STUDYING THE OPTIMAL CONDITIONS FOR GLUCOSE AND BIO-ETHANOL PRODUCTION FROM *SPIROGYRA AFRICANA*

BY

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FACULTY OF ENGINEERING

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DECEMBER, 2016

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DEPARTMENT OF CHEMICAL ENGINEERING

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NIGERIA.

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DECLARATION

I declare that the work in this dissertation entitled ” STUDYING THE OPTIMAL CONDITIONS FOR GLUCOSE AND BIO-ETHANOL PRODUCTION FROM *SPIROGYRA AFRICANA* ” has been carried out by me in the department of chemical engineering under the supervision Of Dr. O. R. Momoh and Prof. B. O. Aderemi. The information derived from the literature has been duly acknowledged in text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

BISIKE CHIDIEBERE EGERE Date

CERTIFICATION

This Dissertation "STUDYING THE OPTIMAL CONDITIONS FOR GLUCOSE AND BIO-ETHANOL PRODUCTION FROM *SPIROGYRA AFRICANA*" meets the regulations governing the award of the degree of Master of Science in Chemical Engineering of the Ahmadu Bello University Zaria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to Sandra, my beloved wife for her support and understanding.

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ABSTRACT

In this research work, the optimal conditions for glucose and bio-ethanol production from *Spirogyra africana* were studied. The *Spirogyra africana* samples collected from the Ahmadu Bello University Zaria Dam were dried, milled and sieved to ≤ 125 µm, and subsequently analyzed for carbohydrate content. Prior to hydrolysis, a set of the samples was pre-treated with NaOH, 0.5, 1.0 and 2.0 % (w/v), another set was blanched for 5 minutes at different temperatures (30 ^{o}C to 100 ^{o}C), while another set served as control (untreated). *Aspergillus niger* and *Saccharomyces cerevisiae* were used for saccharification and fermentation processes, respectively. Untreated biomass sample at two (2) days of hydrolysis with glucose yield of 29.13 wt/wt % was chosen as ideal for saccharification in this study. The result from the proximate analysis showed that *Spirogyra africana* contains 39.72 % carbohydrate. On the first day of hydrolysis, the blanched sample gave a relatively superior glucose yield of 15.94 wt/wt % in comparison to the values of 14.33 and 12.03 wt/wt % for NaOH treated sample and the control, respectively. However, on the second day, the glucose yield from the three set ups (blanched, NaOH treated and the control) all gave a comparative glucose yield of 29.15, 29.41 and 29.13 wt/wt %, respectively. Only marginal increments were recorded on the third day, corresponding to 30.72, 32.28 and 30.66 wt/wt % respectively. The optimum Saccharification conditions obtained in this work were: reaction temperature of 25-30 ^{o}C, pH of 4.5, residence time of 48 hrs., substrate concentration of 50 g/l and *A. niger* concentration of 0.6 % (w/v). Similarly, the optimal conditions for the fermentation were: reaction temperature of 35 ^{o}C, pH of 5.5, reaction time of 72 hrs., and *yeast* concentration of 1.0 % (w/v), which gave a bio-ethanol yield of 21.4 % (v/v).

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF PLATES

LIST OF APPENDICES

LIST OF SYMBOLS AND ABBREVIATIONS

ABV	Alcohol by Volume
AFEX	Ammonia fibre expansion
CRAC	China Resources Alcohol Corporation
FG	Final gravity
GHG	Greenhouse gas emissions
HMF	Hydroxymethyl furfural

OG	Original gravity
SMB	Simulated Moving Bed
Sp	Species
SPORL	Sulphite pre-treatment to overcome Recalcitrance of lignocellulose

CHAPTER ONE

INTRODUCTION

1.1 Background

Bioethanol is a fuel being used as gasoline substitute. It is either produced by the sugar fermentation process, or by the chemical process of reacting ethylene with steam (Matthew, 2013). The main source of sugar required to produce ethanol comes from fuel or energy crops. These fuel crops are normally grown specifically for energy use and they include maize, reed canary grass, cord grasses, Jerusalem artichoke, myscanthus, sorghum plants, and wheat crops. Other feedstocks are: waste straw, willow, and sawdust. These energy crops are cultivated on land, thereby they compete for space with food crops (Matthew, 2013).

Currently, algae is being investigated by researchers across the globe, as a substitute to the first and second generations energy crops because among other things, it grows very rapidly in water bodies, therefore there is no competition for land space with food crops (Somma *et al*. 2010). Chiu *et al*. (2009) in a study reported that Algae have the potentials for increasing the fuel production due to its rapid growth, carbohydrate and lipid accumulation. Eshaq *et al.* (2010) further assert that the yield of alcohol using algal biomass is more when compared to alcohol from other sources like agro based raw materials. Hirano *et al*. (2012) investigated a particularly interesting method of micro algae bio-ethanol production. They demonstrated that under anaerobic conditions even in the dark, the starches within the cells of micro algae were fermented. The potentials for simple, low cost bio-ethanol production are apparent. Razif and Michael (2011), studied the influence of acid pre-treatment on microalgal biomass for bioethanol production and the results showed that the highest bio-ethanol concentration obtained was 7.20 g/L and this was achieved when the pre-treatment step was performed with 15 g/L of microalgae at

140 ^{o}C using 1% (v/v) of sulphuric acid for 30 min. On the other hand, Eshaq *et al.* (2010), earlier observed that higher yield of ethanol was recorded for untreated Spirogyra when compared to chemically pretreated biomass. This apparent conflict suggests the need for more systematic works to be done on the role of pretreatment (physical and/or chemical) in algae conversion.

Secondly, there are varied strains (species) of algae around the world, direct research works on bio-fuel production from *Spirogyra africana,* which is the common strain in Nigeria are very scarce. Hence this work focuses on the effect of pretreatment on glucose yield and other process optimal conditions for glucose and bio-ethanol production from *Spirogyra africana*. The optimal conditions will save cost, time, and energy.

1.2 Problem Statements

Generally, there is a steady increase in demand for green energy, and the limitations of first and second generation biomass as substitute to fossil fuels have been severally documented. Therefore, search light on the third generation biomass is inevitable. Although information on third generation biomass has started gathering, there are several species that are yet to enjoy significant attention because they are geographically dependent for e.g. *Spirogyra africana* species.

Determinations of the optimal conditions for the production of glucose and subsequently fermentation to bio-ethanol, which are indispensible to its commercialization, have to be determined for these newcomers. More so, apparent conflicts still exist on general processing conditions (i.e. the relevance of pretreatment as obtained generally for the second generation biomasses) of these third generation biomasses, which need to be resolved before its processing technology crystallizes.

1.3 Aim and Objectives

Aim: To study the effect of pre-treatment and other processing parameters on the production of glucose and bio-ethanol from *Spirogyra africana.*

Objectives:

The Specific objectives are to;

1. Characterize (Proximate Analyse) *Spirogyra africana* biomass to ascertain if it has sufficient carbohydrate content for glucose and bio-ethanol production.
2. Study the effect of pre-treatment on Saccharification and fermentation of *Spirogyra africana.*
3. Determine the optimum processing conditions for saccharification of *Spirogyra africana* such as: pre-treatment type, temperature, pH, substrate concentration and cell loading.
4. Produce bio-ethanol from the glucose and to determine the optimal fermentation conditions such as: pH, temperature and yeast concentration for bio-ethanol production from *Spirogyra africana*.

1.4 Justification

The determination of the optimal process conditions for the production of glucose and bio-ethanol will enhance the commercialization of the process and likewise save cost, time and energy. Farming this aquatic plant will inevitably create innovative jobs.

1.5 Scope

The scope of this work include collection of *spirogyra africana* sample from ABU Zaria dam, sun drying and reducing the size of the collected sample to $\leq$ 125μm, characterization to ascertain the carbohydrate content, conversion of the carbohydrates to fermentable sugar using *Aspergillus niger* cells, and subsequent fermentation to bio-ethanol with *Saccharomyces cerevisiae*. The effects of blanching and NaOH as pre-treatment agents on the glucose and bioethanol yield were also studied.

CHAPTER TWO

LITERATURE REVIEW

2.1 Bioethanol

Bio-ethanol is the principal fuel used as a gasoline substitute. It is mainly produced by the sugar fermentation process, although it can also be produced by the chemical process of reacting ethylene with steam. The main source of sugar required to produce ethanol comes from fuel or energy crops. These fuel crops are normally grown specifically for energy use and include maize, corn and wheat crops, waste straw, willow, sawdust, reed canary grass, cord grasses, Jerusalem artichoke, myscanthus and sorghum plants (Mathew, 2013).

According to Michael Wang of Argonne National Laboratory (2014), one of the benefits of ethanol is that it reduces greenhouse gas emissions (GHG) by 85% over reformulated gasoline. By contrast, starch ethanol (e.g., from corn), which most frequently uses natural gas to provide energy for the process, may not reduce GHG emissions at all depending on how the starch-based feedstock is produced (Schemer, 2008). According to Schemer of the National Academy of Sciences (2008), there is no commercially viable bio-refinery in existence to convert lignocellulosic biomass to fuel. Absence of production of cellulosic ethanol in the quantities required by the regulation was the basis of a United States Court of Appeals for the District of Columbia decision announced January 25, 2013, voiding a requirement imposed on car and truck fuel producers in the United States by the Environmental Protection Agency requiring addition of cellulosic biofuels to their products (Matthew, 2013). These issues, along with many other difficult production challenges, led George Washington University policy researchers to state that "in the short term, [cellulosic] ethanol cannot meet the energy security and environmental goals of a gasoline alternative" (Somma, *et al.*, 2010).

2.2 History of Bioethanol

The French chemist, Henri Braconnot, was the first to discover that cellulose could be hydrolyzed into sugars by treatment with sulphuric acid in 1819 (EERE, 2009). In 1898, Germany made the first attempt to commercialize the process of producing ethanol from wood (EERE, 2009). This involved the use of dilute acid to hydrolyze the cellulose to glucose, and was able to produce 7.6 litres of ethanol per 100 kg of wood waste (18 US gal (68 L) per ton). The Germans soon developed an industrial process optimized for yields of around 50 gallons (190 L) per ton of biomass. This process soon found its way to the US, culminating in two commercial plants at South Carolina and Louisiana during WWI. These plants used what was called "the American Process" — a one-stage dilute sulphuric acid hydrolysis, the throughput of the American process was much higher, though the yields were half that of the original German process (25 gallons (95 L) of ethanol per ton versus 50). A drop in lumber production forced the plants to close shortly after the end of World War I (EERE, 2009).

Before the World War II, about 90 million gallons of grain alcohol was used as liquid fuel for lamps, the best-selling lamp fuel was camphene, made of turpentine, grain-alcohol and camphor oil (Abebe, 2008). It sold for 50 cents a gallon, making it cheaper than whale oil and lard oil. During the World War II, the US again turned to cellulosic ethanol, for conversion to butadiene to produce synthetic rubber (Abebe, 2008).

The Vulcan Copper and Supply Company was contracted to construct and operate a plant to convert sawdust into ethanol. The plant was based on modifications to the original German Scholler process as developed by the Forest Products Laboratory. This plant achieved an ethanol yield of 50 gal (190 L) per dry ton, but was still not profitable and was closed after the war (Katzen and Schell, 2006).

With the rapid development of enzyme technologies in the last two decades, the acid hydrolysis process has gradually been replaced by enzymatic hydrolysis. Chemical pre-treatment of the feedstock is required to prehydrolyze (separate) hemicellulose, so it can be more effectively converted into sugars. The dilute acid pre-treatment is developed based on the early work on acid hydrolysis of wood at the USFS's Forest Products Laboratory. Recently, the Forest Products Laboratory together with the University of Wisconsin–Madison developed a sulphite pre-treatment to overcome the recalcitrance of lignocellulose (Zhu, *et al*. 2009), for robust enzymatic hydrolysis of wood cellulose.

One time US President, George W. Bush, in his State of the Union address delivered January 31, 2006, proposed to expand the use of cellulosic ethanol. Furthermore, in his State of the Union Address on January 23, 2007, President Bush announced a proposed mandate for 35 billion US gallons (130,000,000 m^3) of ethanol by 2017. It is widely recognized that the maximum production of ethanol from corn starch is 15 billion US gallons (57,000,000 m3) per year, implying a proposed mandate for production of some 20 billion US gallons (76,000,000 m^3) more per year of cellulosic ethanol by 2017. Bush's proposed plan included $2 billion funding (from 2007 to 2017) for cellulosic ethanol plants, with an additional $1.6 billion (from 2007 to 2017) announced by the USDA on January 27, 2007 (Dirk, 2007).

In March 2007, the US government awarded $385 million in grants aimed at jump-starting ethanol production from non-traditional sources like wood chips, switchgrass, and citrus peels. Three (3) of the six (6) projects chosen were to use thermochemical methods and the remaining three (3) were to use cellulosic ethanol methods (Dirk, 2007).

In July 2007, the American company, Range Fuels, announced that it was awarded a construction permits from the state of Georgia to build the first commercial-scale 100-million-US-gallon (380,000 m^3)-per-year cellulosic ethanol plant in the US. Construction

began in November, 2007. The Range Fuels plant was built in Soperton, GA, but was shut down in January 2011, without ever having produced any ethanol. It had received a $76 million grant from the US Department of Energy, plus $6 million from the State of Georgia, and an $80 million loan guaranteed by the U.S. Biorefinery Assistance Program (Dirk, 2007). Work on bioethanol production from Algae have started gathering but little have been done on Spirogyra especially on *Spirogyra africana,* hence this research work.

2.3 Classification of Biofuels

2.3.1 First Generation Biofuels

First-generation biofuels which have attained economic levels of commercial production, have been mainly extracted from food and oil crops such as: barley, maize, rapeseed oil, palm oil, sugarcane, sugar beet, wheat, etc. (Nigam and Singh, 2010). There is daily increase of liquid biofuels production and consumption but their impacts towards meeting the overall energy demands in the transport sector will remain limited due to: competition with fibre and food production, high water and fertiliser requirements, the use of arable land, poor agricultural practices in emerging economies, biodiversity conservation and regionally constrained market structures. Global biofuel production has been increasing rapidly over the last decade, (Eisentraut, 2010) but the expanding biofuel industry has recently raised important concerns. In particular, the sustainability of many first generation biofuels (primarily from food crops such as grains, sugar cane and vegetable oils) has been increasingly questioned over concerns such as reported displacement of food crops, effects on the environment and climate change. In general, there is growing consensus that if significant emission reductions in the transport sector are to be achieved, biofuel technologies must become more efficient in terms of net lifecycle GHG emission reductions while at the same time be socially and environmentally sustainable (Eisentraut, 2010).

The increasing criticism of the sustainability of many of the first-generation biofuels has raised attention to the potential of the so called second-generation biofuels.

2.3.2 Second-Generation Biofuels

These are the type of biofuels produced from lignocellulosic and non-food crops. Depending on the feedstock choice and the cultivation technique, second-generation biofuel production has the potential to provide benefits such as consuming waste residues and making use of abandoned land, promote rural development and improve economic conditions in emerging and developing regions (Evans, 1997).

However, second-generation biofuel crops and production technologies are more efficient because they don't compete with food but their production could become unsustainable if they compete with food crops for available land. Thus, their sustainability will depend on whether producers abide by with criteria like minimum lifecycle GHG reductions, including land use change, and social standards (Eisentraut, 2010).

The limitations of first-generation biofuels produced from food crops have caused greater emphasis to be placed on second generation biofuels produced from lignocellulosic feed stocks, although significant progress continues to be made to overcome the technical and economic challenges, second-generation biofuels production will continue to face major constraints to execute commercial deployment (Sims *et al.,* 2010).

The logistics of providing a competitive supply of biomass feedstock to a commercial plant is challenging, as is improving the performance of the conversion process to reduce costs. The most positive impact of biofuels is the reduction of the GHGs emissions in its production and consumption. This is because biomass production utilized atmospheric CO_2 and biomass is renewable. On the other hand, mass production of biofuels can lead to the increase of GHG emissions by the utilization of fossil transportation fuels in the

complicated logistics needed for biomass cultivation, collection, transportation and distribution (Sims *et al.,* 2010).

2.3.3 Third Generation Biofuels

Biodiesel and bioethanol produced from terrestrial plants have attracted the attention of researchers as possible replacement. Several biofuels have been proposed to replace fossil fuels in order to eliminate the vulnerability of the energy sector. However, due to food versus fuel competition as well as land consumption of these biofuels, there is much controversy and debate on their sustainability (Goh and Lee, 2010).

In this vain, cultivation of algae at sea water or industrial or other waste water provides a possible solution for this energy problem. Microalgae are single-cell, photosynthetic organisms known for their rapid growth and high energy content. Some algal strains are capable of doubling their mass several times per day. Biomass doubling times during exponential growth are commonly as short as 3.5 h. In some cases, more than half of that mass consists of lipids or triacylglycerides (Chisti, 2007).

