Lipids from Autotrophically Grown *Chlamydomonas reinhardtii* and their Potential for Use In Bio-diesel Production

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DECLARATION

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THIS THESIS FOR MASTER OF SCIENCE DEGREE IN MICROBIOLOGY IS MY ORIGINAL WORK AND HAS NOT BEEN PRESENTED IN ANY OTHER UNIVERSITY FOR THE AWARD OF A DEGREE.

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DEDICATION

This thesis is dedicated to my dad Mr. Stephen Ngeno and my mom Mrs. Pauline Ngeno for their love, moral and financial support during the entire period of my MSc studies at the University of Nairobi and to beloved Rose, Brian and June whose love, belief and wishes helped me realize my potential.

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ABBREVIATIONS

3N-BBM+V-Bold- Basal Medium with 3-fold Nitrogen and Vitamins

ACCase- Acetyl CoA Carboxylase

APHA- American Public Health Association

ANOVA- Analysis of variance

ASTM- American Society for Testing and Materials

DO- Dissolved Oxygen

EC- Electrical Conductivity

PH- Potential of Hydrogen Ions

PPM- Parts Per Million

TAG- Triacylglycerol

DEFINITION OF TERMS

Bio-diesel- Any vegetable oil- or animal fat-based diesel fuel consisting of long-chain alkyl (methyl, propyl or ethyl) esters made by chemically reacting lipids with an alcohol.

Biomass- The total mass of living matter in a given unit area.

Dissolved Oxygen- The measure of the amount of oxygen that is dissolved in soil or bodies of water and is essential for survival of aerobic micro-organisms and other aquatic organisms.

Electrical conductivity- Measure of the ability of water to conduct an electric current. It is sensitive to variations in dissolved solids, mostly mineral salts.

Eutrophication- The process of nutrient enrichment of water bodies. This leads to rapid growth of phytoplankton, algae and macrophytes followed by more organic matter accumulation, silting up of the bed of the water body.

Feedstock- Plant materials and animal waste used as raw materials for bio-fuel production.

Green house gasses- A gas in the atmosphere, carbon dioxide, methane and ozone that absorbs and emits radiation within the thermal infrared range thereby causing the heating up of the atmosphere.

Photoinhibition- inhibition of photosynthesis caused by excessive irradiance.

Photoperiod- the duration of exposure to light.

Self-shading- a phenomenon where cells rapidly increase in size and prevent light from reaching other cells in the culture, thereby necessitating agitation.

Transesterification- This is the reaction of a fatty acid and an alcohol, usually in presence of an acid or base catalyst, to give methyl or ethyl ester and glycerin.

ABSTRACT

The ever rising prices of oil in the world markets has led to the need to develop cheap, clean and locally available sources of energy such as the bio-fuels, wind and geothermal energy. Bio-diesel from oil crops may offer a solution to this problem; however, oil crops cannot realistically satisfy the demand for transport fuels, since they compete with food crops compromising food security. Under optimum growth conditions microalgae can yield sufficient amounts of oils to meet the demand for bio-diesel. This study therefore sought to determine the optimum growth conditions for biomass and lipid production for *Chlamydomonas reinhardtii* grown in varying environmental conditions of light intensity, photoperiod, carbon dioxide, urea and nitrogen. Screening and isolation of the alga was carried out from samples collected from Nairobi Dam and grown in Modified Bold Basal Media. Harvesting and subsequent extraction of lipids was done after twenty days of growth. The extracted lipids were then converted to bio-diesel through transesterification process by reacting the lipids with absolute methanol and KOH catalyst. The results indicated an optimum growth at light intensity of 240 μmol m^{-2} s^{-1} in an 18-hour photoperiod. The yield data on biomass of *C. reinhardtii* grown at this photoperiod had a mean dry weight of 53.5±1.29g after a 3 week growth period while a 6-hour photoperiod recorded 25±1.09g translating to daily increase of 2.5g/day and 1.2g/day respectively. *Chlamydomonas reinhardtii* showed a tolerance response of 15% CO_2 concentration and a robust growth in urea of 2.4g/l, giving lipid yield of 4.9ml/g of the dry weight. Growth in 1.2g/l of urea also gave a yield of 5ml/g of dry weight. The yield of lipids in algae grown in 100gms of nitrogen was 0.592g in contrast to 0.102g for those grown in 250gms. Under all these optimum growth conditions it produced 98±1.79g on harvest, which on extraction gave lipid yield of 22 ml. Upon transesterification it gave bio-diesel yield of 14 ml with a density of 0.89 g/ml and a soap content

of 78ppm. These results implied that by optimizing these growth conditions, higher biomass density can be obtained from this species, thereby giving high lipid yield. The results from transesterification gave evidence for using this species as a potential source for high quality bio-diesel.

Keywords: *Chlamydomonas reinhardtii,* Bio-diesel, microalgae, transesterification, autotrophic growth.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background and Rationale

The quest for alternative sources of energy has provided many ways to produce electricity, such as wind farms, hydropower, or solar cells. To meet the world's transport needs, energy-dense liquid fuels such as gasoline, diesel fuel or kerosene are needed. These fuels are all obtained by refining petroleum, whose dependency has a lot of drawbacks, major of which is the deterioration of the environment as a result of accumulation of CO_2. The demand for energy is continually increasing and to protect the environment and maintain a sufficient energy for the supply, a concerted effort is being made to advance renewable energy alternatives which include the bio-fuels.

This project focused on trying to find a substitute for petroleum, a non-renewable environmental pollutant which is getting depleted, leading to an urgent need to find a substitute. Most plants contain oil as a part of their biomass but since their oil contents vary, an attractive source for biodiesel should contain high amount of oils, have least impact on the environment and have low cost of production. Currently most of the crops being used such as Jatropha, soy bean, and sunflower do not meet these requirements (Schubert, 2006). This has ignited interest in the use of algae for making bio-diesel. Microalgae offer a good source for bio-diesel since it can produce up to 30 times more oil per unit of growth area than land plants, (Haag, 2007). In terms of the optimal conversion of the sun's energy, microalgae achieve a photosynthetic efficiency of 11.6% whereas most plants have a rate of 1-2% (Vasudevan and Briggs, 2008) thus resulting high biomass and lipid content. Therefore they have the most potential as a bio-diesel source. Technical challenges, however, exist for the cultivation of high lipid content algae. These

include finding the most appropriate growth conditions and optimization of lipid yield which will give a faster growth and a higher lipid yield. These include optimum light intensity, CO_2 concentration, temperature, *p*H and appropriate nutrients. When algae are subjected to a stressful environment, such as nutrient starvation, carbon uptake is used for storing energy rather than reproduction, thus producing more lipids. Yet, this may be a limiting factor in the optimization of overall oil yield due to the lack of cell reproduction. Overall biomass yield frequently decreases under environmentally stressed conditions. By optimizing the growth conditions we are likely to achieve a faster growth and a higher lipid yield in *Chlamydomonas reinhardtii.*

1.2 Justification

The optimization of the yield is the main factor in mass culture technology of microalgae. Thus, it is necessary to understand the behavior of algal species under different environmental factors that determine different growth parameters. The study of the interactions between these factors and the growth modeling parameters allows for finding the optimal conditions for selected species in large-scale productivity.

There is very scanty data existing on the use of *Chlamydomonas reinhardtii* for bio-diesel production. This is because it is thought to contain little amount of lipids compared to other high yielding species like the *Scenedesmus spp* and as such is rarely used in the study of microalgae as feedstock for bio-fuels (Chisti, 2007). It is, however, a very sturdy organism and can survive under extreme environmental conditions (Mark, 2003) and is relatively easy to isolate. By establishing the optimum growth conditions, one of the biggest contributions will be a detailed documentation of the feasibility of the use of lipids from *C. reihardtii* as a bio-diesel feedstock in a bid to find an environmental friendly substitute for fossil fuels.

1.3 Objectives of the Study

1.3.1 Overall Objective

- To extract lipids from autotrophically grown *Chlamydomonas reinhardtii* and investigate their potential for use in bio-diesel production.

1.3.2 Specific Objectives

- To isolate *Chlamydomonas reinhardtii* from Nairobi Dam
- To compare growth rates and oil yields of *C. reinhardtii* grown under different conditions of light, CO_2, Urea and nitrogen starvation
- To extract, transesterify and determine the purity of resultant biodiesel

1.4 Hypothesis

- Optimum growth conditions of light intensity, CO_2 concentration, urea and a limited supply of nitrogen significantly increases growth rate of *Chlamydomonas reinhardtii* and yields sufficient oils for use in biodiesel production.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Conventional Energy

The global economy depends on the infrastructure of air, land and sea transport networks to move basic goods and people across borders. Similarly, the manufacturing industry thrives due to the ability to transport goods and services cheaply all over the world. To achieve this, a cheap source of energy is needed. For years, fossil fuels have been used for this purpose. The volatile nature of oil prices has, however, shaken the very foundation of the transport-oriented global economy and sent ripple effect throughout numerous sectors leading to decrease in profits and increasing the cost of production of goods and services which are eventually passed down to the consumer.

Fossil fuels are non-renewable energy sources that are found in the earth's crust. They include oil, coal and natural gas. They were formed millions of years ago from the organic remains of prehistoric plants and animals after decomposition (Mann *et al.*, 2009). The fuels are made up of hydrocarbons which store chemical energy in atomic bonds of hydrogen and carbon. Burning the fuels breaks the bonds releasing the energy, which makes them valuable to our society (Skov, 1998).

With the advent of industrial revolution in the 1800s, there was an increased use of fossil fuels (Veziroglu and Sahin, 2008) after the invention of the internal combustion engine.

The main renewable energy sources include hydro-electricity, biomass, geothermal, solar, and wind. Nuclear and fossil fuels are non-renewable as there is a finite amount of deposits beneath the earth and consumption is occurring at a rate that will deplete these sources over the next few centuries (Veziroglu and Sahin, 2008).

The main products of fossil fuel burning are carbon dioxide, nitrous oxides, and hydrocarbons (Huntley and Redalje, 2004). These gases are included in the mixture of gases commonly referred to as "greenhouse gases." Greenhouse gases are heating up the earth's atmosphere by trapping the infrared radiation that would otherwise be reflected back towards space (Green, 2000). This has led to global warming which is imparting many harmful effects on the earth's ecosystem. Levels of several important greenhouse gases have increased by about 25 per cent ever since large-scale industrialization began around 150 years ago (Green, 2000). During the past 20 years, about three-quarters of human-made carbon dioxide emissions were from burning fossil fuels. Among the gases emitted by fossil fuels, carbon dioxide is one of the most significant. Carbon dioxide in the atmosphere has increased by 25% since the beginning of fossil fuel burning and is predicted to further increase by an additional 1.9% each year (O'Driscoll and Vergano, 2007).

The earth's ecosystem provides a natural carbon cycle, but humans are producing more carbon dioxide than the earth can sequester or remove from the atmosphere through natural means. The burning of fossil fuels has led to many negative environmental impacts. During exploration, preparation and transportation of fuels, there is acid drainage from abandoned coal mines, air pollution from refineries and land and water pollution from oil spills. Burning of fossil fuels impacts on health and environment from smog, acid rain and toxic air pollution..

2.2 Bio-diesel Feedstock

Pollution of the environment by petroleum fuels and their high prices have ignited interest in searching for other sources of energy like hydropower, wind energy and the bio-fuels which have so many advantages over the fossil fuels. New sources of energy are

needed, and technology is racing to find alternatives to coal and oil. Of particular interest are biological sources of energy, such as microalgae, which can be used directly for power generation, or processed to create fuels such as bio-diesels, ethanol or methanol (Becker 1994; Illman *et al.*, 2000).

With just a few minor adjustments to a petroleum diesel engine, a car can run on bio-diesel with ease (Addison, 1998.; Tickell and Tickell, 2000). Not only is this fuel similar to petroleum diesel in functionality, but it also has other benefits. Bio-diesel can be manufactured with relative ease, and is a renewable energy source (Sheehan *et al.*, 1998). However, bio-diesel is somewhat expensive currently, the reason being that it is still difficult to produce enough fuel to be competitive with petroleum diesel on a large scale. According to Canakci and van Gerpen (2001), bio-diesel can be made from any oil/lipid source; the major components of these sources are triacylglycerol molecules (TAG), which consist of three long chains of fatty acids attached to a glycerol backbone. TAG molecules can be obtained from a variety of sources which include animal fats, waste cooking oil and pure vegetable oils.

Animal fats include fats and tallow derived from animals. Compared to other sources, these fats frequently offer an economic advantage because they are priced favorably lower and are easy to get. The main disadvantage of this feedstock, however, is that it contains high amounts of saturated fats and their bio-diesel tends to gel, limiting widespread use, particularly for winter-time use (Wen *et al.*, 2006).

Waste cooking oils are recycled oil and grease from restaurants and food processing plants. Their use is good because besides recycling of waste, it utilizes waste products that could otherwise present disposal problems. Waste cooking oils, however, have a

disadvantage in that they have so many impurities that require pre-processing to ensure a bio-diesel product of pure and consistent quality (Brock, 2007; Canakci and Van Gerpen, (1999). Another problem with processing waste cooking oils is that they usually contain large amounts of free fatty acids that cannot be converted to bio-diesel using an alkaline catalyst due to formation of fatty acid salts (soap). The soaps can prevent separation of the bio-diesel from the glycerin fraction. An alternative method is to use acid catalysts, which are able to esterify free fatty acids (Freedman and Pryde, 1982; Liu, 1994).

Pure vegetable oils are the oils derived from various crops and plants such as soybean, canola (rapeseed), corn, cottonseed, flax, sunflower, peanut, palm and jatropha. The oil composition from vegetable crops is pure and this cuts down on pre-processing steps and makes a bio-diesel product of a better quality. This feedstock is, however, not being regarded as the best route forward as source of bio-diesel in that some of these crops take a long time to grow and their lipid yield is low compared to microalgae (Schubert, 2006). The competition for land between these crops and food crops can also lead to food insecurity (Chisti, 2007). This, therefore, shows that these sources of lipids for bio-diesel production are less suitable since they cannot realistically meet the demand. Research into microalgae lipids is now being taken seriously because it is one of the most appropriate sources (Michael, 2004).

2.3 Algae as Feedstock for Bio-diesel

There are approximately 8,000 species of green algae estimated to be in existence. They have chlorophyll *a* and chlorophyll *b* (Jonathan *et al.*, 1971). They are made up of eukaryotic cells and have plastids, the bodies with chlorophyll that carry out photosynthesis. They use light and carbon dioxide to create biomass and use starch as their primary storage component. N-deficiency, however, promotes the accumulation of

lipids in certain species, a property which is being studied by researchers as a way of boosting lipid production (Shen *et al*, 2009). Green algae are the evolutionary progenitors of higher plants and, as such, they have received more attention than other groups of bio-fuel feedstock (Sheehan *et al*, 1998). Green algae may be unicellular, multi-cellular, colonial (living as a loose aggregation of cells) or coenocytic (composed of one large cell without cross-walls; the cell may be uninucleate or multinucleate). They have membrane-bound chloroplasts and nuclei. All green algae are photosynthetic (i.e. autotrophic), which means that they get all of their energy (organic carbon) from photosynthesis. Green algae are generally fast growing and sturdy. They reproduce both asexually (by division of cells) and sexually (Guiry, 2009). Algae have been known to grow in diversified aquatic habitats including lakes, ponds, pools, streams, marshes, and swamps. They are also found in soil, animal and plant substrates, on rocks and in the mud.

