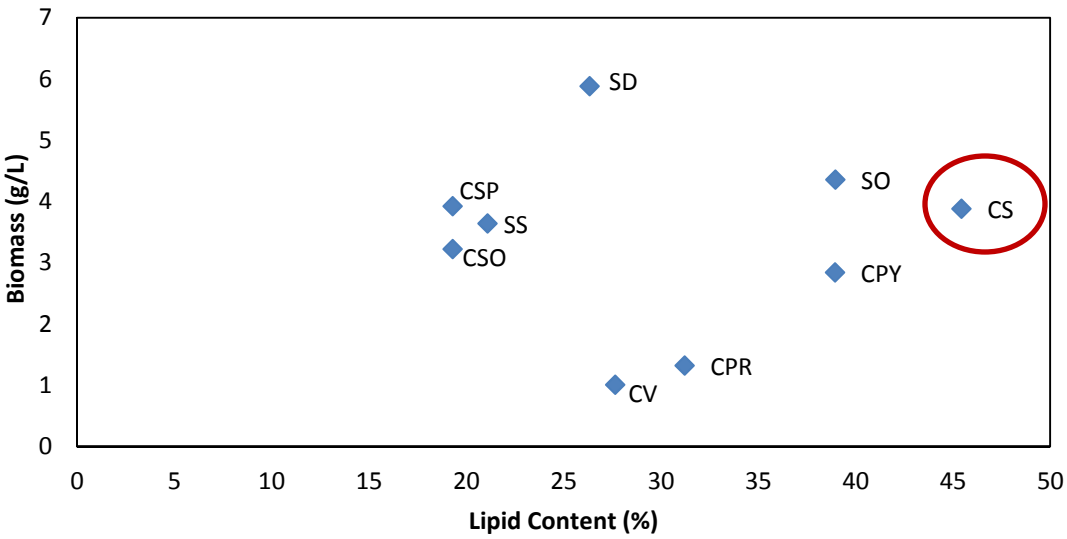


Figure 7.1. Biomass vs. lipid content of freshwater microalgae species. BB:*Botryococcus brauni*, CPR:*Chlorella protothecides*, CS:*Chlorella saccharophila*, CSO:*Chlorella sorokiniana*, CV:*Chlorella vulgaris*, CSP:*Chlorococcum* sp., SD:*Scenedesmus dimorphus*, SO:*Scenedesmus obliqus*, SS:*Scenedesmus sp*.

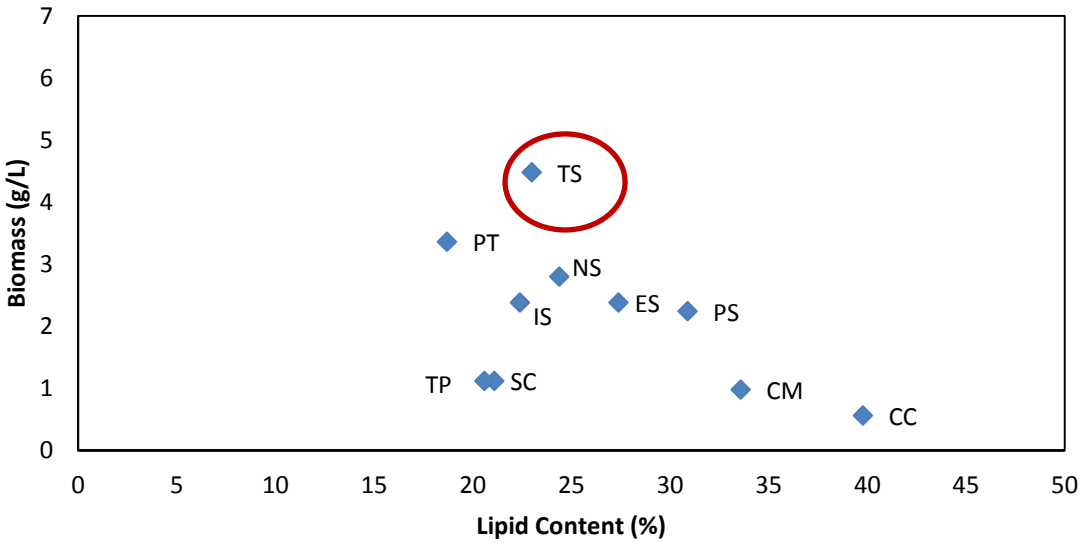


Figure 7.2. Biomass vs. lipid content of marine microalgae species. CC:*Chaetoceros calcitrans*, CM:*Chaetoceros muelleri*, SC:*Skeletonema costatum*, TP:*Thalassiosira pseudonana*, PS:Pavlova salina, ES:*Ellipsoidion sp*., IS:*Isochrysis sp*., NS:Nannochloropsis sp., PT:*Phaeodactylum tricornutum*, TS:*Tetraselmis suecica*, TP:*Thalassiosira pseudonana*.

sulfate L^{-1} and combination of all three at 70 mg nitrogen L^{-1}, 22.1 mg phosphorous $L^{-1}$and 22.8 mg sulfate L^{-1}), three light durations (9, 16 and 24 h) and sodium bicarbonate as the carbon source. The results are shown in Table 7.2. Determination of cell yield was carried out by measuring the optical density of *Chlorella saccharophila* at 484 nm and *Tetraselmis suecica* at 750 nm.

Analysis of the variance (ANOVA) was performed on the cell yield data as shown in Table 7.3 using Minitab statistics software (Minitab® 16.2.2., Minitab Inc., Canada). The effects of microalgae type, light duration and nitrogen source on the biomass yield were significant at 0.001 level. There was also significant interactions between the parameters at the 0.010 level.

The means obtained from the Tukey's grouping are shown in Table 7.4. The two algae (the freshwater and marine water microalgae) were significantly different from one another at the 0.05 level. The results indicate that the highest biomass yield ($2.89x10^6$ cells mL^{-1}) was obtained from the marine microalgae *Tetraselmis suecica*. The nutrient types ammonium nitrate, ammonium phosphate and ammonium sulfate were not significantly different from one another, but were significantly different from the combination of nutrients at the 0.05 level. The highest mean biomass yield of $4.01x10^6$ cells mL^{-1} was achieved using the combination of nutrients. The levels of light exposure 16 and 24 h were not significantly different from one another, but were significantly different from the 9 h exposure at the 0.05 level. However, the highest mean biomass yield of $2.28x10^6$ cells mL^{-1} was achieved at 24 h light exposure.

7.2.1.1. Effect of Microalgae Type: The effect of microalgae type (freshwater and marine) using different nutrient types (ammonium nitrate at 70 mg nitrogen L^{-1}, ammonium phosphate 70 mg nitrogen L^{-1} and 77.5 mg phosphorous L^{-1}, ammonium sulfate at 70 mg nitrogen L^{-1} and 80 mg sulfate L^{-1} and combination of all three at 70 mg nitrogen L^{-1}, 22.1 mg phosphorous L^{-1} and 22.8 mg sulfate L^{-1}) at different light durations (9, 16 and 24 h) on the microalgae cell yield are shown in Figures 7.3-7.5. The results indicate that the marine microalgae (*Tetraselmis suecica*) achieved high cell yields using all nutrient types at all light durations than the freshwater microalgae.

Table 7.2. Average cell yield of *Chlorella saccharophila* (freshwater) and *Tetraselmis suecica* (marine) microalgae using various nitrogen sources at different light exposures with sodium bicarbonate as the carbon source.

Species	Nutrient Type	Light (h)	Cell Yield (cells mL^{-1})
Freshwater	Ammonium nitrate	9	$0.279 \times 10^6 \pm 0.07 \times 10^6$
		16	$0.336 \times 10^6 \pm 0.05 \times 10^6$
		24	$0.460 \times 10^6 \pm 0.04 \times 10^6$
	Ammonium phosphate	9	$0.138 \times 10^6 \pm 0.03 \times 10^6$
		16	$0.186 \times 10^6 \pm 0.05 \times 10^6$
		24	$0.489 \times 10^6 \pm 0.08 \times 10^6$
	Ammonium sulfate	9	$0.139 \times 10^6 \pm 0.02 \times 10^6$
		16	$0.157 \times 10^6 \pm 0.03 \times 10^6$
		24	$0.507 \times 10^6 \pm 0.09 \times 10^6$
	Combination	9	$0.295 \times 10^6 \pm 0.02 \times 10^6$
		16	$0.630 \times 10^6 \pm 0.03 \times 10^6$
		24	$0.689 \times 10^6 \pm 0.01 \times 10^6$
Marine	Ammonium nitrate	9	$0.266 \times 10^6 \pm 0.08 \times 10^6$
		16	$0.619 \times 10^6 \pm 0.02 \times 10^6$
		24	$0.750 \times 10^6 \pm 0.02 \times 10^6$
	Ammonium phosphate	9	$1.664 \times 10^6 \pm 0.05 \times 10^6$
		16	$2.122 \times 10^6 \pm 0.58 \times 10^6$
		24	$2.354 \times 10^6 \pm 0.10 \times 10^6$
	Ammonium sulfate	9	$0.664 \times 10^6 \pm 0.02 \times 10^6$
		16	$0.978 \times 10^6 \pm 0.01 \times 10^6$
		24	$2.793 \times 10^6 \pm 0.02 \times 10^6$
	Combination	9	$2.689 \times 10^6 \pm 0.02 \times 10^6$
		16	$9.415 \times 10^6 \pm 0.06 \times 10^6$
		24	$10.342 \times 10^6 \pm 0.13 \times 10^6$

Table 7.3. Analysis of the variance for biomass yield using $NaHCO_3$ as a carbon source.

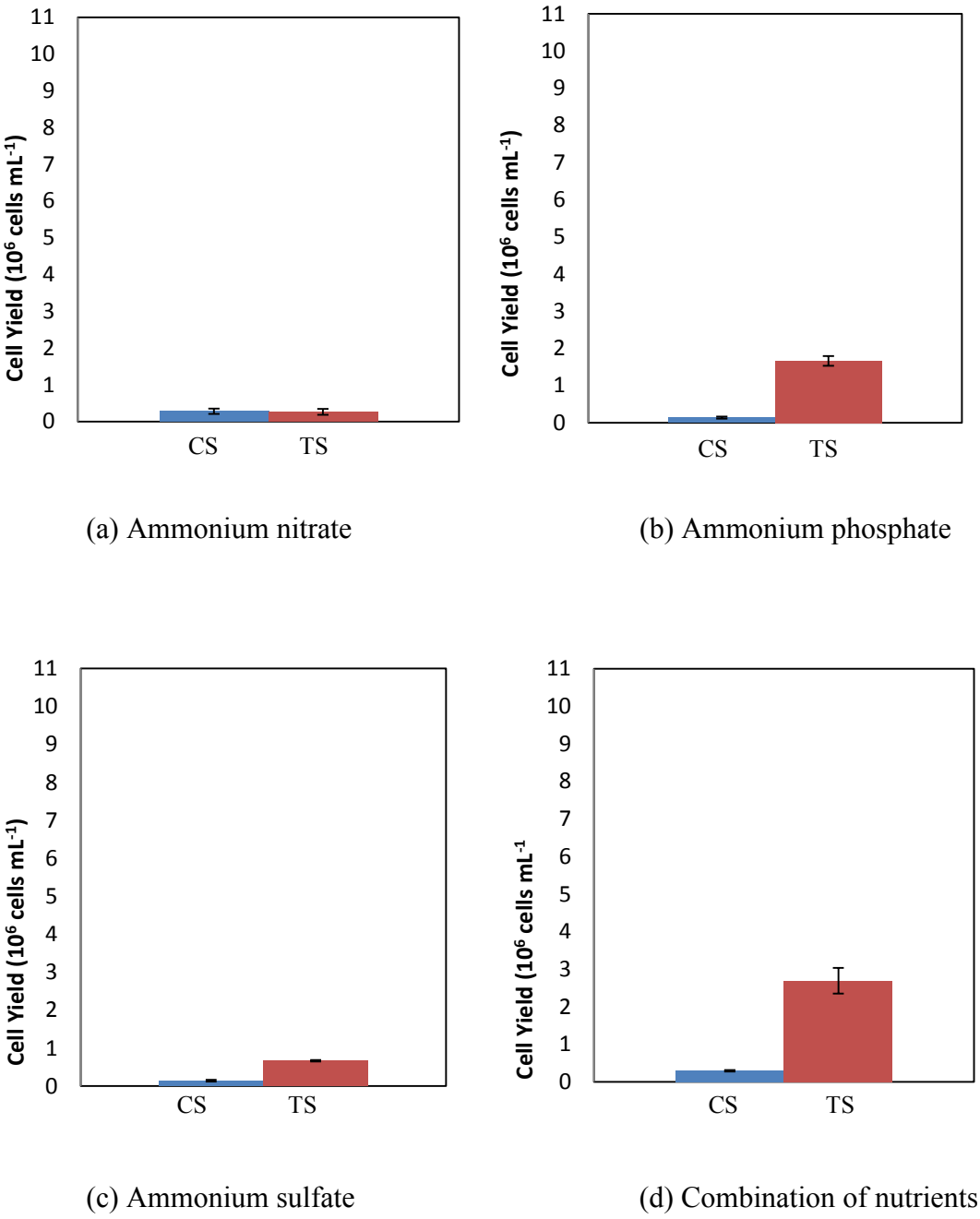
Source	**DF**	**SS**	**MS**	**F**	**P**
Total	71	5.854×10^{14}			
Model					
Species (S)	1	1.152×10^{14}	1.151×10^{14}	60.29	0.001
Nutrient (N)	3	1.413×10^{13}	4.709×10^{13}	24.65	0.001
Light (L)	2	2.896×10^{13}	1.448×10^{13}	7.58	0.001
S*N	3	1.228×10^{14}	4.092×10^{13}	21.42	0.001
S*L	2	1.894×10^{13}	9.469×10^{12}	4.96	0.010
N*L	6	3.546×10^{13}	5.909×10^{12}	3.09	0.010
S*N*L	6	3.116×10^{13}	5.193×10^{12}	2.72	0.020
Error	48	9.169×10^{13}	1.910×10^{12}		

DF: Degrees of Freedom
SS: Sum of Squares
MS: Mean Sum of Squares
F: F Distribution
P: Probability-Value
R^2= 84.34%
CV= 176.85%

Table 7.4. Tukey's grouping for biomass yield using $NaHCO_3$ as a carbon source.

Factors	Level	N	Mean Yield	Tukey's Grouping
Species	Marine water	36	2888406	A
	Freshwater	36	358921	B
Nutrient	Ammonium nitrate	18	451837	A
	Ammonium phosphate	18	1159062	A
	Ammonium sulfate	18	873226	A
	Combination	18	4010527	B
Light (h)	9	24	766963	A
	16	24	1821984	B
	24	24	2282043	B

Groups with the same letter are not significantly different from each other at the alpha significance level of 0.05.