Oil content in microalgae can be up to 80% of dry biomass. Depending on species, microalgae produce many different kinds of lipids, hydrocarbons and other complex oils (Banerjee *et al*., 2002; Guschina and Harwood, 2006). However, not all algal oils are satisfactory for making biodiesel (Chisti, 2007).

The large environmental footprint of algae cultivation is driven predominantly by upstream impacts, mainly, the CO_2 demand and fertilizer. They suggested that these impacts can be reduced by using flue gas and wastewater, to offset most of the environmental burdens associated with algae. However, it is still not proven that this high efficiency can be maintained after scaling-up the technology to a large production plant. Biodiesel production from algae is the promising technology (Chiu *et al*., 2009). The growth medium must provide the inorganic elements that comprise the algal cell. Essential elements include phosphorus, nitrogen, iron and in some cases silicon (Chisti, 2007).

Third generation technology is based on algae or cyanobacteria that contain a high oil mass fraction grown in ponds. Micro-organisms can convert almost all of the energy in biomass residuals and wastes to methane and hydrogen. Certain algae and cyanobacteria have high lipid contents (Spiertz and Ewert, 2009). Under suitable conditions, these micro-organisms can manufacture lipids for biodiesel with yields per unit area that are much higher than those with any plant (Chisti, 2008; Rittmann, 2008). The study reported an increase in the biomass production and lipid accumulation with a CO_2 concentration increase in the aeration.

In order to demonstrate the benefits of algae production coupled with wastewater treatment, Clarens *et al.,* (2010) expanded a model to include three different municipal wastewater effluents as sources of nitrogen and phosphorus. Each provided a significant reduction in the environmental burdens of algae cultivation, and the use of source-separated urine was found to make algae more environmentally beneficial than the terrestrial crops. Algae can be cultivated in farms absorbing CO_2 from the air. They contain oils that can be used as raw material for biodiesel production (Petrou and Pappis, 2009). They have the advantage that they do not conflict with food production. Furthermore, they have the potential to cover the global demand for transportation fuels (Chisti, 2008). The impacts associated with algae production using a stochastic life cycle model and compared with switchgrass, canola and corn was studied by Clarens *et al*. in 2010 and the results of the study show that these crops have lower environmental impacts than algae in greenhouse gas emissions, energy use, and water regardless of cultivation location. Only in total land use and eutrophication potential do algae perform favourably.

2.4 Algae

2.4.1 Algae Classification

Most algae contain chloroplasts that are similar in structure to cyanobacteria. Chloroplasts contain circular DNA like that in cyanobacteria and presumably represent reduced endosymbiotic cyanobacteria.

However, the exact origin of the chloroplasts is different among separate lineages of algae, reflecting their acquisition during different endosymbiotic events. The Table 2.1 details the carbohydrate composition of the major groups of algae. Many of these groups contain some members that are no longer photosynthetic. Some retain plastids, but not chloroplasts, while others have lost plastids entirely.

Table 2.1; Carbohydrate Composition of Some Algae Strain on a Dry Matter Basis (%)

STRAIN	**CARBOHYDRATES**
Scenedesmus obliquus	10-17
Scenedesmus quadricauda	-
Scenedesmus dimorphus	21-52
Chlamydomonas rheinhardii	17
Chlorella vulgaris	12-17
Chlorella pyrenoidosa	26
Spirogyra sp.	**33-64**
Dunaliella bioculata	4
Dunaliella salina	32
Euglena gracilis	14-18
Prymnesium parvum	25-33
Tetraselmis maculata	15
Porphyridium cruentum	40-57
Spirulina platensis	8-14

Source: http://www.oilgae.com/algae/comp/comp.html#sthash.kdixdzcp.dpuf

2.4.2 Commercial Initiatives of Algal Fuel

In April 2006, the start-up company, Solix Biofuels set up in Fort Collins, Colorado USA developed a microalgae reactor technology which could be used in conjunction with existing power stations, running the carbon dioxide in closed cycles. The company is involved in optimizing the process and finding the best species of algae for such reactors. In June 2006, an oil company Petro-Sun, started a wholly owned subsidiary called Algae Biofuels to operate in the US and Australia, to investigate the production of biodiesel, ethanol, methanol, methane, and hydrogen from microalgae. The company recently announced a feedstock supply agreement with the company Bio-Alternatives, under which Petro-Sun supplies half of its algae production (or up to 150 million gallons per year) to Bio-Alternatives as a feedstock for fuel production. The partners plan to locate algae production and biodiesel refinery facilities in Louisiana. In October 2007, the Chevron Corporation announced a collaborative research project with the DoE's National Renewable Energy Laboratory (NREL) to explore the production of transport fuels, including jet fuel, from algae. Now Shell wants to make use of the existing expertise of HR Bio-petroleum, which is already growing algae on the coasts of Hawaii, and test the technology for its efficiency and commercial viability (Wang *et al*., 2008).

It is essential to select strains capable of growing in variety of wastewaters and producing feedstock for biofuels that can compete with biodiesel, biomethane and bioethanol in terms of land and water use, carbon sequestration, and GHG emission savings, etc. Capability of algae to consume large amounts of CO_2 also makes it an attractive option as the process could be carbon neutral (Wang *et al*., 2008).

2.4.3 Harvest, Processing and Uses of Algae

Harvest

The algae store starch mainly in the cells, and the biomass can be harvested at regular intervals from photobioreactors or shallow raceway ponds for the extraction of starch. The starch can be extracted from the cells with the aid of mechanical means (e.g., ultrasonic, explosive disintegration, mechanical shear, etc.) or by dissolution of cell walls using enzymes (Matsumoto *et al*., 2003).

Processing

The starch can be separated by extraction with water or an organic solvent and used for bio-ethanol production. This is done by extracting the intracellular microalgal starch which can be fermented to ethanol using the technology similar to other starch-based feedstocks and it involves two processes, saccharification and fermentation (Rubin, 2008).

The fermentation of starch to ethanol can be carried out in a single step or double steps. Prior to fermentation the starch need to be hydrolyzed to simple sugars and this process is called saccharification. Acid or enzymatic (alpha- and glucoamylase) hydrolysis can be used for the conversion of starch to simple sugars. In the next step, the sugars are fermented to ethanol by a suitable yeast strain. Both these processes can be simultaneously carried out in a single step if an amylase producing strain can be used for ethanol fermentation. Utilization of starch degrading ethanol producers can preclude the cost incurred for acid or enzymatic saccharification of starch. Finally, the ethanol is purified by distillation to remove water and other impurities in the diluted alcohol product (10–15% ethanol). The concentrated ethanol (95% ethanol) is drawn off and condensed into liquid form, which can be blended with fossil fuels or directly used as fuel (Demirbas, 2001; Nigam and Singh, 2010; Brennan and Owende, 2010).

Uses

1. Most algae are rich in oil and are used for bio-diesel production however some strain are low in lipids and can be used for bio-ethanol production (Brennan and Owende, 2010).
2. The residual biomass obtained after oil extraction may also be used as substrate for ethanol fermentation (Harun, *et al*., 2014).
3. The solid residue left over from the bio-fuel processing can be used as cattle-feed (McKendry, 2002).

2.5 Cellulose

2.5.1 Introduction

Cellulose is an organic compound with the formula $(C_6H_{10}O_5)_n$, a polysaccharide consisting of a linear chain of several hundred to many thousands of β(1→4) linked D-glucose units (Crawford, 1981). Cellulose is an important structural component of the primary cell wall of green plants, many forms of algae and the oomycetes. Some species of bacteria secrete it to form biofilms, (Klemm *et al*, 2005). Cellulose is the most abundant organic polymer on earth (Klemm *et al*, 2005). The cellulose content of cotton and algae is 70%, that of wood is 40–50% and that of dried hemp is approximately 45% (Piotrowski *et al.*, 2011).

2.5.2 Cellulosic Ethanol

This is a biofuel produced from wood, grasses, or the inedible parts of plants. It is a type of biofuel produced from lignocellulose, a structural material that comprises much of the mass of plants. Lignocellulose is composed mainly of cellulose, hemicellulose and lignin. Corn stover, Panicum virgatum (switchgrass), Miscanthus grass species, wood chips and the by-products of lawn and tree maintenance are some of the more popular cellulosic materials for ethanol production (Matthew, 2013).

Production of ethanol from lignocellulose has the advantage of abundant and diverse raw material compared to sources such as corn and cane sugars, but requires a greater amount of processing to make the sugar monomers available to the microorganisms typically used to produce ethanol by fermentation. Switchgrass and Miscanthus are the major biomass materials being studied today, due to their high productivity per acre. Cellulose, however, is contained in nearly every natural, free-growing plant, tree, and bush, in meadows, forests, and fields all over the world without agricultural effort or cost needed to make it grow. According to Michael Wang of Argonne National Laboratory, one of the benefits of cellulosic ethanol is that, it reduces greenhouse gas emissions (GHG) by 85% over reformulated gasoline. (McAloon *et al*, 2000).

By contrast, starch ethanol (e.g., from corn), which most frequently uses natural gas to provide energy for the process, may not reduce GHG emissions at all depending on how the starch-based feedstock is produced . According to Schemer of the National Academy of Sciences in 2008, there is no commercially viable bio-refinery in existence to convert lignocellulosic biomass to fuel (Matthew 2013).

2.5.3 Chemistry of Cellulose

Cellulose molecule consists of several chains of glucose as show in figure 2.1

i. Cellulose Molecule

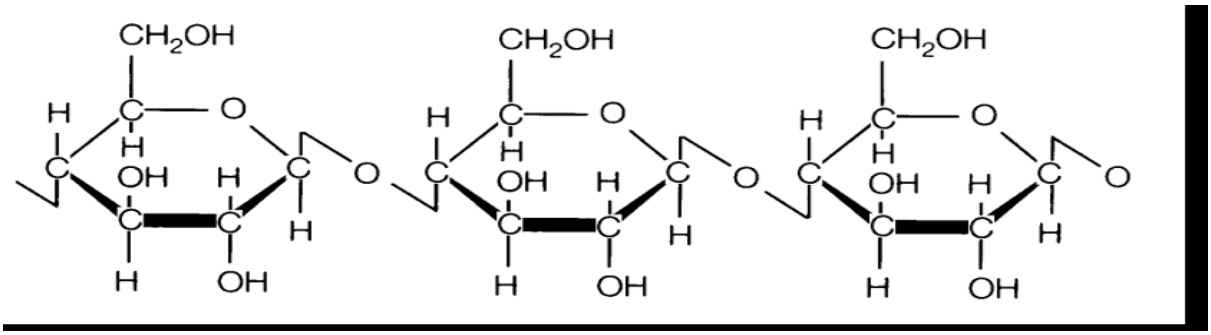


Figure 2.1: Cellulose Chain (http://www.greenspirit.org.uk/resources/cellulose.gif).

ii. Cellulose to Glucose Conversion

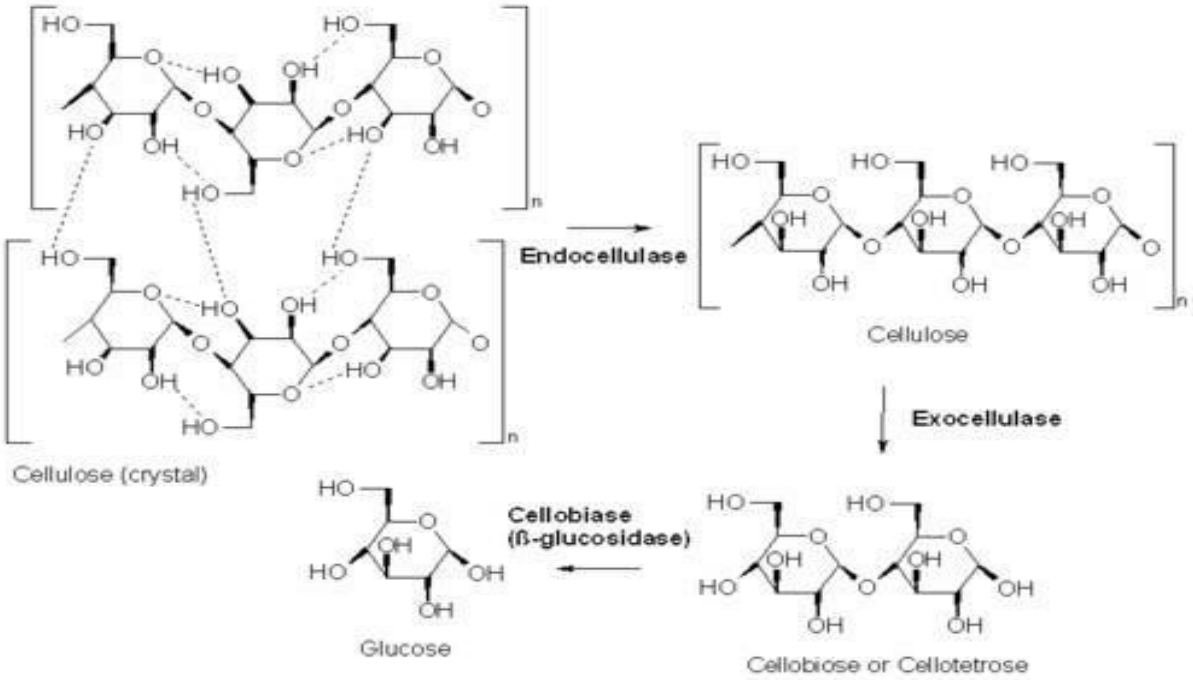


Figure 2.2: Reaction pathway from cellulose to glucose (Enzymeindia, 2008)

iii. Glucose to Ethanol Conversion.

The glucose to bio-ethanol conversion takes place in the presence of yeast while oxygen is librated as shown in equation one (1) below.

$$C_6H_{12}O_6 \xrightarrow{\text{Yeast}} 2CH_3CH_2OH + 2CO_2 \quad \ldots\ldots\ldots\ldots (1)$$

2.5.4 Methods of Producing Bio-Ethanol from Cellulose

The two ways of producing ethanol from cellulose are:

1. Cellulolysis Processes: This consists of hydrolysis on pre-treated lignocellulosic materials, using acid or enzymes to break complex cellulose into simple sugars such as glucose, followed by fermentation and distillation.
2. Gasification**:** This transforms the lignocellulosic raw material into gaseous carbon monoxide and hydrogen (syn gases). These gases can be converted to ethanol by chemical catalysis.

2.6 Cellulolysis (Biological Approach)

2.6.1 Ethanol Production Stages

The stages to produce ethanol using a biological approach are:

1. A "pre-treatment" phase, to make the lignocellulosic material such as wood or straw amenable to hydrolysis

2. Cellulose hydrolysis (cellulolysis), to break down the molecules into sugars

3. Separation of the sugar solution from the residual materials, notably lignin

4. Microbial fermentation of the sugar solution

5. Distillation to produce roughly 95% pure alcohol

6. Dehydration by molecular sieves to bring the ethanol concentration to over 99.5% (Zhu, *et al.*, 2009).

In 2010, a genetically engineered yeast strain was developed to produce its own cellulose-digesting enzymes (Galazka, *et al.*, 2010). Assuming this technology can be scaled to industrial levels; it would eliminate one or more steps of cellulolysis, reducing both the time required and costs of production.

2.6.2 Pre-treatment

Although lignocellulose is the most abundant plant material resource, its usability is curtailed by its rigid structure. As a result, an effective pre-treatment is needed to liberate the cellulose from the lignin seal and its crystalline structure so as to render it accessible for a subsequent hydrolysis step (Zhu, *et al*., 2009).

By far, most pre-treatments are done through physical or chemical means. To achieve higher efficiency, both physical and chemical pre-treatments are required. Physical pre-treatment is often called size reduction. Chemical pre-treatment is to remove chemical

barriers, so the enzymes can have access to cellulose for microbial destruction. The available pre-treatment techniques include acid hydrolysis, steam explosion, ammonia fibre expansion, organosoly, sulphite pre-treatment, (Zhu, *et al*., 2009), SO_2-ethanol-water (Lakovley, 2010), alkaline wet oxidation and ozone pre-treatment (Klinke, *et al.*, 2004). Besides effective cellulose liberation, an ideal pre-treatment has to minimize the formation of degradation products because of their inhibitory effects on subsequent hydrolysis and fermentation processes (Olsson, 1996). The presence of inhibitors will not only further complicate the ethanol production but also increase the cost of production due to entailed detoxification steps. Even though pre-treatment by acid hydrolysis is probably the oldest and most studied pre-treatment technique, it produces several potent inhibitors including furfural and hydroxymethyl furfural (HMF) which are by far regarded as the most toxic inhibitors present in lignocellulosic hydrolysate (Palmqvist and Hahn-Hägerdal, 2000). Ammonia Fibre Expansion (AFEX) is a promising pre-treatment with no inhibitory effect in resulting hydrolysate (Lynd, 1996).

