PRODUCTON AND OPTIMIZATION OF BIOETHANOL FROM FRESH WATER MICROALGAL BIOMASS

BY

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DECLARATION

I hereby declare that this work is the product of my research efforts undertaken under the supervision of Dr. Muntari Bala and has not been presented anywhere for the award of degree or any certificate(s). All sources have been duly acknowledged.

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CERTIFICATION

This is to certify that the research work for this Dissertation and the subsequent write-up by Aminu Abdullahi (SPS/16/MBC/00046) were carried out under my supervision.

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I dedicated this humble effort to my Parent Mal. Abdullahi Bawa and Mal. Hafsat Isiyaku who are the fruit of my thought and whose affections are with me from cradle up till now.

TABLE OF CONTENTS

LIST OF TABLES

Table **Page No**

LIST OF FIGURES

FIGURE **Page No.**

LIST OF ABBREVIATIONS

i. RSM = Response surface methodology

ii. CCD= Central composite Design

LIST OF PLATES

ABSTRACT

Bio-ethanol from different kinds of biomass is one way to reduce both consumption of crude oil and environmental pollution. This study aimed at harnessing the potentials of microalgae as third generation biomass for bioethanol production. The hydrolysis of microalgal biomass was carried out using dilute acid, amylase and combine acid and enzyme hydrolysis. Fermentation of the algal hydrolysate was done using *Saccharomyces cerevisiae* isolated from sugarcane juice. The highest ethanol yield of 13.5% was obtained in hydrolysates of co-combination of dilute acid and enzyme complex while least ethanol concentration of 11.2% was observed in acid hydrolysis. Some important process conditions (pH, temperature and incubation time) were subjected to one factor at a time method in order to assessed their effect in relation to the bioethanol yield. Maximum bioethanol production was attained at pH 5.5, Temperature 35 ℃ and incubation period of 120hrs. However, when the process conditions were subjected to optimization using response surface methodology, the maximal bioethanol yield of 12 % was obtained when pH, temperature and incubation time were set at 5.2, 37.5℃ and 152hrs respectively. So also three dimensional contour surface interaction of the parameters shows positive effect of incubation time with less effect of pH and temperature ranges used respectively. Subjecting the produced bioethanol to FT-IR revealed the presence of single carbon bond (2981cm^{-1}), methyl (1417cm^{-1}) and OH group (3331 cm^{-1}) and boiling of 78.73 ± 0.03℃. Based on the result of this study fresh water *Spirogyra spp*, could serve as low cost biomass for bioethanol production using combined acid and enzyme hydrolysis at optimized pH, temperature and incubation time.

CHAPTER ONE

INTRODUCTION

1.1 Background

A development is 'sustainable' if it "meets the needs of the present without compromising the ability of the future generations to satisfy their own needs. The world population reached 7.3 billion in 2015, and projected to increase by 33% to reach 9.7 billion in 2050, and by 53% to cross 11.2 billion in 2100 (Thirumvengadathen and Thrimalai, 2017). This rapid growth of human population has led to mounting energy demands, which is projected to increase by 50% or more by 2030 and the fossil fuels cannot meet-up the current consumption rate, which is already reported to be 105 times faster than nature can create (Eyasu *et al*., 2018). Fossil fuels are non-renewable sources of energy and their global supplies are unlikely to last more than 120 years if they are to be used at even current rate of consumption (International Energy Agency 2007).

The search for 'clean' energy as an alternative has become the most overwhelming challenges of the 21st century but currently, several alternatives are being studied and implemented (Eyasu *et al*, 2018). Biofuels, (fuels from living organisms) as one of the promising alternatives, provide environmental benefits, since their use leads to a decrease in the harmful emissions of CO_2 and hydrocarbons with a consequent decrease in the greenhouse effects (Kyoung *et al.*, 2013).

Biofuels are liquid or gaseous fuels produced from plants, including microalgae, and seaweeds, municipal wastes and agricultural or forest by-products. Among biofuels, bioethanol, a renewable source of energy, has been accepted more widely as an alternative to fossil fuels (Sudhakar *et al*., 2016).

While biofuel production is cost ineffective and environmentally friendly. Biofuel can therefore be considered as the best alternative of decentralized energy source for developing countries including Nigeria especially in this era of insecurity and unpredictability in fossil fuel supply (Bugaje and Muhammad, 2008).

Nigeria's energy sector is largely dependent on fossil fuels. The major source of energy easily available for use in homes and industries are the various forms of refined fossil fuels such as petrol, gasoline and Dual Purpose Kerosene (DPK)., obtained through fractional distillation of crude oil. Finding sustainable alternatives or substitutes for these sub-sectors from renewable energy resources is necessary for a sustainable economic development of Nigeria (Idris and Awofu 2016).

There are two main global biologically derived transportation fuels: bioethanol and biodiesel (Nguyen *et al*., 2012). However, production of ethanol from sugars and starch-containing materials, referred to as first generation of ethanol, suffers from the debates in competing with human food. United States and Brazil are the leaders in making bioethanol from corn and sugarcane, respectively. In Europe and China, mainly cereals and sugars are used as the feedstock (Junying *et al*, 2015).

In contrast, the second generation of ethanol, produced from lignocellulosic materials, does not have a direct negative impact on food resources, although it may indirectly affect it by using agricultural lands for preparation of the lignocellulosic materials. In addition, due to the complex structure of lignocellulosic materials, different costly pretreatment techniques have to be employed to make the materials susceptible to biological conversions (Sulfahri *et al*., 2011).

Micro Algal feedstocks are regarded as one of the most promising non-food feedstocks for biofuels and that Algae based technologies could be a key tool for reducing greenhouse gas emission (Mamta and Rajiv, 2011). Zhenyi (2013) reported that microalgae is the dominant algae being researched for biodiesel production and can also be utilize for ethanol production by converting their storage material to fermentable sugars. The absolute absence or near absence of lignin makes the enzymatic hydrolysis of algal cellulose less costly and time saving in bioethanol production (Karunakaran *et al*., 2018). In addition, Micro algae have fast growing ability and requires much less water than the traditional cereal plant, produces more biomass, can be grown in salt water or in sewage water with minimal impact on freshwater resources, easily biodegradable and relatively harmless to the environment if spilled (Shukhadar *et al.,* 2016). Generally, microalgae (red, brown, and green) is obtained from natural and cultivated resources (Nguyen *et al.,* 2012). The harvested microalgae is mainly used for production of different hydrocolloids, e.g., agar and alginate and small amounts of these materials are also used for production of food (Yazdani *et al*., 2014).

Traditionally, the yeast *Saccharomyces cerevisiae* has been used all over the world as the major ethanol fermenting microorganism. The larger size, thicker cell wall, better growth at low pH, less stringent nutritional requirement and greater resistance to contamination give yeast advantages over bacteria for commercial fermentation (Tiwari *eta al,* 2015). Several microorganisms, including *Clostridium* sp., have been regarded as ethanologenic microrganisms, but the yeast *Saccharomyces cerevisiae* and facultative bacterium *Zymomonas mobilis* are better candidates for industrial alcohol production (Yazdani *et al*., 2014).

In general, the steps for bioethanol production from biomass include pretreatment, enzymatic hydrolysis, fermentation, and distillation (Sulfahri *et al*., 2016). Almost all kinds of microalgae can be converted to bioethanol by degrading their polysaccharides into corresponding monosaccharide, followed by fermentation with suitable microorganisms (Junying *et al*., 2015). However, the development of macroalgal conversion technology is still at an early stage, and the researches were conducted mainly on lab-scale (Junying *et al*, 2015).

1.2 Statement of Research Problem

One of the alternative renewable-based sources of energy being considered worldwide is bioethanol. Majority of the biomass for bioethanol production comes from terrestrial biomass which is essentially food and this has brought issues on food security, the usage of pesticides, and fresh water in their cultivation (Yazdani *eta al*, 2014). Problems associated with the bioethanol production from these sources have led to the development of a more sustainable alternative feedstock. Lignocellulosic biomass such as wood, agricultural, or forest residues has the potential to be an alternative bioenergy source. But due to its structural complexity, some form of pretreatment is necessary to make biomass more susceptible to enzymatic degradations. Moreover, current pretreatment methods employed on lignocellulosic materials are relatively costly and low yield (Junying, *eta l.*, 2015).

1.3 Justification

Although extensive efforts have been put in place to evaluate the potential of microalgae as a biofuel feedstock during the past 4–5 years but there is currently limited information on the state of Microalgal biomass conversion to ethanol (Nguyen *et al.,* 2012), despite

the fact that freshwater microalgae have numerous characteristics that favour its potential use as an alternative feedstock for biofuels production such as high carbohydrate content, and their tendency to form dense floating mats with very low or no lignin content (Karunakaran *et al*., 2018). That is why this research is designed to produce bioethanol from microalgal biomass.

1.4.0 Aim and Objectives

The aim of this study is production and optimization of bioethanol from microalgal biomass.

1.4.1 Objectives

The specific objectives are:

1. To identify and select the best microalgal biomass and *Saccharomyces cerevisiae* with bioethanol production potential.
2. To assess microalgal ethanol yield under different hydrolyzing agent (Acid, enzymatic and combine acid- enzyme hydrolysis.
3. To optimize pH, temperature and incubation time for bioethanol production from microalgal biomass.
4. To characterize the bioethanol produced.

CHAPTER TWO

LITERATURE REVIEW

2.1 Biofuels

At present, biofuels play an important role in the global energy scenario and have continuously attracts attention over the last few decades (Nguyen *et al.,* 2012). As fuels made from locally grown renewable sources, they have been considered as an alternative to expensive fossil fuels (Idris and Awofu, 2016). Interest in biofuels was reinforced in the later decades of the 20th century by various legislative and political acts (EPA, 1970). The oil embargo by the Organization of the Petroleum Exporting Countries (OPEC) in 1973-1974, which led to a sharp increase in crude oil prices, also led to worldwide interest in alternative energy sources, including biofuels.

2.2 Classification of Biofuel Feedstock

The classification for liquid biofuels are based on the feedstock from its produced. The basic classes include; first-generation, second-generation and third generation fuels. There are no strict technical definitions for these terms. The main distinction between them is the feedstock used.

2.2.1 First Generation Biofuel

A first-generation fuel is the one made from sugars, grains, or seeds, i.e. one that uses only a specific (often edible) portion of the above biomass produced by a plant, and relatively simple processing is required to produce a finished fuel (Idris and Owofu, 2014). First-generation fuels are already being produced in significant commercial quantities in a number of countries. The most well-known first-generation biofuel is

ethanol made by fermenting sugar extracted from sugar cane or sugar beets, or sugar extracted from starch contained in maize kernels or other starch-laden crops (Agwa *et al,* 2018). Global production of first-generation bio-ethanol in 2006 was about 51 billion litres, with Brazil (from sugar cane) and the United States (from maize) each contributing about 18 billion litres, or 35 per cent of the total (RFA, 2012).

2.2.2 Second Generation Biofuel

Second-generation fuels are those made from non-edible lignocellulosic biomass, either non-edible residues of food crop production (e.g. corn stalks or rice husks) or non-edible whole plant biomass (e.g. grasses or trees grown specifically for energy). The fuel properties of second-generation ethanol are identical to those of the first generation equivalents, but because the starting feedstock is lignocelluose, fundamentally different processing steps are involved during production (Idris and Owofu, 2013).

2.2.3 Third Generation Biofuels

Algae are gaining wide attention as an alternative renewable source of biomass for the production of bioethanol, which is grouped under the third generation biofuels‖ (Nigam *et al*., 2010). Algae used in the field of biofuels are typically referred to as microalgae, which are usually unicellular plant-like microorganisms capable of performing photosynthesis. The major drawbacks of first and second generation biofuels are overcome to a greater extent by third generation biofuels. First, they have 5–30 times higher biomass production per unit surface area. Second, they do not compete with traditional agricultural resources as they can be cultivated on non-arable land or on wastewater (Sulfahri *et al.,* 2016). Third, they are rich in oil being 20-50% dry weight of biomass in many species (Chisti, 2007).

2.3.1 Bioethanol

Bioethanol refers to ethanol that is produced from biological materials (biomass). It is colorless clear, water-free liquid with mild characteristic odor produced from the fermentation of sugar or converted starch. It is volatile, miscible with both water and non-polar solvents at ordinary conditions and has density of 0.792 g/cm3 at 15.5˚C (Offosu *et al.,* 2013). It has been proven to limit particulate emissions in compression ignition and spark-ignition engines (Idris and Owofu, 2016). Though bioethanol has a relatively lower energy density, it has a higher octane number (113) than gasoline (87-93) (Renewable Fuels Association 2012). Apart from its environmental benefits, use of bioethanol as fuel or as a low-cost octane boosting additive for gasoline also has economic benefits, as it can create new jobs, support agriculture based economy and help meet the energy needs of developing countries that lack sufficient fossil fuel reserves (Agwa *et al*. 2018). The main source of sugar required to produce ethanol comes from fuel or energy crops and plant (Offosu *et al.,* 2013). These fuel crops are normally grown specifically for energy use and include maize, wheat, waste straw of rice, sorghum and algae which is basically feedstock used in this research. Bioethanol produced from pretreatment and microbial fermentation of biomass has great potential to become a sustainable transportation fuel in the near future (Idris and Owofu, 2016).

2.3.1 Feedstock for bioethanol Production

Ethanol can be produced from different kinds of raw materials. Demiirba (2007)., classify raw material for ethanol production into; simple sugars starch and cellulose. Raw materials containing simple sugars, suitable for direct processing through fermentation include white beet, sugar cane, citrus fruits, tropical plants such as punk, and juice of trees like birches and maple and honey (Offosu *et al.,* 2013). Raw materials containing starch used for the production of ethanol include cereals such as rye, barley, corn, oat, wheat, sorghum etc. Most of the ethanol researches have used raw materials that contain starch. Hermann *et al.,* (1986) obtained maximum ethanol of 41-42 g/l from concentrated deproteinized whey having a lactose content of 23% using *Zymomonas mobilis,* immobilized with sodium alginate. Nimbkar *et al.,* (1989) successfully fermented unsterilized juice of sweet sorghum by using *Saccharomyces cerevisiae* strain 3319 and obtained maximum alcohol of 12.45% (v/v). Co-fermentation of sweet sorghum juice and grain was investigated by Gibbons and Westby (1989) for production of bioethanol and obtained 3.5% (v/v) ethanol. Marszałek and Kamiński (2008)., classify cellulosic biomass materials into four sub groups based on type of resource:

i. wood
ii. municipal solid waste
iii. waste-paper and
iv. crop residue resources

The lignocellulosic biomass comprises of cellulose, hemicelluloses and lignin. Cellulose is a linear, crystalline homo polymer with repeating units of glucose held together by beta-glucosidic linkages. The structure is rigid pretreatment is required to break it down

(Offosu *et al.*, 2013). Although many technologies are investigated but ethanol production from lingo-cellulosic biomass is not yet to commercial scale.

2.3.2 Microalgae as potential feedstock for bioethanol production

Algae are photosynthetic organisms comprised generally of macroalgae and microalgae (phytoplankton). Some microalgae species contain high amount of starch (37% dry weight), which can be converted to ethanol by fermentation (Hwang *et al.*, 2008). Anaerobic digestion (AD) is another biological conversion technique involving the breakdown of the organics in biomass into methane gas. Algae contain no lignin and are high in moisture content, making them a good material for AD and bioethanol production (Verma *et al.*, 2000).

2.4.1 Bioethanol production process

Currently there are a number of advanced technologies for bioethanol production in the world, depending on the raw material subjected to fermentation. Starchy materials require a reaction of starch with water (hydrolysis) to break down the starch into fermentable sugars (saccharification). According to Idris and Awofu (2016) bioconversion of biomass to ethanol requires four principal stages;

i. Pretreatment
ii. Hydrolysis (saccharification)
iii. Fermentation and
iv. product separation/ distillation.