Algae grow rapidly and can double their biomass as much as eight times in a day during exponential growth (Chisti, 2007). Not only are algae efficient producers of natural oils, they are also a part of earth's natural way of sequestering carbon dioxide, thus significantly reducing greenhouse gases. Unlike other bio-diesel sources, algae do not compromise food stock or deplete nutrients in the soil though they still require nutrient sources. Compared with terrestrial crops—which take a season to grow and only contain a maximum of about 5 percent dry weight of oil—microalgae grow quickly and contain high oil content (Chisti, 2007). This is why they are the focus in the algae-to-bio-fuel arena. Microalgae have become the exclusive focus in the development of a sustainable feedstock for bio-diesel production, because bulk of the natural oil they make is in the form of TAGs, which is the right kind of oil for bio-diesel production (Hu *et al.*,

2008). Lipid content of algae is an important parameter that determines the economy of algae bio-diesel production (Chisti, 2008). Depending on the species, microalgae contain a variety of different lipids, hydrocarbons and other complex oils. Most species of microalgae contain relatively high percentages of lipids, with average contents 20-40% dry weight, whereas some species, most notably *Botryococcus braunii,* produce up to 75% of their dry weight as hydrocarbons (Borowitzka, 1988). It has been observed that algae produce enormous amount of lipids when put into stressful environments such as nutrient deprivation, (Piorreck *et al.*, 1984). For instance, a strain of algae put into a nutrient deficient environment can go from 22% to 58% oil content per dry mass (Sheehan *et al.*, 1998) but some strains can reach as high as 80% (Metting, 1996; Spolaore *et al.*, 2006). Some strains can grow heterotrophically without light. High biomass and high lipid yield have been found when heterotrophic algae are placed in low light and supplied organic carbon rather than carbon dioxide as carbon source (Miao and Wu, 2004; Xu *et al.*, 2006). Miao and Wu (2004) found autotrophic *Chlorella protothecoides* possessed a lipid content of 14.57% compared to 55.2% when grown heterotrophically. Xu *et al.* (2006) also reported the lipid content of the *Chlorella protothecoides* which showed an exponential growth in heterotrophic conditions. Thus owing to fast growth and high lipid content, microalgae are considered the best feedstock for the production of bio-fuels.

The production of bio-diesel is based upon the quantity of lipids that the algae produce during its growth. Microalgae are autotrophic organisms and as such they do photosynthesize which allows them to create their own food. It is during photosynthesis that microalgae consume carbon dioxide in the presence of sunlight to grow its biomass

and produce oxygen; biomass is composed of carbohydrates, proteins and lipids. Lipids are esters, composed largely of carbon, oxygen, and hydrogen (Sheehan *et al.*, 1998). They store energy and are essential for cell growth; its chain length is usually between C-12 to C-20. The main importance in lipid production is that they can be converted via a process called transesterification, into bio-diesel fuel, (Vasudevan and Briggs, 2008). Acetyl CoA Carboxylase (ACCase) enzyme has been found to catalyze steps in lipid synthesis in microalgae. Ideally, if genetically manipulated, this enzyme can increase lipid yield in algae though no commercial application has been reported (Leon-Banares *et al.*, 2004). The simplest way, however, is to manipulate the growth nutrient of algae such as nutrient starvation. Nitrogen source and concentration in the growth media greatly influence algae lipid yield (Shen *et al.*, 2009). In nitrogen limited situations, algae lipid content usually increases as a mechanism of survival, which make cells stop their divisions and start to store energy in the form of lipids though biomass growth is consequently expected to be inhibited. Growing of microalgae is one of the biggest challenges that need to be overcome before realization of full benefits of algae-to-bio-fuel project. Currently they can be grown in open ponds (Terry and Raymond, 1985) and in photo-bioreactors (Molina *et al.*, 1999). Open ponds are perceived to be less expensive than photo-bioreactors, since they cost less to construct and operate but have a low biomass productivity compared to photo-bioreactors. Because they are open-air systems, they often experience a lot of water loss due to evaporation. Efficient utilization of carbon dioxide is reduced thus limiting biomass production (Chisti, 2007). Contamination from bacteria, protozoa and other algal species is also another big problem. In addition, growth conditions cannot be controlled in open ponds. On the other hand, photo-

bioreactors offer the best alternative to open ponds. Virtually any translucent container could be called a photo-bioreactor, ranging from small beakers and flasks in the laboratory (Tredici, 1999) to complex tubular photo-bioreactors which consist of arrays of straight transparent tubes for capturing sunlight (Carvalho *et al*, 2006). However, the term is more commonly used to refer to a closed system, as opposed to an open tank or pond. One advantage of photo-bioreactors over open ponds is that they can be employed to overcome the contamination and evaporation problems encountered in open ponds (Molina *et al*., 1999). Optimum environmental growth conditions of temperature, pH, and light can also be achieved since these conditions can easily be controlled and be precisely monitored. Every strain of algae has different growth requirements. As such, to obtain maximum growth and high lipid yield in a photo-bioreactor, these conditions must be determined for each species to be used. The main disadvantage of the photo-bioreactors is that they are costly to scale up. Moreover, light limitation cannot be entirely overcome because light penetration is inversely proportional to the cell concentration (Perner-Nochta and Posten, 1978). That is, as the cell density increases, light penetration reduces due to self-shading. Attachment of cells to the tubes' walls may also prevent light penetration. In a photo-bioreactor, however, it is easy to manipulate the growth conditions to achieve the highest biomass production and lipid yield. Among the growth conditions which can be optimized are light, carbon dioxide, temperature, pH and growth nutrients. There is scanty data on use of lipids from *Chlamydomonas reinhardtii* for bio-diesel production because it is thought that its lipid yield is not sufficient for use (Mellis, 2002). It has however been shown that nutrient deprivation in any microalgae leads to accumulation in lipids (Shen *at al*, 2009; Xiong *at al*, 2000). This property can be utilized

in the growth of *C. reinhardtii* to maximize its lipid production. Determining the optimum growth conditions for this alga may also lead to a higher biomass yield and hence higher lipid production.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Sampling Site

Water samples were collected at the Nairobi dam (1^0 16' S latitude and 36^0 48' E longitude), which is about 4km south west of Nairobi City at the slums of Kibera. At the Dam, several points were identified based on turbidity, distance from the edge of the dam and depth of water, and marked points 1-10. Some chemical and physical properties of water were measured at these points. Water samples were collected from each of these points using clean plastic sampling bottles which were pre-washed using detergent and rinsed with distilled water. A clean jar attached to a long stick was dipped in water below the surface at the depth of approximately 10cm and the collected samples were transferred into containers and transported to laboratory for treatment and analyses.

3.2 Physio-Chemical Water Quality Parameters

During sampling, some physio-chemical parameters of water were first taken in Nairobi Dam in order to establish the environmental conditions in which algae grow in-situ. Among the parameters which were taken included pH, Electrical Conductivity of water, Temperature, Dissolved Oxygen, Phosphorous, Nitrates, Ammonia and Chlorides (Alpha, 1992). Measurements were taken for a period of ten days.

Electrical conductivity was measured by the use of conductivity meter model LF 90, pH, by pH meter model Jenwey pH 90. While dissolved oxygen and temperature were measured by the use of combine-oxygen meter model OXY 10, which also measures temperature.

3.2.1 Chlorides

Chlorides were determined by the use of the MOHR (argentometric) method (Apha, 1992). 50cm^3 of the sample was placed in a 250ml-conical flask on a white reflecting surface. Two drops of phenolphthalein were added into the flask. The sample was adjusted by $^1/_2$N H_2SO_4 and $^1/_2$N NaOH so that it just colours the mixture. 1 cm^3 of standard potassium chromate indicator (i.e. 5g of potassium chromate in 100ml of distilled water) was added to each sample, which then produced a yellow color. Titration was done using standard silver nitrate solution (3.546 grammes of AgNO3 in 1 litre of distilled water) until the color changed from bright yellow to a faint pinkish yellow. The amount of chloride in the samples was determined using the following equation: -

$$Cl^- (mg/l) = \frac{\text{Vol. of } AgNo_3 \text{ x } 3.546g/l \text{ x } 1000}{\text{Vol. of the sample used in mg/l}}$$

(Allen, 1989)

3.2.2 Nitrates

Cadmium reduction method was used in determination of nitrate concentration in water. DR/2000 Spectrophotometer for high range nitrate was used in getting the nitrate levels. The sample cell was filled with 25ml of the water sample and Nitra-ver 5 nitrate reagent powder was added and immediately swirled to mix and left to react for five minutes. A blank sample of 25 ml of distilled water in a sample cell was used to calibrate the spectrophotometer to zero (0.00mg/l NO_3^-). The amount of NO_3^- was then determined by getting the difference between test sample and the blank readings at a wavelength of 500 nm and dial number of 490 (Hach, 1992).

3.2.3 Phosphates

Ascorbic acid method was used in the determination of phosphates. DR/2000 Spectrophotometer for high range PO_4^{3-} was used for this purpose. The sample cell was then filled with 25ml of the water sample and a phosver 3 reagent was added to the sample and swirled immediately to mix and allowed to react for two minutes followed by a 10-minute reaction. A blank sample of 25mg/l of distilled water in a sample cell was used to calibrate the instrument to zero (0.00mg/l- PO_4^{3-} concentration of phosphate was then determined by getting the difference between sample and blank readings at a wavelength of 496nm. (Hach, 1992).

3.2.4 Ammonia

Nessler method was used in the determination of ammonia. DR /2000 Spectrophotometer for high range ammonia were used for this purpose. A liquot of 25ml of the water sample was transferred into a sample cell, and three drops of mineral stabilizer were added to each sample cell and inverted several time to mix, followed by the addition of 3 drops of polyvinyl alcohol (dispersing agent) and further inverted repeatedly to homogenize the mixture. 1ml of Nessler reagent was pipetted into each sample cell with the mixture stoppered and inverted several times and left to react for 1 minute. A blank was prepared using distilled water and given same treatment .The prepared blank was used to calibrate the instrument to zero (0.00mg/l NNH_3). Concentration of ammonia was then determined by getting the difference between the test sample and blank readings at wavelength of 425nm (Hach, 1992).

3.3 Isolation and Culture of *Chlamydomonas reinhardtii.*

3.3.1 Isolation of the species

Of the several methods available for isolation, Capillary Pipette Method was used. This is because method of isolation depends on thallus type and size.

Several small drops of a suitably diluted culture medium were put on a sterile glass-cover slip by a sterile pipette drawn to a capillary. Each drop was then examined under the microscope until only one drop that contained *Chlamydomonas reinhardtii* was found. This drop was removed with a sterile capillary pipette and inoculated into a fresh sterile modified Bolds media as used by Powell *et al*, (2008). *C. reinhardtii* was identified using its morphological characteristics. It is a green motile unicell with 2 apical flagella, sheathed by the plasma membrane that is extruded from the cell wall. It has a single large chloroplast with one or more pyrenoids and starch granules. An "eyespot" region within the chloroplast at the anterior end allows the organism to seek out areas of appropriate light intensity. It also has 2 or more contractile vacuoles at the anterior end of the cell function to remove excess water and waste products.

3.3.2 Culture Conditions

After isolation axenic cultures of *C. reinhardtii* was grown in modified Bold Basal media (Stein, 1973) which was previously sterilized by autoclaving for 20 mins at 120^0C and 15 psi. Stock cultures were maintained at 20^0C $\pm 0.5^0$C in cotton stoppered 2800 ml flasks (Grobbelaar, 2004).

Illumination was provided by overhead fluorescent ''warm white'' 40 W lights located above the growing algae and the height adjusted to obtain an appropriate irradiance level for all experiments. Carbon dioxide was obtained from a CO_2 generator which utilized the process of fermentation of sugar and yeast to give CO_2 gas. The gas was then channeled through a CO_2 meter which was used to measure the concentration. Adjustments were then made of the inlet gas to give desired concentration of carbon dioxide in percentage. Light intensity was measured using the Lux meter. The experiment

was replicated four times. Cultures were maintained at between 20^0C and 25^0C using a waterbath and a pH range of 6.5-7.5

3.3.3 Media Preparation

The media used was modified Bold-Basal medium with 3-fold nitrogen and vitamins (3N-BBM+V). To prepare the media, stock solutions were prepared as given in Table 3 below. Aliquots from these stocks were then carefully measured and added to a given volume of water (Stein, 1973). Table 3 below shows the stock solutions used in formulation of the media. The stock solutions were made to 1 liter with deionized water and autoclaved at 15 psi for 15 minutes.

Table 3: Media formulation

Stock solution	Chemical species	Quantity/L	Quantity/L of Stock Solution Used
1.	KNO_3	25.0 g	30.0 ml
2.	$MgSO_4.7H_2O$	7.50 g	10.0 ml each
	NaCl	0.50 g	
	$K_2HPO_4.3H_2O$	7.50 g	
	KH_2PO_4	17.50 g	
	$CaCL_2.2H_2O$	2.50 g	
3. (Add to 1 L of distilled water 0.75g NaEDTA and these minerals in order from top to bottom)	$FeCl_3.6H_2O$	97.0 mg	6.0 ml
	$MnCl_2.4H_2O$	41.0 mg	
	$ZnCl_2.6H_2O$	5.0 mg	
	$CoCl_2.6H_2O$	2.0 mg	
	$NaMo_4.2H_2O$	4.0 mg	
4.	Vitamin B1 (Thiamin hydrochloride)	0.12 g (in 100 mL distilled water)	1.0 ml each
5.	Vitamin B12 (Cyanocobalamin)	0.1 g (in 100 mL distilled water)	

The algal cells were inoculated following the steps of Smith and Chanley, (1975). The culture vessels were first sterilized followed by nutrient enrichment. New culture from a pure algal species was then inoculated and microscopic observations and cell counts were made on a daily basis to monitor the growth.

3.4 Quantifying Algal Biomass

To quantify the algal biomass, cells were counted under a microscope using the hemacytometer (Sheldon and Parsons, 1967). A colorimeter was also used to measure the optical density of the cultures from which the growth rate was calculated.

The algal cells were counted daily in all the four replicates under a light microscope with a haemacytometer. This is a thick glass slide with a counting chamber which has a microscopic grid etched on the glass surface and are overlaid with a glass cover slip that rests on pillars exactly 0.1 mm above the chamber floor. Thus, the volume of fluid above each square of the grid is known with precision. Motile *Chlamydomonas* cells were killed by adding 0.1 mls Lugol's iodine to 0.9 mls culture to prevent movement and diluted before loading into the hemocytometer. This step was necessary to give accurate results. The dilution factor was recorded to allow calculating the concentration.