Most pre-treatment processes are not effective when applied to feedstocks with high lignin content, such as forest biomass. Organosolv, SPORL (sulphite pre-treatment to overcome recalcitrance of lignocellulose) and SO_2-ethanol-water processes are the three processes that can achieve over 90% cellulose conversion for forest biomass, especially those of softwood species. SPORL is the most energy efficient (sugar production per unit energy consumption in pre-treatment) and robust process for pre-treatment of forest biomass with very low production of fermentation inhibitors. Organosoly pulping is particularly effective for hardwoods and offers easy recovery of a hydrophobic lignin product by dilution and precipitation. SO_2-ethanol-water process effectively fractionates all types of lignocellulosics into clean highly digestible cellulose, undegraded hemicellulose sugars,

reactive lignin and lignosulfonates, and is characterized by efficient recovery of chemicals (Retsina and Pylkkanen, 2004).

2.6.3 Cellulolytic Processes

Cellulolytic Processes is a process for producing cellulolytic and/or hemicellulolytic enzymes by using the residue from the ethanolic fermentation of enzymatic hydrolyzates of cellulosic or ligno-cellulosic materials. This process may be integrated into a process for the production of ethanol from cellulosic or ligno-cellulosic materials (Warzywoda *et al*, 2006).

2.6.4 Chemical Hydrolysis

In the traditional methods developed in the 19th century and at the beginning of the 20th century, hydrolysis is performed by attacking the cellulose with an acid. In 2014, Dussán *et al.*, reported that dilute acid may be used under high heat and high pressure, or more concentrated acid can be used at lower temperatures and atmospheric pressure. A decrystalized cellulosic mixture of acid and sugars reacts in the presence of water to complete individual sugar molecules (Dussán *et al.*, 2014). The product from this hydrolysis is then neutralized and yeast fermentation is used to produce ethanol. As mentioned, a significant obstacle to the dilute acid process is that the hydrolysis is so harsh that toxic degradation products that can interfere with fermentation are produced (Dussán *et al.*, 2014). The BlueFire Renewables uses concentrated acid because it does not produce nearly as many fermentation inhibitors, but must be separated from the sugar stream for recycle [simulated moving bed (SMB) chromatographic separation, for example] to be commercially attractive (Olsson, 1996).

Agricultural Research Service scientists found they can access and ferment almost all of the remaining sugars in wheat straw. The sugars are located in the plant's cell walls, which are notoriously difficult to break down. To access these sugars, scientists pre-treated the

wheat straw with alkaline peroxide, and then used specialized enzymes to break down the cell walls. The method produced 93 US gallons (350 L) of ethanol per ton of wheat straw (Updated Energy and Greenhouse Gas Emissions Results of Fuel Ethanol 2005). Enzymatic hydrolysis cellulose chains can be broken into glucose molecules by cellulase enzymes. The reaction occurs at body temperature in the stomachs of ruminants such as cattle and sheep, where the enzymes are produced by microbes. This process uses several enzymes at various stages of this conversion. Using a similar enzymatic system, lignocellulosic materials can be enzymatically hydrolyzed at a relatively mild condition (50 °C and pH 5), thus enabling effective cellulose breakdown without the formation of by-products that would otherwise inhibit enzyme activity. All major pre-treatment methods, including dilute acid, require an enzymatic hydrolysis step to achieve high sugar yield for ethanol fermentation. Currently, most pre-treatment studies have been laboratory-based, but companies are exploring means to transition from the laboratory to pilot, or production scale (Sunopta press release, 2007).

Various enzyme companies have also contributed significant technological breakthroughs in cellulosic ethanol through the mass production of enzymes for hydrolysis at competitive prices. The fungus *Trichoderma reesei* is used by Logen Corporation to secrete "specially engineered enzymes" for an enzymatic hydrolysis process. Their raw material (wood or straw) has to be pre-treated to make it amenable to hydrolysis. Another Canadian company, Sunopta, uses steam explosion pre-treatment, providing its technology to Verenium (formerly Celunol Corporation)'s facility in Jennings, Louisiana, Abengoa's facility in Salamanca, Spain, and a China Resources Alcohol Corporation in Zhaodong. The CRAC production facility uses corn stover as raw material (Sunopta press release, 2007).

2.6.5 Microbial Fermentation

Traditionally, baker's yeast (*Saccharomyces cerevisiae*), has long been used in the brewery industry to produce ethanol from hexoses (six-carbon sugars). Due to the complex nature of the carbohydrates present in lignocellulosic biomass, a significant amount of xylose and arabinose (five-carbon sugars derived from the hemicellulose portion of the lignocellulose) is also present in the hydrolysate. For example, in the hydrolysate of corn stover, approximately 30% of the total fermentable sugars are xylose. As a result, the ability of the fermenting microorganisms to use the whole range of sugars available from the hydrolysate is vital to increase the economic competitiveness of cellulosic ethanol and potentially biobased proteins.

Metabolic engineering for microorganisms used in fuel ethanol production has shown significant progress (Jeffries and Jin, 2009). Besides *Saccharomyces cerevisiae*, other microorganisms such as *Zymomonas mobilis* and *Escherichia coli* have been targeted through metabolic engineering for cellulosic ethanol production.

Engineered yeasts have been reported to efficiently ferment xylose and arabinose (Karhumaa, 2006). Yeast cells are especially attractive for cellulosic ethanol processes because they have been used in biotechnology for hundreds of years, are tolerant to high ethanol and inhibitor concentrations and can grow at low pH values to reduce bacterial contamination (Becker and Boles, 2003).

2.6.6 Combined Hydrolysis and Fermentation

Some species of bacteria have been found capable of direct conversion of a cellulose substrate into ethanol. One example is *Clostridium thermocellum*, which uses a complex cellulosome to break down cellulose and synthesize ethanol. However, *C. thermocellum* also produces other products during cellulose metabolism, including acetate and lactate, in addition to ethanol, lowering the efficiency of the process. Some research efforts are

directed to optimizing ethanol production by genetically engineering bacteria that focus on the ethanol-producing pathway.

2.7 Gasification Process (Thermochemical Approach)

The gasification process does not rely on chemical decomposition of the cellulose chain (cellulolysis). Instead of breaking the cellulose into sugar molecules, the carbon in the raw material is converted into synthesis gas, using what amounts to partial combustion (Sakamoto *et al*., 2012). The carbon monoxide, carbon dioxide and hydrogen may then be fed into a special kind of fermenter instead of sugar fermentation with yeast. This process uses *Clostridium ijungdahlii* bacteria (Bolyes *et al.,* 2010).This microorganism will ingest carbon monoxide, carbon dioxide and hydrogen and produce ethanol and water. The process can thus be broken into three steps

1. Gasification — Complex carbon-based molecules are broken apart to access the carbon as carbon monoxide, carbon dioxide and hydrogen.
2. Fermentation — Convert the carbon monoxide, carbon dioxide and hydrogen into ethanol using the *Clostridium ijungdahlii* organism.
3. Distillation — Ethanol is separated from water (Sakamoto *et al*., 2012).

2.8 Hemicellulose to Ethanol

Studies are intensively conducted to develop economic methods to convert both cellulose and hemicellulose to ethanol. Fermentation of glucose, the main product of cellulose hydrolyzate, to ethanol is an already established and efficient technique. However, conversion of xylose, the pentose sugar of hemicellulose hydrolyzate, is a limiting factor, especially in the presence of glucose. Moreover, it cannot be disregarded as hemicellulose will increase the efficiency and cost-effectiveness of cellulosic ethanol production (Sakamoto *et al*., 2012).

Sakamoto *et al.,* (2012) showed the potential of genetic engineering microbes to express hemicellulase enzymes. The researchers created a recombinant Saccharomyces cerevisiae strain that was able to:

1. Hydrolyze hemicellulase through codisplaying endoxylanase on its cell surface,
2. Assimilate xylose by expression of xylose reductase and xylitol dehydrogenase.

The strain was able to convert rice straw hydrolyzate to ethanol, which contains hemicellulosic components. Moreover, it was able to produce 2.5 time more ethanol than the control strain, showing the highly effectiveness process of cell surface-engineering to produce ethanol (Sakamoto *et al*., 2012).

2.9 Beer Lambert Law

The basic premise is that specific wavelengths of light will be absorbed by substances across a certain distance. The more light absorbed, the more concentrated the sample (Bolyes *et al.,* 2010). This can be applied in the calibration of a glucose meter with spectrophotometer absorbance.

CHAPTER THREE

MATERIALS AND METHODS

In this chapter, the materials, equipment as well as the various pre-treatment methods and other processes employed in this work are elaborated on.

3.1 List of Equipment / Apparatus

Table 3.1: List of Equipment/ Apparatus

S/No	INSTRUMENT	MODEL	MANUFACTURER
1.	Weighing balance	144	Jenway England
2.	Digital pH meter	3150	Jenway England
3.	Immersion Thermometer	-	Gallen kamp
4.	Incubator / fermenter	57731	Gallen kamp
5.	Laboratory mill	5057	Retsch KG West Germany
6.	Hot plate	1620	Gallen kamp
7.	Spectrophotometer	6051	Jenway England
8.	Alcoholmeter	5092	Jenway England
9.	Measuring Cylinder	-	Pyrex England

3.2 List of Materials, Chemicals and Reagents

Table 3.2: List of Materials, Chemical and Reagents

S/No	MATERIALS	GRADE	SOURCE / MANUFACTURER
1.	*Spirogyra africana*	-	ABU Zaria Dam
2.	Distilled Water	-	Chemical Engineering Department ABU Zaria
3.	*Aspergillus niger*	-	Microbiology Department ABU Zaria
4.	*Saccharomyces cerevisiae*	-	Microbiology Department ABU Zaria
5.	NaOH	Analytical	Changsha Yonta Industry Co., LTD China
6.	H_2SO_4	,,	Changsha Yonta Industry Co., LTD China
7.	Quick Lime (CaO)	,,	Changsha Yonta Industry Co., LTD China

3.3 *Spirogyra africana*

The algae, *Spirogyra africana* was collected from Ahmadu Bello University Zaria Dam. The sample was collected in sterile containers and transferred to the laboratory.

Plate 1: Spirogyra africana bloom at ABU Zaria Dam.

3.4 Methodology

The Methodology for the Production of Bio-Ethanol from *Spirogyra africana* is shown in Figure 3.1

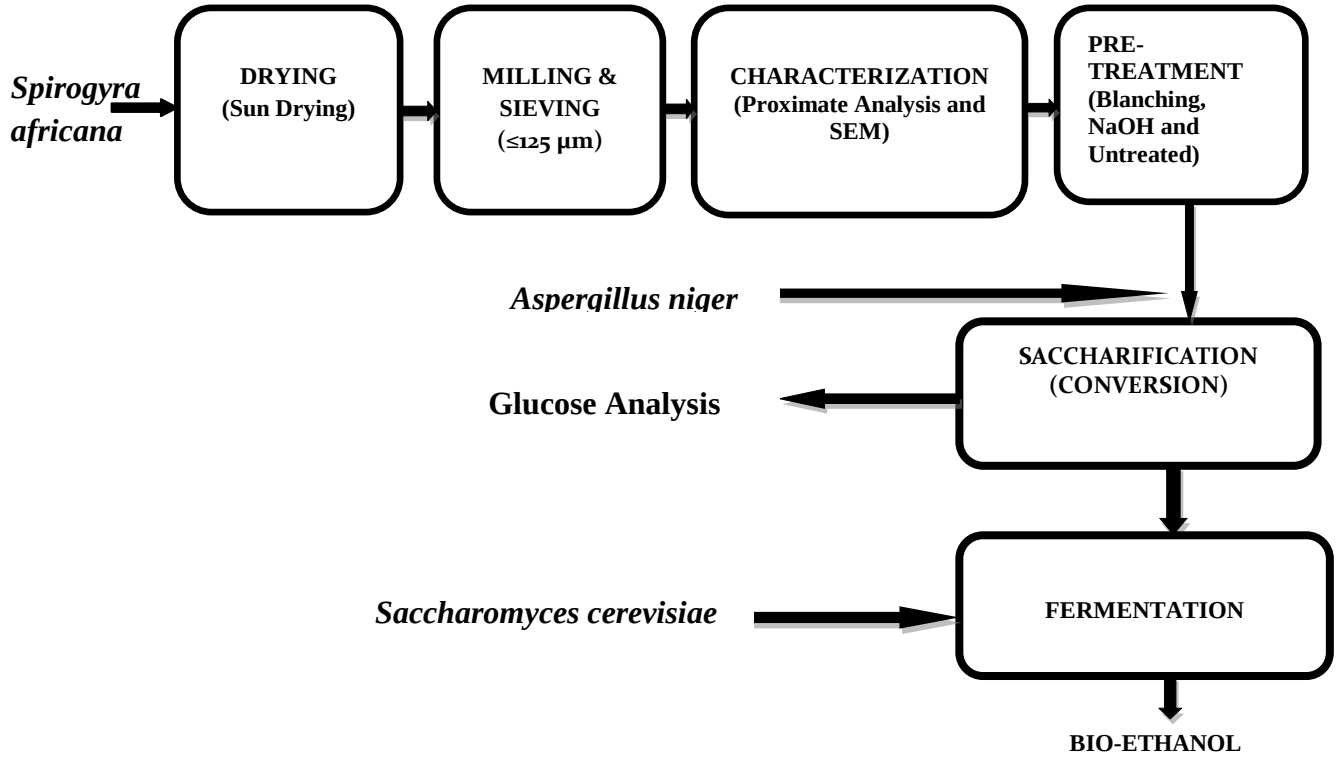


Figure 3.1: Block Diagram for bioethanol production from *Spirogyra africana*

3.5 Pre-Treatment of the *Spirogyra africana*

The pre-treatment of *Spirogyra africana* includes; cleaning, preparation, blanching and NaOH treatments of the substrate (sample) prior to hydrolysis.

3.5.1 Physical Pre-treatment (Drying, Milling and Sieving)

The *Spirogyra africana* sample was sun dried for seven (7) days to reduce the water content and to make the milling easy. The dried sample was then milled and sieved, this was done to increase the surface area and to make the cellulose extraction more effective. The milled feedstock is called mash**.** The size of the biomass was reduced to ≤ 125 µm because Eshaq *et al*. (2010) recorded that further reduction in size, to much less 125 µm, had no effect on the amount of sugar produced.

3.5.2 Characterization of *Spirogyra africana*

Characterization of *Spirogyra africana* was done to ascertain the carbohydrate content and the particles arrangement of the *Spirogyra africana*.

3.5.3 Determination of Suitable Pre-treatment

Physical and Chemical pre-treatment were performed on the *Spirogyra africana* biomass of ≤ 125 µm particle size thus: The biomass were blanched with water (30 ^{o}C to 100 ^{o}C) for a period of 5 minutes (Sobukola *et al.*, 2008) and chemically pre-treated with 0.0 %(w/v) NaOH, 0.5 % (w/v) NaOH, 1.0 % (w/v) NaOH & 2.0 % (w/v) NaOH for a period of 2 hr (Eshaq *et al*. 2010) and the initial free sugar concentrations were recorded.

3.5.4 Enzymatic Conversion / Saccharification

The chemical pre-treated, thermal pre-treated and untreated feedstocks were converted to fermentable sugar (glucose) by treatment with *Aspergillus niger* of concentration between 0-1.3 % wt/wt. This was done after pH adjustment of the mash.

3.5.5 Fermentation

All the pre-treatment steps are necessary before fermentation. The *A. niger* and the cooled, pH-adjusted mash were put into the fermentation tank. The optimal temperature for fermentation, which is 35 ^{o}C, was ensured throughout the operation. Likewise, the pH was adjusted, between 5.0-5.5, throughout the experiment to ensure good alcohol yield. The process was monitored until fermentation was completed. The resident time of the process was three days. Fermentation was completed when the bubbling ceased and the yeast cake, which formed on the top, sank to the bottom. At this point the fermented liquor, known as "beer" was ready to be distilled.

3.5.6 Bacterial Control of the Mash

Bacterial contamination was controlled throughout the operation because bacterial contamination of the mash, before or during fermentation, is one of the biggest factors that affect alcohol yield.

3.5.7 Enzymes Culturing (Fungal)

Two fungal cultures *Aspergillus niger* and *Saccharomyces cerevisiae* were obtained from the Department of Microbiology Ahmadu Bello University Zaria, Nigeria.

The fungi *Aspergillus niger* was cultured and maintained on potato dextrose agar medium at 30 ^{0}C.

