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Optimization of dilute acid and dilute base pretreatment of tropical sawmill dust for bioethanol production

By

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MASTER OF SCIENCE IN BIOTECHNOLOGY

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DECLARATION

I hereby declare that this submission is my own work towards the degree of MSc. Biotechnology and that to the best of my knowledge, it contains no material previously published by another person, nor material which have been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the context.

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ABSTRACT

There is abundance of lignocellulose biomass in Ghana. Currently, there are a lot of sawmill factories and carpentry shops in the country. The use of sawmill dust for the production of bioethanol will go a long way to alleviate environmental pollution by the reduction in the emission of harmful gases which result by burning of these waste products. Most of these gases lead to air pollution and can also contribute to the greenhouse effect. Using sawmill dust will also help minimize the use of land and foodstuffs for the generation of bio-energy. In this study, the optimum amount of glucose was determined by using different concentrations of dilute acids and bases for the pretreatment of different sawmill dust from two different plant species to release glucose for bioethanol production. Concentrations of 0.25 M, 0.5 M and 1 M each of sodium hydroxide, ammonia, sulphuric acid and hydrochloric acid were used for the pretreatment of sawmill dust from Mahogany (*Swietina macrophylla*) and Kapok (*Ceiba pentandra*) plant species at different temperatures and resident times. The temperatures used for the sodium hydroxide and ammonia were 75°C, 90°C and 100°C at resident times of 30, 60 and 90 minutes and 121°C at resident times of 10, 15 and 20 minutes. The temperature used for both the sulphuric acid and hydrochloric acid was 121°C at resident times of 30, 60 and 90 minutes. The optimum amount of glucose (3.37 g/l) for the base was achieved by the use of 1 M sodium hydroxide at 100°C for 90 minutes with *Ceiba pentandra*. The optimum amount of glucose (0.74 g/l) for the acid was attained by the use of 1 M hydrochloric acid at 121°C for 30 minutes with Mahogany plant species. The optimum amount of glucose (5.27 g/l) was attained by the use of cellulase enzyme at 55°C for 30 minutes with Mahogany plant species. The highest amount of ethanol (3.48×10^{-2} % w/v) was attained by carrying out fermentation with *Saccharomyces cerevisia*e at a temperature of 50°C at a pH of 4.0 for 5 days. The use of high temperatures and concentrations produced higher glucose concentrations with the chemical pretreatments and hence provide an alternative way of producing bioethanol**.**

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DEDICATION

This work is dedicated to my family and friends who have always stood by me in both happy and trial moments. Without you I could not have reached the level that l am today. I am indeed very grateful and may the Lord give you long life and strength, so that you can always continue to support me in all my endeavours.

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CHAPTER ONE

1.0. INTRODUCTION

The use of bioethanol as a source of biofuel accounts for about ninety percent (90%) of the total biofuel used globally (Demirbas, 2008). The conventional production process is normally based on the use of enzymes to convert plant biomass into sugars followed by the fermentation of the sugars into ethanol which is finally distilled for usage. Agricultural residues such as cereal crops, corn (maize), sugar cane, sugar beets and cassava among others can be used to produce bioethanol through fermentation. The use of agricultural residues has many advantages over the other products. One important advantage is the reduction of the emission of greenhouse gases because of the reduction in the use of oil in the process (Macedo *et al*., 2008). The process of bioethanol from lignocellulose normally begins with the pretreatment which involves the hydrolysis of the hemicellulose followed by hydrolysis of the cellulose using enzymes and fermenting the sugars to produce ethanol using yeast (Galbe and Zacchi, 2007). To minimize the use of enzymes which is very costly, modifying the methods of pretreatment and developing new strains of microbes such as *Saccharomyces cerevisiae* to ferment pentoses has achieved some success (Madhavan *et al.*, 2012). Bioethanol is produced on a large scale by countries such as Brazil (use sugarcane to produce ethanol) and the United States (use corn to produce ethanol).

Ethanol can be used in 5%-10% blends with gasoline (Hansen *et al.*, 2005).

1.1. SAWDUST

Sawdust constitutes the main waste products which are mostly obtained from wood. It can be obtained during sawing and cutting of wood. It can be obtained in large quantities from the saw mill factories where varieties of wood are processed. It contains lignocellulose and some amount

of moisture. It can serve as a source of silvichemicals. This refers to chemicals which are derived from wood similar to petroleum chemicals. It can serve as an energy source and can also be used to produce organic compounds such as ethanol and methanol (Opera, 2002). The use of waste materials for producing ethanol using conventional processes is being developed (Sarkar *et al.*, 2012). This is relatively cheaper as compared to using other biomass feedstock. Currently most of these waste materials pose a threat to the ecosystem and the environment. Since the use of some of these wastes as raw materials will not compete with the use of food stuffs but serve as source of fuel, this gives an advantage of using some of these waste materials rather than using grains or sugarcane as source of alcohol (Von Sivers *et al*., 1994).

1.2. PRETREATMENT OF LIGNOCELLULOSE

The challenge involved in processing of lignocellulosic biomass to produce ethanol is the pretreatment. The purpose of pretreatment is to break down lignocellulose complex, which is made up of cellulose and lignin to enhance the rate of enzymatic hydrolysis; particularly on the cellulose. The pretreatment should be able to hydrolyze the hemicellulose and degrade the lignin. There are different pretreatment procedures such as mechanical combination (Cadoche and Lopez, 1989), steam explosion (Gregg and Saddler, 1996), ammonia fiber explosion (Kim *et al*., 2003), acid or alkaline pretreatment (Damaso *et al*., 2004; Kuhad *et al.*, 1997), and biological treatment (Keller, *et al.*, 2003). When pretreatment is not done, the amount of cellulose which will be hydrolyzed is often less than 20%, whereas the amount of cellulose hydrolyzed is often greater than 90% when pretreatment is done (Lynd, 1996). The main objective of pretreatment is to remove lignin and hemicellulose and reduce the crystalline structure of cellulose to make them more porous. Moreover, the pretreatment method should be able to increase the quantity of sugars

or enhance its ability to form the sugars during enzymatic hydrolysis, and prevent the formation of inhibitors during the subsequent hydrolysis and fermentation processes. In pretreatment involving lignocelluloses, there are several physical, chemical and biological procedures which have been proposed and developed (Sun and Cheng, 2002). The use of acids or bases for hydrolysis often improve the yield of glucose recovery from the cellulose by removing hemicellulose or lignin in the course of pretreatment (Ye sun and Jiayang,2002). Some of the acids and bases which are commonly used are sulphuric acid (H_2SO_4) and sodium hydroxide (NaOH). Alkaline chemicals (e.g., NaOH or NH_3), are able to break down cellulose, hemicellulose and lignin. This makes it possible for the cellulose to swell above the normal water-swollen stages which increases the pore size, the intra particle porosity and the capillary size. This also leads to a phase change in the structure of the cellulose (Mittal *et al.*, 2011).

1.3. PRODUCTION OF ETHANOL FROM LIGNOCELLULOSE BIOMASS

Ethanol from lignocellulose biomass can be produced through hydrolysis and fermentation process. To be able to produce sugars from lignocellulose, the biomass is first pretreated in order to reduce the size and to open up its structure (Demain and Newcomb 2005). The hemicellulose and the cellulose components are broken down (hydrolyzed) with enzymes or dilute acids into sugar before it is finally fermented to produce ethanol. The processing of lignocellulose to produce ethanol involves four major steps which include Pretreatment, Hydrolysis, Fermentation, and Separation/Purification (Vikari *et al.*, 2012).

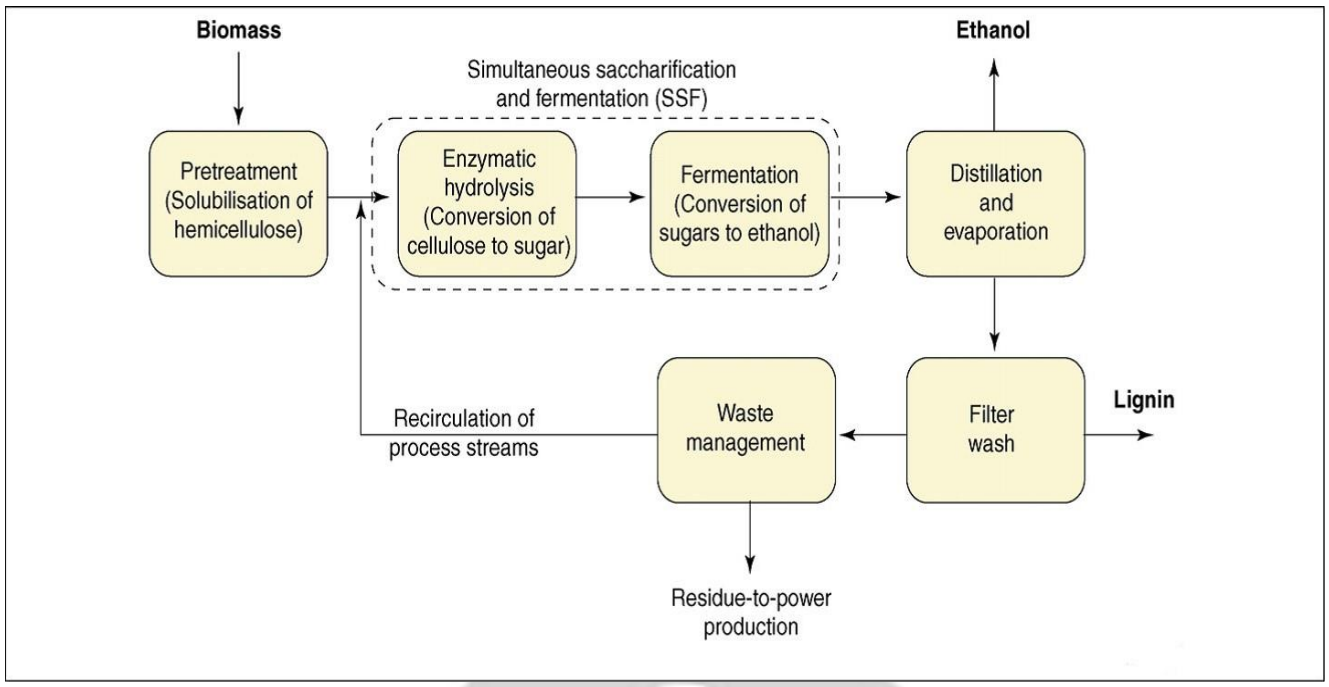

Fig.1.1. Schematic diagram of the production of Bioethanol from lignocellulose (Courtesy: Hahn-Hagerdal and Galbe, 2006)

The general requirements of a microorganism to be used in the production of ethanol is that it should be able to produce a high yield of the ethanol and to tolerate the high concentration of the ethanol in order to keep the cost of distillation low. Advances in genetic manipulations of bacteria (Ingram *et al.*, 1998, 1999) and yeasts (Ho *et al.*, 1998, 1999) has made it possible to produce strains that are capable of fermenting both pentose and hexose sugar units to produce ethanol and other valuable products with high yields. In fact, a fermentation process involving a wild type yeast strain has been patented (Carrascosa, 2006). The most promising bacteria that is able to produce high yield of ethanol is *Zymomonas mobilis*. However, it has a narrow range to fermenting substrates (Claassen *et al*., 1999). Another disadvantage in using this bacterium during the fermentation of sugar cane syrup and other sucrose based media is that it is capable of forming the polysaccharide levan which is made up of fructose units. This can lead to increase in the viscosity

of fermentation broth, and sorbitol (Lee and Huang, 2000). Ethanol can be used as an alternate source of fuel in the future. Several microorganisms including *Clostridium* spp. have been used to produce ethanol, especially, the yeast such as *Saccharomyces cerevisiae* and facultative bacterium such as *Zymomonas mobilis* have been proven to be the better candidates for industrial alcohol production. The bacterium *Zymomonas mobilis*, which is a Gram-negative bacterium, can be used as an alternative microorganism for producing ethanol on the large scale. Generally *Zymomonas mobilis* is preferred over yeasts due to the following advantages ((Lee *et al*., 2007).

i. It has a higher sugar uptake and ethanol yield,

ii. It has lower biomass production, iii. It has a higher ethanol tolerance, iv. It does not require additional oxygen , and

v. It is amenable to various genetic modifications

The bacterium *Zymomonas mobilis* has several advantages compared to S. *cerevisiae* with regards to its ability to produce ethanol and tolerance; therefore most researchers prefer the exploitation of Z. *mobilis* to use glucose through the Entner-Doudoroff pathway. This pathway therefore gives a higher yield of ethanol with low biomass yield compared to *S. cerevisiae* (Lee *et al*., 2007). However, the natural strain of *zymomonas mobilis* is not able to ferment pentose sugar and it is also not robust. Moreover, it is not able to tolerate inhibitors and therefore various steps are required for detoxification before carrying out fermentation. Traditionally, ethanol has been produced by using batch fermentation employing yeast strains that are not able to tolerate the high concentrations of the ethanol. Therefore, there is the need for strain improvement programme to obtain an alternative microorganism that is more tolerant for fermentation.

1.4 PROBLEM STATEMENT

It has been estimated that fossil fuels will be running out by the next few years (Bentley, 2002; Cavallo, 2002), therefore, attention has currently been shifted towards using biomass to produce ethanol which can be used as fuel. The production of bioethanol from foodstuffs and lignocellulose biomass provides an alternative to the use of fossil fuels. It can also be mixed with some quantity of petroleum products. The production of the first generation of ethanol derived from biofuel has been widely used and commercialized in the U.S and Brazil. The ethanol which is mostly used in the U.S is produced from corn, while the one used in Brazil is basically produced from sugarcane (Marcos *et al.,* 2012). Using these foodstuffs as raw materials create a direct competition with humans and animals which depend on these resources as food and also the use of land to produce them for food instead of fuel. On the other hand, using the second generation of ethanol which is produced from lignocellulosic biomass can be obtained from the residues. Moreover, the production of ethanol from sugar or starch has a negative impact on the economy. This makes it more expensive than using fossil fuels. Therefore the need to produce ethanol is gradually shifting towards the use of lignocellulosic materials to minimize the cost of production (Ye Sun and Jiayang, 2002). Organic acids, vitamins and bacterial and fungal polysaccharides such as xanthan can also be produced by fermentation using glucose but this can also be produced theoretically from lignocellulosic waste instead of using the foodstuff (Godliving and Mtsui, 2009).

The total rate of energy obtained from a variety of sources has been increasing since the industrial revolution (Marjurndar, 2013). The use of energy has increased at a rate of 2.5% per year as compared to the total increase with the worldwide population growth of 1.7% per year (doubling every 40 to 60 years (Pimentel and Patzek, 2007). There have been reports (‘out of time? the end of oil’) indicating that the world has reached its peak oil and natural gas availability

(Heinberg, 2007). These energy resources will continue to decrease gradually and continuously until there is little or no oil and gas resources left (Heinberg, 2007; Youngquist and Duncan, 2003), hence the need to depend on an alternative way of producing a sustainable energy. The use of bioethanol provides the solution to global energy crisis, however; it is faced with other challenges. The production of ethanol is faced with many constraints such as availability of land and also competition with food for the land which can also increase both ethanol and food prices (Pimentel and Patzek, 2007). The transport of biomass is also another barrier that limits the production of ethanol, however, producing more biofuels from conventional feedstock could also create a problem with conservation of biodiversity which normally increase the amount of pesticides and fertilizers, thus making it difficult to sustain. Therefore, there is the need to develop cost effective ethanol production from lignocellulose by using enzymatic hydrolysis which could increase the variety and availablility of feedstock and increase the production of biofuels (Eriksson *et al.*, 2002).

1.5. MAIN OBJECTIVE

The main aim of this work was to optimize conditions for the pretreatment of tropical sawmill dust using dilute acid and dilute base for bioethanol production.

1.6. SPECIFIC OBJECTIVES

- Determine the proximate composition of tropical timber sawmill dust.
- Determine the best pretreatment option and neutralization of inhibitors produced during hydrolysis of tropical sawmill dust

- Determine the best combination of parameters (concentration, temperature and time) for the production of high yield of bioethanol.

1.7. JUSTIFICATION

There has been much interest shown in biofuels in recent years when oil prices rose from about US$19 per barrel in the late 1990s to reach a peak of about US$140 per barrel in 2008 (Wyman, 1994). The prices of oil in the world market had not been stable until from 2010 to mid-2014 when the prices had been fairly stable, around US$110 a barrel. The price of oil at the world market is recently hovering around US$ 36 per barrel but keeps on fluctuating. Biofuels were therefore thought to be an alternative to fossil fuel, and a possible solution to the global emerging energy crisis (Keshav *et al.*, 2011). This perception however, was dampened because of the great concerns on the increase in food prices, and possible food security threat that is attributed to the use of biofuels as the main cause (Tatsuji, 2013). Recent studies show that only about 1% of total agricultural area will be used in planting the so called energy crops (Luis *et al*., 2014). The use of just about 50% of these lands for the production of biofuel can provide a great advantage to farmers to diversify their farming which will help them to gain an extra income. The establishment of biofuel processing plants can also create employment for those living in the rural areas (Xu *et al*., 2009). This will go a long way to alleviate poverty since it is in these areas where majority of poor farmers live (Kim and Dale, 2004). Biofuel provides an optional way of producing fuel rather than depending on fossil fuels. This gives several benefits such as reducing the dependence on foreign oil, carbon neutral process which does not emit greenhouse gases and also provides profits to farmers (Lin *et al.*, 2009).

Currently, there are many waste materials that pose a negative effect on the ecosystem (Bin *et al*., 2004). Most of these wastes can be used for the production of alcohol as a source of fuel. The use of agricultural residues has many advantages over their food products. One important advantage is the reduction of the emission of greenhouse gases because of the reduction in the use of oil in the process. Moreover, the use of lignocellulose biomass such as sawdust will also help reduce the use of land and foodstuffs for the production of biofuel. Hence the production of bioethanol is seen as an imminent solution to the global energy crisis. The use of waste products will help reduce the rate of environmental pollution and will further enhance the proper management of these waste materials by harnessing them as raw materials for industrial production.

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. BIOETHANOL

Bioethanol is a renewable source of fuel which is mostly derived from plants. It is good for the environment because of the reduction in carbon dioxide (CO_2) emissions. It does not generate waste and harmful emissions during its production. Bioethanol can also lead to the production of

useful by- products and does not pollute water, air, and land (Sarkar *et al.,* 2012). The production of ethanol from biomass is one of the ways which help to minimize both environmental pollution and the consumption of crude oil (Silverstein, 2007).

The bioethanol consists of two carbons which is produced from glucose by using the yeast *Saccharomyces cerevisiae*. Xylose which is a five carbon sugar can also be converted to ethanol by using certain bacteria and certain genetically modified strains of yeast (Krishnan *et al*., 1999; van Maris *et al*., 2007; Zhang *et al*., 1995). Lignocellulose biomass produces a mixture of hydrogen, carbon dioxide and carbon monoxide, generated as a result of gasification of the biomass but can be converted to ethanol by using yeast for fermentation or by chemical reaction using a catalyst (Henstra *et al*., 2007; Galvita *et al*., 2001). When bioethanol is mixed with gasoline it produces oxygen, resulting in a complete combustion. It is therefore used as a combustion fuel because it burns cleaner than gasoline. The main problem with ethanol is that it turns to absorb a lot of moisture (hygroscopic) and also has about 66% of its energy as the same volume as that of gasoline. Ethanol can however, transport a vehicle about the same distance compared to the same volume of gasoline (Turner, 2013).

2.2. LIGNOCELLULOSIC BIOMASS

Lignocellulose constitutes the main structural component of both woody and non woody plants. It has an enormous biotechnological value because of its chemical properties (Malherbe and Cloete, 2003). Large quantities of lignocellulosic waste are mostly produced through forestry and agricultural practices, paper-pulp industries, timber industries and many agro industries. These wastes pose a problem by polluting the environment. Majority of these waste products are often disposed of by burning the biomass. This is not restricted only to the developing countries, but can

also be considered as a global phenomenon (Levine, 2013). The large amount of these wastes can however, be used to produce biofuels, chemicals, and serve as cheap source of energy, improved animal feeds and human nutrients (Mussato and Teixera, 2010).

Lignocellulosic feedstock provides a possible solution to some of the problems which confront sugar and starch feed stocks. Lignocellulose is a biomass which is made up of three polymers; hemicellulose, cellulose, and lignin. Hemicellulose consists of a five carbon sugar xylose which forms the backbone with side chains of other five and six carbon sugars such as arabinose, glucose, and galactose. Cellulose consists of a polymer of glucose which contains β-1-4 glycosidic bonds. It has a crystalline structure and very difficult to degrade into glucose monomers. Lignin, on other hand, consists of a polymer which is made up of different aromatic subunits (Perez *et al*., 2002).

Agricultural, food processing and municipal wastes, perennial grasses, and woody biomass are some of the examples of lignocellulosic feedstocks (Tan *et al.*, 2008). Lignocellulosic biomass is almost ubiquitous on earth. This makes it possible for local bioethanol production, which could also keep the cost of transportation very low. Lignocellulosic feedstocks do not constitute food resources and therefore do not require the use of land hence there is no competition of lignocellulosic feedstock for land availability. It is not currently used because there are some problems associated with the use of some of these feedstocks for the production of bioethanol. One such problem is the enzyme that is required to break down the cellulose into glucose is more costly than amylase which is used to break down starch (Lynd *et al.*, 2002). Another problem associated with it is that the biomass component which is made up of xylose cannot be fermented with *Saccharomyces cerevisiae* which is the principal microorganism used for starch based ethanol production. It is important to ferment the xylose to make bioethanol production from lignocellulosic material very economical and feasible (Sun and Cheng, 2002). Research has led to

the engineering of some bacteria and yeast strains that are capable of fermenting xylose (Zhang *et al.*, 1995; Krishnan *et al.*, 1999; van Maris *et al*., 2007).

One of the biggest obstacles which makes lignocellulosic biomass not feasible to be used as a source of bioethanol feedstock is that it is very recalcitrant. This makes it difficult to get access to the cellulose and hemicellulose components. Therefore an important process that can disrupt the biomas is required, hence the need for pretreatment (Mosier *et al.*, 2005).

