POTENTIAL USE OF ALGAE FOR NUTRIENT REMEDIATION OF WASTEWATER AND PRODUCTION OF CLEAN ENERGY

BY

MARY RONO

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY OF THE DEPARTMENT OF ENVIRONMENTAL BIOLOGY AND HEALTH, UNIVERSITY OF ELDORET, KENYA

2014

DECLARATION

DECLARATION BY CANDIDATE

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…… ……

MARY RONO **DATE**
SES/D.PHIL/27/04

DECLARATION BY UNIVERSITY SUPERVISORS

This thesis has been submitted with approval as University supervisors.

…….. ……

PROF. J.B. OKEYO-OWUOR **DATE**
SCHOOL OF ENVIRONMENTAL STUDIES
DEPARTMENT OF ENVIRONMENTAL BIOLOGY AND HEALTH
UNIVERSITY OF ELDORET

…….. ……

PROF. OPIPO OSANO **DATE**
SCHOOL OF ENVIRONMENTAL STUDIES
DEPARTMENT OF ENVIRONMENTAL BIOLOGY AND HEALTH
UNIVERSITY OF ELDORET

DEDICATION

This work is dedicated to my parents for their encouragement, moral, material and spiritual support throughout my schooling life. They have always believed in me, and their daily special prayers have continued to sustain me.

ABSTRACT

The reliance on fossil fuels as a source of energy has led to environmental degradation and a myriad of health problems leading to a need for renewable source of energy that is economical and sustainable. Likewise, eutrophication of water bodies is a matter of concern because it disturbs normal functioning of such an ecosystem. The release of improperly treated wastewater is one major cause of this problem. The objective of this study was to utilize nutrients present in wastewater for algal growth and later produce biofuel from the harvested biomass of the algae. Individual algae cultures of locally dominant genera *Spirogyra, Oedogonium* and *Zygnema,* collected in Kesses area of Uasin Gishu County were grown in laboratory conditions at Moi University. A 10g portion of the algae were grown in 250 ml Erlenmeyer flasks containing 100ml of sewage in an incubator set at 26^0C with continuous light provided by a 5 W fluorescent tube. The flasks were stirred once daily to prevent settling of algal cells. 0.05 mg of CO_2 was bubbled to the flasks once. The control consisted of algae grown in Bold Basal media. Algal growth was monitored for seven days using daily measurements of chlorophyll a. Daily uptake of nitrate from the wastewater by the algae was also determined through automated hydrazine reduction method. After the seven days, the algae were harvested and dried. Lipids were obtained from the dried biomass by Soxhlet extraction using a mixture of hexane and ethanol, and weighed in grams. The quantities of starch were estimated using enzymatic-colorimetric method and given as a percentage of the sample. The lipids obtained were utilized to determine the calorific value of the fuel using a bomb calorimeter. All the data obtained were statistically analyzed using the SPSS program. Friedman test was utilized to check whether there was a statistical difference exhibited by the various taxa in amounts of nitrates reduced, as well as in the accumulation of chlorophyll *a*. Wilcoxon test was employed to check whether there was a statistical difference in the amounts of lipids, starch and calorific values between those algae that grew in sewage and those that had been grown in the growth medium. Results showed that all the algae genera grew better in Bold Basal Medium than on sewage. For all the genera that were grown on sewage, growth declined after the fourth day and *Zygnema* showed the highest growth in sewage. On nitrate removal from the wastewater, the length of contact time between the algae and wastewater was of paramount importance. After four days the studied *Spirogyra* decreased the quantities of nitrate in 100 mL sewage by 90.7%, *Oedogonium* by 89.48% and *Zygnema* by 83.84%. On lipid productivity, results obtained showed that growth of algae on sewage led to accumulation of higher quantities of lipid. *Spirogyra* increased lipid quantities when it was cultured on sewage by 73.99%, *Oedogonium* by 91.38 and *Zygnema* by 89.04%. However, those results acquired from estimation of starch illustrated that growth of the algae on sewage led to accumulation of less amounts of starch. Growth of *Zygnema* on the sewage reduced the quantities of starch by 82.11%, 79.63% in *Spirogyra*, and 58.13% in *Oedogonium.* In *Spirogyra* and *Zygnema*, lipids obtained from algae that grew in sewage had higher calorific values than those that had grown in the growth medium. From the results of this study, it can be concluded that sewage may be used as an alternative growth media for algae. The problem of eutrophication can also be counteracted using local genera of algae. The results also illustrate that sewage grown algae can produce biomass suitable for production of biofuel.

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ACKNOWLEDGEMENTS

First and foremost I would like to thank God because without Him this study would not have been possible. I would also wish to express my gratitude to the following persons for their invaluable contributions to my study; my supervisors Professor Odipo Osano and Professor Okeyo Owuor. Indeed I am grateful for their enthusiasm, advice and patience. I learnt so much from them and it was a pleasure working with them.

My further gratitude goes to Professor Vincent Sudoi, Dr. Gelas Simiyu and all the staff and friends from School of Environmental Studies, University of Eldoret, for their sincere commitment in helping me accomplish this work.

Am also thanking my parents and siblings for their unwavering support and guidance throughout my studies. To my husband and children; thank you for being unending source of inspiration. Your love and understanding have seen me through my education.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

The world is entering a period of declining non-renewable energy resources, while energy demand is increasing. The world's oil production is expected to decline in between one and ten decades (Crookes, 2006). As a result of this impending energy crisis, both governments and private industry are examining alternative sources of energy.

Our reliance on fossil fuels has caused carbon dioxide (CO_2) enrichment of the atmosphere, and is the primary contributor to the generally-accepted phenomenon called global warming. In order to realize a stable energy alternative that will meet world demand while mitigating climate change, it is necessary to develop renewable clean fuels. Ironically, most renewable energy initiatives are focused on electricity generation, while the majority of world energy consumption, about two thirds, is derived from liquid fuels (Hankamer *et al*., 2007). The need for renewable sources of portable, liquid fuel is starting to receive greater attention, and much of this attention has been focused on biomass-derived liquid fuels, or biofuels (Haag, 2006; Schneider, 2006).

Biofuels produced from plants have the potential to replace a portion of our fossil fuel consumption with a renewable alternative. However, there is growing concern that the

use of food crops for biodiesel and other renewable fuels may be an uneconomical long term solution (Pimentel and Patzek, 2005).

Urbanization in Kenya has increased the problem of eutrophication in the water bodies. Over thirty two percent of the Kenyan population lives in urban areas and it is estimated that by 2015 urban dwellers will constitute over fifty percent of the total population (KNBS, 2010; UN-Habitat 2008). The uncontrolled growth in urban areas has made planning and expansion of water and sewage systems very difficult and expensive to carry out. The effluents generated from domestic and industrial activities constitute the major sources of the natural water pollution load. This is a great burden in terms of wastewater management and can consequently lead to a point-source pollution problem, which not only increases treatment cost considerably, but also introduces a wide range of pollutants and contaminants to water sources (Amir *et al.,* 2004). The prevention of pollution of water sources and protection of public health by safeguarding water supplies against the spread of diseases, are the two fundamental reasons for treating wastewater. Nutrients such as nitrogen and phosphorous can stimulate excessive, often unwanted, aquatic plant growth. This may exclude other life forms as well as affecting aesthetics (EPA, 2000). Incorporating algae into wastewater treatment system has the potential to alleviate this problem because algae can assimilate nitrogen and phosphorus. Although the deliberate application of algae in wastewater treatment is still fairly limited, algae are found naturally throughout the world in wastewater treatment systems, especially waste stabilization ponds where they work together with microorganisms to reduce organic and inorganic pollutant loads.

In this thesis, use of local genera of algae to remove nitrates from domestic wastewater and their use as a source of biomass for energy production was studied. Local algae have long been naturally selected to their region thus are evolutionary primed for local bioresource production. The lipid content of three genera of fresh water filamentous green algae was extracted and quantified as a possible feedstock for biodiesel. Similarly, the carbohydrate fraction was estimated as a possible feedstock for ethanol. So far, many studies on biofuel production from algae have concentrated on biodiesel production only, whereas there are many other fuel sources from the same biomass, such as alcohol fuel production, biogas production and hydrogen production (Alonso *et al.,* 2000; Bigogno *et al.,* 2002; Darmen *et al.,* 2003; Hu *et al.,* 2008; Rodolfi *et al.,* 2008).

The biomass used as biofuel feedstock was produced from algae grown on domestic wastewater. Domestic sewage contains nutrients which can be turned into a resource to produce energy. While growing in the sewage, the algae were able to take up nutrients from the water, making the water suitable to be released into the environment. While algae have been recognized for their ability to purify wastewater in conventional oxidation ponds, when they die the nutrients in their biomass are recycled back into the wastewater oxidation pond through decomposition (Belle, 2007; Larsdotter, 2006; Mara, 2006; Wells, 2005). Also, algae in an oxidation pond can also cause high levels of total suspended solids in the final effluent and prevent the treatment facility meeting the required total suspended solids standards. This study therefore used algae which had removed nutrients from wastewater for biofuel production in an attempt to find an environmentally suitable use of algae that has played its role in wastewater treatment.

1.2 Statement of the problem

The occurrence of nutrients in water bodies and consequent eutrophication is a widespread environmental problem. The nutrients cause increased growth of microscopic plants, algae and dense mats of floating plants such as water hyacinths. When these plants die, the process of bacterial decomposition uses up oxygen in the water, leading to anoxia. This causes death of fish and invertebrates. Moreover, ammonia and hydrogen sulfide originating from bacterial activities can be released from sediments under conditions of anoxia, affecting plants and animals. High nitrate levels in water that exceeds 10 mg/l NO_3-N can cause methemoglobinemia or blue baby syndrome, a condition found especially in infants less than six months. The stomach acid of an infant is not as strong as in older children and adults. This causes an increase in bacteria that can readily convert nitrate to nitrite. Nitrite is absorbed in the blood, and hemoglobin (the oxygen-carrying component of blood) is converted to methemoglobin. Methemoglobin does not carry oxygen efficiently. This results in a reduced oxygen supply to vital tissues such as the brain. Methemoglobin in infant blood cannot change back to hemoglobin, which normally occurs in adults. Severe methemoglobinemia can result in brain damage and death.

Improper treated domestic wastewater is a source of nitrate. This thesis addresses this issue by using algae to remove nitrates from domestic wastewater, thereby averting the problem of eutrophication and methemoglobinemia.

Fossil fuels are the primary source of energy used in several sectors of the economy such as transport, industry and electricity generation. However, burning of these fuels for energy releases a number of chemicals into the atmosphere including carbon dioxide and nitrous oxide, which are major contributors of global warming and ensuing climate change. Other by-products include sulfur oxides and nitrogen oxides, both of which contribute to acid rain formation that causes damage to plants and buildings. In addition to environmental harm, some by products can cause health problems to humans. Nitrogen oxides irritate the lungs while particulate matter contributes to respiratory illnesses and cardiac problem. This study contributes to the development of alternative sources of energy using local species of sewage-grown algae to produce lipids and starch that are feedstock for biodiesel and bioethanol respectively.

1.3 Objectives of the Study

The overall objective of this study was to investigate the potential of local genera of algae to remove nitrates from wastewater and produce energy.

1.3.1 Specific objectives were:

i. To investigate the capacity of the selected algae genera to remove nitrates from wastewater.

ii. To determine productivity of the selected algae growth in secondary-treated wastewater.

iii. To determine lipid and starch production of the alga grown in sewage

1.4 Justification of the Study

Conventional municipal wastewater treatment can partially remove nitrogen and phosphorus concentrations, leading to a discharge of effluent containing nitrogen and phosphorus. This leads to a deterioration of water quality of natural water bodies. Additional treatments are expensive due to the requirements of additional chemicals and energy. A variety of aquatic ecosystems may be incorporated into a wastewater treatment system for the benefit of nutrient removal, such as marshes and wetlands. However, studies have shown that wastewater treatment with algae is particularly attractive because they play complex multiple roles in the purification process. Besides accumulating and converting of wastewater nutrients into algal biomass, they are also able to induce the precipitation of phosphates and stripping of ammonia through photosynthetically created high pH values. This counteracts eutrophication of surface waters. Further, in organically enriched wastewater, release of oxygen through high photosynthetic rates promotes aerobic degradation of bacteria and other microbes. Organic carbon is oxidized to free carbon dioxide, serving as carbon source for the algae. However the reduction of wastewater Biochemical Oxygen Demand (BOD) occurs at the expense of an increase in algal biomass. Although the benefits of algae in wastewater treatment systems have been studied, the deliberate application in wastewater treatment is limited. Algae are found in wastewater stabilization ponds where they occur naturally and aid in the reduction of

organic and inorganic pollutants. Currently, little use is made of algal biomass produced from such wastewater treatment systems. After treatment, the algae are left to decompose in the system leading to recycling back of the pollutants into the water. Also, presence of algae in the final effluent of the wastewater treatment plant can cause high levels of suspended solids thereby reducing the standards of the final effluent.

This study was able to fill three gaps of knowledge; one, on the use of algal biomass generated after wastewater treatment. Such a biomass would not be suitable as a source of human or animal feed due to variety of toxic compounds accumulated from sewage. It would not be fit for use as a soil amendment and fertilizer due to heavy metal accrual. In this study, after use of algae to remove nutrients from wastewater, the biomass created from the process was utilized to produce lipids and starch, which are feedstock for clean energy sources. Another gap in knowledge that this study fulfilled was on the ability of local species of algae to remove nutrient from wastewater. Thirdly, many studies on biofuel production from algae have focused on biodiesel production only, whereas there are many other fuel sources from the same biomass, such as alcohol fuel production, biogas production. This study filled this gap by quantifying the carbohydrate fraction of the algae as a feedstock for ethanol.

1.5. Null Hypotheses of the Study

H_{01}: The algae genera are not able to grow in wastewater

H_{02}: The algae are not able to remove nitrates from wastewater

H_{03}: The algae do not have potential for renewable energy

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

This chapter presents literature review on the production of biofuel from algae grown on sewage and the reasons why such a source of energy is economical and sustainable and therefore this review is based on the study objectives.

2.2. Problems with Conventional Energy Generation

Since energy is necessary for economic growth and development, it can be said that the progress of a country is related to its energy consumption. Energy affects social, economic, and environmental aspects of development. The economic development is affected by high costs and unreliable energy services, which constrain economic activity in many countries. Also, while energy services, by themselves, are not sufficient to eradicate extreme poverty, they are necessary for creating the conditions for economic growth and improving social equality (Nkomo, 2005).

Fossil fuels are fuels formed by natural processes such as anaerobic decomposition of buried dead organisms, for that reason they contain high percentages of carbon. They include coal, petroleum and natural gas. Fossil fuels are non-renewable sources of energy because they take millions of years to form and reserves are being depleted much faster than new ones are made (Berner, 2003). Since they are made up of hydrocarbons which store chemical energy in atomic bonds of hydrogen and carbon, burning the fuel breaks

the bonds releasing the energy, which makes them valuable to our society (Awosolu, 2007; Lin and Tanaka, 2006; Wackett, 2008). They are consequently useful in transport, generation of electricity and in industry, among other uses. During burning of fossil fuels, 21.3 billion tonnes of carbon dioxide per year are released. Natural processes such as photosynthesis absorb about half of that, leaving a net increase of 10.65 billion tonnes of atmospheric carbon dioxide per year (IPCC 2007). Combustion of fossil fuels also generates sulfuric, carbonic and nitric acids, which fall to the earth as acid rain, impacting both natural areas and built environment. Harvesting, processing and distribution of fossil fuels can create environmental concerns such as air pollution and water pollution from oil refineries.

As Kenya aspires to be a middle income economy as envisaged in Vision 2030, it faces an enormous task of meeting energy needs due to the high expectations in growth to power the economy.