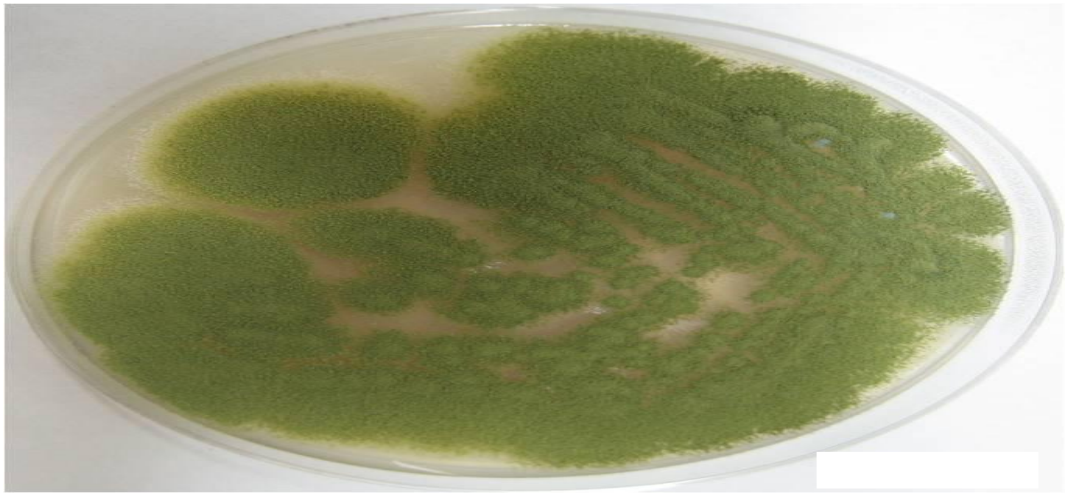


Plate II: Aspergillus niger cells

The yeast *Saccharomyces cerevisiae* was cultured and maintained on YPD (Yeast extract, peptone and dextrose) agar media at 30 ^{0}C.

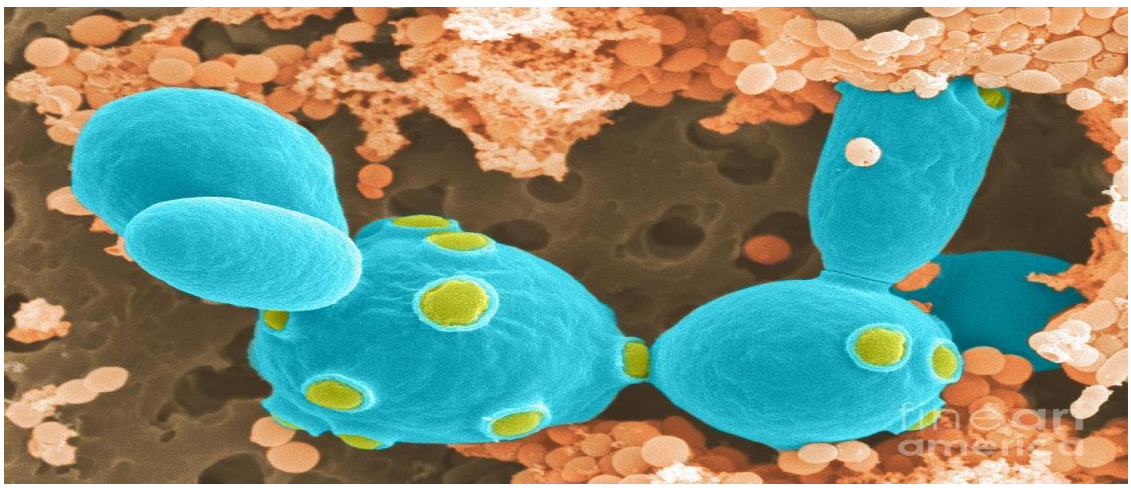


Plate III: SEM image of Saccharomyces cerevisiae.
(Source:www.http://fineartamerica.com/featured/saccharomyces-cerevisiae-sem-scimat.html)

3.5.8 Saccharification of *Spirogyra Africana* Biomass by *Aspergillus Niger*:

For the saccharification of the algal biomass developed mycelia mat of *Aspergillus niger* from the Microbiology Department ABU Zaria was used.

Aspergillus niger is cellulolytic and amylolytic in nature as it produces cellulases and amylases, (Eshaq *et al.*, 2010). These enzymes hydrolyze the cellulose and starch present in *Spirogyra* and release free sugars (glucose). The saccharification was carried out for a period of six days at temperatures ranging between 20-40 ^{0}C and the process was monitored every 24 hrs for sugars released by Hydrazided method of P-hydroxy Benzonic Acid (Blakerey and Mutton, 1980).

3.5.9 Uv-spectrophotometer

The quantitative determination of glucose for bio-ethanol production was done using the UV-spectrophotometer at constant wavelength of 550 nm, using distilled water as blank. UV-spectrophotometer uses discrete wavelengths of light to determine the concentration of certain compounds in a sample.

3.5.10 Glucose Calibration Curve

Figure 3.2: shows the relationship between glucose concentration and light absorbed using spectrophotometer. These were determined by varying different glucose concentration while ascertaining their corresponding absorbance using the method of Hydrazide method of P-hydroxy Benzonic Acid (Blakeney and Mutton, 1980). The calibration curve was used to determine the glucose concentration corresponding to the spectrophotometer readings recorded.

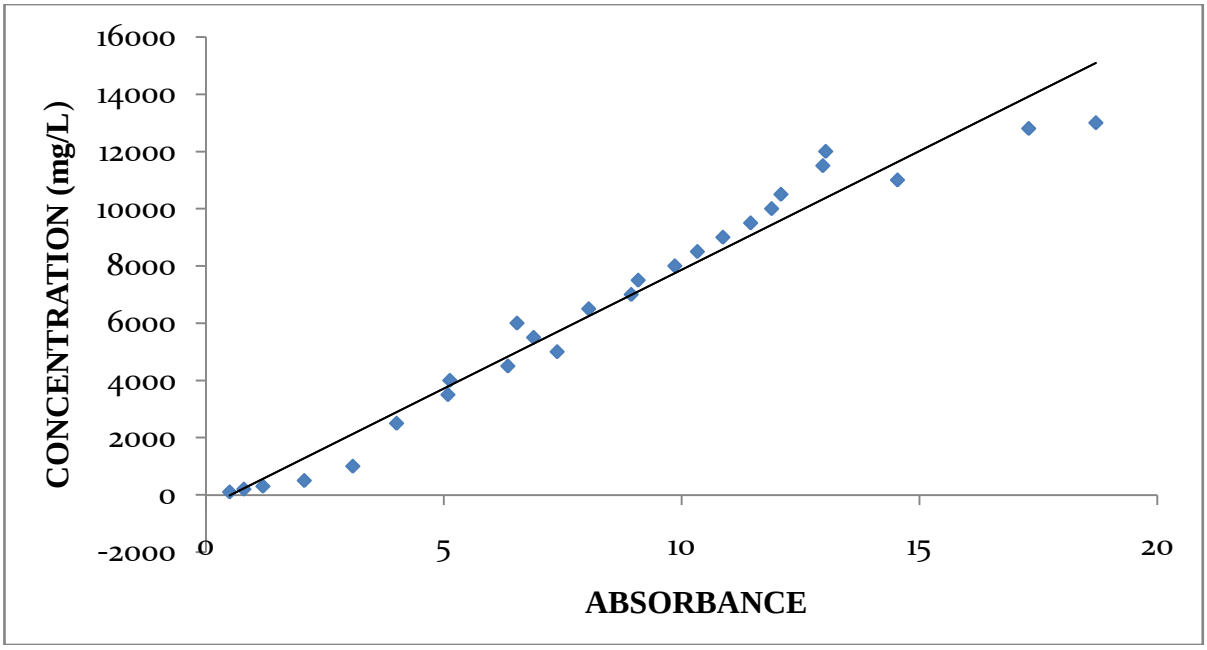


Figure 3.2: Glucose Calibration Curve

3.5.11 Beer Analyzer

A precise beer analyzer was used to determine the ethanol content of the beer. It has a high accurate beer analysis system which determines the alcohol content of all types of beers, beer mixtures, ciders, etc. To determine the ethanol content of a brew, the sugars content at the beginning of brewing and at the very end were measured. The difference tells us how much sugar was consumed and therefore how much alcohol (bio-ethanol) is in the beer.

Plate IV: Ethanol Determination Apparatus (Ethanol Metre)
Source: www.anto-paar.com/my-en/product/group/beverage-analysis

3.6 Process Optimization Stage- Saccharification

The *Spirogyra africana* substrate was hydrolyzed using *Aspergillus niger* cells while the process parameters were varied in order to determine the optimum.

3.6.1 Determination of the Optimal Contact Time for Glucose Production.

This was studied at constant temperature of 30 ºC, pH 4.5, 50 g/L substrate concentration, and 0.6 % (w/v) *A.niger* concentration. The sugar concentrations were observed and recorded daily for six days.

3.6.2 Determination of the Optimal Substrate Concentration for Glucose Production.

Saccharification was studied at different *Spirogyra africana* concentrations between 1.0 - 8.0 % (w/v), at 2.0 % (w/v) interval and at constant temperature of 30 ºC, pH of 4.5, substrate concentration of 50 g/L, residence time of 2 days and *A. niger* concentration of 0.6 % (w/v). The glucose concentrations were observed and recorded.

3.6.3 Determination of the Optimal Cell Loading For Glucose Production.

Saccharification was carried out at different *Aspergillus niger* concentrations between 0.2 – 1.0 % (w/v) at 0.2 % (w/v) interval, and at constant temperature of 30 ºC, pH 4.5, 2 days residence time and 50 g/L substrate concentration while the glucose concentrations were observed and recorded.

3.6.4 Determination of the Optimal Temperature for Glucose Production.

The experiment was carried out at different temperature of 25 ^{o}C, 30 ^{o}C, 35 ^{o}C, 40 ^{o}C and 45 ^{o}C using the incubator at constant pH of 4.5, 50 g/L substrate concentration, 2 days residence time and 0.6 % (w/v) *A. niger* concentration while the glucose concentrations were observed.

3.6.5 Determination of the Optimal pH for Glucose Production.

This was investigated by carrying out the saccharification at initial pH adjusted to 3.5, 4.0, 4.5, 5.0, and 6.0 using sodium acetate buffer at constant temperature of 30 ºC, 50 g/L

substrate concentration, 2 days residence time and *A. niger* concentration of 0.6 % (w/v) while the glucose concentrations were observed.

3.7 **Process Optimization Stage- Fermentation**.

Here the glucose produced was converted to bio-ethanol using *S. cerevisiae* and various process parameters for fermentation were studied in order to determine the optimum conditions.

3.7.1 Determining the Optimal Residence Time for Bio-Ethanol Production.

The experiment was carried out using incubator at 35 ^{o}C, pH 5.5, and yeast concentration of 1.0 % (w/w). The bio-ethanol concentrations were observed daily for six days.

3.7.2 Determining the Optimal Yeast (*S. Cerevisiae*) Concentration for Bio-Ethanol Production.

The experiments were carried out at different *S. cerevisiae* concentration of 0.5 %(w/v), 1.0 % (w/v), 1.5 %(w/v), 2.0 %(w/v) and 2.5 %(w/v) at constant temperature of 35 ^{o}C, pH 5.5 and for three days and the bio-ethanol concentrations were recorded.

3.7.3 Determining the Optimal Temperature for Bio-Ethanol Production

The experiments were carried out at different temperature of 25 ^{o}C, 30 ^{o}C, 35 ^{o}C, 40 ^{o}C and 45 ^{o}C using the incubator at constant pH of 5.5, yeast concentration of 1.0 % (w/w) and for three days, and the bio-ethanol concentrations were recorded.

3.7.4 Determining the Optimal pH for Bio-Ethanol Production

This was studied by carrying out the fermentation at various pH values; 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0, at constant condition of 35 ^{o}C, 5.5 pH, and yeast concentration of 1.0 % (w/w) for 3 days while the bio-ethanol concentrations were recorded.

CHAPTER FOUR

RESULTS AND DISCUSSION

This chapter presents the results of this research work on studying the optimal conditions for production of glucose and bioethanol production from *Spirogyra africana.*

4.1 Proximate Analysis of the *Spirogyra africana*

Table 4.1 shows the results obtained from the proximate analysis of *Spirogyra africana* on dry basis. It contains 39.72 wt% carbohydrates, which makes it a good candidate for bioethanol production.

Table 4.1: Proximate Analysis of the *Spirogyra africana*

Sample Name	% Moisture	% Ash	% Lipid	% Protein	% fibre	% CHO
Spirogyra africana	1.05	31.03	3.00	5.05	20.05	**39.72**

The carbohydrate in *Spirogyra africana* was further broken down as show in Table 4.2. It contains starch and cellulose, which makes the choice of *A.niger* appropriate.

Table 4.2: Break down of carbohydrate content of the *Spirogyra africana*

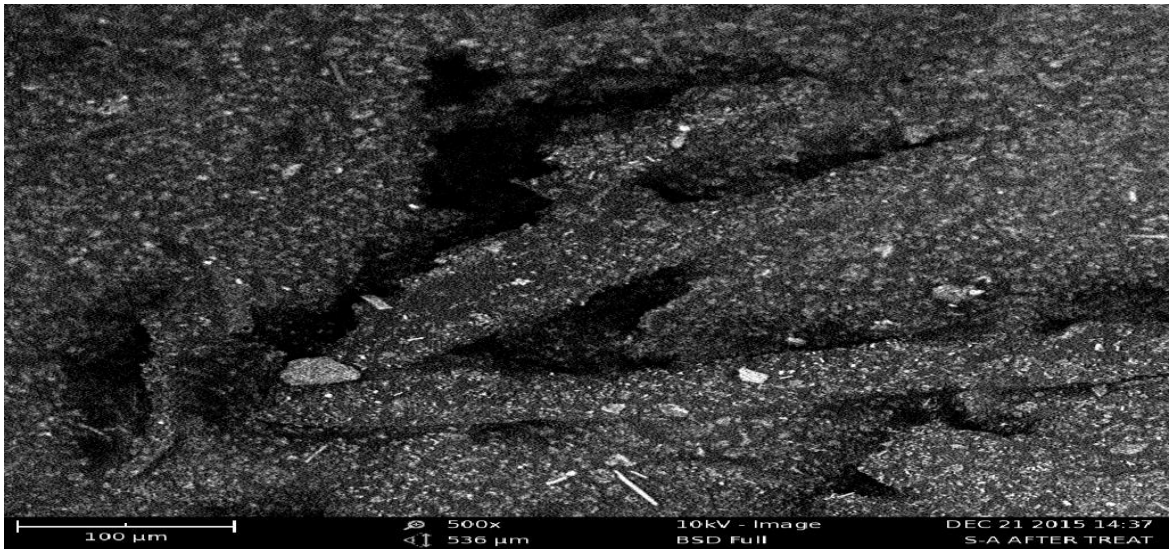
Carbohydrate (CHO)	**Component**
Cellulose	28.8 %
Starch	6.83 %
Free Sugar	4.09 %

Analysed at the Agricultural Research Institute Ahmadu Bello University, Zaria

4.2 Morphology of Blanched and Unblanched Samples

Plate V (a & b) show the images of 80 ^{o}C blanched and unblanched samples of *Spirogyra africana.* From the images, the blanched samples have more interactions with the hydrolyzing agent *Aspergillus niger*. This is because the sample becomes tender after blanching making more carbohydrate to glucose conversion possible.

500X (a)



500X (b)

Plate V SEM Images of Spirogyra africana Sample (a) before and (b) after thermal pre-treatment

4.3 Effect of Pre-Treatments on Glucose Production

4.3.1 Effect of Blanching Temperature on Glucose Production

The effect of blanching temperature on glucose production was carried out to determine the best blanching temperature. Figure 4.1 shows that a rapid increase in the concentrating of glucose produced was observed from 30 ^{o}C to 80 ^{o}C and a decline at temperature

beyond 80 ^{o}C. The maximum concentration of glucose was observed at 80 ^{o}C and this conformed to the SEM analysis in Plate IV which shows that more particles where exposed for hydrolysis in blanched sample than in the unblanched sample. Thus samples blanched at 80 ^{o}C were used for further studies in this work.