2.2.1. Cellulose

Cellulose [$(C_6H_{10}O_5)_n$] which is a polysaccharide consists of several hundred to over ten thousand linear chains of (1-4) linked D-glucose units. The carbon component accounts for 40%-60% of its weight which depends mostly on the type of biomass (Ye sun and Jiayang, 2002). It constitutes the structural component of plant cell wall, algae and oomycetes. Its structure is crystalline, making it resistant to hydrolysis (Liu *et al.*, 2010).

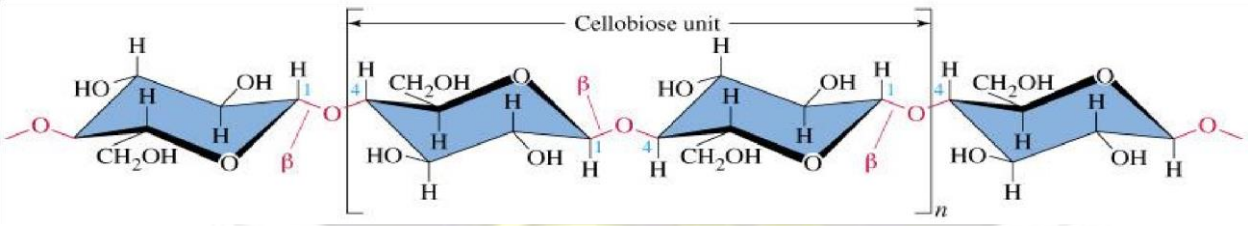

Fig. 2.1. Structure of cellulose

(Courtesy: Varga *et al.*, 2003)

2.2.2. Hemicellulose

It consists of a polysaccharide that is mostly derived from five and six carbon sugars. Hemicellulose, unlike cellulose has an amorphous and random structure which is very weak and

can therefore be easily hydrolyzed by using dilute acid or hemicellulase enzyme. It is made up of several polysaccharrides, such as arabinoxylans, which is found in cellulose and also present in most cell walls of plants. The difference between hemicellulose and cellulose is that hemicellulose has shorter chains, has a lot of branches and consists of several sugar units (Saha, 2003).

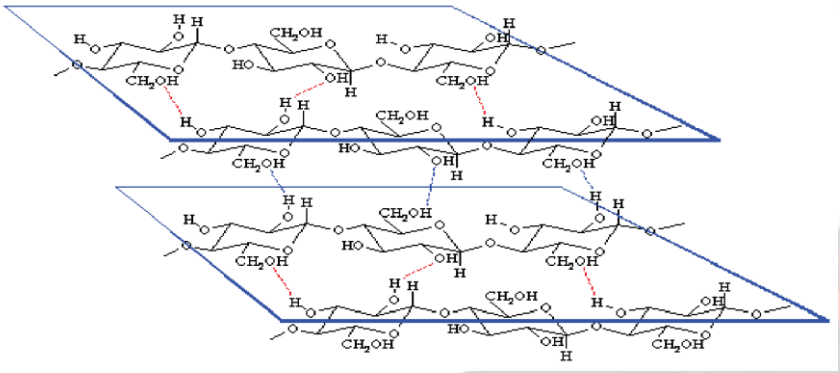

Fig. 2.2. Structure of hemicellulose

(Courtesy: Varga *et al.*, 2003)

2.2.3. Lignin

Lignin consists of a three-dimensional polymer which is made up of different phenyl propane units, which have been joined together by ether and carbon-carbon bonds. It constitutes one of the abundant and important polymeric organic substances that are found in plants. Lignin lacks a defined primary structure and also very unusual because of its heterogeneity. There are very few lignin that have their structures known, but generally most of their structures are unknown. Even though most microorganisms are capable of using hemicellulose and cellulose, some few strains have the ability to break down lignin effectively (Laser, 2002).

2.3. PRETREATMENT OF LIGNOCELLULOSE BIOMASS

The main challenge in bioprocessing lignocellulosic biomass to produce ethanol is pretreatment. This is due to the resistant lignin barrier which reduces the accessibity of the enzymes to hydrolyze cellulose and hemicellulose (Mosier *et al.*, 2005). Lignocellulose consists of a complex matrix and lignin with chains of hemicellulose. Pretreatment is done to break the matrix in order to reduce cellulose which is crystallized thereby increasing the surface area for enzymatic reaction. The hemicellulose is usually hydrolyzed to degrade the lignin and due to the many physical, chemical and structural factors, pretreatment is often required to convert the biomass to ethanol. When pretreatment is done effectively it causes the breakup of the lignocellulose structure, hence making the carbohydrates more accessible for enzymatic saccharification (Xu *et al.*, 2010b). Generally, after pretreatment, the yield of cellulose hydrolysis often exceeds 90% whereas the yield of cellulose is usually less than 20% if pretreatment is not carried out (Lynd, 1996). Therefore, the main aim of pretreatment is to remove lignin and hemicellulose and thus reduce the crystallized structure of the cellulose to increase the porosity of the materials. Moreover, pretreatment should reduce the formation of inhibitors prior to fermentation. For the pretreatment of lignocellulose, several physical, chemical and biological processes have been proposed and developed (Sun and Cheng, 2002). Pretreatment is not only considered as a crucial step in bioconversion process, it also has been described as one of the most costly steps, accounting for up to 18% of the total projected cost (Yang and Wyman, 2008). Different lignocellulosic materials have different structural components and therefore there is the need to develop a suitable pretreatment technology based on the unique characteristics of the type of biomass. Moreover, the use of certain types of pretreatment has great impact on the subsequent steps. For example, the production of inhibitory compounds such as furfural affects ethanol fermentation (Galbe and Zacchi, 2002). Given the big

impact of pretreatment on process cost, there are many key attributes to take into consideration for low-cost pretreatment technologies (Yang and Wyman, 2008).

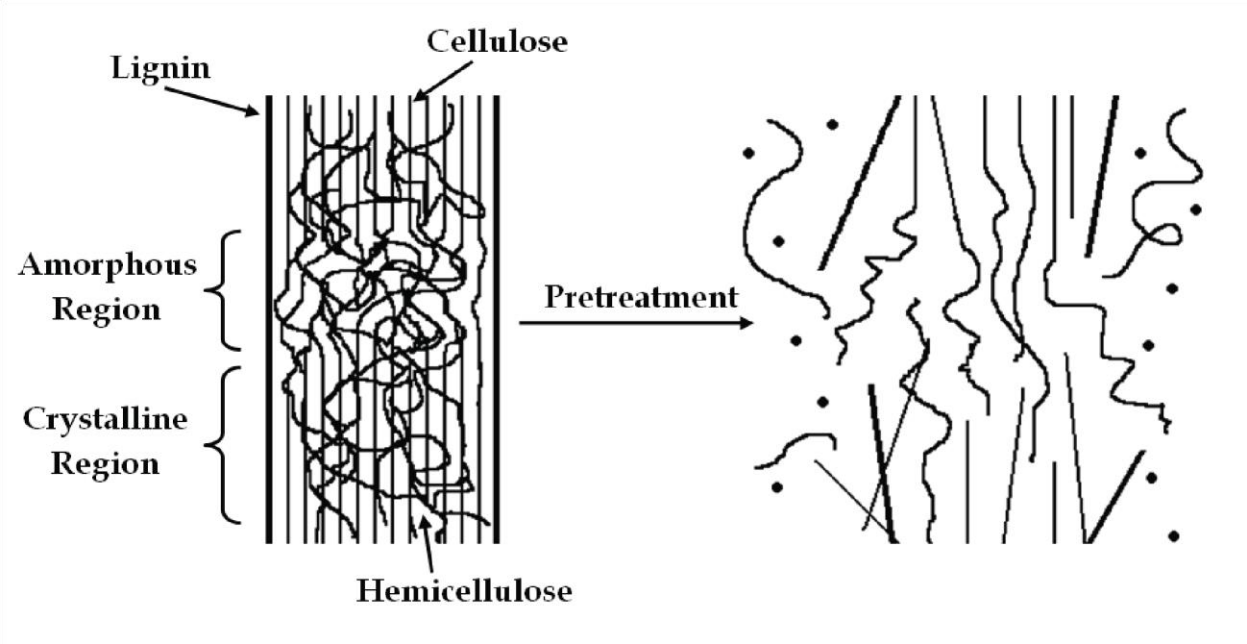

Fig. 2.3. Conversion of biomass to release glucose

(Courtesy: Mosier *et al*., 2005)

2.3.1. Physical Pretreatment

Physical pretreatment refers to the act of reducing the physical size of biomass to increase surface area for enzymatic action (Zhu *et al.,* 2009). It involves two steps; the first step involves mechanical size reduction that processes the wood into chips and the second step involves reducing the size further to particles, fibers and fiber bundles. This can also be achieved by thermo-chemical means such as steam explosion, organosolv process and chemical pulping process (Maha *et al*., 2011). It can also be achieved through grinding or milling which uses more energy than the first step and involves mechanical reduction of the coarse size. Other milling technologies such as hammer milling can also be used to break the wood chips into particles or fibers such as production of wood flour. Disk milling technology is one of the best fiberization processes for woody

feedstock which involves commercial production at the scale of 1000 tons per day (Zhu *et al.*, 2009). This process had been practiced in the pulp and paper industry for several decades and makes it efficient for enzymatic saccharification (Zhu *et al.*, 2009).

Based on the fiberization process and the extent of milling, mechanical energy consumption for disk-milling wood chips to fibers and/or fiber bundles is between 150 and 800Wh/kg wood (Zhu *et al.*, 2009). The energy consumption for producing ethanol from wood using thermal energy is about 10-40% depending on the size of the wood. The use of thermal energy for the production of ethanol from wood is just sufficient for reducing the size of the wood to decrease the consumption of energy, preferably a factor of 5-10 to about 0.1-0.4MJ/kg, is required to achieve a net output for wood ethanol production (Kim and Dale, 2004). This is a significant challenge to cellulosic ethanol production from woody biomass. Generally, there are three factors which affect the energy consumption for reducing the size of wood through disk milling; that is, the extent of size reduction, the fiberization process, and chemical or biological pretreatment of the wood (Yu *et al.*, 2009).

2.3.2. Dilute Acid Pretreatment.

It is the most studied pretreatment process for agriculture biomass (Wyman *et al.*, 2005). The use of dilute acid for pretreatment of woody biomass can achieve some success. Dilute acid pretreatment makes use of acid solutions with concentrations of less than 2% (w/v) at temperatures above 100 °C to break down hemicellulose, disrupt lignin structure and increase the surface area (Mosier *et al*., 2005). Dilute acid pretreatment is normally carried out either continuously in a flow-through reactor (Grethlein and Converse 1991; Zhu *et al*., 2004) or in a

batch reactor (Esteghlalian *et al.,* 1997; Chung *et al.,* 2005; Lloyd and Wyman 2005). Dilute acid pretreatment has been used for the pretreatment of a variety of feedstocks (Chung *et al*., 2005; Esteghlalian *et al*., 1997). It was reported that dilute acid pretreatment helps improve cellulose digestibility of poplar and corn fiber (80%) and switch grass and corn stover (90%) (Grohmann and Bothast 1997; Chung *et al*., 2005).

It can also lead to cellulose conversion with certain types of hardwood species. A study conducted at the US National Renewable Energy Laboratory with dilute acid of a poplar wood resulted in a total sugar recovery of 82.8% with a cellulase loading of 15 FPU/g cellulose (Wyman *et al.,* 2009). Mild steam explosion process was further used to reduce the size after dilute acid pretreatment. Wang *et al.,* (2009) also reported 80% cellulose conversion from Eucalyptus substrate. The substrate which was obtained by direct dilute acid pretreatment of wood chips was done at 180°C with a sulfuric acid charge of 1.84% on wood which was followed by a disk-milling process to reduce the size. On the other hand, dilute acid pretreatment was not able to achieve a better enzymatic cellulose conversion for softwood. Cellulose conversion was only about 40% when spruce wood chips were pretreated at a temperature of

180°C with an acid charge of 1.84% on wood which was followed by disk milling (Zhu *et al*., 2009). The cellulose saccharification improved by using a two-stage acid hydrolysis pretreatment with steam. The recovery of glucose was about 80% which was achieved at the expense of the increase in thermal energy cost (Monaveri *et al.,* 2009a). One of the advantages of dilute acid pretreatment is that about 90% of Xylan can be recovered as xylose during pretreatment and therefore do not require the use of xylanase enzymes (Chen *et al.,* 2012).

Acid-catalyzed steam explosion is the most common steam pretreatment method which is used for woody biomass (Galbe and Zacchi, 2002; Wingren *et al*., 2003; Cullis *et al*., 2004; Monavari *et*

al., 2009b). The acid catalyst is used to impregnate into the wood chips in the gas phase using sulphuric acid before pretreatment is done. Actually steam pretreatment is also another form of dilute acid pretreatment which is done in the vapour phase rather than in the aqueous phase. There has been good recovery of sugar from hardwood with total glucose recovery from 65% to 80% from poplar species (Esteghlalian *et al*., 1997; Lloyd and Wyman 2005). However, xylose from pretreatment when combined with high temperatures and low pH in dilute acid pretreatment can lead to the formation of furfural, which is a strong inhibitor in fermentation. Generally, dilute acid pretreatment is not effective for pretreatment of softwoods and must be therefore combined with steam explosion to produce higher yield.

2.3.3. Alkali Pretreatment

Alkaline pretreatment normally works by the removal of lignin, changing the lignin structure, and also increasing the surface area for increased biomass reactivity, leading to efficient enzymatic hydrolysis (Sun and Cheng, 2002; Wang and Cheng, 2011; Xu *et al*., 2011). It also removes acetyl and some uronic acid substitutions on the hemicellulose that usually reduce accessibility of the enzyme to the hemicellulose and cellulose surface (Chang and Holtzapple, 2000). The essence of using alkalis such as NaOH or lime as catalysts for pretreatment of biomass has been examined in a number of studies which in some cases showed great effectiveness of the pretreatment on various lignocellulosic feedstocks under various conditions

(Bjerre *et al.,* 1996; Wang *et al*., 2010; Wang and Cheng, 2011). It involves the use of lime (Ca $(OH)_2$) to increase the pH, which allows the lignin to be dissolved and eventually removed. This

also allows the preservation of sugars due to the high pH and mild temperatures (Mosier *et al.*, 2005). Lime is commonly used, but other bases such as sodium hydroxide and potassium hydroxide have also be used (Chang *et al.*, 1997; Mosier *et al*., 2005). Lime is mostly preferred over the other bases because it is relatively cheaper compared to the other bases, it is easier to recover and also because it is safe to use (Chang *et al*., 1997). Alkaline pretreatment normally uses lower temperatures compared to other pretreatments; however, the reaction takes a longer time. In one study, the pretreatment time was four (4) weeks with temperatures at 55 ^{0}C and below (Kim and Holtzapple 2005), but some studies used temperatures above 100°C and pretreatment times from 2 to 6 hours (Chang *et al*., 1997; Sierra *et al*., 2009). It is reported that alkaline hydrolysis mechanism is usually based on the saponification of intermolecular ester bonds cross-linking xylan, hemicelluloses and other components such as lignin (Sun and Cheng,

2002). This usually involves the use of calcium or sodium hydroxide. Using these components leads to the formation of salts that can be incorporated in the biomass and hence need to be removed (Alonso and Palomo, 2001). The conditions used are relatively mild but the reaction times can be long. These conditions prevent the condensation of lignin, which results in high lignin solubility, especially for biomass which has low lignin content such as grasses. Because it involves the use of mild conditions, the breakdown of sugars to furfural, and organic acids is normally reduced. The addition of air or oxygen to the reaction mixture generally enhances delignification (Chang and Holtzapple, 2000).

Using aqueous ammonia at high temperatures can also lead to a reduction of the lignin content and also further help in removing some of the hemicellulose while decrystallising the cellulose. Some of the techniques used in ammonia pretreatment include the ammonia fibre explosionmethod (AFEX), ammonia recycles percolation (ARP) and soaking in aqueous ammonia (SAA)

(Holtzapple *et al.,* 1992). The ammonia pretreatment technique normally involves the pretreatment of biomass with aqueous ammonia in a flow-through column reactor. The liquid flows at high temperature through the reactor column which is packed with the biomass. The reactor system is slightly pressurized to prevent flash evaporation (Kim *et al*., 2003). The solid reaction which is rich in cellulose and hemicellulose can then be separated from the liquid. The liquid portion is then sent into a steam-heated evaporator to recover the ammonia and lignin and also to separate the sugar. The ammonia is then recycled in the reactor inlet and the separated fraction is sent into a crystallizer. Washing is done in order to extract the sugar that is retained in the solid matrix. Soaking in aqueous ammonia (SAA) at low temperature effectively removes the lignin in the raw material by reducing its interaction with hemicellulose. This leads to an increase in the surface area and pore size, thus the hemicellulose and cellulose which are retained can be hydrolyzed to fermentable sugars by using most commercial xylanase and cellulase enzymes.

Kim *et al.,* (2008) evaluated SAA as a pretreatment method of biomass. In their study, destarched barley hull was treated with 15-30% aqueous ammonia, at 30°C-75°C for 12 h to 77 days with no agitation and a solid-to-liquid ratio of 1:12. When soaking was done, the solids were recovered through filtration. It was then washed and analyzed; 66% of lignin solubilization was obtained and observed saccharification yields of 83% for glucan and 63% for xylan when biomass was treated with 15% aqueous ammonia at 75°C for 48 hours. Ammonia is expensive and it is also difficult to recover, making this pretreatment option more expensive (Holtzapple *et al.*, 1991).

2.3.4. Biological Pretreatment

Researchers have also used fungi that are able to naturally degrade lignin for pretreatment of biomass before performing enzymatic hydrolysis (Keller *et al*., 2003). The Basidiomycetes also

known as white-rot fungi, produce enzymes that are able to degrade lignin giving the fungi access to the carbohydrates in the cell wall (Taniguchi *et al*., 2005; Bak *et al*., 2010)**.** The process normally involves allowing the fungus to feed on the biomass for some weeks, and then subjecting it directly to a mild form of pretreatment (Keller *et al*., 2003). The use of biological pretreatment has several advantages over other methods. There is low energy cost, no chemical is usually involved and the capital cost and operational cost are usually low. However, the slow rate of biological hydrolysis during the course of pretreatment is the main drawback, compared to other pretreatment technologies (Sun and Cheng, 2002). Also, it requires long periods of time for the pretreatment and complete degradation and consumption of carbohydrates (Balan *et al*., 2009). Fungi such as *Cyathus stercolerus* and *Pleurotus ostreatus* have been examined on different types of lignocellulosic biomass, but their low efficiency and slow growth rate make them unsuitable pretreatment process for commercial purposes (Shi *et al.,* 2009). Due to the lignin-degrading enzyme system, an array of peroxidases and oxidases is generated to carry out spontaneous cleavage reactions (Shi *et al*., 2009). *Phanerochaete chrysosporium* has nearly no effect on cellulose of the wood, which makes it very promising for lignocellulosic biomass pretreatment. Other white-rot fungi such as *Ceriporia lacerata, Ceriporiopsis subvermispora, Pycnoporus cinnarbarinus,* have also been used for the pretreatment of different biomass indicating relative high efficiency (Kumar *et al*., 2009). The combination of a mild physical (ultrasonic) or chemical step (H_2O_2) with subsequent biological treatment using *Pleurotus ostreatus* led to a significant increase in the degradation of lignin and total yield of the sugar (Taniguchi *et al*., 2005). Meanwhile, it is possible to shorten the pretreatment time from 60 days with one step biological pretreatment to 18 days with combined pretreatment (Yu *et al*., 2008).

2.4. ENZYMATIC HYDROLYSIS

The most important commercial enzymes which are used for the conversion of biomass to sugar are cellulase, pectinase, hemicellulase and ligninase. Most of these commercial enzymes are not pure, for example, preparations marketed as cellulase generally contain considerable hemicellulase activity (Hespell *et al.,* 1996; Den *et al.,* 2007b). Cellulases are by far the most important of these enzymes because of their ability to convert large amount of cellulose which is present in the biomass to glucose. The complete degradation of the cellulose requires the acitvities of three separate glycosyl hydrolases; the endoglucanase which hydrolyse the internal gylcosidic bonds, the exoglucanases that bind at the ends of the strands and the cellobiohydrolyases which remove molecules of cellobiose from the ends of cellulose chains

(Withers, 2001).

The commercial production of cellulases for the conversion of biomass for example, the conversion of biomass to appropriate products is derived from the fungus *Trichoderma reesei* (Nieves *et al.,* 1998). It produces five endoglucanase enzymes, two cellobiohydrolyases, one non-reducing enzyme cellobiohydrolyases and two 1-3 gylcosidic linkage enzymes (Vinzant *et al.,* 2001). Cellobiohydrolase enzymes are involved in the hydrolysis of the crystalline cellulose and far more than 60% of the total protein secreted when it is induced for the production of cellulase. The main problem with cellulase is that it is very sensitive to end-product inhibition (Berlin *et al.,* 2006).

Hemicellulase enzymes usually play two major roles in bioconversion. Most pretreatments apart from those using mineral acid catalysts only partially degrade xylan and hemicellulose which is most likely needed to complete saccharification (Guo *et al.,* 2011). Currently there is no commercial hemicellulase which is marketed for the complete xylan hydrolysis and therefore hemicellulases are commercialized for applications that require only partial xylan hydrolysis. Secondly, removal of xylan is often correlated to the digestion of cellulose. Therefore it can be

possible to reduce the severity of the pretreatment by additional xylanases. This has been determined to be the case for a variety of steam-treated biomasses (Berlin *et al.,* 2005; Berlin *et al.,* 2006; Berlin *et al.,* 2007). Developing an effective hemicellulase for the conversion of biomass can be challenging because its mode of preparations can be as complex as the hemicelluloses since each of the unique chemical bonds requires its own enzyme for hydrolysis (Saha, 2003).

The pectinases are used to break down the pectin, which consists of a linear chain of α-(1-4)linked D-galacturonic acid forming the pectin backbone. Pectin as xylan has multiple side groups. Pectinases are effective for degrading corn pericarp xylan, indicating that pectin preparations may have application beyond hydrolyzing pectin (Wu and Lu-Kwang, 1998; Den *et al.,* 2007a).

Examples of ligninase enzymes include lignin peroxidase, manganese peroxidase and laccase. These enzymes have not been used in any other pretreatment except indirectly in biological pretreatments. Laccases help to increase slightly the effectiveness of cellulase and help in the detoxification of the hydrolysates by condensing the lignin related aromatics (Claus, 2004).