2.4.2 Pretreatment

This refers to the solubilization and the separation of one or more of the four components of biomass ie hemicelluloses, cellulose, lignin and extractives to make the remaining solid biomass more accessible to further chemical or biological treatment (Saravamurugan and Rajendran, 2016). Thus pretreatment is required to alter the biomass macroscopic and microscopic size and structure as well as its chemical composition and structure (Moiser *et al*., 2005; Chandel *et al*., 2007). For pretreatment to be effective certain criteria have to be met; these include avoiding size reduction, preserving hemicelluloses fractions, limiting formation of inhibitors due to degradation products, minimizing energy input, and most of all cost effectiveness and moreover recovery of high value co-products (e.g. lignin and protein), pretreatment catalyst, catalyst recycling, and waste treatment are also considered (Zheng *et al*., 2009). Pretreatment is considered the most expensive processing step in the conversion of biomass to ethanol (Moiser *et al*., 2005). Therefore, in conversion of cellulosic material to bioethanol, pretreatment remains the major challenge (Chandel *et al*., 2007). However, in relation to microalgal biomass, very less or no pretreatment is required for bioethanol production due to very low or no lignin content in the microalgal cell wall. Idris and Awofu (2013) reported an increase in amount of bioethanol produced with the un-pretreated *Spirogyra* biomass compared to the pretreated spirogyra biomass. According to Zheng *et al.,* (2009) pretreatment techniques are divided into three basic groups; physical, chemical and biological pretreatments.

2.4.3 Physical pretreatment

Physical pretreatment includes chopping, grinding and milling and can be applied to reduce the size of substrate and increase the surface area for enzyme activity. Corn ethanol producers use grinding and milling; these reduce the size of the corn kernels and open them for enzymatic hydrolysis (Zheng, 2009). Unlike macro and micro algae cellulosic and lignocellulosic raw materials require much more intense physical pretreatment (Sulfahri *et al.,* 2016).

Pyrolysis is also another physical method of pretreatment used to treat lignocellulose materials and involves treating cellulose at temperature greater than 300^{o}C to decompose the cellulose to produce gaseous products and residual char (Kilzer and Broido, 1965). Lignocellulose materials due to their high lignin content require the combination of both physical and chemical pretreatment methods and most commonly used physicochemical method is the steam explosion (Offosu *et al.,* 2013).

2.4.4 Chemical pretreatment

Reported chemicals used for pretreatment of cellulosic materials include dilute acid, alkaline, organic solvent, Ammonia, Sulphur dioxide, Salts like sodium carbonate $NaCO_3$ and sodiumsulphate $NaSO_2$. Both concentrated and dilute acids such as HCl and H_2SO4 have been used in the pretreatment of lignocellulose materials (Offosu *et al.,* 2013). However, both acids are toxic, corrosive and hazardous and therefore require reactors that are resistant to corrosion, as such their use as agents of pretreatment has not been limited (Siveres and Zacchi, 1995; Sun and Cheng, 2002). Saha *et al*., (2005) reported success using dilute acid pretreatment and many researches have been done with different substrate such as wheat straw wheat bran (Palmarola-adrados and Galbe 2005). Bases

such as NaOH have also been used in the pretreatment of lignocelluloses materials. Krishnn *et al*., (1999) used alkali (NaOH) to treat sugarcane leaves for bioethanol production. Organic solvents such as methanol, acetone, ethylene, glycol, triethylene and tetrahydrofurfural alcohol (Chum *et al*., 1988) have also been used in pretreatment of lignocelluloses. Dilute NaOH was also found to be effective in treating straw with low lignin content (Bjerre *et al*.,1996). Sulfahri *et al.,* (2016) suggested the use of dilute NaOH in the pretreatment microalgal biomass (*Spirogyra hylina*).

2.4.5 Biological pretreatment

Most of the pretreatment techniques described require expensive instruments or equipment and high energy and majority of the waste generated from chemical pretreatment can be hazardous to the environment (Offosu *eta al.,* 2013). Thrivangadathan and Thrimulai (2017) recommended the use of microorganisms and their enzymes for pretreatment which is confirmed to be cheap and environmentally friendly. Brown rots, white rot and soft rot fungi are known to attack different components of cellulose and lignocellulosic materials. Brown rot fungi attack only cellulose while imparting minor modifications to lignin, while white and soft rot fungi attack both cellulose and lignin. The ligninolytic enzyme system is an extracellular enzymatic complex that includes peroxidases, laccases and oxidases responsible hydrolyzing all the component of Lignocellulosic material (Martinez *et al,* 2000). These enzymes systems are found to possessed different characteristics depending on the species, strains and culture conditions. Hwang *et al.,* (2004), studied the biological pretreatment of wood chips using four different white-rot fungi for 30 days and found that the glucose yield of pretreated wood by *Trametes versicolor* MrP 1 reached 45% by

enzymatic hydrolysis while 35% solid was converted to glucose by the fungi. A Japanese red pine *Pinusden siflora* (softwood) was pretreated biologically by white-rot fungi of *Ceriporia lacerata, Stereum hirsutum,* and *Polyporus brumalis.* It was found that *S. hirsutum* was the most effective in degrading lignin and improving the enzymatic digestibility of wood (Lee *et al.*, 2007). Keller *et al*. (2003), reported a 3- to 5-fold improvement in enzymatic digestibility of corn stover after pretreatment with *Cyathus stercoreus*

2.5.1 Hydrolysis

Hydrolysis or Sacchrification of the cellulose and hemicellulosic components to monomeric sugar is next step in bioethanol production after pretreatment. There are two types of hydrolysis commonly used: Chemical hydrolysis (dilute and concentrated) and enzymatic hydrolysis.

2.5.2. Acid hydrolysis:

Basically there are two types of acid hydrolysis; dilute and concentrated acid hydrolysis. Dilute acid hydrolysis is conducted at high temperature and pressure and has short reaction time. The biomass is mixed with dilute sulphuric acid and held at temperatures ranging from 120-220^{o}C for short period of time to hydrolyse the hemicellulose Cellulose and to xylose and other reducing sugars respectively (Agwa *et al,* 2018). The disadvantage of using dilute acid hydrolysis is that at high temperature or long residence time the monosaccharides produced degrade and give rise to fermentation inhibitors like furan compounds, weak carboxylic acids and phenolic compounds (Offosu *et al.,* 2013). These inhibitors affect the performance of the ethanol producing microorganism (Chandel *et al*., 2007). To remove these inhibitors and increase the hydrolysate

fermentability, several chemicals and biological methods have been used. These methods include over liming (Martinez *et al*., 2000), charcoal adsorption (Chandel *et al*., 2007), ion exchange, detoxification with laccase and other biological detoxification. The cost is however higher than the benefits achieved (Offosu *et al,* 2014). Concentrated acid hydrolysis uses concentrated acid followed by dilution with water to dissolve the hydrolysed sugar (Offosu *et al,* 2014). The process allows for complete and rapid conversion of cellulose to glucose and hemicelluloses with a little degradation (Chandel *et al*., 2007). The concentrated acid process uses 70% sulphuric acid at between 40 – 50^{o}C for 2 to 4 hours.

2. 5.3 Enzymatic hydrolysis

Lignocellulosic materials can be hydrolyzed enzymatically to get fermentable sugars (Agwa *et al.,* 2018). Bacteria and fungi are a good source of cellulases and hemicellulase that could be used for the hydrolysis of pretreated lignocelluloses. This involve the use of enzymatic cocktail consisting of different hydrolytic enzymes such as cellulases, xylanases and mannanases (Chandel *et al*., 2007). Large numbers of microorganisms are capable of degrading cellulose (Offosu *et al.,* 2013). For fungi, members of the genera that have received considerable attention under aerobic conditions are *Chaetomium,* and *Helotium* (Ascomycetes); *Coriolus, Phanerochaete, Poria, Schizophyllum* and *Serpula* (Basidiomycetes); *Aspergillus, Cladosporium, Fusarium, Geotrichum, Myrothecium, Paecilomyces, Penicillium,* and *Trichoderma* (Deuteromycetes) and *Mucor* (Zygomycetes) (Offosu *et al.,* 2013). However, Rajesh *et al.* (2008), reiterated that only a few of these microorganisms are known to produce significant quantities of cell-free enzymes capable of completely hydrolyzing crystalline cellulose *in vitro.*

2.6.0 Fermentation

A common element essentially to all proposed processes for producing ethanol from cellulosic biomass is microbial fermentation. A variety of microorganisms, generally bacteria, yeast, or fungi ferment carbohydrates to ethanol under oxygen-free conditions (Thrivangadathan and Thrimulai, 2017). According Stewart *et al.* (1983), microorganisms carrying out fermentations do so to acquire energy inform of ATP and are thus dependent upon ethanol production for growth and long-term survival.

2.6. Product separation techniques

2.6.1.1 Distillation

Fermentation by-products are mostly removed by distillation. Distillation is the most commonly and recognized industrial purification technique of ethanol. It utilizes the differences in boiling point of components in a mixture. The principle of operation is by heating a mixture so that low boiling point components are concentrated in the vapor phase. By condensing this vapor, more concentrated less volatile compounds are obtained in liquid phase. Distillation is one of the most efficient separation techniques. However, it contains several problems. One is separation of volatile compounds. In ethanol production, a distillation tower is designed to separate water and ethanol effectively. Water is obtained from the bottom of the tower and ethanol is obtained from the top of the tower. It is expected that impurities with similar boiling points to ethanol lodges in ethanol even after distillation.

2.6.1.2 Adsorption

Adsorption is a separation technique utilizing a large surface area of adsorbent. Compounds are simply adsorbed on the adsorbent depending on their physical and

chemical properties. In general, bigger particles tend to be adsorbed more due to their low diffusivities. Also, compounds with the similar polarity to the adsorbent surface tend to be adsorbed more. When purification of ethanol is considered, non-polar surface and wide ranging pore distribution are favorable since ethanol is polar compounds and various sizes of particles could be contained in ethanol as impurities. From water treatment, activated carbon) and activated alumina are the most expectable adsorbents (Demirbas *et al.,* 2008).

2.7.1 Potential Microbes for Ethanol Production

Microorganisms which have received attention in industrial alcohol production include a wide range of yeasts, some molds and a number of specialized bacteria. According to Stewart *et al*. (1983), the microorganism being employed in the production of bioethanol should possess the following important characteristics;

i) rapid and relevant carbohydrate fermentation ability, ii) appropriate flocculation and sedimentation characteristics, iii) genetic stability, iv) osmotolerance (i.e., the ability to ferment carbohydrate solutions), v) ethanol tolerance and the ability to produce elevated concentration of ethanol, vi) high cell viability for repeated recycling and vii) temperature tolerance. In addition to these characteristics a good candidate should be able to use a wide range of sugar sources.

2.7.2. Yeast

Many types of yeasts with potential ability to convert simple sugars to ethanol have being reported. Different species of yeast require different simple sugar, for example genus the *Saccharomyces cerevisiae* converts glucose, fructose, galactose, maltose, maltotriose and xylose to ethanol while *S. rouxii* utilizes glucose, fructose, maltose and sucrose as carbon

source (Thrivangadathan and Thrimulai, 2017). Some species of yeast have also been found to utilize aldo-pentose and ketopentose. For example, *Candida utilis* is able to utilize xylose aerobically for growth but not for anaerobic fermentation**.**

At present over 95% of the ethanol produced globally involves the use of the yeast species *Saccharomyces cerevisiae* and its related species. *Saccharomyces* and a number of other yeast produce ethanol from carbohydrate via the Entner-Deodoroff pathway where theoretical yield of 0.51g of ethanol and 0.49g of CO2 are produced from 1g of glucose (Offosu *et al.,* 2013).

Yeasts will require addition of gluco-amylases to utilize starch completely. Yeasts cannot also utilize cellulose, hemicelluloses and cellobiose. This inability of yeast to ferment a diversity of cheaper and readily available substrates is a major obstacle to lowering the cost of alcohol. The utilization of sugar to produce ethanol by yeast starts with either its passage into the cell across the cell membrane, or its hydrolysis outside the cell membrane followed by entry into the cell by some or all of the hydrolysis products (Stewart *et al.*, 1983). For example, maltose and maltotriose are absorbed directly across the cell membrane while melibioses and sucrose are hydrolysed by extracellular enzymes and the products taken into the cell (Offosu *et al.,* 2013). The uptake and metabolism of sugars in a mixture occurs in an order determined by regulatory mechanism at the level of gene expression (Agwa *et al.,* 2018). For instance, glucose is the preferred substrate; therefore, the presence of glucose suppresses the induction of other sugar permeases. These substrates are therefore fermented sequentially rather simultaneously (Agwa *et al.,* 2018).

2.7.3 Bacteria

Several bacteria, including *Clostridium* sp, *Zymomonas mobilis*, *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella oxytoca* have been reported as ethanologenic microbes. However, *Zymomonas mobilis*, a gram-negative bacterium, is considered as alternative organism to yeast in large-scale bioethanol production (Agwa *et al.*, 2018). Comparative laboratory and pilot-scale studies on kinetics of batch fermentation of *Z. mobilis* versus a variety of yeast by have indicated the suitability of *Z. mobilis* over yeasts due to the following: its higher sugar uptake and ethanol yield, its lower biomass production, its higher ethanol tolerance, it does not require controlled addition of oxygen during the fermentation, and its amenability to genetic manipulations (Gunasekaran and Raj, 1999). However, compared to the yeast, its utilizable substrate range is restricted to glucose, fructose, and sucrose. Also under anaerobic conditions, *Z. mobilis* produces by-products such as acetoin, glycerol, acetate, and lactate, which result in reduced production of ethanol from glucose (Gunasekaran and Raj, 1999). Itis one of the few facultative anaerobic bacteria which metabolizes glucose and fructose via the Entner–Deudoroff (E–D) pathway, which is usually present in aerobic microorganisms (Agwa *et al.*, 2018).

2.8.0 Factors affecting Fermentation Process

A number of factors known to affect fermentation of sugars to ethanol were studied. Among these important parameters are; pH, Temperature, incubation time, osmotic pressure, Ethanol tolerance etc.

2.8.1 Effect of pH

pH is regarded as one of the most important fermentation parameters due to its effect on growth of microorganism, fermentation rate and by-product formation (Thrivangadathan

and Thrimulai, 2017). Maintenance of pH is therefore of great very importance in fermentation. Offosu *et al.,* (2013) measure changes in pH during the fermentation process by *Zymomonas mobilis* and the results showed that pH fluctuates during the fermentation process. Sulfahri *et al.,* (2016) evaluated the efficiency of *Saccharomyces cerevisiae* and *Zymomonas mobiles* in the pH range of 4.0 to 6.5. In both organisms, ethanol concentration increased with increasing pH until the optimum pH was reached. Yeast and fungi tolerate a range of pH 3.5 to 5.0. Srivastava *et al.* (1997) showed that the optimum initial pH of guava pulp medium for the production of bioethanol was l5.0 for all the three strains of *S. cerevisiae* employed and obtained maximum yield of 5.8% during 36hours of fermentation.

2.8.2 Effect of temperature

Temperature is known to play crucial role in the growth and activities of microorganisms. All organisms thrive best at a particular optimum temperature. The optimum temperature depends on whether the organism is mesophilic or thermophilic. Mesophilic organisms function between 25° to 40°C while thermophilic function between 40-75^{o}C (Lamed and Zeikus 1980). Operation at greater temperatures is desirable for a number of reasons: high fermentation temperature increases growth rate and productivity exponentially when the ethanol producing organism can thrive at such temperature; plant capital cost is less due to higher productivity per unit volume of fermentor vessel and cooling equipment investment is lowered; operating costs are less since less energy is required to maintain desired fermentation temperature and recover the ethanol. Contamination risk is less as fewer organisms exist at high temperatures (Agwa *et al.,* 2018). Enzymatic saccharification can operate up to 55°C and may be combined with fermentation, further

reducing capital and glucose inhibition (Srivastava *et al.* 1997). Krishnan *et al.* (1999) studied a simultaneous saccharification and fermentation in the fluidized bed reactor system. The hydrolysis and fermentation steps were performed at the optimum temperature of 55 and 30º C respectively and were able to obtained ethanol of 19 to 25 g l^{-1}.

2.8.3 Incubation Time

Mixture of damaged sorghum and rice grains were utilized by Suresh *et al.*, (1999) for bioethanol production and obtained ethanol yield of 2.90% (V/V) at 30º C after 5 days of fermentation. Higher ethanol yield was achieved in 3 days during fermentation of yam to ethanol by *S. cerevisiae* as observed by Ramanathan (2000). An experiment was conducted for conversion of raw starch to bioethanol and was 72.2 g l^{-1} bioethanol was produced in 120 minutes (Krishnan *et al.*, 1999). The effect of four different fermentation periods *viz*., 24, 48, 72 and 96 hours on ethanol production from starch medium was studied by Verma *et al.,* (2000). A maximum ethanol concentration of 24.8 g l^{-1} at 48 hours was achieved as compared to 13.7 and 21.6 g l^{-1} at 24 and 96 hours respectively.