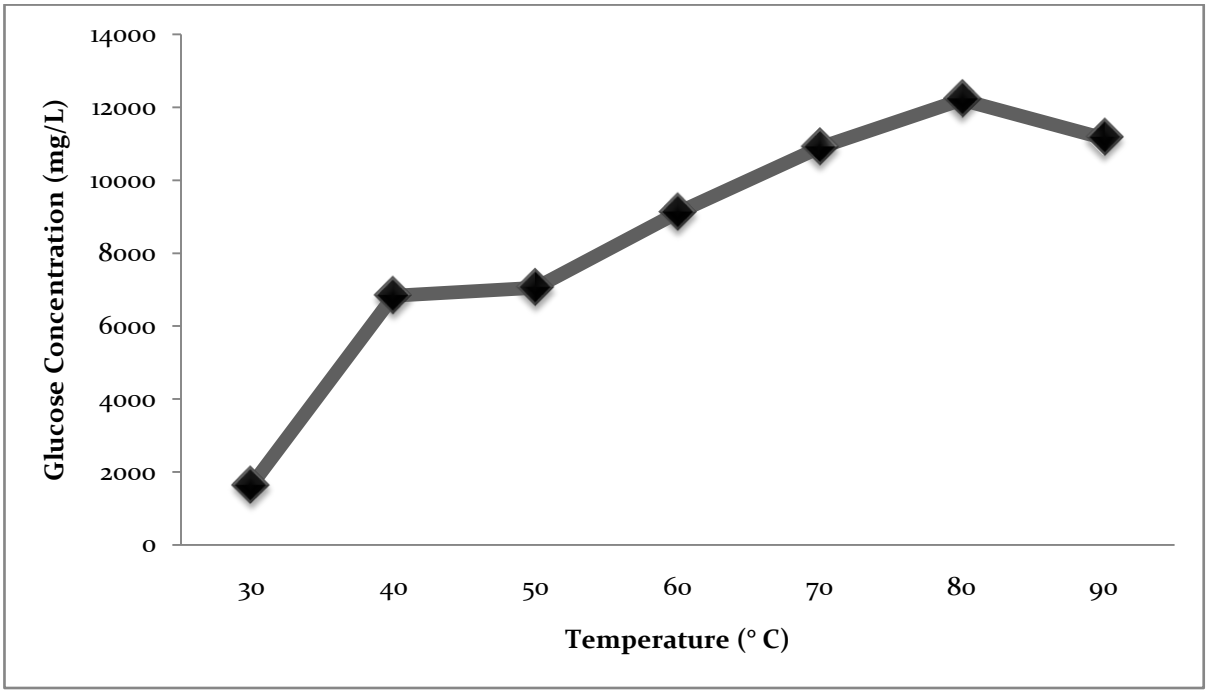


Figure 4.1: Effect of Blanching Temperature on Glucose Production at following hydrolysis conditions: temperature 30 ^{o}C, pH 4.5, substrate concentration 50 g/L, *A. niger* concentration 0.65 % (w/v), and particle size ≤ 125 µm.

4.3.2 Effect of NaOH Concentration on Glucose Production

This was carried out to determine the NaOH concentration for substrate pre-treatment. As can be seen in Figure 4.2, 1.0 % (w/v) NaOH pretreated sample gave the highest concentration of glucose produced when compared with 0.5 and 2.0 % (w/v) NaOH pre-treated, thus samples pre-treated with 1.0 % (w/v) NaOH were used for further experiments.

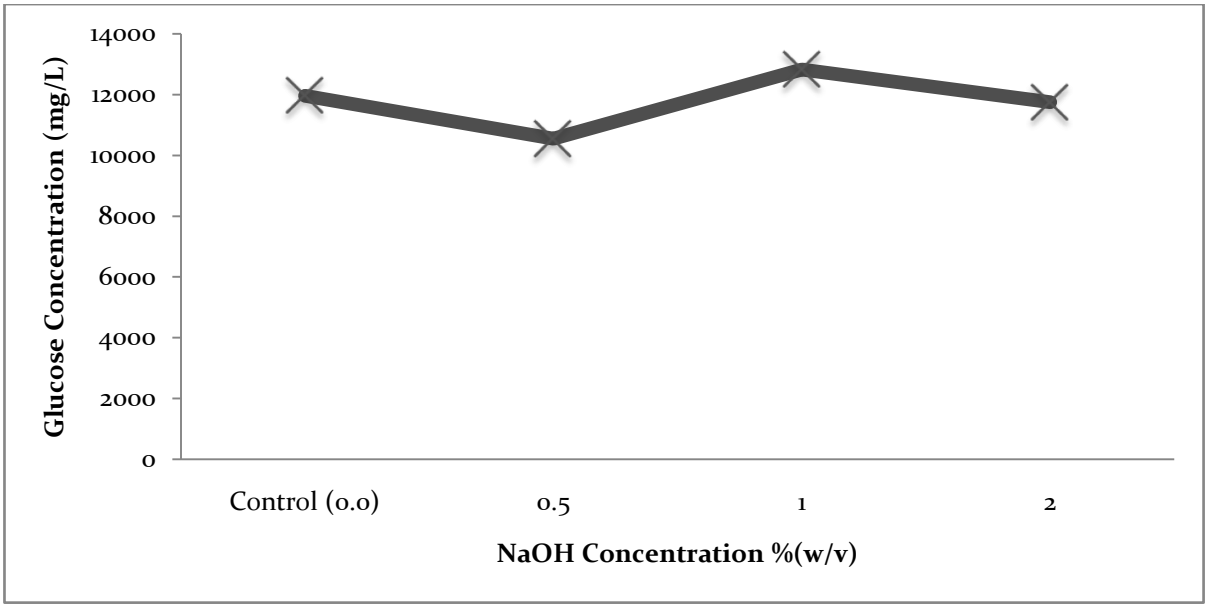


Figure 4.2: Effect of NaOH Concentration on Glucose Production at following hydrolysis conditions: temperature 30 ^{o}C, pH 4.5, substrate concentration 50 g/L, *A. niger* concentration 0.65 % (w/v), and particle size ≤ 125 µm.

4.4 Saccharification

In this experiment the carbohydrate content of the *Spirogyra africana* was hydrolyzed to reducing sugar using *Aspergillus niger* cells. The optimal conditions for glucose production were also studied.

4.4.1 Effect of Pre-treatment on Saccharification

Figure 4.3 shows the effect of pre-treatment on saccharification studied using various concentrations of NaOH ranging from 0.5 (w/v) % to 2.0 (w/v) %, blanching at various temperature ranging from 30 ^{o}C to 100 ^{o}C with untreated substrate serving as control. The results of the tests showed that there was a rapid increase in the glucose production from day one (1) to day three (3) for each of the setups. The sample blanched at 80 ^{o}C produced more glucose than the untreated and the NaOH pre-treated in day one, as there was statistical significance ($P < 0.05$) between glucose produced from it when compared to others. This observation may not be unconnected with the ability of water at that temperature to swell up the starch present in the substrate, making it more accessible to

the hydrolysing enzymes, when compared to the untreated sample. The same is likely true of the sample pre-treated with NaOH, although to a slightly lesser extent. However, on the day two, the results show that there was no statistical significance ($P > 0.05$) between glucose produced from NaOH pre-treatment, blanching and untreated substrates. This finding is of importance because the economic difficulties on commercialization of 2nd generation biofuel substrates border on the biomass pre-treatment step. Hence, avoiding this step (pre-treatment) in the 3rd generation biomass conversion to fuel is an added advantage to its commercialization. This inference agrees with the findings of Wurdack and Mary (2014) that there is no need for pre-treating *Spirogyra* biomass because the cell wall of Spirogyra contains no Lignin; it only has Pectose in the outer layer and Cellulose in the inner layer.

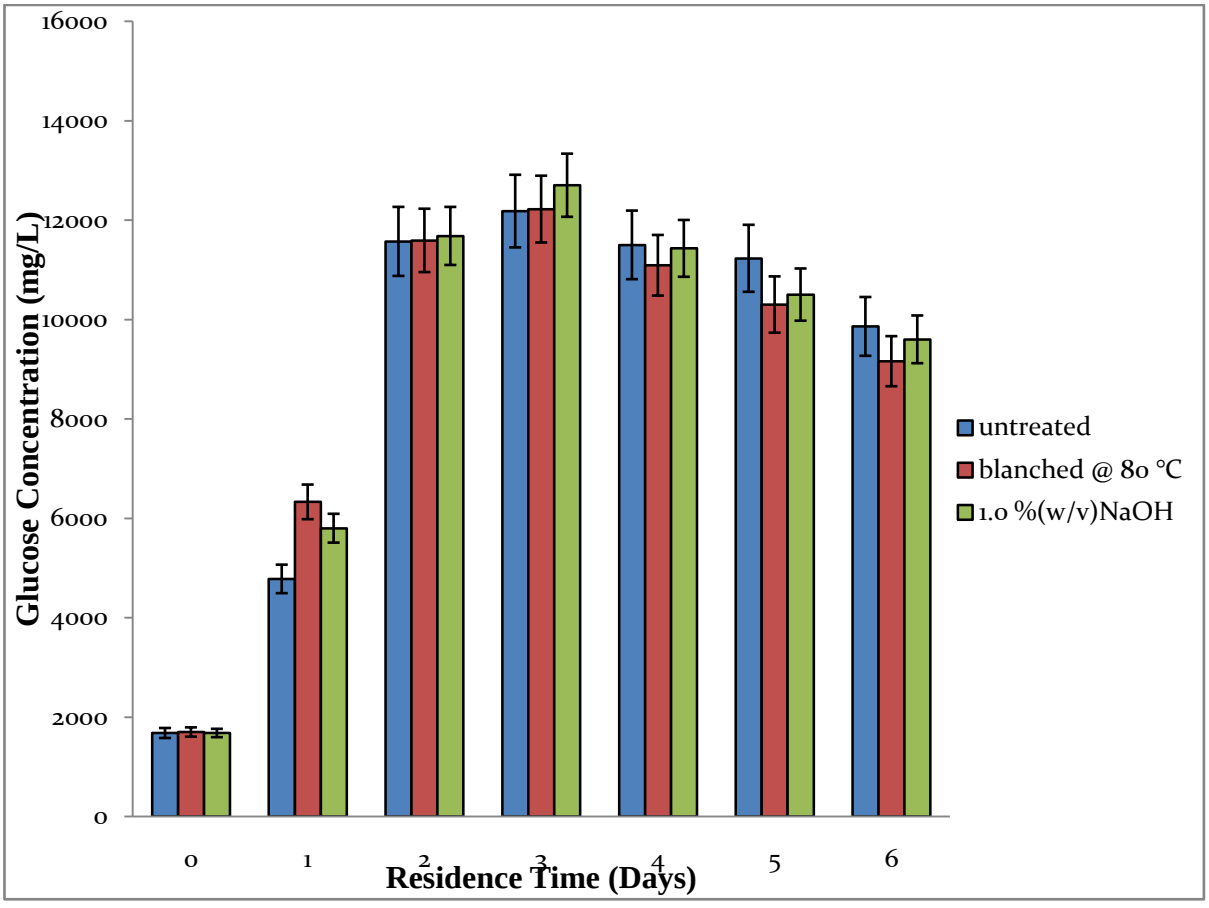


Figure 4.3: Effect of pre-treatment on saccharification at following hydrolysis conditions: temperature 30 ^{o}C, pH 4.5, substrate concentration 50 g/L, *A. niger* concentration 0.65 % (w/v), and particle size ≤ 125 µm.

4.4.2. Effect of Substrate Concentration on Saccharification

Based on the result shown in Figure 4.4 the glucose concentration was relatively constant as substrate concentration increased from 10 g/L to 40 g/L. The highest glucose concentration of 12820 mg/L was achieved at substrate concentration of 50 g/L. At concentrations beyond 50 g/L, no significant change in amount of glucose produced was observed. There is no benefit in increasing the amount of substrate beyond 50 g/L. Eshaq, *et al.* (2010) also reported 50 g/L as the optimum substrate concentration for Spirogyra saccharification whereas Razif and Michael (2011) reported 10 g/L as the optimum substrate concentration for bio-ethanol production from microalgal.

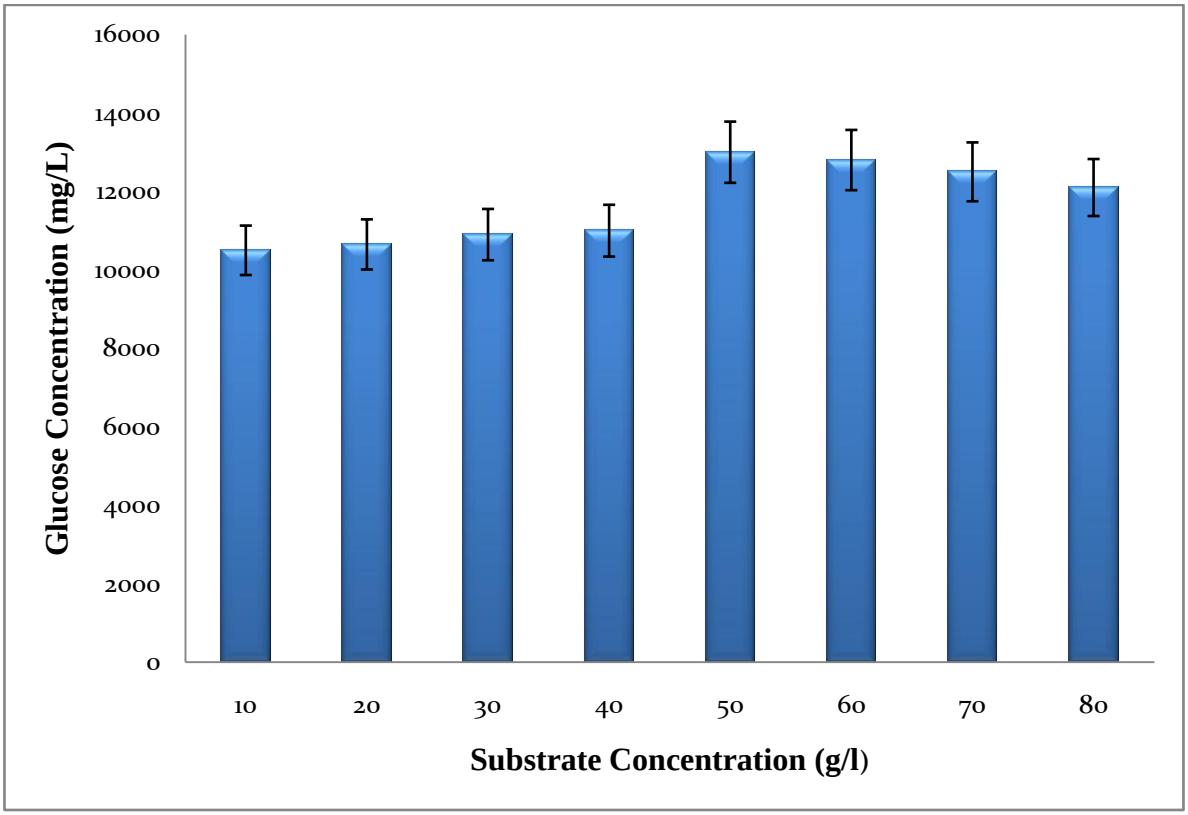


Figure 4.4: Effect of substrate concentration on saccharification at a fixed *A. niger* concentration 0.6 % (w/v), pH 4.5, particle size ≤ 125 µm, temperature 30 ^{o}C and contact time of two (2) days.

4.4.3. Effect of Temperature on Saccharification

The effect of temperature on saccharification is presented in Figure 4.5. The result shows that at 15 ^{o}C the concentration of glucose production was 1680 mg/L, the glucose production increased gradually from 15 ^{o}C to 25 ^{o}C, then stayed relatively constant at 30 ^{o}C before declining with further increase in temperature. This shows that *A. Niger* performs best at the temperature range of 25-30 ^{o}C when used to hydrolyse *Spirogyra africana* biomass. It implies that hydrolysis can be done at room temperature, hence reduced energy requirement. This compares favourably with the work of Eshaq *et al*., (2010) that reported 30 ^{o}C as the optimum temperature for Spirogyra saccharification. However, Ayejagbara, (2015) and Highina and Hofreiter, (2006) reported 45 ^{o}C as their optimum temperature for elephant grass and rice straw hydrolysis using the same fungus respectively. This discrepancy is likely due to high lignin in both elephant grass and rice straw, which needs a higher temperature for unbundling, Aiyejagbara (2015).