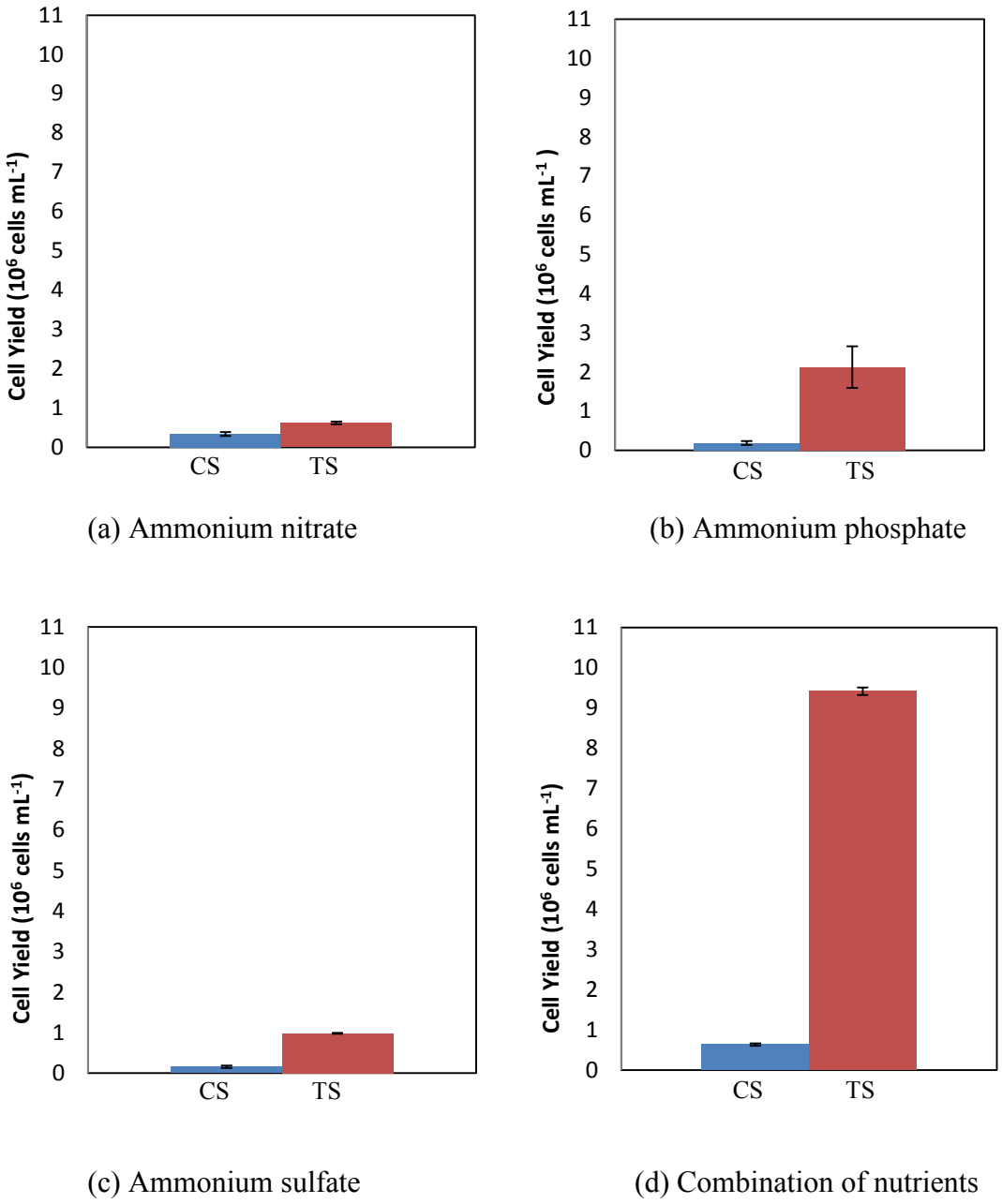
(a) Ammonium nitrate

(b) Ammonium phosphate

(c) Ammonium sulfate

(d) Combination of nutrients

Figure 7.3. Effect of microalgae type on the cell yield (cells mL^{-1}) using different nutrients at 9 h light exposure, CS-*Chlorella saccharophila*, TS- *Tetraselmis suecica*.



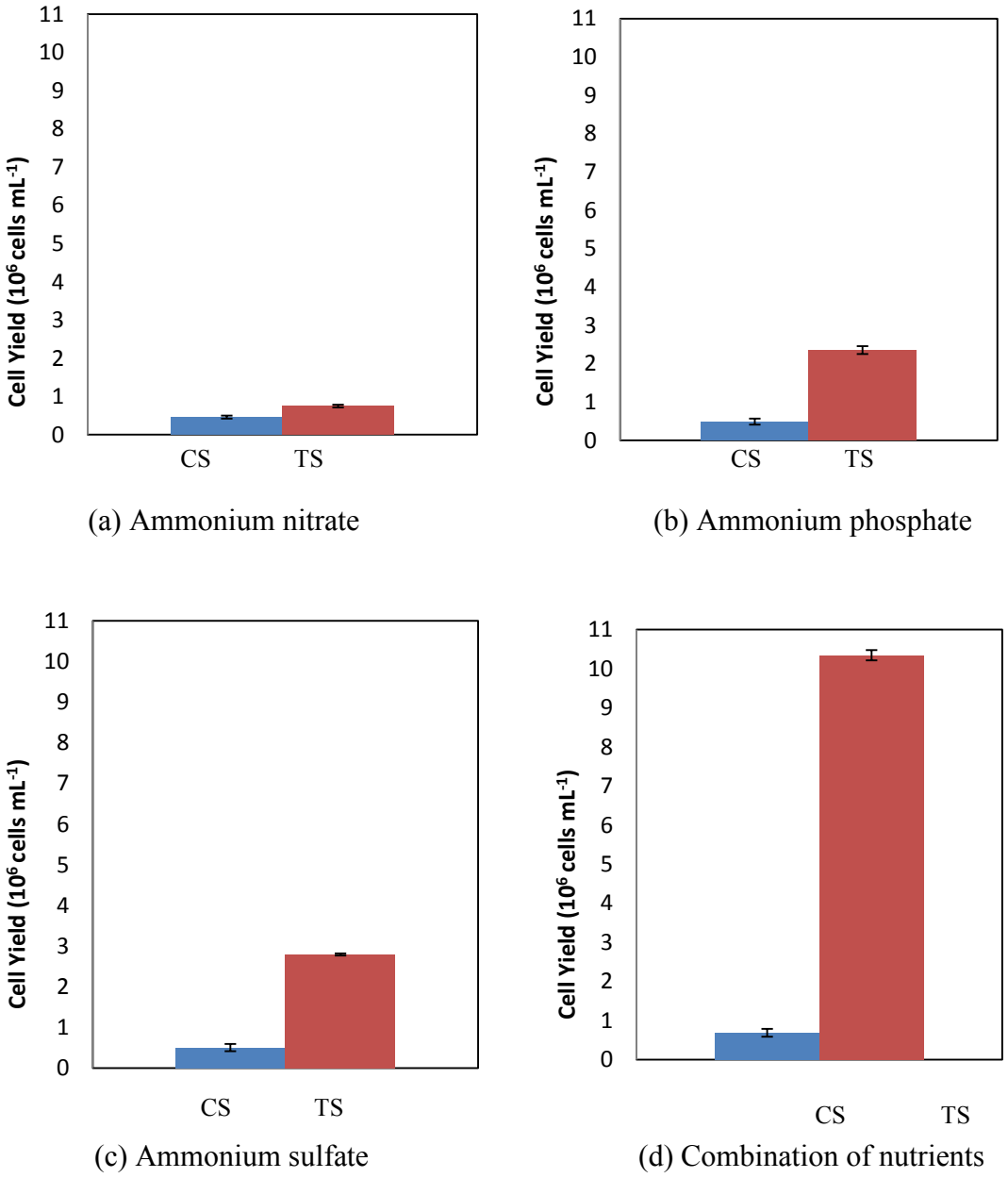
(a) Ammonium nitrate

(b) Ammonium phosphate

(c) Ammonium sulfate

(d) Combination of nutrients

Figure 7.4. Effect of microalgae type on the cell yield (cells mL^{-1}) using different nutrients at 16 h light exposure, CS-*Chlorella saccharophila*, TS- *Tetraselmis suecica*.



(a) Ammonium nitrate

(b) Ammonium phosphate

(c) Ammonium sulfate

(d) Combination of nutrients

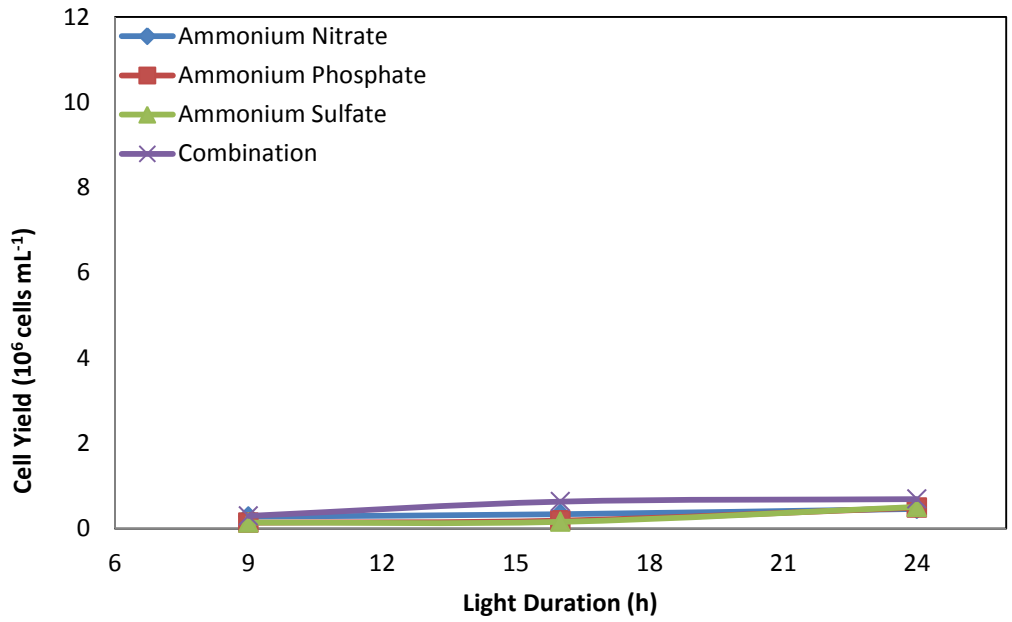
Figure 7.5. Effect of microalgae type on the cell yield (cells mL-1)using different nutrients at 24 h light exposure, CS-*Chlorella saccharophila*, TS- *Tetraselmis suecica*.

The marine microalgae (*Tetraselmis suecica*) resulted in cell yields of $0.266x10^6$, $1.664x10^6$, $0.664x10^6$ and $2.689x10^6$ cells mL^{-1} while the freshwater microalga (*Chlorella saccharophila*) resulted in cell yields of $0.28x10^6$, $0.14x10^6$, $0.14x10^6$ and $0.30x10^6$ cells mL^{-1} at 9 h light exposure, using nutrients ammonium nitrate, ammonium phosphate, ammonium sulfate and combination of nutrients, respectively. Similar trends were observed at the other light exposures (16 and 24 h), higher yields were observed for the marine microalga (*Tetraselmis suecica*) as opposed to the freshwater (*Chlorella saccharophila*).

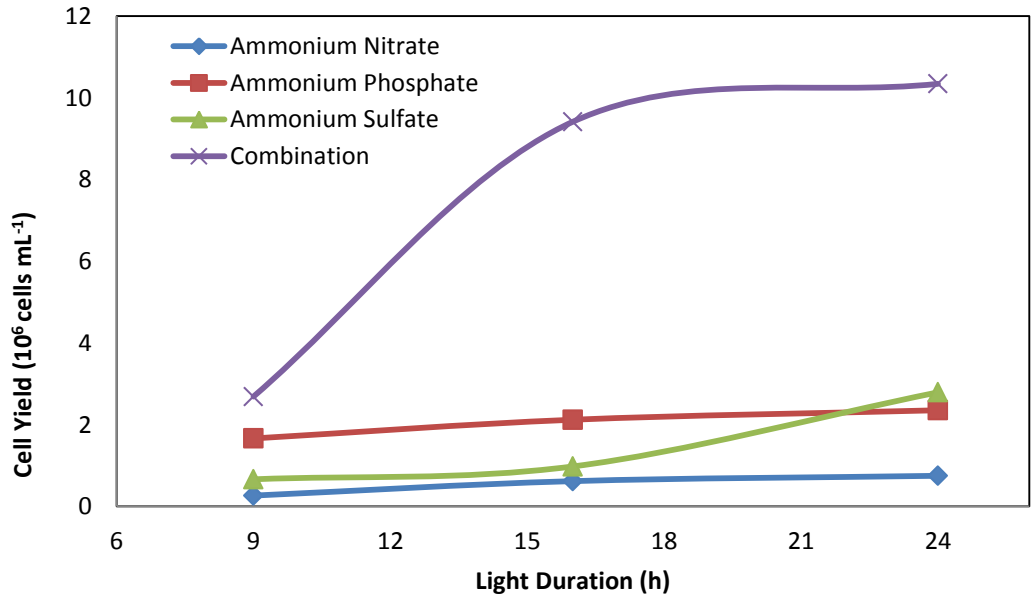
7.2.1.2. Effect of Light: The effect of light duration (9, 16 and 24 h) on the cell yield of *Chlorella saccharophila* and *Tetraselmis suecica* using different nutrients (ammonium nitrate, ammonium phosphate, ammonium sulfate and mix of all three) is shown in Figure 7.6. The results indicate a slight increase in cell yields as the light duration is increased for both microalgae species. However, Tukeys statistical analysis (Table 7.4) indicated that the mean yields achieved using light durations of 16 and 24 h were not significantly different from one another, however they were significantly different from the yields achieved using 9 h light exposure.

For the ammonium sulfate nutrient, an increase in light duration from 9 to 24 h, increased the cell yields from $0.138x10^6$ to 0.506x106 ($0.368x10^6$) and from $0.664x10^6$ cells mL^{-1} to $2.79x10^6$ cells mL^{-1} ($2.13x10^6$ cells mL^{-1}) for the freshwater (*Chlorella Saccharophila*) and marine (*Tetraselmis suecica*) microalgae, respectively. Similar trends were observed using the other nutrients for the freshwater microalgae. Microalgae cell yields increased by $0.180x10^6$, $0.352x10^6$ and $0.394x10^6$ cells mL^{-1} for the freshwater microalgae using the nutrients ammonium nitrate, ammonium phosphate and combination, respectively. Similarly, the marine microalgae cell yield increased by $0.353x10^6$, $0.689x10^6$ and $7.651x10^6$ cells mL^{-1} for the nutrients ammonium nitrate, ammonium phosphate and combination of nutrients.

7.2.1.3. Effect of Nutrient Type: The effect of nutrient type (ammonium nitrate, ammonium phosphate, ammonium sulfate and mix of all three) on the cell yield of freshwater (*Chlorella Saccharophila)* and marine (*Tetraselmis suecica)* microalgae at different light durations is shown in Figure 7.7. The results indicate that the cell yields

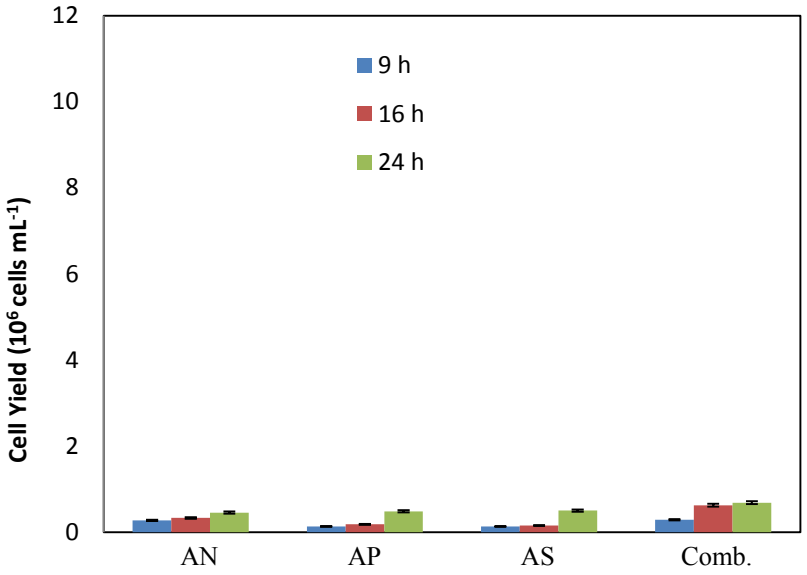


(a) *Chlorella saccharophila*

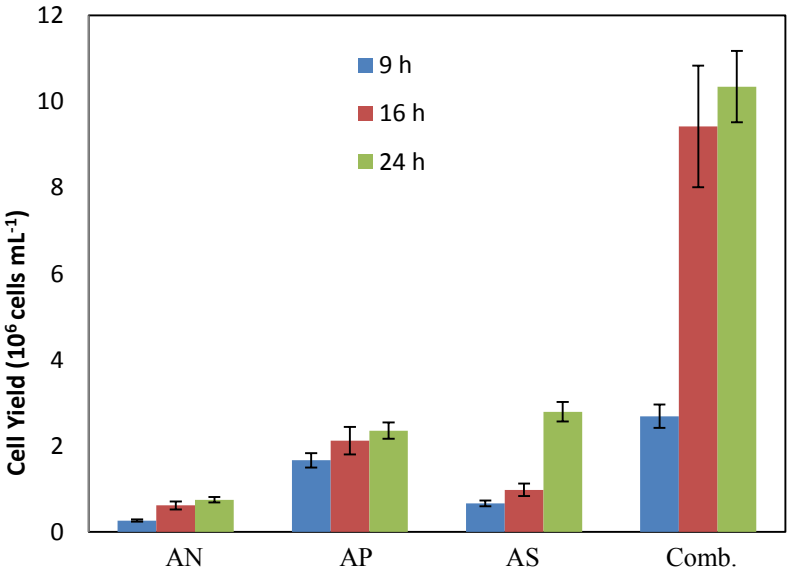


(b) *Tetraselmis suecica*

Figure 7.6. Effect of light duration on the cell yield (cells mL^{-1}) of freshwater (*Chlorella saccharophila*) and marine (*Tetraselmis suecica*) microalgae, using different nutrients.