2.8.4 Alcohol Tolerance

Some fermenting organisms cannot tolerate ethanol concentrations above 10 to15% (w/v) becouse enzymatic proteins become denatured (Offosu *et al.*, 2013). Higher temperature lowers the tolerance of the organism. High alcohol concentration may also disrupt the structure of microbial membrane and makes it less stable. Although ***S. cerevisiae*** and related species produce ethanol with apparent ease, it is toxic to the cell at levels ranging between 8 and 18% (w/v) ethanol, depending on the strain of yeast and the metabolic state of the culture. One of the groups of chemicals that have been known to affect the

tolerance of yeast to ethanol has been unsaturated fatty acids. Susan *et al.,* (1978) have shown that cells grown in the presence of linoleic acid are more tolerant to added ethanol than those grown in the presence of oleic acid.

2.9.0 Optimization of bioethanol production using Response Surface Methodology(RSM)

Response Surface Methodology (RSM) is a collection of mathematical and statistical techniques useful for the modeling and analysis of problems in which a response of interest is influenced by several variables and the objective is to optimize this response (Montgomery 2005). The response surface methodology (RSM) is extensively used in bioethanol production as this model predicts experimental modifications like changes in operational conditions, various processing steps, which ultimately help in designing an experimental setup with minimum requirements and maximum yields (Elibol, 1999).

The traditional method for optimization of parameters involves optimizing one parameter at a time. This is not only a time-consuming process, but often misses the alternative effects between components. Limitations of one-at-a-time-parameter optimization can be eliminated by employing Response surface method (RSM) which is used to explain the combined factors in a fermentation process (Elibol, 1999). Generally, RSM defines the effect of the independent process variables, alone or in combinations, and generates a mathematical model that describes the entire process. Also, the RSM summarizes mathematical methods and statistical inference for an approximate functional relationship between a response variable and a set of design variables. The most popular RSM is the Central composite design (CCD) which is an efficient and flexible technique that provides sufficient information on the effects of process variables and overall

experimental error with a minimum number of experiments. In the purpose of present work, response surface methodology was used to optimize the culture conditions.

2.9.1 Advantages and Disadvantages of bioethanol over other fuel types

Bioethanol as a transport fuel tables numerous advantages over traditional fuel such as:

i. Premature ignition and prevention of cylinder knocking due to the higher octane number and higher heat of vaporization compared to traditional fuel (Balat *et al*., 2008.

ii. Reduction in hydrocarbon and carbon monoxide exhaust emission based on the higher oxygen content of bioethanol (Demirbas, 2008).

iii. In an internal combustion engine, the lower energy content of bioethanol fuel blend as the compression ratio is higher and burn time is shorter (Lucia, 2010).

iv. The blending or mixing of bioethanol with traditional or other kinds of fuel is compatible with current engine designs. (Demirbas, 2008).

v. Bio-ethanol is chemically miscible in petrol (Demirbas, 2007).

However, there are disadvantages associated with bio-ethanol which are as follows:

i. Combustion of bio-ethanol when blended with petrol, releases formaldehyde and acetaldehyde, which are toxic to human (Demirbas, 2011).

ii. The use of agricultural products such as cereal grains will limit food and feed reserves in developing countries, leading to food crisis. (Chakauya *et al.*, 2009).

2.9.2 Economic Implication of Bioethanol Production

There exists today a significant demand in industrialized countries for biofuels, driven largely by regulatory mandates for blending of biofuels into petroleum fuels. This demand is likely to grow considerably in the years ahead, driven by increasingly ambitious regulatory mandates, sustained high oil prices, and energy security concerns (Idris and Awofu, 2014). Biofuel demands in many developing countries will also grow, driven by similar factors. Opportunities for trade in biofuels or biofuel feedstock will be expanding. Ethanol production from renewable sources such as corn, sugarcane and microphytes may bring important benefit to the entire world economy (Idris and Owofu, 2014). More specifically, production of ethanol will prove to be extremely beneficial to the countries with developing economy (Idris and Owofu, 2014). For the local community investment in both developed and developing countries associated with constructions production facilities bioethanol production will leads to generation of new high-paying jobs for the people and generate income for the households and also boost the general economic activities of the area. One way of improving rural economies is adding value to farm product locally rather than selling those products raw (Offosu *et al.*, 2013). According to Swenson, (2008) is very straightforward: processing of the agricultural products in the area helps to substantiate and stabilize local production and, through the processing, adds economic value to the commodities as additional income to farmers and workers in any given area. The use of agricultural waste for bioethanol production will increase market opportunities for the farmers in addition to selling their product, the wastes from their crops can also be sold to the ethanol producing industries. Farmers can also become stakeholders in the bioethanol producing companies, thus

creating more income for them (Swenson 2008). To some countries like Nigeria, ethanol producing industries will stimulate capital investment. Mrbanchuk (2008)., reported that, as in 2007 bioethanol producing industries has provided employment to 238,000 workers in all sectors of the U.S economy, adding $47.6 billion to the nation's GDP, and $12.3 billion from the pockets of consumers.

2.9.3 Environmental Impact of Bioethanol Production

When ethanol is combusted, the released carbon dioxide is recycled into plant material being it the major ingredient to synthesize carbohydrate during photosynthesis cycle; ethanol production is therefore a closed carbon dioxide cycle (Wyman, 1999). Becouse bioethanol production process utilizes only energy from renewable sources there is no net carbon dioxide added to the atmosphere, this is what makes bioethanol an environmentally friendly energy source (Foody, 1988). In addition, the toxicity of the exhaust emissions from ethanol is lower than that of petroleum sources (Idris and Awofu, 2016). Ethanol derived from biomass is the only liquid transportation fuel that does not contribute to the greenhouse gas effect (Foody, 1988). This will hence reduce the threat of global warming and its consequence which include transformation of forest into desert. The release of carbon dioxide from combustion of fossil fuel in automobiles is the biggest single concern. Therefore, as energy demand increases, the global supply of fossil fuels will cause more harm to human health by contributing to the greenhouse gas (GHG) emission. The main advantage of utilizing biomass conversion into ethanol is the reduction of GHG pollution of the atmosphere (Demirbas, 2007). Wyman and Hinman, (1990)., reported that, the amount of carbon dioxide released when fuel is produced and then burned is equal to the amount of ethanol needed to replenish the plant needed to

produce the ethanol. The ethanol blended diesel (E10 and E30 simultaneously decreases octane number, high heating value, aromatic fractions and kinematic viscosity of the blended fuels and changes distillation temperatures (Bang-Quatn *et al.*, 2003). These factors, according to Chandel *et al.*, (2007), are what leads to the complete combustion of ethanol with less emission. Bioethanol has the ability to reduce ozone precursors by 20-30% (Idris and Owofu, 2014). However, one draw-back associated with the use of ethanol as fuel is the emissions of aldehyde, predominantly acetaldehydes, which are higher in ethanol than those of gasoline. However, acetaldehydes emissions pose less adverse health effects in comparison to formaldehydes emitted from gasoline engines (Gonsalves, 2006). Another issue of environmental benefit of the production of ethanol is the energy balance of ethanol. An amount of energy is required to produce ethanol; i.e. energy is required to grow, harvest, transport and prepare the feedstock for ethanol production. Energy is also required for fermenting the feedstock to ethanol. The total amount of energy input into the ethanol production process compared to the energy released by burning the resulting ethanol fuel is known as energy balance. According to critics, the non-renewable energy required to grow and convert feedstock into ethanol is greater than the energy value present in the ethanol fuel (Pimentel, 2001). However, report by Shapouri *et al.* (2002) revealed that production of corn-ethanol is energy efficient, in that it yields 34 percent more energy than it takes to produce it, including growing the corn, harvesting, transporting, and distilling it into ethanol.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Chemicals and Reagents

All chemicals and reagents used in this study were of analytical grade and were obtained from Laboratory Chemicals of Departments of Biochemistry, Chemistry, Microbiology and Center for Dry Land Agricultural Research, Bayero University Kano.

3.2 Study Design

Water sample and sugarcane were collected from Ajiwa Dam and Yan-rake Market respectively. The water sample was examined microscopically for microalgae. *Spirogyra sp* being the dominant species was cultured for bioethanol production. Following post-harvest processing the *Spirogyra* biomass was hydrolyzed prior to fermentation. Yeast (*Saccharomyces cerevisiae)* was isolated from the sugarcane juice and inoculum was prepared for fermentation of microalgal biomass for bioethanol production. Effect of production pH, temperature and incubation time was also assessed and same conditions were also optimized using response surface optimization process. The physicochemical characteristics of produced ethanol was determined and compared with commercially produced ethanol. The schematic representation of overall process is shown in (figure 3.1).

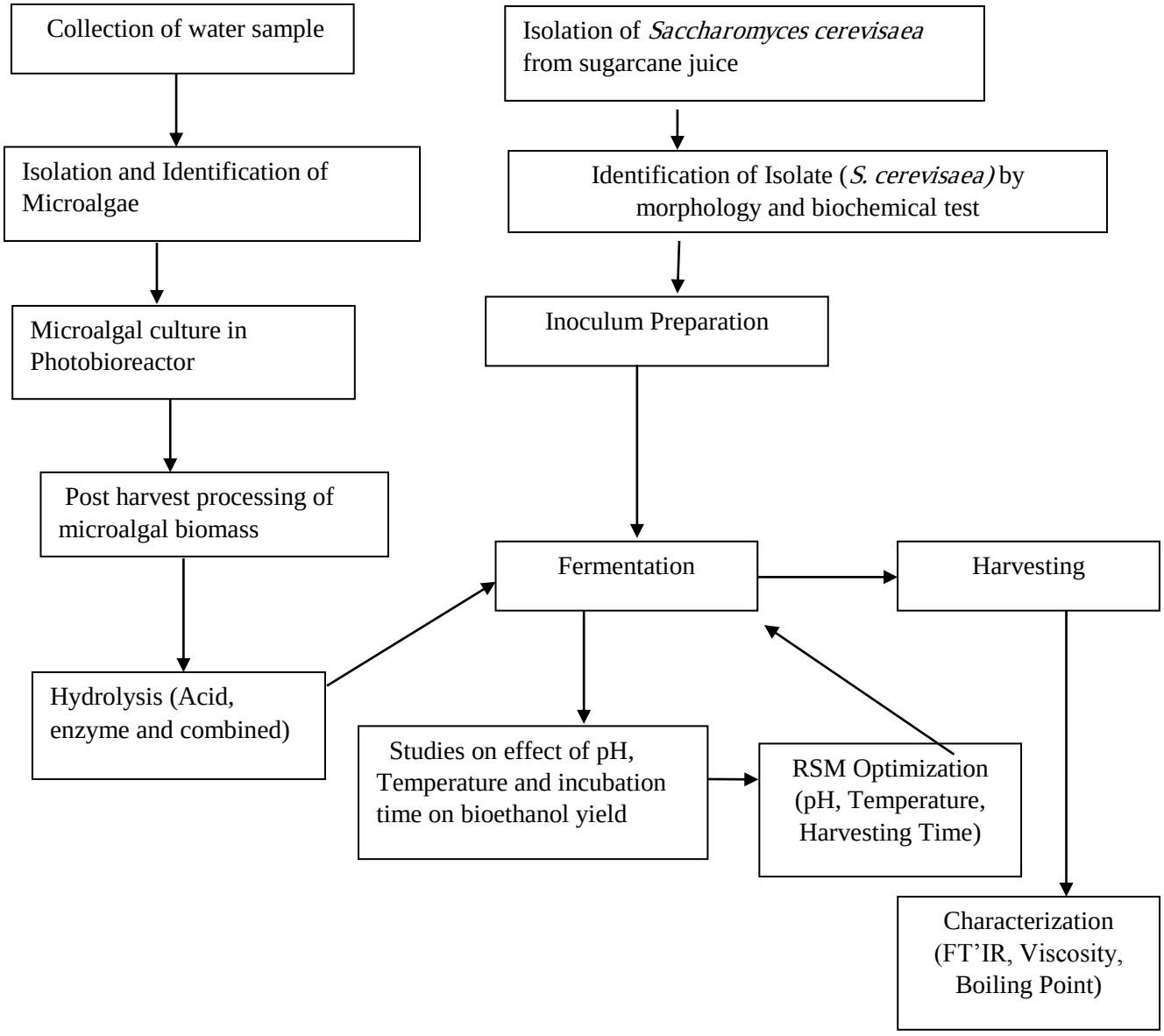


Figure 3.1: flow chart of the overall study design on bioethanol production from microalgal biomass

3.3 Collection of Water sample

Water sample was collected from Ajiwa Dam Katsina state. Ajiwa Dam is located at Batagarawa Local Government Area of Katsina state and lies between latitude 12°30-13° North longitude 7 ° 30-80 East in the Sudan savannah ecological zone of Nigeria. The elevation of the site is about 518m above sea level. The Dam has catchment area of 1678km with 12 metres height and spill ways of 60 meters, lift pump of 1040km/hr capacity. The water sample was collected using a 100ml brown bottle container as described by Indabawa (2014). The bottles were rinsed with the water to be sampled prior to collection. The samples were transported in a clean transparent plastic container with ice to Department of Plant Biology, Bayero University Kano for microscopic screening of microalgae.

3.4 Isolation and Identification of microalgae

The micro algae were isolated using pipetting method (Sulfahri, 2009). From each plastic container a drop of water sample were mounted on clean grease free glass slide and covered with cover slip. The slide was then viewed under low (x40) and high power (x100) objective lenses. The micrograph of individual microalgal cell was identified using microalgae identification guide developed by (Pelmer,1990). And the dominant Spirogyra sp was selected and subjected to culture in photobioreactor for bioethanol production.

3.4.1Medium Preparation for Microalgal Culture

The media was prepared in 100ml capacity conical flask by taking exactly 10ml from stock solution of $NaNO_3$ and 1ml each of K_2HPO_4.3.H2O, $MgSO_4.7H_2O$, $CaCl_2$. $2.H_2O$, Citric Acid + Ferric Ammonium Citrate, EDTA, and Na_2CO_3 (Table 3.1). Exactly 1ml of trace element solution was also added. (Table 3.2). Each of the substances was dissolved separately before adding together. After the medium preparation, the flask was plug with cotton wool and autoclaved at 121 ℃, 15psi pressure for 15 minutes. The combined stock was adjusted to pH 7.0 using 1M NaOH and 1M HCl.

Table 3.1: Media composition

Stock	Stock solution	ml/Litre
$NaNO_3$	150g/L	10ml
$K_2HPO_4.3.H_2O$	40g/L	1ml
$MgSO_4.7H_2O$	75g/L	1ml
$CaCl_2$. $2.H_2O$	36g/L	1ml
Citric Acid + Ferric Ammonium Citrate	6g/L	1ml
EDTA	1g/L	1ml
Na_2CO_3	20g/L	1ml

Table 3.2: Trace metal solution

Substances	g/L
H_3BO_4	2.8
$MnCl_2$. $4H_2O$	1.8
$NaMoO_4$. $5H_2O$	0.39

$CuSO4.\ 5H_2O$	0.07
$Co(NO_3)_2.\ 6H_2O$	0.049

3.4.2. Microalgal Culture

Individual cell of *Spirogyra sp.* being the most dominant species was picked using capillary tube and inoculated in to the medium (BG-11). The culture was allowed to grow and bloom in locally made photo-bioreactor for three weeks in the Department of Biochemistry Bayero University Kano before harvesting for bioethanol Production.

3.5.1 Post-harvest Processing of the Biomass

The cultured algae were harvested and subjected to sun drying to remove moisture content for 72hrs. The dried biomass was then ground and sieved with 1mm pore size. Fine powder of the Spirogyra biomass was used for all fermentation and optimization experiments.

3.6.0 Isolation of yeasts

Exactly 3kg of sugarcane stalk was collected from Yan rake market in Kano state Nigeria. The stalk was squeezed using clean mortar and the resulting juice were collected in clean Petri dish. After 2hrs of exposure to air 1ml of sugarcane juice was taken aseptically into a test tubes. The samples were then serially diluted 10-fold in sterilized distilled water. One ml of the serially diluted sample was inoculated by streaking on plates of standard yeast extract peptone dextrose agar media (YPD) (supplemented with chloramphenicol (0.05 mg/l) (Nwachukwu, 2001) and incubated at 28°C for 24 hours (Offosu *et al.*, 2013).