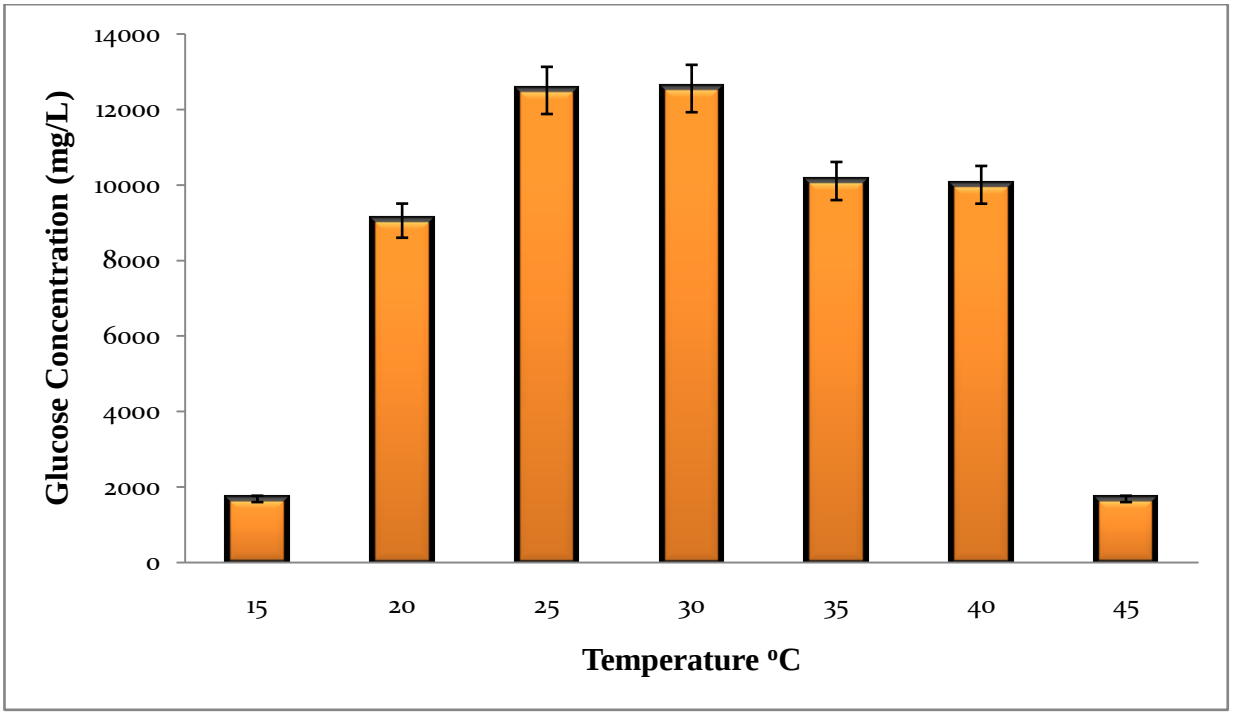


Figure 4.5: Effect of temperature on saccharification at *A. niger* concentration of 0.6 % (w/v), pH of 4.5, particle size of ≤ 125 µm, substrate concentration of 50 g/L, and residence time of two (2) days.

4.4.4 Effect of contact time on saccharification

It was observed as shown in Figure 4.6 that the initial free sugar was about 1680 mg/L which increased to 6090 mg/L after 1 day and further increased to 11680 mg/L another day. After 3 days, there was only a slight increase in the concentration of glucose produced which is statistical insignificant ($P > 0.05$) when compared with the glucose produced after two (2) days saccharification. Hence, contact time of 2 days was taken as the optimum hydrolysis duration. At residence time of 4, 5 and 6 days there is a decline in the concentration of glucose produced which may be due to the glucose formed being consumed or the process being inhibited. Eshaq *et al.*, (2010) reported a steady decline in the amount of sugar released after 1 day (24 hrs) while hydrolysing Spirogyra with Lactose.

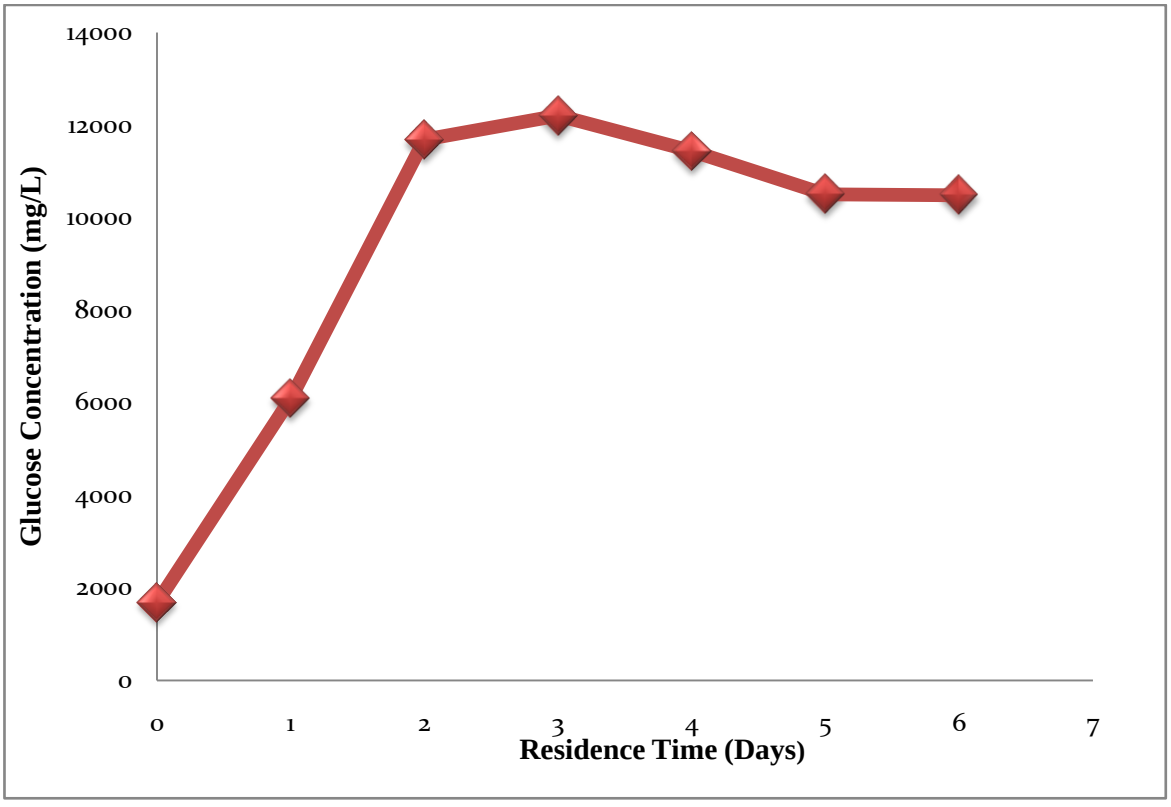


Figure 4.6: Effect of contact time on saccharification at a fixed *A. niger* concentration of 0.6 %(w/v), pH of 4.5, particle size of ≥ 125 µm, substrate concentration of 50 g/L, and temperature of 30 ^{o}C.

4.4.5 Effect of pH on Saccharification

The effect of pH on saccharification was studied and its result in Figure 4.7 shows that at pH of 3.0 the glucose produced was 1690 mg/L, between pH of 3.0 and 4.0 there was a gradual increase in the concentration of glucose produced, at pH 4.5 the maximum concentration of glucose was produced and a decline in glucose concentration at 5.0 pH and beyond was observed. This shows that *A. Niger* cells perform best at pH 4.5. Likewise, the work of Ayejagbara, (2015) reported pH of 4.5 as the best saccharification pH for elephant grass biomass using *A. niger*.

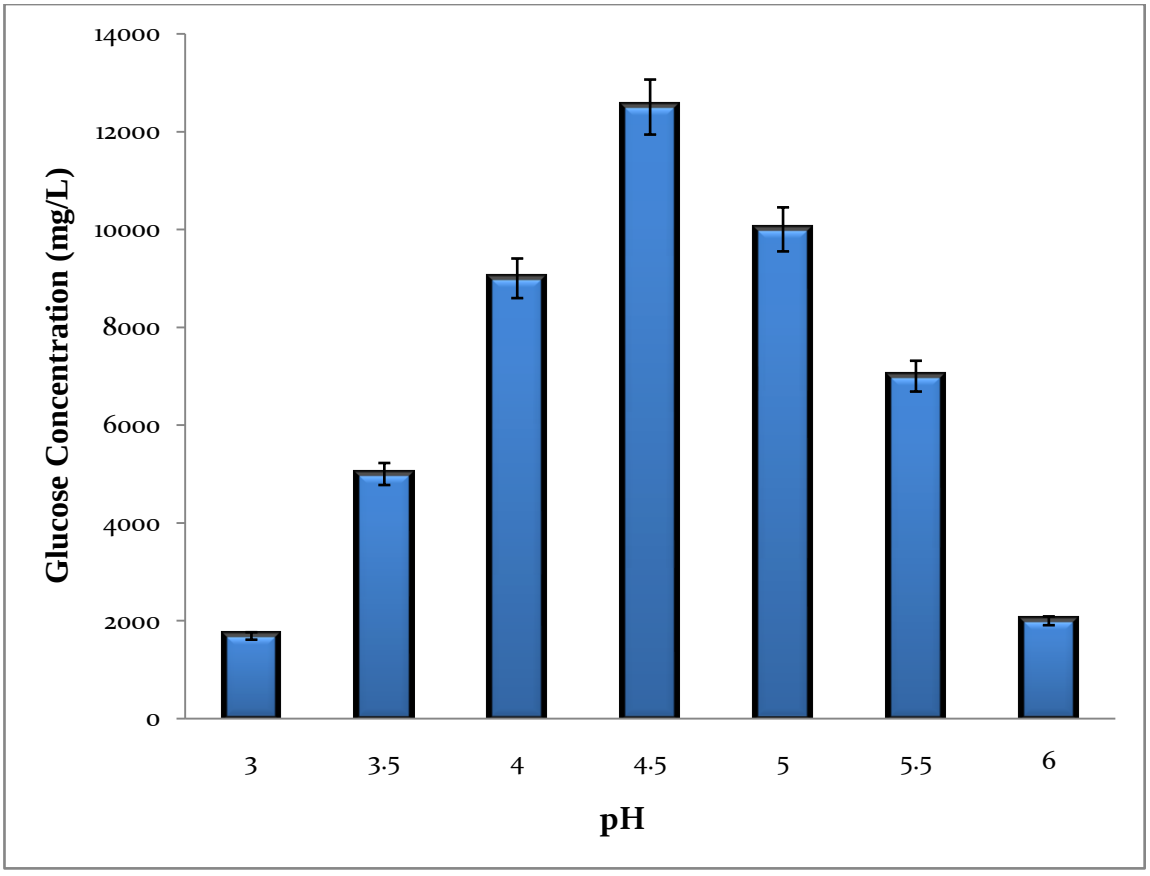


Figure 4.7: Effect of pH on saccharification at a fixed *A. niger* concentration of 0.6 % (w/v), contact time of two (2) days, particle size ≤ 125 µm, substrate concentration of 50 g/L, and temperature of 30 ^{o}C..

4.4.6. Effect of Aspergillus niger Conc. On Saccharification

The effect of *Aspergillus niger* concentration was studied. The results are presented in Figure 4.8 shows that the sample contained initial glucose (free sugar) concentration of 1680 (w/v) %. At cell loading of 0.2 (w/v) %, 4000 (mg/L) of glucose was produced. There was gradual increase in the concentration of glucose produced as cell loading increased from 0.2 w/v % to 0.4 w/v %, whereas glucose production was very rapid at cell loading of 0.4 w/v % and 0.6 w/v %, maximum production was observed at a cell loading of 0.6 w/v. Thus 0.6 w/v % was taken as the optimal cell loading. Cell loading beyond 0.6 w/v % produced a slight decline in the glucose concentration. This may be due to cells activity inhibition as a result of high toxicity of glucose in the media. This is very similar to the work of Ayejagbara, (2015) who reported that 0.6 w/v % is the best cell loading for elephant grass biomass saccharification using *A.niger*.

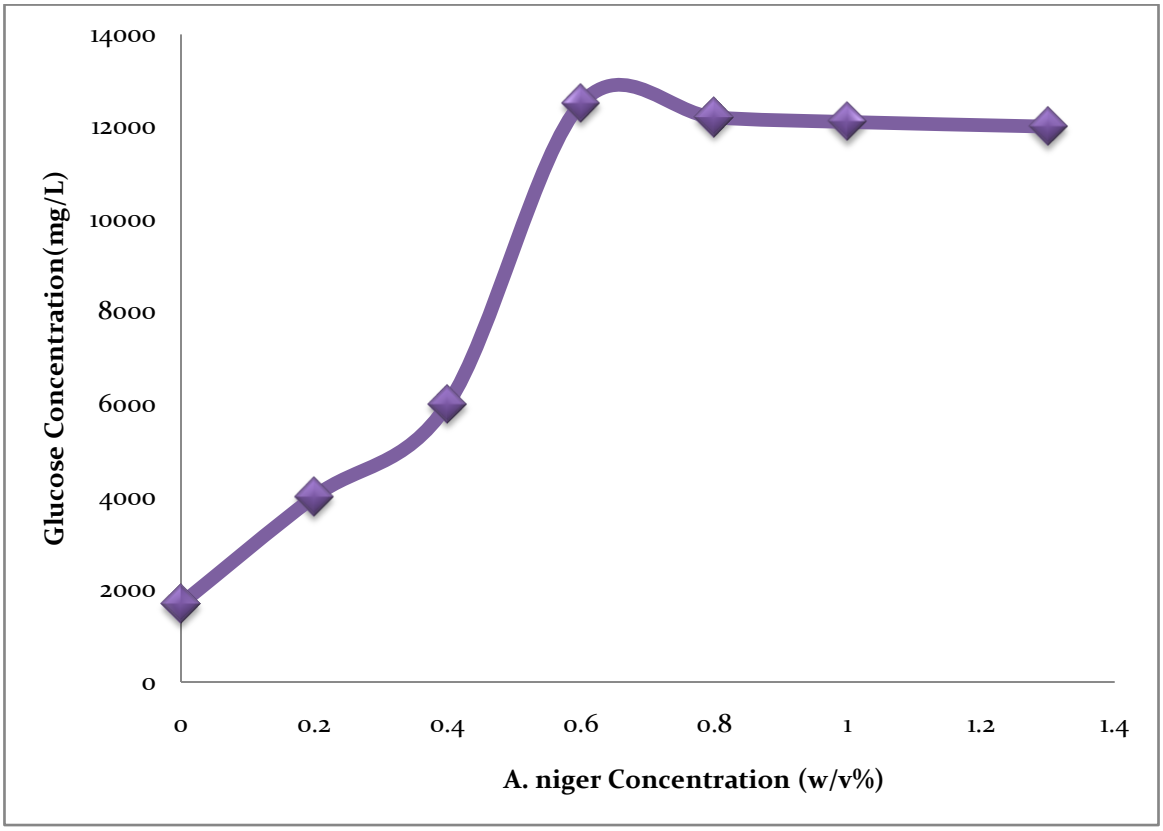


Figure 4.8: Effect of *A. niger* Concentration on Saccharification at a fixed pH of 4.5, residence time of two (2) days, particle size of ≤ 125µm, substrate concentration of 50 g/L, and temperature of 30 ^{o}C.

In summary, the best saccharification conditions are:

- ✓ Soaking temperature of 25-30 ^{o}C
- ✓ pH of 4.5
- ✓ Contact time of Two (2) days for untreated sample
- ✓ Substrate Concentration of 50 g/l
- ✓ A. *niger* concentration of 0.6 % (w/v)
- ✓ Particle size of ≤ 125 µm.

4.5 Fermentation

The fermentation studies were carried out using glucose solution obtained from the untreated *Spirogyra africana* hydrolysed for two (2) days. The following process parameters were studied during the fermentation to determine the optimum conditions for bioethanol production from *Spirogyra africana*; temperature, pH, yeast concentration and residence time.

4.5.1 Effect of Temperature on Fermentation

The effects of temperature on fermentation were investigated at different incubation temperatures between 20 - 50 ^{o}C at 5 ^{o}C interval and it could be established from Figure 4.10 that fermentation at 35 ^{o}C produced the highest concentration of bio-ethanol (8.72 % ABV). This shows that yeast is more temperature sensitive, when compared to *Aspergillus niger* for the hydrolysis reaction.