(a) *Chlorella saccharophila*



(b) *Tetraselmis suecica*

Figure 7.7. Effect of nutrient type on the cell yield (cells mL^{-1}) of freshwater (*Chlorella saccharophila*) and marine (*Tetraselmis suecica*) microalgae at varying light durations. AN: Ammonium nitrate, AP: Ammonium phosphate, AS: Ammonium sulfate, Comb: Combination of nutrients.

for the different microalgae were highest using the combination of nutrients at varying light durations. Tukeys statistical analysis (Table 7.4) revealed that the mean yields achieved using ammonium nitrate, ammonium phosphate and ammonium sulfate nutrient systems were not significantly different from one another for both microalgae species. However the combination of nutrient system was significantly different from the other three nutrient systems.

For the freshwater microalgae (*Chlorella saccharophila*) the ammonium sulfate and ammonium phosphate nutrients did not vary significantly from one another at all light durations, but did however vary significantly from ammonium nitrate system at the 9 and 16 h but not at the 24 h duration. The highest cell yields of $0.295x10^6$, $0.630x10^6$ and $0.689x10^6$ cells mL^{-1} were achieved using combination of nutrient system at 9, 16 and 24 h, respectively. The lowest cell yields of $0.138x10^6$ and $0.156x10^6$ cells mL^{-1} resulted using ammonium sulfate nutrient at 9 and 16 h, respectively. However the lowest cell yields at 24 h of $0.460x10^6$ cells mL^{-1} resulted from ammonium nitrate.

For the marine microalgae (*Tetraselmis suecica*) the lowest cell yields were $0.266x10^6$, $0.750x10^6$ and $0.619x10^6$ cells mL^{-1} resulted from using ammonium nitrate at 9, 16 and 24 h, respectively. The highest cell yields at of $2.689x10^6$, $9.41x10^6$ and $10.34x10^6$ cells mL^{-1} were achieved using the combination of nutrient system at 9, 16 and 24 h, respectively. Ammonium sulfate system cell yields were not significantly different from ammonium nitrate system at 9 and 16 h, but were significantly different at 24 h. However, the ammonium sulfate achieved a 4 fold increase than that of ammonium nitrate.

7.2.2. Microalgae Oil Content

Microalgae oil yield was determined for two types of microalgae *Chlorella saccharophila* (freshwater) and *Tetraselmis suecica* (marine) using four types of nitrogen sources (ammonium nitrate at 70 mg nitrogen L^{-1}, ammonium phosphate 70 mg nitrogen L^{-1} and 77.5 mg phosphorous L^{-1}, ammonium sulfate at 70 mg nitrogen L^{-1} and 80 mg sulfate L^{-1} and combination of all three at 70 mg nitrogen L^{-1}, 22.1 mg phosphorous L^{-1} and 22.8 mg sulfate L^{-1}), three light durations (9, 16 and 24 h) and sodium bicarbonate as the carbon source. The results are shown in Table 7.5. Determination of yield was

Table 7.5. Average oil yield of *Chlorella saccharophila* (freshwater) and *Tetraselmis suecica* (marine) microalgae using various nitrogen sources at different light exposures with sodium bicarbonate as the carbon source.

Species	**Nutrient Type**	**Light (h)**	**Oil Yield** (%)
Freshwater	Ammonium nitrate	9	14.162±4.85
		16	13.176±2.58
		24	7.714±3.44
	Ammonium phosphate	9	29.075±13.40
		16	27.353±1.43
		24	7.861±2.24
	Ammonium sulfate	9	13.247±0.58
		16	7.168±1.00
		24	3.782±3.56
	Combination	9	22.443±17.6
		16	18.781±0.89
		24	12.907±7.85
Marine	Ammonium nitrate	9	2.742±0.76
		16	1.482±1.03
		24	1.102±0.67
	Ammonium phosphate	9	1.876±0.78
		16	1.213±1.38
		24	0.640±0.55
	Ammonium sulfate	9	2.158±0.43
		16	1.589±1.70
		24	1.175±0.31
	Combination	9	0.450±0.21
		16	0.435±0.16
		24	0.270±0.06

carried out by measuring the amount of oil obtained with respect to the biomass recovered (Equation 5).

Analysis of the variance (ANOVA) was performed on the oil yield data as shown in Table 7.6. The effects of microalgae type and nitrogen source on oil yield were significant at the 0.01 level. However the interactions between light duration, species type and nitrogen source were not significant at the 0.01 level.

The means obtained from the Tukey's grouping are shown in Table 7.7. The two algae (the freshwater and marine water microalgae) were significantly different from one another at the 0.05 level. The results indicate that the highest mean oil yield (15.91 %) was obtained from the freshwater microalgae *Chlorella saccharophila*. The nutrient types ammonium nitrate, ammonium sulfate and the combination of nutrients were not significantly different from one another, but were significantly different from the ammonium phosphate nutrient system at the 0.05 level. However, the ammonium phosphate system was not significantly different from the combination of nutrient system. The highest mean oil yield of 13.56 % was achieved using the ammonium phosphate system. The levels of light exposures at all varying durations of 9, 16 and 24 h were not significantly different from one another.

7.2.2.1. Effect of Microalgae Type: The effects of microalgae type (freshwater and marine) using different nutrient types (ammonium nitrate at 70 mg nitrogen L^{-1}, ammonium phosphate 70 mg nitrogen L^{-1} and 77.5 mg phosphorous L^{-1}, ammonium sulfate at 70 mg nitrogen L^{-1} and 80 mg sulfate L^{-1} and combination of all three at 70 mg nitrogen L^{-1}, 22.1 mg phosphorous L^{-1} and 22.8 mg sulfate L^{-1}) at different light durations (9, 16 and 24 h) on the microalgae oil yield are shown in Figures 7.8-7.10. The results indicate that the freshwater microalgae (*Chlorella saccharophila)* achieved the highest oil yields using all nutrient types at all light durations and the marine microalgae achieved the lowest oil yields at all nutrient types and all light durations.

The freshwater microalgae (*Chlorella saccharophila)* resulted in oil yields of 13.17, 29.08, 7.17 and 22.44% while the marine microalga (*Tetraselmis suecica)* resulted in oil yields of 1.48, 1.21, 1.17 and 0.45% at 9 h light exposure using nutrients ammonium nitrate, ammonium phosphate, ammonium sulfate and combination of nutrients,

Table 7.6. Analysis of the variance for oil yield using $NaHCO_3$ as a carbon source.

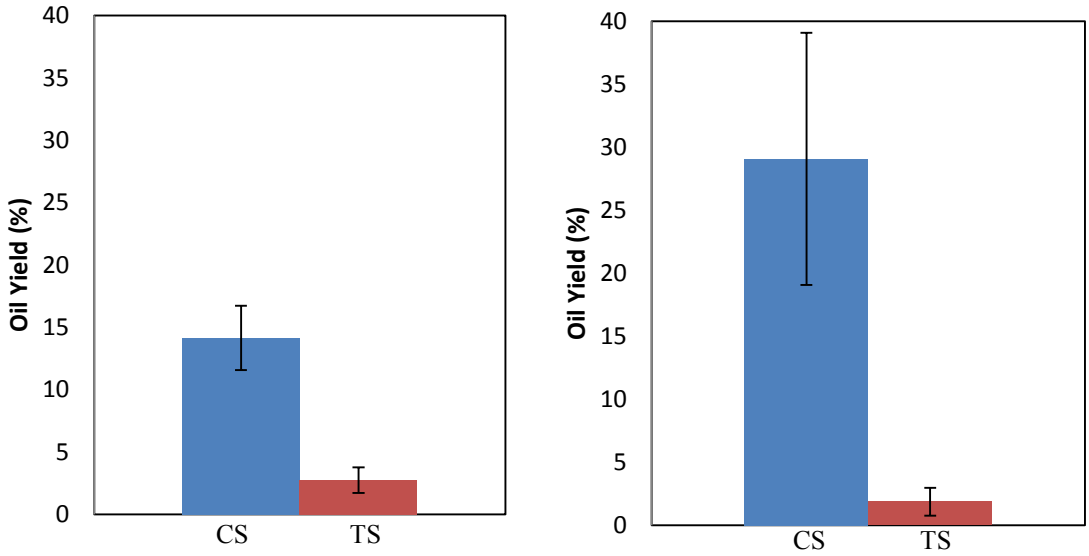
Source	**DF**	**SS**	**MS**	**F**	**P**
Total	71	8296.85			
Model					
Species (S)	1	3866.82	3866.82	80.37	0.000
Nutrient (N)	3	765.33	255.11	5.30	0.003
Light (L)	2	31.62	15.81	0.33	0.722
S*N	3	895.03	298.34	6.20	0.001
S*L	2	50.24	25.12	0.52	0.597
N*L	6	210.31	35.05	0.73	0.629
S*N*L	6	168.01	28.00	0.58	0.743
Error	48	2309.49	48.11		

DF: Degree of freedom
SS: Sum of square
MS: Mean of square
F: F Distribution
P: Probability-Value
$R^2 = 0.72$
CV= 125.85 %

Table 7.7. Tukey's grouping for oil yield using $NaHCO_3$ as a carbon source.

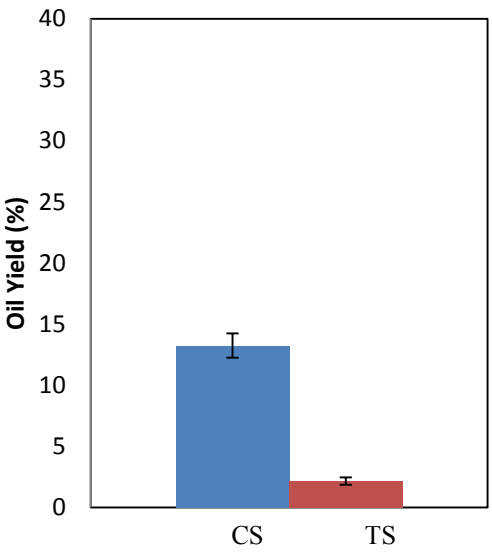
Factors	Level	N	Mean Yield (%)	Tukey's Grouping
Species	Marine water	36	1.261	A
	Freshwater	36	15.918	B
Nutrient	Ammonium nitrate	18	6.730	A
	Ammonium phosphate	18	13.560	B
	Ammonium sulfate	18	4.853	A
	Combination	18	9.214	A B
Light (h)	9	24	9.523	A
	16	24	8.194	A
	24	24	8.051	A

Groups with the same letter are not significantly different from each other at the alpha significance level of 0.05.

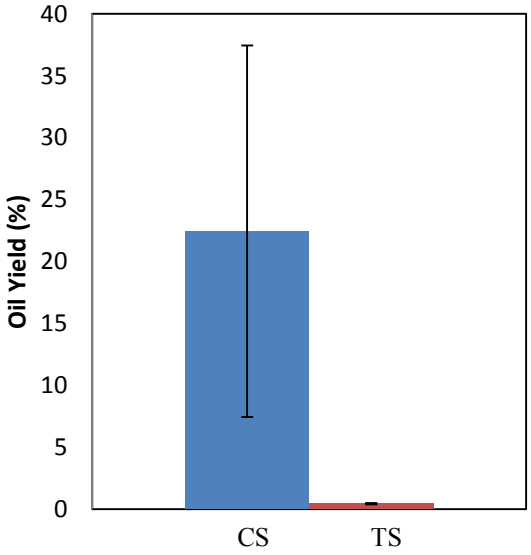


(a) Ammonium nitrate

(b) Ammonium phosphate

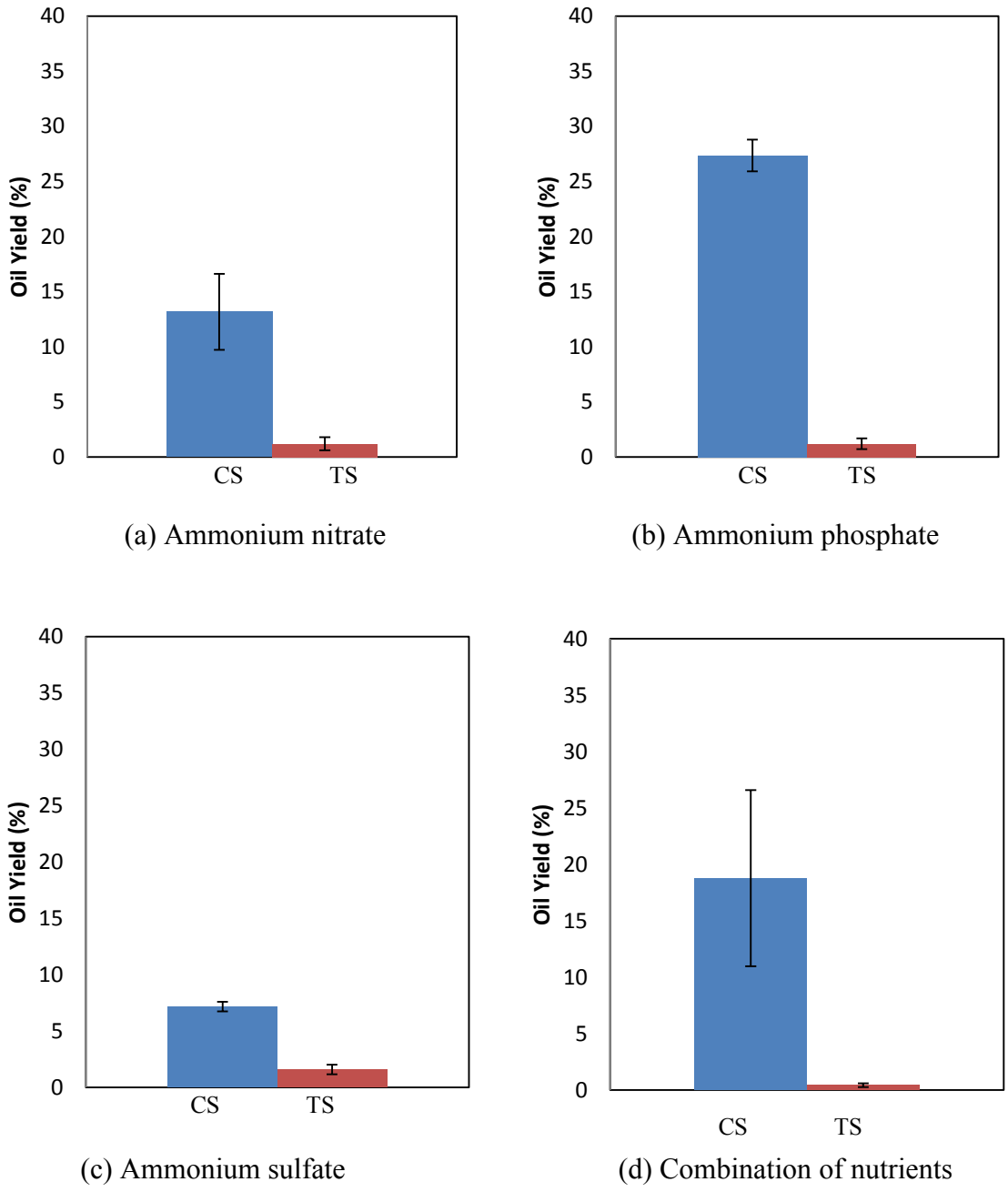


(c) Ammonium sulfate



(d) Combination of nutrients

Figure 7.8. Effect of microalgae type on the oil yield (%) using different nutrients at 9 h light exposure, CS-*Chlorella saccharophila*, TS- *Tetraselmis suecica*.