3.6.1 Identification of yeast isolates

Colonies suspected to be yeast (*Saccharomyces sp.*) were isolated using spread plates method and purified by streaking on freshly prepared media and incubated for 1 day at 30°C. Isolates from such fresh plates was subjected to characterization on the basis of; morphology and fermentative tests (Biochemical) of sugars such as glucose, lactose and sucrose (Offosu *et al,* 2013). The cultured and identified organisms was maintained on Patato dextrose agar media (PDA) at 30ºC.

3.6.2 Preparation of Inoculum

Cell suspension (10ml) of *Saccharomyces cerevisiae* prepared from 2 days old slant culture was inoculated in to 100ml of medium and incubated at 30ºC for 48hours on a rotary shaker at 200 rpm. The *S. cerevisiae* cells were then collected by centrifugation and then dried. The inoculum concentration of 0.3 % (dry weight/volume) was utilized for the fermentation.

3.7. Hydrolysis of Microalgal Biomass *(Spirogyra sp.)*

To study the effect of hydrolysis on ethanol yield from microalgal *(Spirogyra spp.)* The biomass was divided into three portions following the method described with slight modification where HCl was used in place of H_2SO_4 (Agwa *et al.,* 2018).

The first portion was subjected to acid hydrolysis. The *Spirogyra spp* biomass was hydrolyzed using 5% 2N HCl, autoclaved at 121°C for 15 min. The solution obtained was neutralized with phosphate buffer and centrifuged at 4000 rpm for 30 min and filtered.

The second portion of the *Spirogyra* biomass was subjected to enzymatic (amylase) hydrolysis. The biomass was mixed with 3% amylase at concentration of 5.26 IU activity per ml of suspension. Amylase preparations and the mixtures were maintained at pH 4.5 and incubated at 50°C for 24 h on rotary shaker at 4000 rpm for 30 minutes. Then the mixture was centrifuged at 3000 rpm for 35 min and filtered for fermentation.

Third portion of the biomass was subjected to combine dilute acid and enzymatic hydrolysis. The biomass was first hydrolyzed with 5% 2N HCl, autoclaved at 121°C for 15 min and then neutralized to pH 4.5 with citrate buffer. The solution was then incubated with 3% amylase enzyme preparations and kept in a water bath for 12 hours at 75 ℃. Then centrifuged at 4000 rpm for 35 min and filtered to obtain clear pellet for fermentation.

All the three hydrolysates were analyzed for total reducing sugars before fermentation with *Saccharomyces cerevisiae* as described by (Agwa *et al.*, 2018).

3.8 Analytical methods for estimation of reducing sugars in hydrolyzed microalgal biomass (*Spirogyra spp)*

The amount of reducing sugars was estimated by dinitrosalicylic acid (DNS) method as described by (Offosu *et al.*, 2013). To evaluate the reducing sugar content exactly 0.5ml of each acid, enzyme and combine acid-enzyme hydrolyzed *Spirogyra sp.* biomass added in a separate test tubes. The volume was made up to 3 ml using distilled water. Three milliliters (3 ml) of DNS reagent were added to each sample, mixed and placed in water bath at 75 ℃ for 10 minutes. The absorbance was measured at 510 nm using UV

Spectrophotometer. The standard glucose was also prepared similarly with concentration in 0-1mg/ml.

3.9 Fermentation

Submerged fermentation was carried out in 250ml Erlenmeyer flask. To each hydrolysate 0.3 % (dry weight/volume) of pre fermentative yeast (*Saccharomyces cerevisiae)* was inoculated and fermentation was carried out with intermittent shaking at 2000 rpm for five days (120 hours) at ambient temperature of 30℃ and pH 4.5. Each experiment was carried out in triplicate and Means standard deviation were calculated.

After five days of fermentation, the resulting culture broth was centrifuged at 4000 rpm for 40 minutes and then distilled at 72- 80℃ (boiling point of ethanol) and fraction was collected in container.

3. 10. Effect of pH on bioethanol yield

Four sets of pH values were used to provide a wide range of selection of an optimum pH condition for ethanol production. Exactly100 ml of hydrolysate was transfered into 250 ml conical flasks and pH was then adjusted by using 1M HCl and 1M NaOH to 4.5, 5.0, 5.5 and 6.0 using digital pH meter. The flasks were then sterilized at 121 ℃ for 15 minutes. Upon cooling, the pre-fermentative *Saccharomyces cerevisiae* measuring 0.3 % (dry weight/volume) was inoculated aseptically to the sterilized media and there after incubated (shaking) with agitation speed of 200rpm at an ambient temperature of 30ºC for 5 (120 hours) days.

3.11.1 Effect of temperature on bioethanol yield

Suitable temperature for maximum production of ethanol by the *Saccharomyces cerevisiae* was studied by incubating the hydrolysate with yeast at 30, 35, 40 and 45 ^{0}C in shaking incubator (200 rpm) for 5 (120 hours) days. This experiment was done using optimum pH value of 5.0 obtained in section 3.11. above.

3.11.2 Effect of incubation time on bioethanol yield

Five flasks were set at pH 5.0 and temperature of 35 ℃ obtained in section respectively. Bioethanol yield was measured at 24 hrs, 48 hrs, 72 hrs, 96 hrs and 120 hrs.

3.12.1 Analytical method for the determination of bioethanol concentration

Resulting ethanol yield was determined by UV-VIS quantitative analysis of alcohol using dichromate reagent as described by Oyeleke and Jibrin (2009). Exactly 1m of standard ethanol was diluted with 99ml of distilled water to give a concentration of 1%. Then each of 0,2,4,6 and 8ml of the 1% ethanol was diluted to 10ml with distilled water to produce 0, 0.2, 0.4, 0.6, and 0.8% of the ethanol. To each of the varying ethanol concentrations, 2ml of diachromate reagent was added and allowed to stand in water bath at 70^{0}C for 15 minutes. The absorbance of each concentration was measured at a wavelength of 600nm using UV-VIS spectrophotometer and the readings were used to developed a standard ethanol curve. Then 4ml of the distillates was put in test tube and treated with 2ml of dichromate reagent. The mixture was allowed to stand for 15 minute in water bath at 70ºC and absorbance was measured at 600nm using UV-VIS spectrophotometer. Ethanol standard concentration curve was used to extrapolate the concentration of ethanol in hydrolysate.

3.12.2 Characterization of the bioethanol

The resulting bioethanol was subjected to analysis using flourier transform infrared spectroscopy (FTIR), boiling point determination, and relative viscosity to ensure the product produced was ethanol. Exactly 0.5ml (converted to solid by FTIR converter) bioethanol was placed on to the diamond attenuated total reflector then a background scan was performed and the sample was then scanned at 650-700 cm^{-1} to a produce spectrum.

3.12.3 Determination of boiling point of bioethanol

The boiling point of ethanol was determined using Thiele tube method as described by (Blank, 1993). Exactly 0.5ml of the ethanol sample was placed in a small tube along with an inverted capillary tube. The set up was attached to a thermometer and heated inside a Thiele tube. As the bubble eventually stopped boiling point was recorded as the temperature when liquid just began to enter the capillary tube. All experiments were carried out in triplicate and average values were recoded.

3.12.4 Determination of relative viscosity of the bioethanol

The relative viscosity of the ethanol produced was determined using a conventional Ostwald viscometer at 30℃ (Maria and Soma, 2014). Flow time was measured and relative viscosity was calculated as follows:

Relative Viscosity $\mu r = \mu/\mu l$

where; μr = relative viscosity, μ= Viscosity of the sample and

μl= is the viscosity of deionized water.

μr= □ x ts / □ x t^o

where;

where ts = is the falling time (sec.) of the sample at 30°Cand

(t^o sec.) = falling time of deionized water under same condition and

□ = Density = mass / volume (kg/m^3)

3.13.1 Optimization of process condition

The statistical based optimization was used to study the effect of pH, temperature and incubation time on ethanol yield using Central Composite Design (CCD). pH, (A), temperature (B) and incubation time (C) were taken as independent variables and ethanol yield was choosen as the dependent variables (Table 3.3). This resulted in twenty experimental runs from the software Design expert software, version 6.0.6. (Table 3.4). The modeling and statistical analysis were performed using Design expert software, version 6.0.6. All fermentation experiments were carried out in 250 ml Erlenmeyer flasks with working volume of 100 ml and agitation speed 200 rpm. Multiple regression analysis of the observed responses in terms of the coded factors resulted in the quadratic model below.

$Y=+8.49-0.057A-0.021B+1.46C-0.086A^2-0.174B^2-0.048C^2-0.087AB-0.081AC-0.064BC$……Equation 1.

A, B and C represents the independent variables (coded form) of pH, temperature and harvesting time respectively.

Table: 3.3 Factors for RSM experimental design.

Factor	Indicator	Low level	High level
Ph	A	4.5	6.0
Temperature (˚C)	B	30	45
Incubation time (hrs)	C	24	120

Table 3.4: RSM experimental design for bioethanol production from microalgal biomass.

Exp. Runs	pH	Temperature (℃)	Incubation Time (hrs)
1	4.5.0	30.0	120
2	5.25	37.5	8.72
3	5.25	37.5	72
4	5.25	50.0	72
5	6.00	45.0	24
6	5.25	37.5	72
7	5.25	37.5	72
8	4.50	45.0	24
9	4.50	30.0	24
10	6.00	30.0	24
11	5.25	37.5	152
12	3.99	37.5	72
13	5.25	37.5	72
14	6.51	37.5	72
15	5.25	24.9	72
16	5.25	37.5	72
17	6.0.	30.0	120
18	6.00	45.0	120

19	5.25	37.5	72
20	4.50	45.0	120

3.13.2 Validation of the second order polynomial model

The second order polynomial model obtained from RSM was validated by conducting a series of experiments randomly selected from the design in Table 3.4. The experiments were done by choosing random values of parameters within the optimized levels as shown Table 3.5. The experimental output was then compared to the values predicted by the second order model obtained from CCD, to estimate the goodness of fit of the model.

Table: 3.5. Experimental set up for model validation of bioethanol production from microalgal biomass (*Spirogyra spp)*

Exp. Run	**pH**	**Temp. (℃)**	**Inc. Time(hr.)**
	A	**B**	**C**
1	5.3	37.5	152
2	5.25	37.5	72
3	4.5	30	120

3.14.1 Statistical analysis

The average data and standard deviations were obtained from the triplicate of experiments for each run using Microsoft Excel (Office, 2010). The standard deviation for each value was 5%. Analysis of variance (ANOVA) was done using Design-Expert software 6.0.6. A confidence level of 95% was used in this study. Any p-values less than 0.05 were considered significant and vice versa.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.0 Results

4.1 Microscopic Identification of the Sample

Table 4.1 showed the results of microalgae species isolated from water sample collected from Ajiwa Dam. Three different microalgae species were identified microscopically and the number of appearance of each specie was recorded. The specie with highest number of appearance was used for bioethanol production. *Spirogyra spp.* cells was found to appears 72 times from the overall water sample followed by *Chlorella vulgaris* with a total of 12 cells while the least number of cells (8) was recorded in *Snesdesmus sp*. *Spirogyra sp.* being the most dominant species was subjected to culture for bioethanol production.

Table 4.1: Isolated micro algal specie from studied water sample

SN	Microalgal Species	Number of cells
1	*Spirogyra spp*	72
3	*Chlorella vulgaris and*	12
3	*Snesdesmus spp*	8

4.2 Isolation of fermentative Yeast (*Saccharomyces cerevisiae)*

Three *Saccharomyces sp.* isolates were identified. On the basis of colony and cell morphology including the growth of isolates in liquid medium. All three isolates were aerobes with creamish colony and spherical shaped (plate 1). Table 4.2 depicts the result of fermentation capacity of some simple sugars by the isolated *Saccharomyces cerevisiae* from sugarcane juice. Nearly, all isolates fermented glucose, fructose and sucrose but none fermented lactose.

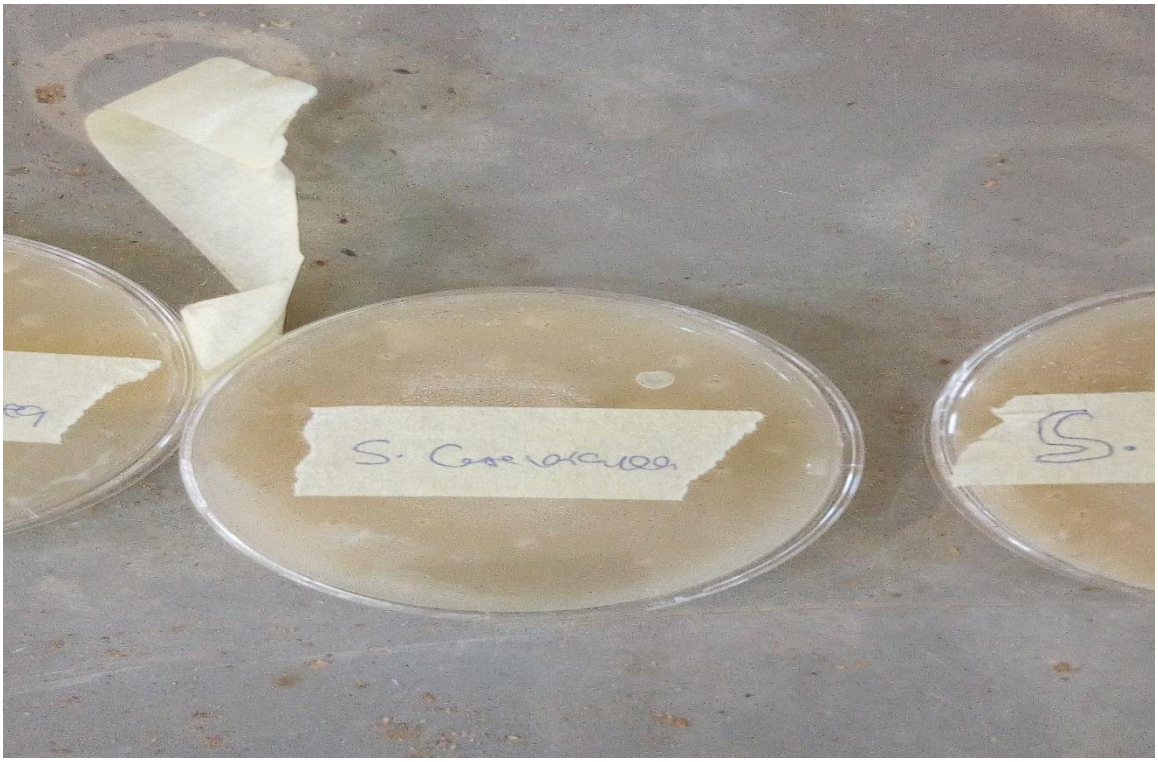


Plate 2: showing *S. cerevisiae* morphology and color on PDA media

Table 4.2: Fermentation of some simple sugars by yeast isolate

Isolate	Glucose	Lactose	Fructose	Sucrose
A	+ Gas	-	+ Gas	+ Gas
B	+ Gas	-	+ Gas	+ Gas
C	+ Gas	-	+ Gas	+ Gas

4.3 Reducing sugar concentration in hydrolyzed microalgal biomass *(Spirogyra sp)*

Figure 4.1 illustrated the amount of reducing sugar content released before and after hydrolysis. The initial sugar content before fermentation of the hydrolysate was found to be 0.43, 0.30 and 0.82 mg/ml after acid, enzyme and combine hydrolysis respectively. After five-days fermentation at 30 ℃ pH 4.5, 0.3% (dry weight/volume) *Saccharomyces cerevisiae* inoculum for 120hrs, the reducing sugar content were found to be reduced significantly (P> 0.05) in combine acid-enzyme and enzyme hydrolysates by 86.5% (0.82-0.11) and 80% (0.30-0.06) and acid hydrolysis respectively (Figure 4.1).