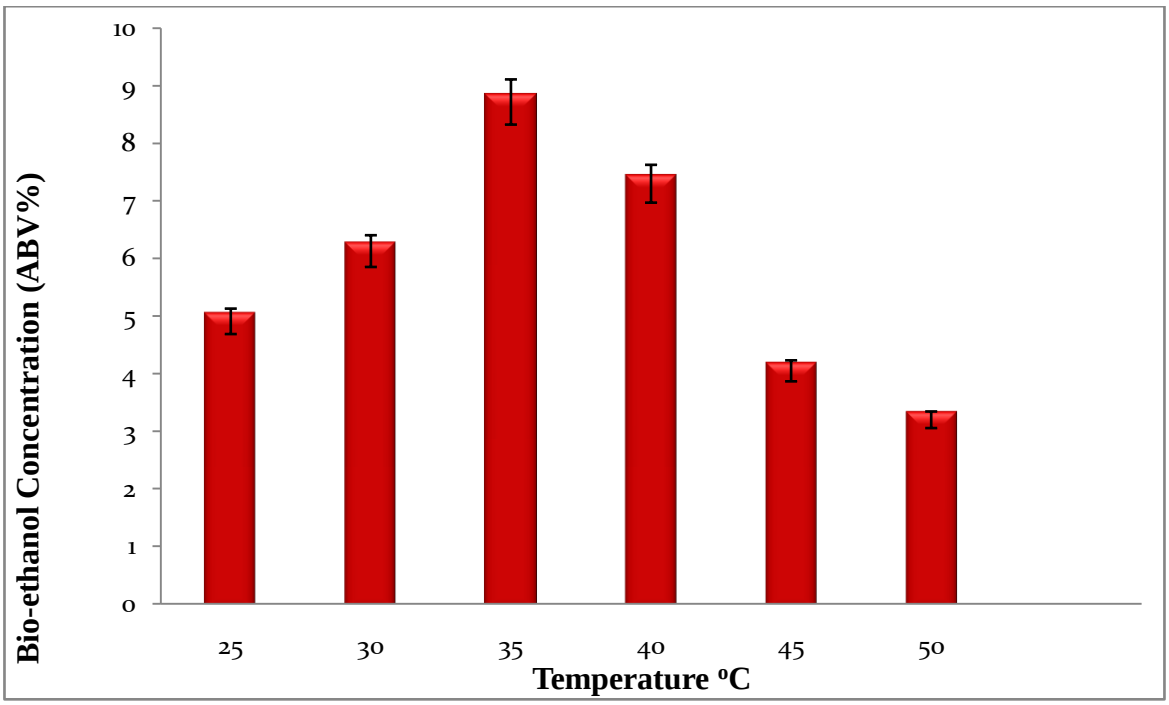


Figure 4.9: Effect of temperature on fermentation at pH 5.5, yeast concentration 1.0 (w/v) % and residence time three (3) days.

4.5.2 Effect of pH on Fermentation

The effect of pH fermentation was investigated at different pH of 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0. It was observed that fermentation at pH of 5.0 to 5.5 produced the highest bio-ethanol of 8.2 %ABV (Figure 4.10 referred). This is because the S. *cerevisiae* strives best under certain pH range and this conforms to the work of Ohgren, *et al.* (2007) which reported 5.5 as best pH for biomass fermentation.

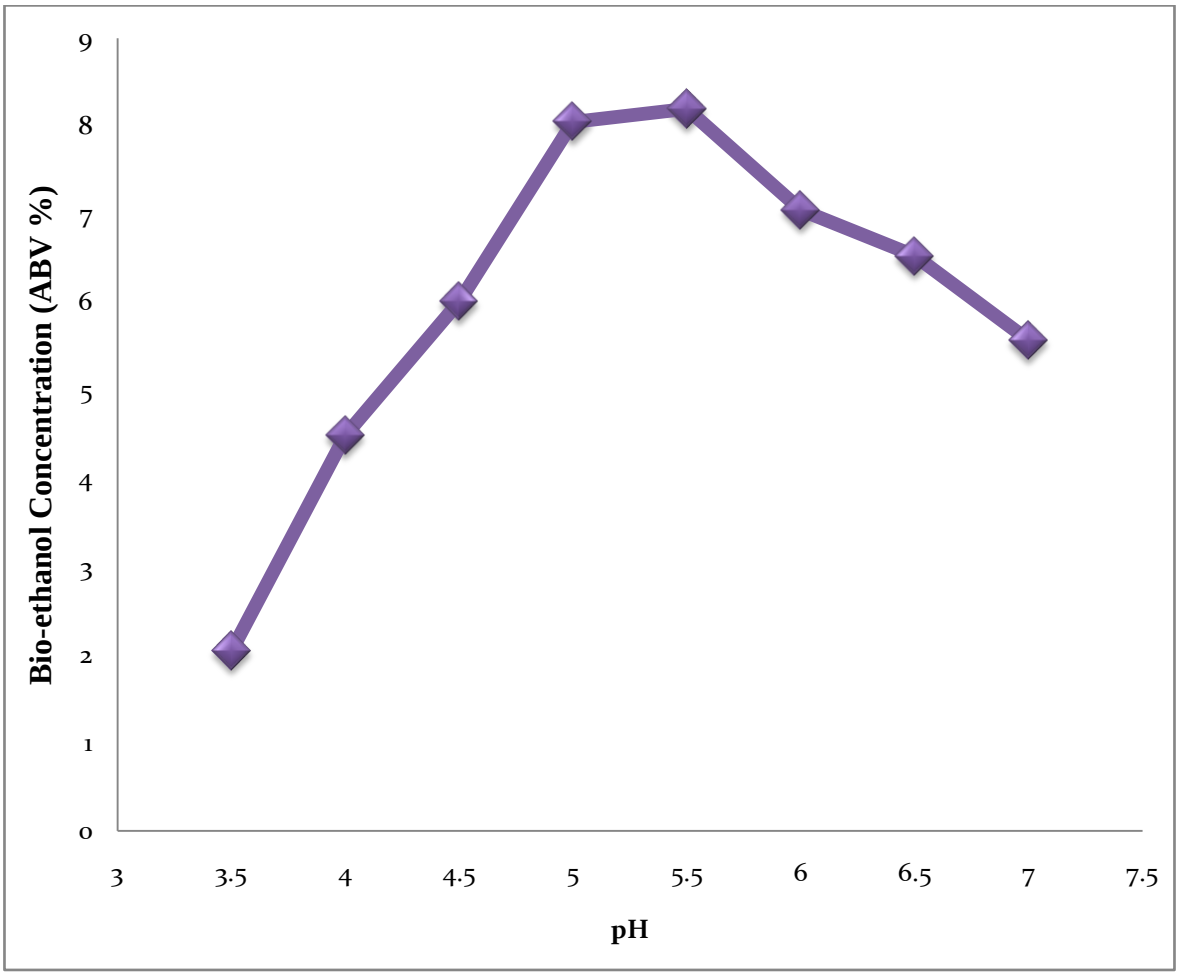


Figure 4.10: Effect of pH on fermentation at temperature, 35 ^{o}C; yeast concentration 1.0 (w/v) % and fermentation period of three (3) days.

4.5.3 Effect of Yeast (*Saccharomyces Cerevisiae*) Concentration on Fermentation

The effect of yeast (S. *cerevisiae*) concentration on fermentation was investigated at different yeast concentration between 2 – 16 w/v % at interval of 2 w/v %. As shown in Figure 4.11 the fermentation at 10 w/v % yeast concentration, produced the highest bio-ethanol (8.52 % ABV). The decreasing trend of bio-ethanol concentration on increasing the cell loading beyond 10 w/v % could be because the produced bio-ethanol act as carbon source in fermentation broth.

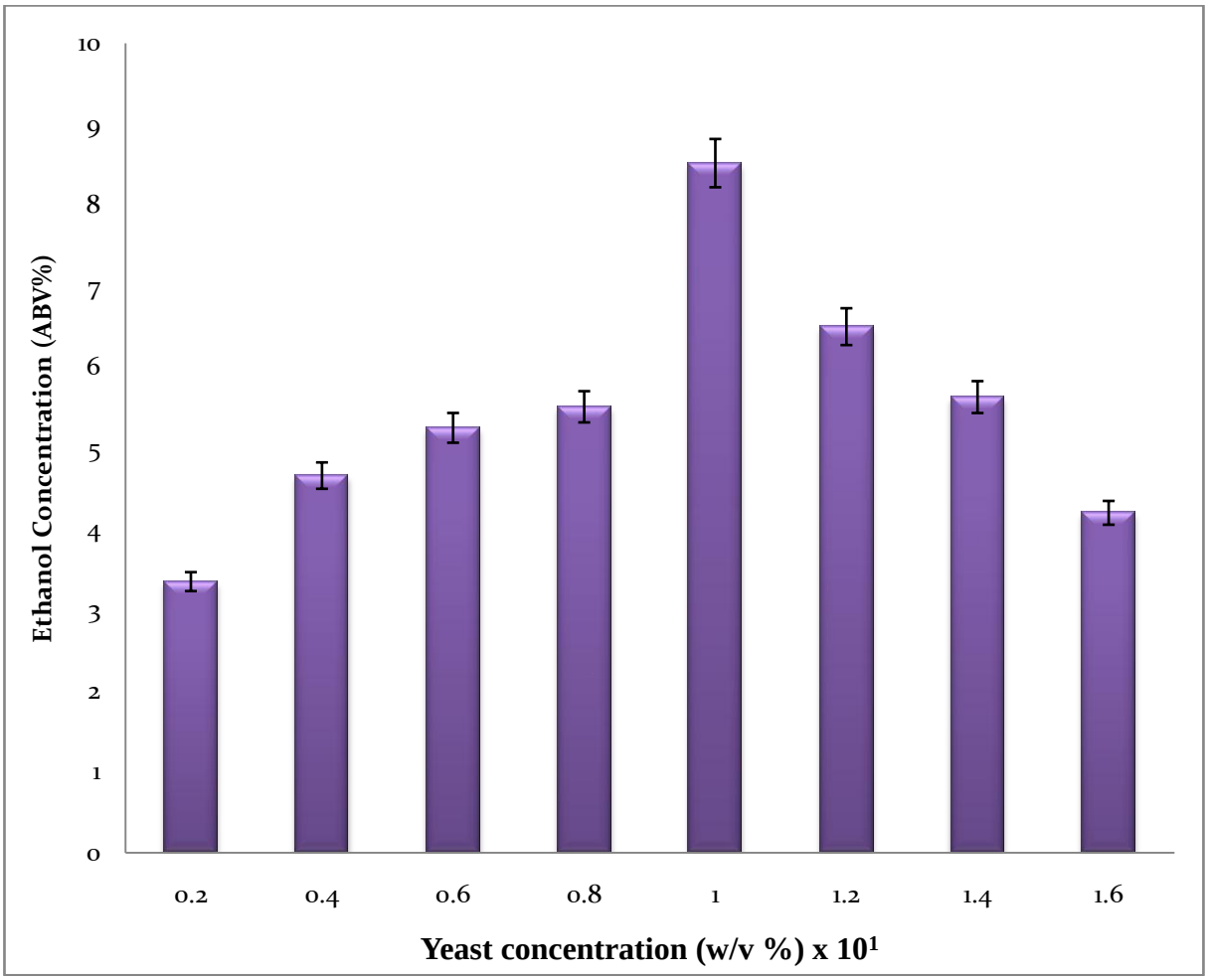


Figure 4.11: Effect of Yeast Concentration on Fermentation temperature 35 ^{o}C, pH 5.5 and residence time three (3) days.

4.5.4 Effect of Residence Time on Fermentation

The effect of residence time on bio-ethanol yield was investigated at different fermentation durations: 1 day, 2 days, 3 days, 4 days, 5 days, and 6 day. It was observed that on the third day (72 hr) the highest bio-ethanol of 8.52 % ABV was produced (Figure 4.12). The optimum fermentation period obtained in this investigation is higher than the 32 hr reported by Jeong-Hoon *et al*., (2012) as the optimum residence time for bio-ethanol production from algae (*Gelidium amansii)* biomass via fermentation. The difference may not be unconnected with the variation in the species of algal involved.

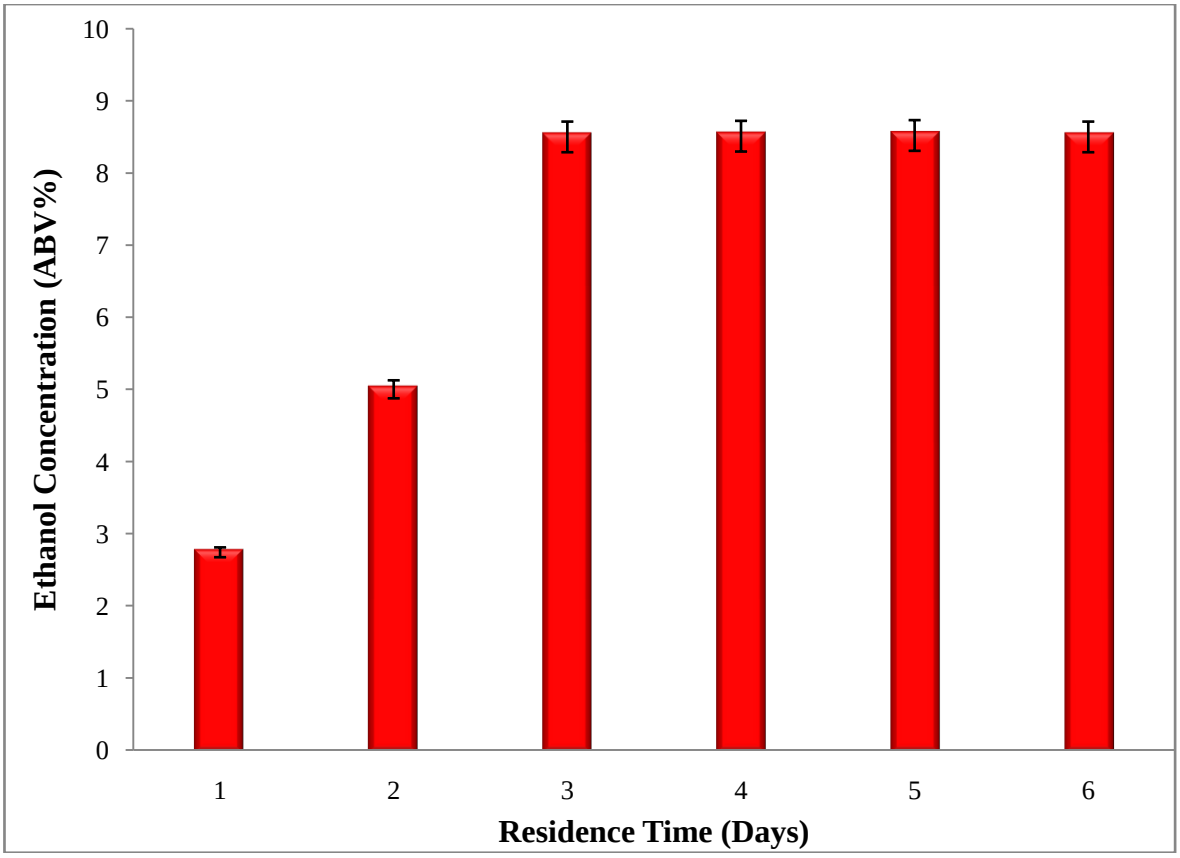


Figure 4.12: Effect of Residence time (days) on Fermentation The following conditions were kept constant during this study: Temperature 35 ^{o}C, pH 5.5 and Yeast concentration 10 (w/v) %.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

1. The proximate Analysis of *Spirogyra africana* biomass showed that it contains 39.79 % carbohydrate (with the ratio of cellulose:starch:free sugar of 28.8:6.83:4.09 respectively) which makes it a good candidate for glucose and bio-ethanol production using *A. niger* and *S. cerevisiae*, respectively.
2. Untreated biomass sample at two (2) days hydrolysis period with glucose yield value of 29.13 wt/wt % was chosen as optimum for saccharification in this study based on economic considerations, because on the first day of hydrolysis, the blanched sample gave a relatively superior glucose yield (15.94 wt/wt %) in comparison to the values of 14.33 and 12.03 for NaOH treated sample and the control respectively, while on the second day, the glucose yield from the three set ups (blanched, NaOH treated and the control) all gave a comparative glucose yield of 29.15, 29.41 and 29.13 respectively. Only marginal increments were experienced on the third day corresponding to 30.72, 32.28 and 30.66 wt/wt %, respectively.
3. The other optimal processing conditions for the hydrolysis are: 25-30 ^{0}C, pH 4.5, 50 g/l Substrate Concentration and 0.6 % w/v *A. niger* concentration.
4. The bio-ethanol yield of 21.40 % wt/v was obtained by fermenting sugars from untreated biomass
5. The optimal conditions for fermentation are: 35^{o}C, 5.0-5.5 pH, 1.0 % (w/v) yeast concentration and three days (72 hours) fermentation period.

5.2 Recommendations

With respect to results and drawn conclusion from this research work, I recommend that:

1. The economic viability of producing bio-ethanol from algae should be studied.

2. Cultivation of green algae (spirogyra) for bio-ethanol production should be encouraged in the renewable energy research institutions (centres) in Nigeria.