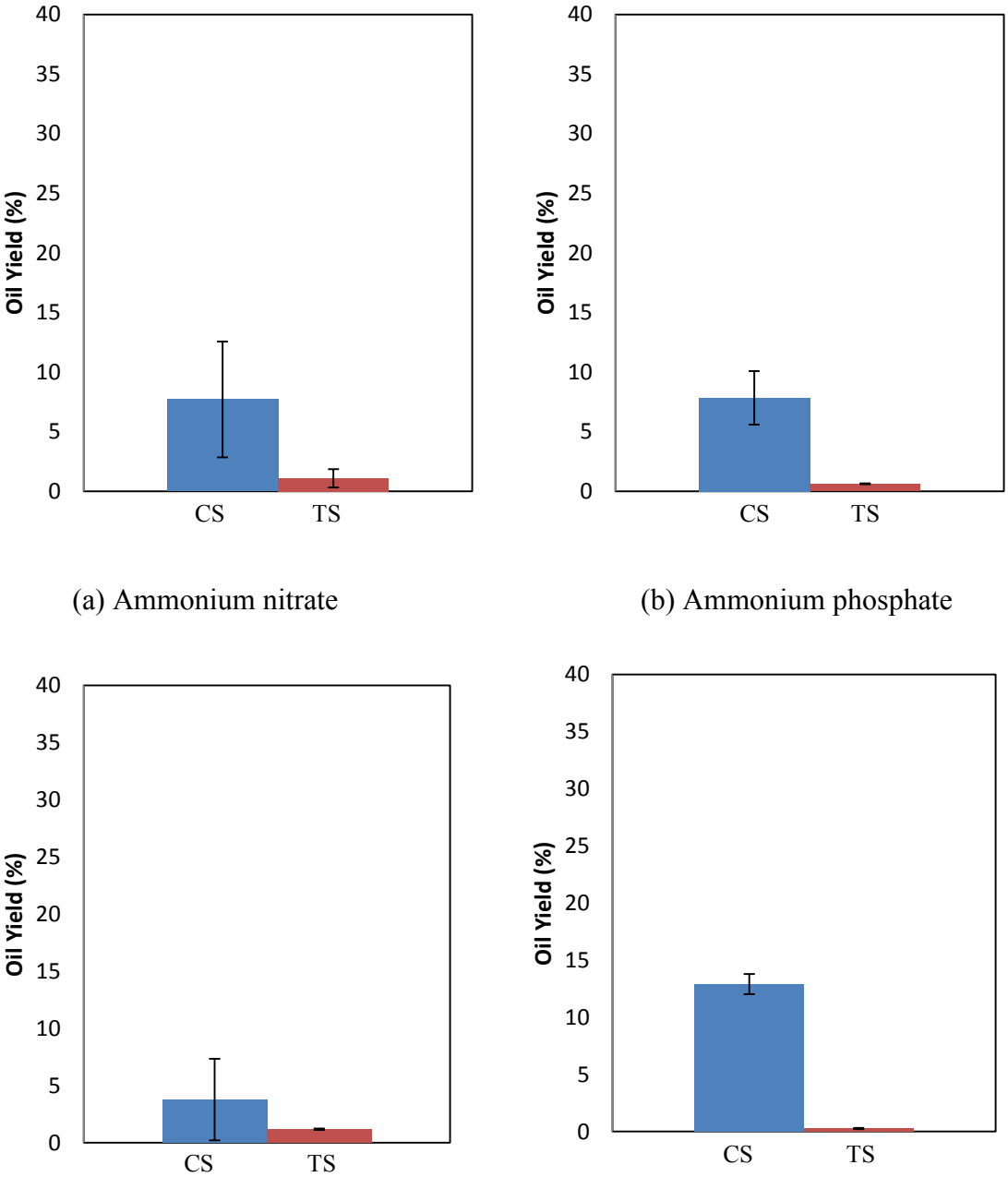
(a) Ammonium nitrate

(b) Ammonium phosphate

(c) Ammonium sulfate

(d) Combination of nutrients

Figure 7.9. Effect of microalgae type on the oil yield (%) using different nutrients at 16 h light exposure, CS-*Chlorella saccharophila*, TS- *Tetraselmis suecica*.



(a) Ammonium nitrate

(b) Ammonium phosphate

(c) Ammonium sulfate

(d) Combination of nutrients

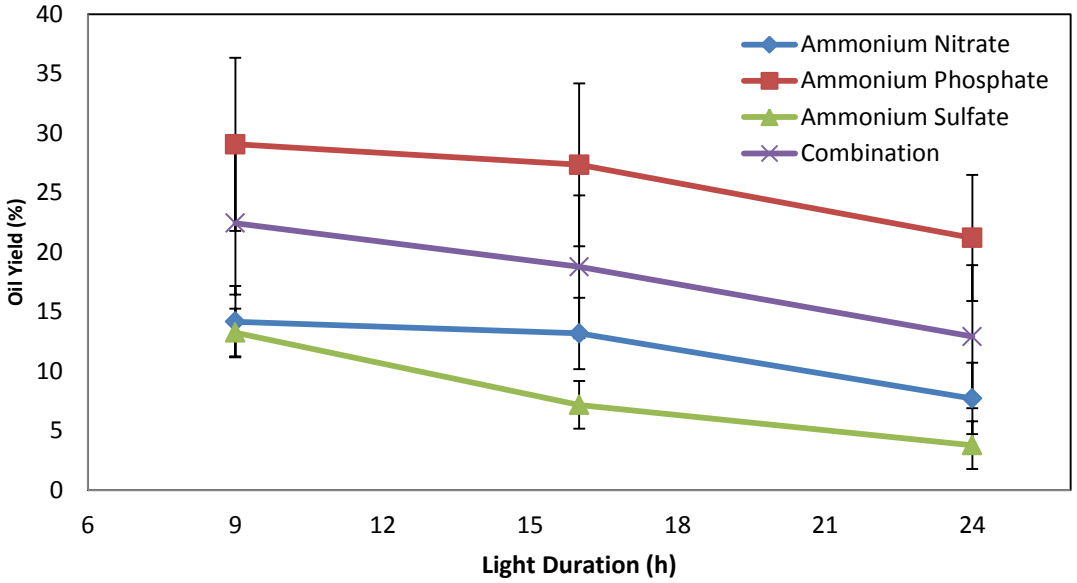
Figure 7.10. Effect of microalgae type on the oil yield (%) using different nutrients at 24 h light exposure, CS-*Chlorella saccharophila*, TS- *Tetraselmis suecica*.

respectively. Similar trends were observed at all other light exposures (16 and 24 h), higher oil yields were observed for the freshwater microalga (*Chlorella saccharophila)* as opposed to the marine (*Tetraselmis suecica)* microalga.

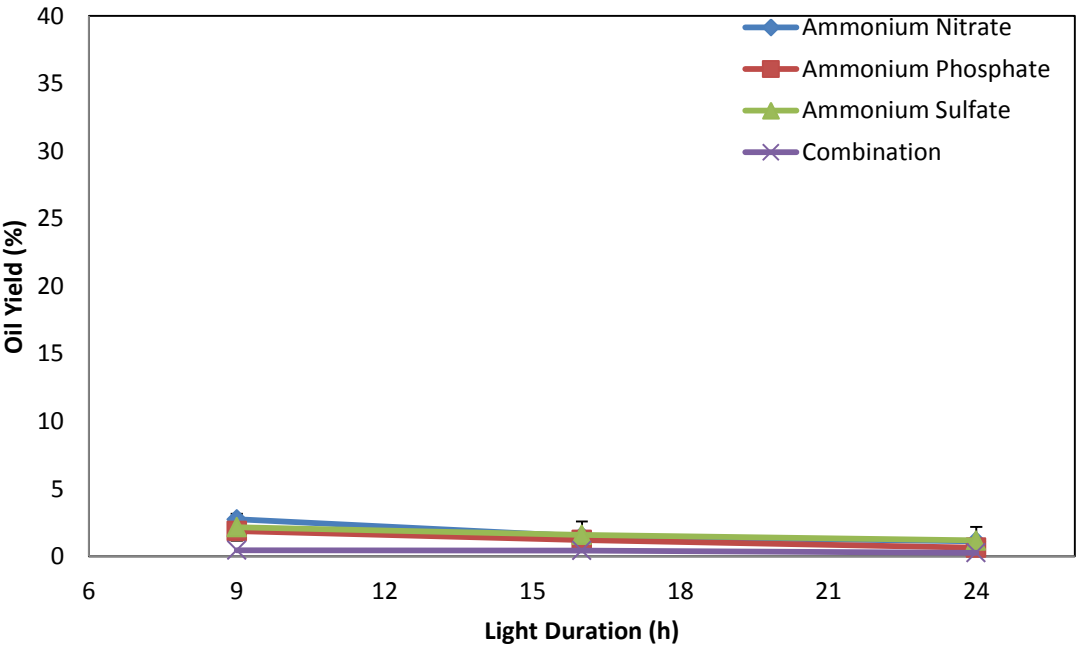
7.2.2.2. Effect of Light: The effects of light duration (9, 16 and 24 h) on the oil yield of *Chlorella saccharophila* and *Tetraselmis suecica* using different nutrients (ammonium nitrate, ammonium phosphate, ammonium sulfate and mix of all three) are shown in Figure 7.11. Statistical analysis from the Tukey's grouping (Table 7.7) indicated that the mean oil yields achieved by varying the light duration from 9 h to 24 h were not significantly different from one another.

The ammonium phosphate system illustrated a decrease in oil yields from 29.08 to 7.86% for the freshwater (*Chlorella saccharophila)* microalgae and increased from 1.18 to 1.89% for the marine (*Tetraselmis suecica*) microalgae as the light duration increased from 9 to 24 h. Similarly, in the ammonium sulfate nutrient, an increase in light duration from 9 to 24 h, decreased the oil yields from 7.17 to 3.78% for the freshwater (*Chlorella saccharophila)* microalgae and increased from 1.18 to 1.59% for the marine (*Tetraselmis suecica*) microalgae. However, ammonium nitrate and combination of nutrient systems illustrated similar trends for both microalgae species. Ammonium nitrate system resulted in increased oil yields as the light was increased from 9 to 24 h from 13.17 to 14.16% and from 1.48 to 2.74% for *Chlorella saccharophila* and *Tetraselmis suecica*, respectively. Combination of nutrients system depicted a decrease in oil yields as the light duration increased from 9 to 24 h from 22.44 to 18.78% and from 0.45 to 0.27% for *Chlorella saccharophila* and *Tetraselmis suecica*, respectively. However, these variations were not statistically different from one another. Since the average mean oil yield achieved by varying the light duration resulted in similar yields.

7.2.2.3. Effect of Nutrient Type: The effects of nutrient type (ammonium nitrate, ammonium phosphate, ammonium sulfate and combination of nutrients) on the oil yield of freshwater (*Chlorella saccharophila)* and marine (*Tetraselmis suecica)* microalgae at different light durations were determined. The results are shown in Figure 7.12. Statistical analysis from the Tukey's grouping (Table 7.7) indicated that the mean oil yields achieved using ammonium nitrate, ammonium phosphate and combination of

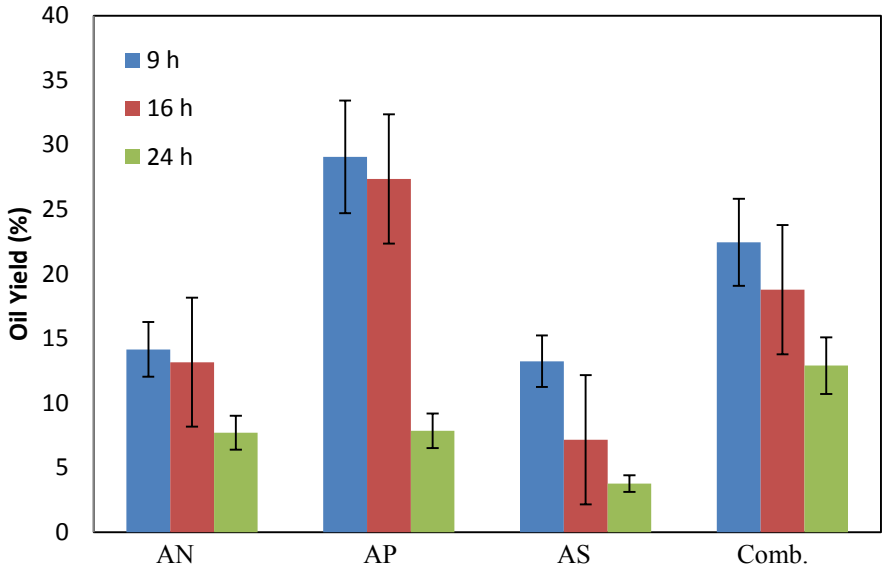


(a) *Chlorella saccharophila*

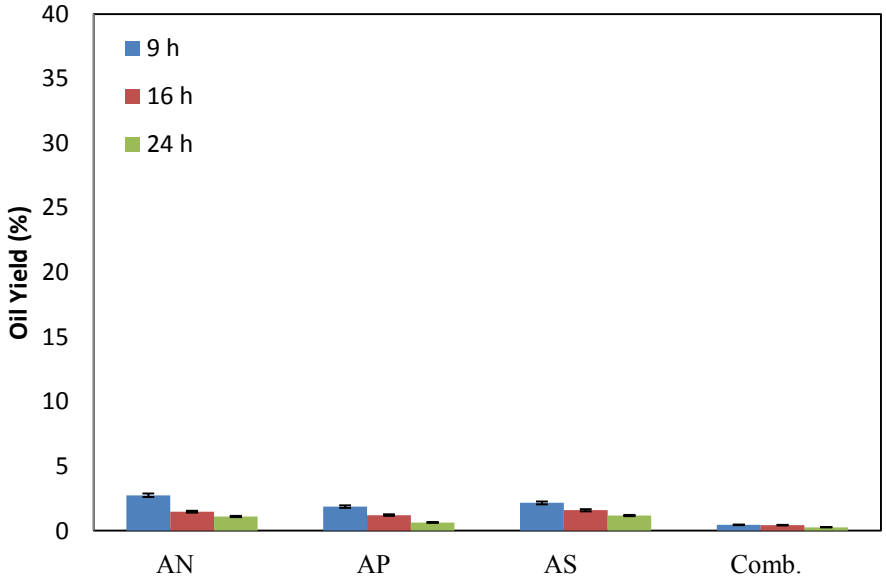


(b) *Tetraselmis suecica*

Figure 7.11. Effect of light duration on the oil yield (%) using different nutrients for two microalgae species.



(a) *Chlorella saccharophila*



(b) *Tetraselmis suecica*

Figure 7.12. Effect of nutrient type on the oil yield (%) of freshwater (*Chlorella saccharophila*) and marine (*Tetraselmis suecica*) microalgae at varying light durations. AN: Ammonium nitrate, AP: Ammonium phosphate, AS: Ammonium sulfate, Comb: Combination of nutrients.

nutrients were not significantly different from one another, but were significantly different from the ammonium phosphate system. In addition the analysis revealed no significant difference between the ammonium phosphate and combination of nutrient system. This can be seen in Figure 7.12 since the variation bars for the combination of nutrients and ammonium phosphate nutrient systems overlap one another. Overlap is also seen for ammonium sulfate, ammonium nitrate and combination of nutrient systems.

The results indicate that the freshwater microalgae achieved higher magnitude of oil yields than those of the marine water microalgae for all nutrients at all light exposures. Oil yields at 9 and 16 h light exposures for the freshwater microalga (*Chlorella saccharophila)* were the highest using ammonium phosphate nutrient system, while at 24 h the highest oil yield was achieved using the combination of nutrients. For the marine microalgae (*Tetraselmis suecica)*, the ammonium nitrate resulted in the highest oil yields at 9 and 24 h, but at 16 h light exposure the highest oil yield resulted using ammonium sulfate system.

For the freshwater microalga (*Chlorella saccharophila*), ammonium nitrate, ammonium phosphate, ammonium sulfate and combination of nutrients resulted in oil yields of 13.18, 7.71 and 14.16%, 29.08, 27.35 and 7.86%, 7.17, 13.24 and 3.78% and 22.44, 12.91 and 18.78% at 9, 16 and 24 h, respectively. For the marine microalga (*Tetraselmis suecica)* ammonium nitrate, ammonium phosphate, ammonium sulfate and combination of nutrients resulted in oil yields of 1.48, 1.10 and 2.74%,1 .21, 0.64 and 1.88%, 1.18, 2.16 and 1.59%, 0.45, 0.44 and 0.27% at 9, 16 and 24 h, respectively.

7.2.3. Optimal Growth Conditions using $NaHCO_3$

The optimal nutrient and light exposure duration for the *Chlorella saccharophila* (freshwater) microalgae were the combination of nutrients and 16 h, respectively. The highest cell yield of 6.89×10^5 cells mL^{-1} was achieved using the combination of nutrients and full light exposure (24 h), which also resulted in 18.9% oil yield, however this was not significantly different from the yields obtained using combination of nutrients at 16 h light exposure of 6.30×10^5 cells mL^{-1}, with an oil yield of 12.9 %. The increase in lipid yield by 146% does not offset the increase in light energy being used of 150% (from 16 to 24h). Thus, the higher cell yield achieved using combination of nutrients with 24 h

light exposure and the higher oil yield do not compensate for the increased light exposure duration, which makes these the most optimal conditions for growth.

The optimal nutrient and light exposure duration for the *Tetraselmis suecica* (marine) microalgae was determined using ammonium nitrate and 24 h light exposure. The highest oil yield of 2.74% was achieved using the ammonium nitrate as the nutrient source with full light exposure (24 h). However, the increase in lipid yield of 1.6% does not offset the increase in light exposure of 150% (from 16 to 24 h). These parameters resulted in a cell yield of 7.50×10^5 cells mL^{-1}, which was not the highest; however, the higher oil yield percentage did offset the lower cell yield. In order to improve the economics of biodiesel production achieving higher total lipid yields in combination with economical energy use is of importance.