To calculate the number of cells, the cells were first counted in the 4 outer squares and calculated as follows:

Cell concentration per millimeter = Total cell count in 4 squares × 2500 × dilution factor

3.5 Harvesting Microalgae

The growth of *Chlamydomonas reinhardtii* was characterized by five phases as shown in the following figure.

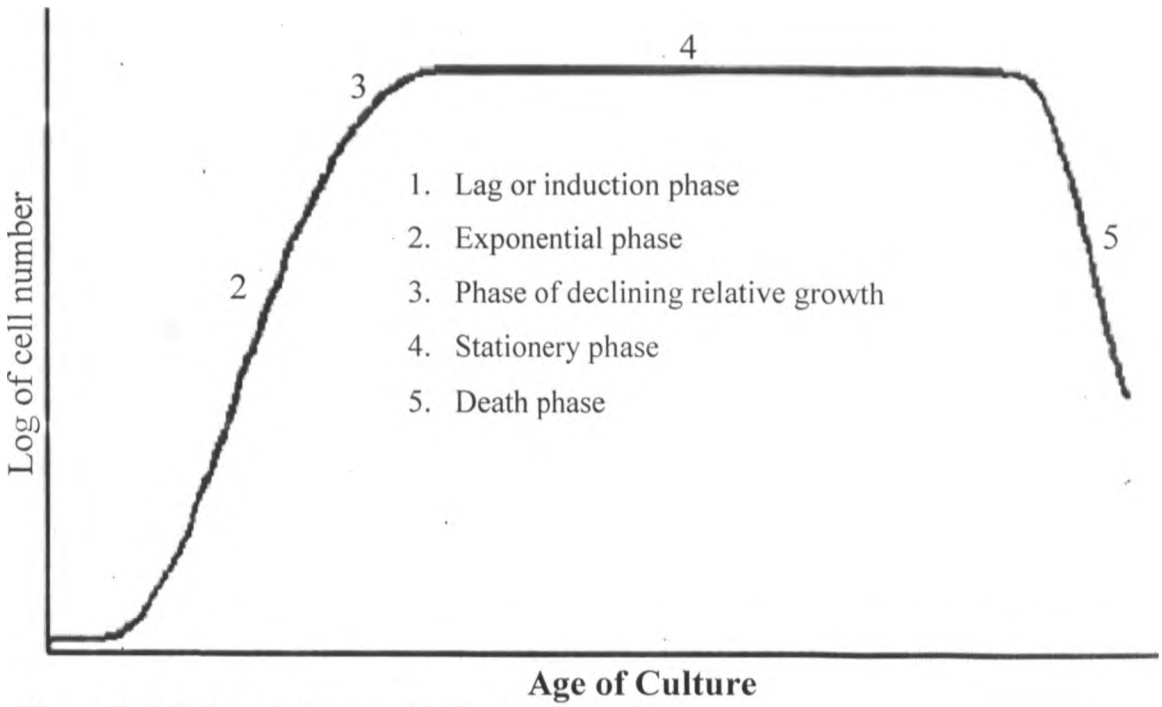


Figure 3-1: Microalgae growth dynamics

In the lag phase, there was little increase in cell density. In the exponential phase, the cell density increase was a function of time, t. Very fast growth was witnessed and growth rate of the algae was calculated from this phase. In the phase of declining growth rate, cell division slowed down when nutrients, light, pH, carbon dioxide or other physical and chemical factors began to limit growth. In the stationary phase the limiting factor and the growth rate were balanced, resulting in a relatively constant cell density.

The process of harvesting of the alga was started when the phase of declining relative growth began, just before the stationary phase. This was on the first day when the growth rate declined. Harvest was achieved by the process of auto flocculation, as reviewed by

Fox (1983) and Barnabé (1990). In this process no chemical flocculants like the ferric chloride and aluminium sulphate were used. At the onset of the phase of declining growth carbon dioxide supply into the culture was stopped completely. This caused the alga to flocculate on its own in 24-48 hours. Though this method was time consuming, it was the least expensive.

3.6 Drying of Harvested Algae

There was a need of dewatering the cultures to a paste and removing the remaining moisture through drying. Of all the drying methods like flash drying, rotary drying, or spray drying, sun drying was chosen for use in this project because it is cheap and not energy intensive and does not present risk of rupturing the cell wall prematurely thereby causing a loss of lipids.

Sun drying of the alga was accomplished by exposing the biomass to direct solar radiation. Presence of water in the alga inhibits transesterification reaction, preventing it from going to completion (Romano, 1982 and Canakci and Van Gerpen, 1999) and would also lead to formation of too much soap, thereby increasing the processing cost (Mohn, 1978). Therefore drying was a very important step in achieving a pure product. Besides water removal, sun drying also caused disintegration of chlorophyll leading to a good color to the final product.

3.7 Extraction of Oils

The extraction of oils was done by use of the soxhlet apparatus with the solvent being the n-hexane, (figure 3.2). 250ml of n-hexane was put into the soxhlet and heated in a water bath. This was done so because the n-hexane is highly flammable and cannot be heated in a naked flame. The microalga, placed in the thimble inside the apparatus was

washed repeatedly with the n-hexane passing through the condenser (Chisti, 2007). The soxhlet apparatus is air tight and no solvent was lost through evaporation or spillage thus enabling the solvent to be reused for each cycle.

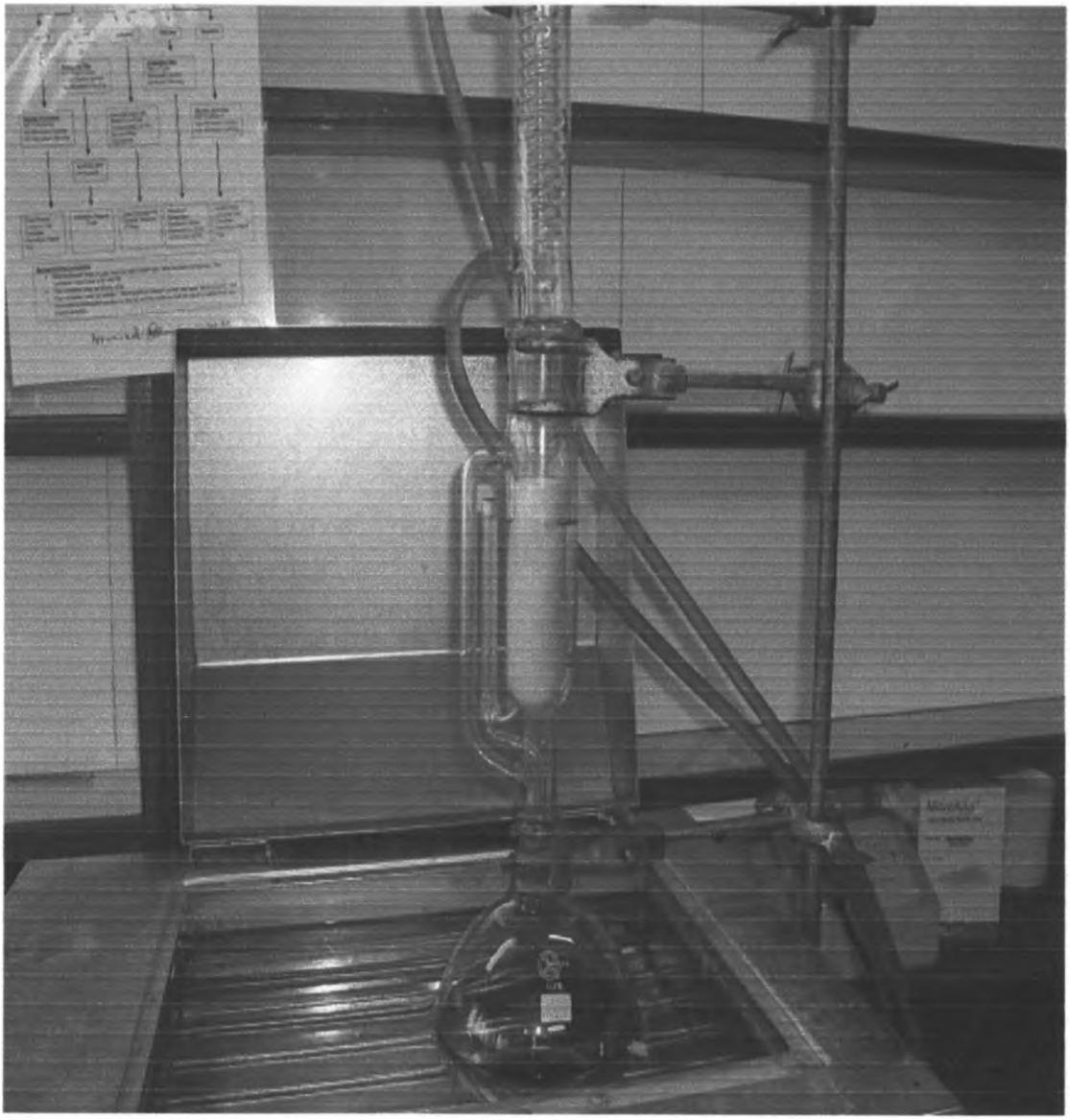


Figure 3-2: Oil extraction using the soxhelet apparatus

3.8 Separation of Oil-Hexane Mixture

Chemical extraction using soxhlet apparatus resulted in a mixture of lipids and the n-hexane solvent. Before transesterification was carried out, the oil was first recovered from the lipid-solvent mixture. A rotavapour was used in this process, (figure 3.3). The mixture was put in a rotavapor and heated in a water bath to 71^0C, slightly above the boiling point of the solvent, until all the solvent evaporated leaving behind the lipids. The amount of n-hexane that was collected from the condenser was measured and found to be 240ml which was almost equal to the amount used in the extraction process. To ensure that complete separation had occurred, the mixture was further centrifuged at 3400 rpm for 5 minutes.

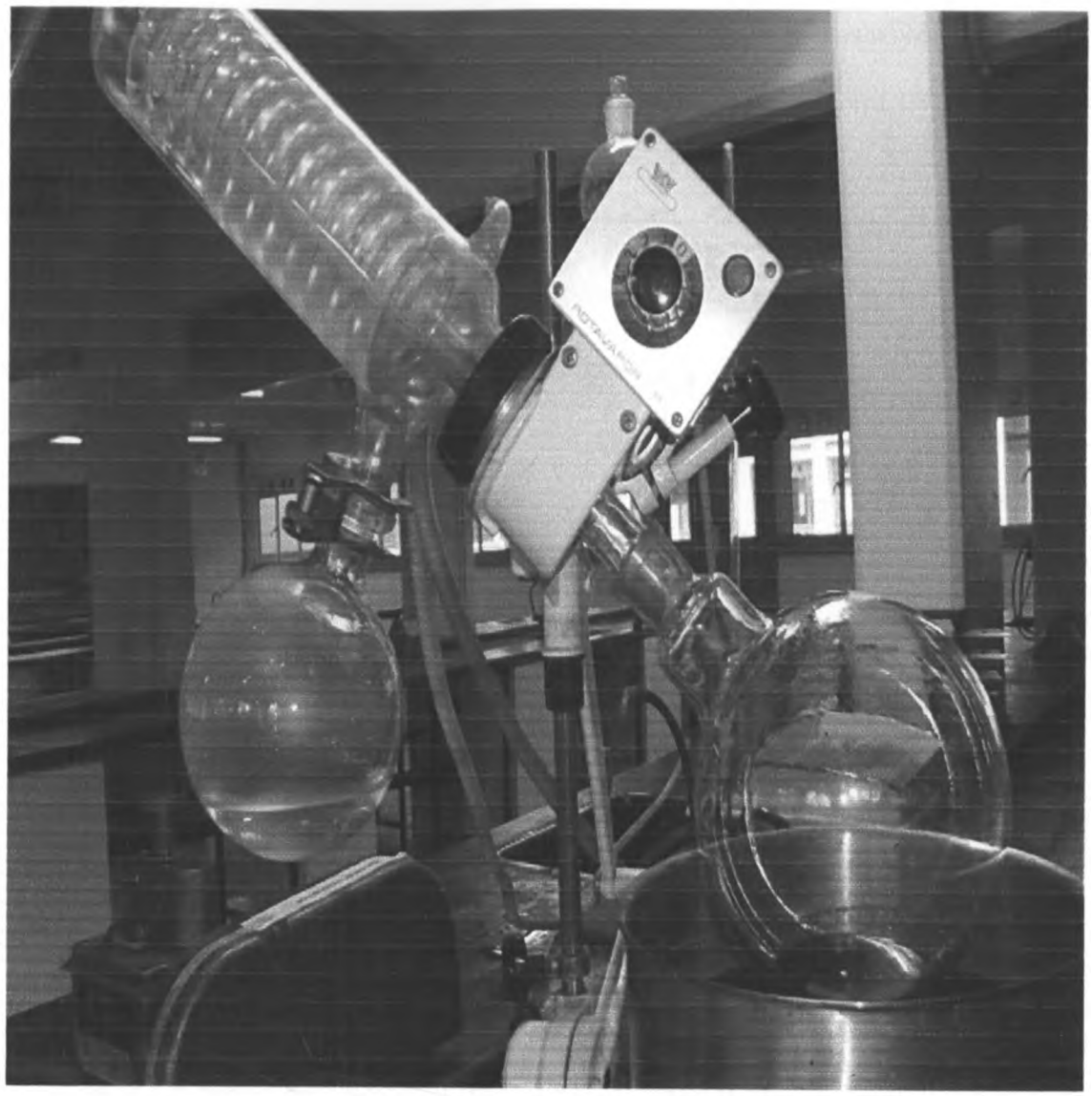
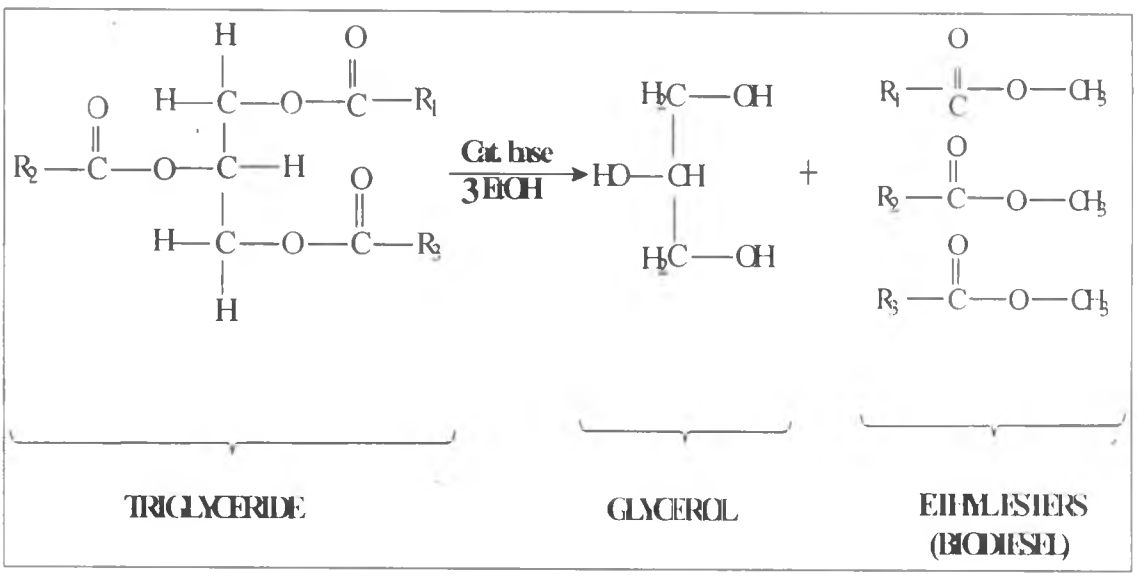


Figure 3-3: Separation of oil-hexane mixture by use of a rotavapour

3.9 Transesterification

Transesterification is the reaction between an alcohol and a triglyceride molecule. It addresses the problem of viscosity associated with bio-diesel. The three fatty acids molecules of the triglycerides are reacted with an alcohol (e.g. methanol or ethanol) to give methyl/ethyl esters of fatty acids (bio-diesel), and glycerol, (figure 3.4).