2.5. FERMENTATION

The most suitable microorganisms which have been used for commercial ethanol production are the yeast *Saccharomyces cerevisiae* and the bacterium *Zymomonas mobilis*. Both microorganisms have exceptional ethanol tolerance and high productivities. These organisms, however, can neither ferment xylose which represents more than 30% of the carbohydrates found in herbaceous plants and hardwoods (Dien *et al.,* 2003). There are only few types of yeasts that are capable of fermenting xylose to ethanol to produce higher yields (Slininger *et al.,* 2006). This has necessitated the need to apply recombinant techniques to develop industrially suitable strains with significant

yields (Jeffries and Jin 2004; Jeffries *et al*., 2007). Most molecular microbiologists have turned to the use of microorganisms to engineer strains that can convert biomass sugar to ethanol. They have constructed Z. *mobilis* (Zhang *et al*., 1995; Deanda *et al*., 1996; Mohagheghi *et al*., 2002) and *S. cerevisiae* strains to metabolise xylose (Kuyper *et al*., 2005). Xylose metabolism has been incorporated into S. *cerevisiae* by exploiting the pathway from native xylose-fermenting yeasts (Ho *et al.,* 1998; Wahlbom *et al.,* 2003; Fujita *et al.,* 2004;

Sedlak and Ho, 2004; Den Haan *et al.,* 2007a; Den Haan *et al.,* 2007b) or more recently by introducing a functional xylose isomerase (Kuyper *et al.,* 2004; kuyper *et al.,* 2005). There has been intensive research on *Saccharomyces* which is being pursued in various laboratories in the world (Chu *et al*., 2007). The other approach is to use bacteria that can normally ferment the xylose and other sugars and produce strains that can selectively produce the ethanol. This is achieved by making use of the two terminal enzymes which are involved in the production of ethanol using *Z. mobilis* and removing the genes which are responsible for the production of other by-products. Examples of microorganisms which have been successfully engineered include the Gram-negative bacterium *E. coli* (Ingram *et al.,* 1987; Ohta *et al.,* 1991a; Hespell *et al.,* 1996; Yomano *et al.,* 1998).

CHAPTER THREE

3.0. MATERIALS AND METHODS

3.1.1. BIOMASS COLLECTION AND PREPARATION

Two different sawmill dust from two different plant species namely *Swietina macrophylla* (Mahogany) and *Ceiba pentandra* (kapok) were collected from K.K sawmill factory and the Catholic Technical Institute respectively within the Sunyani Municipality of the Brong Ahafo Region. The samples were dried in the sun for three days and subsequently kept in a solar drier for twenty four hours to reduce the moisture content as much as possible. It was then sieved to obtain the finest particles with a sieve having a pore size of 1-2 mm. The resultants were then put in black polythene bags and kept in a cool dry place.

Plate 1: Samples under solar dryer

3.1.2. COMPOSITION OF BIOMASS

Each of the two plant species were analyzed for their nutrient composition. The carbon, nitrogen and phosphorus components of each species were determined. The moisture and the ash content were also determined (Vassilev *et al.*, 2010).

3.2. PREPARATION OF SOLUTIONS

3.2.1. Preparation of Dilute Acid Solutions

A volume of 500 ml of dilute hydrochloric acid (HCl) of 0.25 M was prepared by measuring 21.5 ml of the stock solution whose concentration was 11.64 M. The volume measured was poured into a 500 ml volumetric flask that was filled partially with distilled water. Distilled water was used to rinse the measuring cylinder into the volumetric flask. It was then topped with distilled water while swirling until it reached the 500 ml mark. The same procedure was carefully followed to prepare the 500 ml of the dilute acid for 0.5 M and 1 M respectively from the same stock solution. The process was used to prepare 500 ml of 0.25 M, 0.5 M and 1 M respectively of dilute sulphuric acid (H_2S0_4) by measuring their corresponding volumes from the stock solution whose concentration was 18.35 M.

3.2.2. Preparation of Dilute Base Solutions

A volume of 500 ml of dilute sodium hydroxide (NaOH) of 0.25 M was prepared by weighing 10 g of sodium hydroxide in a beaker. It was then dissolved with distilled water by stirring continuously with a stirring rod. The dilute solution was poured into a 500 ml volumetric flask which was partially filled with distilled water. The beaker was rinsed with distilled water into the volumetric flask and topped up with distilled water while swirling until it reached the 500 ml mark. The same procedure was used to prepare 0.5 M and 1 M by weighing their corresponding masses.

The dilute ammonia solution on the other hand was prepared the same way the dilute acids were prepared. A volume of 500 ml of the dilute ammonia (NH_3) of 0.25, 0.5 and 1 M each was prepared by measuring the appropriate volumes of the stock solution whose concentration was 18.29 M.

3.3. PRETREATMENT

A mass of 0.1 g each of the two samples namely *Swietina macrophylla* (Mahogany) and kapok (*Ceiba pentandra*) were weighed on an electronic balance and put in well labelled test tubes. A volume of 2.5 ml each of the different concentrations of the dilute acids and bases were measured and poured into the labelled test tubes. The test tubes were put in a water bath which was set according to respective temperatures and incubation periods. The concentrations used were 0.25 M, 0. 5 M, and 1 M. Each of the samples at the different concentrations was incubated at temperatures of 75°C, 90°C, and 100°C at resident times of 30, 60 and 90 minutes respectively. For the samples incubated at 121°C the manual autoclave was used and the resident times used were 10, 15, and 20 minutes for the bases. The resident times used for the acids were 30, 60 and 90 minutes.

After the resident times, the labelled test tubes were removed and cooled under running tap water and the hydrolyzates were measured for their glucose levels using a glucose meter. All samples were triplicated and their averages were used (Kumar *et al*., 2009).

Plate 2: Samples in water bath

3.4. ENZYMATIC HYDROLYSIS

Cellulase enzyme obtained from Sukahan (Weifang) Bio-technology Company limited (China), was used for the enzymatic hydrolysis. The pH of the enzyme was 4.85. A mass of 0.1 g each of the two different plant species were weighed using electronic balance and 2.5 ml of the cellulase enzyme was measured and poured into labelled test tubes. The labelled test tubes were incubated at 55°C and 100°C for 30, 60 and 90 minutes respectively. For sample incubated at 121°C, the manual autoclave was used and the resident times used were 10, 15, and 20 minutes respectively. The labelled samples were removed after their respective resident times, cooled under running tap water and filtered. The hydrolyzates were measured for their respective glucose levels using a glucose meter. All samples were triplicated and their averages were used (Yang *et al*., 2011).

3.5.1. Moisture Content Determination

This method involves drying a representative sample in an oven at 95°C-110°C for 24 hours. Crucibles were first weighed and approximately 5.0 g of the samples were weighed and allowed

to dry overnight in hot air oven. The crucibles plus the samples were cooled in a dessicator and re-weighed. The weight loss was calculated as moisture weight multiplied by 100 over the sample weight. The formula used is represented as

% Moisture = Moisture weight X 100
Sample weight (fresh weight)

Moisture content

Calculations

(A + B) - A = B

(A + B) - (A + C) = B – C = D
% Moisture = D/B × 100

Where A = crucible weight, B = sample weight, C = dry sample weight, D = moisture weight

3.5.2. Ash Content Determination

Ash refers to the inorganic residue obtained by burning a sample at 600°C. The ash crucible was removed from the oven and placed in desiccators to cool and weighed. It was then put in a furnace for 4 hours at 550°C. It was then allowed to cool below 200°C for 20 minutes. The crucible was put in a desiccator and covered with a stopper top, cooled and then weighed. The percentage weight was calculated as weight of ash multiplied by 100 over the sample weight. The formula used is represented as

% **Ash = Ash weight x 100**
Sample weight

Calculations

(A + B) – A = B

(A + C) – A = C

% Ash = C/B × 100 where A = crucible weight, B = sample weight, C = ash weight.

3.5.3. Determination of carbon (Walkley-Black wet oxidation method)

A 0.1667 M potassium dichromate was prepared by dissolving 49.04 g of reagent grade potassium dichromate dried at 105°C in distilled water. The solution was made up to 1000 ml. A 0.5 N ferrous sulfate was also prepared by dissolving 278 g of ferrous sulphate in 500 ml distilled water. A volume of 40 ml of conc. H_2SO_4 was added and the volume made up to 2000 ml. Diphenylamine was also prepared by dissolving 0.5 g of diphenylamine in a mixture of 100 ml conc. H_2SO_4 and 20 ml distilled water. A mass of 0.05 g of sample (shell/nib) was weighed into an Erlenmeyer flask and 10 ml of 1.0 N $K_2Cr_2O_7$ solution was added followed by 20 ml of conc. H_2SO_4. The mixture was swirled and allowed to stand for 30 minutes for complete digestion. A volume of 200 ml of distilled water, which was followed by 10 ml orthophosphoric acid and 2 ml diphenylamine indicator were added. The solution was then titrated against 0.5 N ferrous sulphate solution until the colour changed to dark blue and then to a green end point. The titre value was then recorded and the titre value for the blank solution was determined.

The % C was calculated as:

$$\% C = \frac{M \times (Vbl - Vs) \times 0.003 \times 1.33 \times 100}{g}$$

Where M = Normality of ferrous sulfate = 0.5 N

Vbl = titre value of blank solution

Vs = titre value of sample solution

g = mass of sample taken

0.003 = milli –equivalent weight of C in grams (12/4000)

1.33 = correction factor used to convert the wet combustion C value to true C value since the wet combustion method is about 75% efficient in estimating C value (100/75)

The organic matter content was determined by using the formula:

% Organic matter content = % organic carbon x 1.724

(1.724 is the Van Bemellean factor)

3.5.4. Determination of nitrogen (Kjeldahl Method)

A mass of 1.0 g of the sample was weighed and transferred into a 500 ml digestion flask and 30 ml of H_2SO_4 and a spatula of Kjeldahl catalyst added. The digestion flask with the mixture were heated in the DK20 heating digester block starting at a temperature of 80°C and then the temperature raised to 350°C. The content of the digestion flask was heated until the volume was reduced to 4 ml. The contents of the digestion flasks were cooled and the volume made up to 100 ml in volumetric flasks. The volumetric flasks were labelled accordingly. Ten (10) milliliters of sample digest was transferred by means of pipette into a Kjeldahl distillation apparatus after the addition of 20 ml of 40 % NaOH and the distillate collected. The presence of nitrogen gives a light blue colour. A volume of 200 ml of the distillate was titrated with 0.1 N HCl till the colour changed from light blue to gray and suddenly flashed to pink. A blank was carried out with the solution sample.

The weight of N was calculated as;

14 g of N contained in one milli-equivalent weight of NH_3

Therefore, weight of N in the sample = $\frac{14\ x\ (A - B)\ x\ concentration\ of\ acid}{1000}$

Where:

A = volume of standard HCl used in the sample titration

B = volume of standard HCl used in the blank titration

The percentage of Nitrogen in the sample is calculated as;

$$\% N = \frac{14 \, x \, (A - B) \, x \, concentration \, of \, acid}{1000} \, x \, 100$$

3.5.5. Determination of Phosphorus (P)

A vanadomolybdate reagent was prepared by dissolving 22.5 g of ammonium molybdate in 400 ml of distilled water and 1.25 g of ammonium vanadate which was also dissolved in 300 ml of boiling distilled water. The vandate solution was then added to the molybdate solution and allowed to cool to room temperature. A volume of 250 ml of analytical grade HNO_3 was then added to the solution mixture and diluted to 1 litre with deionized water. The standard phosphate solution was also prepared by dissolving 0.2195 g of analytical grade KH_2PO_4 in 1000 ml distilled water. This solution contained 50 µg P/ml. A standard curve was prepared by pipetting

1, 2, 3, 4, 5 and 10 ml of standard solution (50 µg P/ml) in 50 ml volumetric flasks. A volume of 10 ml of vanadomolybdate reagent was added to each flask and the volume made up to 50 ml. This gave a P content of the flasks as 1, 2, 3, 4, 5, and 10 µg P/ml. These concentrations were measured on the Jenway 6051 colorimeter to give absorbance measurements at a wavelength of 430 nm. A plot of absorbance against concentration was used to prepare the calibration curve. A volume of 5 ml of the sample solution was put into a 50 ml volumetric flask and 10 ml of vanadomolybdate reagent added and the volume made up to 50 ml. The sample was kept for 30 minutes for colour development. A stable yellow colour that developed was read on the colorimeter at 430 nm. The observed absorbance was used to determine the P content from the standard curve.

The % P was calculated as:

P content (g) in 100 g sample (% P) = $\frac{C \times df \times 100}{1\,000\,000} = \frac{C \times 1000 \times 100}{1\,000\,000} = \frac{C}{10}$

Where C = concentration of P (µg /ml) as read from the standard curve;

df = dilution factor, which is 100 *10 = 1000, as calculated below:

1 g of sample made to 100 ml (100 times);

5 ml of sample made to 50 ml (10 times)

1 000 000 = factor for converting µ

3.6.1 SIMULTANEOUS SACCHARIFICATION AND FERMENTATION (SSF)

A mass of 2.5 g of each sample was put into three conical flasks and 60 ml of 1 M HCl added to each of the samples in the three conical flasks. The conical flasks were covered with a cotton wool and autoclaved at 121°C for 30 minutes. After the pretreatment the glucose levels were measured and the pHs were adjusted to 4.0 by adding sodium hydroxide. The glucose levels were also measured at this stage to determine whether the addition of the sodium hydroxide will have a significant impact on the level of glucose. A mass of 0.8 g of *Saccharomyces cerevisiae* was then measured and added to each of the conical flasks. The vessels were then put into incubators and the temperatures were regulated at 30°C, 40°C and 50°C respectively. It was then left for 5 days for fermentation to occur (Oseghale and Sideso 2011, Vasquez *et al.*, 2014).

3.6.2 Determination of Ethanol Concentration

To determine the ethanol concentration two things were measured

1. The volume of the starting culture
2. The volume of the gas ($C0_2$) produced during fermentation

To obtain the rate of fermentation, the volumes produced at specific times were also required.

The volume of gas (CO_2) produced during fermentation was converted in moles of CO_2

The volume/mole of CO_2 at S.T.P (Standard Temperature and Pressure) is 24 litres/mole (1/24=0.042)

The volume of CO_2 produced from the yeast experiments. (For eg. at 50°C the volume of CO_2 was 3.24 ml (0.00324 litres) of CO_2 in the glucose fermentation)

The volume produced is equivalent to 0.00324 litres X 0.042 mol/litre= 0.0001361 moles of CO_2

If 0.0001361 of CO_2 are produced, then how much ethanol was produced?

$$\mathbf{C_6H_{12}O_6 \longrightarrow 2CH_3CH_2OH + 2CO_2}$$

Glucose yields two ethanol molecules and $2CO_2$ molecules. For every ethanol molecule produced, 1 $C0_2$ gas molecule is released.

So if 0.0001361 moles of CO_2 are produced in our fermentation, then 0.0001361 moles of ethanol are produced.

To find out the concentration of ethanol, the volume of the fermentation medium is required. If it was 18 ml (0.018 litres) then the concentration of ethanol ⟶ 0.0001361/0.018= 0.00756 moles/ litres or 7.56mM ethanol

What is the % ethanol?

In fermentation, ethanol percentage (% w/v) or gm/100ml are usually used rather than concentrations. To calculate % (w/v), the molecular weight of ethanol (46 g/mol) is used. From fermentation: 0.00756 mol/litre X 46 g/ mol = 0.34776 g/litre or 0.034776 g/100 ml which is equal to 3.48×10^{-2} % (w/v)

3.7 RESPONSE SURFACE ANALYSIS, THEORY AND METHOD

The response surface methodology was introduced in the 1950s. It was developed between 19761999 to be used in basic experimental designs for fitting linear surface response models and also for describing methods which can be used for determining the optimum levels. The modern method make use of response surface models which have random effects, generalized linear models and graphical techniques for comparing response surface designs. One of the main aim of this method is to determine the optimum values of variables resulting in a maximum response over a certain region of interest. This makes use of a 'good fitting model' that provides a good representation of the mean response which is to be used to determine the optimum level.

Giovannitti-Jensen and Myers (1989) proposed the graphical technique of variance dispersion graphs (VDGs) which consist of two-dimensional plots that show the maximum, minimum and average of the prediction variance on concentric spheres, chosen within the experimental region against their radii. Zahran *et al.*, (2003) also proposed fraction of designed space (FDS) plots where the prediction variance is plotted against the fraction of the design space that has prediction variance at or below the given value. Khuri *et al.,* (1996) proposed the quantile plots (QP) approach for estimating and comparing several response surface designs using their linear models. Quantile dispersion graphs (QDGs) were proposed by Khuri (1997) for comparing designs for evaluating variance components used in an analysis of variance (ANOVA) situation.

CHAPTER FOUR

4.0. RESULTS

Table 4.1: Mean (±SD) Moisture content and proximate composition of sawdust

Sample	Moisture content (%)	Ash (%)	Carbon (%)	Nitrogen (%)	Phosphorus (%)
Ceiba pentandra	9.25±0.15	1.08±0.21	0.14±0.19	0.07±0.12	61.45±0.11
Mahogany	9.75±0.13	2.63±0.31	0.14±0.15	0.04±0.12	69.03±0.15

Table 4.1 shows the proximate composition of sawdust. From the table, Mahogany had a higher moisture and ash content than *Ceiba pentandra*. The carbon content for both Mahogany and *Ceiba pentandra* were the same. However, *Ceiba pentandra* had a slightly higher nitrogen content than Mahogany while the Phosphorus content for Mahogany was higher than *Ceiba pentandra*

4.1. PRETREATMENT USING DILUTE BASES

The Mahogany(*Swietina macrophylla*) and kapok(*Ceiba pentandra*) were both treated with different concentrations of the dilute bases namely, sodium hydroxide (NaOH) and Ammonia (NH_3) at different resident times and temperatures of 30, 60 and 90 minutes at 75°C, 90°C and 100°C. The concentrations used were 0.25 M, 0.5 M and 1 M.

A one way analysis of variance conducted on the three different concentrations and their glucose levels showed that there was a significant difference ($P<0.05$).

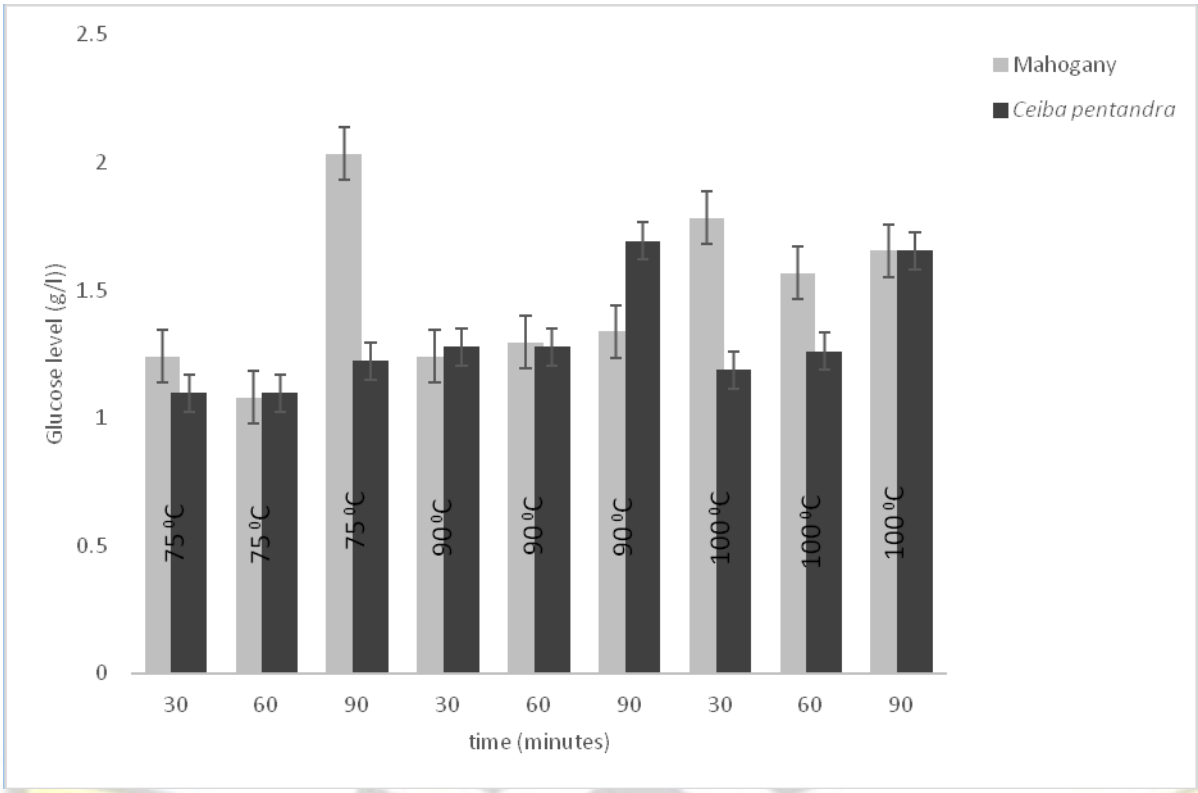

Fig.4.1: Pretreatment using a concentration of 0.25 M NaOH at temperatures of 75°C, 90°C and 100°C

From Figure 4.1, after pretreatment with 0.25 M NaOH at a temperature of 75°C 90°C and 100°C, the glucose concentration on average was higher for Mahogany than the *Ceiba pentandra* even though the *Ceiba pentandra* had a higher significant glucose concentration ($P<0.05$) of 1.1 g/l at a temperature of 75°C for 60 minutes compared to 1.08 g/l for Mahogany. The *Ceiba pentandra* also had a higher significant glucose concentration ($P<0.05$) at 90°C for 90 minutes with 1.69 g/l compared to 1.34 g/l for Mahogany. The concentration increased significantly ($P< 0.05$) with both temperature and time up to a temperature of 90°C for 90 minutes with a concentration of 1.69 g/l and decreased significantly ($P<0.05$) at 100°C for 30 minutes with a glucose concentration of 1.19 g/l before finally increasing significantly ($P<0.05$) from 100°C for

60 minutes to 100°C for 90 minutes from 1.26 g/l to 1.66 g/l for *Ceiba pentandra*.