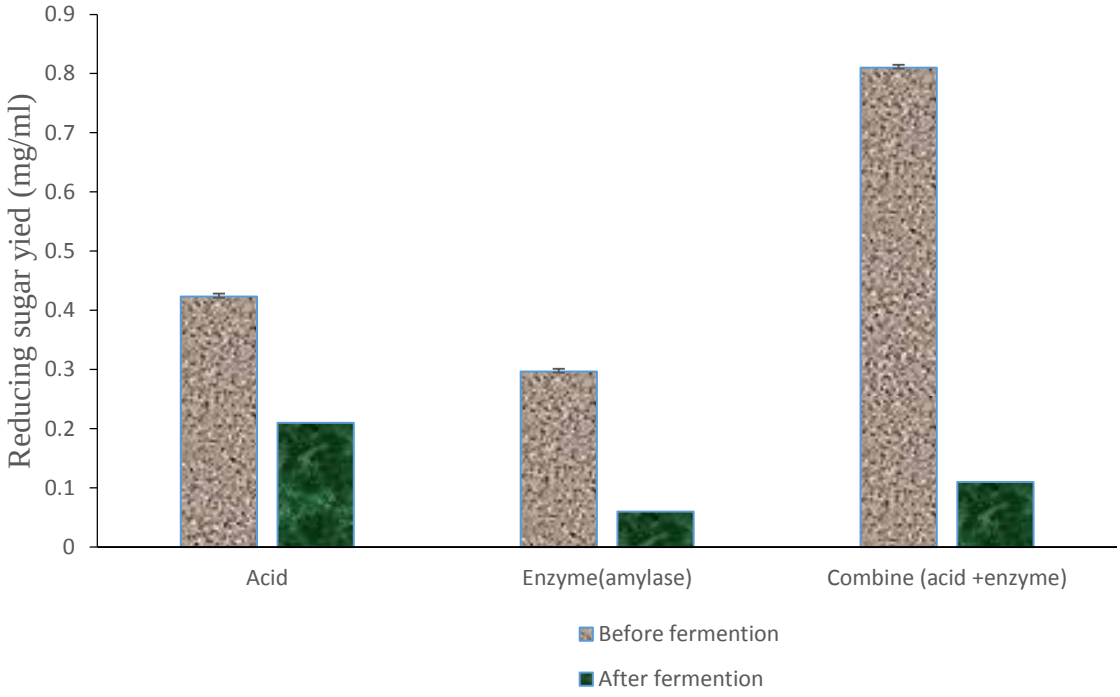


Figure 4.1: Concentration of reducing sugar using different hydrolytic agent before and after fermentation.

4.4 Effect of hydrolytic agent on ethanol yield from microalgal *(Spirogyra spp.)* biomass

Figure 4.2 illustrated the effect of different hydrolytic conditions on the bioethanol yield. The effect of hydrolytic agent was studied by subjecting the spirogyra biomass to acid, enzyme and combine acid and enzyme hydrolysis respectively. From the result obtained high ethanol yield of 13.5% was recorded in combined dilute acid followed by enzymatic(amylase) hydrolysis with 12.9% while the least bioethanol concentration of 11.2% was observed in dilute acid hydrolysis. However, analysis of varience shows significant difference ($p > 0.05$) in ethanol yield between acid, enzyme and combined hydrolysis but no difference observed ($p < 0.05$) between acid and enzyme hydrolysis.

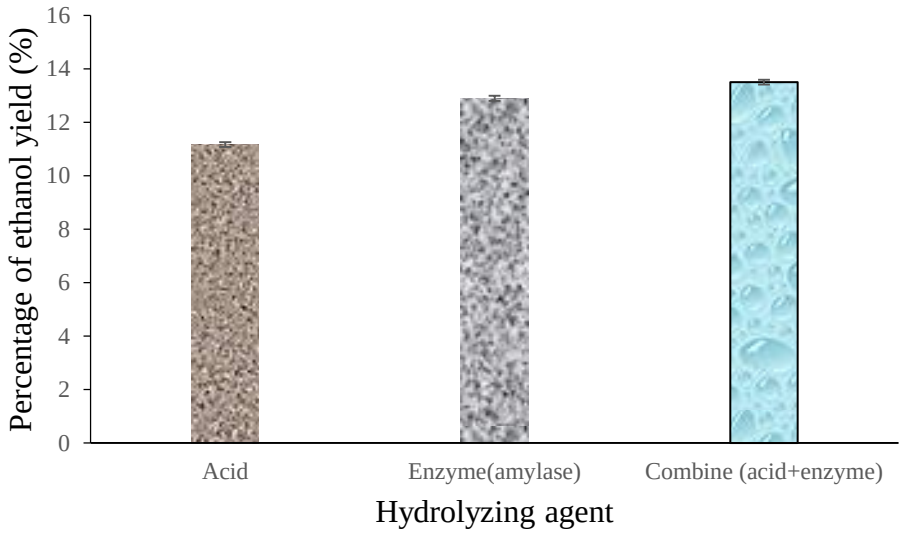


Figure 4.2: Effect of hydrolyzing agents on ethanol yield using *Spirogyra sp.* biomass

4.5. Effects of pH, temperature and incubation time on bioethanol yield from microalgal biomass *(Spirogyra sp.)*

Figure 4.3,4.4 and 4.5 showed the effect of initial pH, temperature and incubation time on bioethanol yield from microalgal biomass. The effect of different pH was studied at pH range of 4.5-6.0. Generally, initial increased in bioethanol yield was observed with increase in pH from 4.5 to 5.0 and later declined at pH 5.5 and 6.0 (Figure 4.3). The increased was more pronounced at pH 5.0 with maximum ethanol yield of 7.5% and least was recorded at pH 6.0 with ethanol yield of 6.5% respectively. Analysis of variance indicated that there was significant difference ($p > 0.05$) in the bioethanol yield at across the pH values.

Figure 4.4 depicts the effect of temperature on ethanol yield from microalgal biomass. Generally initial increased in bioethanol yield was observed with increase in temperature from 30 to 35 ℃. However, the yield declined from 40 to 45 ℃. The increase was more pronounced at 35 ℃ with maximum bioethanol yield of 7.1% beyond which increase in temperature leads to decrease in bioethanol yield. Analysis of variance indicated that there was no significant ($p > 0.05$) difference in the ethanol produced across each temperature.

Effects of incubation time on ethanol yield was also determined subjecting the flask to incubation at different period intervals ie; 24,48,72, 96 and 120 hrs. As depicted in Figure 4.5. There was general gradual increase in bioethanol yield with increasing incubation time. The Maximum ethanol yield was recorded at 120 hr (6.4%) followed by 96hrs with 6.3% and the least ethanol yield of 5.8% was observed at 24hours of

incubation. Analysis of variance indicated that there was significant difference ($p > 0.05$) in the bioethanol yield between the incubation time of 24-72hr, 48- 96hr, and 72-120hr respectively. However, there was no significant ($p > 0.05$) difference in ethanol produced at incubation time 24-48hr, 48- 72hr, 72-96hr and 96-120hr respectively.

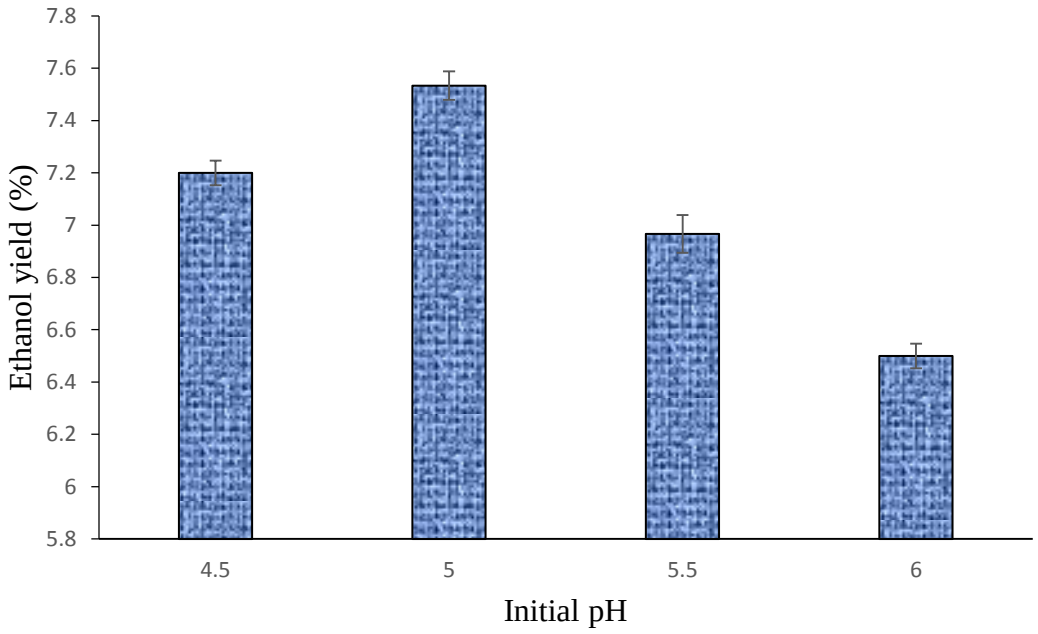


Figure 4.3: Effect of pH on bioethanol yield from microalgal biomass *(Spirogyra sp.)*

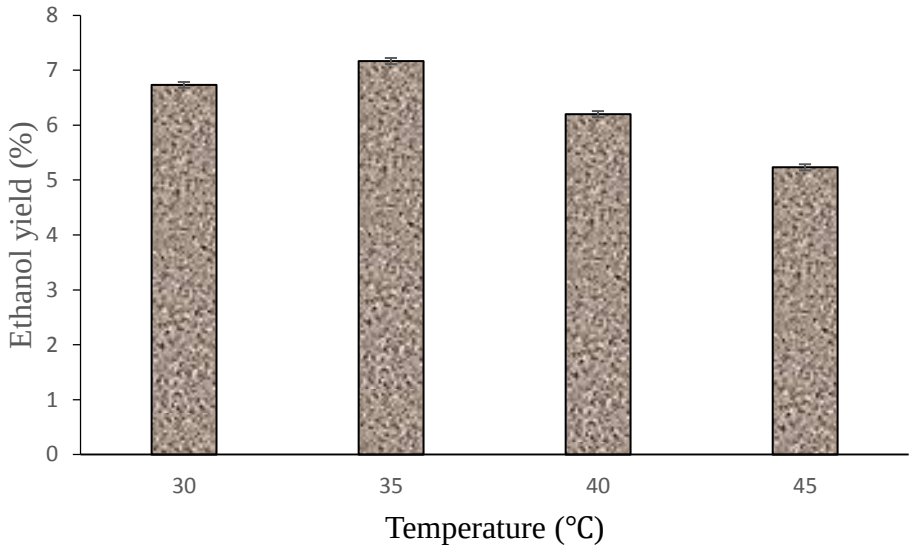


Figure 4.4 Effects incubation temperature on bioethanol yield from microalgal biomass *(Spirogyra spp)*

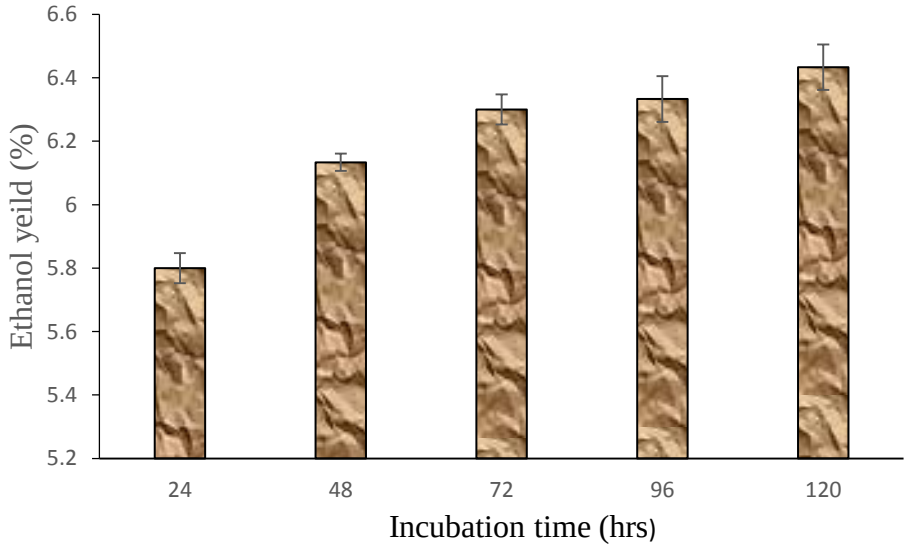


Figure 4.5: Effect of incubation time on ethanol yield from microalgal biomass *(Spirogyra sp.)*

4.6.1 Optimisation of bioethanol production condition

Table 4.3 shows the result of actual bioethanol yield and predicted bioethanol obtained for different conditions of pH, temperature and incubation time. The responses obtained for each experimental run and the predicted responses were closer to each other. It can be observed that increased in temperature and pH led to decrease in bioethanol yield. However, the yield increased positively with increase in incubation time. Maximum ethanol yield of 14% was achieved at 152 hours of incubation at 37.5 ℃ and pH 5.2. (Run 11, Table 4.3) while the least bioethanol yield was recorded at 72hrs of incubation at 50 ℃ and pH 5.2 (Run 4, Table 4.3).

Table 4.3: Actual and predicted bioethanol yield at different condition of pH, temperature and incubation time

Exp. Runs	pH	Temperature (℃)	Incubation Time (hrs)	Experimental yield (%)	Predicted yield (%)
1	4.5	30	120	8.7	9.43
2	5.25	37.5	8.72	5.0	4.65
3	5.25	37.5	72	9.2	8.49
4	5.25	50	72	3.1	3.19
5	6	45	24	5.5	4.96
6	5.25	37.5	72	9.2	8.49
7	5.25	37.5	72	9.2	8.49
8	4.5	45	24	5.0	4.65
9	4.5	30	24	5.1	3.63
10	6	30	24	3.2	3.10
11	5.25	37.5	152	12.0	12.0
12	3.99	37.5	72	5.8	7.01
13	5.25	37.5	72	7.6	8.49
14	6.51	37.5	72	6.6	5.10
15	5.25	24.9	72	4.3	3.92
16	5.25	37.5	72	8.2	8.49
17	6	30	120	6.3	6.85
18	6	45	120	3.3	4.29
19	5.25	37.5	72	7.4	8.49
20	4.5	45	120	8.7	7.91

Table 4.4 shows the experimental and fitted model results of bioethanol yield from microalgal biomass. From the result and temperature range of has no significant ($p > 0.05$) effect on the bioethanol yield with P value of 0.14 and 0.56> 0.005 respectively. However, incubation time has a P value = 0.002 indicating its significance at $P < 0.05$

As illustrated in Table 4.4, the analysis of variance for a P-value < 0.05 indicates a significant effect on the response. The Model F-value of 6.01 implies the model is significant. There is only 0.49% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.05 indicate model terms are significant. In this case C, A^2, B^2 are significant model terms. The "Lack of Fit F-value" of 4.15 implies there is a 7.23% chance that could occur due to noise. Regression analysis produced the following second-order polynomial fit with a satisfactory coefficient of determination ($R^2 = 0.84402$).

Ethanol. yield = -90.92112 +17.51818A +2.50683B +0.21818 C -1.53018 * A^2-0.031015 * B^2 -2.06406E-005 * B-0.015556 * A * B. -0.022569 * A * C-1.77083E-003 * B * C……..Equation ii.

where, A, B and C represents pH, temperature and incubation time respectively. AB, AC and BC are the interactions, and A^2, B^2 and C^2 are the quadratic terms.

Table: 4.4 Analysis of the bioethanol yield from microalgal biomass *(Spirogyra sp.)*

Source	**Squares**	**Square**	**Value**	**Prob> F**	
Model	0.9370	0.10422	6.0123	0.0049	Significant
A	0.0440	0.044032	2.5401	0.1421	
B	0.0062	0.006235	0.3597	0.5620	
C	0.2892	0.289172	16.6820	0.0022	
A^2	0.1067	0.106767	6.1592	0.0324	
B^2	0.4386	0.438632	25.3043	0.0005	
C^2	0.0003	0.000326	0.0188	0.8937	
AB	0.0006	0.000613	0.0353	0.8547	
AC	0.0528	0.052813	3.0467	0.1115	
BC	0.0325	0.032513	1.8756	0.2008	
Lack of Fit	0.1396	0.027932	4.1462	0.0723	not significant
R-Squared	0.8440	0.70364	Adeq Precision	8.17056	
Predicted R-squire	0.0479				

KEY: A = pH, B = temperature C = incubation time

Figure 4.6 depicts a correlation between the observed bioethanol yield and the predicted values. The cluster distribution between observed and predicted bioethanol yield signifies satisfactory correlation between the observed values and the predicted values in the parity plot. Additionally, Fig.4.7 shows the parity graph showing the distribution of residual and predicted values of bioethanol yield. The clustered points around the diagonal line indicate goodness of fit of the model since there is less deviation between the observed and predicted values.