Where R_1, R_2, and R_3 are alkyl or aryl groups.

Figure 3-4: Equation for the transesterification reaction.

The "R" groups are the fatty acids which are usually 12 to 22 carbons in length. This large oil molecule is reduced to about one third its original size, lowering the viscosity and making it similar to diesel fuel (Fukuda *et al* 2001). KOH was used in this reaction because it is easier to dissolve in methanol much more easily than NaOH though the later is cheaper. Before transesterification of the fatty acids, titration on the oils was first done so as to find out how much alkaline was needed to completely neutralize any free fatty acids present, thus ensuring a complete reaction.

3.9.1 Titration of the Lipids

Titration of lipids was done using the Better Titration Method (Addison, 1998). 1g of KOH was dissolved in 1000ml of water and this was recorded as the KOH solution. Next, 1ml of oil was dissolved in 10ml isopropyl alcohol. This was the lipid solution.

With a dropper the KOH solution was gradually added into the lipid solution, a milliliter at time. The number of drops was counted and after each drop the pH level was checked with a standard pH paper. The number of drops of KOH needed for the pH to reach 8-9 was recorded and used to calculate the volume of KOH needed to neutralize the oil. This procedure was repeated three times to get the correct amount. Accuracy in finding the volume needed was important because inadequate KOH would leave some unreacted oils which will be mixed with bio-diesel and glycerin and too much of it would result in a gel.

Since the reaction is reversible the alcohol (methanol) was added in excess to drive the reaction forward and ensure complete conversion, as observed by Fukuda et al., (2001). The reaction was carried out in a water bath and the temperature kept at 70^0 C, slightly above the boiling point of methanol. The reaction time was 8 hours after which the mixture was left to stand overnight in the separating funnel to allow for separation to take place. Though methanol is expensive and toxic, it was chosen over ethanol because it is more effective due to physical and chemical properties (small chain and polarity). Ethanol also requires larger quantities of reagents thus larger quantities of products. Recovery of ethanol and products is more complex and costly.

3.10 Separation of Biodiesel-Glycerin Mixture and Removal of Alchohol

Once the reaction was complete, there were two major products: glycerin and bio-diesel. Each had a substantial amount of the excess methanol that was used in the reaction. The glycerin phase, being much denser than the bio-diesel phase, settled at the bottom and bio-diesel at the top and thus was separated with glycerin simply being drawn off the bottom of the separating funnel as shown in figure 3.5 below.

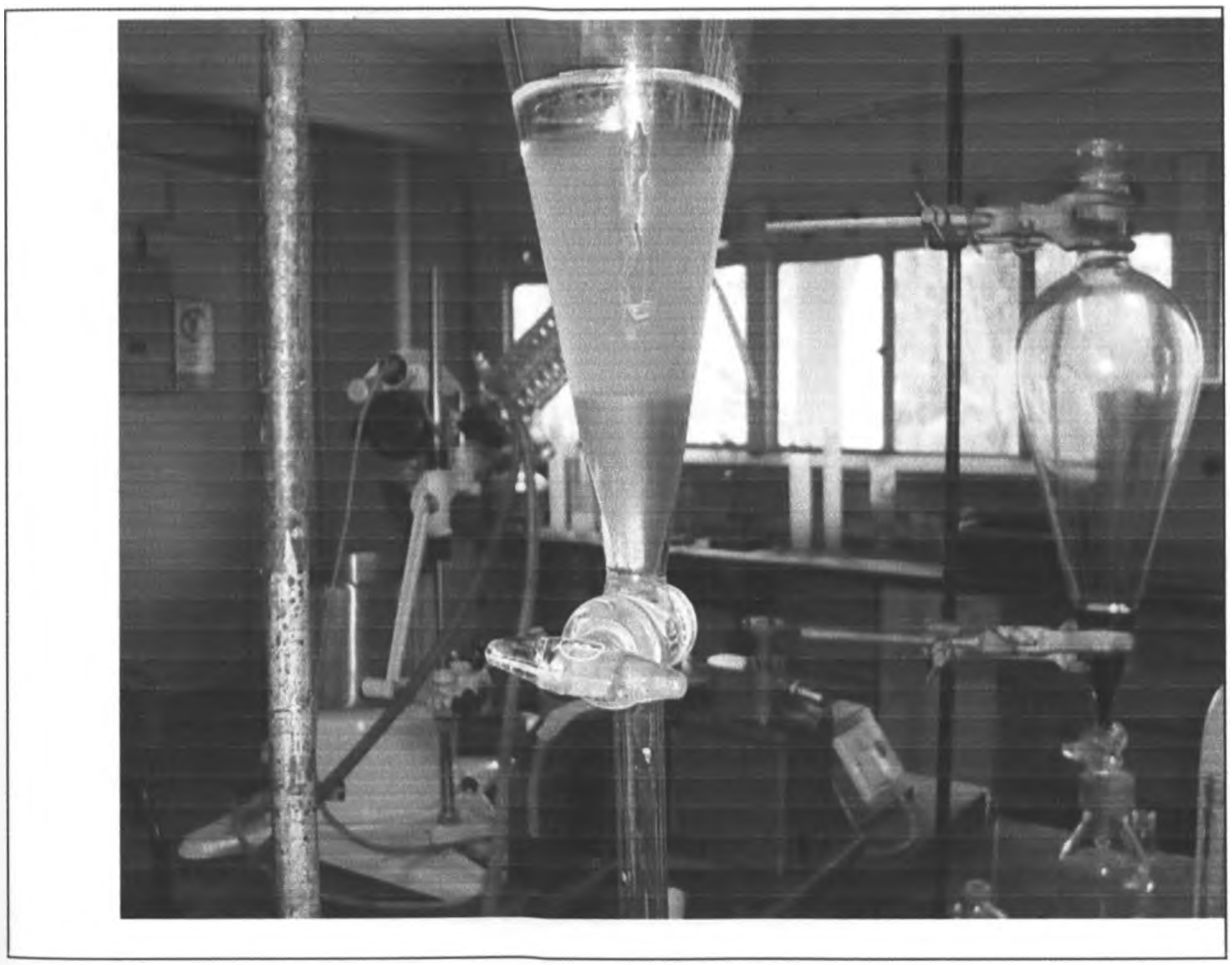


Figure 3-5: Bio-diesel-glycerin mixture after being left overnight

Once the glycerin and bio-diesel phases were separated, the excess alcohol in the bio-diesel phase was then removed by distillation in a rotavapour. With a boiling point of 65 ^{0}C, methanol evaporated first leaving the bio-diesel behind (bio-diesel boiling point, 330 ^{0}C)

3.11 Testing the Purity of the Bio-diesel

After the last step of alcohol removal, there was what was now considered pure bio-diesel. Glycerin, methanol and soaps may have been present in the bio-diesel as impurities and needed to be removed. To ascertain the purity, therefore, some further experiments were carried out on the bio-diesel. These included washing to get rid of the remnant soaps, titration to determine whether all the soaps had been removed, testing for the completion of the reaction, determining the density of the bio-diesel and the pH of the final product.

3.11.1 Washing the Bio-diesel

Once separated from the glycerin, the bio-diesel was then purified by washing repeatedly with water to remove residual catalyst and soaps. Water and bio-diesel were mixed in the ratio 1:1 and shaken gently for 5 minutes and thereafter allowed to settle for 5 hours. Since bio-diesel has a lower specific gravity than water, the water eventually separated and settled at the bottom and the bio-diesel at the top. After settling the water and the impurities, mainly the remaining alcohol and catalyst, was drained from the bottom of the separating funnel. At the first wash, the water turned cloudy due to formation of soap when water came into contact with catalyst in the bio-diesel. It turned clear as the soap was washed out with subsequent washings.

After each wash, the water was drained off and another batch of clean water was used. This was repeated until the water was totally clear; showing that all the catalyst and soaps

had been removed. It was then allowed to stand for four days to dry. The result was a clear amber-yellow liquid as shown in the figure 3-6.

3.11.1.1 Titrating for Soaps in Bio-diesel

Titrating for soaps in the bio-diesel can help in understanding the success of the bio-diesel processing before and after washing (Schafer, K. 1998). When used after washing, quite simply, it tells one how well the bio-diesel has been washed. When used before washing, it gives an idea of how close the process is to perfection and even more importantly, how much washing will need to be done. Before titrating for soaps, the presence of left over catalysts used in the reaction were first tested. This was important as these catalysts may give inaccurate results. This was done as follows:

100 ml of isopropyl was poured into 250 ml beaker and placed in a scale, tared to zero. 10 g of the bio-diesel was then added. Next, 5 drops of 1 % phenolphthalein solution was added. Clear liquid indicates absence of the catalyst while magenta color indicates its presence and neutralization is necessary. This was done by adding 0.01N HCL and stirring until the solution turned clear (ASTM Standard D6751, 2009).

After establishing the absence of catalyst, titration for soaps was then carried out as follows:-

0.01N HCL was added to test the soap content in the bio-diesel. While stirring the liquid in beaker about 20 drops of Bromophenol blue indicator solution was added and the solution turned dark blue. It was then weighed and recorded. After this, 0.01N HCL solution was added slowly while stirring until it turned from blue to yellow. It was then weighed again and the final weight recorded (ASTM Standard D6751, 2009).

3.11.1.2 Calculation to Determine Amount of Soap in PPM

The weight difference of the two weights recorded was equivalent to the number of grams of 0.01N HCL added. Since the solution is basically water 1 gram = 1 ml. To calculate amount of soaps in ppm, the amount of 0.01N HCL used in the titration was multiplied by the Catalyst Factor (320.56 for KOH and 304.4 for NaOH) (ASTM Standard D6751, 2009).

3.11.2 Testing for the completion of the reaction

The testing for the completion of the reaction was done after washing to further ascertain the purity. This was done by mixing 25 ml of the bio-diesel with 225 ml of methanol. It was then shaken gently for 20 minutes and left to stand for one hour.

3.11.3 Density of the Bio-diesel

Further to the above tests, the density of the bio-diesel was measured at 25 ^{0}C using two methods. In one, an empty 10 ml graduated cylinder was weighed and noted down. The bio-diesel was then added to the graduated cylinder and the weight measured again and from this the weight of the bio-diesel alone calculated by subtracting the weight of the empty cylinder from that of the cylinder and the bio-diesel. The density (which is the mass per unit volume) of the bio-diesel was then calculated. Secondly, a hydrometer was used to measure the specific gravity. The graduated cylinder was filled with the bio-diesel up to about three quarters full. The hydrometer was then carefully placed inside and swirled gently to remove any bubbles clinging to the bottom of the cylinder. The hydrometer was then allowed to settle for about 15 minutes. The level the bio-diesel surface reached on the hydrometer scale is the specific gravity of the bio-diesel

Figure 3-6: Pure bio-diesel (B-100) after being washed (soap content-75ppm, density 0.89 g cm^{-1}, pH 6.9)

The whole process of biodiesel production from microalgae is summarized in the flow chart shown in figure 3-7 below.

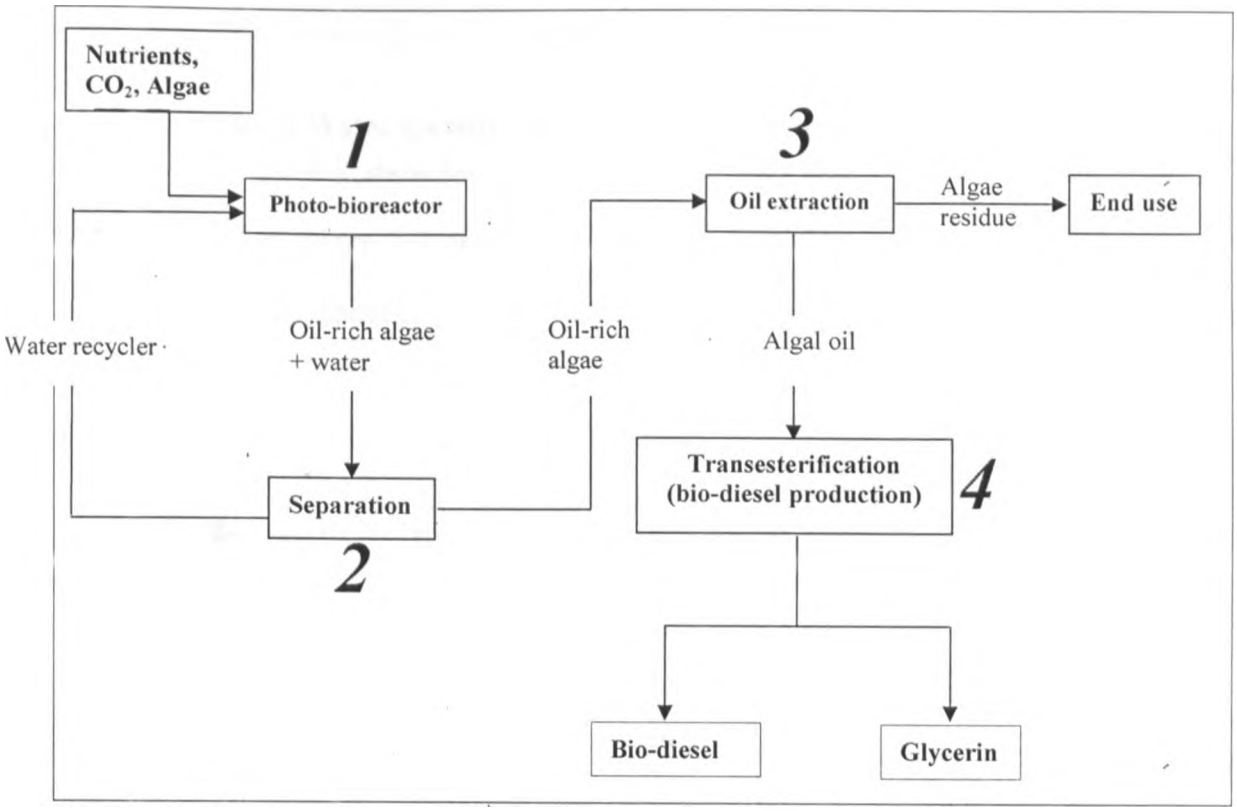


Fig3.7: The flow chart of biodiesel production from *Chlamydomonas reinhardtii*

Steps:

1. Microalgae are grown in a photobioreactor, which is a closed vessel that allows light to penetrate to a growth medium which consists of water, nutrients and algal cells
2. Microalgae harvesting separates oil-rich algae from water and the water is recycled back to the photo-bioreactor and be reused
3. Oil extraction from harvested algae. This was done by solvent extraction. After this step the solvent is normally recovered and reused
4. Bio-diesel production was done by transesterification with alcohol (methanol) and an alkali catalyst.