The Mahogany, on the other hand, started initially with 1.24 g/l at 75°C for 30 minutes and decreased significantly ($P<0.05$) to 1.08 g/l at 75°C for 60 minutes and increased significantly ($P<0.05$) to 2.03 g/l at 75°C for 90 minutes. The glucose concentration declined to 1.24 g/l at 90°C for 30 minutes and increased significantly ($P<0.05$) to 1.78 g/l at 100°C for 30 minutes and again to 1.56 g/l at 100°C before finally increasing significantly ($P<0.05$) to 1.66 g/l at 100°C for 90 minutes.

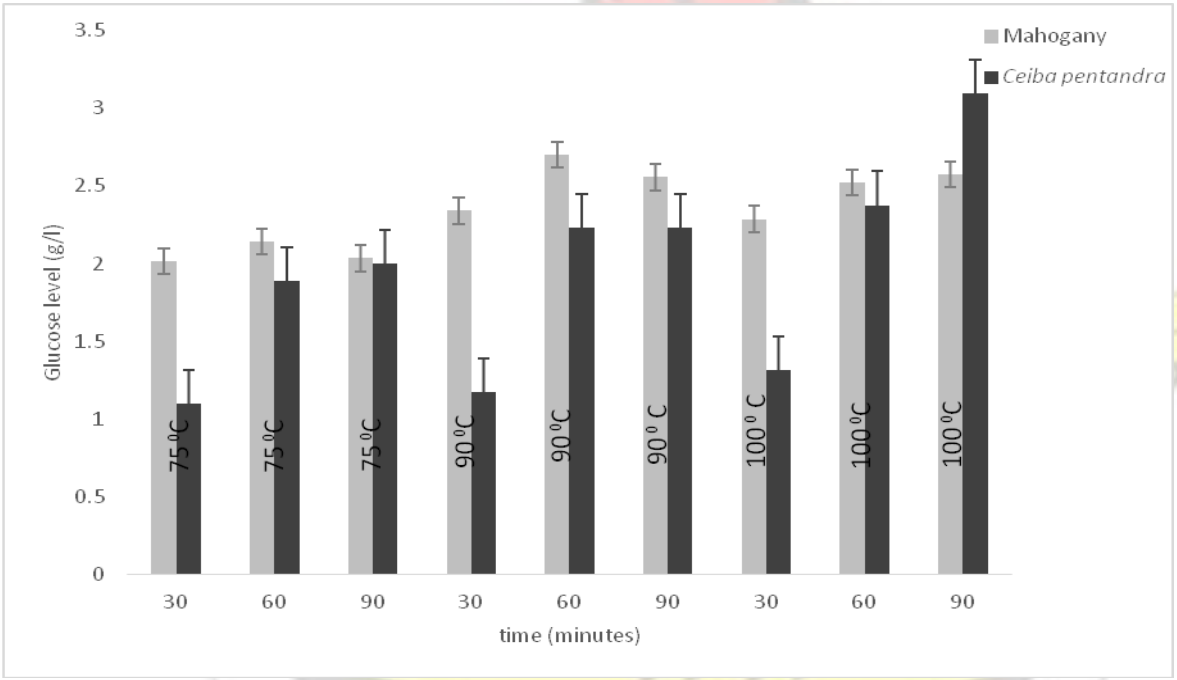

Fig. 4.2: Pretreatment using a concentration of 0.5 M NaOH at a temperature of 75°C, 90°C and 100°C

From Figure 4.2, after pretreatment with 0.5 M NaOH at a temperature of 75°C, 90°C and 100°C, Mahogany had a significant higher glucose concentration($P<0.05$) than *Ceiba pentandra* even though the highest concentration(2.57 g/l.) was recorded by *Ceiba pentandra* at 100°C for 90 minutes.

The *Ceiba pentandra*, on the other hand, increased significantly (P<0.05) with both time and temperature, but the level decreased significantly (P<0.05) to 1.17 g/l at 90°C for 30 minutes and increased significantly (P<0.05) to 1.31 g/l at 100°C for 30 minutes.

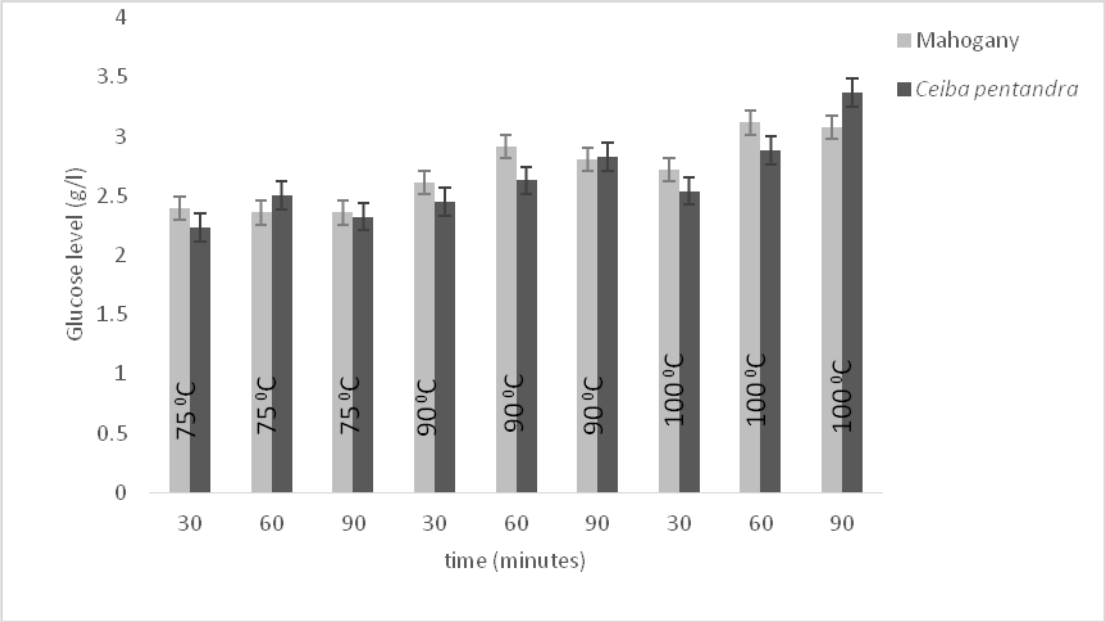

Fig. 4.3: Pretreatment using a concentration of 1 M NaOH at a temperature of 75°C, 90°C and 100°C

From Figure 4.3, after pretreatment with 1 M NaOH at temperatures of 75°C, 90°C and 100°C, Mahogany had higher glucose concentration than *Ceiba pentandra*, but the highest concentration was recorded by *Ceiba pentandra* at 100°C for 90 minutes at 3.37 g/l. The Mahogany increased significantly (P<0.05) with both time and temperature up to 90°C for 60 minutes at 2.92 g/l and declined significantly (P<0.05) to 2.72 g/l at 100°C for 30 minutes and increased significantly (P<0.05) at 100°C for 60 minutes to 3.11 g/l.

The *Ceiba pentandra* also increased significantly (P<0.05) with both temperature and time, but the concentration decreased significantly (P<0.05) to 2.54 g/l at 100°C for 30 minutes and increased significantly (P<0.05) to the highest level of 3.36 g/l at 100°C for 90 minutes.

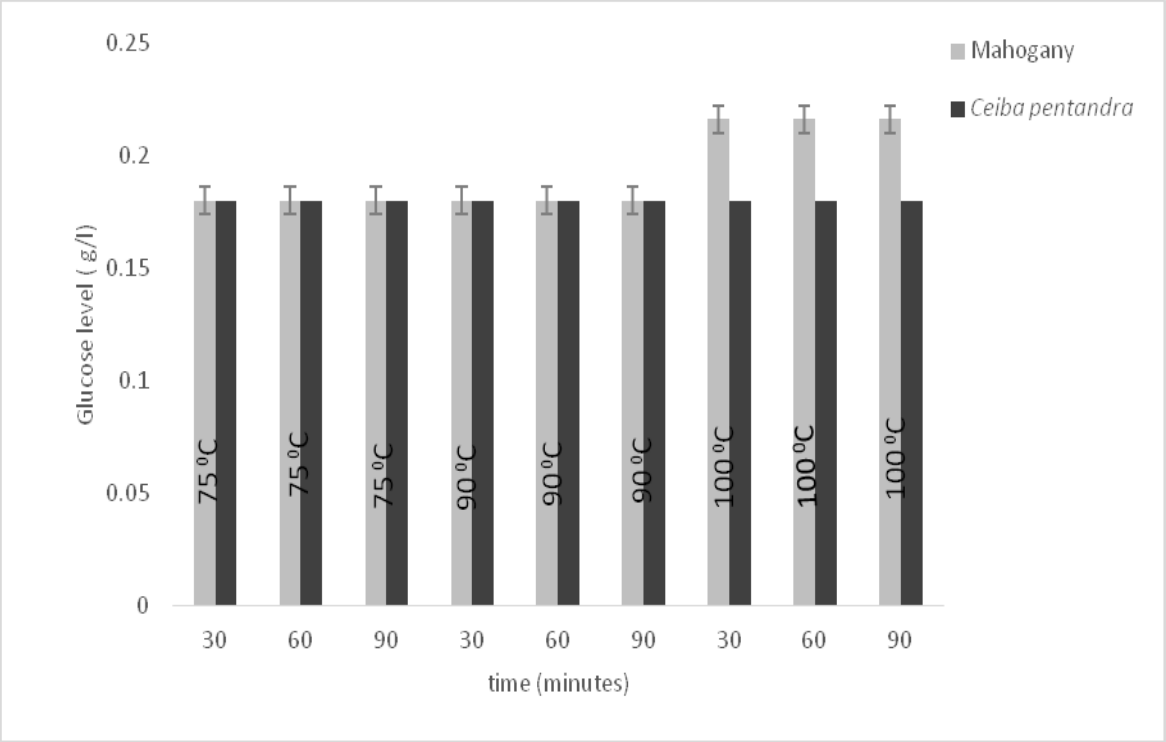

Fig. 4.4: Pretreatment using a concentration of 0.25 M NH_3 at a temperature of 75°C, 90°C and 100°C

From Figure 4.4, after pretreatment with 0.25 M Ammonia at temperatures of 75°C, 90°C and 100°C the glucose concentration remained the same for both Mahogany and *Ceiba pentandra* at 0.18 g/l until at 100°C when the concentration increased significantly (P<0.05) to 0.22 g/l for Mahogany but *Ceiba pentandra* remained constant.

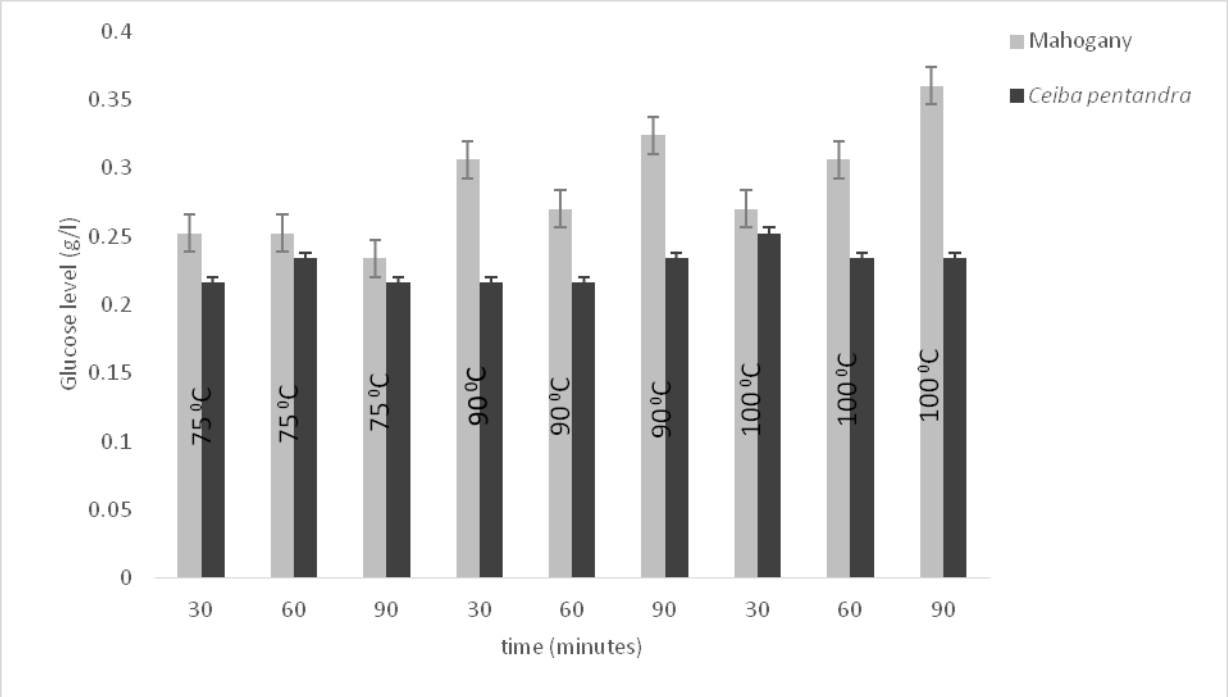

Fig. 4.5: Pretreatment using a concentration of 0.5 M NH_3 at a temperature of 75°C, 90°C and 100°C

From Figure 4.5, after pretreatment with 0.5 M Ammonia at a temperature of 75°C, 90°C and 100°C, Mahogany had a significant higher glucose concentration ($P<0.05$) than *Ceiba pentandra*. The glucose concentration was constant at 75°C for 30 and 60 minutes at 0.25 g/l and declined significantly to 0.23 g/l at 90 minutes for Mahogany. It increased significantly ($P<0.05$) to 0.31 g/l at 90°C for 30 minutes and decreased significantly ($P<0.05$) to 0.27 g/l at 90°C for 60 minutes before increasing significantly ($P<0.05$) again to 0.32 g/l at 90°C for 90 minutes. It decreased significantly ($P<0.05$) to 0.27 g/l at 100°C for 30 minutes and increased significantly ($P<0.05$) to the maximum level of 0.31 g/l at 100°C for 90 minutes.

For the *Ceiba pentandra*, the glucose concentration increased significantly ($P<0.05$) at 75°C for

60 minutes to 0.23 g/l and remained constant at 0.22 g/l from 75°C for 90 minutes to 90°C for 60 minutes and increased significantly ($P<0.05$) to 0.25 g/l at 100°C for 30 minutes and remained constant at 100°C for both 60 minutes and 90 minutes at 0.23 g/l.

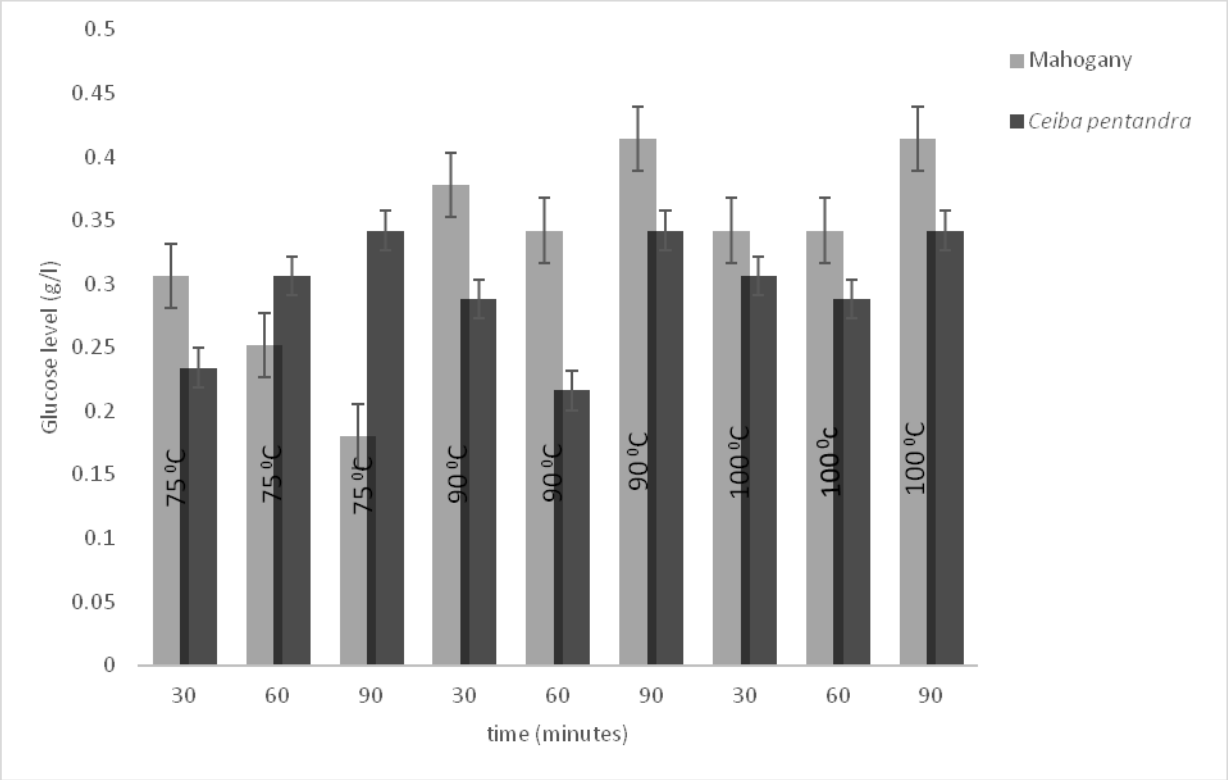

Fig. 4.6: Pretreatment using a concentration of 1 M NH_3 at a temperature of 75°C, 90°C and 100°C

From Figure 4.6, after pretreatment with 1 M Ammonia at a temperature of 75°C, 90°C and 100°C, Mahogany had a significant higher glucose concentration ($P<0.05$) than *Ceiba pentandra* except at 75°C for 60 and 90 minutes when the *Ceiba pentandra* had a significant higher glucose concentration ($P<0.05$) than Mahogany.

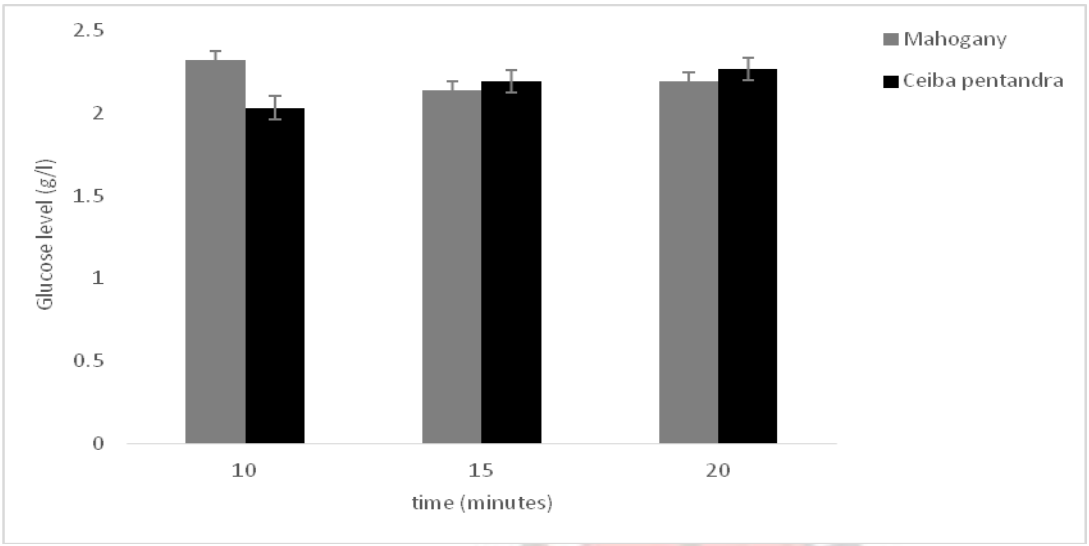

Fig. 4.7 Pretreatment using a concentration of 0.25 M NaOH at a temperature of 121°C

Figure 4.7, shows pretreatment with 0.25 M NaOH at a temperature of 121°C. After pretreatment it was observed that the glucose concentration for *Ceiba pentandra* was significantly ($P<0.05$) higher than Mahogany except at 10 minutes when the Mahogany had a significant higher glucose concentration ($P<0.05$) then *Ceiba pentandra*.

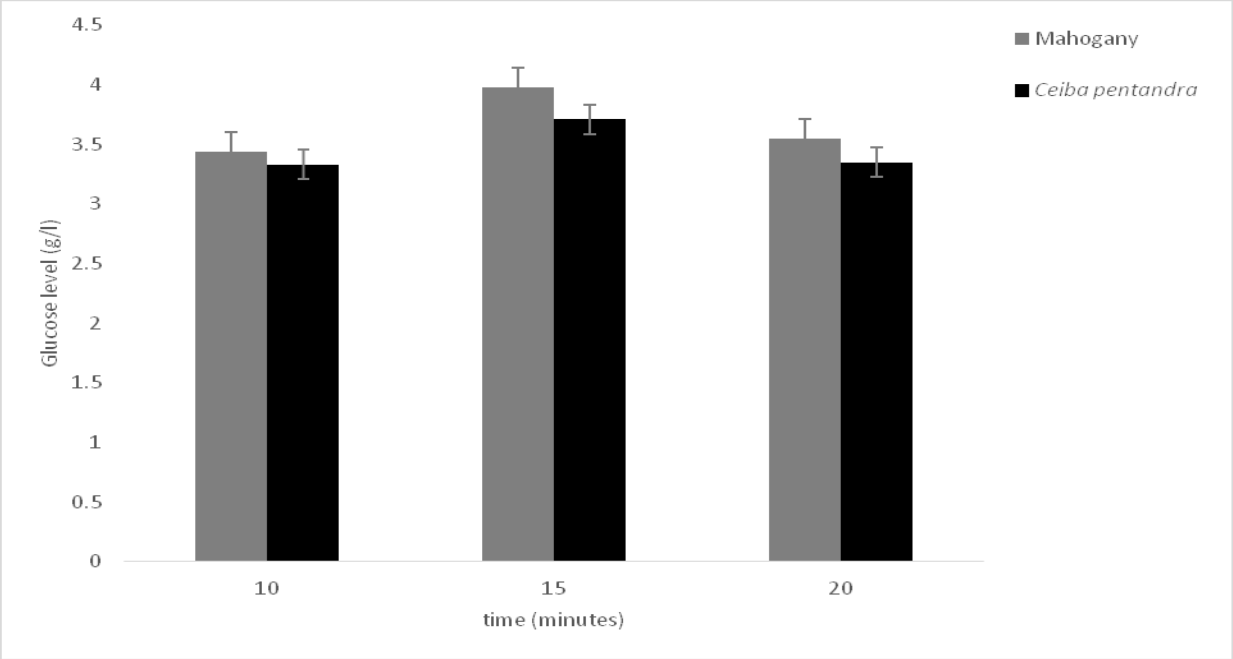

Fig. 4.8: Pretreatment using a concentration of 0.5 M NaOH at 121°C

From Figure 4.8, after pretreatment with 0.5 M NaOH at 121°C, the glucose concentration for both Mahogany and *Ceiba pentandra* increased significantly ($P<0.05$) to 3.98 g/l and 3.71 g/l respectively at 15 minutes and decreased significantly ($P<0.05$) to 3.55 g/l and 3.35 g/l respectively at 20 minutes.

