OPTIMIZATION OF THE PRETREATMENT AND HYDROLYSIS OF PLANTAIN STEM *(MUSA PARADISICA)* USING CELLULASE FROM SNAIL *(ACHATINA MARGINATA)* FOR THE PRODUCTION OF BIOETHANOL

BY

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MTECH/SSSE/2008/2113

DEPARTMENT OF BIOCHEMISTRY

FEDERAL UNIVERSITY OF TECHNOLOGY

MINNA, NIGER STATE.

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A THESIS SUBMITTED TO THE POSTGRADUATE SCHOOL, FEDERAL UNIVERSITY OF TECHNOLOGY, MINNA IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF THE DEGREE OF MASTERS OF TECHNOLOGY (M.TECH) IN BIOCHEMISTRY

DEPARTMENT OF BIOCHEMISTRY

FEDERAL UNIVERSITY OF TECHNOLOGY, MINNA

NIGER STATE, NIGERIA.

MAY, 2012

DECLARATION

I hereby declare that this thesis titled Optimization of the pretreatment and hydrolysis of plantain stem (*Musa paradisiacal*) using cellulase from snail *(Achatina marginata*) for the production of bioethanol is a collection of my original research work and has not been presented for any other qualification anywhere. Information from other sources (published or unpublished) has been duly acknowledged.

.... 9/06/12

OSEKI OMO PETER SIGNATURE/DATE

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FEDERAL UNIVERSITY OF TECHNOLOGY,

MINNA,NIGERIA

CERTIFICATION

This thesis titled Optimization of the pretreatment and hydrolysis of plantain stem (*Musa paradisiacal*) using cellulase from snail (*Achatina marginata*) for the production of bioethanol by Oseki Omo Peter, MTECH/SSSE/2008/2113 meets the regulations governing the award of the degree of Masters of Technology of the Federal University of Technology, Minna and it is approved for its contribution to scientific knowledge and literary presentation.

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DEDICATION

This work is dedicated to God Almighty for His grace towards the successful completion of this work.

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ABSTRACT

The efficacy of crude cellulase from the haemolyph of a giant African Snail (*Achatina marginata*) on the hydrolysis of plantain stem biomass (*Musa paradisacal)* was investigated. The biomass were pretreated with $Ca(OH)_2$ and HCl optimized at 1N and 5N for 10, 20 and 30mins.The pretreated biomass were hydrolyzed with crude snail cellulase optimized at 30, 40 and 50^0C for 30mins, 60mins, 120mins and 180mins and the result showed that, glucose had an optimal yield of 197± 0.01mg/100g sample at 1N-30mins $Ca(OH)_2$ pretreatment (optimal pretreatment condition) before and an optimal yield of 291 ± 0.22mg/100g sample at 180mins hydrolytic time (optimal hydrolytic time) . There was no significant difference in glucose yield at 30, 40 and 50^0c incubation temperature. The ethanol distillate obtained was 76% (volume by volume) with methane, ethane, propane and butane as constituent. Thus, snail could be a good source of cellulase for hydrolysis of plantain stem to ethanol and other value added products via $Ca(OH)_2$ pretreatment.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

1.0 INTRODUCTION

Biofuel is a renewable fuel that can be synthesized from edible, non-edible and waste oils. Due to the diminishing petroleum reserve and the alarming environmental consequences arising from fossil fuel combustion, non – edible waste have attracted attention as a potential renewable source of energy to the petroleum based diesel fuel. A number of processes have been developed for biofuel production involving chemical or enzyme analysis or supercritical alcohol treatment (Fukuda *et al.*, 2001). Example of such process is separate hydrolysis and fermentation that has proven effective in the production of biofuel and hence was adopted in this experiment (Brown *et al.*, 2000).

Many starting materials such as soya bean oil, sunflower oil ,cotton seed oil, rage seed oil, palm oil and restaurant kitchen waste have been evaluated for preparation of biodiesel by enzymatic route ,but due to their edible nature (e.g. palm oil) ,it has been greatly discouraged in many African (Nigam, 2001). Hence the search for alternative starting material such as plantain stem that is not widely used as food.

Bioethanol is mainly produced from fermentation of sugar containing materials such as molasses, sugar cane (cane juice or cane syrup), series crops sugar beef and sweet sorghum. Recent biotechnological developments have led to an increased focus on utilization of lingo cellulose biomass as a resource for the production of liquid fuels and other chemicals. Multiple biomass substrates have been identified to hold a great potential due to their high content of cellulose and hemicelluloses combined with abundant annual production (Wiselogel, *et al.*, 1996).