CHAPTER FOUR

4.0 RESULTS

4.1 Physico-Chemical Water Quality Parameters at the Nairobi Dam

Tables 4-1 and 4-2 show the physical and chemical properties of the water at the Nairobi dam from where the sampling of algae was done. The mean values and standard errors of electrical conductivity, pH, temperature and Dissolved Oxygen at different sampling points are as shown in Table 4-1 below. The electrical conductivity of the water varied significantly at different sampling points ($df=10$, $F= 256.797$ $p<0.05$). The lowest electrical conductivity mean values were recorded at point 10 with the value of $32.94\pm1.74\mu Scm^{-1}$. There was a significantly slight increase in electrical conductivity at points 2, 3 and 4 with mean values of $37.55 \pm 3.85\mu Scm^{-1}$, $38.10\pm2.09\mu Scm^{-1}$ and $38.1\pm 2.09\mu Scm^{-1}$ respectively. The pH for the water also showed significant increase at different sampling points ($df=10$, $F=44.195$, $p<0.05$) with the highest being 7.84 ± 0.72 and lowest 6.47 ± 0.25. The data showed a significant increase in temperature ($df=10$, $F=20.082$, $p<0.05$) in all points ranging from 18.3 ± 1.3^0C at point 10 to 24.37 ± 2.8^0C at point 5. It was observed from the data that the dissolved oxygen values varied significantly ($df=10$, $F=2.304$, $p<0.05$) between 3.32 ± 0.7 mg/l at point 9 and 5.85 ± 0.41 mg/l at point 7. The Points with 6 and 7 had the highest values of dissolved oxygen.

Table 4-1: Mean values and standard errors of electrical conductivity (μScm^{-1}), pH, temperature (^0C) and Dissolved Oxygen (mg/l) of water from Nairobi dam.

SAMPLING PNTS	PARAMETERS			
	EC(μScm^{-1}) ±SE	pH ±SE	TEMP ^0C±SE	DO (mg/l) ±SE
PNT1	32.94±1.74	6.47±0.25	19.65±0.46	4.4±0.33
PNT2	37.55±3.85	6.57±0.24	18.51±1.49	4.28±0.46
PNT3	38.10±2.09	6.69±0.23	18.59±1.49	3.11±0.56
PNT4	38.10±2.09	6.59±0.28	20.45±0.51	4.46±0.59
PNT5	431.70±99.77	7.84±0.72	24.37±2.81	5.21±1.81
PNT6	474.50±123.88	7.08±0.56	20.34±1.4	5.83±0.61
PNT7	453.00±125.22	7.57±0.31	20.91±1.24	5.85±0.41
PNT8	112.20±36.33	7.05±0.29	19.51±0.76	5.47±0.57
PNT9	69.65±19.58	6.81±0.27	18.78±1.9	3.32±0.7
PNT10	59.2±13.63	6.70±0.22	18.30±1.3	3.45±0.51
Mean	170.9±32.09	7.14±0.33	20±1.5	4.5±0.65

The mean values and standard errors of NO_3, PO_4, NH_3 and Cl^- in mg/l for the different sampling points in the Nairobi Dam are as shown in table 4-2. The recorded value of nitrates varied significantly ($df=10$, $F=43.223$, $p<0.05$) and showed an increase from 4.23±2.31 mg/l at point 1 to the highest of 47.21±10.5mg/l at point 5. The data also shows a significant variation of phosphate levels in the 10 sampling points ($df=10$, $F=72.161$, $p<0.05$) and increase from point 3 to 8. The levels of ammonia varied

significantly (df=10, F=88.143, p<0.05) between 0.39±0.61 mg/l in point 1 to 37.35±10.91 mg/l in point 5. The variation showed a sharp increase from point 4 and 5 and a drop from point 6 to point 3. The chloride levels also showed a significant variation from 21.06±12.52 mg/l at point 1 to 385.88±92.72 mg/l at point 5 (df=10, F=53.237, p<0.05) with an increase from point 1 to 6 and a drop from point 6 to 10

Table 4-2: Mean Values of Nitrates (mg/l), phosphates (mg/l) ammonia (mg/l) Chlorides (mg/l) and Their Standard error at the sampling points.

SAMPLING POINT	PARAMETER			
	NO_3 (mg/l)	**PO_4 (mg/l)**	**NH_3 (mg/l)**	**CL^- (mg/l)**
PNT1	4.23±2.31	0.19±0.1	0.39±0.61	21.06±12.52
PNT2	7.72±4.4	0.24±0.1	0.37±0.29	40.56±27.95
PNT3	7.2±4.42	0.23±0.11	0.31±0.27	40.49±24.81
PNT4	14.61±7.11	2.29±0.15	30.11±0.19	71.57±31.02
PNT5	47.21±10.8	2.43±0.65	37.35±10.91	358.88±92.72
PNT6	24.56±6.88	2.47±0.68	24.22±9.29	210.51±67.50
PNT7	22.28±5.83	2.55±0.69	22.05±8.22	195.91±56.08
PNT8	11.62±3.57	0.76±0.22	3.86±2.37	78.04±37.64
PNT9	11.61±4.31	0.37±0.15	1.56±0.59	59.65±30.72
PNT10	13.23±3.36	0.31±0.13	1.18±0.59	52.88±29.55
Mean	15.7±5.3	1.2±0.2	12.14±3.33	112.96±41.05

4.2 Effect of Light and photoperiod on Growth Rate of *Chlamydomonas reinhardtii*

The results of the effect of light and photoperiod on growth rate of the alga are as shown in table 4-3 below.

The ANOVA output showed a highly significant difference ($F=25.213$, $df=3$, $p<0.05$) in cell counts for *C. reinhardtii* grown under different lengths of illumination time.

Grown in light for 18 hours, the alga showed the highest growth rate and attained a high stationary phase cell yield of 3.8294×10^6 cells/ml, whereas 6 hour illumination period showed the least growth rate and reached the lowest stationary phase cell yield of 9.0×10^5 cells/ml. Those grown for 12 hours attained a yield of 2.1879×10^6 cells/ml at the stationary phase. With no other source of carbon provided, *C. reinhardtii* grown in the dark showed the least growth. Those grown in 6 hours of light reached stationary phase after 19 days of growth while those exposed for 18 hours reached stationary phase after 20 days. Although the cultures had an almost similar cell density at the beginning, the cultures exposed to light for 18-hours had a higher growth rate and large biomass increase than those grown for 6 hours or 12 hours. This was mostly evident in the exponential phase during which the growth rate was highest. Using the formula presented by Levasseur *et al.,* (1993), the growth rates of *C. reinhardtii* grown in different durations of light are as shown in the table 4-4 below and from this table it can be seen that 18-hour photoperiod produced the highest growth rate. Although the cultures were inoculated with the same biomass density, the stationary phase biomass yield varied considerably for the various treatments applied. Figure 4-1 illustrates the mean dry weights of the alga grown under different durations of exposure to light. It is seen from the figure that those exposed to 18 hours of light had the highest biomass yield compared to 12-hour and 6-

hour exposure. The figure showed a progressive increase in biomass yield as the duration of illumination was increased. In absence of light only very minimal growth was recorded as shown in the figure

Table 4-3: Cell counts for algae grown in illumination times of 6, 12 and 18 hours and without light

Day	Cell Count (cells/ml)			
	With Light			Without Light
	6 hrs.	12 hrs.	18 hrs.	
1	1.57×10^{4}	1.57×10^{4}	1.57×10^{4}	1.57×10^{4}
2	1.57×10^{4}	1.57×10^{4}	1.57×10^{4}	1.57×10^{4}
3	1.57×10^{4}	1.57×10^{4}	1.57×10^{4}	1.57×10^{4}
4	1.73×10^{4}	1.78×10^{4}	1.78×10^{4}	1.69×10^{4}
5	1.88×10^{4}	1.99×10^{4}	2.06×10^{4}	1.89×10^{4}
6	2.14×10^{4}	2.33×10^{4}	2.69×10^{4}	2.06×10^{4}
7	2.54×10^{4}	2.64×10^{4}	3.58×10^{4}	2.39×10^{4}
8	2.89×10^{4}	2.91×10^{4}	5.89×10^{4}	2.58×10^{4}
9	3.63×10^{4}	4.02×10^{4}	8.98×10^{4}	3.18×10^{4}
10	5.85×10^{4}	6.47×10^{4}	1.198×10^{5}	3.96×10^{4}
11	7.71×10^{4}	$845.\times10^{4}$	2.012×10^{5}	4.58×10^{4}
12	8.42×10^{4}	1.223×10^{5}	3.92×10^{5}	5.6×10^{4}
13	1.304×10^{5}	1.992×10^{5}	5.69×10^{5}	6.89×10^{4}
14	2.903×10^{5}	3.712×10^{5}	1.089×10^{6}	7.82×10^{4}
15	3.781×10^{5}	5.593×10^{5}	1.9908×10^{6}	8.94×10^{4}
16	5.992×10^{5}	9.259×10^{5}	2.9872×10^{6}	9.52×10^{4}
17	7.899×10^{5}	1.6865×10^{6}	3.2294×10^{6}	1.089×10^{5}
18	8.92×10^{5}	1.878×10^{6}	3.4294×10^{6}	1.159×10^{5}
19	9.038×10^{5}	2.049×10^{6}	3.6294×10^{6}	1.179×10^{5}
20	9.038×10^{5}	2.1879×10^{6}	3.8294×10^{6}	1.199×10^{5}
21	9.038×10^{5}	2.1879×10^{6}	3.8294×10^{6}	1.199×10^{5}
22	9.038×10^{5}	2.1879×10^{6}	3.8294×10^{6}	1.199×10^{5}
23	9.0×10^{5}	2.1879×10^{6}	3.8294×10^{6}	1.199×10^{5}

Table 4-4: Growth rates (div/day) of algae under 0, 6, 12 and 18 hours of exposure to light

Treatment	No Light	6 Hours	12 Hours	18 Hours
Growth Rate (div/day)	0.2	0.384	0.49	0.60

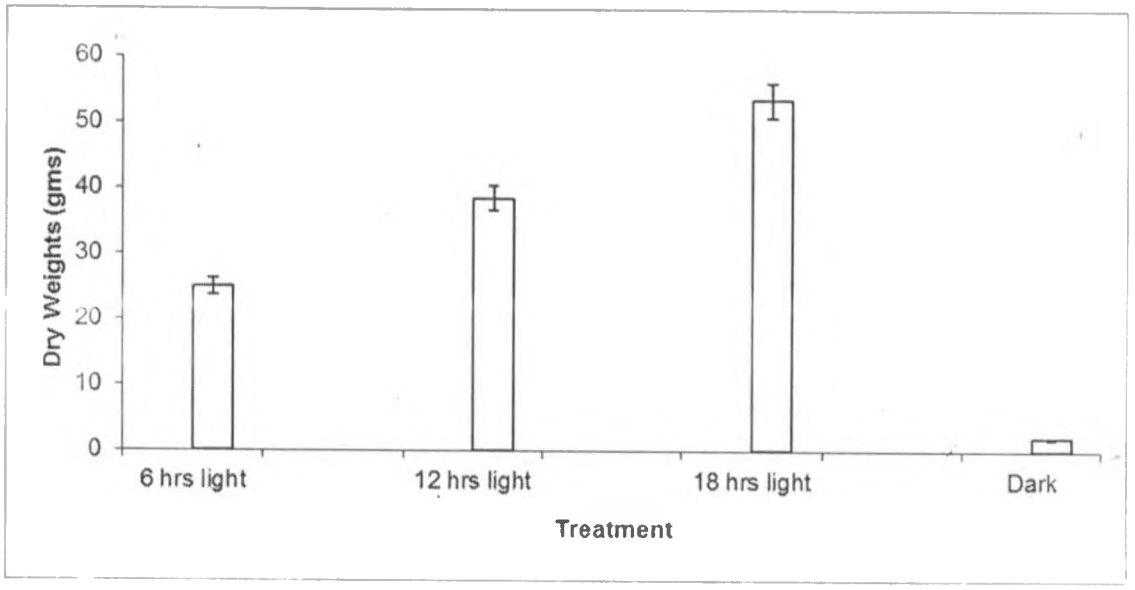


Fig 4-1: Comparison of the mean dry weights of algae grown in 0, 6, 12 and 18 hours of illumination. Error bars represent standard error.

4.3 Effect of Varying Light Intensity on Growth Rate of *Chlamydomonas reinhardtii*

Figure 4-2 below shows the mean doublings per day for the alga grown under varying light intensities with all other environmental conditions kept constant. There was a highly significant difference ($df=10$, $F=178.467$, $p<0.05$) in growth rate of the alga under different light intensities.

From the results, the growth rate for *C. reinhardtii* grown with a light intensity of 240 µmol m^{-2} s^{-1} produced the highest growth. Growth above and below this range was diminished.

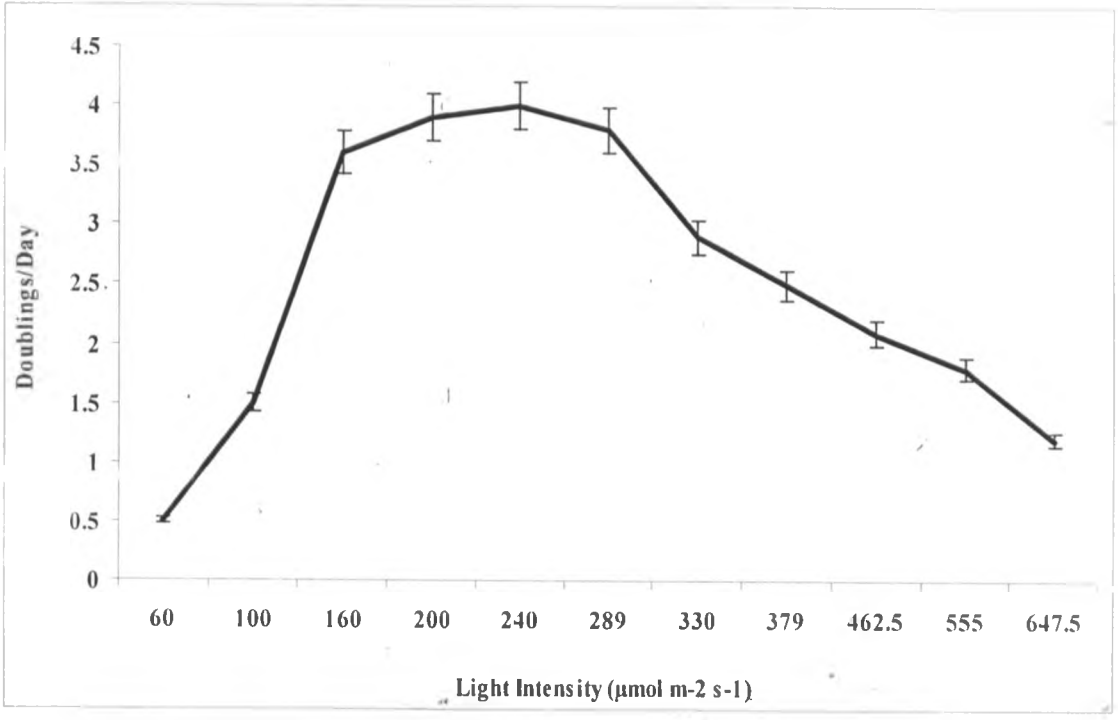


Fig 4-2: Effect of light intensity on growth rate of *Chlamydomonas reinhardtii*. Error bars represent standard error.