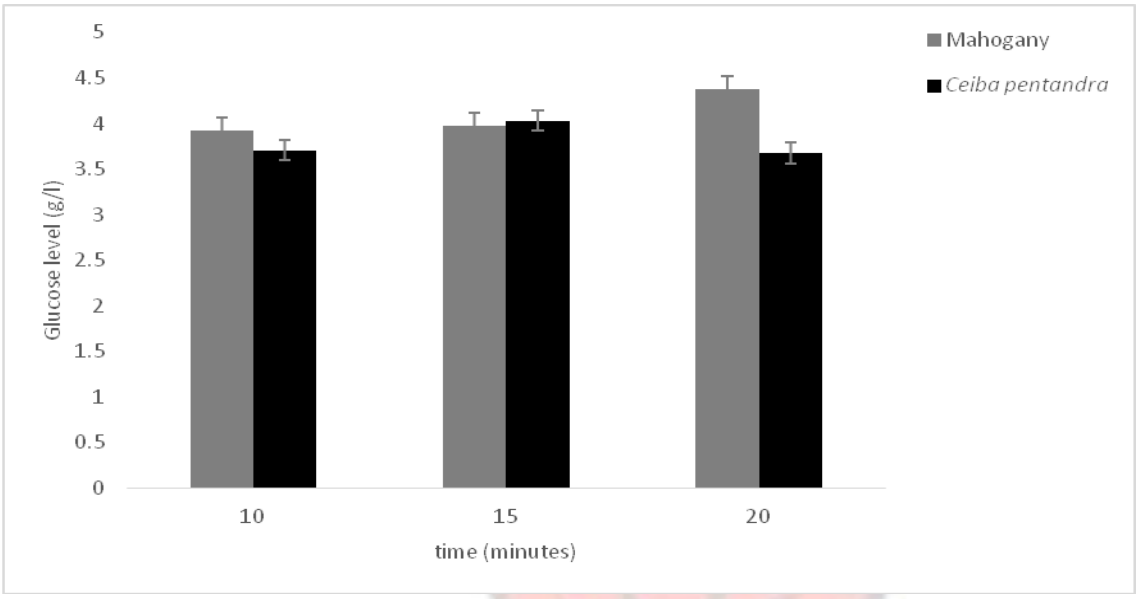

Fig. 4.9: Pretreatment using a concentration of 1 M NaOH at a temperature of 121°C

From Figure 4.9, after pretreatment with 1 M NaOH at a temperature of 121°C the glucose concentration for Mahogany increased significantly with time with the highest level of 4.37 g/l recorded at 20 minutes while for *Ceiba pentandra* it increased significantly ($P<0.05$) to 4.03 g/l at 15 minutes and decreased significantly ($P<0.05$) to 3.67 g/l at 20 minutes.

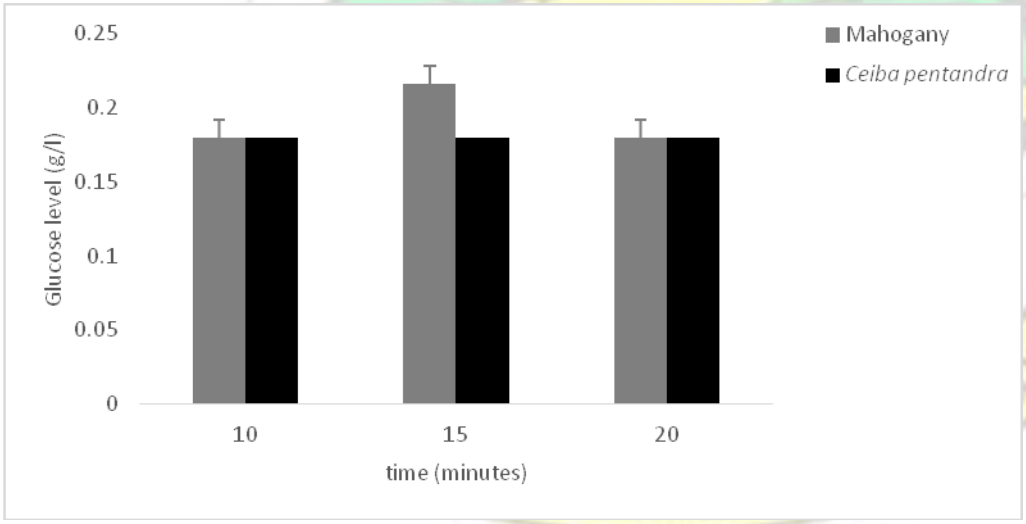

Fig. 4.10: Pretreatment using 0.25 M NH_3 at 121°C

Figure 4.10, shows pretreatment with 0.25 M Ammonia at 121°C. After pretreatment, the glucose concentration for Mahogany increased significantly ($P<0.05$) to the highest level of 0.22 g/l at 15 minutes and decreased significantly ($P<0.05$) to 0.18 g/l at 20 minutes while the concentration for *Ceiba pentandra* remained the same at 0.18 g/l.

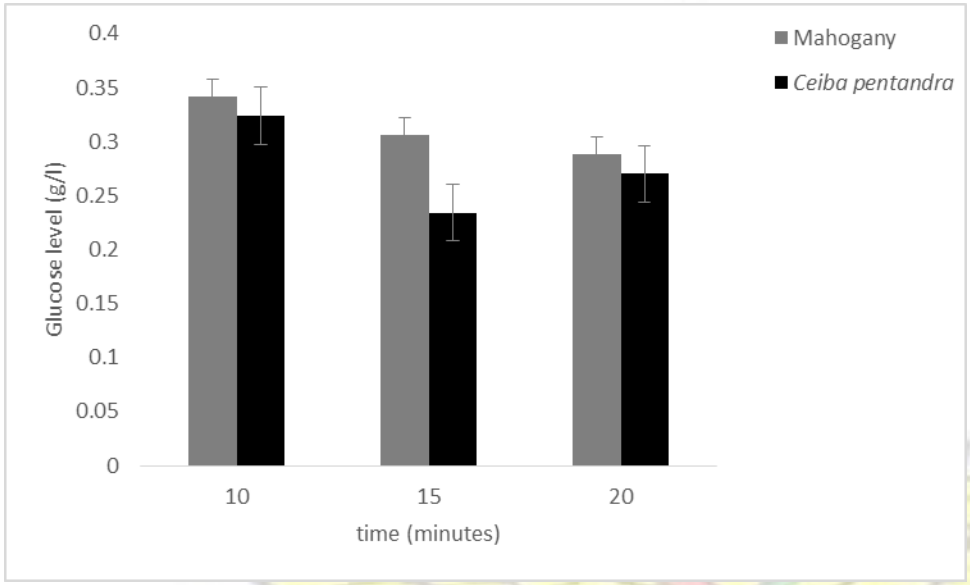

Fig. 4.11: Pretreatment using a concentration of 0.5 M NH_3 at 121°C

From Figure 4.11, after pretreatment with 0.5 M Ammonia at 121°C, the glucose concentration for Mahogany decreased significantly ($P<0.05$) with time while for *Ceiba pentandra* it decreased significantly ($P<0.05$) from 0.32 g/l at 10 minutes to 0.23 g/l at 15 minutes and increased significantly ($P<0.05$) to 0.27 g/l at 20 minutes.

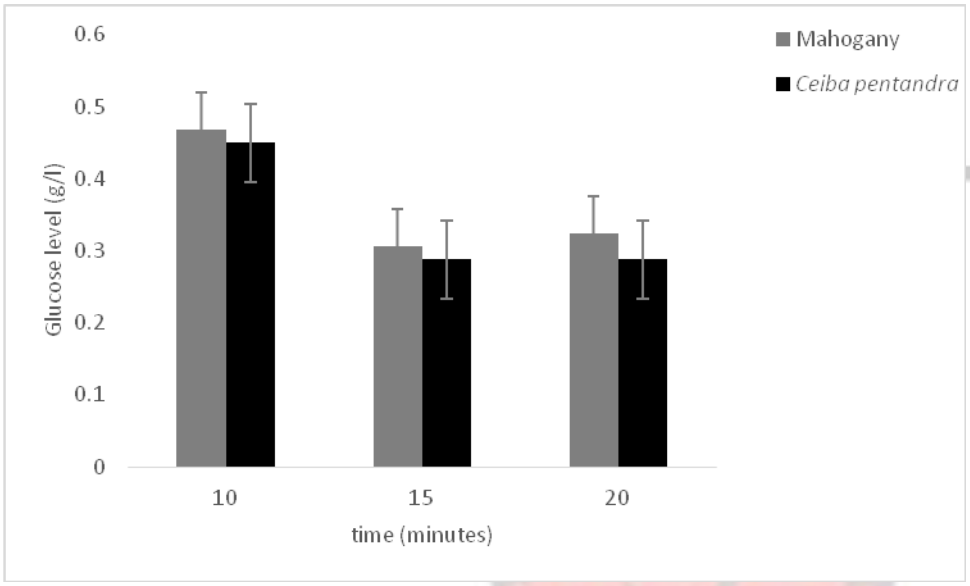

Fig. 4.12: Pretreatment using a concentration of 1 M NH_3 at a temperature of 121°C

From Figure 4.12, after pretreatment with 1 M Ammonia at a temperature of 121°C, the glucose concentration for Mahogany decreased significantly ($P<0.05$) from 0.47 g/l at 10 minutes to 0.31 g/l at 15 minutes and increased significantly ($P<0.05$) to 0.33 g/l at 20 minutes while for *Ceiba pentandra* it decreased significantly ($P<0.05$) from 0.45 g/l at 10 minutes to 0.29 g/l at 15 minutes and remained constant at 20 minutes.

4.2. PRETREATMENT USING DILUTE ACIDS

The Mahogany and *Ceiba pentandra* were both treated with different concentrations of the dilute acids namely, Hydrochloric acid (HCl) and Sulphuric acid (HSO_4) at different reaction times.

The concentrations were 0.25 M, 0.5 M and 1 M respectively and the glucose levels measured. A one way analysis of variance conducted on the three different concentrations and their glucose levels showed that there was no significant difference (P>0.05).

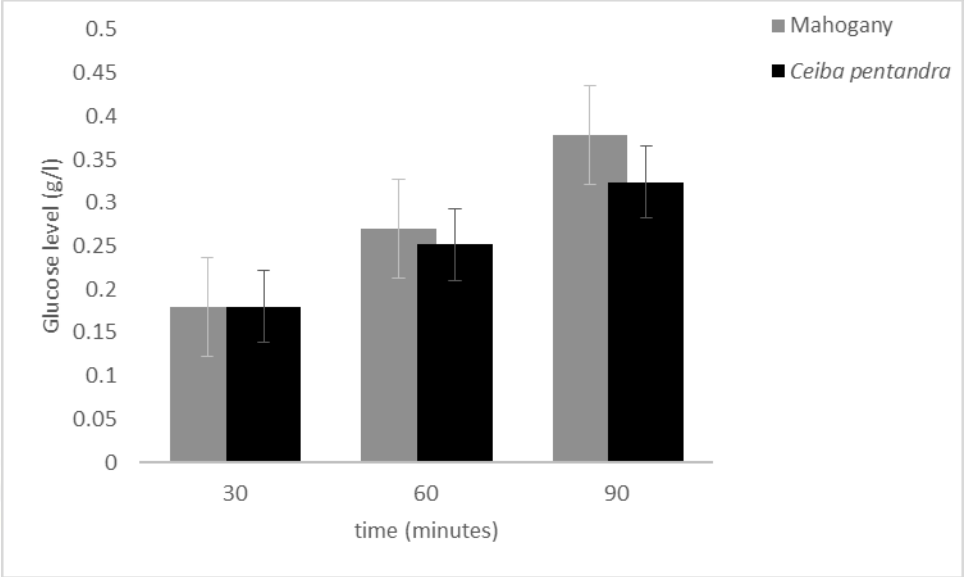

Fig. 4.13: Pretreatment using a concentration of 0.25 M HCl at a temperature of 121°C

From Figure 4.13, after pretreatment with 0.25 M HCl at a temperature of 121°C, the glucose concentration for both Mahogany and *Ceiba pentandra* increased with time but the increase was not significant (P>0.05).

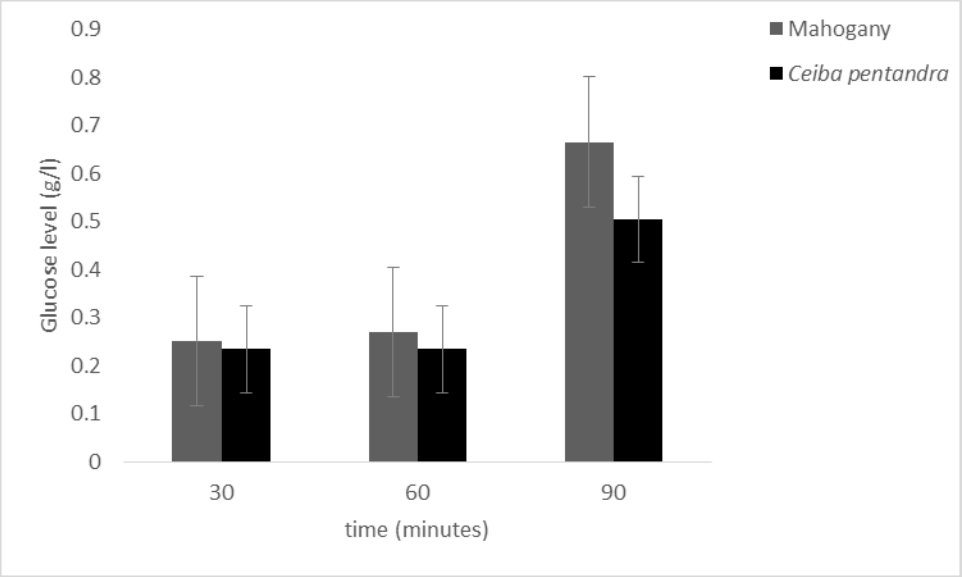

Fig. 4.14: Pretreatment using a concentration of 0.5 M HCl at a temperature of 121°C

From Figure 4.14, after pretreatment with 0.5 M HCl at a temperature of 121°C, the glucose concentration for both Mahogany and *Ceiba pentandra* increased with time. The glucose concentration for Mahogany increased from 0.25 g/l at 30 minutes to 0.27 g/l at 60 minutes and increased again to 0.66 g/l at 90 minutes while the glucose concentration for *Ceiba pentandra* was constant at 0.23 g/l from 30 minutes to 60 minutes and increased to 0.50 g/l at 90 minutes.

There was no significant difference between the glucose levels ($P>0.05$).

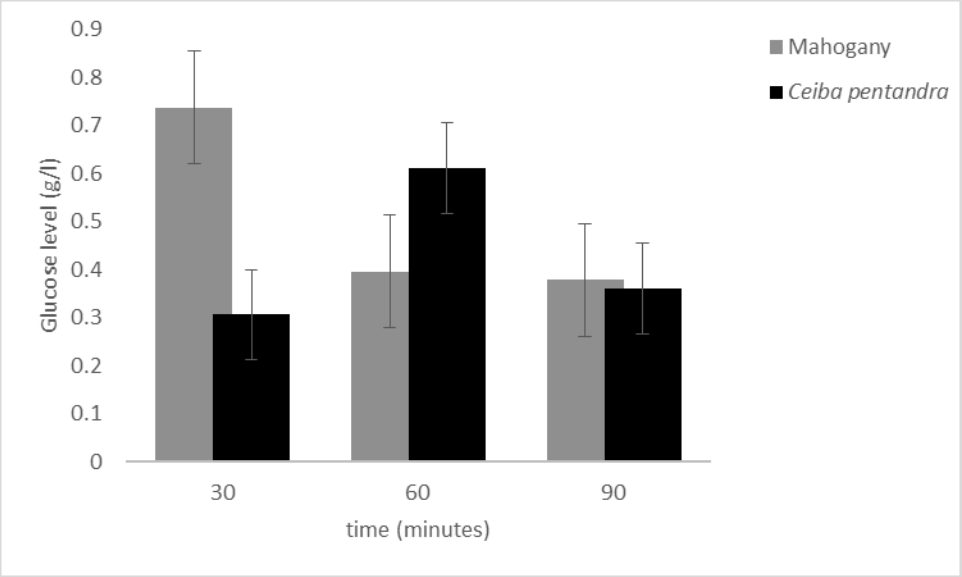

Fig. 4.15: Pretreatment using a concentration of 1 M HCl at a temperature of 121°C

From Figure 4.15, after pretreatment with 1 M HCl at a temperature of 121°C, the glucose concentration for Mahogany decreased with time while that for *Ceiba pentandra* increased from 0.31 g/l at 30 minutes to 0.61 g/l at 60 minutes and decreased to 0.36 g/l at 90 minutes. There was no significant difference between the glucose levels (P>0.05).

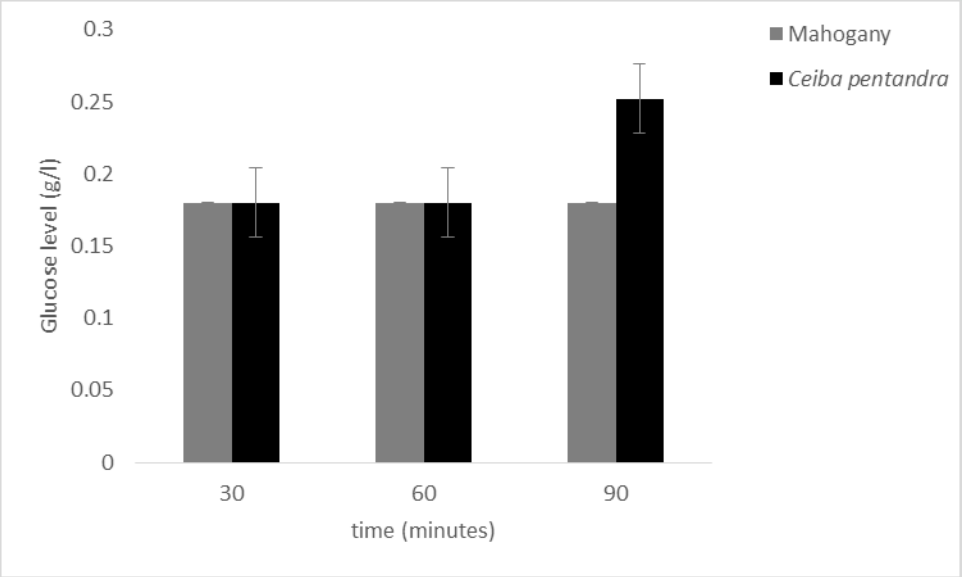

Fig. 4.16: Pretreatment using a concentration of 0.25 M H_2S0_4 at a temperature of 121°C

From Figure 4.16, after pretreatment with 0.25 M H_2S0_4 at a temperature of 121°C, the glucose concentration for both Mahogany and *Ceiba pentandra* remained constant at 0.18 g/l from 30 minutes to 60 minutes. The glucose concentration for *Ceiba pentandra* increased to 0.25 g/l at 90 minutes while that of Mahogany still remained the same. The increase was not significant (P>0.05).

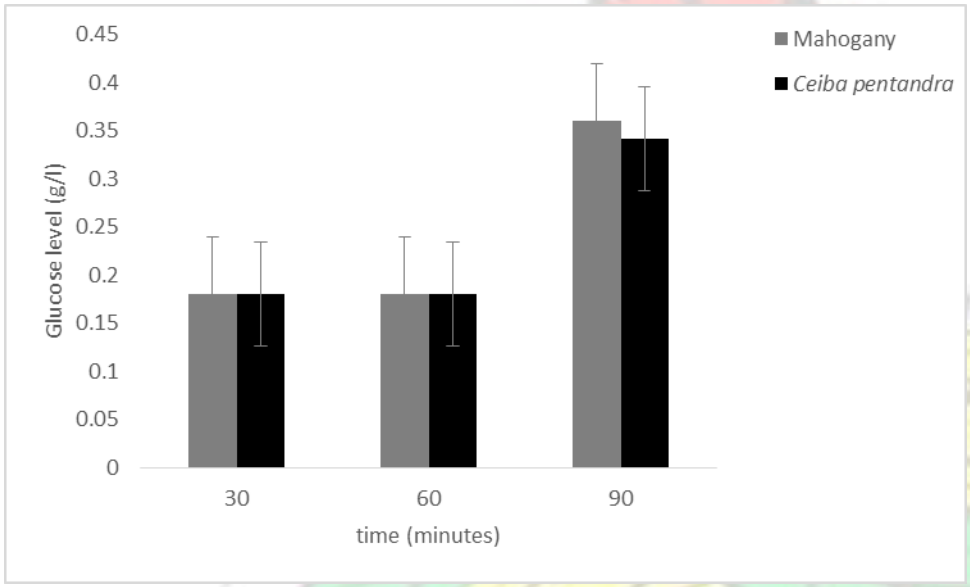

Fig 4.17: Pretreatment using a concentration of 0.5 M H_2S0_4 at a temperature of 121°C

From Figure 4.17, after pretreatment with 0.5 M H_2S0_4 at a temperature of 121°C, the glucose concentration for both Mahogany and *Ceiba pentandra* was constant at 0.18 g/l from 30 minutes to 60 minutes but increased to 0.36 g/l and 0.34 g/l respectively at 90 minutes but the increase was not significant (P>0.05).