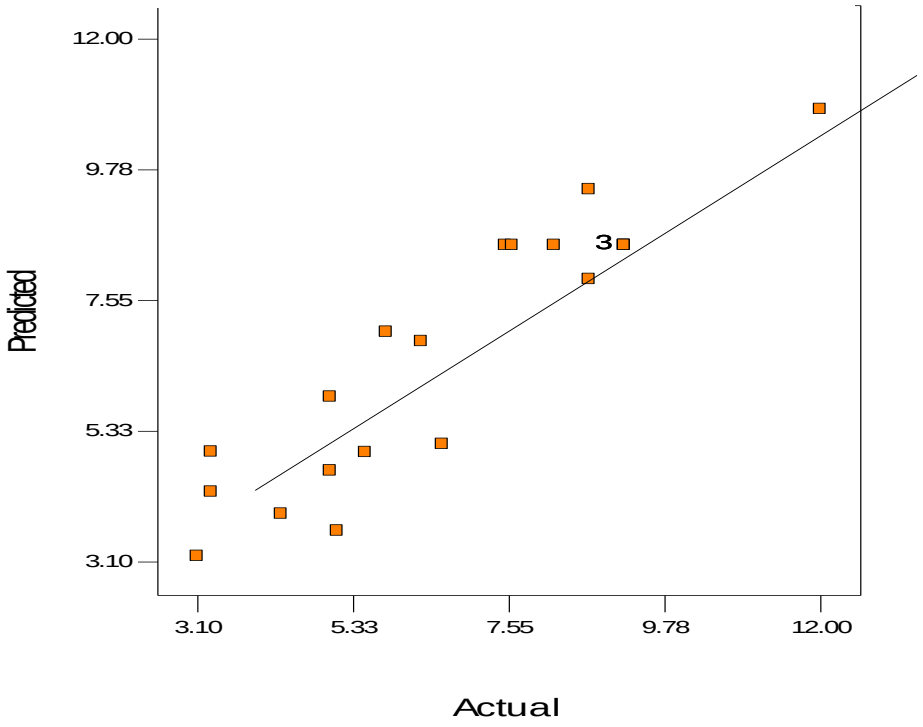


Figure 4.6. Parity graph showing the distribution of actual vs. predicted values of ethanol yield.

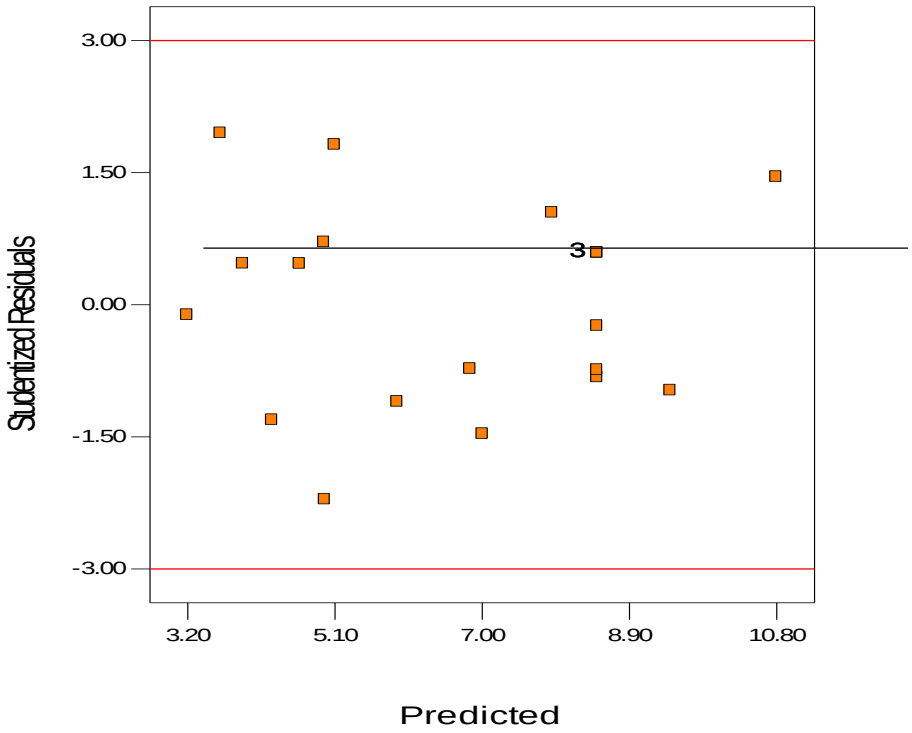


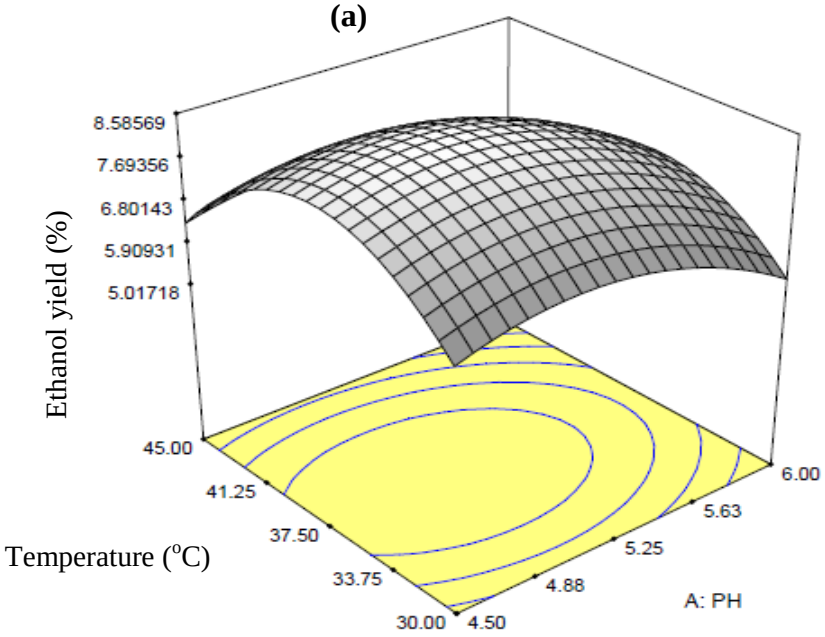
Figure 4.7. Parity graph showing the distribution of residual vs. predicted values of ethanol yield from microalgal biomass.

4.6.2 Interaction of pH, temperature and incubation time for optimization of ethanol yield from microalgal biomass

Response surface was generated by plotting the response (bioethanol yield) on the y-axis against any two independent variables on the x-axis, while keeping the other independent variables at its centre part. Therefore, three response surfaces were obtained by considering the possible combinations.

Figure 4.8 (ai, ii, bi, ii, and ci, ii) represents the three-dimensional and contour surface plots for the optimization conditions. The plot illustrates the main and the interactive effects of the independent variables on the dependent ones. The response surface plots were generated by plotting the response on the y-axis. Figure 4.8 (ai, ii) shows the effect of temperature and pH on ethanol production keeping the other variable (incubation period) constant (72hr) level. Bioethanol yield was found to increase with the increased in temperature and pH. However, the ethanol yield was more pronounced at 37.5ºC and pH 5.25 but beyond these the yield declined. The elliptical 3-dimentional surface shows the interactive effect of pH and temperature on bioethanol yield. As shown in (Figure 4.8 bi, ii and ci, ii), harvesting time exert a positive effect on the bioethanol yield showing a linear increased significantly with time as shown by its P value of 0.002 The linear graph of time shows time is independent of pH, and temperature in relation to bioethanol yield ((Figure 4.8 bi, ii and ci, ii).

(a)


8.58569
7.69356
6.80143
5.90931
5.01718
Ethanol yield (%)
45.00
41.25
37.50
33.75
30.00
Temperature (°C)
4.50
4.88
5.25
5.63
6.00
A: PH

(b)

.

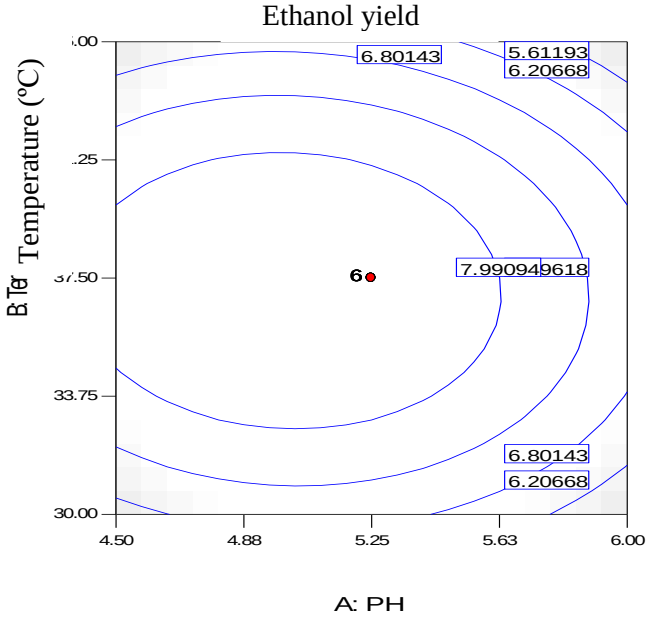
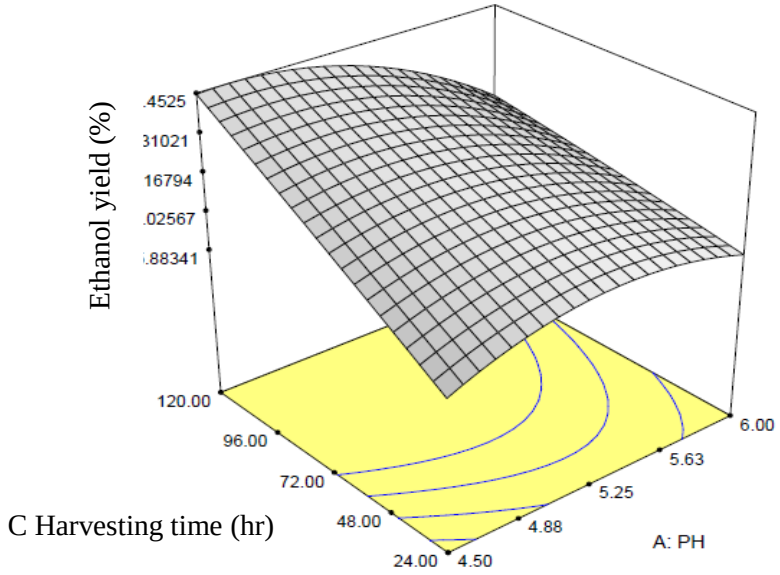

Ethanol yield
6.80143
5.61193
6.20668
7.990949618
6.80143
6.20668
Temperature (°C)
33.75
30.00
4.50
4.88
5.25
5.63
6.00
A: PH

Figure 4.8

Figure 4.8: (a). 3-dimensional and contour (b) response surface plot of temperature vs. pH on ethanol yield (incubation time kept constant at 72 hr).

(a)



(b)

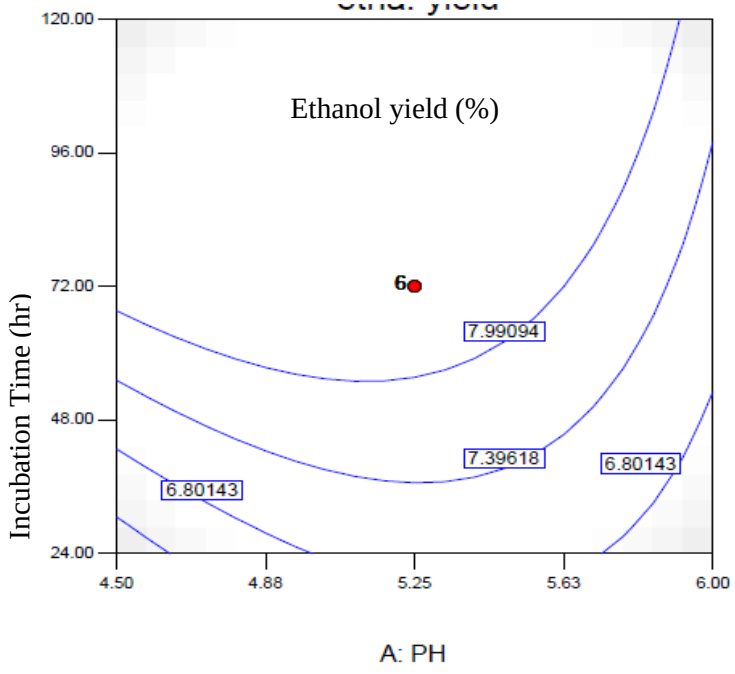
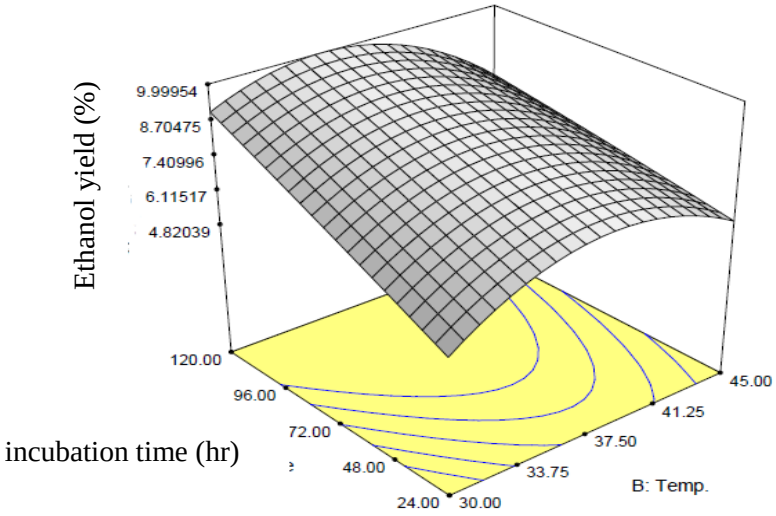


Figure 4.8. 3-Dimentional (a) and contour Response surface plot Incubation time vs. Temperature on ethanol yield (pH was kept constant at 5.0).

(a)



(b)

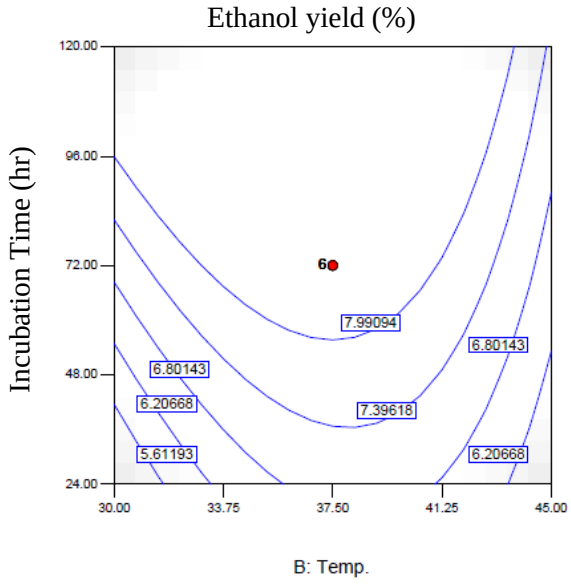


Figure 4.8 c. 3-Dimentional (a) and contour (b) Response surface plot of incubation time vs. pH on ethanol yield

4.6.3 Validation of second order polynomial model between observed and predicted ethanol yield from microalgal biomass

Table 4.6 represents the result of validation runs with observed and predicted values. The observed values of bioethanol yield were compared with the values predicted by the second order model. The result indicated that there was very good correlation between experimental and predicted values and in turn proves the validity of the models.

Table 4.6: Validation runs with observed and predicted bioethanol yield from *Spirogyra* biomass.

Exp. Run	pH A	Temp. (°C) B	Inc. Time(hr.) C	Observed yield (%) ±0.05	Predicted yield (%)
1	5.30	37.5	152	13.85	13.98
2	5.25	37.5	72	8.75	8.49
3	4.50	30.0	120	9.05	9.43

4.7.1 Characterization of bioethanol produced from microalgal biomass

The FT-IR spectra of the produced bioethanol was compared with that of a commercially available ethanol (Table 4.7). The examination of the spectra reveals the presence of some functional groups found on the fermentation distillates. The FT-IR spectrum of the produced bioethanol reveals peak at 3331cm-^{1} which was slightly lower than that of commercial ethanol with 3335 cm^{-1} attributed to OH stretching. Peak found at 2981cm-^{1} which is exactly like that of commercial ethanol was due to single carbon bond C-O (SP^3) stretching while peak at 1714 cm^{-1} was attributed to the presence of methyl group (CH_3) which was lower than that of commercially produced ethanol as shown in Table 4.7.