Current energy demand in Kenya is met from four broad sources. Biomass is the largest form of energy consumed in the country, accounting for about 68 % of the national total. Petroleum is the next most important accounting for 22 % followed by electricity 9%, and coal at upto1% (Ndegwa *et al.,* 2011). However, use of traditional stoves that use firewood and charcoal for cooking in a poorly ventilated area leads to air pollution and development of respiratory diseases. Smoke emission from burning of biomass (wood, charcoal, agricultural residues, and dung) is largely particulate matter and other invisible hazardous emissions, including, carbon monoxide, nitrogen dioxide sulphur oxides, formaldehyde, and polycyclic organic matter, including carcinogens such as benzo [*a*]

pyrene, and dioxins (ITDG, 2002). Exposure to indoor air pollution, especially to particulate matter, from the combustion of biofuels (wood, charcoal, agricultural residues, and dung) has been implicated as a causal agent of respiratory diseases in Kenya. Other diseases associated with indoor air pollution include chronic obstructive pulmonary disease, asthma, cancer of the nasopharynx and larynx, tuberculosis, perinatal conditions, low birth weight, and diseases of the eye such as cataract and blindness (ITDG, 2002).

Charcoal continues to be harvested from trust lands and gazette forests, an annual business worth Ksh 17 billion (Ministry of Energy, 2004). This consequently has a negative impact on the environment and leads to climate change (NEMA, 2005). Kenya is estimated to be emitting 14.4 million tonnes of carbon-dioxide per year through deforestation (Karekenzi *et al.,* 2008).

2.3. Problems with Biofuel Production Using Crops

There are several crops currently grown in Kenya that can serve as alternative sources of energy. These include jatropha, castor, croton, sunflower, sweet sorghum, sugarcane and cassava. The problem with use of the crops as sources of biofuels is that they compete with food production for land.

Jatropha is gaining attention as a biofuel feedstock that avoids competition with food because it is claimed that it can be grown on marginal lands that would otherwise not support food crops (Tomomatsu and Swallow, 2007). This would have tremendous potential in Kenya since approximately 50% of the land is currently un-arable (Government of Kenya, 2010). However, evidence suggests that jatropha will not be

viable as a biofuel feedstock if grown on marginal lands without irrigation and fertilizer inputs. While it has been claimed that Jatropha plants can survive in areas with as little as 250 mm of rainfall per year and can withstand long periods of drought by shedding most of its leaves to reduce transpiration loss, it is becoming increasingly accepted that irrigation would be required in such conditions to produce economically viable yields for biofuel production (Katwal and Soni, 2003: Kuman and Sharma, 2008). Achten *et al.* (2008) found that a minimum annual average rainfall of 500-600mm per year is required to yield a harvestable amount of seeds. It is thus well supported that jatropha grown for biofuel production would require additional water inputs if grown on marginal lands. This is of significant concern to an immensely water-scarce country like Kenya. Agriculture in Kenya is already highly constrained by limited water resources; with only 12% of the country suitable for agriculture because the rest is too dry. It can, therefore, be concluded that jatropha cultivation for biofuel will also be severely constrained by water in Kenya and could put immense stress on water resources. Planting jatropha on marginal lands with irrigation would indirectly compete with other agricultural crops by increasing the demand for such a scarce resource. This illustrates that even if jatropha is planted on marginal lands in Kenya, there is a strong potential for it to compete with food crops for water resources, yielding significant consequences for food security.

Sorghum and cassava form the main diet for populations that live in arid parts of the country. If these crops are switched to biofuel, it will affect the food security of these people. Since Kenya is already a net importer of food and agricultural goods crop

switching would make Kenya more dependent on other countries for a consistent and sufficient supply of food (FAO, 2005).

2.4 Algae as Feedstock for Biofuel

Algae fuel (also known as algal fuel) is a biofuel made from algae. "Algaculture" (farming of algae) can be a route to making biodiesel, bioethanol, biogas, and other biofuels (Hu *et al*., 2008). Algae are a diverse group of eukaryotic organisms that belong to the Phylum Protista. These organisms use light energy to convert carbon dioxide and water into carbohydrates and other cellular products. During this process oxygen is released. Algae contain chlorophyll a, which is required for photosynthesis (Benning and Pichery, 2008; Chisti, 2007).

Organisms that are classified as algae are quite diverse. They include both microscopic unicellular and macroscopic multicellular organisms. These organisms differ from other eukaryotic photosynthetic organisms like land plants due to the fact that they lack an organized vascular system and they have relatively simple reproductive procedures. As one of the primary producers of carbohydrates and other cellular products, algae are essential in the food chains of the entire world. A large portion of the oxygen in the atmosphere is produced by algae (Chisti, 2007).

Organisms that are considered algae are grouped together by a number of properties. These include the main photosynthetic pigments of each group, the structure of the cell wall, the type of storage products, the mechanisms of motility, and the mode of reproduction. A number of algal groups derive their name from the major color displayed

by most of the algae in the group, for example Chlorophyceae, the green algae (Nester *et al.* 2004). Algae are found anywhere there is water – fresh water, salt water, and in the soil. Due to the fact that the oceans cover over 70% of the earth's surface, aquatic algae are major producers of oxygen and important users of carbon dioxide. Phytoplankton is predominantly made up of unicellular algae. This phytoplankton is a major source of food for many animals, large and small (Nester *et al.* 2004).

All algae are primarily made up of proteins, carbohydrates, fats, and nucleic acids in varying proportions. While the percentages can vary with the type of algae, some types of algae are made up of up to 40% fatty acids based on their overall mass. It is this fatty acid that can be extracted and converted into biofuel (Hu *et al.,* 2008).

Chlorophyceae, green algae, are the strain most favored by researchers. However, green algae tend to produce starches instead of lipids and require nitrogen to grow. They have the advantage that they have very high growth rates at 30^{o}C and at high light levels in aqueous solution (Hu *et al.,* 2008).

Unlike terrestrial crops such as corn and soybean, which require a full growing season to yield crops, algae can be harvested day after day (Christi, 2007). Because algae consumes carbon dioxide and produces oxygen through photosynthesis, it is particularly attractive as a means to curtail carbon emissions along with producing fuel. Furthermore, algae can be used to clean up waste by processing nitrogen from wastewater and carbon dioxide from power plants (Stepan *et al.,* 2002). Unlike other biomass sources, algae do not

compromise a food stock. It can be grown on marginal lands that are useless for ordinary crops (Rodolfi *et al.,* 2008, Stepan *et al.,* 2002).

There are three energy production options from algae (i) production of methane gas from the whole biomass (ii) production of ethanol via fermentation from the carbohydrates and (iii) production of biodiesel from algal oil (Boechat and Giani, 2000; Christi, 2007). The economics of fuel production from algae demands that we utilize all the biomass as efficiently as possible (Hu *et al.*, 2008).

2.4.1 Algae as feedstock for biodiesel

Oil rich algae can be found among diverse taxonomic groups, and the total lipid content may vary noticeably among individual species or strains within and between taxonomic groups. Oil rich green algae show an average total lipid content of 25.5% dry cell weight. The lipid content increases considerably (doubles or triples) when the cells are subjected to unfavorable culture conditions, such as photo-oxidative stress or nutrient starvation. The intrinsic ability to produce large quantities of lipid and oil is species/strain-specific, rather than genus-specific (Hu *et al*., 2006). According to Tonon *et al.*, (2002), the increase in total lipids in aging algal cells or cells maintained under various stress conditions consisted primarily of neutral lipids, mainly triacylglycerols. This was due to the shift in lipid metabolism from membrane lipid synthesis to the storage of neutral lipids. *De novo* biosynthesis and conversion of certain existing membrane polar lipids into triacylglycerols may contribute to the overall increase in triacylglycerols. As a result, triacylglycerols may account for as much as 80% of the total lipid content in the cell.

Algae synthesize fatty acids as building blocks for the formation of various types of lipids. The most commonly synthesized fatty acids have chain lengths that range from C16 to C18, similar to those of higher plants (Hu *et al.,* 2006). Fatty acids are either saturated or unsaturated, and unsaturated fatty acids may vary in the number and position of double bonds on the carbon chain backbone. In general, saturated and mono-unsaturated fatty acids are predominant in most algae (Kumar *et al.,* 2011). Specifically, the major fatty acids are C16:0 and C16:1 in the Bacillariophyceae, C16:0 and C18:1 in the Chlorophyceae, C16:0 and C18:1 in the Euglenophyceae, C16:0, C16:1 and C18:1 in the Chrysophyceae, C16:0 and C20:1 in the Cryptophyceae, C16:0 and C18:1 in the Eustigmatophyceae, C16:0 and C18:1 in the Prasinophyceae, C16:0 in the Dinophyceae, C16:0, C16:1 and C18:1 in the Prymnesiophyceae, C16:0 in the Rhodophyceae, C14:0, C16:0 and C16:1 in the Xanthophyceae, and C16:0, C16:1 and C18:1 in cyanobacteria (Kumar *et al.*, 2011).

Polyunsaturated fatty acids (PUFAs) contain two or more double bonds. Based on the number of double bonds, individual fatty acids are named dienoic, trienoic, tetraenoic, pentaenoic and hexaenoic fatty acids (Basova, 2005)

Biodiesel, produced by the trans-esterification of triglycerides with methanol, yielding the corresponding mono-alkyl fatty acid esters, is an alternative to petroleum-based diesel fuel (Durrett *et al*., 2008).

Lipid metabolism, particularly the biosynthetic pathways of fatty acids and triacylglycerol, has been poorly studied in algae in comparison to higher plants. Based upon the sequence homology and some shared biochemical characteristics of a number of genes and/or enzymes isolated from algae and higher plants that are involved in lipid metabolism, it is generally believed that the basic pathways of fatty acid and triacylglycerol biosynthesis in algae are directly analogous to those demonstrated in higher plants. It should be noted that because the evidence obtained from algal lipid research is still fragmentary, some broad generalizations are made in this section based on limited experimental data (Hu *et al*., 2008)

In algae, the *de novo* synthesis of fatty acids occurs primarily in the chloroplast. Overall, the pathway produces a 16- or 18-carbon fatty acid or both. These are then used as the precursors for the synthesis of chloroplast and other cellular membranes as well as for the synthesis of neutral storage lipids, mainly triacylglycerols, which can accumulate under adverse environmental or sub-optimal growth conditions.

The committed step in fatty acid synthesis is the conversion of acetyl CoA to malonyl CoA, catalyzed by acetyl CoA carboxylase (ACCase). In the chloroplast, photosynthesis provides an endogenous source of acetyl CoA (Baud *et al.,* 2007; Ruuska *et al.,* 2002; Schwender and Ohlrogge, 2002). In green algae, as glycolysis and pyruvate kinase (PK), which catalyzes the irreversible synthesis of pyruvate from PEP, occur in the chloroplast in addition to the cytosol, it is possible that glycolysis-derived pyruvate is the major photosynthate to be converted to acetyl CoA for *de novo* fatty acid synthesis (Andre *et al.,* 2007). An ACCase is generally considered to catalyze the first reaction of

the fatty acid biosynthetic pathway – the formation of malonyl CoA from acetyl CoA and CO_2. This reaction takes place in two steps and is catalyzed by a single enzyme complex. In the first step, which is ATP-dependent, CO_2 (from HCO_3^-) is transferred by the biotin carboxylase prosthetic group of ACCase to nitrogen of a biotin prosthetic group attached to the ε-amino group of a lysine residue. In the second step, catalyzed by carboxyltransferase, the activated CO_2 is transferred from biotin to acetyl CoA to form malonyl CoA (Ohlrogge and Browse, 1995).

According to Ohlrogge and Browse (1995), malonyl CoA, the product of the carboxylation reaction, is the central carbon donor for fatty acid synthesis. The malonyl group is transferred from CoA to a protein co-factor on the acyl carrier protein (ACP). All subsequent reactions of the pathway involve ACP until the finished products are ready for transfer to glycerolipids or export from the chloroplast. The malonyl group of malonyl ACP participates in a series of condensation reactions with acyl ACP (or acetyl CoA) acceptors. The first condensation reaction forms a four-carbon product, and is catalyzed by the condensing enzyme, 3-ketoacyl ACP synthase III (KAS III) (Jaworski *et al.*, 1989). Another condensing enzyme, KAS I, is responsible for producing varying chain lengths (6–16 carbons). Three additional reactions occur after each condensation. To form a saturated fatty acid the 3-ketoacyl ACP product is reduced by the enzyme 3-ketoacyl ACP reductase, dehydrated by hydroxyacyl ACP dehydratase and then reduced by the enzyme enoyl ACP reductase. These four reactions lead to a lengthening of the precursor fatty acid by two carbons. The fatty acid biosynthesis pathway produces saturated 16:0- and 18:0-ACP. To produce an unsaturated fatty acid, a double bond is

introduced by the soluble enzyme stearoyl ACP desaturase. The elongation of fatty acids is terminated either when the acyl group is removed from ACP by an acyl-ACP thioesterase that hydrolyzes the acyl ACP and releases free fatty acid or acyltransferases in the chloroplast transfer the fatty acid directly from ACP to glycerol-3-phosphate or monoacylglycerol-3-phosphate (Ohlrogge and Browse, 1995). The final fatty acid composition of individual algae is determined by the activities of enzymes that use these acyl ACPs at the termination phase of fatty acid synthesis.

Triacylglycerol biosynthesis in algae has been proposed to occur via the direct glycerol pathway (Hu *et al.,* 2006). Fatty acids produced in the chloroplast are sequentially transferred from CoA to positions 1 and 2 of glycerol-3-phosphate, resulting in formation of the central metabolite phosphatidic acid (PA) (Ohlrogge and Browse, 1995). Dephosphorylation of PA catalyzed by a specific phosphatase releases diacylglycerol (DAG). In the final step of triacylglycerol synthesis, a third fatty acid is transferred to the vacant position 3 of DAG, and this reaction is catalyzed by diacylglycerol acyltransferase, an enzymatic reaction that is unique to triacylglycerol biosynthesis. PA and DAG can also be used directly as a substrate for synthesis of polar lipids, such as phosphatidylcholine (PC) and galactolipids. The acyltransferases involved in triacylglycerol synthesis may exhibit preferences for specific acyl CoA molecules, and thus may play an important role in determining the final acyl composition of triacylglycerol (Hu *et al.,* 2008).

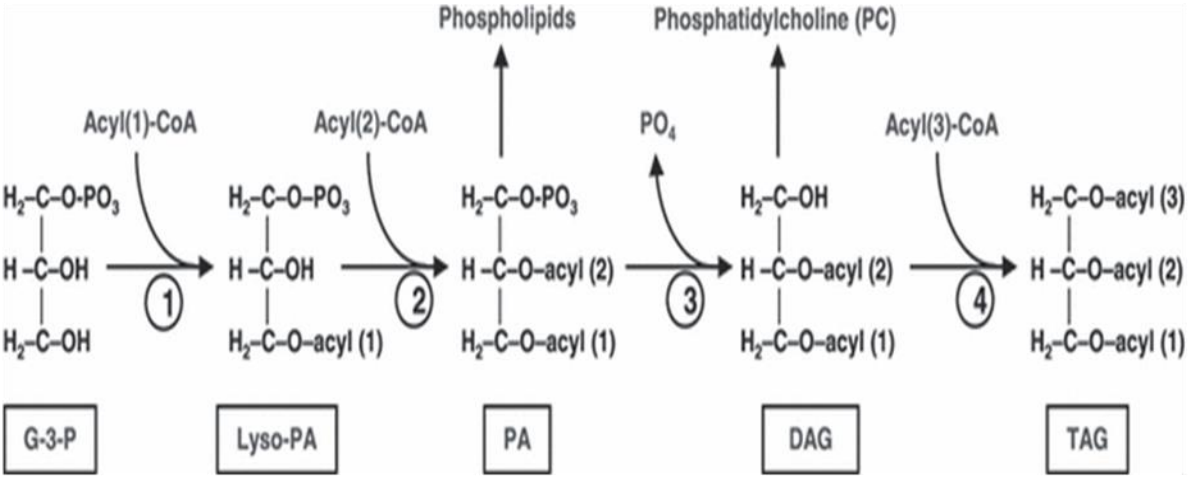

Figure 1 showing process of triacylglycerol formation

In most of the algal species/strains examined, triacylglycerols are composed primarily of C14–C18 fatty acids that are saturated or mono-unsaturated (Harwood, 1998). As exceptions, very-long-chain (>C20) PUFA synthesis and partitioning of such fatty acids into triacylglycerols have been observed in the green alga *Parietochloris incise* (Trebouxiophyceae) (Bigogno *et al*., 2002) and the freshwater red microalga *Porphyridium cruentum* (Cohen *et al*., 2000) among others.