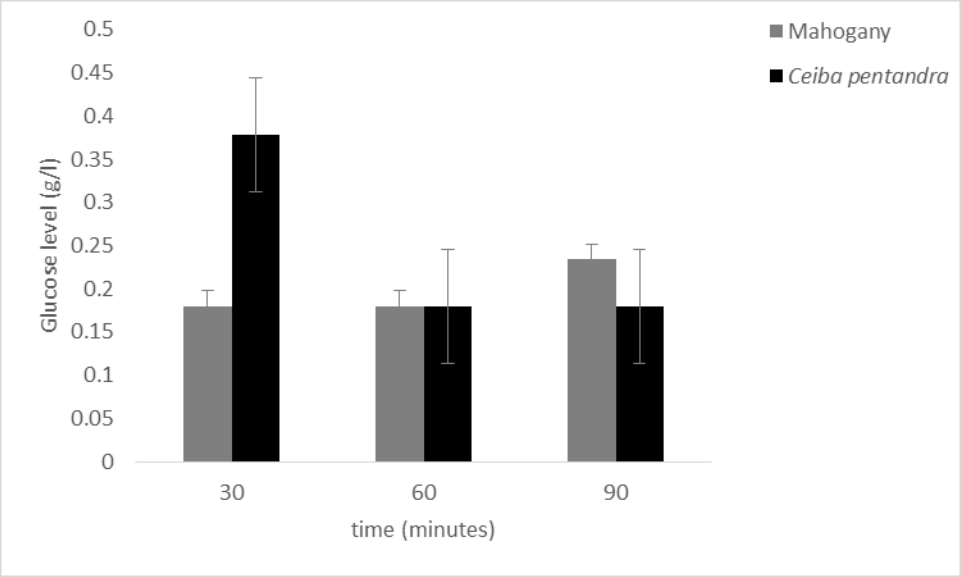

Fig. 4.18: Pretreatment using a concentration of 1 M H_2S0_4 at a temperature of 121°C

Figure 4.18 shows pretreatment with 1 M H_2S0_4 at a temperature of 121°C. After pretreatment the glucose concentration for Mahogany was constant at 0.18 g/l from 30 minutes to 60 minutes but it increased to 0.23 g/l at 90 minutes while that of *Ceiba pentandra* decreased from 0.38 g/l at 30 minutes to 0.18 g/l at 60 minutes and remained constant at 90 minutes. There was no significant difference between the glucose levels ($P>0.05$).

4.3. ENZYMATIC HYDROLYSIS

The Mahogany and *Ceiba pentandra* were both treated with cellulase enzyme at different temperatures and resident times and the amount of glucose released were measured.

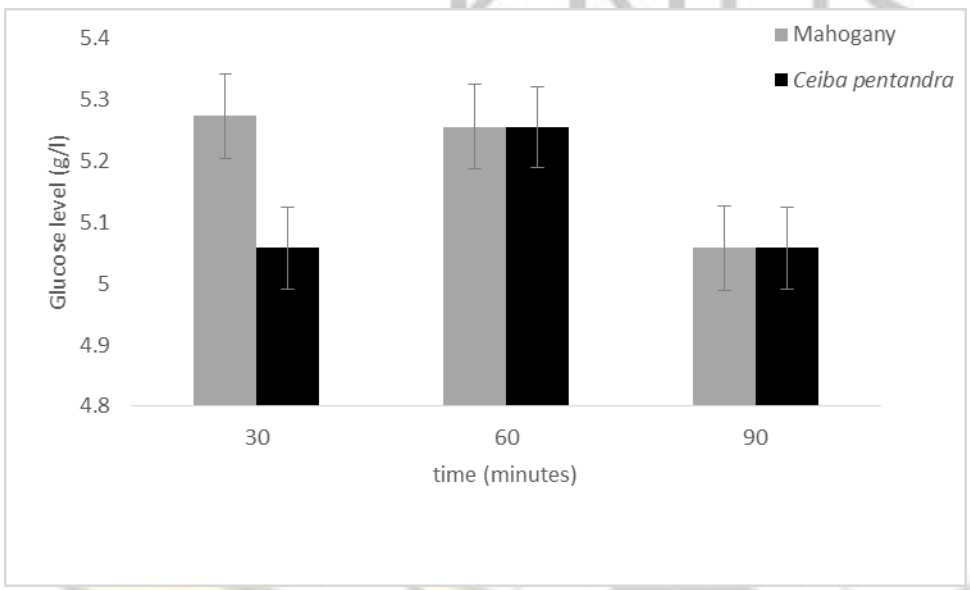

Fig.4.19: Pretreatment with cellulase enzyme at 55 ^{0}C

From Figure.4.19, after pretreatment with cellulase enzyme, the glucose level for Mahogany decreased significantly ($P<0.05$) with time while for the *Ceiba pentandra* the glucose level increased significantly ($P<0.05$) from 5.06 g/l at 30 minutes to 5.26 g/l at 60 minutes and decreased significantly ($P<0.05$) to 5.06 g/l at 90 minutes.

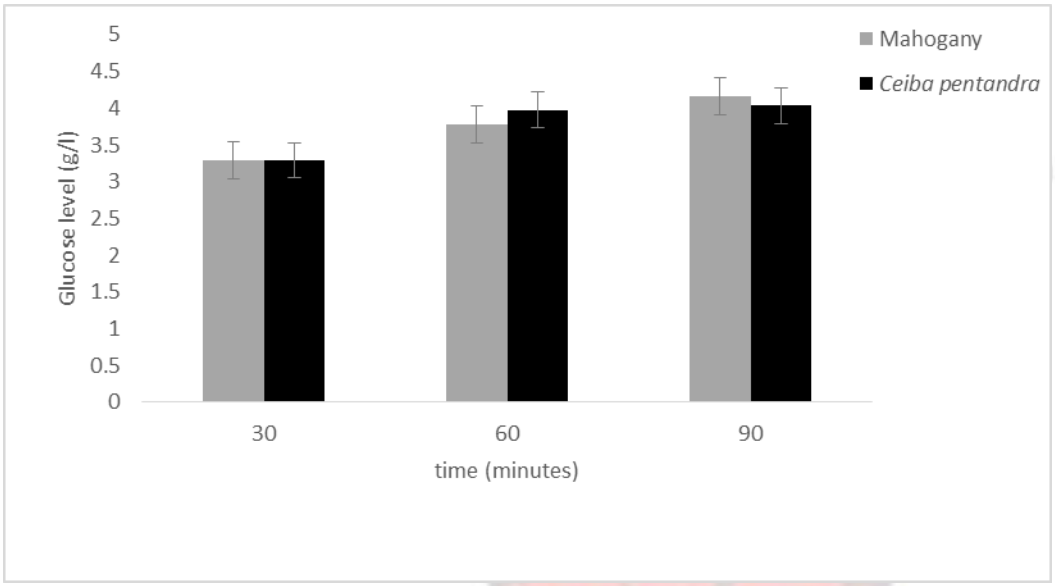

Fig. 4.20: Pretreatment with cellulase enzyme at 100°C

From Figure 4.20, after pretreatment with cellulase enzyme at 100°C, the glucose concentration for both Mahogany and *Ceiba pentandra* increased significantly ($P<0.05$) with time.

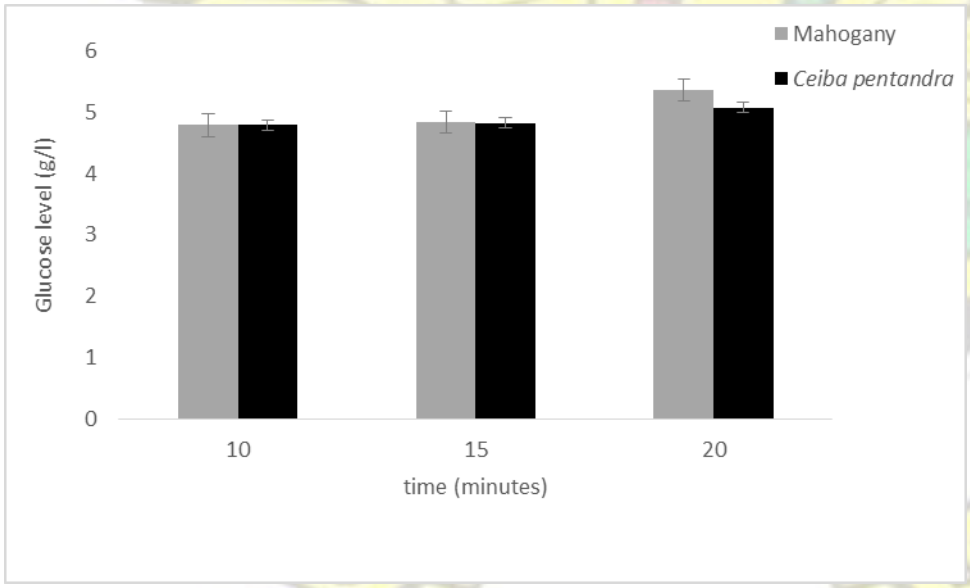

Fig. 4.21: Pretreatment with cellulase enzyme at 121°C

From Figure 4.21, after pretreatment with cellulase enzyme at 121°C, the glucose concentration for both Mahogany and *Ceiba pentandra* increased significantly ($P<0.05$) with time.

4.4.1 Dilute Base Pretreatment Analysis

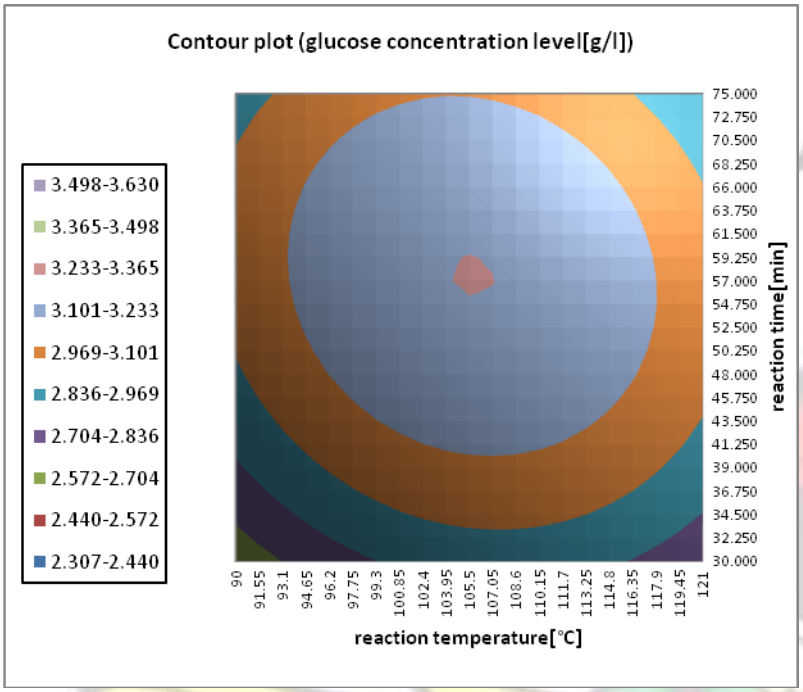

Fig. 4.30: Contour plot (2D) of glucose concentration against temperature and time

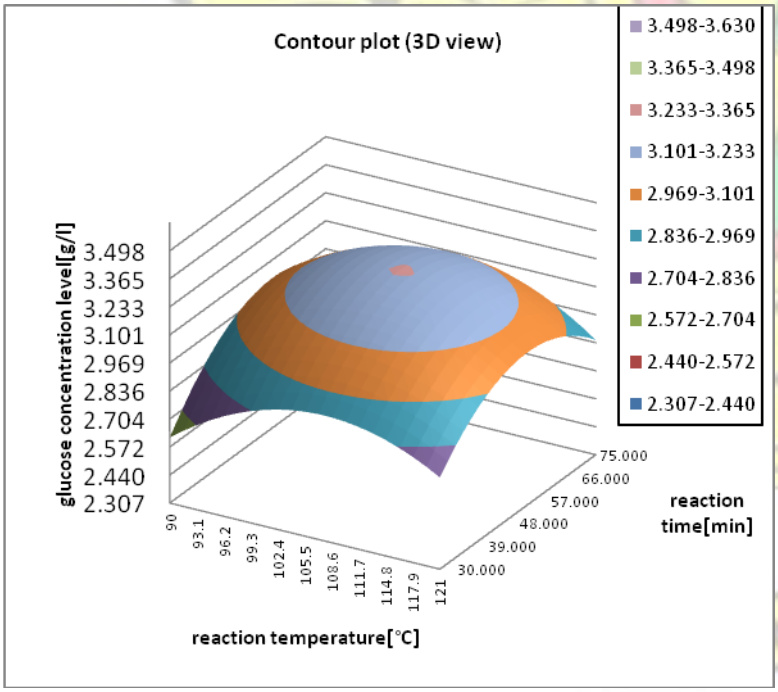

Fig. 4.31: Contour plot (3D) of glucose concentration against temperature and time Fig 4.30 and Fig 4.31 represent the surface response area which is graphically displayed. This is done by a 2d and 3d contour plot. It can be seen easily that the optimal area is situated in the center (red area) as shown. The optimum can be located around 105.50°C and 57 minutes and has a value of more than 3.36 g/l. The optimum was reached during the use and test of *Ceiba pentandra* plant species in Sodium hydroxide solution. In other words, the use of sodium hydroxide solution for this chemical reaction yields the optimum concentration levels for *Ceiba pentandra* plant species. Finally the two following diagrams show the traces of each factor with regards to the answer variable which are shown

The important indicators for the model are R^2 and Q^2. When the value is close to 1 it indicates that the model has a good fitting to the data. This ANOVA and its model was able to describe the data very well, because $R^2 = 0.887$ as shown (goodness of fit statistics) in Table A-4 in appendix A. Further details about the model are available in the two following sections with the model parameters and the model equation shown in Appendix A.

Analysis of variance was also done to assess the impact of different base concentration levels on glucose levels measured by the test. Subjects were divided into three groups according to their concentration levels (Group 1: 0.25, Group 2: 0.50, and Group 3: 1.0). Statistically there was a significant difference at the $p < 0.05$ level in glucose levels for the three different base concentration levels (i.e. 0.25, 0.50, and 1.0) used for the experiment ($F > 4.0$, $P \leq 0.05$) [ANOVA table (Table A-10) in Appendix A].

4.4.2. Dilute Acid Pretreatment Analysis

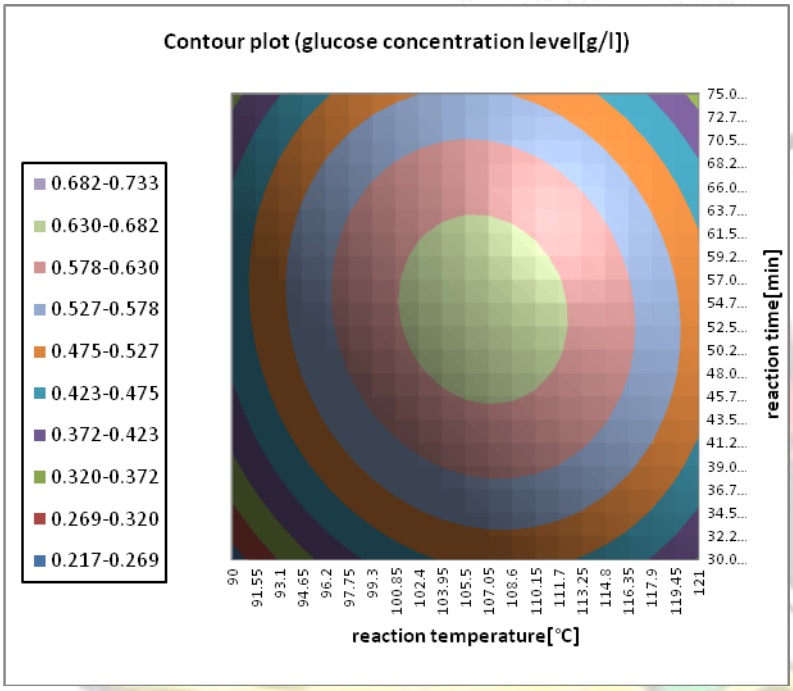

Fig 4.32: Contour plot (2D) of glucose concentration against temperature and time

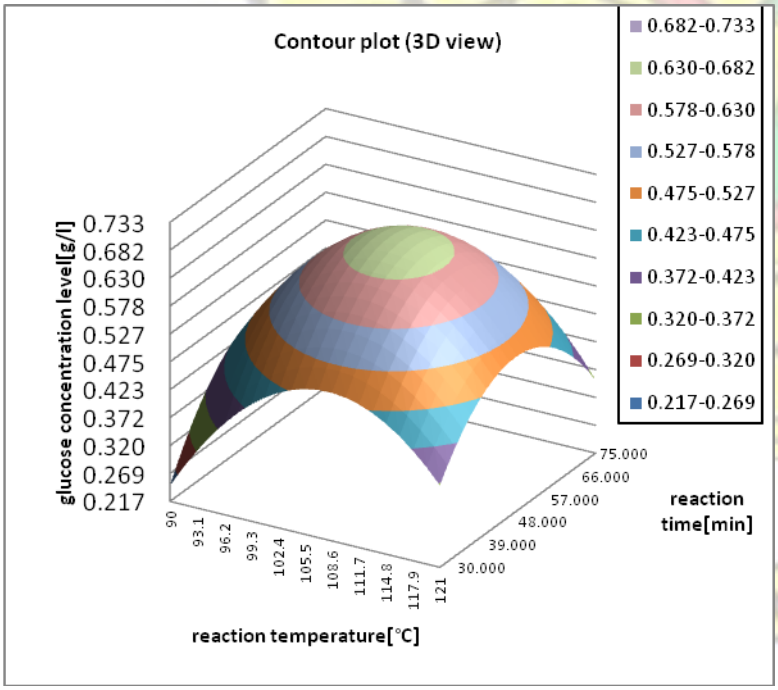

Fig 4.33: Contour plot (3D) of glucose concentration against temperature and time Fig 4.32 and fig. 4.33 represent the surface response area which is graphically shown. This is shown by a 2d and a 3d contour plot. The optimal area can easily be located in the green area as shown. The optimum can be located around 107.03^0C and 54.75 minutes and has a value of more than 0.68 g/l. The optimum was reached during the use and test of Mahogany plant species in hydrochloric acid solution. In other words, the use of hydrochloric acid solution for this chemical reaction yields the optimum concentration levels for Mahogany. Finally the traces of each factor with regards to the answer variable are shown

The important indicators for the model are R^2 and Q^2. When the R^2 value is close to 1 it indicates that the model has a good fitting to the data. The ANOVA used in this model describe the data very well, because $R^2 = 0.885$ as indicated in goodness of fitness statistics in table B-4 in appendix B. More details about this model can also be found in the two following sections with the model parameters and the model equation shown in Appendix B.

A one-way between groups analysis of variance was also used to assess the effect of different acid concentration levels on glucose levels measured by the test. Subjects were divided into three groups according to their concentration levels (Group 1: 0.25, Group 2: 0.50, and Group 3: 1.0). Statistically there was no significant difference at the $P > 0.05$ level in glucose levels for the three different acid concentration levels (i.e. 0.25, 0.50 and 1.0) used for the experiment ($F< 3.1$, $P> 0.05$)ANOVA table (Table B-11) in Appendix B].

4.5. Ethanol concentration

Table 4.2: Relationship between temperature and the concentration of ethanol

Temperature(^{0}C)	Glucose concentration after fermentation (%)	Ethanol concentration % (w/v)
30	36.71±0.40	$1.68x10^{-4}$
40	41.18±0.29	$3.86x10^{-4}$
50	66.18±0.12	$3.48x\ 10^{-2}$

From Table 4.2, it was observed that as the temperature increased, the ethanol concentration also increased. The highest ethanol concentration of $3.48x\ 10^{-2}$ % (w/v) was attained at a temperature of 50°C with the highest amount of glucose after fermentation while the lowest ethanol concentration of $1.68x10^{-4}$ % (w/v) was recorded at a temperature of 30°C with the minimum amount of glucose after fermentation.

CHAPTER FIVE

5.0. DISCUSSION

Sawmill dust is a major waste material obtained after the processing of wood. It is often burnt as a waste product which causes air pollution. It can be used as a raw material for mulching but majority are thrown away as waste. It is made up of components such as of lignin, hemicellulose and cellulose which can be degraded to fermentable sugars. These sugars can be exploited by some microorganisms to produce ethanol. It also contains important minerals such as carbon, nitrogen and phosphorus which can also be utilized by microorganisms.

In this study, an important process known as pretreatment was performed to produce fermentable sugars to ethanol. The pretreatment of Mahogany and *Ceiba pentandra* with different concentrations of sodium hydroxide and ammonia at different temperatures and resident times were analyzed. High glucose level of 3.37 g/l and 3.08 g/l were recorded using a concentration of 1 M NaOH at 100°C for 90 minutes time with *Ceiba pentandra* and Mahogany respectively (Fig. 4.3). The ammonia also recorded high glucose level of 0.41 g/l and 0.34 g/l for Mahogany and *Ceiba pentandra* respectively at 100°C for 90 minutes with 1 M NH_3 (Fig. 4.6). The highest glucose level was recorded at 4.37 g/l at 121°C for 20 minutes with Mahogany using sodium hydroxide (Fig 4.9), but this was different for the ammonia which released 0.32 g/l at the same conditions compared to the highest glucose level of 0.47 g/l recorded at 121°C for 10 minutes with Mahogany (Fig. 4.12). Thus increasing the concentration, temperature and resident time yielded higher levels of glucose at certain situations. Kim and Holtzapple (2005), reported that dilute bases release high amount of glucose when pretreated at high concentrations, at mild temperatures and at long resident times. It is therefore, not always necessary to increase the resident time at high temperatures because extending the resident time at moderate temperature could also increase the

glucose level. The different plant species recording different glucose levels for both the sodium hydroxide and ammonia at the same conditions could be attributed to the chemical compositions and properties of the two species (Vassilev *et al*., 2010). Prevalent among these factors is the moisture variations among the two plant species (Table 4. 1). The high moisture content of the Mahogany could have interfered with the glucose level thereby reducing the concentration (Turner, 2013). This could have accounted for the reason why the *Ceiba pentandra* at some point had a higher glucose concentration than Mahogany, even though the overall performance of Mahogany was better than the *Ceiba pentandra*. At a concentration of 0.25 M the glucose level was lower than the concentration at 0.5 M and 1 M for both the sodium hydroxide and the ammonia. The glucose level increased as the concentrations of the bases increased. The sodium hydroxide pretreatment released more glucose than pretreatment with ammonia at the three different concentrations. Varga *et al*., (2003), reported that pretreatment with sodium hydroxide can cause swelling, which can lead to an increase in internal surface area, a decrease in the polymerization, a decrease in crystallinity and further disruption of the lignin structure and separation of the structural linkages between the lignin and the carbohydrates. Silverstein, (2007) also demonstrated that Sodium hydroxide pretreatment results in high level of delignification and cellulose conversion. The *Ceiba pentandra* produced the highest glucose concentration with sodium hydroxide at 1 M and 0.5 M at 100°C for 90 minutes, except at 0.25 M where the highest glucose level of 2.03 g/l was produced by Mahogany at 75°C for 90 minutes compared to 1.1 g/l for *Ceiba pentandra* (Fig. 4.1).