4.4 Effect of Carbon Dioxide on Growth Rate of *Chlamydomonas reinhardtii*

There was a highly significant difference in the number of cells for *C. reinhardtii* grown with CO_2 (with a concentration of 0.03%) and without CO_2 (df=1, F= 48.166, $p<0.05$) as shown in figure 4-3

Though the initial cell density was similar, the exponential phase was robust for those grown in carbon dioxide, reaching stationary phase cell yield of 2.2×10^6 compared to 1.5×10^5 without CO_2 on the 20^{th} day growth.

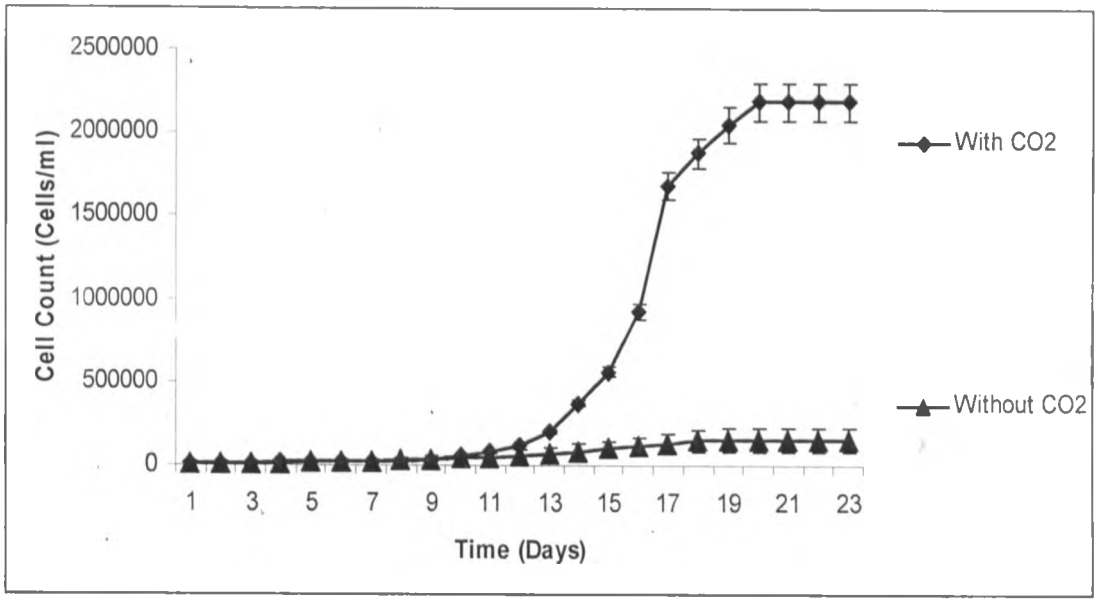


Fig 4-3: Growth rates of *Chlamydomonas reinhardtii* grown in CO_2 and without CO_2 for 23 days of growth. Error bars represent standard error.

4.5 Effect of Varying Carbon Dioxide Concentrations on Growth of *Chlamydomonas reinhardtii*

Figure 4-4 shows the doublings per day for *C. reinhardtii* grown in carbon dioxide of concentrations ranging from 0.03% to 15%. There was a highly significant difference in the growth rates of the alga under different concentrations of carbon dioxide (df=3, F=4.492, $p<0.05$).

From the results it was noted that *C. reinhardtii* grown in 15% CO_2 had the highest growth rate compared to 10%, 5% and the ambient CO2 concentration of 0.03%.

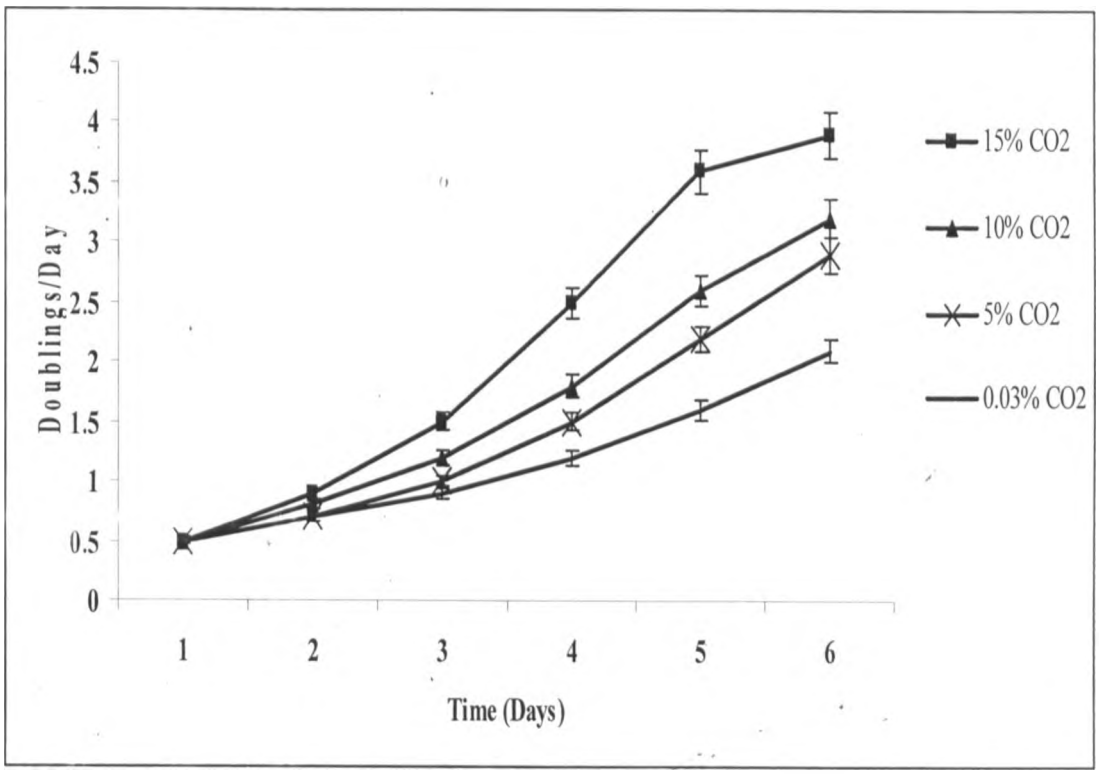


Fig 4-4: Graph of growth rate of *C. reinhardtii* grown in different concentrations of CO_2. Error bars represent standard error.

4.6 Effect of Urea on Growth Rate of *Chlamydomonas reinhardtii*

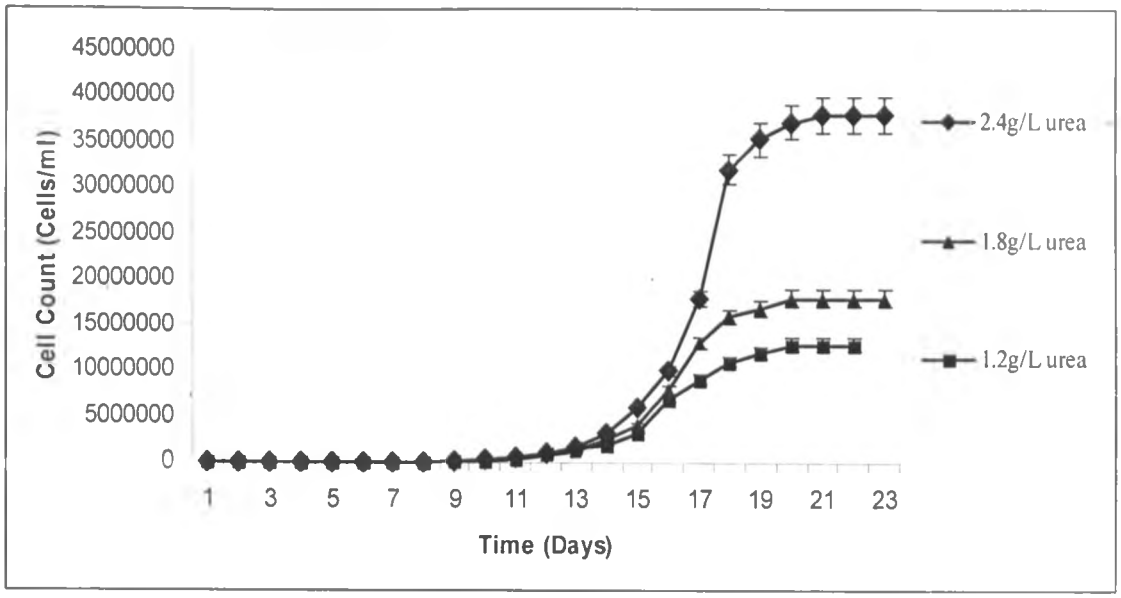


Fig 4-5: Cell counts for the alga grown in different concentrations of urea. Error bars represent standard error.

Figure 4-5 shows the cell counts for *Chlamydomonas reinhardtii* grown in different concentrations of urea. There was significant difference ($df=2$, $F=13.162$, $p<0.05$) in the growth rate of the alga grown in 2.4 g/l urea, 1.8 g/l urea and 1.2 g/l urea. The alga exhibited good/optimal growth in 2.4 g/l urea, reaching a high stationary phase cell yield of 3.79909×10^{7} after three weeks of growth. Growth in 1.2 g/l reached a maximum cell yield of 1.09002×10^{7} at the stationary phase while that grown in 1.8 g/l had a cell yield of 1.7999×10^{7} at the stationary phase.

Figure 4-6 below shows the comparison of dry weights for *C. reinhardtii* grown in varying concentration of urea. There was a significant difference in the weights of the biomass ($df=2$, $F=5.467$, $p<0.05$) for the alga grown in different concentrations of urea.

That grown in 2.4 g/l urea, it had the highest biomass yield giving a dry weight of 98.4 g whereas 1.8 g/l of urea gave a dry weight of 66.8g. The alga grown in 1.2 g/l of urea gave a biomass yield of 45g. The increase in levels of urea was directly proportional to the biomass yield. 1.8g/l of urea resulted in an increase of 45.8% of while 2.4g/l resulted in an increase of 48.1%.

Figure 4-7 shows the weight of lipids expressed as percentage of the dry weight of the alga. There was, however, no significant difference in the weight of the lipids ($df=3$, $F=4.987$, $p>0.05$). Growth in 1.2g/l urea resulted in a comparably higher yield of the lipids as compared to 1.8g/l and 2.4g/l of urea. Growth in 1.2g/l urea gave lipid yield of 22% of the dry weight, 1.8g/l urea gave 21.53% and 2.4g/l gave 21.04% of the dry weight. Increase in nitrogen levels was inversely proportional to the lipid yield. An increase of 0.6g/l of urea from 1.2g/l to 1.8g/l resulted in corresponding decrease of 0.47% in lipid yield whereas an increase of a similar margin from 1.8g/l to 2.6g/l urea resulted in a decrease of 0.49% in lipid yield.

Figure 4-8 is a photograph of the four replicates of *C. reinhardtii* growing in 2.4 g/l of urea at the end of the exponential phase, just before harvest. The deep green color indicates the dense biomass resulting from a vigorous growth.

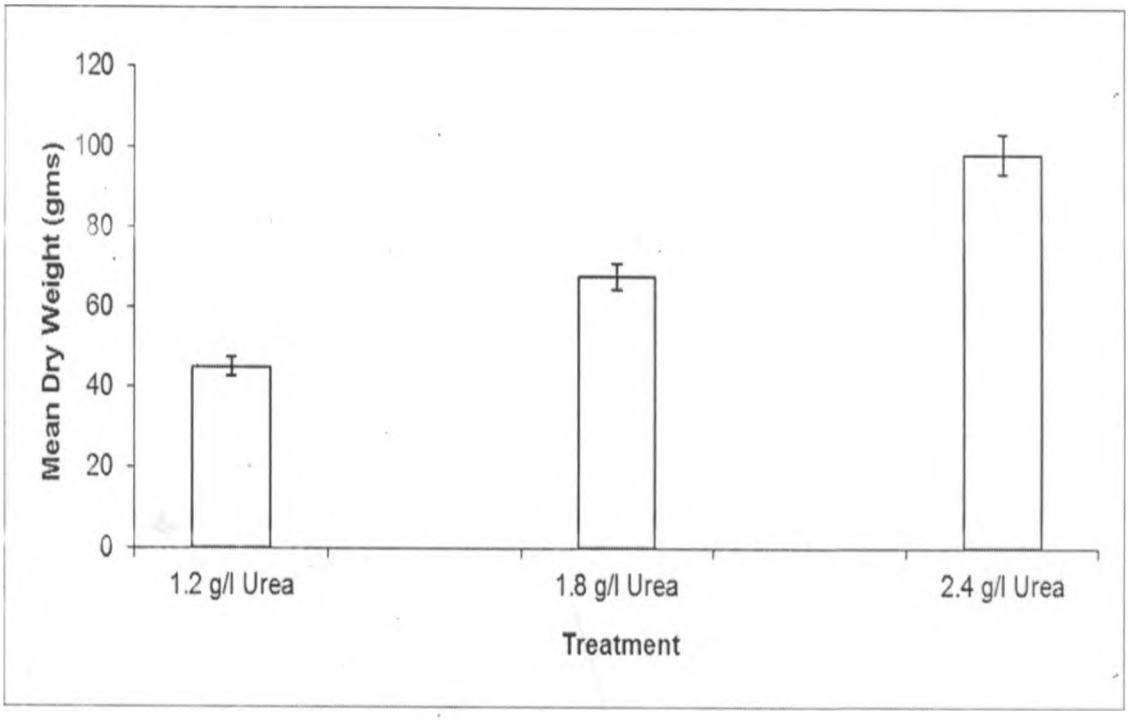


Fig 4-6: Comparison of the dry weights of *C. reinhardtii* grown under different concentrations of urea. Error bars represent standard error.