The main challenge in the conversion of biomass into ethanol is the pretreatment step.Due to the structure of the lignocelluloses complex, the pretreatment is required for its degradation, the removal of lignin, the partial or total hydrolysis of hemicelluloses and the decrease in the fraction of crystalline cellulose(amorphous cellulose) the most suitable form for the subsequent hydrolysis step (Sander *et al.*, 2007).

After pretreatment, cellulose is subjected to enzymatic hydrolysis in order to obtain glucose that is transformed into ethanol by the effect of micro organism. Eventually the sugar released during the hydrolysis of hemicellulose can be converted into ethanol. Industrially, the pretreated materials is mainly thought to be hydrolysed and fermented in two different steps: Separate Hydrolysis and Fermentation (SHF) or in a single step – Simultaneous Saaccharification and Fermentation (SSF). A few microbial species such as Neurospora, Monilla, Paecilomyces and Fusarium sp. have been reported to hold the ability to ferment cellulose directly to ethanol (Sander *et al*, 2007) and hence in the course of this work, SHF was adopted using yeast enzyme as a fermenter.

Conclusively, since lignocelluloses materials are by product of agricultural activities, industrial residues and domestic waste, it holds a chance of becoming the main feed stock for ethanol production in the near future

1.1 Aim of the Research

The research is aimed at producing biofuel from the stem of a plantain (*Musa paradisica*) using cellulase from snail (*Achatina marginata)*.

The objectives of the research is as follows:-

(i) To determine the cellulase activity in snail and show the location (Gut or haemolyph) with the highest cellulolytic activity.

(ii) To study the effect of Ca $(OH)_2$ and HCl pretreatment on the plantain stem by estimating the % Lignin, Hemicelluloses, cellulose and total carbohydrate before and after pretreatment.

(iii) To study the effect of Ca $(OH)_2$ and HCl pretreatments on the fermentable sugar by estimating the concentration of Glucose, Xylose, Arabinose, Mannose and Galactose before and after pretreatment.

(iv) To study the hydrolytic effect of snail cellulase on the fermentable sugar of the plantain stem by estimating the increase in the concentration of the resulting sugars after Ca $(OH)_2$ and HCl optimized hydrolysis.

(v) To estimate the % yield of ethanol produced from the acidic (HCl) and Basic [Ca $(OH)_2$] pretreated plantain stem biomass.

(vi) To estimate other constituent present in plantain stem ethanol and suggest if it will be a good source of industrial biofuel or not.

1.2 Justification

The quest for alternative sources of fuel has persisted and the need to turn agro waste into useful product via chemical and enzymatic processes has become of keen interest (Klinke *et al.*, 2003). This is so majorly in Africa where our non-renewable fuel is constantly decreasing couple with insufficient food for the populace. Hence, this research is aimed at converting lignocellulosic waste into biofuel for man's use.

1.3 Scope of the Research

The present study which shows the bioconversion of plantain stem hydrolysate into ethanol using cellulase from snail involves 5 steps as highlighted below:

i. Pretreatment

The plantain stem biomass was pretreated with 1N and 5N $Ca(OH)_2$ and HCl optimized for 10, 20 and 30mins and there after, several analysis was carried out to evaluate the best pre-treatment option.

ii. Hydrolysis

The pretreated sample was hydrolysed with snail cellulase to study the effect of the .snail cellulase on the fermentable sugar and confirms it hydrolytic ability.

iii. Fermentation / Distillation / GC Analysis

The hydrolysed sample was fermented with yeast and the resulting solution crude (ethanol) was distilled to obtained a specific % ethanol which was subjected to Gas chromatography analysis using Flame Ionisation Detector (with Helium carrier at 5PSI) to estimate other constituent of the biofuel.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Plantain Biomass

Plantain botanically called *Musa paradisiaca* is generally used for cooking in contrast to the soft, sweet bananna (which is sometime called dessert bananna). Plantain tends to be firmer and lower in sugar content than dessert banana and is a staple food in the tropical regions of the world. Regions with plantain crops include the Southern United States, Hawaii, Caribbean, Central America, Bolivia, Southern Brazil, Nigeria, Columbia, India, Malaysia, Egypt, Cameroon, Ghana, and Thailand among others. (Omojasola *et al.*, 2008)

2.1.1 Plantain Flowers

Each pseudostem of a plantain plant will flower only once and all the flowers grow at the end of its shoot in a larger bunch consisting of multiple hands with individual fingers (the fruits). Only the first few hands will become fruits.

In Vietnam, the young male flower at the end of the bunch is used in salad. In the cuisine of laos, the plantain flower is typically eaten raw in vermicelli soups.