Table 4.7: Characteristics of bioethanol produced from microalgal spirogyra biomass

Bioethanol from Microalgal biomass		
Functional group	**cm^{-1}**	**Commercial Ethanol (Sigma Aldrich co.)** **cm^{-1}**
OH	3331	3335
C-H(SP^3)	2981	2794
CH_3 (Methyl)	1417	1454
Asymmetric CO	1089	1089
Symmetric CO	1048	1048

4.7.2 Determination of Relative viscosity and boiling point of the produced bioethanol

Table 4.8 shows some physicochemical characteristics of produced bioethanol from spirogyra biomass. According to the result obtained the relative viscosity of the produced bioethanol was found to be 1.35 ± 0.02 mPas which was higher than that of commercially produced ethanol with 1.32 ± 0.02 mPas. Also the boiling point of the produced bioethanol was found to be 78.81± 0.2 ℃ which was higher than that of commercial ethanol 78.62± 0.2 ℃. Independent paired T-test reveals no significant difference between the boiling point and relative viscosity of produced and commercial ethanol with p- value of 0.2 and 0.1 which is greater than 0.05.

Table: 4.8: Relative viscosity and boiling point of bioethanol produced from Microalgal biomass

Test	Produced bioethanol from Microalgal biomass	Commercial ethanol (Sigma Aldrich co.)
Relative viscosity (mPas)	1.35 ± 0.02	1.32 ± 0.02
Boling point (℃)	78.81± 0.2	78.62± 0.2

4.8.1 Discussion

The increasing over usage of fossil fuels is also one of the leading causes of several other concerns, such as high levels of pollution and environmental catastrophes (National Research Council, 1999). These are what prompt scientists to develop new renewable energy resources that are viable, clean, and sustainable.

Biofuels such as biodiesel, biohydrogen and bioethanol are used as alternative to fossil fuels, the latter is currently the most produced biofuel in the world, with almost 110 billion litres in 2011 (RFA, 2012). Due to this reason many scientists have tried to find out various sources for production of bioethanol. In the present study the potential of bioethanol production using fresh water microalgal. biomass isolated from Ajiwa Dam was studied.

The isolated yeasts from sugarcane juice appear as unicellular, large spherical individual cells with creamish appearance and all isolates fermented glucose, fructose and sucrose, but not lactose. These observations were similar to those reported by Offosu *et al.*, (2013) and Elijah *et al*., (2010) who reported the isolation of *Saccharomyces cerevisiae* and other yeast from palm wine.

Hydrolysis of biomass to fermentable sugar is a major key step for bioethanol production. Depending on the nature of the biomass different agents such as chemical, thermal, enzyme and mechanical can be employed for biomass hydrolysis or sacchrification. In the present study effect of hydrolyzing agents on bioethanol yield from microalgal biomass was assessed. The highest reducing sugar yield observed in combined (acid + enzyme) hydrolysis may be attributed to lack of sufficient activity of acid or amylase alone for entire hydrolysis of *Spirogyra spp* biomass. So also this may be linked to

presence of some negligible amount of lignin on *Spirogyra spp* biomass. Agwa *eta al.*, (2018) worked on saccharification and bioethanol fermentation of carbohydrate extracted from microalgal biomass using genetically identified organisms and reported higher reducing sugar and bioethanol yield of 0.23g/l in combined acid and enzyme(cellulase) hydrolysis. Previous studies like that of Sukumaran (2009). showed that the low ethanol yield observed in acid hyrolysate may be due to some degradative by-products like hydroxyfurfural and other organic substances produced by the acid hydrolysis which may likely inhibit fermentation process. So also higher salt concentration formed during pH adjustment may inhibit growth and metabolism of the fermenting organism *S. cereviecea* (Salman and Ali 2014). However, the enzymatic hydrolysate is not known to contain any inhibitory by-product that may inhibits growth of the fermenting organisms. Moreover, the highest yield of bioethanol in combined acid and enzyme hydrolysis observed may be attributed to their capacity to break down undisrupted cell wall carbohydrates of microalgal biomass.

Physical factors such as pH, temperature and incubation time are considered among the most important fermentation parameters due to their effect on growth of microorganism, fermentation efficiency and by-product formation. Therefore, maintenance of these parameters is therefore of great significance in fermentation for better yield.

In the present study the decreased in bioethanol yield observed with increase in pH could be due to lesser enzyme activity of the fermenting organism *S. cerevisiae* at pH greater than 5.5. This agrees with observation of Hwang *et al.*, (2004) who reported that the activities of bioethanol producers are slightly suppressed at pH below 4.5 and 6.0 above.

Srivastava *et al*. (1997) showed that the optimum, initial pH of guava pulp medium was 5 where maximum ethanol yield of 5.8 %. Periyasamy *et al*. (2009) obtained the maximum bioethanol at pH 4.8 from sugar molasses using *S. cerevisiae.* Ado *et al.* (2009) studied bioconversion of cassava starch into ethanol and found the maximum yield of ethanol at pH 5. Asli (2010) also studied efficient parameters in batch fermentation of ethanol using *S. cerevisiae* in red grapes substrate, and achieved maximum concentration of bioethanol at pH 4.5.

Moreover, this study also revealed gradual increase in bioethanol concentration with increasing incubation time. This in line with the findings of Marakis and Marakis (1996) who obtained maximum ethanol concentration of 5.8 % at pH 4.5 from aqueous carob pod extract after 120 hr of incubation. Neelakandan and Usharani (2009) produced bioethanol from cashew apple juice using immobilized yeast and reported maximum bioethanol yield at 32 ^{0}C after140 hr of incubation.

The production of ethanol from Microalgal biomass was optimized by response surface methodology. The maximal 14% bioethanol was obtained with pH of 5.25, at 37.5°C for 152 hrs. Production of ethanol from banana peels was optimized by Tiwari (2015) using response surface methodology in a two-step process and reported maximal ethanol yield of 9.2 %v/v ethanol with 4 %w/w yeast, an initial pH of 4.8, at 28°C for 192 hrs. Dash *eta al,* (2017) observed optimum values of temperature at 30 °C incubation time for 84 h, and at a medium pH of 5.0 were determined by the point prediction tool of the software (Design expert) with 98.93% validity.

Generally, the different optimizations results obtained by different researchers using different substrates give an indication that the optimization conditions for optimum ethanol production depend on the substrate used and thus it's one of the critical determining factor in relation to bioethanol production.

Fourier transform infrared (FTIR) spectrometers have become a common feature of most laboratories, and is used in a wide variety of research. Over the last 50 years, quantitative analysis of mixtures using FTIR has progressed from matching peaks at a few wavenumbers to matching entire spectra (Coldea *eta al.,* 2011). The spectrum of the produced bioethanol from microalgal biomass reveals the presence of functional groups that are similar with commercially produced ethanol. This agrees with the findings of Mendes *eta al.,* (2003) who worked on determination of ethanol in fuel ethanol and beverages by fourier transform (FT)-near infrared and FT-raman spectrometries and reported the presence of CH stretching modes (2800–3050 cm^{-1}), OH stretching (3100–3500 cm^{-1}) and CH_3 (1097 cm^{-1}).

The relative viscosity and boiling point of the produced bioethanol from Microalgal biomass were also determined to show the similarity between produced and commercial ethanol and were found to be comparable and thus confirming what was produced were bioethanol.

CHAPTER FIVE

SUMMARY, CONCLUSION AND RECOMMENDATION

5.1 Summary

Bioethanol was produced from fresh water microalgal biomass (*Spirogyra sp.*) using *Saccharomyces cerevisiae* isolated from sugar cane juice. In case of hydrolysis with acid, enzyme and combination (acid and enzyme). Reducing sugar concentration was found to be higher when a combination of acid and enzyme (amylase) was employed sequentially. Same hydrolysate gave highest bioethanol yield of 13.5%. Determination of effect of some production condition revealed high bioethanol yield at pH 5.0, at 35^0C and incubation period of 120hr. However, when same conditions were subjected to optimization using response surface methodology (RSM) the near optimal conditions for the fermentation were found to be 5.2 pH at 37.5°C and incubation period of 152 hr, providing the maximal ethanol concentration of (14%). Three dimensional and contour surface interaction of the three parameters show the positive effect of incubation time on the ethanol yield. The FT-IR result of the produced bioethanol confirmed the presence of single carbon hydrogen bond, methyl and OH group. The boiling point and relative viscosity of produced bioethanol from microalgal biomass and that of commercial ethanol were found to be relatively close.

5.2 Conclusion

Based on the result from this study microalgal biomass possessed potential for bioethanol production. When subjected to hydrolysis using acid, enzyme (amylase) and combine acid and enzyme hydrolysis, combined acid and enzyme hydrolysis was found to successfully produce high level of reducing sugars and bioethanol yield from microalgal

biomass. Both pH, temperature and incubation time were found to exert effect on bioethanol yield. By optimizing these conditions incubation time were found to exert more positive effect on bioethanol yield compared to pH and temperature ranges used. Therefore, by exploiting the potentials of low-cost substrate such as *spirogyra sp.* as it is available abundantly in fresh water and more importantly it has very low lignin content may open new road map for the bioethanol-production technology which is regarded to be eco-friendly and sustainable fuel.

5.3 Recommendations

i. Different hydrolysis agent should be explored on the *Spirogyra sp.* biomass to increase the yield of reducing sugars yield for fermentation.
ii. Development of improved strains like thermos tolerant yeast is required to enhance the overall fermentation efficiency and also to reduce harvesting time.
iii. Nutrient supplementation may lead to further increase in fermentation efficiency and hence increase bioethanol yield.

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APPENDICES

APPENDIX I: LIST OF EQUIPMENT, GLASS WARES AND CHEMICALS

MATERIALS	Specification
1. Basket	Plastics
2. Bucket	Plastics
3. Laboratory trail	Plastics
4. Mortor and pistil	Ceramic
5. Siever	
6. Incubator	
7. Photo bioreactor	

REAGENTS/MEDIA

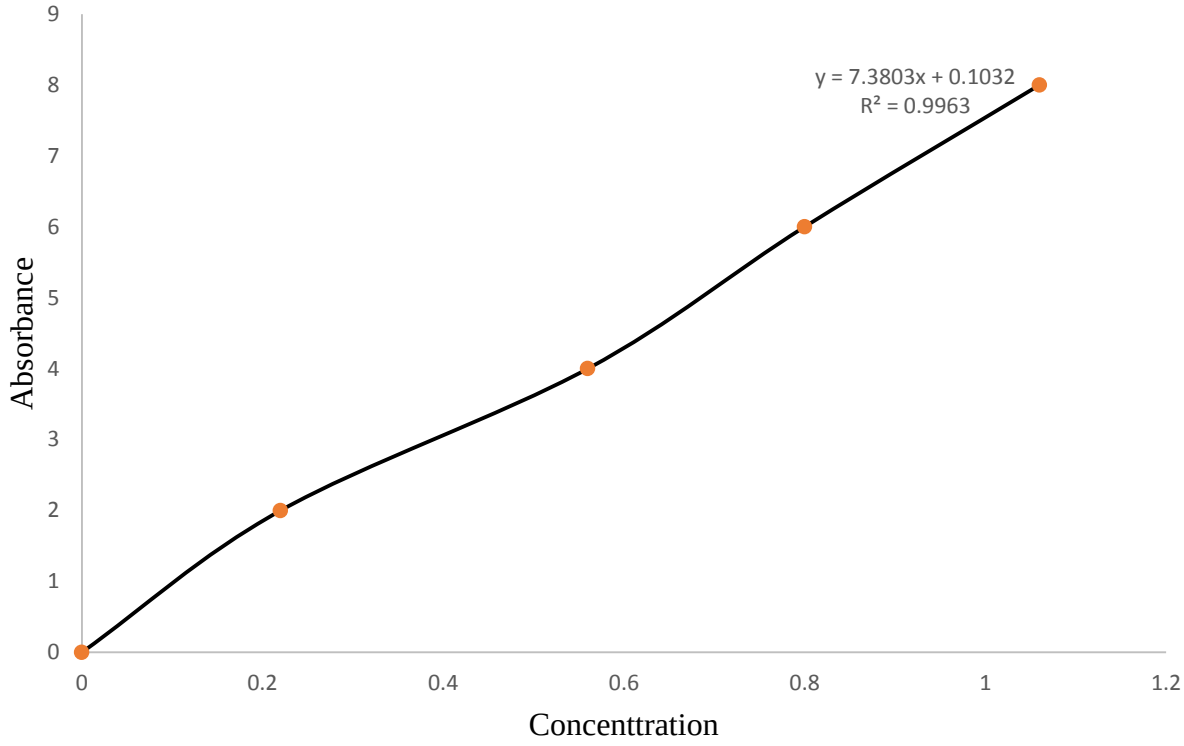
1. Algae biomass
2. Potato dextrose agar (PDA) medium
3. Yeast extract, peptone and dextrose agar media (YPD)
4. Distilled water
5. Ammonium nitrite
6. Potassium hydrogen tetraoxophosphate
7. Magnesium tetraoxosulphate VI (Heptahydrate)
8. Iron tetraoxosulphate VI (Heptahydrate)
9. Manganese tetraoxosulphate VI (Tetrahydrate

GLASS WARES/APPARATUS

1. Conical flask

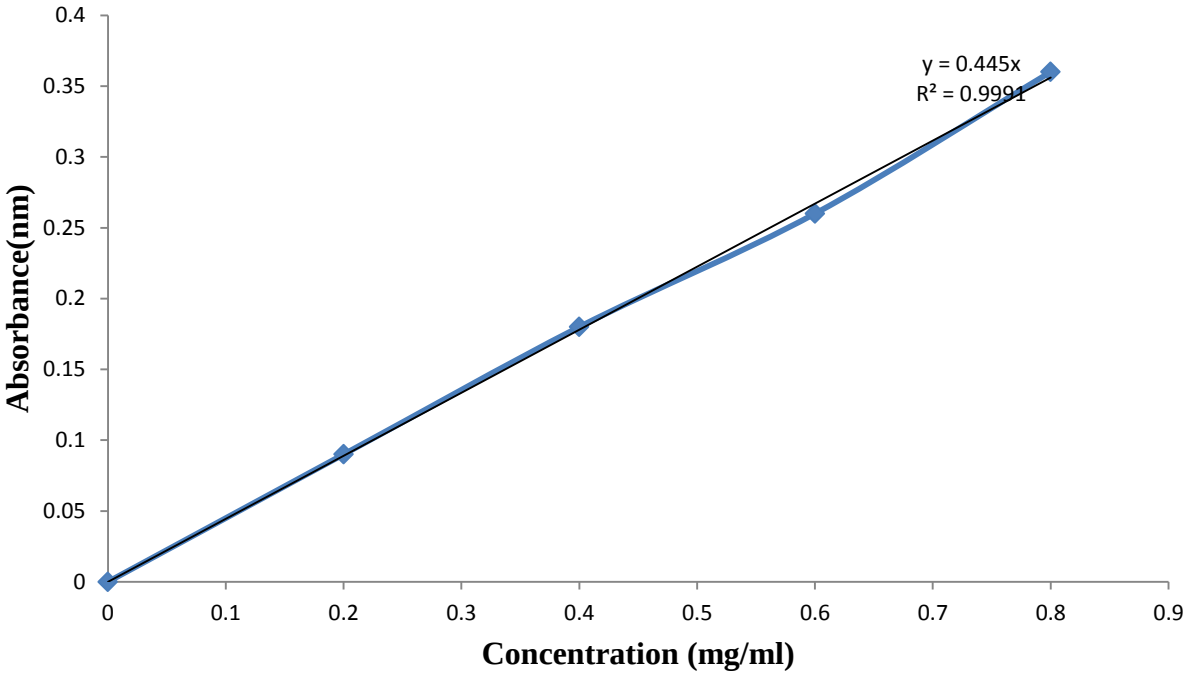
2. Beaker
3. Glass rod
4. Erlenmeyer
5. Autoclave
6. Thermometer
7. Stop watch
8. Petri dish
9. Pipette
10. Test tubes
11. Spatula
12. Electric washing balance
13. Inoculating needle
14. Capillary tube
15. Measuring cylinder 10ml
16. Cotton wool
17. Foil paper
18. Spirit lamp.