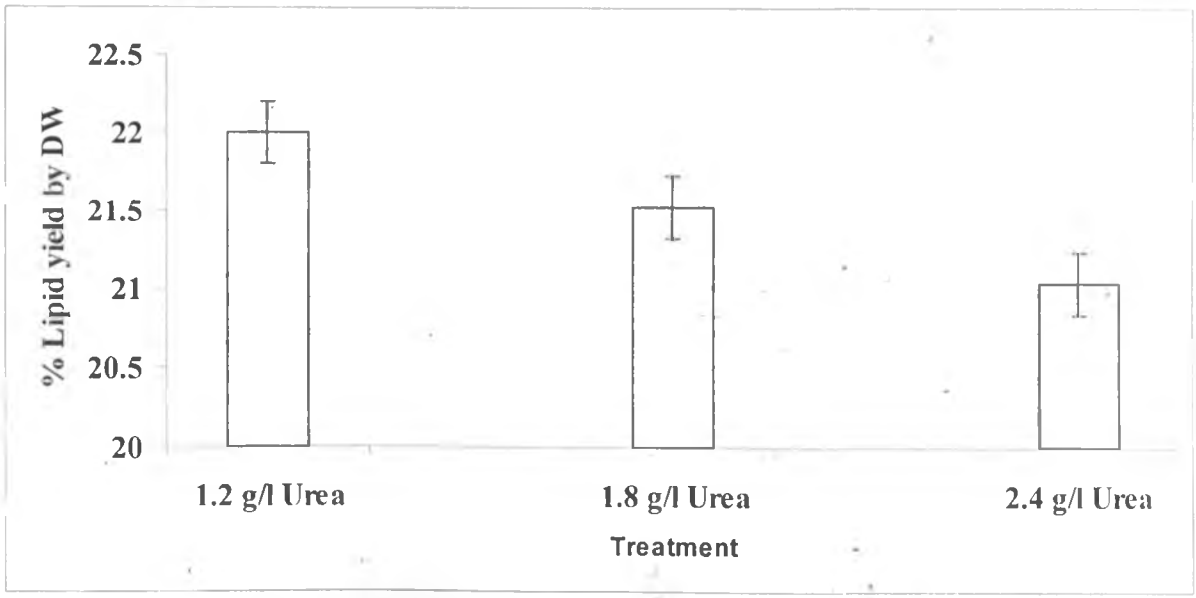


Figure 4-7: Lipid yield of *C. reinhardtii* grown in 1.2g/l, 1.8g/l and 2.4g/l urea expressed as percent of the dry weight. Error bars represent standard error.

Fig 4-8: Twent three days old *C. reinhardtii* grown in 2.4 g/l urea .

4.7 Effect of Varying Nitrogen Levels on Lipid Yield from *Chlamydomonas reinhardtii*

Figure 4-9 below shows the mean lipid yield and standard errors for *C. reinhardtii* in the 9 replicates grown in various concentration of nitrogen. The growth under varying nitrogen levels was highly significant ($df=2$, $F=9.994$, $p<0.05$). 100g/l of nitrogen had the highest lipid yield compared to 150g/l and 200g/l of nitrogen.

The mean weights of the lipids showed significant variation for the different levels of nitrogen ($df=3$, $F=6.897$, $p<0.05$). Growth in 100g/l of nitrogen gave mean yield of 0.363±0.14g whereas 150g/l of nitrogen gave a 0.135±0.04g a reduction of 63%. There was a similar reduction of 45% in lipid yield for 200g/l nitrogen. 100g/l of nitrogen gave the highest yield compared to the other two groups.

It can be seen from the data that growth in 100g of nitrogen gave the highest lipid yield across all the replicates more than all the other experimental groups, giving a mean weight of 0.363±0.14g. Those grown in 150g of nitrogen yielded a mean of 0.135±0.04g, while the growth in 200g of nitrogen gave the lowest yield of 0.073±0.05g.

The dry biomass weights however showed a totally different scenario from weights of the lipids. Growth in 100g of nitrogen gave a mean yield of 1.8±0.06g while the growth in 200g gave a yield of 2.8±0.5g.

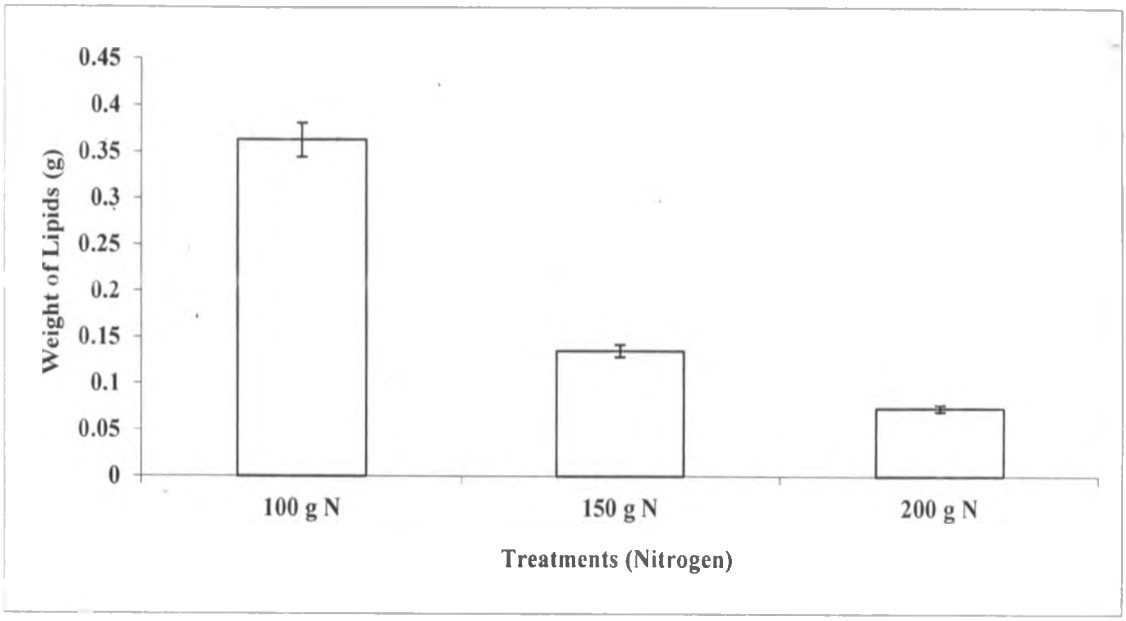


Fig4-9: Chart showing mean weights in grams of lipids from *C. reinhardtii* grown in varying levels of nitrogen (g/l). Error bars represent standard error.

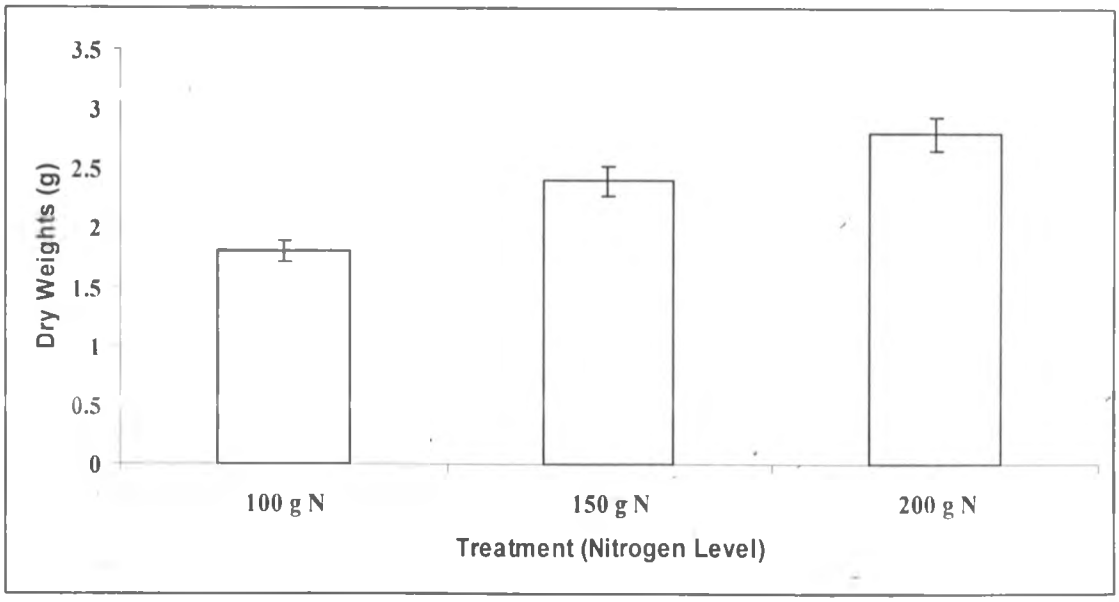


Figure 4-10: Dry weights of lipids harvested from *C. reinhardtii* grown in different concentrations of nitrogen. Error bars represent standard error.

4.8 Test of the Purity of the Bio-diesel

The results of the test of the purity of the bio-diesel indicated that the reaction proceeded to completion resulting in a pure product.

In two of the four replicates, the bio-diesel dissolved completely in methanol forming a clear bright phase and had a mean density of $0.89\pm0.10 g/cm^3$ and a mean pH of 6.9 ± 0.13. After four washings, the result was a clear amber yellow liquid with soap content of 75ppm and a density of $0.89\ gm^{-1}$ as shown in (figure 3-6).

CHAPTER FIVE

5.0 DISCUSSION

Bio-diesel, which is derived from transesterification of lipids, has become very attractive as an alternative transportation fuel due to the continued depletion of fossil oil reserves and associated environmental concerns with its use. Currently, most bio-diesel is derived from oil/lipid feedstock extracted from terrestrial food crops. Productivity of the lipids is a function of the environmental factors in which the intended feedstock grows. Among these pertinent environmental factors are light, photoperiod, carbon dioxide, nitrogen, temperature and pH. The target feedstock should occupy a small growing space, grow faster and yield high amount of lipids. Crop based feedstock cannot fulfill these conditions. Microalgae represent a potential alternative (Gerpan. 2005) and are a more sustainable feedstock for production of next generation bio-fuels as its cultivation does not compete with food production. They have a higher photosynthetic efficiency, biomass productivity and growth rate compared to other crop-based feedstock (Klass, 1998). There is a need, however to search for appropriate species to characterize various environmental conditions that apportion resources for growth and development of the feedstock and equally characterize the feedstock with respect to its growth. In this work therefore the optimum growth conditions of the algal species was assessed.

The most appropriate sources of the feedstock would be found in ecosystems like water in which organisms like algae, one of best feedstock for bio-diesel; inhabit. Such ecosystem should be capable of supplying appropriate growth nutrients thus influencing the growth of organisms in it.

The elevated level of nutrients in the Dam explains the presence of so many aquatic weeds and algal species. This increased presence of aquatic weeds and algal species is due to eutrophication as a result of municipal and industrial wastewater discharge into the Dam. The high levels nutrients which favor growth of algae like Phosphates and Nitrates have occasioned eutrophication, an indication of pollution. Phosphorus, present as phosphates, has contributed immensely to the large population of algae since it is one of the essential minerals required for growth. The dam is a sink for nutrients; consequently the slightly acidic conditions are attributable to the dynamics of these nutrient especially balance between CO2, carbonate and bicarbonate ions as well as other natural compounds such as humic and fulvic acids. The slightly acidic pH greatly favored the growth of the *Chlamydomonas reinhardtii* as witnessed by their thriving populations in the Dam.

The large algal population of algae in the dam may have been influenced by the temperature of the water as it determines their availability because it affects their physical, chemical and biological processes. The temperature of the water at the Dam seemed to favor the growth of *Chlamydomonas spp* as it was the most abundant microalgal species in the samples collected.

The abundance of the algal populations in the dam was also attributed to high levels of Dissolved Oxygen, which allows aerobic micro-organisms to stabilize any biodegradable organic material present in water thus making such nutrients like carbon available for algae and other aquatic organisms in the Dam.

The ability of *Chlamydomonas reinhardtii* to grow under such conditions at the Dam, therefore, implied that in open pond cultivation, polluted waters can be used for growing

microalgae provided other environmental conditions are kept within the range which will promote optimum growth. In the dam which is basically an open pond, however, environmental conditions are hard to control hence algal growth is limited by the fluctuations of these conditions depending on the prevailing weather conditions. As such, when isolating algae from such an environment, the prevailing climatic conditions should be taken into consideration since it may affect the availability, hence ease of isolation of the algal species of interest. The in-situ growth conditions can form a basis for determination of the optimum growth conditions in the laboratory.

Most microalgae are strictly photosynthetic, that is, they need light and carbon dioxide as energy and carbon sources. Light is one of the major environmental factors affecting the growth rates of unicellular algae because in most cases it is always at an improper level. In many laboratory experiments for physiological research of algae the light intensity is either too low or high to permit logarithmic growth. In nature the intensity is well above saturation and may be high enough to inhibit growth during much of the day. Light that directly influences photosynthesis mechanism is an important factor in defining optimal conditions for the culture (Falkowski *et al*., 1985). In the presence of non-limiting nutrients, the efficiency of microalgal culture remained controlled mainly by the intensity of light and photoperiod. Light and photoperiod are two important parameters which, at optimum levels, enable the microalgae to produce energy-rich carbon compounds which can be harnessed for bio-energy production (Casadevall *et al*,. 1985). Light energy is the growth limiting substrate and thus low or high light intensity below or above the optimum level will decrease productivity. Similarly, optimum level of light intensity will increase the yield. The results of the experiment to study the effect of

light and photoperiod on growth rate of *Chlamydomonas reinhardtii* grown at 25 ^{0}C, indicated that as light availability increased, so did algal biomass. Day length (photoperiod) is one of the determinant factors in the growing of this microalgae. This was evident in the productivity of the alga *C. reinhardtii* grown in varying levels of photoperiod whereby 18-hour exposure to light produced the fastest growth rate and a highest biomass yield. Similarly the 6-hour exposure produced the least biomass yield of all the three illumination periods. Indeed, the day length influences photosynthesis, respiration, cellular division, and the growth rate as indicated by enhanced growth at higher times of irradiation. These results are supported by the findings of Hobson *et al.*, (1979) who found that day length factor has a positive affect on the enzymatic activities and macromolecule syntheses, therefore resulting in high biomass yield. The influence of day length on the growth rate at a constant incident light of 240μ mol m^{-2} s^{-1} shows that growth was maximum under the illumination up to 18-hours. Accordingly, growth depends on the quantity of energy received by the cultures. The longer the duration of illumination, the shorter the generation time. It was generally noted that the alga exhibited a growth rate that was proportional to the duration of the effective light period. These results show that growth rate was not constant but increased with day length with a maximum at longer periods of illumination and confirm the real effect of the photoperiod on the growth rate of *C. reinhardtii*. It is expected, consequently, that continuous illumination would achieve the maximum growth rate recorded. Several researchers have reported this to be actually true (Foy & Gibson 1993; Foy *et al.*, 1976; and Nielsen, 1992). However, most works generally suggest the use of light/dark cycles instead of continuous light, which seems to be inappropriate. Indeed, a light/dark alternation allows

for either an increase in final concentration or a lowering of production costs. The necessity of a dark phase is explained by the photosynthesis being governed by two reactions, a photochemical phase that is light dependent and another, a biochemical dark phase that is light independent. Whereas the 18-hour treatment for the algal culture had the robust growth resulting in a dense biomass of deep green color, growth without light (0-hour photoperiod) did not produce any quantifiable growth at all and the culture turned yellow after barely a week, indicating a total collapse of the culture. *C. reinhardtii* has, however been found to grow in the dark (heterotrophic growth) with acetate added to the growth media as carbon source (Harris, 2001). These results showed the importance of the photoperiod especially in the use of a photo bioreactor for growth (Peeters & Eilers, 1978).