A type of poriyah (dry curry) is made from plantain flowers in Tamil Nadu. Thoran is made in kerala with the end of the bunch and is considered to be highly nutritious. (Osagie and Eka, 1998)

2.1.2 Plantain Leaves

Plantain leaves can exceeds two meters in length. They are similar to banana leaves but are larger and stronger, thus reducing waste in cooking. In Latin America, plantain

leaves are lightly smoked over an open fire which improves storage properties, flavour and aroma. In Venezuela, they are fairly widely available in grocery stores or open air markets and are used as wrapper in hallacas. In Africa, plantain leaves are dried and used to wrap corn and bean dough before it's boiled to fanti kenkey, a Ghanaian dish eaten with ground pepper, onions, tomatoes and fish (Milala *et al.*, 2005).

Traditionally, plantain leaves are used like plates while serving South Indian thali or during sadya and are said to have a religious significance in many Hindu rituals as they help add essential aroma to the dish. (Baig *et al.*, 2004)

2.1.3 Plantain Shoot / Stem

After harvesting the fruit, the plantain plant can be cut, and the layers peeled (like an onion) to get a cylinder shaped soft shoot. In the South Indian state of Tamil Nadu, plantain shoot is chopped into fine pieces and made as a salad or dry curry. Plantain shoot is considered rich in fibres and is considered as a very good remedy for avoiding constipation. Regular intake of the juice squeezed from the shoot or the shoot consumed as a salad is considered by the locals as sure cure for various ailments such as stomach ulcer and kidney stones. The dried stem peels are slit into fine threads and are used for weaving mats, stringing garlands and packaging wrapper. Juice from the stem and peel have also been used traditionally as a first aid for burns and minor abrasion while some claims it has some medical properties (Baig *et al.*, 2004).

2.2 Snail – A Natural Source of Enzyme

Snail is a common name for almost all member of the molluscan class gastropoda that have coiled shells in the adult stage. When the word is used in a general sense, it includes sea snails, land snails and fresh water snails. Otherwise snail – like creatures that lack a shell (or have only a very small one) are called slugs.

One species of land snails, the giant African snail, can grow to be 15 inches (38cm) snout to tail, and weigh 2 pounds (0.91kg). the largest living species of sea snail is syrinx aranus which has a shell that can measure up to 91cm (36in) in length, and the whole animal with the shell can weigh up to 18kg (40 1b).

Snail can be found in a very wide range of environments including ditches, deserts, and the abyssal depths of the sea. Although many people are familiar with terrestrial snails, land snails are in the minority. Marine snails constitute the majority of snail species, and have much greater diversity and a greater biomass. Numerous kinds of snail can also be found in fresh water and they are mostly herbivourous.Several works have been done on snail which revealed its digestive and cellulolytic abilities. Krishna (2009) studied the digestive enzyme and cellulolytic bacteria in the gut of giant land snail (*Achatina fulica*) and revealed the following; that

i Amylase is present in the saliva, gastric,intestine, digestive glands and in small amount in the stomach and rectum

ii Protease is present in the gastric juice, stomach, intestine and digestive gland

iii. Saliva and rectum contain traces of protease

iv. Lipase is present in gastric juice, stomach, intestine, rectum and digestive gland but not in saliva.

v. Cellulase is present in the digestive gland and

vi. Cellulolytic bacterias are present in the intestine

Hence in this work, cellulase activities were investigated in the snail and haemolyph to find out which was of better source of cellulase.

2.3 Lignocelluloses- A Valuable Resource

Cellulosic biomass provides a low cost and abundant resource that has the potential to support large-scale production of fuels and chemicals. Lignocellulosic biomass include materials such as agricultural residues (e.g. corn stover and wheat straw), forestry residues (e.g. sawdust, thinnings, and mill wastes), portions of municipal solid waste (e.g. waste paper) and various industrial wastes. Herbaceous (e.g. switchgrass) and woody crops can also be used.

Lignocellulosic materials are formed from three main components: cellulose, hemicelluloses, and lignin. A variety of fungi and bacteria can break down lignocelluloses by using a battery of hydrolytic and oxidative enzymes. Cellulose is the major component in plant cell walls and constitutes up to 50% of the dry weight of wood. Cellulose and hemicelluloses can be hydrolysed to sugar that can be further converted, either microbially or chemically, into energy carriers such as ethanol and butanol, or various other products such as organic acids, acetone, or glycerol (Galbe, 2002). The hexose sugars glucose, galactose, and mannose can be easily metabolized by conventional yeast, *Saccharomyces cerevisiae.* The hydrolysis products of xylans require organisms capable of fermenting both hexoses and pentoses.