Although the occurrence and the extent to which triacylglycerol is produced appear to be species/strain-specific, and are ultimately controlled by the genetic make-up of individual organisms, oil rich algae produce only small quantities of triacylglycerol under optimal growth or favorable environmental conditions (Christi, 2007; Hu, 2004). Synthesis and accumulation of large amounts of triacylglycerol accompanied by considerable

alterations in lipid and fatty acid composition occur in the cell when oil rich algae are placed under stress conditions imposed by chemical or physical environmental stimuli, either acting individually or in combination. The major chemical stimuli are nutrient starvation, salinity and growth-medium pH. The major physical stimuli are temperature and light intensity. In addition to chemical and physical factors, growth phase and/or aging of the culture also affects triacylglycerol content and fatty acid composition (Christi, 2007; Dahmen *et al.,* 2003; Lynn *et al.,* 2000).

Lipid content and fatty acid composition are subject to variability during the growth cycle. In many algal species in the literature, an increase in triacylglycerols is often observed during stationary phase, for example, in the chlorophyte *Parietochloris incise*, triacylglycerols increased from 43% (total fatty acids) in the logarithmic phase to 77% in the stationary phase (Bigogno *et al.,* 2002), and in the marine dinoflagellate *Gymnodinium* sp., the proportion of triacylglycerols increased from 8% during the logarithmic growth phase to 30% during the stationary phase (Mansour *et al.*, 2003).

Culture aging or senescence also affects lipid and fatty acid content and composition. The total lipid content of cells increased with age in the green alga *Chlorococcum macrostigma* (Collins and Kalnins, 1969) in: Hu *et al.,* 2008). An exception to this was reported in the diatom *P. tricornutum,* where culture age had almost no influence on the total fatty acid content, although triacylglycerols were accumulated and the polar lipid content was reduced (Alonso *et al.,* 2000).

2.4.3 Algae as a feedstock for bioethanol

2.4.3.1 Starch production in green algae

Starch is the most widespread and abundant storage carbohydrate in plants. Humans depend upon starch for nutrition, exploit its unique properties in industry, and use it as a feedstock for bioethanol production (Keeling and Myers, 2010). Starch is only found in eukaryotes and serves as an important store of energy that is captured by plants using sunlight, water, carbon dioxide, and soil nutrients. Two different kinds of starches exist: first, the floridean starch in the cytoplasm of red algae, second, green algae and higher plants always make starch within the plastid from ADPglucose (Ball and Morell, 2003). Of all algae, those in the division Chlorophyta (green algae) display the closest relationship to the vascular plants. Chlorophytes harbor a chloroplast that contains the same types of photosynthetic pigments as land plants (Ball and Morell, 2003; Hicks *et al.* 2001). At variance with other divisions of algae, starch is produced within the chlorophyte plastids and displays a structure very similar to that of vascular plants.

Starch is a huge complex structure composed of two polymers of glucose; amylose and amylopectin, which together form semicrystalline, insoluble granules with an internal lamellar structure. Both of these glucan biopolymers are composed of clusters of an alternating pattern of linear α-1, 4 linked glucose units, branched by α-1, 6 linkages. When the α-1, 6-glucosidic linkages are infrequently dispersed in a polymer of approximately 100- 10,000 glucosyl residues, the polymer is amylose. When the α-1, 6-glucosidic linkages are more frequently and regularly dispersed in a polymer of

approximately 10,000-100,000 glucosyl residues, the polymer is amylopectin (Ball and Morell, 2003; Keeling and Myers, 2010; Myers *et al.,* 2000; Zeeman *et al.,* 2010). Amylopectin, being the major component makes up 75% or more of the starch granule and is responsible for the granular nature of starch. Amylose, being smaller than amylopectin is lightly branched and exists primarily in an unorganized form within amorphous regions of the granule.

2.4.3.1.1 The mechanism of starch granule biosynthesis; Amylopectin synthesis

The basic structure of the starch granule is dictated by the packing of amylopectin molecules in organized arrays. The substrate for starch synthesis in green algae and higher plants is ADPglucose. This ADPglucose is synthesized by the enzyme ADPglucose pyrophosphorylase (AG-Pase). This enzyme utilizes glucose-1-P produced from photosynthesis to synthesize ADPglucose (Ball and Morell 2003). The synthesis of α 1, 4 and α 1, 6 linkages occur by means of starch synthases and starch-branching enzymes. Starch synthases transfer glucosyl units from ADPglucose to the non-reducing ends of growing polymers via new α 1, 4 linkages. These starch synthases are encoded by five gene classes, GBSS (Granule-bound starch synthases), SSI, SSII, SSIII and SSIV. GBSS is responsible for amylose synthesis. The SS isoforms generate the chains in amylopectin and genetic and biochemical data indicate that each SS isoform has different properties and a distinct role in amylopectin synthesis. The SSI, SSII, and SSIII classes elongate short, medium and long chains respectively.

The branching of amylopectin proceeds concurrently with chain elongation. Branching is catalyzed by branching enzymes (BE; α -1, 4-glucan: α-1, 4-glucan-6-glycosyltransferase; EC: 2.4.1.18), which cut existing α-1, 4-glucan chains and transfer the cut segment of six or more glucose units to the C6 position of a glucosyl residue of another or same glucan chain.

2.4.3.1.2. Amylose synthesis

The amylose component of the starch is synthesized by GBSS. Amylose-free starch granules are normal in appearance, illustrating that only amylopectin is necessary for starch granule formation. GBSS transfers glucosyl residues from ADP-glucose to its glucan substrate in series, generating long chains. This occurs within the semicrystalline matrix formed by amylopectin. GBSS can use soluble malto-oligosaccharides as substrates for amylose production or it also act on existing side chains of amylopectin and contribute to the formation of long chains of amylopectin. Amylose synthesis may render starch denser and improve the efficiency of carbon storage.

Algae, similar to any plant, accumulate carbohydrates as an assimilatory product of photosynthesis and intracellular storage material in several forms, such as starch, several sugars including glucose specifically under autotrophic conditions (Choix *et al.*, 2012).

2.5 Sewage Treatment

Domestic wastewater includes typical wastes from the kitchen, bathroom, and laundry, as well as any other wastes that people may accidentally or intentionally pour down the drain (UNEP, 2000). Sanitary wastewater consists of domestic wastewater as well as those discharged from commercial, institutional, and similar facilities (Von Sperling, 2007). In general, the volume of sanitary wastewater generated is about 400 liters per capita per day (UNEP, 2000). There's no available data on the volume of sewage generated in Kenya per day.

Physically, wastewater is usually characterized by a gray color, musty odor, and a solids content of about 0.1% (Liu and Liptak, 2000). Chemically, wastewater is composed of organic and inorganic compounds as well as various gases. Organic components may consist of carbohydrates, proteins, fats, greases, surfactants, oils, pesticides and phenols. Inorganic components may consist of heavy metals, nitrogen, phosphorus, sulfur, chlorides (UNEP, 2000). In domestic wastewater, the organic and inorganic portions are approximately 50% for each category (Liu and Liptak, 2000). However, since wastewater contains a higher portion of dissolved solids than suspended, about 85 to 90% of the total inorganic component is dissolved and about 55 to 60% of the total organic component is dissolved. Gases commonly dissolved in wastewater are hydrogen sulfide, methane, ammonia, oxygen, carbon dioxide, and nitrogen. The first three gases result from the decomposition of organic matter present in the wastewater (Liu and Liptak, 2000). Biologically, wastewater contains various microorganisms but the ones that are of

concern are protista that includes bacteria, fungi, protozoa, and algae (Liu and Liptak, 2000).

The world is faced with problems related to the management of wastewater. This is due to extensive industrialization, increasing population density and high urbanized societies (McCasland *et al.,* 2008). It is estimated that 32.3% of the Kenyan population lives in urban areas (KNBS, 2010). Urbanization continues in Kenya and it is estimated that by 2015 urban dwellers will constitute over 50% of the total population (UN-Habitat (2008). The uncontrolled growth in urban areas has made planning and expansion of water and sewage systems very difficult and expensive to carry out.

The prevention of pollution of water sources and protection of public health by safeguarding water supplies against the spread of diseases, are the two fundamental reasons for treating wastewater. This is accomplished by removing substances that have a high demand for oxygen from the system through the metabolic reactions of micro organisms, the separation and settling of solids to create an acceptable quality of wastewater effluents and the collection and recycling of microorganisms back into the system, or removal of excess microorganisms from the system (Amir *et al.,* 2004).

At present, conventional municipal wastewater undergoes primary, secondary and tertiary treatments before it is discharged. Primary treatment includes screening the wastewater for removing the suspended large or small particles, settling in the settling tanks. After the primary treatment, the wastewater undergoes secondary treatment, which may be one or more combinations of treatments by activated sludge, filters or lagoons. Secondary

treatment is mostly a biological process wherein microorganisms in wastewater convert the non-settleable solids to settleable solids. Following the secondary treatments, the wastewater undergoes a tertiary treatment, which may involve the use of intermittent sand filters for increased removal of suspended solids from the wastewater. In other cases, tertiary treatment involves the processes which remove plant nutrients, primarily nitrogen and phosphorous, from wastewater (Tchobanoglous, 2003).

The problem with the conventional municipal wastewater treatment is that it can partially remove nitrogen and phosphorus concentrations in effluent wastewater (Trecidi *et al.*, 2010). Discharge of untreated water with the presence of residual nitrogen and phosphorus leads to deterioration in water quality and eutrophication of natural water bodies (Smith, 2009; Cornel *et al.,* 2011). Additional treatments in the municipal wastewater treatment plant can double the cost with the requirement of additional chemicals and energy. Often, tertiary chemical treatment is not preferred due to additional problems from the sludge being contaminated from the by-product of the treatment. For example, sludge can get contaminated by aluminum from the tertiary treatment for phosphorus removal, where sludge disposal raises safety concerns. Algal culture has been proposed as a solution for wastewater treatment (Wang *et al.,* 2009)). The dual purpose system is highly attractive since nitrogen and phosphorus can be eliminated and algal biomass can be produced in one step. The biomass produced can be harnessed and converted into energy through thermochemical conversion (gasification, direct combustion and pyrolysis), biochemical conversion and transesterification to produce biodiesel (Brennan and Owende, 2010).

2.5.1 Optimization of Wastewater Treatment Using Algae

For wastewater treatment, a great variety of aquatic ecosystems may be used, for example, marshes, wetlands and ponds in which the combined or separate activity of various organisms such as bacteria, algae, zooplanktons, fish, macrophytes are acting as real processing units. Wastewater treatment with algae has been particularly attractive because of their photosynthetic capabilities, transforming sunlight into useful biomass (Pittman *et al.,* 2011). Under appropriate conditions, the waste-born algae develop biomasses equivalent or superior to higher plants transforming the waste into a new substance with many potential uses (Christi 2007). In this process, the wastewaters are no longer considered wastes, but valuable substrates for the production of biological materials.

Although the deliberate application of algae in wastewater treatment is still fairly limited, algae are found throughout the world in wastewater treatment systems, especially waste stabilization ponds where they work together with microorganisms to reduce organic and inorganic pollutant loads.

Studies have shown that algae can be used to treat domestic wastewater where they are efficient at removing nitrogen and phosphorus from the wastewater (Bhatnagar *et al.,* 2010; Ruiz-Marin *et al.,* 2010; Shi *et al.,* 2007; Wang *et al.,* 2009). Research has also shown that algae can reduce nutrients from agricultural waste, in spite of it containing higher levels of nitrogen and phosphorus than domestic sewage (An *et al.,* 2003; Wang *et*

al., 2010; Wilkie and Mulbry 2002; Woertz 2007). Some algae have been shown to have the ability to remediate industrial-derived wastewater, predominantly for the removal of heavy metal pollutants and organic chemical toxins such as hydrocarbons, biocides and surfactants rather than nitrogen and phosphorus (de-Bashan and Bashan 2010; Mallick 2002). But because industrial wastewaters have low nutrient concentration, the algal biomass generated is quite low.

2.5.2 The Role of Algae in Wastewater Treatment

Algae play a complex multiple roles in the purification process of wastewater. In organically enriched wastewater, release of free oxygen is of major significance, promoting aerobic degradation processes of bacteria and other microorganisms. Organic carbon is partly oxidized to free carbon dioxide, serving as carbon source for the algae. However, reduction of wastewater BOD occurs at the expense of an increase in algal biomass. It is here that the possibility of resource recovery is created via algal harvesting (Rawat *et al.,* 2011).

A second important role of algae is the accumulation and conversion of wastewater nutrients (mainly dissolved salts of nitrogen and phosphorus) into algal biomass (Martinez *et al*., 2000). Algae can also intervene in reduction of nutrients by inducing precipitation of phosphates and stripping of ammonia through photosynthetically created high pH values (Garcia *et al.,* 2006). Both precipitation and stripping, together with incorporation of nutrients are important for tertiary treatment of wastewater to counteract

eutrophication of surface waters (Olguin, 2003). The nutrients, instead of being a waste, become feed for the algae, which in turn become either feed or a fuel source.

Algae also reduce wastewater malodors by creating alkaline conditions in upper parts of the treatment pond and chemically trapping hydrogen sulfide, mercaptans and volatile fatty acids (Wells, 2005). Besides this, algae are also known for their bactericidal capabilities, seriously reducing the propagation of pathogenic bacteria (Ansa *et al.*, 2008). Many different mechanisms are used in the disinfection of sewage in conventional wastewater treatment. Algal photosynthesis causes an increase in the pH due to the simultaneous removal of CO_2 and H+ ions and the uptake of bicarbonate when the algae are carbon-limited (Ansa *et al.*, 2008; Araki *et al.*, 200; Craggs *et al.*, 2004).

Finally, accumulation of heavy metals and toxic compounds by the algae is an additional advantage of an algal wastewater treatment system, but contaminating the algal biomass produced and excluding the algae for certain uses. Several conventional methods are available for the removal of heavy metal pollutants from aqueous solutions or industrial effluents, such as chemical precipitation, electroplating, ion exchange, and membrane processes. However, these methods are either inefficient or expensive when heavy metals are present in the wastewater at low concentrations, and thus more effective and environmentally benign technologies are still in demand (Chen *et al.*, 2012). Biosorption by passive binding to microorganisms (bacteria, fungi, and algae) has much potential for treating industrial effluents due to the feature of environmental friendly, low cost, high

metal binding capacity, low sludge generation, and high efficiency in removing heavy metals from dilute effluents (Marcano *et al.,* 2009). Algae biomass are of particular interest as biosorbents since they have high metal binding capacities due to the polysaccharides, proteins or lipids on their surfaces, thereby providing numerous metal binding sites (such as carboxyl, hydroxyl, carbonyl, sulfhydryl, thioether, sulfonate, amine, imine, amide, imidazole, phosphonate and phosphodiester groups) (Chen *et al.,* 2012). Algae that have been used to remove heavy metals include *Spirogyra* spp for biosorption of chromium and *Scenedesmus acutus* for removal of cadmium (Bishnoi *et al.*, 2007; Chen *et al.*, 2012).