7.3. CO_2 as a Carbon Source

7.3.1. Microalgae Biomass Yield

Microalgae yield for the two types of microalgae *Chlorella saccharophila* (freshwater) and *Tetraselmis suecica* (marine) using CO_2 as the carbon source at three concentrations (3:97, 6:94 and 9:91 CO_2:Air v/v), and the optimal nutrient conditions (determined using $NaHCO_3$ as the carbon source) and 24 h (highest lipid content produced) are illustrated in Table 7.8. Determination of cell yield was carried out by measuring the optical density of *Chlorella saccharophila* at 484 nm and *Tetraselmis suecica* at 750 nm.

ANOVA was performed on the cell yield data as shown in Table 7.9. The effect of microalgae type on the cell yield is significant at the 0.01 level. However, the effect of carbon source and the interactions between the species and carbon source were not significant at the 0.05 level.

The results obtained from the Tukey's grouping are shown in Table 7.10. The two microalgae (freshwater and marine water) were significantly different from one another at the 0.05 level. The results indicate that the highest mean biomass yield (1.47×10^6 cells mL^{-1}) was obtained from the marine microalgae species. The CO_2 concentrations (3%, 6% and 9%) were not significantly different from one another at the 0.05 level.

Table 7.8. Average cell yield of *Chlorella saccharophila* (freshwater) and *Tetraselmis suecica* (marine) microalgae using optimal nutrient and light conditions with carbon dioxide gas as the carbon source.

Species	**Nutrient Type**	**CO_2:Air Ratio (v/v)**	**Cell Yield (cells mL^{-1})**
Freshwater	Combination	3:97	$0.159 \times 10^6 \pm 0.04 \times 10^6$
		6:94	$0.890 \times 10^6 \pm 0.06 \times 10^6$
		9:91	$0.818 \times 10^6 \pm 0.04 \times 10^6$
Marine	Ammonium nitrate	3:97	$1.878 \times 10^6 \pm 0.06 \times 10^6$
		6:94	$1.522 \times 10^6 \pm 0.07 \times 10^6$
		9:91	$1.872 \times 10^6 \pm 0.01 \times 10^6$

Table 7.9. Analysis of the variance for biomass yield using CO_2 as the carbon source.

Source	DF	SS	MS	F	P
Total	17	9.664×10^{12}			
Model					
Species (S)	1	5.796×10^{12}	5.795×10^{12}	26.28	0.01
Carbon Source (C)	2	3.228×10^{11}	1.614×10^{11}	0.78	0.50
S*C	2	9.000×10^{11}	4.500×10^{11}	2.04	0.17
Error	12	2.646×10^{12}	2.205×10^{11}		

DF: Degree of freedom
SS: Sum of square
MS: Mean of square
F: F Distribution
P: Probability-Value
$R^2 = 0.73$
CV= 61.2 %

Table 7.10. Tukey's grouping on microalgae yield using CO_2 as the carbon source.

Factors	Level	N	Mean Yield	Tukey's Grouping
Species	Marine Water	12	1757247	A
	Freshwater (v/v)	12	622356	B
CO_2:Air	3:97	6	1018168	A
	6:94	6	1206299	A
	9:91	6	1344936	A

Groups with the same letter are not significantly different from each other at alpha significance level of 0.05 .

7.3.1.1. Effect of Microalgae Type: The effect of the microalgae type (freshwater and marine) using varying CO_2:air ratio (3:97, 6:94 and 9:91) for the optimal nutrient conditions for each species and 24 h light exposre on the microalgae cell yield are shown in Figure 7.13. The results indicate that the marine microalgae (*Tetraselmis suecica*) achieved the highest cell yields at all carbon dioxide to air ratios, while the freshwater microalgae (*Chlorella saccharophila*) resulted in the lowest cell yields at all carbon dioxide concentrations.

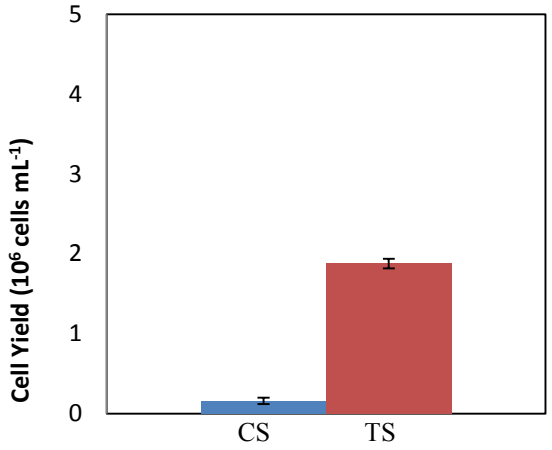
The marine microalgae (*Tetraselmis suecica)* resulted in cell yields of $1.877x10^6$, $1.522x10^6$, and $1.871x10^6$ cells mL^{-1} while the freshwater microalga (*Chlorella saccharophila)* resulted in cell yields of $0.159x10^6$, $0.890x10^6$ and $0.818x10^6$ cells mL^{-1}, using CO_2:Air ratios of 3:97, 6:94 and 9:91, respectively.

7.3.1.2. Effect of Carbon Dioxide Concentration: The effect of carbon dioxide concentration (3:97, 6:94 and 9:91 v/v) on the biomass yield of the marine and freshwater microalgae is illustrated in Figure 7.14. Statistical analysis results from Turkey's grouping (Table 7.10), indicate that increasing the carbon dioxide concentration had no significant effect on the microalgae cell yields. This can be seen in Figure 7.14 since the data between varying the concentration overlap one another and are all within the error bars that indicate the data variation. As the carbon dioxide concentration increased from 3% to 9% in air for the freshwater microalgae (*Chlorella saccharophila*), there was no significant increase in cell yields.

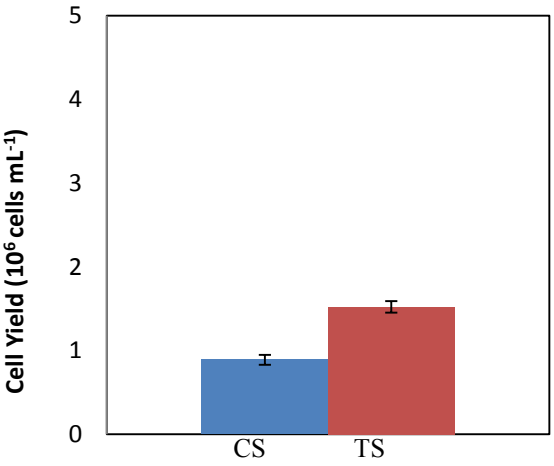
As the carbon dioxide concentration increased from 3% to 9% in air for the marine microalgae (*Tetraselmis suecica*), no significant effect on the microalgae cell yields were seen. Statistical analysis results from Turkey's grouping (Table 7.10), indicate that increasing the carbon dioxide concentration had no significant effect on the microalgae cell yields. This can be seen in Figure 7.14 since the data between varying the concentration of carbon dioxide overlap one another and are all within the error bars.

7.3.2. Microalgae Oil Content

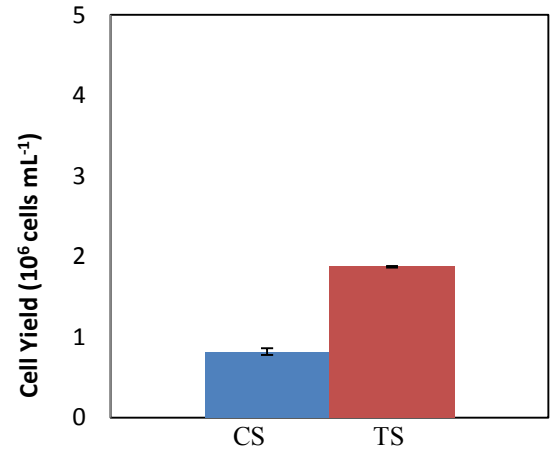
Microalgae oil yield was determined for two types of microalgae *Chlorella saccharophila* (freshwater) and *Tetraselmis suecica* (marine) using the three concentrations of CO_2:air (3:97, 6:94 and 9:91) at optimal nutrient and light conditions



(a) CO_2:Air (3:97)



(b) CO_2:Air (6:94)



(c) CO_2:Air (9:91)

Figure 7.13. Effect of microalgae type on the cell yield using varying CO_2:Air (v/v) concentrations, CS-*Chlorella saccharophila*, TS- *Tetraselmis suecica.*

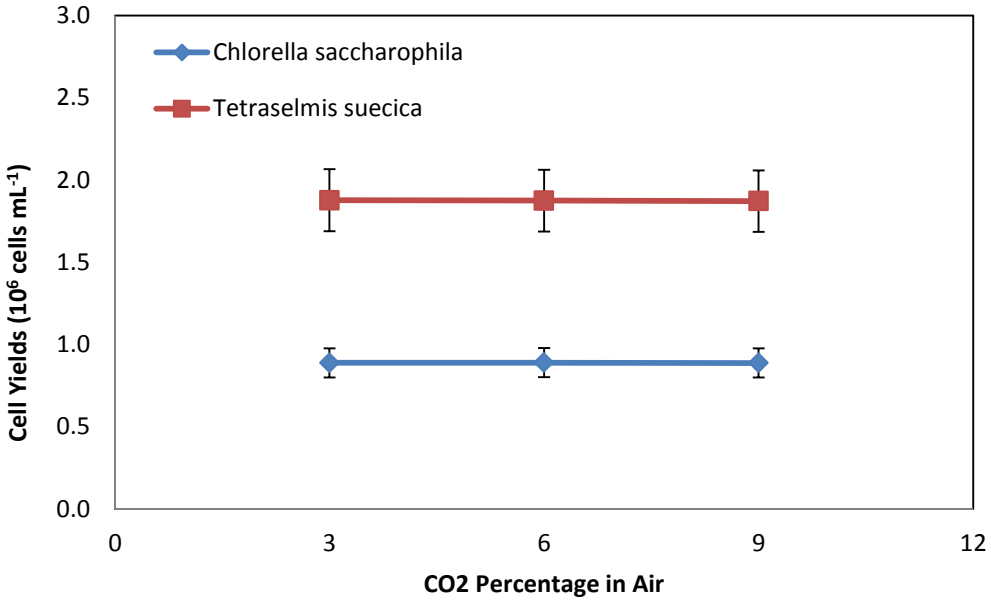


Figure 7.14. Effect of carbon concentration on the cell yield of freshwater (*Chlorella saccharophila*) and marine (*Tetraselmis suecica*) microalgae, using different concentrations.

are illustrated in Table 7.11. Determination of yield was carried out by measuring the amount of oil obtained with respect to the biomass recovered (Equation 5).

Analysis of the variance (ANOVA) was performed on the oil yield data as shown in Table 7.12. The effects of microalgae type on oil yield were significant at the 0.005 level. However, the effect of carbon source and the interactions between species type and carbon source were not significant at the 0.005 level.

The results obtained from the Tukey's grouping are shown in Table 7.13. The two microalgae (freshwater and marine water) were significantly different from one another at alpha significance level of 0.05. The results indicate that the highest mean oil yield (7.75%) was obtained from the freshwater microalgae species. The CO_2 concentration of 3%, 6% and 9% CO_2 were not significantly different from one another at the 0.05 level.

7.3.2.1. Effect of Microalgae Type: The effect of the microalgae type (freshwater and marine) using varying CO_2:air (3:97, 6:94 and 9:91) at the optimal conditions for each species on the microalgae oil yield are shown in Figure 7.15. The results indicate that the freshwater microalgae (*Chlorella saccharophila*) achieved the higher oil yields at all concentrations of carbon dioxide (3%, 6% and 9%), while the marine microalgae (*Tetraselmis suecica*) resulted in the lowest oil yields at all carbon dioxide concentrations (3%, 6% and 9%).

The freshwater microalgae (*Chlorella saccharophila)* resulted in oil yields of 4.47%, 3.84% and 3.93%, while the marine microalga (*Tetraselmis suecica)* resulted in oil yields of 1.63%, 1.08% and 2.43%, using CO_2:Air of 3:97, 6:94 and 9:91, respectively.

7.3.2.2. Effect of Carbon Dioxide Concentration: The effect of carbon dioxide concentration (3:97, 6:94 and 9:91) on the oil yield of the marine and freshwater microalgae is illustrated by Figure 7.16. Statistical analysis results from Turkey's grouping (Table 7.10), indicate that increasing the carbon dioxide concentration had no significant effect on the microalgae oil yields. This can be seen in Figure 7.16 since the data between varying the concentration overlap one another and are all within the variation in data bars.

As the carbon dioxide concentration increased from 3% to 6% (in air), the freshwater

Table 7.11. Average oil yield of *Chlorella saccharophila* (freshwater) and *Tetraselmis suecica* (marine) microalgae using optimal nutrient and light conditions with carbon dioxide gas as the carbon source.

Species	Nutrient Type	CO_2:Air Ratio	Oil Yield (%)
Freshwater	Combination	3:97	4.467±2.253
	Combination	6:94	3.841±1.873
	Combination	9:91	3.927±0.717
Marine	Ammonium nitrate	3:97	1.625±0.987
	Ammonium nitrate	6:94	1.082±0.359
	Ammonium nitrate	9:91	2.430±2.122

Table 7.12. Analysis of the variance for oil yield using CO_2 as the carbon source.

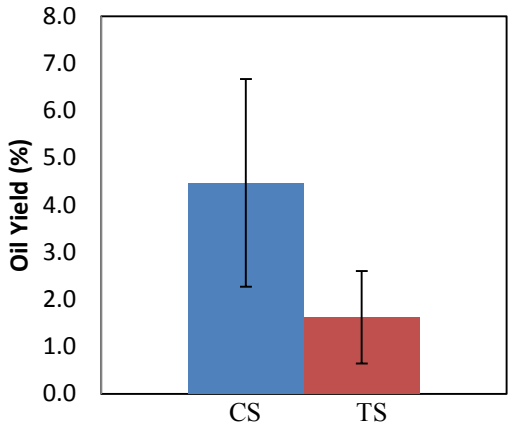
Source	**DF**	**SS**	**MS**	**F**	**P**
Total	17	55.738			
Model					
Species (S)	1	27.875	27.875	13.00	0.004
Carbon Source (C)	2	1.255	0.628	0.29	0.751
S*C	2	0.881	0.441	0.21	0.817
Error	12	25.727	2.144		

DF: Degree of freedom
SS: Sum of square
MS: Mean of square
F: F Distribution
P: Probability-Value
$R^2 = 0.54$
CV= 63.9%

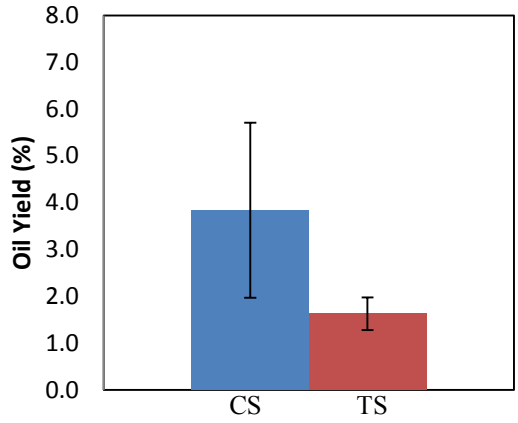
Table 7.13. Tukey's grouping on oil yield using CO_2 as the carbon source.

Factors	Level	N	Mean Yield	Tukey's Grouping
Species	Marine Water	12	1.878	A
	Freshwater	12	7.754	B
CO_2:Air	3:97	6	3.047	A
	6:94	6	2.462	A
	9:91	6	2.994	A

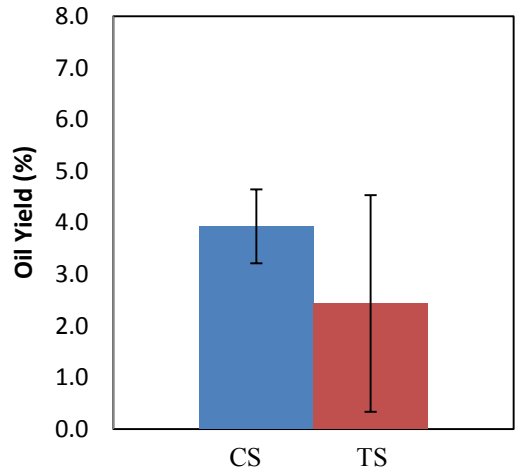
Groups with the same letter are not significantly different from each other at alpha significance level of 0.05 level.