APPENDIX II: STANDARD CURVES



Standard Ethanol Concentration Curve

Standard curve of reducing sugars



Ethanol standard curve

APPENDIX III:

Reducing sugar Yield from Spirogyra hydrolysis

Hydrolysis Conditions	Reducing sugar concentration (mg/ml)			Mean	SD±
Acid	0.43	0.42	0.42	0.423333	0.004714
Enzyme	0.3	0.29	0.3	0.296667	0.004714
Combine	0.82	0.81	0.8	0.81	0.008165

Effects of Hydrolysis on Ethanol Yield

Hydrolysis Conditions	Ethanol Yield (%)			Mean	SD±
Acid	11.1	11.3	11.1	11.16667	0.094281
Enzyme	11.4	11.3	16.0	12.9	2.192411
Combine	13.2	14.0	13.3	13.5	0.355903

APPENDIX IV

Effects of pH on ethanol yield

pH	Ethanol Yield (%)			Mean	SD±
4.5	7.2	7.3	7.1	7.2	0.08165
5	7.4	7.6	7.6	7.533333	0.094281
5.5	7.0	7.1	6.8	6.966667	0.124722
6	6.5	6.6	6.4	6.5	0.08165

Effects of Time on Ethanol Yield

Incubation Time (hrs)	**Ethanol Yield (%)**			**Mean**	**SD±**
24	5.9	5.8	5.7	5.8	0.08165
48	6.1	6.2	6.1	6.133333	0.04714
72	6.2	6.3	6.4	6.3	0.08165
96	6.2	6.3	6.5	6.333333	0.124722
120	6.3	6.4	6.6	6.433333	0.124722

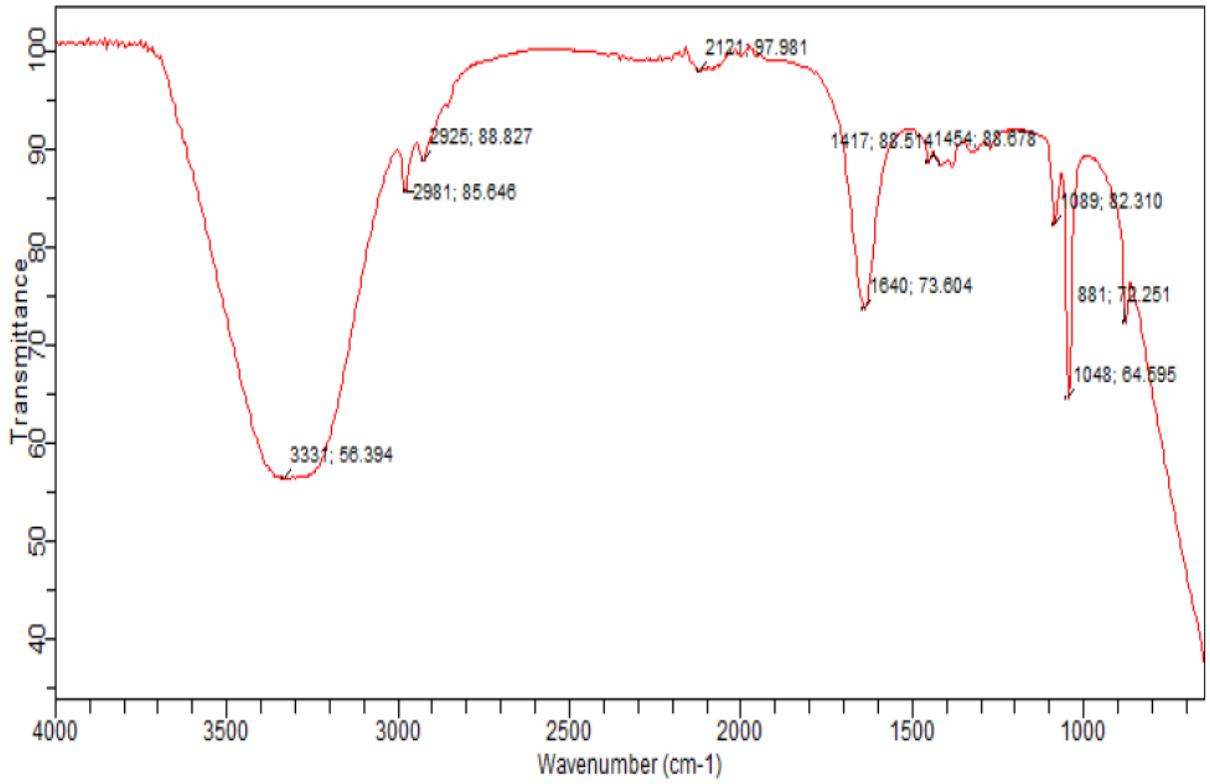
APPENDIX V

Background Scans: 32 Date/Time: 10/23/2018 2:40:11PM

Resolution: 8 cm-1 Range: 4,000.00 - 650.00

System Status: Good Apodization: Happ-Genzel

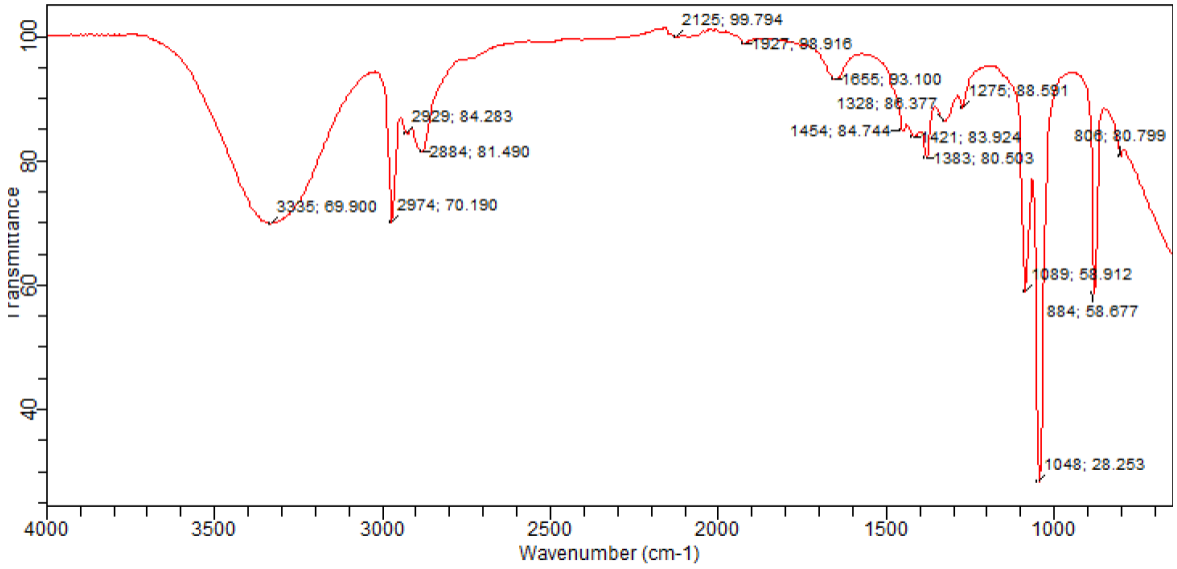
File Location: C:\Program Files\Agilent\MicroLab PC\Results\Sample A_2018-10-23T14-41-15.a2r



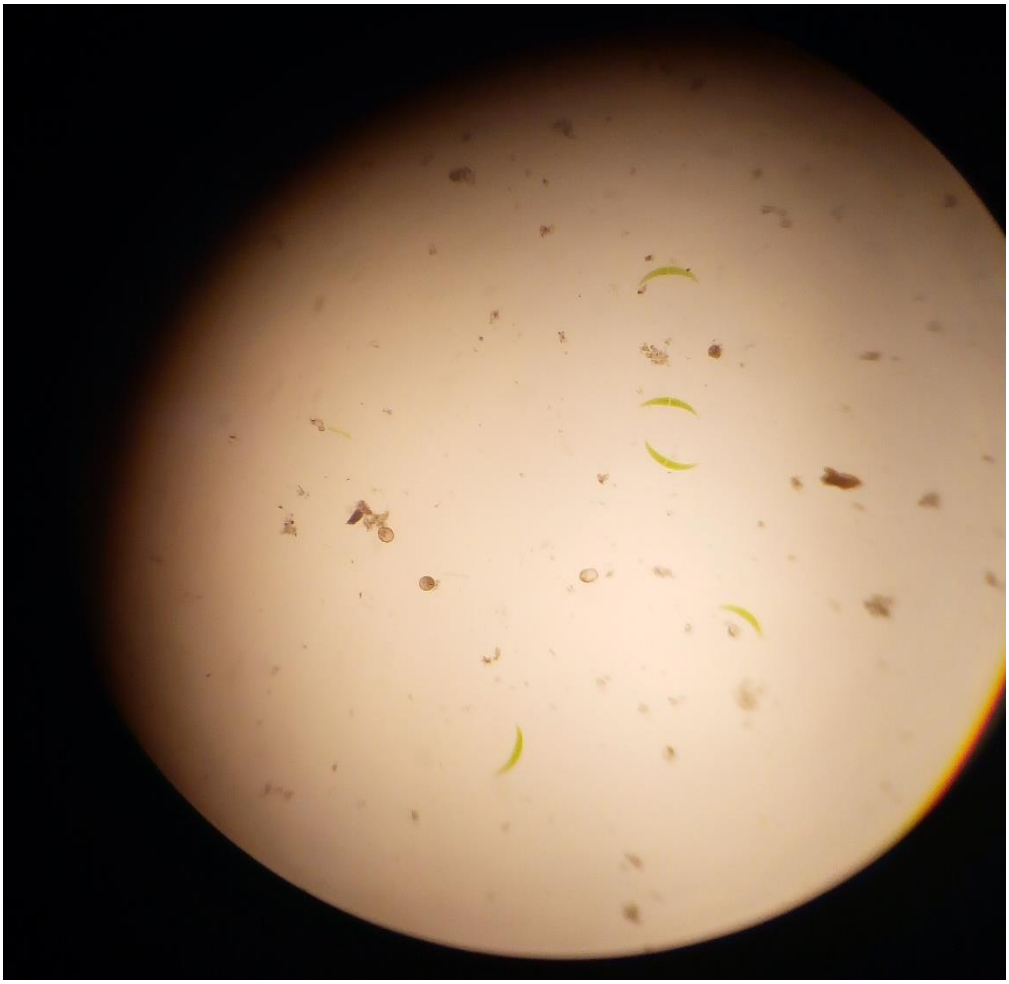
FT-IR result of the produced bioethanol

FT-IR of the commercial ethanol

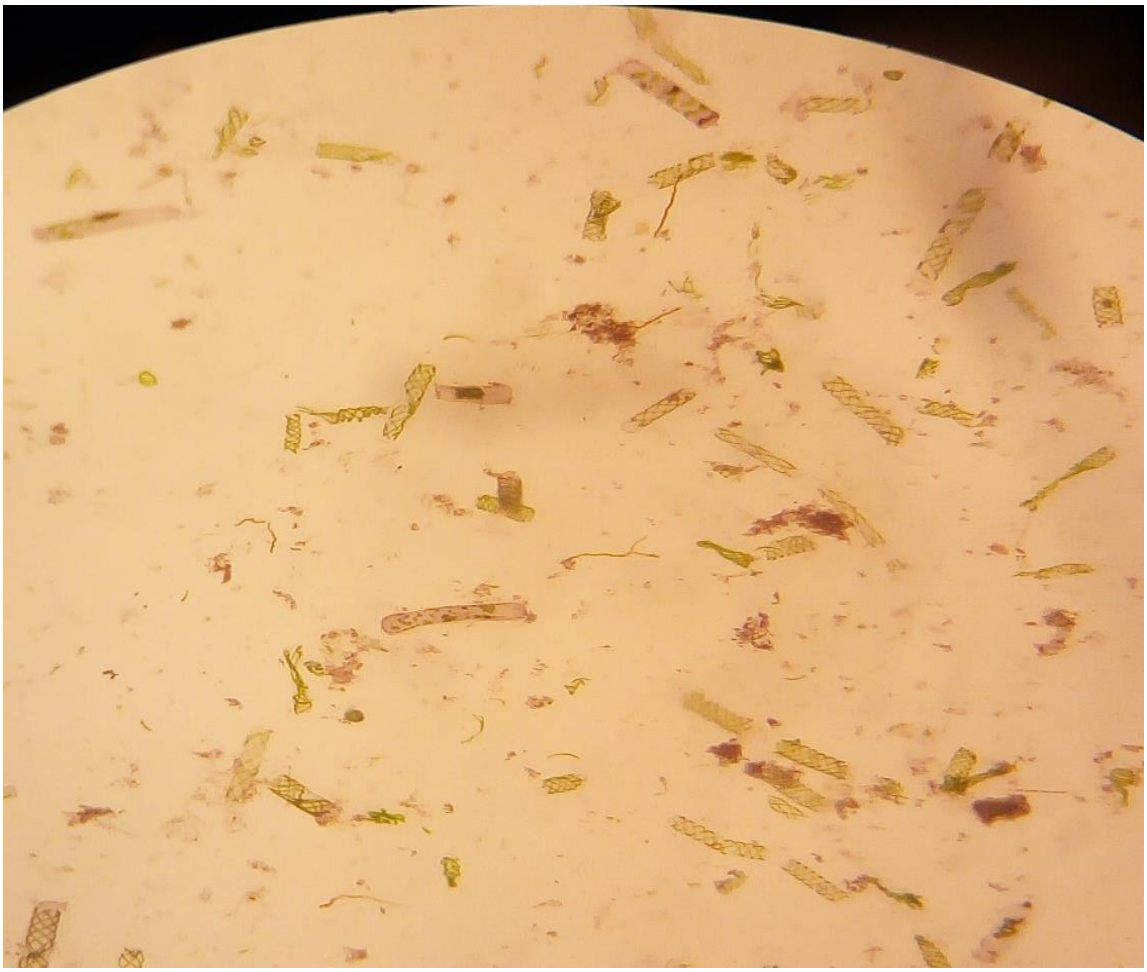
File Location: C:\Program Files\Agilent\MicroLab PC\Results\Commercial Ethanol_2019-04-18T10-06-49.a2r



APPENDIX VII: Micrographic picture of microalgae isolated from water sample



Micrographic picture of *chlorella vulgaris* isolated from water sample



Micrographic picture of *Spirogyra sp.* isolated from water sample

APPENDIX IX

Dried powder of Microalgal biomass

APPENDIX X

ANOVA Table for effect of hydrolytic agent on reducing sugar and bioethanol yield

		Sum of Squares	**Df**	**Mean Square**	**F**	**Sig.**
Reducing sugar	Between Groups	0.429	2	0.215	3861.6	0.00
	Within Groups	0	6	0		
	Total	0.429	8			
Ethanol Yield	Between Groups	8.809	2	4.404	1.782	0.247
	Within Groups	14.827	6	2.471		
	Total	23.636	8			

ANOVA Table for effect of pH and temperature on reducing sugar and bioethanol yield

ANOVA						
		Sum of Squares	Df	Mean Square	F	**Sig.**
pH	Between Groups	1.697	3	0.566	39.922	**0.00**
	Within Groups	0.113	8	0.014		
	Total	1.81	11			
Temp	Between Groups	6.247	3	2.082	118.984	**0.00**
	Within Groups	0.14	8	0.018		
	Total	6.387	11			

ANOVA for effect of incubation time on bioethanol yield

Incubation Time					
	Sum of Squares	Df	Mean Square	F	**Sig.**
Between Groups	0.74	4	0.185	13.214	**0.001**
Within Groups	0.14	10	0.014		
Total	0.88	14			

T-test for boiling point and viscosity of produced ethanol vs commercial ethanol

		F	Sig.	T	df	Sig. (2-tailed)	Mean Dif	Std. Error Difference	
Boiling point	Equal variances assumed	0	1	1.569	4	0.192	0.2666667	0.1699673	
	Equal variances not assumed			1.569	4	0.192	0.2666667	0.1699673	
Viscosity	Equal variances assumed	0.114	0.752	2.228	4	0.09	0.04	0.01795	
	Equal variances not assumed			2.228	3.958	0.09	0.04	0.01795	