Algae have also been used to remove oil contaminants. Algal species such as *Oscillatoria salina, Plectomena terebrans*, *Aphanocapsa sp.* (Raghukumar *et al*. 2001), and *Synechococcus* (El-Bestawy *et al.* 2007) have been successfully used in bioremediation of oil contaminants in different parts of the world, as mixed population or using algal mats.

2.5.3 Commercial applications of wastewater-harvested algae

In order to add value to the wastewater treatment process, a means of further utilizing the biomass is needed. To date, algae biomass has found a variety of uses, one of the foremost being as a source of feed for fish, crustacea, shell-fish, poultry, cattle, rabbits, pigs and humans (Spolaore *et al.,* 2006). The human consumption of algae biomass is however restricted to very few species due to the strict food safety regulations, commercial factors, market demand and specific preparation (Pulz and Gross, 2004).

Although the predominant reason for utilizing algae as a food source is due to its protein content, a variety of nutrients, namely carbohydrates, lipids, vitamins, pigments, minerals and trace elements, are also present (Becker, 2007). *Chlorella, Spirulina and Dunaliella* dominate the market. Algae biomass is marketed in tablet or powder form as food additives generally in the health food market, which is expected to remain a stable market (Spolaore *et al.,* 2006). *Chlorella* is also used for medicinal value such as protection against renal failure and growth promotion of intestinal lactobacillus. *D. salina,* is exploited for its b-carotene content of up to 14%.

In small doses (5-10% feed supplement) algae are reported to positively affect the immune system, fertility, weight control, and condition of the skin and coat (Pulz and Gross, 2004; Spolaore *et al.,* 2006). Currently the predominant use of algae as a feed supplement is in poultry production to increase the fatty acid composition and carotenoid content of egg yolks, and the docosahexaenoic acid (DHA) content of the flesh (Fredriksson *et al.,* 2006; Pulz and Gross, 2004). In aquaculture algae are used as a food source for molluscs at all growth stages, crustaceans and some fish species in the larval stage, and for culturing of zooplankton which feed late-larvae and juveniles of some crustacean and fish species (Ferreira *et al.,* 2008; Mitra *et al.,* 2007). Of the total world production of algae it is estimated that 30% is sold for animal feed applications (Becker, 2007; Spolaore *et al.,* 2006).

Other applications of algae include use as a fertilizer and soil amendment, as well as for the production of pigments, vitamins, polysaccharides, sugars, pharmaceuticals, amino acids, phytol, essential oils, enzymes and bioflocculants (Lehmann *et al.,* 2006). As a soil

amendment, algae have been used in coastal regions all over the world to increase water binding capacity and mineral composition (Pulz and Gross, 2004). Algae in soil ecosystems contribute to the soil fertility by producing polymers which assist with particle adherence and water storage. Some soil algae are also able to fix nitrogen and produce bioactive compounds which influence higher plants by promoting germination, leaf or stem growth, and flowering (Pulz and Gross, 2004). As a soil additive dry algae biomass can be used as a slow release fertilizer and shows comparable results to synthetic fertilizer with respect to dry weight production and nutrient composition (Mulbry *et al.*, 2005).

Algae produce pigments such as chlorophylls, phycobiliproteins and carotenoids. In some species such as *Dunaliella salina* and *Haematococcus pluvialis*, secondary pigments, β-carotene and astaxanthin respectively, may be produced in larger quantities than the primary chlorophyll pigments (Guerin *et al.*, 2003). Carotenoids have a variety of uses as "natural" food colorings and feed additives. As a feed additive, it is used to enhance, for example, the color of salmonid flesh and the colour of egg yolks, and also to improve the health and fertility of lot-fed cattle (Guerin *et al.*, 2003).

Whilst naturally produced astaxanthin cannot compete commercially with the synthetic form, there are a few applications where the naturally produced product is preferred due to enhanced deposition in tissues (Spolaore *et al.*, 2006). Biliproteins occur in Rhodophyceae, Cryptophyceae and Cyanophyceae and are sold as a natural blue pigment to be used in health foods and cosmetic products (Guerin *et al.*, 2003). Additionally, they

are used in industry and research immunology laboratories as powerful and highly sensitive fluorescent reagents (Spolaore *et al.,* 2006).

Carotenoids are natural antioxidants produced to counteract the cell damaging effects of reactive oxygen species generated as by-products of photosynthetic activity (Pulz and Gross, 2004). In humans the free-radical scavenging capacity of antioxidants protects against numerous life-threatening diseases including various forms of cancer, coronary heart disease, diabetes, premature aging and arthritis. Humans are unable to synthesize the full complement of required antioxidants and therefore are dependent on obtaining these from the diet (Dufosse *et al.,* 2005). For this reason algal carotenoids appear in the nutraceutical market and even though synthetic carotenoids are cheaper to manufacture, carotenoids from microalgae have the advantage of supplying natural isomers in their natural ratio (Spolaore *et al.,* 2006). In addition to its antioxidant activity, β-carotene is a provitamin A. Vitamin A is important in the body as it acts as an immunostimulator, it protects against eye diseases and helps to maintain healthy skin (Dufosse *et al.,* 2005; Spolaore *et al.,* 2006).

Other carotenoids produced by microalgae which have good antioxidant properties include lycopene, zeaxanthin, and lutein (Granado *et al.,* 2003). Zeaxanthin and lutein are xanthohpylls (oxygenated carotenoids) with no provitamin A activity. However, their consumption is directly associated with a reduction in the risk of arteriosclerosis, cataracts, age-related muscular degeneration, multiple-sclerosis and cancers (Bhosale and Bernstein, 2005).

Another important group of compounds derived from algae are polyunsaturated fatty acids

(PUFAs) which are essential components of cell membranes in higher eukaryotes as they confer flexibility, fluidity and selective permeability (Pulz and Gross, 2004; Ward and Singh, 2005). Animals lack the enzymes required for the synthesis of PUFAs of more than 18 carbons and therefore these need to be obtained through diet (Spolaore *et al.*, 2006). The most common source of PUFAs in the human diet is fish and fish oils. The primary source of these PUFAs however are algae, either through direct consumption or through consumption of zooplankton which feed on microalgae (Spolaore *et al.*, 2006). Due to some of the disadvantages associated with fish oils such as fishy smell, unpleasant taste, presence of environmental pollutants, instability and presence of mixed fatty acids, algae have been considered for the direct production of PUFAs (Ratledge, 2004; Spolaore *et al.*, 2006; Ward and Singh, 2005). The major targets of production are γ-linolenic acid (GLA), arachidonic acid (ARA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) (Ward and Singh, 2005).

Currently DHA is the only commercially available algae-derived PUFA and is used in the preparation of infant milk formulas in the US (Spolaore *et al.*, 2006). DHA is a major structural component of grey matter in the brain and in infants it is necessary for the development of normal neural and retinal functions, and supports good cardiovascular health (Ratledge, 2004; Ward and Singh, 2005).

Carbohydrates accumulate in algae cells as storage materials, are present as osmoregulators and make up a significant proportion of biomass in the cell walls (Dismukes *et al.*, 2008). Polysaccharides have been harvested from algae and are used as viscosifiers (thickening agents), flocculants and lubricants (Courtois, 2009). Some algal polysaccharides have shown pharmacological importance in having anti-cancer properties and the ability to stimulate the immune system (Courtois, 2009; Masojidek and Torzillo, 2008).

Antibiotics produced by algae are largely unidentified but are thought to include fatty acids, phenolic substances, polysaccharides, alcohols, tannins and terpenoids, and are toxic to other algae, bacteria, fungi, viruses and protozoans (Hanaa *et al.*, 2009). Some useful clinical compounds have been isolated from algae. For example, an antihelminthic has been isolated from *Digenea simplex* for treatment of roundworms (Ebadi 2006).

2.5.4 Reuse of wastewater effluent

After undergoing treatment and harvesting of the biomass, wastewater that has been treated using an algal based treatment system can be reused for various activities without it causing environmental and public health problems. This is because during treatment, algae are able to remove organic and inorganic compounds as well as pathogenic microbes (Brennan and Owende, 2010). Reuse of wastewater effluent is an attractive endeavor for Kenya for the following reasons;

Water scarcity

Kenya is classified as a water scarce country with only 647 cubic metres of renewable freshwater per capita (Ngigi and Macharia, 2006; UN-WATER/WWAP 2006). Water scarcity in many parts of Kenya is a limiting factor against development activities. Hence, there is need for water saving and water enhancement strategies. With dropping per capita freshwater availability, there is increasing dominance of wastewater in the water balance and this makes wastewater a very important source of reclaimed water (Githuku, 2009).

Water Supply Gap

Kenya is a water scarce country where most municipal councils are unable to supply their population with sufficient water. The current population is about 38.6 million and the prevailing birthrate of about 4% per year (Kenya National Bureau of Statistic, 2010). This means that water scarcity will get worse over time. In 2006, the estimated total water available in Kenya was 20,291 million m^3 (UN-Water, 2006). Potable water is used to flush toilets and wash cars, which are activities that could utilize water of much lower quality, such as treated wastewater.

Kenya's Vision 2030 has attempted to address this lack of equity in the supply of potable water by advocating for the conservation of water sources, rainwater harvesting, and enhancing the utilization of ground water (Government of the Republic of Kenya, 2007). Kenya's Ministry of Water and Irrigation goal to avail water in sufficient quantity and quality by 2010 has not been achieved (Ministry of Water and Irrigation, 2007). This

existing gap needs to be bridged to meet Kenya's industrial, domestic and agricultural water needs. One way of doing that is by wastewater reuse.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

The following apparatus and instruments were used in the study;

Microscope (Olympus), Incubator (Gallenkamp), Nickel chromium, Bomb Calorimeter (Gallenkamp), Soxhlet apparatus, Rotary evaporator (Buchi, Germany), Spectrophotometer (Jenway, UK), Bench centrifuge (Gallenkamp), Boiling water bath (Memmert), Vortex mixer (Stuart), Magnetic stir plate (Memmert), pH meter (Model pH 90), Top-loading balance that is capable of weighing accurately to +0.01 g (Mettler, Holland), Analytical balance that is capable of weighing accurately to +0.0001 g (Denver instruments Co.), Laboratory oven (Memmert, Germany), 5W fluorescent tube (Philips), 250 ml Erlemeyer flasks (Pyrex), Whatman GF/C filter paper of 0.45µm mesh size, Plastic strainer, Grinding mill with 0.5 mm screen (Retsch), Stop clock timer (digital), Pipettes, Dispenser 1000 mL or greater capacity capable of accurately delivering 20 and 30 Ml, Glass test tubes – 16 x 100 mm (Pyrex), 25 x 150 mm Glass tubes with polytetrafluoroethylene (PTFE) - lined screw caps, Plastic film.

Reagents used in the study were as follows; Sodium nitrate ($NaNO_3$), brand; Sigma, purity; 98%, Calcium chloride hydrate ($CaCl_2 \cdot 2H_2O$), Sigma, purity; 95%, Magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$), Sigma, purity; 99.5%, Dipotassium phosphate anhydrous ($K_2HPO_4 \cdot 3H_2O$), Sigma, purity; 98%, Monopotassium phosphate (KH_2PO_4), Sigma, purity; 95%, Sodium chloride (NaCl), Sigma, purity; 95%, Sulphanilamide ($H_2NC_6H_4SO_4NH_2$), brand: Sigma-Aldrich, purity: 99%, N-(1-naphthyl)-ethylenediamine

(NED) ($C_{12}H_{14}N_2 \cdot 2HCl$), brand: Sigma-Aldrich, purity: 99%, Ammonium chloride (NH_4Cl), brand: Sigma-Aldrich, ACS reagent with purity of 99.5%,

Disodium ethylenediamine tetraacetate ($C_{10}H_{14}N_2Na_2O_8$), brand: Sigma, purity 98.5%, Hydrochloric acid (HCl), brand; Sigma-Aldrich, purity; 95%, Hexane, anhydrous [$CH_3(CH2)_4$], brand; Sigma-Aldrich, purity; 95%, Ethanol (C_2H_6O), brand; Sigma-Aldrich, purity; 99%, Glacial acetic acid (CH_3CO_2H); Sigma, purity; 98%, Sodium hydroxide (NaOH), Sigma, purity; 95%, α-amylase (Sigma-Aldrich), Amyloglucosidase (Sigma-Aldrich), Glucose oxidase (Sigma-Aldrich), Peroxidase (Sigma-Aldrich), 4-aminoantipyrine (Sigma-Aldrich), Sodium phosphate anhydrous (Na_2HPO_4), Sigma-Aldrich, purity; 98%, Phenol (ACS grade), Glucose oxidase (Sigma-Aldrich), Glucose Sigma-Aldrich, purity 99.5%, Benzoic acid (Sigma, purity; 98%), Sodium acetate ACS reagent (Sigma, purity; 99%) and Oxygen 99%.

3.2. Methods

3.2.1 Sewage Sampling

Sewage samples were obtained from Moi University stabilization ponds, that are made up of a series of anaerobic, facultative and maturity ponds.

Composite samples made up of several grab samples were obtained from the different sampling points, following sampling protocol by EPA (2007). Sampling was systematic and three replicates were made at each point.

Prior to sewage sampling, the containers to be used to sample and transport the sample to the laboratory were washed with a brush and phosphate- free detergent. They were then rinsed three times with cold tap water. Rinsing with 10 % hydrochloric acid followed before rinsing three times with de-ionized water. At the sampling site, the bottles were rinsed three times with portions of the sample before being filled. Sampling was directly into 250 ml plastic containers. A sampling rod made of stainless steel pole with a clamp on one end on which the sampling bottle was securely attached was used to collect the samples. Sampling was done on the subsurface, about 30 cm depth because the ponds were shallow. Samples were collected about one meter from the edge, as edge sewage is not typical of the majority of the wastewater. For each pond, there was a sampling point at the inlet area (A), another at the outlet section (B) and two other points at the two other opposite sections of the pond (C, D) (figure 2). After sampling from these four points, the wastewater was mixed and one liter from this composite sample was taken to the laboratory. This sampling was done in triplicate. Preliminary sampling involved all the ponds, but after the initial laboratory analyses on the nutrient status of wastewater, subsequent sampling was done on the first maturity pond because the nutrient levels of the wastewater from the pond was sufficient to culture algae (Brennan and Owende, 2010).

3.2.2 Identification of Algae in Kesses

Green filamentous algae were sampled from Kesses area, mainly along the edges of Sambul River and in ponds (figure 3). The locality was chosen because of the proximity

to Moi University, where the laboratory is based. The study was also interested in local species of algae.

Photosynthetic algae represent a large and diverse group of organisms that have only a limited history of characterization and exploitation. The application of resource exploitation from algae is relatively untapped, with the potential to produce fuel, food, fibers and pharmaceuticals on a large scale. It is important to identify or characterize local species of algae as they may often offer significant but currently unknown benefits for bioresource production. This is because indigenous algae are adapted to prevailing abiotic and biotic factors thus are evolutionarily primed for local bioresource production.

3.2.2.1. Sampling of Algae

Sampling of algae was done twice a week between January and March 2009 before onset of the long rains which normally wash away the algae. Sampling was done after mid-morning because the algae migrate to the surface of the water with the presence of sunshine. Sampling was normally concluded on the same day so that the samples could be taken to the laboratory on the same day.