(a) CO_2:Air (3:97)



(b) CO_2:Air (6:94)



(c) CO_2:Air (9:91)

Figure 7.15. Effect of microalgae type on the oil yield (%) using varying CO_2:Air (v/v) concentrations, CS-*Chlorella saccharophila*, TS- *Tetraselmis suecica.*

microalgae (*Chlorella saccharophila*), decreased in oil yield from 4.47% to 3.84%. Further increase in CO_2 concentration to 9% illustrated a slight increase to 3.93%. As the carbon dioxide concentration increased from 3% to 6% (in air), the marine microalgae (*Tetraselmis suecica*) decreased in oil yield from 1.62% to 1.08%. However a further increase in CO_2 concentration to 9% illustrated an increase to 2.43%. Although a slight variation in oil yields was noted, statistical analysis revealed no significant difference.

7.3.3. Optimal Growth Conditions using CO_2

The optimal carbon dioxide to air ratio for both the *Chlorella saccharophila* (freshwater) and *Tetraselmis suecica* (marine) microalgae using combination of nutrients at 24 h light exposure duration is 3%. These are the most economical conditions since there was no significant increases seen when the carbon dioxide concentration was increased to 9%. The costs associated with increasing carbon dioxide concentration would not be justified since similar biomass and oil yields were achieved using 3% carbon dioxide.

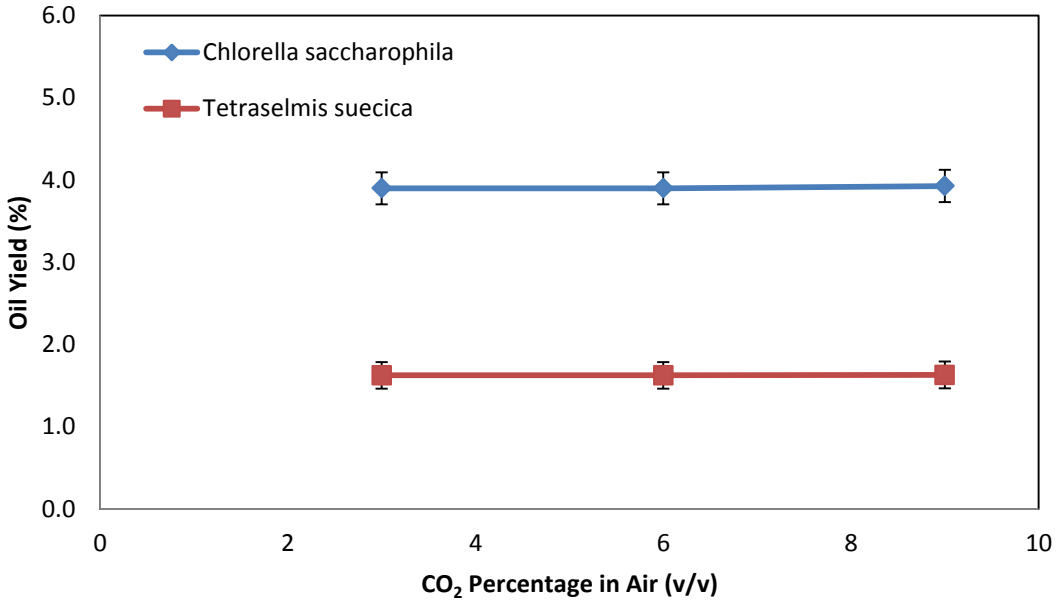


Figure 7.16. Effect of carbon concentration on the oil yield of freshwater (*Chlorella saccharophila*) and marine (*Tetraselmis suecica*) microalgae using different concentrations of CO_2:air (v/v).

CHAPTER 8. DISCUSSION

8.1. $NaHCO_3$ as a Carbon Source

8.1.1. Microalgae Biomass Yield

The main factors that affect the biomass productivity of photoautotrophic microalgae cultures are light duration and nitrogen source. The nitrogen sources play a role in the cell growth and metabolism. Nitrogen sources are ammonium and nitrate salts. Light acts as the main energy source for microalgae growth. Achievement of high microalgae yield is based on the utilization efficiency of the light energy and nitrogen source (Pratoomyot et al., 2005; Aslan et al., 2006; Go et al., 2012).

In this study, the effects of various nitrogen sources (ammonium nitrate, ammonium phosphate, ammonium sulfate and a mix of all three) and different light exposures (9, 16 and 24 h) were investigated in an open pond system in order to determine the parameters that yield the highest microalgae biomass. The results indicate that the microalgae strain, nitrogen source used and light duration had significant effects at an alpha significance level of 0.01, on the microalgae cell yield. The highest cell yields for *Chlorella saccharophila* obtained using sodium bicarbonate as the carbon source were 29546, 63080 and 68980 cells mL^{-1} at the 9, 16 and 24 h light durations and with the combination of all three nutrients as the nitrogen source, respectively. The highest cell yields for *Tetraselmis suecica* obtained using sodium bicarbonate as the carbon source were $941x10^4$, $268x10^4$ and $1{,}034x10^4$ cells mL^{-1} at the 9, 16 and 24 h light durations and with the combination of all three nutrients as the nitrogen source, respectively.

8.1.1.1. Effect of Microalgae Type: The selection of the type of microalgae to be used as a potential for biodiesel production is of great importance (Doung et al., 2012). Several researchers noted that the freshwater microalgae *Chlorella saccharophila* (Herrera-Valencia et al., 2011; Isleten-Hosoglu et al., 2012; Singh et al., 2013) and the marine microalga *Tetraselmis suecica* (Thomas et al., 1984; Nieves et al., 2005; Michels et al., 2013) have great potential for being used as a source for biodiesel production. In this study, the marine microalgae *Tetraselmis suecica* resulted in cell yields higher than those of the freshwater microalgae. Literature findings clearly indicate that both the

productivity and the cell yields (as dry weight or cell count) were much higher for the marine microalga *Tetraselmis suecica* as opposed to the freshwater microalga *Chlorella saccharophila*.

Herrera-Valencia et al. (2011) noted a biomass productivity of 154.3 mg/L/d for *Chlorella saccharophila* at 16 h light exposure for cultures grown for a total of 17 days. Singh et al. (2013) noted a dry cell yield of 378 mg/L using the microalga species *Chlorella saccharophila*. Isleten-Hosoglu et al. (2012) noted a dry cell weight yield of 138 mg/L for *Chlorella saccharophila* grown using sodium nitrate. Chinnasamy et al. (2010) noted a biomass productivity of 23 mg/L/d for *Chlorella saccharophila* grown in industrial wastewaters. In this study the biomass yield obtained for *Chlorella saccharophila* was 175 mg/L with a biomass productivity of 17.5 mg/L/d. These results are comparable to those obtained in the literature since they do not vary significantly. The differences in the results can be attributed to the different growth conditions used and the varying harvesting techniques.

Nieves et al. (2005) noted cell concentrations in the range of 0.6-0.7 $x10^6$ cells mL^{-1} at the end of the exponential growth phase (day 3) for *Tetraselmis suecica* species. Michels et al. (2013) achieved a biomass productivity for *Tetraselmis suecica* of 350 mg/L/d. Thomas et al. (1984) reported of a biomass productivity of 192 mg/L/d using *Tetraselmis suecica* microalga. Moheimani (2013) achieved a biomass productivity of 320 mg/L/d for the marine microalga *Tetraselmis suecica.* Bondioli et al. (2012) noted a biomass productivity for *Tetraselmis suecica* of 237 mg/L/d. Danquah et al. (2010) reported a biomass yield 1.29 g/L for the marine microalga *Tetraselmis suecica* grown in F medium for a growth period of 10 days. In this study the biomass yield obtained for *Tetraselmis suecica* was 2.48 g/L with a biomass productivity of 247 mg/L/d. These results are in agreement with those obtained in the literature since they do not vary significantly. The differences in the results can be attributed to the different growth conditions used and the varying harvesting techniques.

8.1.1.2. Effect of Light Exposure: The duration of light exposure was directly related to the microalgae biomass yields obtained in this study. Over the ten day growth period, increases in the light exposure periods from 9 h to 24 h resulted in higher microalgae

yields when the light duration increased from 9 h to 24 h. The ammonium nitrate, ammonium phosphate, ammonium sulfate and combination systems resulted in biomass yields for *Chlorella saccharophila* of $0.127x10^6$, $0.352x10^6$, $0.368x10^6$ and $0.394x10^6$ cells mL^{-1}, respectively. Similarly the marine microalga *Tetraselmis suecica* exhibited an increase in biomass yields as the light duration increased from 9 to 24 h for all nutrient systems. Increasing the light duration increased the biomass due to the increased photosynthetic activity. This relationship was similar to that reported in the literature by several authors.

Al-Qasmi et al. (2012) stated that the biomass yield is directly linked with the increase in light duration. Khoeyi et al. (2012) reported cell numbers for *Chlorella vulgaris* of $39x10^6$ cells mL^{-1}, $60x10^6$ cells mL^{-1} and $75x10^6$ cells mL^{-1} at light exposures of 8, 12 and 16 h, respectively. Jacob-Lopes et al. (2009) noted that the microalgae *Aphanothece microscopia Nageli* resulted in a maximum biomass yields of 0.343, 1.640 and 5.100 g L^{-1} at light exposures of 8, 16 and 24 h, respectively. Mata et al. (2012) reported biomass yields for *Scenedesmus obliquus* of 0.30 and 0.45 g L^{-1} at light exposures periods of 12 and 24 h, respectively. Larsdotter (2006) noted that higher rates of nitrate uptake were achieved with increased illumination periods. Bouterfas et al. (2006) reported on *Selenastrum minutum* achieving a cell growth rate of 1.1/d at 9 h light exposure which increased to 1.85/d with 24 h light exposure. Wahidin et al. (2013) noted that *Nannochlorpsis sp*. achieved an increase in cell yield from $1.3x10^7$ to $2.1x10^7$ cells mL^{-1} as the light duration was increased from 12 to 18 h. Larsdotter (2006) noted large fluctuations in algal biomass and removal of nutrients efficiencies due to seasonal variations in light and temperature. These findings clearly illustrate that there exists a relationship between microalgae biomass yields and light duration exposures. Higher biomass yields achieved using longer light exposures are a result of increased photosynthetic cell activity which causes the cells to uptake more nutrients causing increased cell division. This explains the increased cell yields obtained in all nutrient systems as the light exposure duration increased.

8.1.1.3. Effect of Nutrient Type: Several nitrogen sources can be used for microalgae growth, including ammonia, urea, nitrate and nitrite (Becker, 1994). The results obtained

from this study indicated that ammonium nitrate, ammonium phosphate, and ammonium sulfate nutrients were not significantly different from one another but were, however, significantly different from the combination of the three nutrients. The system using the combination of nutrients resulted in the highest cell yields at all light exposures. The ranking of the nutrient systems in increasing order (worst to best) for biomass yield (lowest to highest) for the freshwater microalga (*Chlorella saccharophila*) were: ammonium sulfate, ammonium phosphate, ammonium nitrate and combination of nutrients. For the marine microalga (*Tetraselmis suecica*) the ranking of the nutrient systems in increasing order (worst to best) for biomass yield (lowest to highest) were: ammonium nitrate, ammonium sulfate, ammonium phosphate and combination of nutrients. The results indicated that microalgae species have preference for nitrogen source and the sulfur and phosphorus are also important nutrients.

Makareviciene et al. (2011) achieved the best biomass productivity of *Chlorella* sp. and *Scenedesmus* sp. using decreased concentrations of sodium nitrate. Matusiak (1976) found that *Chlorella vulgaris* grew well in industrial wastewaters with 600 mg L^{-1} of ammonium as the nitrogen source. Costa et al. (2001) noted *Spirulina platensis* biomass yields of 1.559, 0.993 and 0.081 g L^{-1} using sodium nitrate, ammonium nitrate and ammonium sulfate, respectively. Their results suggested that ammonium was the preferential source of nitrogen nutrient, but not the best for high biomass yields. Becker (1994) stated that ammonia assimilation in microalgae is easier due to the simplicity of the molecule, and its presence in a solution inhibits the nitrogenase activity. Odum (1983) noted that microalgae are still capable of using nitrate because of its presence in nature.

Phosphorous uptake is an energy intensive process, which comes from photosynthesis (light). Longer light exposures results in higher yields due to higher phosphorous uptake by the cells (Kaplan et al., 1986; Martinez et al., 1999; Hessen et al., 2002; Sato and Murata, 1980). Larsdotter (2006) noted that additional illumination in the winter for algal culture increased the uptake of phosphorus. Abe et al. (2002) noted that *Trentepohlia aurea* biomass was 1.5 times higher in culture grown with sufficient nitrogen and phosphorus than in an ordinary medium, but removal efficiency of

phosphate was higher in media grown with phosphate alone as appose to combination. Wang et al. (2013) reported of ammonium and phosphorus reductions by *Oedogonium sp*. of 95.9 and 92.9%, respectively. Pai and Lai (2011) noted an increase in freshwater oleaginous algae biomass from 28.3 to 254 mg L^{-1} with ammonium and phosphate removal efficiencies of 84.8 and 36.2%, respectively. Totsch et al. (2006) noted that the growth of *Chlamydomonas sp*. and *Ochromonas sp*. was stimulated with the addition of phosphorus into the media. Li et al. (2010) noted that the species *Scenedesmus sp*. produced a higher growth rate of $2.21x10^6$ cells/mL/d with nitrogen and phosphorous contents of 12.1 and 0.27 mg/L, respectively, as opposed to medium with nitrogen or phosphorous limitations. Abe et al. (2002) noted biomass yields for *Trentepohila aurea* of 200, 580 and 600 mg L^{-1}, using nitrate, phosphate and combination of ammonium, nitrate and phosphate. Fried et al. (2003) stated a significant positive effect on algae growth with both nitrogen and phosphorus as nutrient sources which were also in agreement with Ryan et al. (1972). Das et al. (2009) noted that phosphorus alone only slight increased the growth of microalgae. Totsche et al. (2006) noted that the growth of *Chlamydomonas sp*. and *Ochromonas sp*. was stimulated with the addition of phosphorus into the media. Li et al. (2008) showed that *N. oleoabundans* increased in biomass from 1.2 to 2.4 g L^{-1} after nitrate had been depleted on day 2.

The ammonium sulfate nutrient system did not prove to be the best for yielding high biomass. The results obtained in this study indicated that it was the worst system for the freshwater microalga and the second worst for the marine microalga, but the yields of ammonium sulfate and ammonium nitrate nutrients did not vary significantly. The low yields achived using ammonium sulfate systems can be an idicaiton that this nutrient is not suitable for cell yields (Cuhel et al., 1984). Costa et al. (2001) noted of the freshwater algae *Spirulina platensis* achieving biomass yields of 0.993 and 0.081 g L^{-1} using ammonium nitrate and ammonium sulfate, respectively. Guzman-Murillo et al. (2007) noted that ammonium sulfate nitrogen source resulted in lower cell yields than ammonium nitrate in *Phaeodactylum tricornutum* species. Rocha et al. (2003) reported of *Nannochloropsis gaditana* increasing in biomass to $4x10^7$ cell mL^{-1} until day 4 using ammonium sulfate after which a decrease to $3.2x10^7$ cells mL^{-1} was noted at day 7. They also showed that an increase in ammonium sulphate concentration from 5 mM to 10 mM

decreased the cell yield from $4x10^7$ to $2.9x10^7$ cells mL^{-1}. These observations indicate that sulfate is not effective nutrient for promoting microalgae cell growth.

The combination of nutrients (ammonium nitrate, ammonium phosphate, ammonium sulfate) resulted in the highest biomass yields at the end of the experimental run (10 days) for both types of microalgae. The results showed significantly higher biomass yields are achieved when the microalgae species are provided with a full spectrum of nutrients (nitrate, phosphate, and ammonium) as opposed to the individual systems containing only ammonium as the nitrogen source with one other nutrient. Many studies have demonstrated effective microalgae biomass production using wastewaters media containing several nutrients (Juan, 2006; Chen et al., 2009; Ji et al., 2013) Different nutrients are responsible for the operation of different cell parts; lack of one nutrient affects the overall efficiency of the cells.

8.1.2. Oil Content

The selection of the type of microalgae to be used as a potential source for biodiesel production is of great importance (Doung et al., 2012). Several researchers noted that the freshwater microalga *Chlorella saccharophila* (Herrera-Valencia et al., 2011; Singh et al., 2013; Isleten-Hosoglu et al., 2012) and the marine microalga *Tetraselmis suecica* (Nieves et al., 2005; Michels et al., 2013; Thomas et al., 1984) have great potential for being used as a source for biodiesel production. In this study the freshwater microalgae resulted in higher oil yields in comparison with that produced by the marine microalgae *Tetraselmis suecica.* This can be attributed to the different microalgae biomass yields obtained. The marine cells resulted in much higher cell yields when compared to those of the freshwater, but resulted in much lower oil content since the cells were using the nutrients for cell division as opposed to storage of the lipids (Pittman et al., 2011). Sharma et al. (2012) noted that the main factors that affect the accumulation of lipids in microalgae are nutrients, light exposure duration, carbon source and microalgae yield. The results obtained for this study indicated that the nutrient type and light duration have significant effect on oil content in both microalgae species.