Comparatively, the Mahogany performed much better with ammonia pretreatment than *Ceiba pentandra*. The glucose concentration increased with sodium hydroxide at 0.25 M when the resident time was increased to 90 minutes at the temperatures of 75°C, 90°C and 100°C

respectively with the highest level of 2.03 g/l attained at 75°C with Mahogany (Fig 4.1). This confirms that alkaline pretreatment requires lower temperatures and long reaction times. Kim and Holtzapple (2005) reported a study in which pretreatment times used was as long as four weeks at 55°C.

The glucose concentration with sodium hydroxide increased again when the time was increased from 60 to 90 minutes; and also when both temperature and resident time were increased. Increasing the temperature alone was not enough to increase the glucose concentration. For example, when the temperature was increased to 100°C at 30 minutes the glucose concentration decreased, but when the time was increased, the glucose level increased, however, at a higher temperature of 121°C when the time was increased the glucose level rather decreased. This means that at a very high temperature, a short resident time is required (Fig. 4.8). The moderate temperature and long resident time allowed for complete hydrolysis of the hemicelluloses, lignin and the crystalline structure of the cellulose to release glucose. At very high temperatures, the rate of reaction increases thereby increasing the entire hydrolysis process, however, increasing the resident time could have allowed for the formation of inhibitors which might have interfered with the glucose concentration. Dilute pretreatment makes use of acid solutions with concentrations of less than 2% (w/v) at high temperatures above 100°C to breakdown hemicellulose, disrupt lignin structure, and increase surface area (Mosier *et al*., 2005).

The study therefore reveals that pretreatment with sodium hydroxide requires mild temperatures at long reaction times and higher temperatures require shorter resident times. Increasing the concentration also increased the glucose concentration; this is because it was able to break the bonds forming the structure. The mechanism of alkaline hydrolysis usually depends on the saponification of the intermolecular ester bonds which crosslink the xylan, hemicellulose and other

components such as lignin (Sun and Cheng, 2002). This usually involves the use of strong alkali such as sodium hydroxide or calcium hydroxide which accelerates the breakdown of the ester bonds to form the corresponding alcohol and acid or salt. Thus, increasing the concentration catalysed the hydrolysis process. Consequently, increasing the time at mild temperatures allowed for complete breakdown of the ester bonds (Lei *et al*., 2009)

After pretreatment with 0.25 M Ammonia the glucose concentrations were relatively lower compared to pretreatment with sodium hydroxide. For example at 100°C for 90 minutes when glucose concentration was 0.22 g/l with ammonia, that of sodium hydroxide was 1.66 g/l for Mahogany (Fig.4.1 and Fig. 4.4) This is because ammonia is not very effective for pretreating biomass with high lignin content as documented by Sun and Cheng (2009). Therefore the low concentration of the ammonia (0.22 g/l) might not have been strong enough to disrupt the hemicelluloses, the crystalline cellulose and lignin to release much glucose. After pretreatment with 0.5 M, however, the glucose level increased. The glucose level also increased when the temperature was increased after a shorter resident time. For example, after pretreatment with 1 M ammonia the highest glucose levels of 0.47 g/l and 0.45 g/l were recorded at 121°C for 10 minutes with both Mahogany and *Ceiba pentandra* respectively. Even though the ammonia pretreatment did not produce much glucose however, when the concentration and temperature were increased the glucose level also increased. The high concentrations and temperatures might have enhanced hydrolysis and subsequent release of the glucose, however, long exposure might have caused the release of certain inhibitory substances which reduced the glucose concentration (Delgenes *et al*., 1996). The optimum glucose level for the base was 1 M sodium hydroxide at a temperature of 100°C and 90 minutes resident time with *Ceiba pentandra* plant species. This was the conditions

at which both the sodium and the ammonia all produced the maximum amount of glucose; however, the sodium hydroxide yielded more glucose concentrations than the ammonia at these same conditions. This was confirmed using the Response surface methodology which is graphically displayed by a 2D and 3D contour in fig 4.30 and fig 4.31 .

The effects of different concentrations of dilute hydrochloric acid (HCl) and dilute Sulphuric acid (H_2SO_4) at different temperatures and resident times were analyzed. The performances of the dilute acid pretreatments were lower than that of the dilute bases; because dilute acid pretreatment requires very high temperatures at short resident time for hydrolysis. At high temperatures, dilute sulphuric acid pretreatment showed high rate of reaction and improved the rate of cellulose conversion (Esteghlalian, 1997), but at mild temperature the saccharification yields of cellulose is quite poor (McMillan,1994). In this study, pretreatment with the hydrochloric acid released more glucose at the different concentrations than the sulphuric acid. The low concentration of the HCl was not powerful enough to dissolve the hemicelluloses and disrupt the lignin to increase the surface area (Mosier *et al*., 2005), however, increasing the reaction time allowed for some level of hydrolysis thus releasing more glucose. This also confirms that when using lower concentrations of acid for pretreatment, a longer resident time is required to release high amount of glucose at a higher temperature (Emmel, 2003). The glucose level increased when the concentration was increased to 0.5M. The glucose level further increased when the resident time was also increased. The increase in the HCl concentration further enhanced the hydrolysis process thus releasing more glucose. The high glucose level attained at 30 minutes with Mahogany could be attributed to the high concentration of the acid. The acid was strong enough to digest the cellulose after a short period; however, increasing the time might have caused the release of certain inhibitory substances

in the hydrolyzate which might have interfered with the glucose concentrations (Delgenes *et al*., 1996). However, the low glucose level recorded initially for *Ceiba pentandra* might have resulted from the structure of the hemicelluloses, cellulose and lignin. A more complex structure requires more time for hydrolysis thus increasing the time allowed for maximum break down of the crystalline structure to release more glucose.

Pretreatment with dilute sulphuric acid (H_2SO_4) yielded very low glucose concentrations (0.18 g/l). The performance of the dilute sulphuric acid (H_2SO_4) was very poor compared to that of hydrochloric acid and the other pretreatments that were studied. The lower concentration of the acid might have accounted for the poor glucose levels because it could not degrade the cellulose to release more glucose. The increase in time could have resulted in some kind of hydrolysis with the *Ceiba pentandra*, hence causing the release of more glucose. According to Wyman *et al*., (2009) and Zhu *et al*., (2009) weak acid pretreatment can achieve only about 40% cellulose conversion to glucose. The increase in concentration and resident time may have resulted in further hydrolysis of the cellulose to release more glucose, hence increasing the glucose levels. The glucose levels recorded at 1 M was constant at 0.18 g/l for both Mahogany and *Ceiba pentandra*, at 60 minutes however, the glucose level recorded for *Ceiba pentandra* was 0.38 g/l at 30 minutes while for Mahogany, the level recorded at 90 minutes was 0.23 g/l (Fig 4.18). The high concentration of the acid could have improved the hydrolysis of the cellulose for the *Ceiba pentandra*, but extending the resident time might have rather resulted in the formation of inhibitors (Johnson *et al*., 2013) thus reducing the glucose level while for the Mahogany increasing the resident time rather led to cellulose hydrolysis which caused the release of more glucose. The optimum amount of glucose for the acid was 1 M hydrochloric acid at a temperature of 121°C at a

time of 30 minutes with Mahogany plant species. These conditions recorded the maximum glucose concentrations for both the hydrochloric acid and the sulphuric acid, however, the hydrochloric acid performed better than the sulphuric acid at these same conditions. This was confirmed using the Response surface methodology which is graphically displayed by a 2D and 3D contour in fig 4.32 and fig 4.33.

The optimum for the enzyme was found to be 55°C. This temperature released high amount of glucose (5.27 g/l), however, extending the temperature reduced the enzymatic activities, hence leading to a reduction in the glucose concentrations. The increase in temperature could have catalyzed the entire reaction process, because temperature generally increases the rate of chemical reactions. Enzymes are however, proteins, hence exposing those to such a high temperature for long periods could have denatured the proteins thereby reducing it activity to hydrolyze the crystalline structure of the cellulose to release more glucose (Andreaus *et al*., 1999). Nevertheless, at 121°C the glucose level increased greatly, this is because the resident times were reduced drastically at this temperature. The higher temperature could have increased the rate of enzymatic reaction allowing the breakdown of the amorphous structure of the cellulose making it more porous to release the glucose which was embedded in it. Long exposure could have destroyed the enzymes and hence reduced its functions. Enzymes are also specific in their actions, thus the cellulase acted specifically on the cellulose which contained the glucose hence increasing the temperature could have further enhanced the degradation of the complex cellulose structure to release more glucose at a very short time (Saha, 2003).

The optimum conditions attained for the acid was used for the fermentation process because of the low pH of the acid. After five days there were generally a drastic reduction in the glucose concentrations, this proved that the yeasts were actually making use of the glucose (Table 4.2). Mahogany had better nutrient content than *Ceiba pentandra*. The nutrients enhanced the growth of the yeasts which increased their utilization of the glucose to produce ethanol, therefore making Mahogany a better choice for fermentation than *Ceiba pentandra*.

The ethanol concentrations measured after fermentation showed an increase with high temperature. This showed that temperature had effect on the concentrations of the ethanol.

Factors such as temperature when not properly regulated can affect the ethanol yield (Adeyinka, 1993). The quantity of ethanol was generally very poor; this may be due to the high moisture content of the Mahogany which could have further reduced the concentration of the ethanol since it is hygroscopic (Turner, 2013). The high ash content could also had accounted for the low concentration since it is also known to have an effect on the concentration of ethanol (Li *et al*, 2011). Moreover, the ethanol concentration was affected by some factors such as the fermentation conditions, the acidity, the reaction vessel, the retention time and pH. Furthermore, lignocellulose biomass such as sawdust is known to have low glucose compared to sugar foodstuffs. Even though much glucose was consumed by the yeasts at 30°C, it produced the lowest concentration of ethanol (Table 4.1). This is because yeasts do not survive well at lower temperatures; however, better yields of 3.86 $x10^{-4}$ % (w/v) and 3.48 x 10^{-2} % (w/v) of ethanol were recorded at 40°C and 50°C respectively. This does not hold for very high temperatures since from literature it is known that most yeast will not survive at very high temperatures and at very acidic conditions (Taherzadeh *et al.*, 2011).

CHAPTER SIX

6.0. CONCLUSIONS AND RECOMMENDATIONS

6.1. CONCLUSIONS

The production of bioethanol from lignocellulose depends mainly on pretreatment. The best parameters during pretreatment of biomass such as sawdust include high concentrations of acid or base and high temperatures. These conditions are able to break the crystalline structure of the cellulose, hemicellulose and lignin to release fermentable sugars. The use of alkaline particularly, the sodium hydroxide provided the best pretreatment option for sawdust. The use of acids such as hydrochloric acid also gave significant amounts of glucose. The use of enzymes for hydrolysis gave the best results in terms of glucose production but because of the high cost and the difficulty

in obtaining the enzymes, optimizing conditions for acids and bases for pretreatment provides an alternate and viable option for the production of bioethanol.

The optimum level of ethanol production for *Ceiba pentandra* sawdust was 1 M sodium hydroxide at a temperature of 100°C and 90 minutes resident time while that of Mahogany sawdust was 1 M hydrochloric acid at a temperature of 121°C at a time of 30 minutes.

6.2. RECOMMENDATIONS

- Higher concentrations of acid or base and higher temperatures should be used during pretreatment of biomass in future works.
- Long resident time is recommended for alkaline pretreatment at moderate temperatures while short resident time is recommended for acid pretreatment at high temperatures.
- Further analysis in the future should be done to ascertain the type, amount and levels of inhibitors produced in the hydrolyzates after hydrolysis.
- In addition, a more analytical method such as a high performance liquid chromatography (HPLC) which can measure both pentose and hexose sugar units could be used to measure the hydrolyzates in future works.
- Finally, an engineered microorganism which can convert both pentose and hexose sugar units to ethanol could be used for the fermentation to produce sufficient amounts of the ethanol.

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APPENDICES

APPENDIX A

DATA ANALYSIS

DILUTE BASE HYDROLYSIS

Table A-1

Variable	Obs.	Obs. +data	Obs. Data	Min	Max	Mean	SD
Glucose concentration level	**13**	**0**	**13**	**2.538**	**3.366**	**2.950**	**0.257**
Reaction temperature	13	0	13	83.580	127.420	105.500	12.656
Reaction time	13	0	13	20.680	84.320	52.500	18.371

Obs. +data = Observations with missing data Obs. –data = Observations without missing data

Table A-2

Variable information:

Short name	Long name	Unit	Unit (symbol)
G1	Reaction temperature	Degree	°C
G2	Reaction time	Minutes	Min

Table A -3

Model name: GLUCOSE

Experimental design (Central composite design):

Observation	Sort order	Run order	Repetition	Reaction temperature	Reaction time	Glucose concentration level
Obs1	1	1	1	90	30	2.538
Obs2	2	2	1	121	30	2.808
Obs3	3	3	1	90	75	2.826
Obs4	4	4	1	121	75	2.912
Obs5	5	5	1	83.57969	52.5	2.88
Obs6	6	6	1	127.4203	52.5	2.718
Obs7	7	7	1	105.5	20.68019	2.628
Obs8	8	8	1	105.5	84.31981	2.922
Obs9	9	9	1	105.5	52.5	3.114
Obs10	10	10	1	105.5	52.5	3.125
Obs11	11	11	1	105.5	52.5	3.225
Obs12	12	12	1	105.5	52.5	3.291
Obs13	13	13	1	105.5	52.5	3.366

Regression of variable glucose concentration level:

Table A-4

Goodness of fit statistics:

Observations	**13.000**
Sum of weights	13.000
DF	7.000
R^2	0.887
Adjusted R^2	0.805
MSE	0.113
RMSE	0.336
MAPE	2.426
DW	1.737
Cp	6.000
AIC	-24.369
SBC	-20.980
PC	0.308
Press RMSE	0.233

Table A

Press	0.381
Q^2	0.519

-5

Analysis of variance:

Source	DF	Sum of squares	Mean squares	F	Pr> F
Model	5	0.702	0.140	10.936	0.003
Error	7	0.090	0.013		
Lack of fit	3	0.043	0.014	1.242	0.405
Pure error	4	0.047	0.012		
Corrected Total	12	0.792			
Computed against model Y=0					

Table A-6

Model parameters:

Source	Value	Standard error	T	Pr> |t|	Lower bound (95%)	Upper bound (95%)
Intercept	3.224	0.051	63.607	< 0.0001	3.104	3.344
reaction temperature	0.016	0.040	0.396	0.704	-0.079	0.111
reaction time	0.101	0.040	2.520	0.040	0.006	0.196
reaction temperature^2	-0.217	0.043	-5.040	0.001	-0.318	-0.115
reaction time^2	-0.229	0.043	-5.319	0.001	-0.330	-0.127
reaction temperature*reaction time	-0.046	0.057	-0.812	0.444	-0.180	0.088

Equation of the model:

Glucose concentration level = 3.2242+1.58621753619448E-02*reaction

Table A

Temperature+0.100972348417211*reaction time-0.2166*reaction

temperature^20.2286*reaction time^2-4.60000000000001E-02*reaction

temperature*reaction time **-7**

Standardized coefficients:

Source	Value	Standard error	T	Pr> |t|	Lower bound (95%)	Upper bound (95%)
reaction temperature	0.050	0.127	0.396	0.704	-0.251	0.352
reaction time	0.321	0.127	2.520	0.040	0.020	0.622
reaction temperature^2	-0.647	0.128	-5.040	0.001	-0.951	-0.343
reaction time^2	-0.683	0.128	-5.319	0.001	-0.987	-0.379
reaction temperature*reaction time	-0.103	0.127	-0.812	0.444	-0.405	0.198

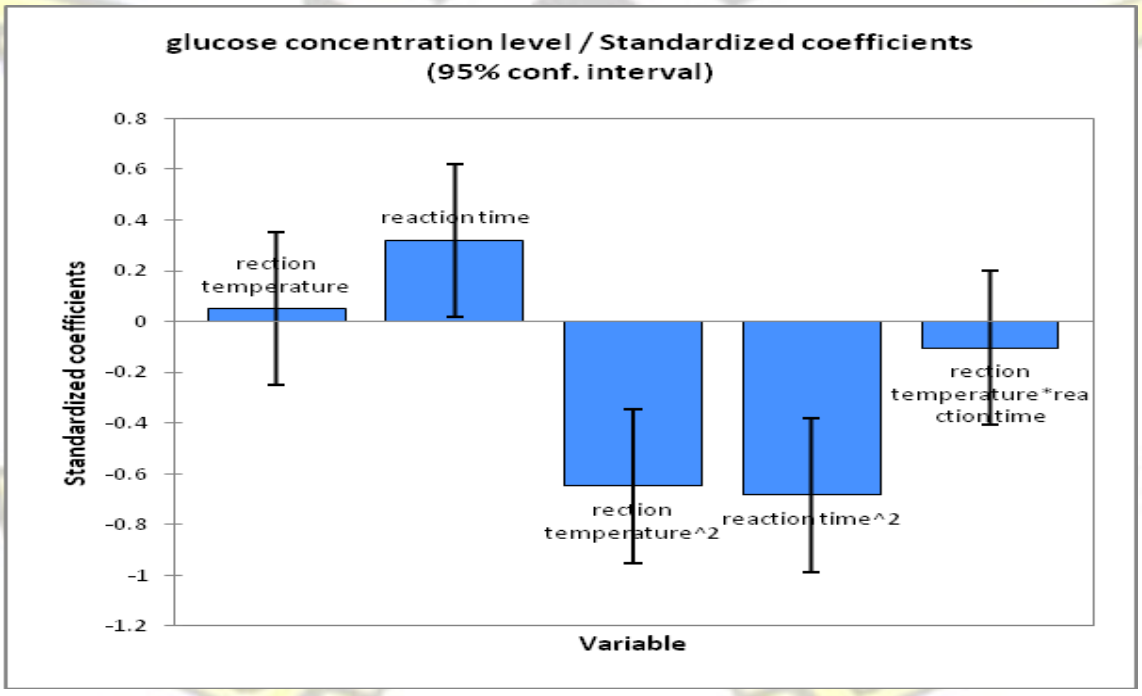

Fig. A-1

Table A

-8

Predictions and residuals:

Observation	Weight	glucose concentration level	Pred(glucose concentration level)	Residual	Std. residual	Std. dev. on pred. (Mean)	Lower bound 95% (Mean)	Upper bound 95% (Mean)	Std. dev. on pred. (Observation)	Lower bound 95% (Observation)	Upper bound 95% (Observation)
1	1	2.538	2.616	-0.078	-0.232	0.090	2.404	2.828	0.144	2.274	2.958
2	1	2.808	2.740	0.068	0.202	0.090	2.528	2.952	0.144	2.398	3.082
3	1	2.826	2.910	-0.084	-0.250	0.090	2.698	3.122	0.144	2.568	3.252
4	1	2.912	2.850	0.062	0.185	0.090	2.638	3.062	0.144	2.508	3.192
5	1	2.880	2.769	0.111	0.331	0.090	2.557	2.981	0.144	2.427	3.110
6	1	2.718	2.813	-0.095	-0.284	0.090	2.601	3.025	0.144	2.472	3.155
7	1	2.628	2.624	0.004	0.011	0.090	2.412	2.836	0.144	2.282	2.966
8	1	2.922	2.910	0.012	0.036	0.090	2.698	3.122	0.144	2.568	3.252
9	1	3.114	3.224	-0.110	-0.328	0.051	3.104	3.344	0.124	2.930	3.518
10	1	3.125	3.224	-0.099	-0.295	0.051	3.104	3.344	0.124	2.930	3.518
11	1	3.225	3.224	0.001	0.002	0.051	3.104	3.344	0.124	2.930	3.518
12	1	3.291	3.224	0.067	0.199	0.051	3.104	3.344	0.124	2.930	3.518
13	1	3.366	3.224	0.142	0.421	0.051	3.104	3.344	0.124	2.930	3.518

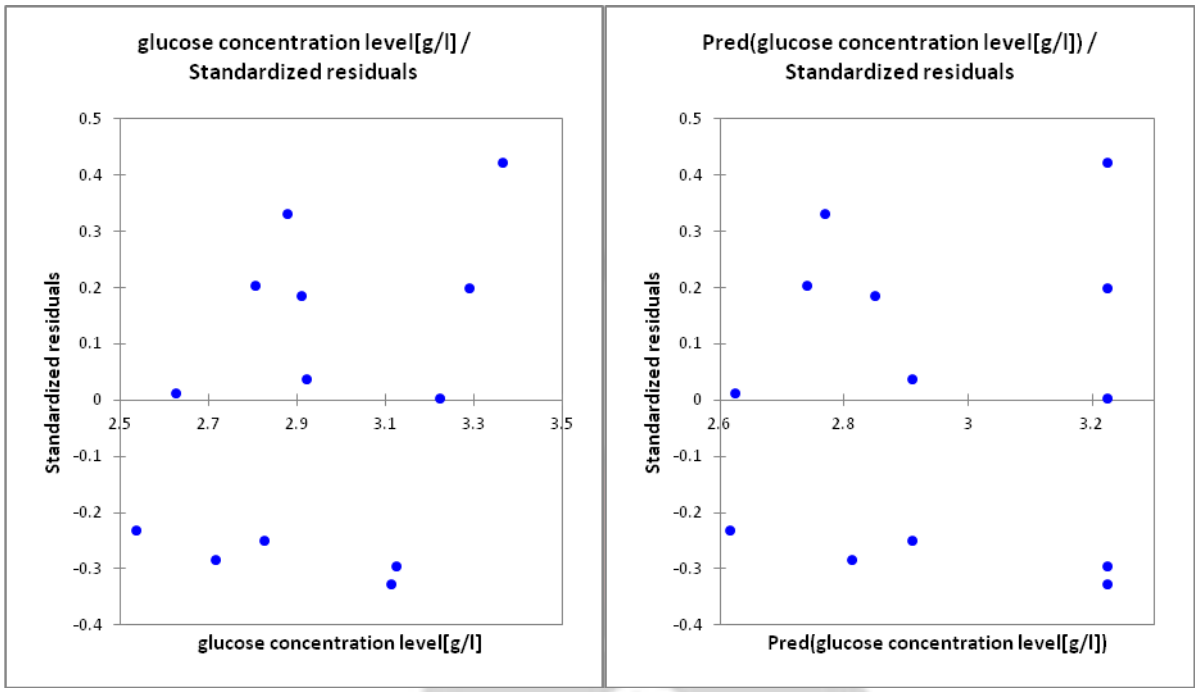
glucose concentration level[g/l] /
Standardized residuals
Standardized residuals
glucose concentration level[g/l]
Pred(glucose concentration level[g/l]) /
Standardized residuals
Standardized residuals
Pred(glucose concentration level[g/l])