Light intensity plays an important role in algal culture, but the requirements vary greatly with the culture depth and the density of the culture. From the results of the experiment on the effect of light intensity on the growth of *Chlamydomonas reinhardtii* the optimal light intensity varied between 160μ mol m^{-2} s^{-1} and 240 μ mol m^{-2} s^{-1} with a peak at 240 μ mol m^{-2} s−1. The growth rate was reduced at light intensity values below or above these ranges. The results show that low or high light intensities cannot sustain the maximum growth rate. At higher light intensities beyond 240 μ mol m^{-2} s^{-1}, the growth was limited due to the phenomenon of photo-inhibition in the microalga. The growth of the alga under increasing light intensities can be divided into three distinct phases. The first is a light dependent phase in which the growth rate rose with the increase in light intensity. Here, growth increased as a function of light intensity. The growth rate however, was slow because the light intensity was still below the optimum

level. The second phase is a light independent portion or plateau. Here, there was minimal response to changes in light intensities. The optimum light intensity of the alga was determined from this phase. The third phase is a light dependent portion in which the rate of growth declined with increase in light intensity. In this phase, further increase in light intensity caused a sharp decrease in growth. The injurious effect of high light intensities upon the growth of *C. reinhardtii* was evident at this phase. The results showed the deleterious effects of light at 25 ^{0}C began at unexpectedly low light intensities, implying that this alga requires relatively low light intensities to attain a high biomass yield. Similar results were obtained by Myers (1953) and Kok (1951) for the light-saturating intensity for *Chlorella pyrenoidosa.* The results obtained in this experiment, however, can vary depending on the light source and the geometry of illumination. It was deduced from the findings that illumination intensity is one of the major parameters that should be controlled in the cultivation of the microalga since too high levels have been found to cause photosystem damage (Vacha, 1995; Barber, 1994) leading to low productivity whereas too low levels will cause slow growth.

Microalgal biomass contains approximately up to 50% carbon by dry weight (Sánchez *et al.*, 2003) which is typically derived from carbon dioxide. Studies done by various researchers suggest that about 1.5 to 2.0 kg of CO_2 is required to produce 1.0 kg of biomass (Mazzucca *et al*, 2000). Welty (2005) found out from various studies that carbon dioxide is an essential part of photosynthesis and will increase the growth rate of algae as algal cells need it to grow autotrophically. Carbon dioxide is the sole source of carbon for the process of photosynthesis and biomass production and oxygen is liberated as a product.

According to previous studies (Benemann *et al.*, 1987), the supply of carbon dioxide to microalgal mass culture systems is one of the main difficulties in growing of algae and the limitations must be solved for the process to succeed. The concentration of CO_2 must not be too high that it produces growth inhibition and, on the other hand, must not be too low that limits growth.

The remarkable effect of carbon dioxide on growth of microalgae was evident from the experiment on the effect of CO_2 on growth rate of *C. reinhardtii.* The growth rate in presence of CO_2 was higher than in its absence. Fast growth was witnessed at the exponential phase where the cells were dividing rapidly since the nutrients were available in plenty. Increased CO_2 concentrations significantly enhanced the growth rates of the alga. It tolerated CO_2 concentrations up to 15% exhibiting faster growth rate than when grown in the ambient concentration of 0.03%. Though the alga inhabits the waters of the Nairobi Dam where CO_2 concentration is roughly 0.03%, it was found that higher growth rate can be achieved at elevated concentrations of CO_2. From the results, growth of the alga in 15% CO_2 doubled exponentially from day 3 to day 5 after which it started to level off when nutrients became limiting. Self-shading and photo-inhibition due to increased cell densities also caused the leveling off. These findings are fundamental in understanding the responses of *C. reinhardtii* to the high CO_2, but hardly lead to any predictions of the ecological impacts of atmospheric CO_2 increase, because CO_2 concentrations in this study are hundredfold of the present atmospheric CO_2 level.

Several species have been tested under CO_2 concentrations of 15% and above can be used in the amelioration of effect of green house gasses as was observed by Yoshihara *et al.*, (1996). *C. reinhardtii* can also be used for this purpose because it showed a CO_2

tolerance of 15%. It is possible to use the CO_2 emitted from industries for algal growth, while at the same time keeping the environment cleaner. That is, carbon dioxide that is released in power plants by burning fossil fuels can be fed into algal growing systems as was reported by Sawayama *et al.*, (1995) and Yun *et al.*, (1997) and thus the algae may be used at the site for green house emission control system. Carbon capture by use of microalgae is developing as a bio-secure, scalable, climate adaptive and highly cost-effective technology for producing bio-fuels from algae and reducing emission of CO_2 using algal photosynthesis (Benemann, 1993).

Urea was also found to have a profound effect on the growth of the alga. Biomass growth was inhibited in nitrogen-lacking situations, so there was a lipid yield peak for the alga at different nitrogen concentrations. This result is in agreement with the findings of Shen *et al*, (2009). *Chlamydomonas reinhardtii* was grown in urea as the nitrogen source. Higher concentrations (2.4g/l) of urea led to faster growth of the alga, reaching an enormous stationary phase cell yield of 10^7cells/ml which upon harvesting gave the highest amount of dry weight. Growth of the alga was explained by Syrett and Leftley, (1976) and Bekheet and Syrett, (1977) to be due to the ability of the alga to metabolize urea due to presence of two enzymes, namely urease and urea amidolyase (UALase) which are induced during higher concentration of urea.

Though the higher concentrations occasioned a high biomass yield, this observation was, however, not commensurate with the extracted lipids (Pietilainen and Niinioja, 2001). This was because the alga may have shifted its metabolism away from lipid production and towards cellular growth since nitrogen was available in excess, as was explained by Iwamoto and Sugimoto, (1958) who also reported similar results with

different species of algae. The faster growth and subsequent low lipid yield in 2.4 g/l urea for *C. reinhardtii* was therefore attributed to the switch to cellular growth at the expense of lipid production leading to low lipid yield in higher concentrations of the urea.

Since *Chlamydomonas reinhardtii* can metabolize nitrogen, it can be used to efficiently remove nitrogen from waste water such as water from the industries. Since presence of nitrogen in the water may encourage eutrophication, they must be removed because they will lead to algal bloom (Benemann, 2004). Just like the removal of carbon dioxide from the atmosphere, *C. reinhardtii* can also be used in microalgal nitrogen treatment of wastewaters due to its ability to metabolize nitrogen (Nakajima *et al*, 1997). Craggs *et al*., (1997) and Lau *at al*., (1994) have studied the use of microalgae as a possible solution to this problem and were successful. Thus besides using *C. reinhardtii* for the production of bio-diesel, they can also be used in the biological wastewater treatments, where nitrogen removal has long been a major problem.

The results of the lipid yields for *C. reinhardtii* grown in varying concentrations of nitrogen showed that growth in 100g/L of nitrogen gave the highest yield of the lipids even though it was shown in experiment of the effect of urea on growth rate of the alga that biomass growth was found to be inhibited in nitrogen lacking-lacking conditions. It was generally concluded that the presence of nitrogen in the media may have greatly influenced lipid yield of *C. reinhardtii.* It was observed that there was a definite decrease in lipid production as nitrogen level increased, which indicated that lipid production decreased with an extreme increase in nitrogen provided due to shift from lipid production to cellular growth (Hu *et al*., 2008). Besides, in nitrogen limited situations, the alga increased its lipid content as a mechanism of survival which made it stop its cell

divisions and start to store energy in the form of lipids for use in extremely harsh growth conditions as observed by Miller, (1962) who also explained that in times of nitrogen scarcity, all of the cellular nitrogen is utilized in synthesis of enzymes and essential cell structures and any carbon dioxide subsequently fixed is converted into carbohydrates or fats rather than into proteins thereby drastically reducing biomass growth. Lipid production is partially regulated by cellular growth, and algae cell growth has been directly linked with nitrogen present (Healey, 1975). Since lipid accumulation takes place in many algae species as a response to exhaustion of the nitrogen supply (Iwamoto and Sugimoto, 1958), it has been suggested that this may provide a means of enhancing the lipid yield for bio-diesel production using algae as a feedstock (Miller, 1962; Myers and Clark, 1944).

The formation of a clear phase from the test of completion of reaction experiment showed that the methyl ester was pure, without any impurities. Any impurities present would have formed undissolved material at the bottom of the flask as was observed by Weiksner, (2003).

The purity of the bio-diesel and hence the success of the transesterification process is important in bio-diesel production. Density measurement is one of the tests for the purity of the bio-diesel because compression ignition engines are designed to inject fuel into the combustion chamber by volume rather than mass; therefore density is one of the important parameters which must be kept at the right standard for diesel fuel injection system.

The pH of 6.9 indicated that the bio-diesel was pure and met the standards of the ASTM,

as was the density of 0.89 g/ml. Ji-Yeon *et al.* (2007), using other sources of bio-diesel feedstock reported similar results of pH and density. This is the ASTM standards density of the unblended bio-diesel (B-100), but if blended with diesel the density of the blend will change depending on the amount of the bio-diesel to diesel ratio as was explained from studies done by Ji-Yeon *et al.*, (2007). In this experiment, some of the replicates had a higher than normal density and this was attributed to experimental errors. These included the presence of soaps, glycerin and unreacted methanol in the bio-diesel. The presence of the soaps was attributed to incomplete removal of the soaps by washing in those particular replicates.

The purity and properties of commercial bio-diesel fuel, however, depends upon the refining practices employed and the nature of the feedstock from which it is produced, as was reported by Weiksner (2003). For example, bio-diesel can be produced from a variety of vegetable oils or animal fats which produces similar volatility characteristics and combustion emissions with varying cold flow properties.

5.1 CONCLUSION

From the results of this research *Chlamydomonas reinhardtii* was found to be very promising as a feedstock for bio-diesel production. This alga was found to be easily isolated, cultured with ease, and gave reasonably high lipid yields. It is a very sturdy organism and can withstand extreme environmental conditions. For this reason it was found to be the most abundant algal species in the water samples collected from the Nairobi Dam. Under optimum growth conditions of temperature, light and carbon dioxide, it was found to grow very rapidly. When subjected to stress, which in this case was nitrogen limitation, it yielded lipids equivalent to 22% dry weight. As demonstrated in this research, the use of lipids from *C. reinhardtii* for bio-diesel production is feasible and production of low-cost microalgal bio-diesel primarily requires improvements to algal growth conditions. Thus, in the context of climactic changes and of soaring prices of petroleum, bio-fuels from algae such as *C. reinhardtii* are now being presented as a renewable energy alternative.

To obtain a pure product which conforms to the set bio-diesel standards care must be taken to avoid contamination during inoculation and growth.

Light, which is essential for the process of photosynthesis, greatly increased the biomass growth and high lipid yield. Duration of illumination was also an important factor since biomass yield increased with increasing photoperiod.

With CO_2 as the carbon source for the alga, the biomass growth was enhanced and this increased with the increase in the concentration which subsequently boosted the lipid yield and the bio-diesel.

5.2 RECOMMENDATION

Illumination, urea, and carbon dioxide concentrations increased growth and lipid yield. Growth in illumination levels above 18 hours and urea concentrations above 2.4 g/l are recommended to improve the biomass and lipid yield of this alga. Equally carbon dioxide concentration of 15% was found to increase growth and is thus recommended for the growth of the alga. Nitrogen concentration of 100 g/l or less is recommended for higher lipid yield.

Positive growth in 15% CO_2 is an indication that this alga may be used for sequestration of the carbon dioxide from the atmosphere. Similarly its ability to metabolize nitrogen may be utilized in the microalgal wastewater treatment system to remove nitrogen from wastewaters before discharging in to dams or rivers.

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APPENDICES

Appendix 1: Analysis of variance (ANOVA) between the 10 sampling points of Nairobi Dam using sytat 9.0 software for different water quality parameters.

Significance level: 0.05

ELECTRICAL CONDUCTIVITY

Dep Var: EC N: 110 Multiple R: 0.981 Squared multiple R: 0.963

Analysis of Variance

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
POINT	9995722.091	10	999572.209	256.797	0.000
Error	385353.438	99	3892.459		

pH

Dep Var: pH N: 109 Multiple R: 0.905 Squared multiple R: 0.819

Analysis of Variance

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
POINT	57.753	10	5.775	44.195	0.000
Error	12.806	98	0.131		

TEMPERATURE

Dep Var: TEMP N: 110 Multiple R: 0.818 Squared multiple R: 0.670

Analysis of Variance

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
POINT	437.488	10	43.749	20.082	0.000
Error	215.669	99	2.178		

DISSOLVED OXYGEN

Dep Var: DO N: 110 Multiple R: 0.435 Squared multiple R: 0.189

Analysis of Variance

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
POINT	63.389	10	6.339	2.304	0.018
Error	272.346	99	2.751		

NITRATES

Dep Var: NO3 N: 110 Multiple R: 0.902 Squared multiple R: 0.814

Analysis of Variance

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
POINT	14242.275	10	1424.228	43.223	0.000
Error	3262.149	99	32.951		

AMMONIA

Dep Var: NH3 N: 110 Multiple R: 0.948 Squared multiple R: 0.899

Analysis of Variance

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
POINT	18343.419	10	1834.342	88.143	0.000
Error	2060.294	99	20.811		

PHOSPHATE

Dep Var: PO4 N: 110 Multiple R: 0.938 Squared multiple R: 0.879

Analysis of Variance

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
POINT	100.319	10	10.032	72.161	0.000
Error	13.763	99	0.139		

CHLORIDE

Dep Var: CL N: 110 Multiple R: 0.918 Squared multiple R: 0.843

Analysis of Variance

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
POINT	1107512.829	10	110751.283	53.237	0.000
Error	205953.751	99	2080.341		

Appendix 2a: ANOVA output for effect of photoperiod on growth rate of the *C. reinhardtii*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	80543367030890.400	3	26847789010296.810	25.213	.000
Within Groups	32903140808 2464.200	309	10648265633 73.671		
Total	40957477511 3354.600	312			

Appendix 2b: ANOVA results for effect of CO2 on growth of *C. reinhardtii*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1985204358 2826.080	1	1985204358282 6.080	48.166	.000
Within Groups	7501324527 0217.300	182	412160688297. 898		
Total	9486528885 3043.400	183			

Appendix 2c: ANOVA result for effect of urea concentration on growth rate of *C. reinhardtii*

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	283498014 6145509.000	2	14174900730 72754.000	13.162	.000
Within Groups	295088339 36663580.000	274	10769647422 1399.900		
Total	323438140 82809090.000	276			

Appendix 2d: ANOVA results for the effect of light intensity on growth rate of *C. reinhardtii*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	58.407	10	5.841	178.467	.000
Within Groups	1.080	33	.033		
Total	59.487	43			

Appendix 2e: ANOVA output for the effect of carbon dioxide concentration on growth rate *C. reinhardtii*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	12.109	3	4.036	4.492	.005
Within Groups	82.656	92	.898		
Total	94.765	95			

Appendix 2f: ANOVA output for the effect of varying nitrogen levels on lipid yield

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.396	2	.198	9.994	.001
Within Groups	.495	25	.020		
Total	.892	27			