8.1.2.1. Effect of Microalgae Type: In this study, the oil yields obtained from the freshwater microalga *Chlorella saccharophila* were 10 fold higher than those obtained

from the marine microalgae *Tetraselmis suecica,* despite the higher biomass yield obtained from the marine microalgae. This can be attributed to the fact that the *Chlorella saccharophila* cells are better at accumulating lipids rather than undergoing cell division, while those of the *Tetraselmis suecica* species are better at cell division at the expense of storing lipids. Sharma et al. (2012) stated that the occurrence and extent to which lipids are produced by microalgae is species/strain specific. Rodolfi et al. (2009) noted that higher biomass yields correspond to lower cellular lipid content. Reports in literature (Herrera-Valencia et al., 2011; Isleten-Hosoglu et al., 2012; Chinnasamy et al., 2010; Tan and Johns 1991) indicate that the freshwater (*Chlorella saccharophila)* microalgae are better at accumulating lipids than the marine microalgae (*Tetraselmis suecica*).

Herrera-Valencia et al. (2011) noted a lipid productivity of 63.3 mg/L/d for *Chlorella saccharophila* at 16 h light exposure for cultures grown for a total of 17 days. Isleten-Hosoglu et al. (2012) reported of a lipid content of 29.5% from *Chlorella saccharophila* grown with 5 and 20 g/L of glycerol and glucose, respectively. Liu et al. (2011) achieved a lipid yield of 45% and a lipid productivity of 153 mg/L/d from *Chlorella saccharophila.* Chinnasamy et al. (2010) noted a lipid content of 12.90% for *Chlorella saccharophila* grown in BG-11 media. Tan and Johns (1991) reported that *Chlorella saccharophila* resulted in a lipid content and lipid productivity of 47% and 0.29 g/L, respectively.

Griffiths and Harrison (2009) noted a lipid productivity in *Tetraselmis suecica* of 32 mg/L/d. Danquah et al. (2010) reported of *Tetraselmis suecica* achieving a total lipid content of 108.7 mg/L. Montero et al. (2011) noted a lipid productivity of 27 mg/L/d using the microalga *Tetraselmis suecica.* Moheimani (2013) noted a lipid productivity of 14.8 mg/L/d for the marine microalga *Tetraselmis suecica.*

These literature findings indicate that the marine microalgae *Tetraselmis suecica* does not produce lipids as effectively as the freshwater microalgae *Chlorella saccharophila.* This can attributed to the fact the different species use their energy for different metabolic pathways. In this case the marine microalga used its energy for cell division while the freshwater used it for lipid accumulation. Rodolfi et al. (2009) noted that *Prophyridum cruentum, Scenedesmus, Chlorella* and *Chaetoceros calcitrans* resulted in biomass

productivities of 0.37, 0.26, 0.23 and 0.04 g L^{-1} d^{-1} and lipid contents of 9.5, 21.1, 18.7 and 39.8%, respectively. Kumar et al. (2012) noted that no linear relationship exists between lipid content and biomass yield for 25 strains of *Spirulina* tested. Pai and Lai (2011) achieved a 9 fold increase in biomass of oleaginous algae (28.3 to 254 mg L^{-1}), but the oil yield only increased by 1.6 folds (20.4 to 33.6%).

8.1.2.2. Effect of Light: Statistical analysis indicated that varying the light duration had no significant effect on the oil yields, since some nutrient systems resulted in higher yields at 9 hour light exposure, while others resulted in lower yields at 9 h light exposure. Thus overall the means were similar to one another indicating no significant difference. However looking at each nutrient system individually, we see that there are significant interactions between the light duration and nutrients. The oil yields fluctuated between the different light and dark exposures with no apparent trend for each of the species grown using the various nutrient systems. The fluctuation in oil yields between the different species can be attributed to the fact that different microalgae species respond differently to the nutrients and light energy provided. Sharma et al. (2012) stated that microalgae biomass and lipid accumulation compete for photosynthetic assimilation.

Parmar et al. (2011) noted that light and dark cycles play an important role in the cell growth rate of microalgae cultivation. The microalgae species use the light energy as a source for synthesizing cell protoplasm. Kitaya et al. (2008) noted that photoperiod affects the microalgal cell growth rates in combination with other environmental parameters such as temperature and nutrient composition. Khotimchenko and Yakovleva (2005) stated that varying light periods stimulates the growth, fatty acid synthesis and the formation of membrane components (chloroplast).

Wahidin et al. (2013) reported on marine microalgae *Nannochloropsis sp*. cultured using a light intensity of 100 μmol m^{-2} s^{-1} at varying photoperiod cycles (12, 18 and 24 h light exposure) and noted that the highest lipid content of 31.3% was achieved at 18 h light exposure. An increase in light exposure to 24 h resulted in a lipid content of 27.9%. Also, a reduction in light exposure to 12 h resulted in a reduction in lipid content to 25.6%.

Goncalvas et al. (2013) reported that varying the photoperiod for the species *Chlorella vulgaris* and *Pseudokirchneriella subcapitata* had no influence on the lipid yields. Harwood (1998) noted that varying light and dark cycles altar the lipid metabolism of the microalgae cells which in turn altars the lipid profile. With varying species the triacylglyceride composition of the cell is different for each species in response to different photoperiods.

Herrera-Valencia et al. (2011) noted a lipid yield of 40% for *Chlorella saccharophila* grown at 16 h light exposure for cultures grown a total of 17 days. Goncalves et al. (2013) noted a lipid content of 22, 19 and 26 mg g^{-1} in *C. vulgaris* and 28, 27.5 and 34 mg g^{-1} in *P. subcapitata* at 10, 14 and 24 h light exposures, respectively. Perez-Pazos and Fernandez-Izquierdo (2011) achieved a lipid yield from *Chlorella sp*. of 0.25 and 0.17 g/L when grown at 6 and 18 h light exposures. Lim and Zaleha (2013) studied the effects of three photoperiods (8, 12 and 24 h) on the fatty acid composition of three marine species (*Chaetoceros calcitrans*, *Chlorella sp*. and *Nannochlorosis*) and noted that the best fatty acid content was achieved using 12 h light exposure. Brown et al. (1996) noted that *Thalassiosira pseudonana* cultures grown in continuous light or 12:12 h light/dark resulted in higher triacylglycerol (TAG) content during exponential phase and higher polyunsaturated fatty acid content in stationary phase, compared to those with less light exposures. Bandarra et al. (2003) noted that a shorter light exposure period increased the oil content in *Isochrysis galbana*.

8.1.2.3. Effect of Nutrient Type: The results obtained from this study for both freshwater and marine microalgae species suggest that nutrient type plays an important role in oil yield. Culture of the freshwater (*Chlorella saccharophila*) microalgae using ammonium nitrate, ammonium phosphate and ammonium sulfate achieved similar cell yields (10 $x10^3$ - 69 x 10^3 cells mL^{-1}), however, the oil yields ranged from 7.71% to 14.71%, from 21.20% to 29.07% and from 3.78% to 13.3%, respectively. Combination of nutrients resulted in higher cell yields, but the oil yield range of 12.9 to 22.5% was lower. Statistical analysis indicated that the ammonium nitrate, ammonium sulfate and combination of nutrients did not vary significantly from one another, while those of ammonium phosphate and combination of nutrients did not vary from one another. This

can be seen, since the oil yield ranges between the varying nutrient systems (ammonium nitrate, ammonium sulfate and combination of nutrients) overlap one another. Also nutrient systems ammonium phosphate and combination of nutrients oil yield ranges overlap one another.

The marine (*Tetraselmis suecica*) microalgae resulted in biomass yields that were higher than the freshwater microalgae. The cell yields for the marine microalgae were (in increasing order form lowest to highest) ammonium nitrate, ammonium sulfate, ammonium phosphate, and combination of nutrient types. The highest oil yields were achieved (in increasing order from lowest to highest) ammonium nitrate, ammonium sulfate, ammonium phosphate and combination of nutrients. Sharma et al. (2012) stated that the occurrence and extent to which lipids are produced by microalgae is species/strain specific. Accumulation of large amounts of lipids can be achieved by altering environmental factors such as chemical and physical stimuli (Hu et al., 2008).

Significant impacts on the growth and lipid accumulation in microalgae by nutrient availability have been noted. As algal growth begins to slow down the algal cells no longer require the synthesis of new membrane compounds, so the cell starts producing lipids as storage compounds. During normal growth conditions (sufficient nutrients provided), ATP and NADPH are the products of photosynthesis that are used to generate biomass, ADP and $NADP^+$ are then available as the photosynthetic acceptor molecules. When nutrients are unavailable the growth of the cells becomes impaired which causes the depletion of the $NADP^+$ pool of unused photosynthetic acceptor molecules. This can be dangerous to the cells and can cause damage to some of the cell components. During lipid synthesis NADPH is consumed which releases $NADP^+$ molecules under nutrient stress (Hu et al., 2008; Thompson, 1996). Rodolfi et al. (2009) stated that oil accumulation in nitrogen deficient environments does not increase the oil productivity due to poor offset by the lower productivities achieved under nutrient deprivation.

In this study, the results indicate the highest oil yields for the freshwater (*Chlorella saccharophila)* microalgae were achieved using ammonium phosphate nutrient, which indicates that it is the lack of nitrogen and the addition of phosphorus that stimulate the production of lipids. This can also be seen in the combination of nutrients which resulted

in the second highest oil yields for this species, due to the presence of phosphorus. The combination of nutrients resulted in higher biomass than ammonium phosphate system which would explain the difference in lipid yields noted. Ammonium phosphate alone was not the best for producing biomass and the combination illustrated better yields for both biomass and lipid yields. Additionally, the higher oil yields achieved at 9 h indicate that less light results in less biomass generation which would be replaced by generating lipid yields.

Similarly, the results for the marine microalgae *Tetraselmis suecica* indicate that the highest lipid yields were achieved with the ammonium nitrate system and the lowest oil yields were achieved with the combination of nutrients. Consequently, the lowest biomass yields for this species were achieved using the ammonium nitrate system and the highest were achieved for the combination of nutrient system which indicates that the ammonium nitrate system was a stressed environment due to lack of sulfur and phosphorous which cause the cell to store lipids instead. Since the biomass was significantly higher in ammonium phosphate and ammonium sulfate systems we can conclude that the cells require these nutrients for biomass production. The significant increase in biomass resulted in relatively high lipid yields. However, for the combination of nutrients the lipid yields were significantly lower than the other systems which can be explained by the significant biomass yields, which indicates that sulfur and phosphorus and nitrogen present in combination is ideal for biomass generation which does not leave room for lipid production.

Mutlu et al. (2011) noted that *Chlorella vulgaris* had increased lipid content from 12.29 to 35.6% when the culture was deprived of nitrogen, but was phosphorus sufficient. Reitan et al. (1994) reported that the microalgae *Nannochloris atomus* and *Tetraselmis sp*. resulted in decreased lipid content due to phosphorous starvation. Matthew et al. (2009) achieved an increase in lipids with sulphur limitation in *Chlorella sp*. and *Chlamydomonas reinhardtii*. Illman et al. (2000) reported a 40% increase in lipids in low nitrogen containing medium. Pai and Lai (2011) noted an increase in microalgae lipid content of 13.2% with ammonia nitrogen and phosphate removal efficiencies of 84.8 and 36.2%. Sato et al. (2000) achieved an increase in lipid content with limitation of sulphur

in *Chlamydomonas reinhardtii*. Kumar et al. (2012) noted increases in biomass (0.9 – 2.9 g L^{-1}) from increased concentrations of nitrogen, but the lipid content decreased (48-32%). Converti et al. (2009) achieved an increase in lipid production upon nitrogen deprivation of 15.31 and 16.41% in *Nannochloropsis oculata* and *Chlorella vulgaris*, respectively. Alonso et al. (2000) noted an increase in TAG content from 69 to 75% in *Phaeodactylum tricornutum* when nitrogen was limited. Hu et al. (2008) stated the sulfate limitation in microalgae promotes lipid accumulation.

8.2. CO_2 as a Carbon Source

8.2.1. Microalgae Biomass Yield

8.2.1.1. Effect of Carbon Source: The marine microalgae (*Tetraselmis suecica)* resulted in higher cell yields than the freshwater microalgae (*Chlorella saccharophila)* at all CO_2 concentrations. These results are similar to those observed using sodium bicarbonate as the carbon source using the various nutrient systems and light exposures.

Using the operating conditions (light and nutrient sourese) that generated the highest lipid yields using the $NaHCO_3$, the CO_2 as a carbon source at varying concentrations of were not significantly different from one another as statistical analysis of the data have revealed. The two different types of microalgae resulted in significantly different yields using carbon dioxide as the carbon source, however varying the concentration showed no significant varation in the data. Carbon dioxide concentrations of 3%, 6% and 9% all resulted in cell yields that were higher than those using $NaHCO_3$ as the carbon source.

Tetraselmis suecica (marine) microalga had a biomass increases of 203%, 145% and 202% over that produced with $NaHCO_3$ as the carbon source when CO_2 was used at concentrations of 3%, 6% and 9%, respectively. The slight varation in the yields were not significant as indicated by statistical analysis.

Devgoswami et al. (2011) studied the microalgae species *Chlorella, Haematococcus* and *Scenedesmus* sp. and noted that the highest biomass yielding species was *Chlorella*. It resulted in biomass yields of 82 and 189 mg/L/d using the sodium bicarbonate and CO_2, respectively, as the carbon source. Moheimani (2012) noted that the *Chlorella sp*.

and *Tetraselmis suecica* grown using CO_2 resulted in biomass yields that were 6 and 23% higher than those obtained using $NaHCO_3$ as the carbon source.

8.2.1.2. Effect of Carbon Dioxide Concentration: Statistical analysis revealed that there was no significant effect in biomass yields as the concentration of carbon dioxide was increased. Similar results are also noted in the literature that illustrate a fluctuation in cell yields as the carbon dioxide concentration is increased, however the differences are not significant. Flocculation in yields can be attributed to the fact that increasing CO_2 concentrations decreases the pH, which is not suitable for growth of some species (Widjaja, 2009).

Salih et al. (2011) noted that the microalgae growth efficiency and productivity is affected by the CO_2 concentration; higher CO_2 concentration resulted in better growth and productivity. Schippers et al. (2004) noted that doubling atmospheric carbon dioxide in nutrient rich media results in an increase in saltwater microalgae biomass of up to 40% and up to 50% in freshwater species. However, there must be an optimal range for CO_2 concentration in the media. Excess CO_2 presents a toxic environment for the cells since it alters the pH of the medium (Widjaja, 2009).

Yue and Chen (2005) reported that the freshwater microalgae *Chlorella* species resulted in an increase in algal growth rate of 200% when CO_2 (1% concentration) was administered into the media as opposed to the ambient air. Additionally they noted that higher concentrations of CO_2 resulted in decline in algal growth. Nakano et al. (1996) noted that the microalgae species *Euglena gracilis* exposed to CO_2 at varying concentrations (5-45%) grew best at 5% CO_2 while concentrations greater than 45% showed that growth was inhibited. Maeda et al. (1995) noted that *Chlorella* sp. microalgae is capable of growing at 100% CO_2 concentrations, however the highest growth rate was achieved at 10% concentration of CO_2. Hanagata et al. (1992) reported that the species *Scenedesmus* sp. can grow at CO_2 concentrations of 80%, but reported that maximum growth was achieved at 10-20% CO_2 concentration.

Low levels of CO_2 inhibit the growth of the cells due to the low carbon source (Kaewkannetra et al., 2012; Goswami et al., 2012). Additionally, excess CO_2 present in the media would be converted to different ions due flocculation in pH, which would in

turn affect the growth of the cells. Thus, higher CO_2 concentrations result in lower pH in the media.

Studies performed by Ho et al. (2010) and Jacob-Lopes et al. (2008) found that excess CO_2 absorbed into the media has a negative effect on the microalgae growth, as was seen in this study. Kaewkannetra et al. (2012) stated that an optimal CO_2 concentration exists in microalgae medium because as the CO_2 concentration is increased, the remaining CO_2 is converted to H_2CO_3 which results in decreasing the pH, thereby affecting the growth of the microalgae (Figure 8.1).