Fig. A-2

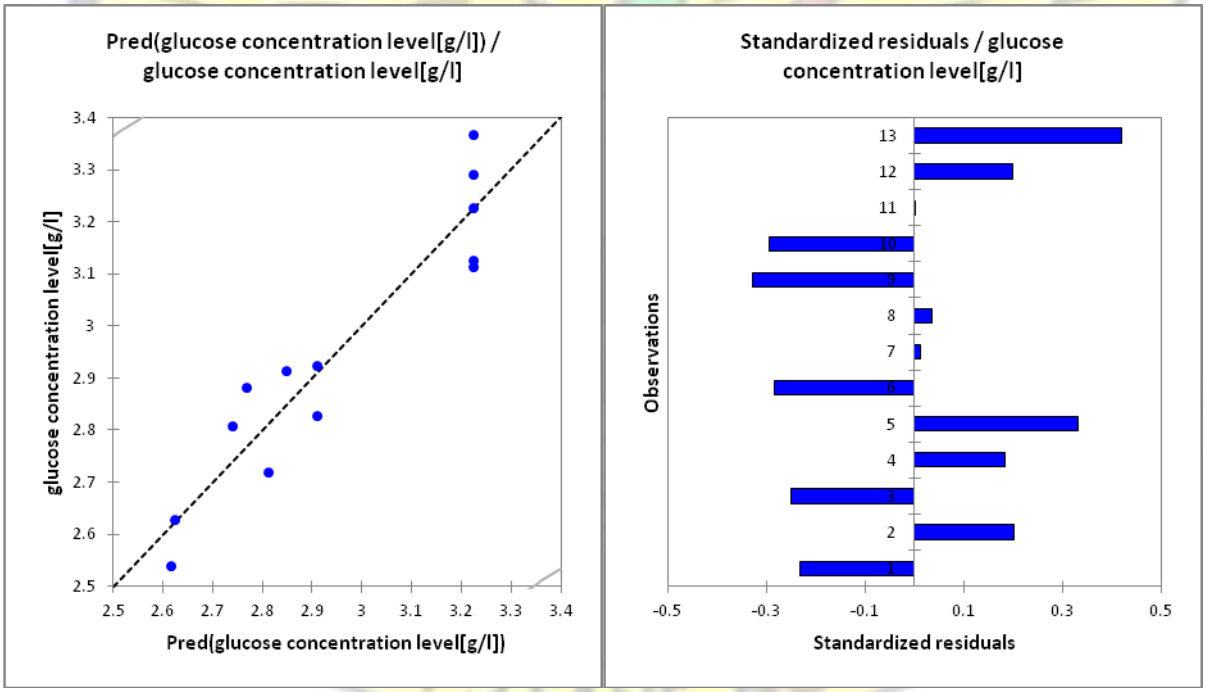
Pred(glucose concentration level[g/l]) /
glucose concentration level[g/l]
glucose concentration level[g/l]
Pred(glucose concentration level[g/l])
Standardized residuals / glucose
concentration level[g/l]
Observations
Standardized residuals

Fig. A-3

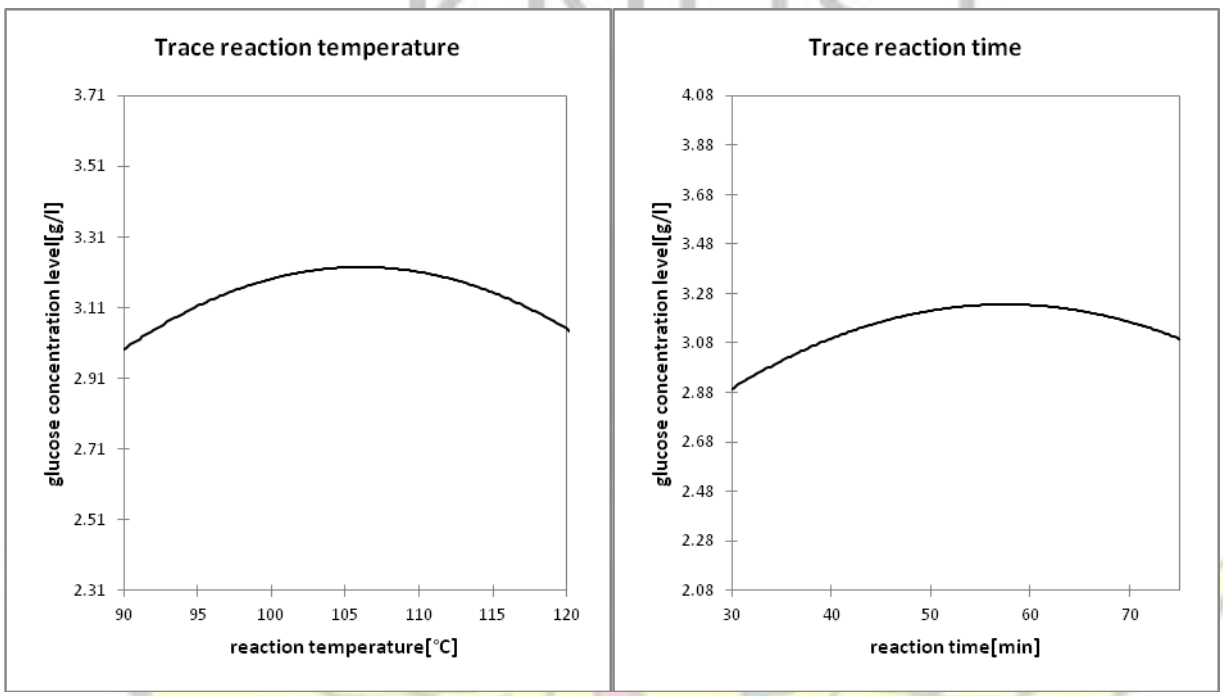
Trace reaction temperature
glucose concentration level[g/l]
3.71
3.51
3.31
3.11
2.91
2.71
2.51
2.31
90
95
100
105
110
115
120
reaction temperature[°C]
Trace reaction time
glucose concentration level[g/l]
4.08
3.88
3.68
3.48
3.28
3.08
2.88
2.68
2.48
2.28
2.08
30
40
50
60
70
reaction time[min]

Fig. A-4

Table A-9

Descriptives											
			N	Mean	SD.	Std. Error	95% Confidence Interval for Mean		Min.	Max.	Between-Component Variance
							Lower Bound	Upper Bound			
G025	0.05		4	1.669	0.9978	0.49	0.08176	3.25724	0.180	2.26	
	0.1		4	0.189	0.0180	0.00	0.16036	0.21764	0.180	0.216	
	0.25		4	1.710	1.0227	0.51	0.08252	3.33748	0.180	2.322	
	Total		12	1.189	1.0503	0.30	0.52214	1.85686	0.180	2.322	
	Model	Fixed Effects			0.8250	0.23	0.65073	1.72827			
		Random Effects				0.50	-.96349	3.34249			0.580990
G050	0.05		4	2.664	1.6054	0.80	0.10934	5.21866	0.270	3.708	
	0.		4	0.292	.04500	0.02	0.22089	0.36411	0.234	0.342	
	0.25		4	2.821	1.6812	0.84	0.14623	5.49677	0.324	3.978	
	Total		12	1.926	1.7130	0.49	0.83761	3.01439	0.234	3.978	
	Model	Fixed Effects			1.3424	0.38	1.04937	2.80263			
		Random Effects				0.81	-1.59363	5.44563			1.556926
G010	0.05		4	2.92500	1.765	0.88	0.11579	5.73421	0.288	4.032	
	0.1		4	0.34650	0.082	0.04	0.21551	0.47749	0.288	0.468	
	0.25		4	3.18150	1.832	0.91	0.26635	6.09665	0.450	4.374	

Table A-10

Test of Homogeneity of Variances				
	Levene Statistic	df1	df2	Sig.
G025	4.214	2	9	0.051
G050	4.017	2	9	0.057
G010	3.937	2	9	0.059

Table A-11

ANOVA						
		Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**
G025	Between Groups	6.009	2	3.005	4.414	0.046
	Within Groups	6.126	9	0.681		
	Total	12.135	11			
G050	Between Groups	16.060	2	8.030	4.456	0.045
	Within Groups	16.219	9	1.802		
	Total	32.278	11			
G010	Between Groups	19.669	2	9.834	4.553	0.043
	Within Groups	19.440	9	2.160		
	Total	39.108	11			

Table A-12

Robust Tests of Equality of Means					
		Statistic[a]	df1	df2	Sig.
G025	Welch	7.561	2	4.003	0.044
	Brown-Forsythe	4.414	2	5.998	0.066
G050	Welch	7.610	2	4.006	0.043
	Brown-Forsythe	4.456	2	5.992	0.065
G010	Welch	7.734	2	4.017	0.042
	Brown-Forsythe	4.553	2	6.004	0.063
a. Asymptotically F distributed.					

APPENDIX B

DILUTE ACID HYDROLYSIS

Table B-1

Summary statistics:

Variable	Obs.	Obs. +data	Obs. data	Min.	Max.	Mean	SD
Glucose concentration level	**13**	**0**	**13**	**0.234**	**0.738**	**0.454**	**0.178**
Reaction temperature	13	0	13	83.580	127.420	105.500	12.656
Reaction time	13	0	13	20.680	84.320	52.500	18.371

Table B-2

Variable information:

Short name	Long name	Unit	Unit (symbol)
G1	Reaction temperature	degree	°C
G2	Reaction time	minutes	min

Table B -3

Experimental design (Central composite design):

Observation	Sort order	Run order	Repetition	reaction temperature	reaction time	glucose concentration level
Obs1	1	1	1	90	30	0.234
Obs2	2	2	1	121	30	0.378
Obs3	3	3	1	90	75	0.378
Obs4	4	4	1	121	75	0.396
Obs5	5	5	1	83.57969	52.5	0.252
Obs6	6	6	1	127.4203	52.5	0.306
Obs7	7	7	1	105.5	20.68019	0.342
Obs8	8	8	1	105.5	84.31981	0.36
Obs9	9	9	1	105.5	52.5	0.504
Obs10	10	10	1	105.5	52.5	0.612
Obs11	11	11	1	105.5	52.5	0.666
Obs12	12	12	1	105.5	52.5	0.738
Obs13	13	13	1	105.5	52.5	0.738

Regression of variable glucose concentration level:

Table B-4

Goodness of fit statistics:

Observations	**13.000**
Sum of weights	13.000
DF	7.000
R^2	0.885
Adjusted R^2	0.803
MSE	0.054
RMSE	0.233
MAPE	8.436
DW	0.896
Cp	6.000
AIC	-33.885
SBC	-30.495
PC	0.311
Press RMSE	0.118

Table B

Press	0.097
Q^2	0.745

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Analysis of variance:

Source	DF	Sum of squares	Mean squares	F	Pr> F
Model	5	0.337	0.067	10.804	0.003
Error	7	0.044	0.006		
Lack of fit	3	0.005	0.002	0.181	0.904
Pure error	4	0.038	0.010		
Corrected Total	12	0.381			
Computed against model Y=0					

Table B-6

Model parameters:

Source	Value	Standard error	T	Pr> |t|	Lower bound (95%)	Upper bound (95%)
Intercept	0.652	0.035	18.436	< 0.0001	0.568	0.735
reaction temperature	0.030	0.028	1.066	0.322	-0.036	0.096
reaction time	0.023	0.028	0.839	0.429	-0.043	0.090
reaction temperature^2	-0.178	0.030	-5.955	0.001	-0.249	-0.108
reaction time^2	-0.142	0.030	-4.753	0.002	-0.213	-0.072
reaction temperature*reaction time	-0.032	0.040	-0.797	0.452	-0.125	0.062

Equation of the model:

Glucose concentration level = 0.6516+2.97959415460184E-02*reaction

Temperature+2.34319805153395E-02*reaction time-0.178425*reaction

temperature^20.142425*reaction time^2-0.0315*reaction temperature*reaction time

Table B

-7

Standardized coefficients:

Source	Value	Standard error	T	Pr> |t|	Lower bound (95%)	Upper bound (95%)
Reaction temperature	0.137	0.128	1.066	0.322	-0.166	0.439
Reaction time	0.107	0.128	0.839	0.429	-0.196	0.410
Reaction temperature^2	-0.769	0.129	-5.955	0.001	-1.074	-0.463
Reaction time^2	-0.614	0.129	-4.753	0.002	-0.919	-0.308
Reaction temperature*reaction time	-0.102	0.128	-0.797	0.452	-0.405	0.201

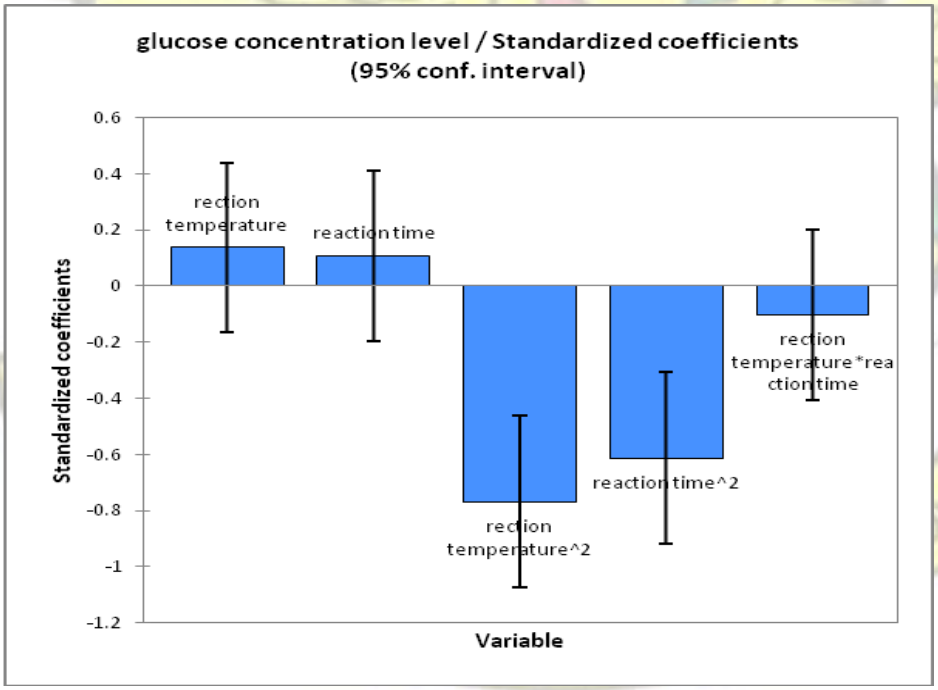

Table B
Figure B-1

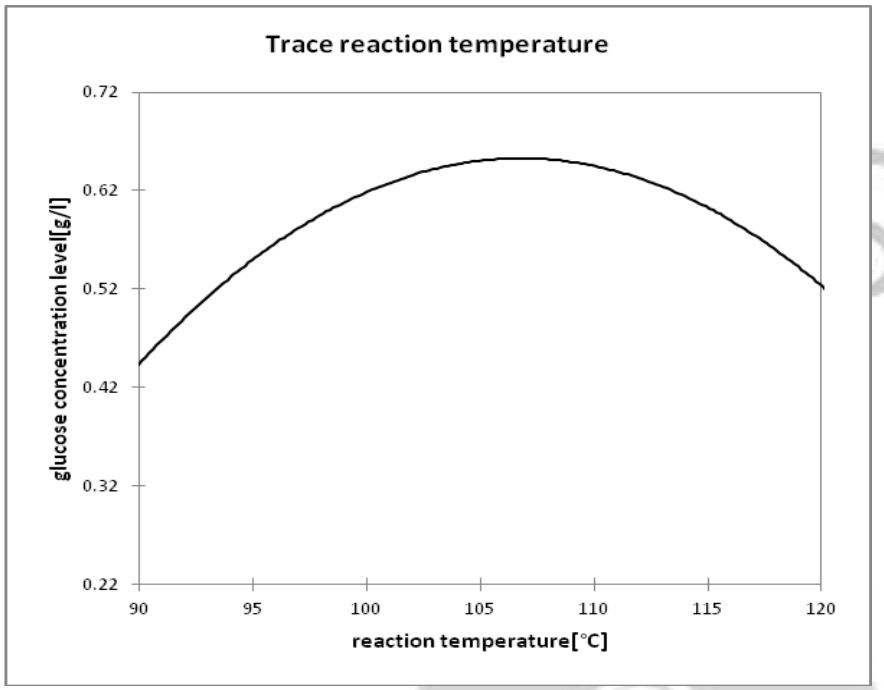
Trace reaction temperature
0.72
0.62
0.52
0.42
0.32
0.22
glucose concentration level[g/l]
90
95
100
105
110
115
120
reaction temperature[°C]

Fig. B-2

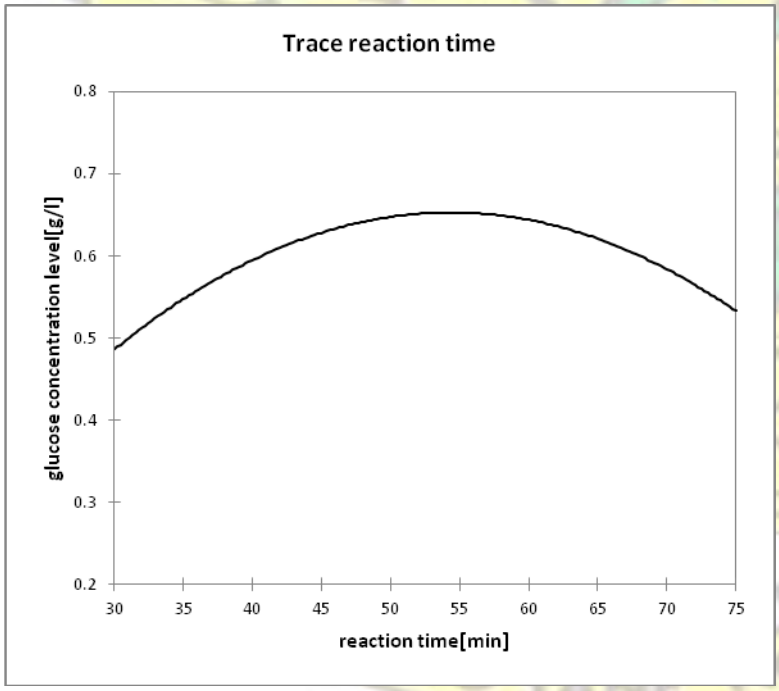
Trace reaction time
0.8
0.7
0.6
0.5
0.4
0.3
0.2
glucose concentration level[g/l]
30
35
40
45
50
55
60
65
70
75
reaction time[min]

Fig.B-3

TABLE B-8 Table B-9

Descriptives

			N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
							Lower Bound	Upper Bound		
G025	0.05		4	0.28800	0.084427	0.042214	0.15366	0.42234	0.180	0.378
	1		4	0.21600	0.041569	0.020785	0.14985	0.28215	0.180	0.252
	0.25		4	0.19350	0.027000	0.013500	0.15054	0.23646	0.180	0.234
	Total		12	0.23250	0.066229	0.019119	0.19042	0.27458	0.180	0.378
	Model	Fixed Effects			0.056524	0.016317	0.19559	0.26941		
		Random Effects				0.028500	0.10987	0.35513		
G05	0.05		4	0.42300	0.198545	0.099272	0.10707	0.73893	0.252	0.666
	0.1		4	0.23400	0.076368	0.038184	0.11248	0.35552	0.180	0.342
	0.25		4	0.23850	0.084906	0.042453	0.10340	0.37360	0.180	0.360
	Total		12	0.29850	0.150884	0.043556	0.20263	0.39437	0.180	0.666
	Model	Fixed Effects			0.132238	0.038174	0.21214	0.38486		
		Random Effects				0.062264	0.03060	0.56640		
G10	0.05		4	0.4680	0.18060	0.09030	0.1806	0.7554	0.36	0.74
	0.1		4	0.3375	0.20543	0.10271	0.0106	0.6644	0.18	0.61
	0.25		4	0.2250	0.05970	0.02985	0.1300	0.3200	0.18	0.31
	Total		12	0.3435	0.17926	0.05175	0.2296	0.4574	0.18	0.74
	Model	Fixed Effects			0.16164	0.04666	0.2379	0.4491		
		Random Effects				0.07021	0.0414	0.6456		

Test of Homogeneity of Variances				
	Levene Statistic	df1	df2	Sig.
G025	2.779	2	9	0.115
G05	5.072	2	9	0.033
G10	2.323	2	9	0.154

Table B-10

ANOVA						
		Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**

G025	Between Groups	0.019	2	0.010	3.051	0.097
	Within Groups	0.029	9	0.003		
	Total	0.048	11			
G05	Between Groups	0.093	2	0.047	2.660	0.124
	Within Groups	0.157	9	0.017		
	Total	0.250	11			
G10	Between Groups	0.118	2	0.059	2.264	0.160
	Within Groups	0.235	9	0.026		
	Total	0.353	11			

Table B-11

Robust Tests of Equality of Means					
		Statistic[a]	**df1**	**df2**	**Sig.**
G025	Welch	2.159	2	5.292	0.206
	Brown-Forsythe	3.051	2	5.073	0.135
G05	Welch	1.467	2	5.564	0.308
	Brown-Forsythe	2.660	2	5.035	0.163
G10	Welch	3.158	2	4.664	0.136
	Brown-Forsythe	2.264	2	6.450	0.180
a. Asymptotically F distributed.					

APPENDIX C

PICTURES OF LABORATORY WORK