Sampling of algae was done following the method by Kelly *et al.*, (2005). The algal filaments were sampled with the help of a plastic forceps and strainer of 0.5 mm pore size and were washed with sampling-site water to clean the entangled detritus and silt. Each lump was then put into a 100 ml wide mouthed plastic bottle with water-tight lids containing some water from the sampling site. The bottles were labeled using a

waterproof marker of sampling date and place. The samples were immediately taken to the laboratory.

In the laboratory, the algae samples were rinsed thoroughly to remove macro-invertebrates, debris and sediment. Dominant taxa analysis was done using a modified method by Lund *et al.*, (1958). Identification of the algae was done using a compound microscope with the aid of algae plates found in APHA (2005).

A representative amount of the sample was obtained by first swirling the sampling bottle containing the sample around to mix the content. A 1 ml of the sample was then removed using a dropper marked in ml and put on a clear slide, covered with a slip and placed on the microscope stage. The number of filaments for each identified taxa was counted in the field of view of the microscope objective. This was continued ten times for each sample and until all taxa had been identified. The total number of filaments for each taxon was tabulated. The total number of all filaments was tabulated and the total was recorded at the bottom of the sheet, and the dominant taxa were defined by the greater number of filaments. In total, seventy two samples were collected and brought to the laboratory. Of eight species identified, only three species were selected based on their abundance, as shown on the Table 2.

However, lack of fruiting bodies in the forms collected restricted their identification up to the genus.

Table 1 Green filamentous algae found in Kesses area and their frequency

Algae genera	Percentages
Spirogyra sp.	28.6
Oedogonium sp.	21.43
Zygnema sp.	19.05
Mougeotia sp.	7.14
Cladophora sp.	4.76
Ulothrix sp.	2.38
Microspora sp.	2.38
Rhizoclonium sp.	2.38
Mixed samples	11.90

(Source: Author, 2013)

The genera that were then selected for the study were *Spirogyra* sp., *Oedogonium* sp. and *Zygnema* sp. due to their abundance.

Spirogyra

Spirogyra is a photosynthetic free-floating filamentous form of green algae, commonly seen as bright green masses on the surfaces of fresh water ditches and ponds. It is a genus of green algae, belonging to the order Zygnematales. The name "spirogyra" is derived

from the spiral arrangement of chloroplasts in its filaments. This feature is unique to this genus, which has almost 400 species.

Spirogyra cells are cylindrical in shape and are connected end to end, forming a long, unbranched filament-like structure. The cell wall is made up of an outer layer of cellulose and an inner layer of pectin. The inner surface of the cell wall is lined with a thin layer of cytoplasm. The spiraled ribbon-shaped chloroplasts are embedded in the cytoplasm lining. The number of chloroplasts in each cell may vary from one to sixteen. Each chloroplast has several round bodies, pyrenoids, which are responsible for the production of starch. Each cell has a prominent nucleus in the centre, suspended by thin strands of cytoplasm from the inner part of the cell wall. The cells are long and thin, and each spirogyra filament measures between 10 and 100 micrometer in width. Every cell in the filament except the basal one is capable of cell division.

Spirogyra is capable of both sexual and asexual reproduction. Asexual reproduction occurs by fragmentation of the filaments, whereas sexual reproduction is by conjugation.

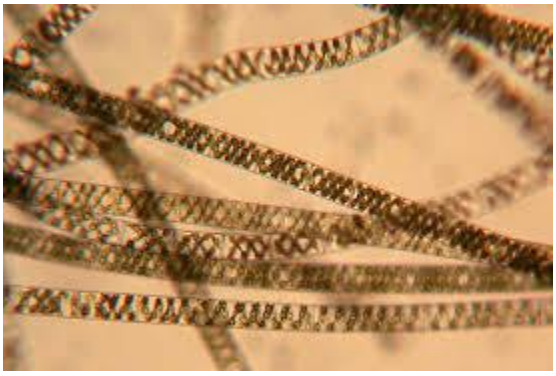

Plate 1. Illustration of *Spirogyra* filaments (Source; Author 2013)

Zygnema

Zygnema is a very common alga and is usually found along side *Spirogyra* in quite still waters. It is a genus of about 140 species, all occurring in freshwater. They form unbranched filaments, with each cell containing a pair of stellate chloroplasts and each chloroplast possessing single, conspicuous pyrenoids. The nucleus is located within the cytoplasmic bridge connecting the two chloroplasts. The basal cells occasionally develop rhizoidal growths for attachment in turbulent waters. Reproduction can occur in three ways: - asexual, sexual and vegetative.

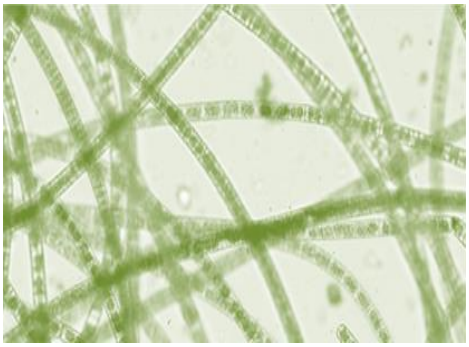

Plate 2 Unbranched filaments of *Zygnema* (Source; Author 2013)

Oedogonium

Oedogonium are freshwater, uninucleate and unbranched filamentous green algae. There is a dense chloroplast, usually filling the cell, with pyrenoids. Cells frequently are wider at one end than the other; occasionally there are bulbous, almost globular cells. The chief diagnostic character is the presence of rings at the wider end, which arise as a consequence of cell division, one ring per division which that cell has undergone, which can usually be seen in the filament by careful focusing under favorable lighting (condenser iris shut down). *Oedogonium* reproduces sexually as well as vegetatively.

Chloroplasts are reticulate, extending from one end of the cell to the other. The many pyrenoids are at the intersections of the reticulum.

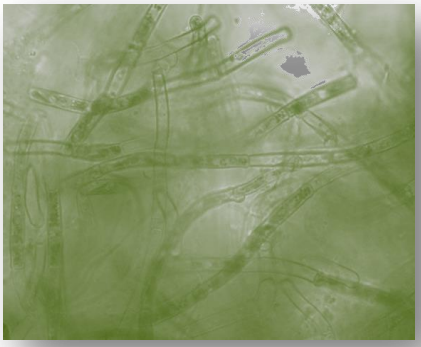

Plate 3 *Oedogonium* filaments (Author 2013)

3.3 Potential Ability of Sewage to Support Algal Growth

Growth was obtained gravimetrically by using periodic chlorophyll *a* measurement that was taken on each day of study for seven days. This study was repeated twice and had two replications. All the glassware that were used to grow the algae were first washed with soap and rinsed severally using tap water before soaking in 10 % Hydrochloric acid solution for 24 hours and rinsed twice with distilled water. Ten grams of each algal genus was cultured independently in 250-ml conical Erlenmeyer flasks containing 100ml pre filtered wastewater as culture medium. Filtration through Whatman GF/C filter paper of 0.45µm mesh size ensured that the wastewater cultures were maintained free of bacteria, fungi and other species of algae contamination.

The Erlenmeyer flasks were plugged with cotton to limit evaporation while allowing gas exchange and randomly arranged in an incubator set at 26^{o}C. Continuous light was

provided by one 5W fluorescent tube. The Erlenmeyer flasks were then stirred once a day to prevent settling of cells. The control experiment consisted of 250-ml conical Erlenmeyer flasks containing 100 ml Bold Basal Medium which contains the following compounds;

Preparation: To 940 ml of distilled water, the following was added:

ml	stock solution	g / 400ml H_2O
10	$NaNO_3$	10
10	$CaCl_2 \cdot 2H_2O$	1
10	$MgSO_4 \cdot 7H_2O$	3
10	$K_2HPO_4 \cdot 3H_2O$	3
10	KH_2PO_4	7
10	NaCl	1

Source; Miao & Wu, 2006.

About 0.05 mg of CO_2/L was bubbled to the Erlenmeyer flasks once using a pressurized carbon dioxide tank with solenoid valve. The experiment lasted for 7 days, but on each day measurement of chlorophyll *a* pigments was carried out. This pigment was extracted from 10 milligrams of algae that was either growing on sewage or in the growth medium. The algae were ground in a porcelain mortar with 50 ml of 90% acetone. The extract was kept in the dark for 24 h before filtered using Whatman number one filter paper. The absorbance of the clear extract was measured at 663.2 nm and 646.8 nm in the Spectrophotometer.

Chlorophyll *a* content µg/ml was calculated using the formula (APHA 2005);

Chl *a* µg/ml = $12.25A_{(663.2)} - 2.79A_{(646.8)}$ ..... (Equation number 1)

3.4 Nitrates Removal by Different Algae Genera

Introduction

Domestic sewage is a rich source of nutrients required by algae. Inorganic nitrogen and phosphorus are the most important of these nutrients, and it is the supply of these that cause eutrophication. Algae have a potent underutilized ability to remove such nutrients. In this study three different alga genera were grown on partially treated sewage in order to assess their efficiency to remove nitrates from the wastewater.

This project was accomplished through experimental study that followed the complete randomized design with two replications and two repetitions. The following procedure was followed during culturing of algae:

Wastewater was collected from Moi University sewage treatment works and nitrate analysis carried out according to APHA (2005). The wastewater was then used to culture the algae. Ten grams of algae was cultured independently in 250-ml conical Erlenmeyer flasks containing 100 ml pre filtered wastewater as culture medium. Filtration through Whatman GF/C filter paper of 0.45μm mesh size ensured that the wastewater cultures were maintained free of bacteria, fungi and other species of algae contamination. The Erlenmeyer flasks were plugged with cotton to limit evaporation while allowing gas exchange and randomly arranged in an incubator set at 26^{o}C. Continuous light was provided by one 5W fluorescent tube. The Erlenmeyer flasks were then stirred once a day to prevent settling of cells. The control experiment consisted of 250-ml conical Erlenmeyer flasks containing the wastewater but without the algae, to estimate nutrient

reduction by oxidation. The result from the control setup was deducted from the experimental results to minimize error in nutrient uptake.

On each day for the seven days that the algae were cultured, nitrate analysis of the wastewater was done. This occurred after algal cells were separated from the growth media using an ordinary tea plastic strainer.

3.4.1 Nitrate-N and Nitrite-N

The method for analyzing Nitrate-N was Automated Hydrazine Reduction Method (APHA 2005).

In order to prepare sulphanilamide solution, 5 g of sulphanilamide were dissolved in a mixture of 50 ml concentrated Hydrochloric acid and 300 ml water, and it was diluted to 500 ml with water. During the preparation of N-(1-naphthyl)-ethylenediamine (NED) solution, 500 mg of NED were dissolved in 500 ml of water. In order to make Ammonium chloride-EDTA solution, 13 g of ammonium chloride and disodium ethylenediamine tetracetate (1.7 g) were dissolved in 900 ml of water. The pH of this solution was adjusted to 8.5 by adding concentrated ammonium hydroxide and the volume of the solution was finally adjusted to 1 L.

Diluted ammonium chloride-EDTA solution was prepared by using 300 ml of Ammonium chloride-EDTA solution was and diluting to 500 ml with water. 1 M hydrochloric acid solution was prepared by using 8.3 ml of 37% concentrated HCl which was diluted to 100 ml with water.

The procedure was as follows: For nitrite determination 25 ml of each sample were diluted to 100 ml with diluted ammonium chloride-EDTA solution. For color development and measurement of absorbance, 2 ml of sulphanilamide solution were added to 50 ml of the former solution, and 2 ml of NED solution were added, under stirring, after 5 min. Absorbance was measured at 540 nm after a reaction period lasting between 10 min and 2 h. For total nitrate and nitrite determination, 1 ml of HCl solution was added to 50 ml of sample and mixed. Absorbance was then read at 220 nm.

Calibration curves for nitrate based on absorbance at 220 nm, and for nitrite based on absorbance at 220 and 540 nm, were generated with standards in the range 0.5 –10.00 mg L^{-1}. Values of nitrite concentration, obtained as described above, were then used to calculate the absorbance of nitrite at 220 nm. The nitrate absorbance was calculated via subtraction of the nitrite absorbance at 220 nm from the total absorbance at the same wavelength, and this value was then converted to nitrate concentration using the calibration curve obtained with the nitrate standards.

After nitrate values had been obtained, nutrient removal rate (mg l^{-1} day^{-1}) were made.

3.5 Determination of Renewable Energy Potentials of the Alga Grown In Sewage

Introduction

Renewable resources can be produced from algae without compromising agricultural land and freshwater resources or promoting land degradation. This occurs when wastewater is utilized to cultivate the desired native species for biomass feedstock production. In this study, algae were grown in wastewater for lipid and starch production, possible feedstocks for biofuel production.

3.5.1 Lipid productivity of algae

This study was accomplished through experimental study that followed the complete randomized design with two replications and two repetitions. Each genus of algae filaments was grown separately in 250-ml Erlenmeyer flasks containing 100 ml wastewater as culture medium, which had been sterilized to remove bacteria and other organisms. Twenty grams of alga was grown in an incubator set at 26^0C. Continous light was provided by one 5W fluorescent tube and the flasks were stirred once a day to prevent settling of cells. The control experiment was grown using Bold Basal culture medium. Each experiment lasted 7 days.

3.5.1.1 Lipid extraction

After harvesting the algae by separating the filaments from the liquid using a plastic strainer, lipid analysis was performed. A solvent mixture of hexane and ethanol were used to extract both polar and nonpolar lipids.

During algal lipid extraction, the dried algal cells were finely ground using mortar and pestle and weighed. Hexane and ethanol mixture, in 2:1 ratio, was used to extract the lipid. After calculating the amount of ethanol required, in a cellulose extraction thimble (Whatman no. 2810-338), 10 grams of the ground biomass was placed with 4 ml of the ethanol to enhance cell disruption and the mixture was allowed to stand for an hour. The thimble was plugged with silanized glass wool and placed in the Soxhlet apparatus. The reservoir was filled with 125 ml of the extraction solvent. The apparatus were assembled and the solvent was allowed to reflux for 8 hours.

After 8 hours, the heater was disconnected and the system was cooled to room temperature. After cooling, the reservoir containing the solvent and the lipids was detached from the Soxhlet apparatus and placed in the evaporation apparatus to recover the solvents. The solvent reservoir was placed in the evaporation apparatus and the evaporated solvent recovered in the collector.

The lipid extracts was transferred to a pre-weighed test tube fitted with a stopper. The lipid extract was dried completely of the residual solvent and weighed. The content of lipids was determined in the sample by weight difference:

Lipid content (%) = amount of lipid extracted (g)/weight of original sample (g) × 100

..... (Equation number 2)

3.5.2 Starch production in algae as a possible feedstock for bioethanol

The algae genera were grown as in section 3.5.1.

3.5.2.1 Estimation of Starch in Algae by an Enzymatic-Colorimetric Method (Modified Bach Knudsen Assay)

The following reagents and solutions were prepared;

(a) During preparation of Acetate buffer 100 mM, pH 5.0., 5.71 mL of glacial acetic acid was pipetted and transferred immediately to a flask and dissolved with water. The volume was brought to 850 mL. While stirring solution on a magnetic plate, the pH was adjusted to 5.0 + 0.1with 1M NaOH solution and diluted to 1 L with distilled water.

(b) While preparing Amyloglucosidase solution 200 U/mL, concentrated amyloglucosidase was diluted with 100 mM sodium acetate buffer **(**a**)** to give 1 mL of solution per sample with 2 to 5 mL excess.