Goswami et al. (2012) noted that the species *Selenastrum* sp. resulted in biomass yields of 0.667 mg/L/d, 0.889 mg/L/d, 0.797 mg/L/d and 0.778 mg/L/d as the carbon dioxide concentration increased from 4,400, 5,200, 7,500 and 8,200 ppm, respectively. Devgoswami et al. (2011) noted that upon increase in CO_2 concentration from 4,400 ppm to 4,758 ppm the biomass (g/L/d) increased from 161 to 188%, while further increase in CO_2 concentration (7,929 ppm) illustrated a decrease of 163% for *Chlorella* green microalgae. Similarly, the *Haematococcus* and *Scenedesmus* species illustrated increased growth rate as the concentration of CO_2 increased from 4,400 to 4,758 ppm, but further increase to 7,929 ppm resulted in lower growth rates. These findings indicate that increasing the carbon dioxide concentration does not result in significantly higher biomass yields.

Kaewkannetra et al. (2012) noted that an increase in CO_2 concentration from 5 to 15% resulted in an increase in biomass yield from 1.7 to 2.3 g/L. However, further increase in CO_2 concentration resulted in decreased yields. Yue and Chen (2005) noted that the microalgae species *Chlorella* grown in CO_2 concentration over the range of 0.035% - 70% resulted in the highest cell concentration of 5.9 g/L at a CO_2 concentration of 10%. Ho et al. (2010) and de Morris and Costa (2007) also noted that an optimum CO_2 concentration for the species *Scenedesmus* exists between 10% to 15%. Widjaja (2009) noted that *Chlorella vulgaris* resulted in increased growth rate as the CO_2 concentration increased from 0-50 mL/min; additionally, an increase in CO_2 concentration resulted in decreased growth rates due to the decrease in pH as the CO_2 concentration is increased.

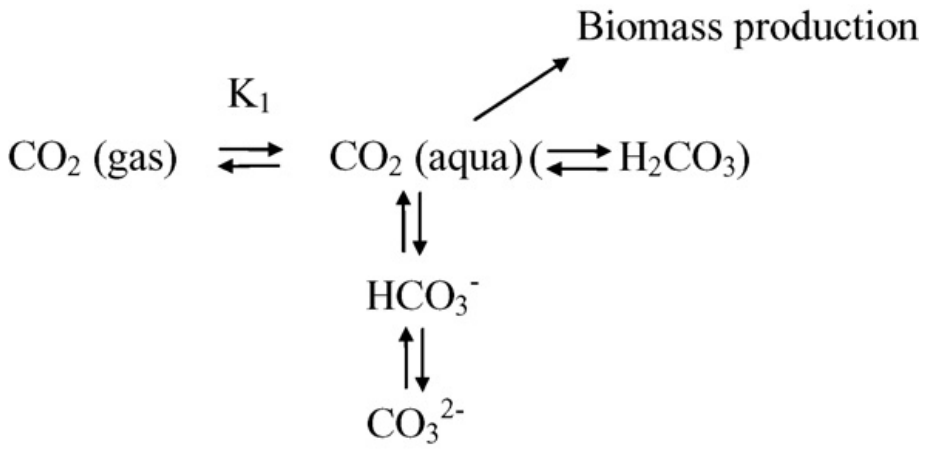


Figure 8.1. Conversion of CO_2 into biomass by microalgae, illustrating the pH fluctuation when excess CO_2 is absorbed into the media (Sorensen et al. 1996).

8.2.2. Oil Yield

8.2.2.1. Effect of Carbon Type: Similar to the results obtained from using sodium bicarbonate as the carbon source, the freshwater microalgae resulted in higher oil yields than those of the marine microalgae at all CO_2 concentration. This is similar to the findings of the study performed by Pittman et al. (20011) which indicates that the marine cells resulted in much higher cell yields when compared to those of the freshwater but resulted in much lower oil yields since the cells were using the nutrients for cell division as opposed to storage of the lipids.

The oil yields achieved by *Chlorella saccharophila* at all concentration of CO_2 were significantly lower than those obtained using $NaHCO_3$ as the carbon source (18.78%). This suggests that $NaHCO_3$ is not the best source for rapid growth rate as the CO_2 carbon source. This can be seen from the increase in cell growth of the freshwater microalgae of up to 29% and cell growth increase of up to 203% for the marine (*Tetraselmis suecica)* microalgae. Since CO_2 results in higher cell yields, it indicates that the cells use their energy for growth as opposed to promoting lipid storage. The lower biomass yields and higher oil yields achieved using $NaHCO_3$ by both species indicate that this carbon source creates an environment that better promotes lipid accumulation.

Mukund et al. (2013) tested *Chlorella*, *Chlorococcum* sp. and *Desmococcus* sp. and noted that the lipid accumulation by these species increased when the growth media contained bicarbonate. Nayak et al. (2013) reported a lipid yield of 16% in *Scenedesmus* sp IMMTCC-6 when supplied with CO_2 only, while addition of $NaHCO_3$ to the medium resulted in an increase in lipid yield to 22%. Dhakal et al. (2013) noted that the freshwater microalgae species *Chlorella vulgaris* resulted in lipid yield of 22% using $NaHCO_3$ as the carbon source.

8.2.2.2. Effect of Carbon Dioxide Concentration: Varying the concentration of carbon dioxide over the range of 3% - 9% did not significantly influence the lipid yield. However, the slight variation in lipid content can be explained by the variation in biomass yield obtained. The higher oil yield obtained for the freshwater microalgae at the 3% CO_2 concentration can be attributed to the trade-off of less biomass produced.

The slight variation in lipid content of the marine (*Tetraselmis suecica)* microalgae species can also be attributed to the slight variation in biomass.

Similar findings were also reported in the literature. Devgoswami et al. (2011) tested CO_2 concentrations of 4,400 ppm, 4,758 ppm and 7,929 ppm on *Chlorella*, *Haematococcus* and *Scenedesmus* sp. and noted that the highest biomass growth rate was achieved at 4,758 ppm which resulted in lipid yields of 31, 29 and 18%, respectively. Widjaja et al. (2009) noted that the microalgae species *Chlorella vulgaris* resulted in lipid yield of 20%, 28% and 25% at CO_2 concentration of 0 mL/min, 20 mL/min and 50 mL/min, respectively. Huang and Su (2013) noted that the microalgae species *Chlorella vulgaris* grown using 0%, 15% and 50% CO_2 concentrations resulted in lipid yields of 34%, 35% and 36%, respectively.

8.2.3. Optimum Conditions

The optimum conditions for high lipid yields for the freshwater *Chlorella saccharophila* microalga were found using an open pond culturing system. The best nutrient was found to be the combination of ammonium nitrate, ammonium sulfate and ammonium phosphate. The optimum light exposure was found to be at 16 h duration. The carbon source for higher lipid yields was $NaCHO_3$. Although higher lipid yields were achieved using light exposure of 24 h, they do not compensate for the higher costs associated with increased light exposure periods. Additionally the increased cell yields achieved using carbon dioxide at the expense of oil production do not offset one another. Thus, using the combination of nutrients, at 16 h with $NaCHO_3$ are much more suitable for biodiesel production as a result of better economics and a balance between high lipid and cell yields.

The optimum conditions for high lipid yields for the marine water *Tetraselmis suecica* microalga were found using an open pond culturing system. The best nutrient was found to be the ammonium nitrate. The carbon source for higher lipid yields was CO_2. Although the light exposure duration of 24 h has been used in this study to determine the best conditions using carbon dioxide as the carbon source, results obtained in this study indicate that the slight increase in lipid yields using higher light exposure

periods does not offset expenses required for higher energy use. Thus running the system using less energy would be much more economically suitable for biodiesel production.

CHAPTER 9. RECOMMENDATIONS

1. Studies revealed that carbon source largely affects the growth production and nitrogen source largely affects the lipid accumulation. Further study on the effect of nitrogen depletion in combination with varying carbon source concentrations should be investigated.
2. Optimal salinity of biomass should be investigated in conjunction to the carbon source and nitrogen limitation, since the study performed by Kaewkannetra et al. (2012) found that higher cell lipid accumulation was achieved under salt stress using NaCl using the freshwater microalga *Scenedesmus obliquus*.
3. Optimal conditions using a combination of $NaHCO_3$ and CO_2 gas that yield high biomass and promote lipid production should be investigated in order to assess the impact that atmospheric CO_2 has on the feedstock for biodiesel production.
4. Investigation of extended microalgae growth beyond 10 days should be evaluated in order to determine the best stage for microalgae oil harvesting and the possibility of continuous operation with interval harvesting.
5. Further investigation into the production of other value added products that can be extracted from the remaining biomass after oil extraction should be investigated to improve the economics of biodiesel production from microalgae.

CHAPTER 10. CONCLUSIONS

The cell growth and oil yields of *Chlorella saccharophila* (freshwater) and *Tetraselmis suecica* (marine) microalga were investigated using various nutrient types (ammonium nitrate, ammonium phosphate, ammonium sulfate and combination of nutrients), light durations (9 h, 16 h and 24 h), and carbon source ($NaHCO_3$ and CO_2) as the carbon source. The following conclusions are made from the results.

1. The *Tetraselmis suecica* resulted in higher cell yields compared to the *Chlorella saccharophila* under all operating parameters.
2. Over the tested range of 9-24 h light exposure, the biomass yields increased with increasing light duration for both the *Tetraselmis suecica* and *Chlorella saccharophila*. However, there were no statistically significant differences between 16 and 24 h.
3. The combination of nutrients resulted in the highest growth yields for both species of microalgae. However, high growth did not necessarily result in high lipid yields, which need to be considered when using oil as feedstock for biodiesel production.
4. Higher oil yields were achieved with the freshwater (*Chlorella saccharophila*) microalgae as opposed to the marine (*Tetraselmis suecica*) microalgae under all growth parameters using $NaHCO_3$.
5. No significant difference between varying light exposure periods using the various nutrients on oil yield was noted.
6. The freshwater (*Chlorella saccharophila)* microalgae resulted in the highest lipid yields using ammonium phosphate nutrient, but this nutrient resulted in lower biomass yield.
7. The marine microalgae achieved the highest oil yields using ammonium nitrate, but the cell yield was the lowest.
8. The inverse relation between oil yield and biomass yield can be understood by the mechanics of the microalgae cell. Cell generation and lipid production both require energy; when the cells use energy for cell generation they are producing less oil for storage.

9. The combination of nutrients at 24 h light exposure resulted in the highest biomass yields for *Chlorella saccharophila* which resulted in the highest total lipid yield, however the economics of increasing the light exposure period from 16 to 24 h is not offset by the slight increase in lipid yield of 5%.
10. The biomass yields achieved using CO_2 at varying concentrations were much higher than those using $NaHCO_3$ for both microalgae species.
11. The results indicate that the oil yields achieved using CO_2 at all concentrations were significantly lower than those achieved using $NaHCO_3$ for the freshwater microalga. The marine microalgae resulted in a slight decrease in lipid yields using CO_2 as opposed to $NaHCO_3$.
12. Varying the concentration of CO_2 did not affect the biomass or lipid yield in both microalgae species.
13. The optimal growth conditions for *Chlorella saccharophila* are the combination of nutrients, the with 16 h light exposure and $NaHCO_3$ as a carbon source and those for *Tetraselmis suecica* are the ammonium nitrate, the 16 h light exposure and CO_2 as a carbon source.

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APPENDIX

Table A1. Biomass and oil yield data for *Chlorella saccharophila* using $NaCO_3$.

Nutrient Type	Light (h)	Biomass Yield (cells mL^{-1})	Weight biomass (g)	Weight of oil (g)	Oil Yield (% biomass)
AN	9	258080	0.32	0.10	15.53
AN	9	359850	0.25	0.34	18.18
AN	9	220800	0.37	0.08	8.78
AN	16	335820	1.64	0.04	12.68
AN	16	286800	1.08	0.04	15.96
AN	16	384800	1.05	0.04	10.89
AN	24	432800	0.66	0.07	4.28
AN	24	444800	1.87	0.12	11.15
AN	24	502800	0.91	0.08	7.71
AP	9	137805	0.70	0.11	15.69
AP	9	104800	0.21	0.09	42.47
AP	9	170800	0.41	0.12	29.07
AP	16	185800	0.38	0.11	28.82
AP	16	134800	0.50	0.13	25.93
AP	16	236800	0.48	0.13	27.31
AP	24	404800	1.11	0.07	6.28
AP	24	508800	1.06	0.10	9.44
AP	24	554800	0.52	0.04	7.68
AS	9	158060	0.98	0.15	13.79
AS	9	119550	0.49	0.15	12.63
AS	9	138800	0.10	0.17	13.32
AS	16	126900	1.09	0.07	7.17
AS	16	186690	1.19	0.04	8.16
AS	16	156800	1.28	0.06	6.18
AS	24	406800	1.27	0.10	7.90
AS	24	536800	2.17	0.04	1.84
AS	24	576800	1.27	0.02	1.61
Comb.	9	108800	0.33	0.17	39.14
Comb.	9	374800	4.20	0.16	4.05
Comb.	9	402800	0.66	0.13	24.14
Comb.	16	752800	2.07	0.70	19.68
Comb.	16	862800	2.51	0.68	17.90
Comb.	16	276800	2.65	0.65	18.78
Comb.	24	689790	3.56	0.35	5.79
Comb.	24	688800	3.80	0.33	11.61
Comb.	24	690800	3.19	0.27	21.33

Table A2. Biomass and oil yield data for *Tetraselmis suecica* using $NaCO_3$.

Nutrient Type	Light (h)	Biomass Yield (cells mL^{-1})	Weight biomass (g)	Weight of oil (g)	Oil Yield (% biomass)
AN	9	193478	5.20	0.11	1.90
AN	9	345652	4.12	0.11	3.39
AN	9	258695	2.45	0.18	2.93
AN	16	463043	5.77	0.02	0.38
AN	16	802173	3.25	0.1	2.43
AN	16	593478	6.13	0.04	1.63
AN	24	771739	5.79	0.04	0.69
AN	24	728260	6.38	0.12	1.88
AN	24	750000	5.42	0.04	0.74
AP	9	1089130	5.35	0.15	2.80
AP	9	1997826	6.16	0.03	0.49
AP	9	1906522	5.76	0.02	0.35
AP	16	771739	6.45	0.16	2.70
AP	16	2815217	5.86	0.1	1.76
AP	16	2780435	6.02	0.07	1.16
AP	24	3293478	5.93	0.08	1.24
AP	24	1310870	5.67	0.03	0.51
AP	24	2458696	6.02	0.01	0.17
AS	9	680434	1.33	0.08	2.50
AS	9	663043	0.90	0.05	1.67
AS	9	650000	1.10	0.07	2.30
AS	16	989130	3.20	0.03	0.78
AS	16	963043	2.99	0.02	0.45
AS	16	984782	3.05	0.1	3.54
AS	24	728260	3.86	0.02	1.51
AS	24	4484783	4.42	0.01	1.11
AS	24	3167391	2.83	0.01	0.91
Comb.	9	2863043	6.23	0.02	0.33
Comb.	9	2436957	5.90	0.02	0.34
Comb.	9	2767391	4.34	0.03	0.69
Comb.	16	2354348	6.80	0.02	0.29
Comb.	16	13436957	6.50	0.04	0.62
Comb.	16	12454348	7.56	0.03	0.40
Comb.	24	10015217	9.85	0.03	0.30
Comb.	24	11793478	10.10	0.02	0.20
Comb.	24	9219565	9.79	0.03	0.31

Table A3. Biomass and oil yields for *Chlorella saccharophila* and *Tetraselmis suecica* using CO_2 at varying concentrations.

CO_2:Air	**Nutrient**	**Light (h)**	**Biomass Yield (cells mL^{-1})**	**Weight biomass (g)**	**Weight of oil (g)**	**Oil Yield(% biomass)**
Freshwater						
3:97	Comb.	24	176800	0.72	0.05	6.92
3:97	Comb.	24	184800	0.80	0.02	2.49
3:97	Comb.	24	114800	0.75	0.03	3.99
6:94	Comb.	24	408800	1.73	0.10	5.77
6:94	Comb.	24	1564800	3.44	0.07	2.03
6:94	Comb.	24	696800	2.42	0.09	3.71
9:91	Comb.	24	416800	0.50	0.02	3.99
9:91	Comb.	24	1140800	1.57	0.05	3.12
9:91	Comb.	24	896800	1.30	0.06	4.61
Marine						
3:97	AN	24	1206522	1.88	0.05	2.66
3:97	AN	24	1993478	3.28	0.05	1.52
3:97	AN	24	2432609	4.33	0.03	0.69
6:94	AN	24	1184783	5.94	0.04	0.67
6:94	AN	24	2271739	5.96	0.08	1.34
6:94	AN	24	1110870	5.69	0.07	1.23
9:91	AN	24	2002174	3.23	0.03	0.93
9:91	AN	24	1858696	2.54	0.10	3.93
9:91	AN	24	1754348	3.02	0.04	1.33