3. Pilot plant for bio-ethanol production from spirogyra should be modelled, designed and fabricated.

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APPENDIX I

Table Ia: Various Pre-Treatment Methods

S/NO	SAMPLE TYPE	INITIAL pH	ADJUSTED pH	INITIAL GLUCOSE CONCENTRATION (mg/l)
	UNTREATED			
1		6.3	4.5	1680
	0.5%(w/v) NaOH			
2		10.2	4.5	1680
	1.0%(w/v) NaOH			
3		11.4	4.5	1680
	2.0%(w/v) NaOH			
4		12.8	4.5	1680
	BLANCHED @ 30, 40, 50, 60, 70, 80, 90 and 100 ^{0}C			
5		6.8	4.5	1680

Table Ib: Glucose Produced by Various Pre-Treatment Samples in mg/l

R.TIME (DAYS)	A	B	C	D	E	F	G	H	I	J	K	L
0	**1680**	**1700**	**1700**	**1700**	**1700**	**1700**	**1700**	**1700**	**1700**	**1670**	**1680**	**1690**
1	**4780**	**2520**	**2750**	**3480**	**4010**	**5520**	**6330**	**6213**	**6210**	**4520**	**6090**	**5850**
2	**11570**	**5820**	**6040**	**6520**	**8950**	**10430**	**11590**	**10804**	**10803**	**9620**	**11680**	**10500**
3	**12180**	**6150**	**6830**	**7050**	**9110**	**10890**	**12700**	**11150**	**11149**	**10550**	**12820**	**11750**
4	**11500**	**5840**	**6215**	**6781**	**8982**	**9915**	**11090**	**10719**	**10718**	**9530**	**11430**	**10820**
5	**11230**	**5432**	**6025**	**6243**	**8420**	**9214**	**10300**	**10082**	**10080**	**8380**	**10500**	**10020**
6	**9860**	**5213**	**5982**	**6012**	**7918**	**8899**	**9160**	**9014**	**9013**	**7020**	**9630**	**9580**

Where:

A= Untreated (Control)

B= 30^{o}C blanched

C= 40^{o}C blanched

D= 50^{o}C blanched

E= 60^{o}C blanched

F= 70^{o}C blanched

G= 80^{o}C blanched

H= 90^{o}C blanched

I= 100^{o}C blanched

J= 0.5 % (w/v) NaOH Treated

K=1.0 % (w/v) NaOH Treated

L=2.0 % (w/v) NaOH Treated

APPENDIX II

Table IIa: Effect of pre-treatment on Saccharification

Time (day)	1st run Untreated (mg/l)	2nd Run Untreated (mg/l)	Av. Glucose Untreated (mg/l)	1st Run 80% Blanched(mg/l)	2nd Run 80% Blanched (mg/l)	Av. Glucose in 80% Blanched(mg/l)	1stRun Glucose 1.0%(w/v) NaOH (mg/l)	2nd Run Glucose 1.0 %(w) NaOH (mg/l)	Av. ucose 1.0%(w/v NaOH (mg/l)
0	1668	1692	1680	1680	1720	1700	1680	1680	1680
1	4772	4788	4780	6320	6340	6330	6080	6100	6090
2	11550	11590	11570	11600	11580	11590	11660	11700	11680
3	12200	12160	12180	12190	12210	12200	12800	12840	12820
4	11510	11490	11500	11080	11100	11090	11440	11420	11430
5	11220	11240	11230	10330	10270	10300	10530	10470	10500
6	9850	9870	9860	9140	9180	9160	9650	9610	9630

Table IIb: ANOVA of the effect of Pre-treatment on saccharification

Time (Day)	Mean of Untreated (mg/l)	Std. Dev. of Untreated (mg/l)	Std. Error of Untreated (mg/l)	Mean of Blanched (mg/)	Std. Dev. of Blanched (mg/l)	Std. Error of Blanched (mg/l)	Mean of NAOH (mg/l)	Std. Dev. of NaOH (mg/l)	Std. Error of NaOH(mg/l)	Level Of Significance
0	1680.00	1.697	1.200	1700	2.828	2.0000	1680.0	0.000	0.000	0.55
1	4780.00	1.131	0.800	6330	1.414	1.0000	6090.0	1.414	1.000	0.0001**
2	11570.0	2.828	2.000	11590	1.414	1.0000	11680.0	2.828	2.000	0.55
3	12180.0	2.828	2.000	12200	1.414	1.0000	12820.0	2.828	2.000	0.0002**
4	11500.0	1.414	1.000	11090	1.414	1.0000	11430.0	1.414	1.000	0.0002**
5	11230.0	1.414	1.000	10300	4.243	3.0000	10500.0	4.243	3.000	0.0002**
6	9860.00	1.414	1.000	9160	2.828	2.0000	9630.00	2.828	2.000	0.0002**
The differences in the values are significant but glucose produced by sample pre-treated with NaOH is most significant. **ABCDEFG means with different letter are significantly different**										

P>0.05 =Not Significant ()
P<0.05= Significant (*)
p<0.01 =Highly Significant (**)

APPENDIX III

Table IIIa: Effect of substrate concentration on saccharification

Substrate concentration (g/l)	1st RunGlucose Concentration (mg/l)	2nd Run Glucose Concentration (mg/l)	Average Glucose Concentration (mg/l)
10	10430	10570	10500
20	10535	10665	10650
30	10712	10688	10700
40	10790	10810	10800
50	12980	13020	13000
60	12851	12749	12800
70	12533	12467	12500
80	12098	12102	12100

Table IIIb: ANOVA of the Effect of substrate concentration on saccharification

Substrate concentration (g/l)	Mean of Glucose Concentration (mg/l)	Std. Dev. of Glucose Concentration (mg/l)	Std. Error of Glucose Concentration (mg/l)	Level of Significance
10	10500	9.899	7.0000	G
20	10600	9.192	6.5000	G
30	10700	1.697	1.2000	E
40	10800	1.414	1.0000	E
50	**13000**	**2.828**	**2.0000**	**A**
60	12800	7.212	5.1000	B
70	12500	4.667	3.3000	C
80	12100	0.283	0.2000	D
P = 0.0001: the differences in value are highly significant but the optimum yield was observed at 50g/l substrate concentration. **ABCDEFG means with different letter are significantly different**				

APPENDIX IV

Table IVa: Effect of Residence Time on Saccharification.

Residence time (days)	0	1	2	3	4	5	6
1st Run Glucose Concentration (mg/l)	1680	6100	11970	10180	11440	10500	10476
2nd Run Glucose Concentration (mg/l)	1680	6080	11990	12220	11420	10500	10484
Average Glucose Concentration (mg/l)	1680	6090	11980	12200	11430	10500	10480

Table IVb: ANOVA of the Effect of Residence Time on Saccharification

Residence time (days)	Mean of Glucose Concentration (mg/l)	Std. Dev. of Glucose Concentration (mg/l)	Std. Error of Glucose Concentration (mg/l)	Level of Signif.
0	1680	0.000	0.000	D
1	6090	1.414	1.000	C
2	11980	1.414	1.000	A
3	11200	144.250	102.000	A
4	11430	1.414	1.000	A
5	10500	0.000	0.000	B
6	10480	0.566	0.400	B
P = 0.0001 meaning the differences in the values are generally highly significant **but there is no significance between day 2, 3, and 4.****ABCDEFG means with different letter are significantly different**				

APPENDIX V

Table Va: The Effect of Temperature on Saccharification.

Temperature (^{0}C)	15	20	25	30	35	40	45
1st run Glucose Concentration (mg/l)	1685	9040	12470	12565	10092	9988	1673
2nd run Glucose Concentration (mg/l)	1675	9060	12530	12535	10108	10000	1687
Average Glucose yield (mg/l)	1680	9050	12500	12550	10100	10000	1680

Table Vb: ANOVA of the Effect of Temperature on Saccharification

Temperature (^{0}C)	Mean of Glucose Concentration (mg/l)	Std. Dev. of Glucose Concentration (mg/l)	Std. Error of Glucose Concentration (mg/l)	Level of Significance
15	1680	0.707	0.500	F
20	9050	1.414	1.000	E
25	12500	4.243	3.000	B
30	12550	2.121	1.500	A
35	10097	0.707	0.500	C
40	9995	0.990	0.700	D
45	1680	0.990	0.700	F
P = 0.0001: the differences in the values are generally highly significant and glucose Produced at 30^{O}c is most significant. **ABCDEFG means with different letter are significantly different**				

APPENDIX VI

Table VIa: the Effect of pH on Saccharification.

pH value	3.0	3.5	4.0	4.5	5.0	5.5	6.0
1st Run Glucose Conc. (mg/l)	1680	1800	10825	12530	11790	10950	10450
2nd Run Glucose Conc. (mg/l)	1700	1800	10775	12470	11810	11050	10550
Average Glucose Conc. (mg/l)	1690	1800	10800	12500	11800	11000	10500

Table VIb: ANOVA of the Effect of pH on Saccharification.

pH value	Mean of Glucose Conc. (mg/l)	Std. Dev. of Glucose Conc. (mg/l)	Std. Error of Glucose Conc. (mg/l)	Level of Significance
3.0	1690	1.414	1.000	G
3.5	1800	0.000	0.000	F
4.0	10800	3.536	2.500	D
4.5	12500	4.243	3.000	A
5.0	11800	1.414	1.000	B
5.5	11000	7.071	5.000	C
6.0	10500	7.071	5.000	E
P = 0.0001: The differences in the values are generally highly significant and glucose produced at 4.5pH is most significant. **ABCDEFG means with different letter are significantly different**				

APPENDIX VII

Table VIIa: Effect of *A.Niger* Concentration on Saccharification

A.niger concentration (w/v) %	0	0.2	0.4	0.6	0.8	1.0	1.2
1st Run Glucose Concentration (mg/l)	1680	4020	4540	12505	12000	12180	12090
2nd Run Glucose Concentration (mg/l)	1680	3980	4460	12495	12000	12220	12110
Average Glucose Concentration (mg/l)	1680	4000	4500	12500	12000	12200	12100

Table VIIb: ANOVA Effect of *A.Niger* Concentration on Saccharification

A.niger concentration (w/v) %	Mean of Glucose Concentration (mg/l)	Std. Dev. of Glucose Concentration (mg/l)	Std. Error of Glucose Concentration (mg/l)	Level of Significance
0	1680.00	0.000	0.000	G
0.2	4000.00	2.828	2.000	F
0.4	4500.00	5.657	4.000	E
0.6	12497.5	0.354	0.250	A
0.8	12000.0	0.000	0.000	D
1.0	12200.0	2.828	2.000	B
1.2	12100.0	1.414	1.000	C
P = 0.0001: The differences in the values are generally highly significant and glucose produced at 0.6 *A. niger* concentrations is most significant. **ABCDEFG means with different letter are significantly different**				

APPENDIX VIII

Table VIIIa: The effect of temperature on fermentation

Temperature ^{o}C	Run 1 Ethanol (ABV %)	Run 2 Ethanol (ABV %)	Average, Ethanol (ABV %)
25	5.14	4.91	5.03
30	6.48	6.13	6.31
35	8.76	8.72	8.74
40	6.40	7.30	6.85
45	2.80	4.05	3.43
50	2.80	3.20	3.0

Table VIIIb: ANOVA of The effect of temperature on fermentation

Temperature ^{o}C	Mean Of Ethanol (ABV %)	Std. Dev. Of Ethanol (ABV %)	Std. Error Of Ethanol (ABV %)	Level of Significance
25	5.03	0.16263	0.11500	C
30	6.31	0.25456	0.18000	B
35	8.74	0.02828	0.02000	A
40	6.85	0.63640	0.45000	B
45	3.43	0.02828	0.02000	D
50	3.0	0.28284	0.20000	E
P = 0.0001: The different values of ethanol produced at different temperature are generally significant and the bioethanol produce @ 35^{o}C is most significance **ABC means with different letter are significantly different**				

APPENDIX IX

Table IXa: The effect of pH on fermentation

pH	Run 1 Ethanol (ABV %)	Run 2 Ethanol (ABV %)	Average Ethanol (ABV %)
3.5	2.12	1.96	2.04
4.0	4.42	4.58	4.49
4.5	6.21	5.81	6.01
5.0	8.30	7.80	8.05
5.5	8.05	8.15	8.20
6.0	7.10	7.00	7.05
6.5	6.76	6.30	6.53
7.0	5.36	5.77	5.57

Table IXb: ANOVA of The effect of pH on fermentation

pH	Mean of Ethanol (ABV %)	Std. Dev. of Ethanol (ABV %)	Std. Error of Ethanol (ABV %)	Level of Significance
3.5	2.04	0.11314	0.08000	F
4.0	4.49	0.11314	0.08000	E
4.5	6.01	0.28284	0.20000	D
5.0	8.05	0.35355	0.25000	A
5.5	8.20	0.07071	0.05000	A
6.0	7.05	0.07071	0.05000	B
6.5	6.53	0.32527	0.23000	B
7.0	5.57	0.26870	0.19000	D
P = 0.0001: The different values of ethanol produced at different temperature are generally significant. The bioethanol produce @ 5.0 and 5.5 are most significance **ABCDEFG means with different letter are significantly different**				

APPENDIX X

Table Xa: The effect of Yeast Conc. on fermentation

Yeast Concentration (w/v) % x 10^1	RUN 1 Ethanol (ABV %)	RUN 2 Ethanol (ABV %)	Average Ethanol (ABV %)
0	0.0	0.0	0.00
0.2	3.18	3.52	3.35
0.4	4.52	4.81	4.66
0.6	4.68	5.81	5.25
0.8	5.09	5.92	5.51
1.0	8.32	8.71	8.52
1.2	6.02	5.89	5.96
1.4	5.34	4.98	5.16

Table Xb: ANOVA of the effect of Yeast Concentration on fermentation

Yeast Concentration (w/v) % x 10^{-1}	Mean of Ethanol (ABV %)	Std. Dev. of Ethanol (ABV %)	Std. Error of Ethanol (ABV %)	Level of Significance
0	0.00	0.00000	0.00000	F
0.2	3.35	0.02828	0.02000	E
0.4	4.66	0.63640	0.45000	D
0.6	5.25	0.19092	0.13500	C
0.8	5.51	0.09192	0.06500	B
1.0	8.52	0.09899	0.07000	A
1.2	5.96	0.12728	0.09000	B
1.4	5.16	0.10003	0.04650	C
P = 0.0001: The different values of ethanol produced at different yeast concentration are generally significant. The bioethanol produce @ 0.6(w/v)% yeast conc. is the most significance **ABCDEFG means with different letter are significantly different**				

APPENDIX XI

Table XIa: The effect of Residence Time on fermentation

Residence Time (days)	RUN 1 Ethanol (ABV %)	RUN 2 Ethanol (ABV %)	Average Ethanol (ABV %)
0	0.0	0.0	0.00
1	3.00	2.48	2.74
2	5.46	4.54	5.00
3	8.39	8.61	8.50
4	8.48	8.54	8.51
5	8.50	8.53	8.52
6	8.49	8.55	8.50

Table XIb: ANOVA of the effect of Residence Time on fermentation

Residence Time (days)	Mean of Ethanol (ABV %)	Std. Dev. of Ethanol (ABV %)	Std. Error of Ethanol (ABV %)	Level of Significance
0	0.00	0.00000	0.00000	D
1	2.74	0.92828	0.02000	C
2	5.00	0.63640	0.45000	B
3	8.50	0.19092	0.33500	A
4	8.51	0.09192	0.4500	A
5	8.52	0.09899	0.37000	A
6	8.50	0.12728	049000	A
P = 0.0001: The different values of ethanol produced at different residence time are generally significant. The bio-ethanol produce @ day 3 is the most significance. **ABCDEFG means with different letter are significantly different**				

APPENDIX XII

Table XIIa: Glucose Yield for Various Pre-treated

RESIDENCE TIME (DAYS)	**UNTREATED %(V/V)**	**BLANCHED AT 80 ^{O}C %(V/V)**	**1.0 (w/v)% NaOH PRE-TREATED %(V/V)**
0	4.23	4.28	4.23
1	12.03	**15.94**	14.33
2	**29.13**	29.15	29.41
3	30.66	30.72	32.28
4	28.95	27.92	28.78
5	28.27	25.86	26.44
6	24.82	23.37	24.24

Table XIIb: Bio-Ethanol Yield for the Blanched and Untreated Sample after One Day Saccharification

TIME (DAYS)	**BIO-ETHANOL YIELD FOR BLANCHED SAMPLE (V/V)%**	**BIO-ETHANOL YIELD FOR UNTREATED SAMPLE (V/V)%**
0	0.00	0.00
1	3.78	3.12
2	7.28	6.53
3	13.37	12.21
4	13.42	12.22
5	13.44	12.23
6	13.44	12.23

Table XIIc: Bio-ethanol Yield for the Untreated Sample After Two Days Saccharification

TIME(DAY)	**ABV%**	**YIELD%(V/V)**
0	0.00	0.00
1	2.74	6.90
2	5.00	12.59
3	8.50	21.40
4	8.51	21.42
5	8.52	21.45
6	8.52	21.45