(c) Glucose oxidase–peroxidase (GOPOD) reagent was prepared using a mixture of glucose oxidase, 7000 U/L; peroxidase, 7000 U/L; and 4-aminoantipyrine, 0.74mM. During preparation, this reagent was prepared by dissolving 9.1 g of sodium phosphate anhydrous (Na_2HPO_4) and 5.0 g of Potassium dihydrogen orthophosphate (KH_2PO_4) in 300 mL distilled water in a volumetric flask. Distilled water was used to rinse chemicals into bulb of flask. The flask was swirled to dissolve completely. To the solution, 1.0 g phenol and 0.15 g 4-aminoantipyrine was added. Distilled water was used to rinse chemicals into bulb of flask and swirled to dissolve completely. Glucose oxidase (7000

U) and peroxidase (7000 U) was added and the enzymes rinsed into flask with distilled water. The volume was brought to 1 L with the distilled water. The reagent was stored in a sealed amber bottle at 4°C.

(d) To make Glucose standard solution, approximately 75, 150, and 250 mg of glucose was weighed and recorded to 0.0001 g. The glucose was rinsed from weigh paper into 250 mL volumetric flask with 0.2% benzoic acid solution and dissolved. The volume was brought in each flask to 250 mL volume with 0.2% benzoic acid solution to give 3 independent glucose standard solutions. To calculate actual glucose concentrations of the solutions, the weight of glucose was multiplied by dry matter percentage and percentage purity as provided by the manufacturer in the certificate of analysis and divided by 250 mL. The solutions were prepared one day before use to allow equilibration of α- and β-forms of the glucose and stored at room temperature.

3.5.2.2. Preparation of Reagent Blanks and Standard Curves

(a) A Reagent blank was prepared for each assay, whereby tubes containing no sample and only the reagents added for each method was carried through the entire procedure. Absorbance values for the reagent blanks were subtracted from sample absorbance values.

(b) Standard curves were made by taking 0.1 mL of 0.2% benzoic acid solution and 300, 600, and 1000 μg/mL standard glucose solutions and pipetting in duplicate into the bottom of 16 x 100 mm glass culture tubes. A 3.0 mL portion of GOPOD reagent was

added to each tube using a positive displacement repeating pipette aimed against wall of tube so it would mix well with the sample.

Tops of tubes were covered with plastic film and incubated in a 50°C water bath for 20 min. Absorbance was read at 505 nm. All readings were completed within 30 min of the end of incubation.

The quadratic equation describing the relationship of glucose μg/mL (response variable) and absorbance at 505 nm (independent variable) was calculated. This standard curve was used to calculate glucose μg/mL in sample solutions. A new standard curve was prepared with each new batch of GOPOD reagent.

During sample preparation, 200 mg of three algae genera was accurately weighed into 25 x 150 mm screw cap glass tubes. A standard was included with each set of determination. 30 mL of 0.1M sodium acetate buffer (pH 5.0) was dispensed into each tube. A 0.1 mL portion of heat-stable, α-amylase was then added and the tubes were capped and mixed. The tubes were incubated for 1 h at 100°C in a boiling water bath and mixed at 10, 30, and 50 min of incubation. The tubes were then removed from the water bath and cooled on bench for 0.5 h.

In order for hydrolysis of starch to glucose to take place, 1 mL of amyloglucosidase solution was added and the tubes mixed. The tubes were then incubated for 2 h at 50°C, mixing at 1 h of incubation. A 20 mL portion of distilled water was added to each of the tubes, recapped, and inverted repeatedly to mix.

The contents of each test tube were then transferred into centrifuge tubes and centrifuged at 1000 revolutions per minute for 10 min. Centrifuged solutions were allowed to cool to room temperature before filtering them though Whatman 54 filter paper. A 1.0 mL portion of the filtrate was diluted to 10 mL with distilled water and mixed thoroughly.

Quantification of Glucose was accomplished by pipetting 0.1 mL of distilled water and sample solutions into the bottoms of 16 x 100 mm glass test tubes in duplicate; using 2 tubes/sample solution. A 3.0 mL portion of glucose oxidase-peroxidase reagent was added to each tube using a positive displacement repeating pipette so it would mix well with the sample.

The tubes were placed in a rack and covered with plastic film and incubated in a 50°C water bath for 20 min. Absorbance, *A*, was read at 505 nm. The spectrophotometer was zeroed using 0 μg / mL standard. *A* values for each sample was averaged and use in calculations.

Free glucose was determined on samples carried through above steps except that no α-amylase was added. Sample solutions are then subject to steps above and the calculations used to convert absorbance values to anhydroglucose as a percentage of dry matter. By expressing free glucose as anhydroglucose the value could be directly subtracted from the value obtained with the inclusion of amylase and amyloglucosidase, thus giving a value for starch corrected for the free glucose in the sample.

Total starch in the sample was calculated as follows:

Starch, % = DA x F x FV/0.1 x 1/1000 x 100/W x 162/180

= DA x F/W x FV x 0.9 (Equation number 3)

Where:

DA = Absorbance (reaction) read against the reagent blank.

F = conversion from absorbance to μg, for example 300 (μg of D-glucose) / absorbance for 300 μg of glucose

FV = Final volume (i.e. = 30 mL acetate buffer + 0.1 mL amylase + 1 mL amyloglucosidase solution + 20 mL H2O = 51.1 mL)

0.1 = volume of sample analyzed

1/1000 = Conversion from μg to mg

100/W = Factor to express "starch" as a percentage of flour weight

W = The weight in milligrams ("as is" basis) of the sample analyzed

162/180 = Adjustment from free D-glucose to anhydro D-glucose (as occurs in starch).

3.5.3 Determination of calorific value of algal lipids

One of the most important characteristic of a fuel is its gross calorific value. This is because the value represents the amount heat produced during complete combustion of a unit quantity of fuel. It therefore determines whether a fuel is efficient and effective or not.

3.5.3.1Determination of gross calorific value of the fuel

A bomb calorimeter was used. Procedure for sample preparation and measurement was as follows:

A 1g portion of lipid from the three genera (*Zygnema*, *Spirogyra* and *Oedogonium)* was put in the crucible. The stirrer was started and the initial temperature noted. The current through the crucible was started and the sample allowed to burn in presence of 25 atmospheric pressure oxygen. Heat released during combustion is normally taken by the water causing its temperature to rise. The final steady state temperature was noted.

Calorific value of the lipid (in j/g) was calculated as follows:

$= \underline{(m_w + W)\, Cw\, (T_2 - T_1)} / mF$ …….. (Equation number 4)

Where m_1 and m_2 are mass of water in copper calorimeter and water equivalent of bomb calorimeter respectively,

mF = mass of the lipid,

T_1 and T_2 = initial and final temperature of water,

C_w = specific heat of water

W = water equivalent of the calorimeter

m_w = mass of water in calorimeter

CHAPTER FOUR

RESULTS

4.1 Potential of Algae to Remove Nitrates from Domestic Wastewater

The wastewater on which the genus *Spirogyra* was growing decreased in nitrates from a mean of 6.56 ± mg/l on the initial day to 0.83 ± mg/l on day four. *Zygnema* reduced the nitrate quantities in the sewage from 6.56 ± mg/l on the initial day to 1.07 ± mg/l on the fourth day. Similarly, there was a decrease in the nitrate content of the wastewater on which genus *Oedogonium* was growing on, from 6.56 ± mg/l on the first day, to 1.01 ± mg/l on day four. These are illustrated in graph below.

Graph showing nitrates removal from wastewater by different species of algae.

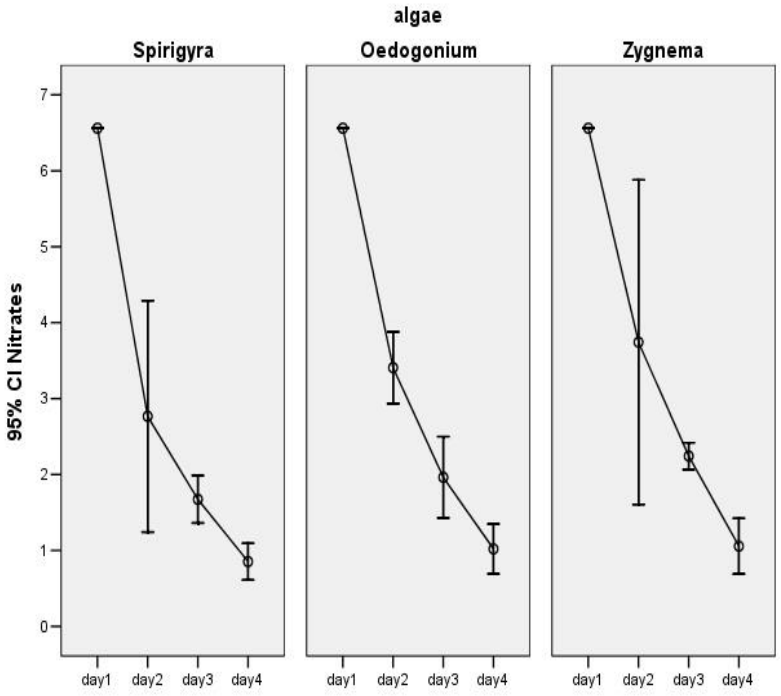

Figure 2. Nitrates reduction by algae

The data size in the study was small so the most appropriate statistical test was Friedman test. A Friedman test was conducted to determine if there was a significant change in levels of nitrate that was reduced by the algae between days 1, 2, 3 and 4. In *Spirogyra* the difference in nitrate removal across the days; day 1(median = 6.56), day 2 (median = 2.96), day 3 (median = 1.69) and day 4 (median = 0.83) was statistically significant (X^2 (N = 3) = 9, p = .02). Kendall's coefficient of concordance of 1 indicated that there was a very strong difference in nitrate removal on each subsequent day.

In *Zygnema*, the difference in nitrate removal across the days day 1(median = 6.56), day 2 (median = 3.42), day 3 (median = 2.04) and day 4 (median = 1.07) was significant (X^2 (N

= 3) = 9, p = .02). Kendall's coefficient of concordance of 1 indicated that there was a very strong difference in nitrate removal on each subsequent day.

In *Oedogonium,* the difference in nitrate removal across the days day 1(median = 6.56), day 2 (median = 3.41), day 3 (median = 2.23) and day 4 (median = 1.01) was significant (X^2 (N = 3) = 9, p = .02). Kendall's coefficient of concordance of 1 indicated that there was a very strong difference in nitrate removal on each subsequent day.

The percentage of nitrate reduced by the algae between the first and second day was as follows; *Spirogyra* at 65.39 %, *Oedogonium* at 48.02 % and *Zygnema* at 42.98 %.The percentage of nitrate reduced by the algae between the second and third days show that *Oedogonium* led at 42.51 %, *Spirogyra* was at 26.43 % while *Zygnema* decreased the nutrient by 40.11 %. Between the third and fourth day, *Zygnema* reduced nitrate by 52.67 %, *Oedogonium* by 47.95 % and *Spirogyra* by 49.1 %.

In order to see if the different genera exhibited any differences in nitrate removal on each day, Mann-Whitney test was done. The results show that on day two of the study, there was no statistically significant difference between the median of nitrates reduced by *Spirogyra* and *Zygnema* (U = 1, p = .127). Similarly, no statistically significant difference was noted between *Zygnema* and *Oedogonium* (U = 4, p = .827), and between *Spirogyra* and *Oedogonium* (U = 1, p = .127).

On day three, the same test showed that there was a statistically significant difference between the median of nitrates removed by *Zygnema and Oedogonium* (U = .000 p = .05) and also between *Spirogyra and Oedogonium* (U = .000 p = .05). However, *Spirogyra*

and *Zygnema* median of nitrates did not statistically differ from each other on this day (U = 1 p = .127).

On day 4, it was observed that across the genera, there was no statistical difference in median of nitrates reduced by the algae. The median for nitrates reduced by *Spirogyra* and *Zygnema* was not statistically different from each other (U = 1 p = .127). Similarly, the median for nitrates reduced by *Zygnema* and *Oedogonium* did not statistically differ from each other (U = 3 p = .513) and also for the median for nitrates removed by *Spirogyra* and *Oedogonium* (U = 1 p = .127).

4.2 Potential Ability of Sewage to Support Algal Growth

The aim of this investigation was to assess the potential suitability of sewage to be an alternative growth media for algae. Growth was measured gravimetrically by using chlorophyll a concentration, a pigment found in all green plants. In the three genera grown on sewage, exponential growth occurred during the first three days. This was then followed by decrease in growth, while those grown on BBM progressed in growth throughout the experiment, as shown in the graph below;

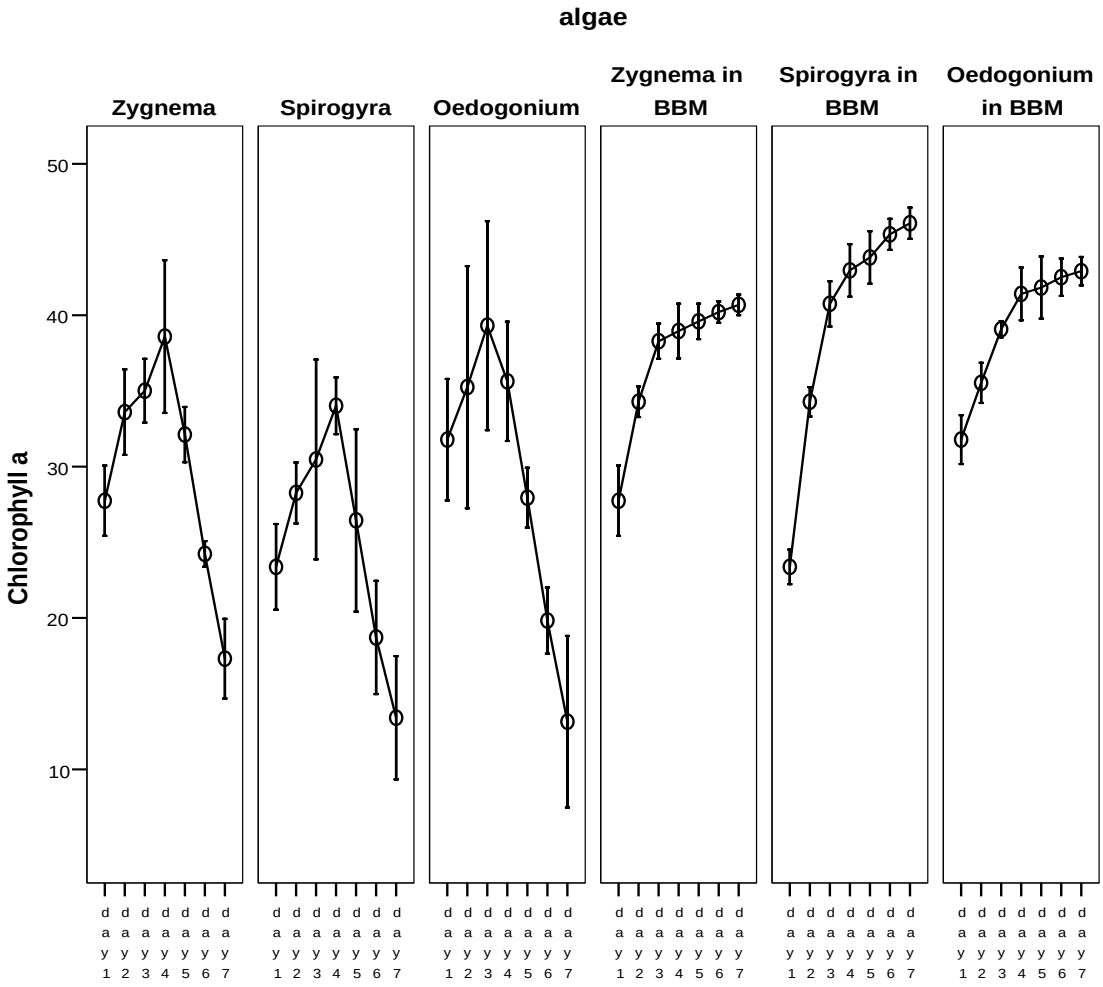

Figure 3 Growth of Algae in sewage

Due to the small data size in this study, the most appropriate statistical test was Friedman test. A Friedman test was conducted to determine if there was a significant change in levels of chlorophyll *a* that was accumulated by the algae between days 1, 2, 3, 4, 5, 6 and 7.

In *Spirogyra* the difference in chlorophyll *a* accumulated across the days day 1(median = 28.23), day 2 (median = 33.71), day 3 (median = 35.11) day 4 (median = 37.84), day 5 (median = 32.14), day 6 (median = 24.09) and day 7 (median = 17.06 was statistically significant (X^2 (N = 3) = 18, p = .006). Between day one and day two there was a percentage increase in chlorophyll *a* of 19.41 %, 3.99 % increase between the second and third day and 7.76 % increase between the third and fourth day. The fourth day had the highest amount of chlorophyll *a.* After day 4, the amount of chlorophyll *a* began to decline. There was a 15.06 % decrease in the pigment between days 4 and 5, 25.05 % decrease between days 5 and 6 and 29.18 % decrease between days 6 and 7. Day 7 had the least amount of chlorophyll *a.*

In *Zygnema*, the difference in chlorophyll *a* accumulation across the days day 1(median = 23.14), day 2 (median = 28.29), day 3 (median = 30.67), day 4 (median = 34.14), day 5 (26.85), day 6 (median = 18.59) and day 7 (median = 13.41) was significant (X^2 (N = 3) = 18, p = .006). There was a percentage increase in chlorophyll *a* of 22.26 % between day 1 and 2, 8.41 % increase between the second and third day and 11.31 % increase between the third and fourth day. The fourth day had the highest amount of chlorophyll *a.* After day 4, the amount of chlorophyll *a* began to decline. There was a 21.35 % decrease in the pigment between days 4 and 5, 30.76 % decrease between days 5 and 6 and 21.86 % decrease between days 6 and 7. Day 7 had the least amount of chlorophyll *a.*

In *Oedogonium*, the difference in chlorophyll *a* accumulation across the days day 1(median = 31.78), day 2 (median = 34.67), day 3 (median = 39.31), day 4 (median = 35.82), day 5 (27.79), day 6 (median = 19.88) and day 7 (median = 13.14) was significant

(X^2 (N = 3) = 17.71, p = .007). Between day one and two there was a percentage increase in chlorophyll *a* of 9.44 %, 11.52 % increase between the second and third day. In this genus, the third day had the highest amount of chlorophyll *a*. After day 3, the amount of chlorophyll *a* began to decline. There was an 8.88 % decrease in the pigment between days 3 and 4, 22.41 % decrease between days 4 and 5, 28.46 % decrease between days 5 and 6, and 33.90 % decrease between day 6 and 7. Just like in the other genera, day 7 had the least amount of chlorophyll *a*.

4.3 Determination of the Renewable Energy Potential of the Alga Grown In Sewage

4.3.1 Quantities of lipids in algae

Harvesting was done after seven days. The algae had turned color from dark green to light yellow, probably as a result of lipid accumulation.

Algae that had been grown on sewage produced more lipids than those grown on growth medium, as shown in figure 4 below;

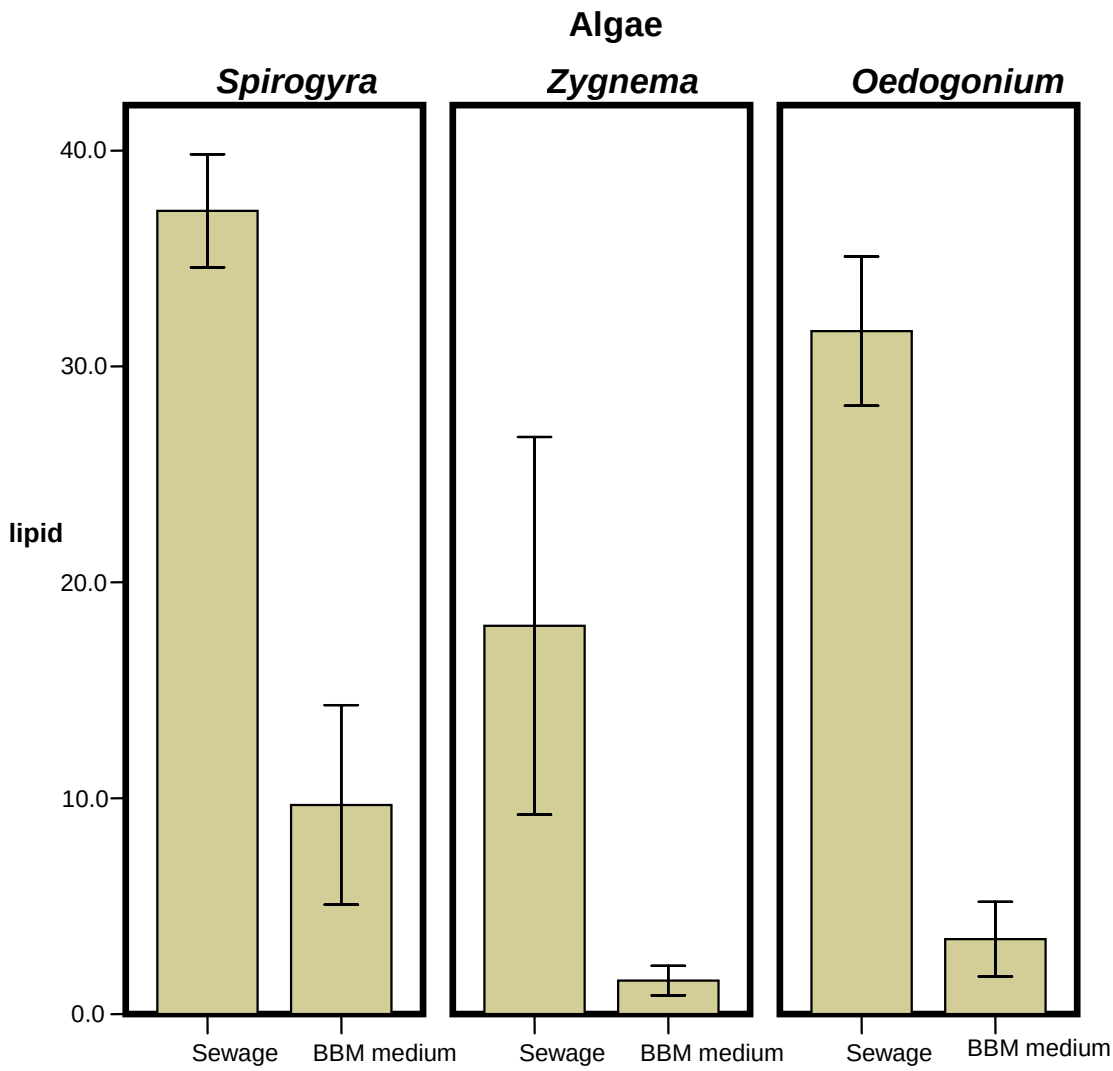

Figure 4 variations of lipid productions by various algae genera

Wilcoxon (Mann-Whitney) rank sum test was used to determine whether there was a statistical difference in lipid production between the algae that grew in sewage and those that grew in growth medium. In the three genera, it was shown that there was a statistically significant difference between the amounts of lipids that were accumulated

by algae that grew in sewage and those that grew in bold basal medium at the end of both treatments W (n_1 = 3, n_2= 3) = 6, p = .05). The algae that grew in sewage had a higher mean rank of 5 than those that grew in the medium, which had a mean rank of 2. This shows that the algae that grew in sewage accumulated higher amounts of lipid.

However, lipid accumulation tended to depend on algae genera. *Spirogyra* produced the highest amount of the lipid with a median of 23.86. *Zygnema* had the least amount of lipid in this study with a median of 7.47. Nevertheless, all the algae grown in the growth medium had similar amounts of the lipids with a median of 1.5.

The algae genera increased their lipid productivity when they were grown in sewage. A 20g portion of *Oedogonium* grown in 100ml of sewage had the highest increase in accumulation of lipid at 91.38%, whereas there was a 73.99% increase in lipid production when *Spirogyra* was grown on sewage. *Zygnema* increased their lipid production by 89.04% on sewage.

4.3.2 Estimates of starch in algae as feedstock for bioethanol

Three species of algae; *Spirogyra, Zygnema* and *Oedogonium* had been individually grown in the laboratory for seven days. The plants on treatment were cultured using secondary-treated sewage obtained from Moi university sewage treatment ponds. Those on control had been cultured in a growth medium (Bold Basal Medium). Starch was hydrolyzed using enzymes alpha-amylase and amyloglucosidase and estimated calorimetrically.

Results obtained show that those algae grown on the medium produced higher percentage of starch than those grown on sewage, as shown in figure 5 below.

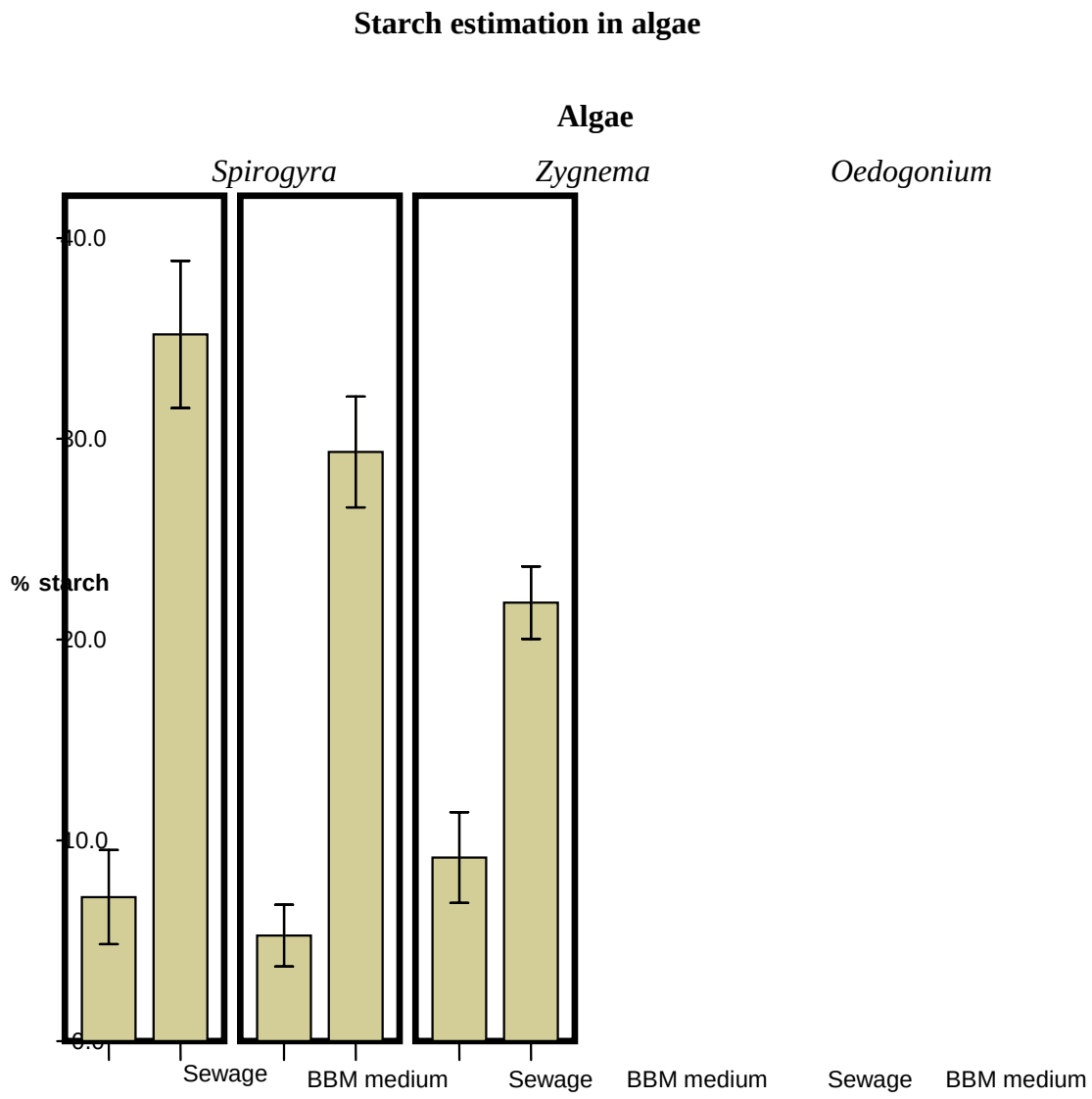

Figure 5. Starch production by algae genera

Wilcoxon sum rank test was used to determine whether there was a statistical difference in starch production between the algae that grew in sewage and those that grew in growth medium. The results showed that all algae genera that grew on the Bold Basal Medium accumulated more starch than those that were grown in sewage as shown by the high mean rank. The algae that grew in the medium had a higher mean rank of 5 than those that grew in the sewage, which had a mean rank of 2. However, in all the genera the algae that grew on sewage had a median of 1.5. On whether there was a statistical significant difference between the amounts of starch accumulated by those algae that grew in sewage and those that grew in the growth medium, the three genera had similar results. It showed that there was a statistical significant difference between the amounts of starch that were accumulated by algae that grew in sewage and those that grew in bold basal medium at the end of both treatments W ($n_1 = 3$, $n_2 = 3$) = 6, $p = .05$). In this study, *Spirogyra* was a high starch producer with a median of 20.81 *Oedogonium* had a median of 15.62, and *Zygnema* of 7.48.

In this study, it was observed that growth of the algae on sewage decreased starch accumulation, probably due to nutrient unavailability. The highest decrease was observed in *Zygnema* where growth in sewage led to a decrease in starch by 82.11%. In *Spirogyra*, the decrease was at 79.63% and *Oedogonium* at 58.13%.

Pearson correlation showed that the accumulation of starch in the algae had a negative but significant correlation with lipid production ($r = -.75$, $n = 18$, $p < .05$ two tails). When the algae accumulated starch, lipid productivity decreased and vice versa. This

observation was noted for all genera that grew in sewage as well as in the growth medium.

4.3.3 Study on energy content of Algae lipid

The energy contents of the lipid extracted from the algae was determined and the results are shown on the graph below.

Gross calorific values of algae

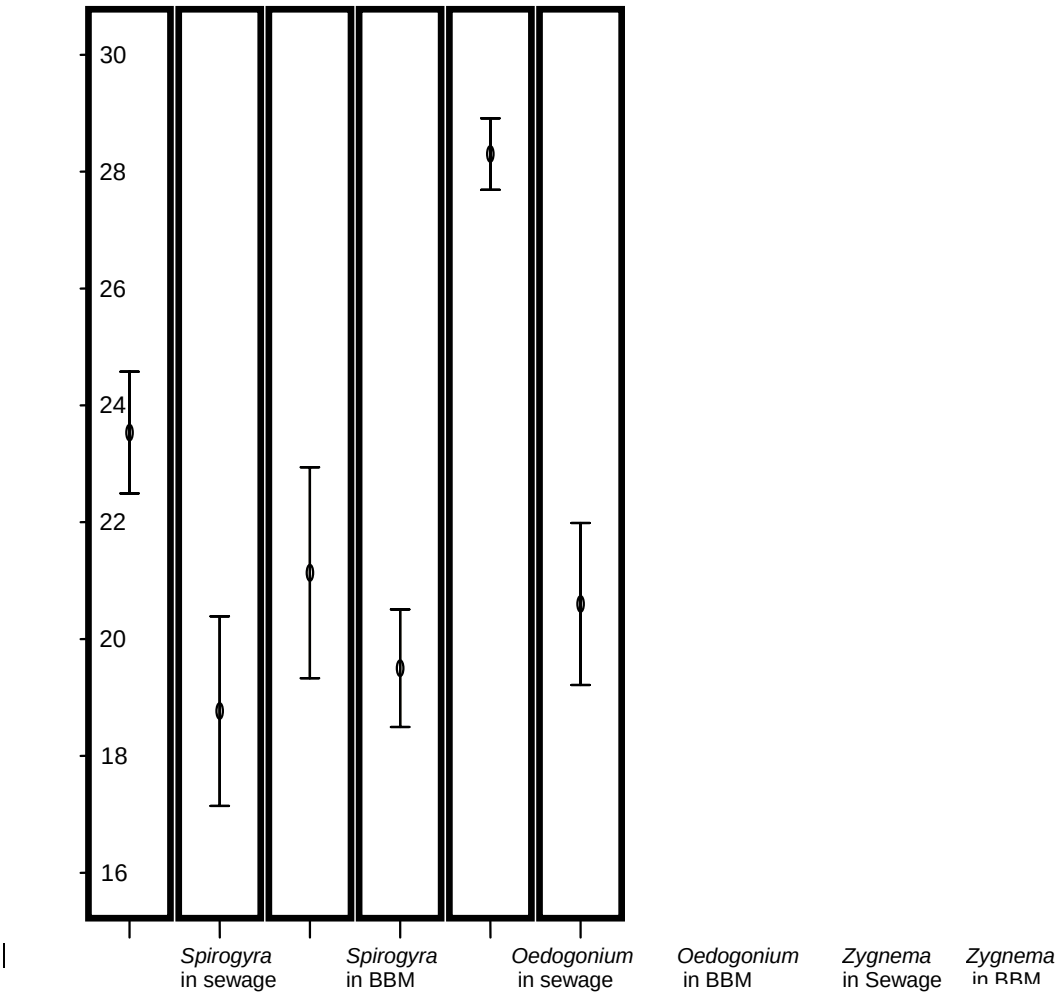

Figure 6. Calorific values of algal lipids

Wilcoxon rank sum test was utilized to determine whether there was a statistical difference in calorific values of lipids produced by the algae that grew in sewage and those that grew in growth medium. Results showed that in *Spirogyra* and *Zygnema*, the

calorific values of lipids from algae that grew in sewage and from those that grew in the medium showed a statistical significant difference W (n_1 = 3, n $_2$= 3) = 6, p = .05). The calorific value of the lipids from algae that grew in sewage was higher with a mean rank of 5, while those of algae that had grown in the medium had a mean rank of 2.

In *Oedogonium* however, the calorific values of lipids from algae that grew in sewage and from those that grew in the medium did not show a statistical significant difference W (n_1 = 3, n $_2$= 3) = 8, p = .275). However, in this genus the calorific value of the lipids from algae that grew in sewage was higher with a mean rank of 4.33, while those of algae that had grown in the medium had a mean rank of 2.67.

Of the three genera, calorific value of the lipid from *Zygnema* was higher at a median of 24.75, *Spirogyra* was at 21.35 while that from *Oedogonium* was 20. On the other hand, in all genera, it was observed that the calorific values of the lipids obtained from the algae grown on the medium had a similar median of 1.5.

Pearson correlation showed that amount of lipid did not correlate significantly with calorific value (r = .43, n = 18, n.s. two tails).

CHAPTER FIVE

DISCUSSION

Introduction

The declining non-renewable energy sources have led to a sharp increase in crude oil prices, which has in turn made the cost of living to rise. As a result both government and private sector are becoming involved in the development of alternative sources of energy. So far, most researches have concentrated on fuel from crop biomass such as maize for bioethanol as well as non-crop plants such as jatropha that use up a lot of land. The main problem with production of this biomass is the rise in global hunger due to conflict in land use between agriculture and biofuel production (Johansson and Azar, 2007). This study sought to use sewage-grown local genera of green algae to produce biomass for biofuel production as well as reduction of the nutrient quantities from the wastewater effluent.

5.1 Capacity of Algae Genera to Remove Nitrate from Wastewater

The results show that the algae genera were able to reduce nitrate in wastewater, leading to rejection of the null hypothesis. The algae were able to achieve this through uptake and assimilation of the nutrients; algae use inorganic nutrients for growth. The length of contact time the algae grew on the wastewater was important as it affected the amount of nitrate removed by the algae genera. *Spirogyra* removed the highest amount of nitrate than the other two genera, probably because it grew better in sewage.

Removal of nitrates from wastewater is significant because it prevents eutrophication of water bodies. Eutrophication is widespread because conventional treatment of wastewater is not efficient; therefore this study has shown that algae can be incorporated into sewage treatment. The specific use of algae in the efficient removal of different forms of nitrogen as well as phosphorus from waste stream has been reported. Of the three genera used in this study, *Spirogyra* has been frequently used in the past compared to the other two species. This can be attributed to the fact that *Spirogyra* is an abundant and widespread genus worldwide. In the study by Lei and Ma (2009), 3 g of *Spirogyra* decreased phosphorus by 96.84%, ammonium-nitrogen by 88.60% and total nitrogen by 85.49% from municipal sewage in 20 days. Similarly, Wen and Fan (2010) found that 80 mg/L of *Spirogyra* removed total nitrogen by 85.4% from eutrophic waters after a 20 day treatment. Other species of algae have also been utilized in the same area of study. Dominic *et al.,* (2009) found that *Chlorella vulgaris* and *Synechocystis salina* decreased nitrate content of water sample by 84 % and 82.5 % respectively within 10 days. In yet another study, *Chlorella vulgaris* decreased nitrates by 62.5% from wastewater (Wang *et al.,* 2009) and total Nitrogen by 92% in the study by Cho *et al.,* (2011). *Chlorella* and *Scenedesmus* have also been shown to remove very high (>80%) and in many cases almost complete removal of ammonia, nitrate and total phosphorus from secondary treated wastewater (Martinez *et al*., 2000; Ruiz-Marin *et al*., 2010; and Zhang *et al.,* 2008).

Research has also been done on the relationship between pond algae found in waste stabilization ponds and reduction in nitrogen in these ponds. Such studies have confirmed

that the reduction in nitrogen is due to algae uptake (Camargo *et al.,* 2007; Camargo *et al.,* 2009; Mara 2006). However, these algae are always left to die and decompose in the pond and the nutrients are recycled back into the wastewater, thus making the treatment ineffective. Furthermore, while algae effectively treat wastewater, their dominance in the oxidation pond ecosystem can cause high levels of total suspended solids in the final effluent and prevent the treatment meeting TSS standards. This study avoided such a scenario by using the algae biomass that had taken up nitrates for energy generation.

5.2 Growth of Algae in Sewage

The results showed a significant difference between growth of algae in sewage and in the Bold Basal growth medium, whereby growth in the medium was higher than in the sewage. However, wastewater was able to sustain growth of algae for a limited period until the nutrients got depleted. For this reason, the null hypothesis is rejected. Nitrogen is an important component of the chloroplast, the site of photosynthesis therefore depletion of the nutrient in the media led to decline in growth. *Zygnema* and *Spirogyra* grew better in sewage than *Oedogonium*.

These results are similar to the study by Singh and Dhar, (2007) in which several species of algae including *Nostoc, Anabaena, Plectonema* and *Chlorella* had lesser growth in wastewater in comparison to Bristol medium. However, another research showed sewage as a better growth medium than agricultural fertilizers for algae growth. Using *Tetraselmis chuii* and *Dunaliella viridis*, Costa *et al.,* (2004) found population densities of the algae grown in urban sewage were higher than those obtained with agricultural

fertilizers. The ability of an algae species to thrive in wastewater appears to be species dependent. This is because while the algae in this study grew better in the growth medium, *Chlorella sp* in the study by Cho *et al.,* (2011) showed a gradual increase in growth with time when they were grown on partial sewage.

5.3 Study on Renewable Energy Potential of the Algae

5.3.1 Estimation of Starch in algae

The algae genera produced starch which can be converted to bioethanol, a renewable energy. Results show that the algae that had been grown in the growth medium had more starch than those that grew on sewage. This is probably because those that grew on sewage depleted the nutrient concentrations in that sewage quickly therefore could not photosynthesize as those that grew on the growth medium. Starch, being products of photosynthesis could not be produced at high rates because their growth levels had slowed. On the other hand the algae that grew in the medium had all optimum nutrients necessary for growth, resulting in better development and accumulation of starch.

Previous researches have not dwelt on estimation of starch produced by algae; they have rather been done on the production of bioethanol from *Spirogyra* through fermentation process. An exception is that by Becker (2006), who found that *Spirogyra* had 64% carbohydrate content. Eshaq *et al.,* (2010 and 2011) on the other hand obtained 9.7% bioethanol by fermenting starch produced by the genera. Similarly, Sulfahri *et al.,* 2011 obtained 11.36% bioethanol through the same process.

In this study, it was observed that growth of the algae on sewage decreased starch accumulation, probably due to nutrient unavailability. This behavior contrasts what Dragone (2011) observed in *Chlorella vulgaris* where the algae increased starch content due to nutrient limitation. Other studies that have utilized *Spirogyra* in the production of bioethanol include those by Fuad *et al.,* (2011) and Sulfahri *et al.,* (2011).

5.3.2 Accumulation of Lipids by algae genera

The algae genera accumulated lipids, which can be transesterified to biodiesel, a renewable energy. However, the algae that grew in sewage produced more lipid that those that were in the growth medium. Of the three genera, *Spirogyra* had the highest amount of lipids irrespective of the growth media. It was followed by *Oedogonium* and *Zygnema*. Further, the study reveals that the algae genera increased their lipid productivity when they were grown on sewage. This shows similarity to the study by Mostafa *et al.,* (2012), where *Nostoc muscorum* increased the lipid productivity by 16.8% when grown on sewage but decreased it by 12.5% when cultured in synthetic media. In the same study however, *Anabaena flousaquae* accumulated higher amounts of lipids at 7.4% when they were grown on synthetic media, but had 5.5% lipid when they grew on sewage. In yet another research, algae accumulated an average lipid yield of 1.07g/100g dry weight when they were grown on wastewater (Zuka *et al.,* 2012).

In this study the variation between algae species in accumulation of lipids may be due to different reactions to abiotic stress such as nutrient depletion, particularly nitrate. A

decrease in the concentration of nitrogen in the medium resulted in a significant change in cell composition, favoring the accumulation of lipids. This altered metabolism towards lipid production is of beneficial role in algal tolerance or defense mechanism (Beer *et al.*, 2009; Hu *et al.*, 2008; Lardon *et al.*, 2009; Rodolfi *et al.*, 2008; Woertz *et al.*, 2009). *Spirulina platensis* that was studied by Uslu *et al.*, (2011) similarly accumulated large amounts of lipids when the concentration of nitrogen in the medium diminished.

Of the three algae genera used in this study, *Spirogyra* and *Oedogonium* have been utilized before for research on lipid production. However, none of the studies utilized sewage for growth. Murugan and Rao (2011) did a search for high biofuel-producing algae in Chennai, and found that *Spirogyra* and *Oedogonium* formed 7.2% and 9.2% total oil respectively. Hossain *et al.*, (2008) used common species *Spirogyra* and *Oedogonium* in Pakistan to compare biodiesel production and found that the former produced 7.3% and the latter 9.2% lipid. Likewise, Kumar *et al.*, (2011), while studying naturally occurring algae in north India found that on oil extraction, *Spirogyra* led with 14.82% total oil. Locally in Kenya, Rutikanga (2011) characterized the freshwater algae of JKUAT for bioethanol and biodiesel potential and found that *Spirogyra* had 2.18% oil content. Other studies that have utilized *Spirogyra* for biodiesel production due to its high lipid production include those by Binnal (2010). However, Woertz *et al*. (2009) determined the lipid content from mixed algae cultures, originally isolated from local wastewater treatment ponds, and grown in anaerobically digested dairy manure wastewater in outdoor batch cultures. After 6 days growth, peak lipid accumulation reached 14% to 29% dry weight.

From this study, it is evident that in the presence of optimum nutrients, these algae genera perform photosynthesis and accumulate starch, but when nutrients become limited, they accumulate lipids.

In conclusion, this study demonstrated that algae can produce lipids and starch over short periods of time without competing for arable land.

5.3.3. Determination of calorific value of algae lipid

Algae lipids in this study had high calorific values that qualify them to be used as raw material for biodiesel production. The results obtained show that the type of growth media in which the algae grew played a significant role in determining the calorific value of the lipids. This is because those algae that grew in sewage, irrespective of the genera, had high calorific values. The algae increased the calorific values when grown in sewage; from 18.77 kJ g^{-1} to 23.5 kJ g^{-1} for *Spirogyra*, 20.6 kJ g^{-1} to 28.3 kJ g^{-1} for *Zygnema* and 19.5 kJ g^{-1} to 21.1 kJ g^{-1} for *Oedogonium.* This is because those algae that grew in sewage produced high amounts of lipids as compared to those that were grown in the growth medium. Those algae had been growing for seven days in sewage and had depleted the entire nitrate in that medium. This deficiency probably led to nitrogen stress, an event known to trigger accumulation of lipids. Lipids in algae play a beneficial role in algal tolerance or defense mechanism. The present results are in agreement with those of Illman *et al.,* (2000), in which five strains of *Chlorella* grown in Watanabe and low-N media had high calorific value of 29 kJ/g when they grew in low-N medium. Besides low

nitrogen, the presence of salt in the growth medium also appears to trigger accumulation of lipids in algae. In the study of Taludkar *et al.,* (2012), calorific value of *Ankistrodesmus falcatus* biomass varied depending on the salinity of the medium, with algae grown under 160mM of NaCl having calorific value of 27.9 kJg^{-1}, while those grown on BG11 control had less calorific values of 19.1 kJg^{-1}.

It was also observed that oil from *Zygnema* had higher energy than that of the other two genera in spite of this genus being a low lipid producer in this study. However, lipid from *Spirogyra* and *Oedogonium* had similar calorific values. This can be attributed to variation between algal species in terms of biotic response to nitrogen depletion that triggered the increased accumulation of lipids.

Even though these calorific values are lower than those of Jatropha oil at 42.47 kJ g^{-1}, castor oil at 35.50 kJ $g^{-1}$and palm oil at 38.00 kJ g^{-1} (Okullo *et al.,* 2012), these other plants use up a lot of land during growth whereas algae have an added advantage as they grow in sewage. While they grow in the wastewater they remove nutrients that cause eutrophication. However, lipids from this study can be used to produce biodiesel because plant oils with calorific values from 5.45 kJ g^{-1} to 45.00 kJ g^{-1} are commonly used as raw materials for biodiesel production (Okullo *et al.,* 2012).

CONCLUSION AND RECOMMENDATION

From the results of this study, it is concluded that:

1. The algae genera *Spirogyra, Zygnema* and *Oedogonium* are capable of reducing nitrates contained in the wastewater, thus preventing eutrophication of receiving water bodies.
2. The algae genera *Spirogyra, Zygnema* and *Oedogonium* are able to grow in sewage.
3. *Spirogyra, Zygnema* and *Oedogonium* grown in sewage can accumulate oil and starch that can be converted into sources of clean energy. The lipids from algae grown in sewage have high calorific values.

The study also noted that there was a relationship between lipid content, growth of the algae and nutrient status of the growth media. There was an inverse relationship between growth and lipid accumulation. Lipid content increased within an alga during nutrient limitation, which also affected growth, thus providing an explanation for the relationship.

The following recommendations are suggested for future studies;

- These algae genera should be incorporated into conventional wastewater treatment for nitrate removal in order to control eutrophication of water bodies.
- Algae used in wastewater treatment should be harvested and used to produce renewable sources of energy.

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