Due to the crystalline structure of cellulose as well as the complex structural organization of cellulose, hemicelluloses, and lignin, lignocellulosic materials are

more difficult to break down than, for example, starch based materials. Sugar monomers can be produced from cellulose and hemicelluloses either by acids or by hydrolytic enzymes. Saccharification of cellulosic biomass by acid has a long history, and both dilute-acid technologies at high temperature and concentrated acids at lower temperatures have been studied (Galbe *et al.*, 2002). Enzymatic degradation of lignocelluloses involves a set of different enzymes. A wide variety of cellulolytic fungi and bacteria have been reported to date and this number is continually increasing.

The most abundant source of raw materials available for energy production in Finland consists of forest residues, of which the final cutting site residues and first thinning comprise the highest potential for bioenergy production. Other interesting sources would be side streams from the pulp and paper industry. Currently, the growing stock volume in Nigeria is about 2billion m^3, consisting mainly of softwood, namely pine (47%) and spruce (34%), and the hardwood birch (15%) , Bark (8million m^3), sawdust (2.3 million m^3) and forest and industrial chips (1.9) million m^3), are used for energy generation in Nigeria, but solely as solid fuel (Sander *et al.*, 2007).

Softwood, the dominating source of lignocelluloses in the Northern hemisphere, had been the subject of interest as a raw material for fuel ethanol production in Sweden, Canada and Western USA (Sander *et al.*, 2007). Ethanol fuel can reduce greenhouse gas emissions and improve air quality as well as offer strategic or economical advantages (Bolin, 1998). Ethanol is already produced (from sugar cane and maize) in large quantities in Brazil and United States to replace petrol in motor vehicles. Today, all cars equipped with a catalyst can run on a mixture of 90% gasoline as fuel

(Bolin,1998). Ethanol is also used to produce an oxygenated fuel additive, ethyl butyl ether (ETBE), which is formed in the reaction between ethanol and isobutylene. France and Spain, the largest fuel ethanol producers in the European Union, produce ETBE from ethanol. Several comprehensive reviews on the economics and challenges of ethanol production from biomass, as well as the use of ethanol as fuel are available (Fukuda *et al.*, 2001).

Over the past two decades the cost of biological conversion of cellulosic biomass to ethanol has been reduced from about 1.22 USD/l to the point where it is becoming economically viable. The present ethanol production cost, estimated by NREL (National Renewable Energy Labouratory, U.S.), is 0.31 USD/l, and is expected to decrease to about 0.22 USD/l by the year 2010 (Classen *et al.*, 1999). This is mainly due to advances in biosciences, leading to decreased cost of cellulolytic enzymes and development of more efficient organisms for fermentation (Classen *et al.*, 1999). Presently, enzymatic hydrolysis is considered the most promising technology for converting biomass into sugars and to be used as raw material for the production of various other biotechnical bulk chemical products.

2.4 Structural Features of Lignocellulosic Material

The major components of lignocellulosic materials are cellulose, hemicelluloses, lignin and extractive. The wood cells are composed of different layers, which differ from one another with respect to their structure and chemical composition. Basically, cellulose forms a skeleton which is surrounded by other substances functioning as matrix (hemicelluloses) and encrusting (lignin) materials. Cellulose, hemicelluloses and lignin are closely associated and covalent cross-linkages have been suggested to

occur between lignin and polysaccharides (lignin-carbohydrate complexes, LCC).The side-groups arabinose, galactose and 4-0- methylglucuronic acid are most frequently perceived as connecting links to lignin (Mosier *et al.*, 2005). It is generally agreed that the hemicelluloses molecules are oriented parallel to the cellulosic fibrils as shown in Figure 1

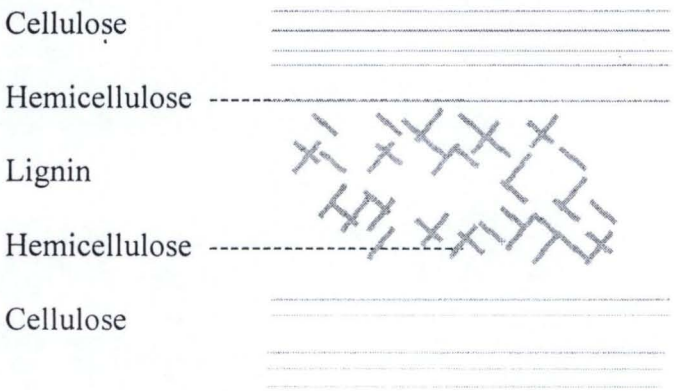

Figure 1: Proposed Model for the Structure of Softwood Lignocellulose

Source: Mosier *et al.*, 2005

The composition of agricultural wastes materials or woody biomass depends on the plant species and age, growth conditions and fractionation or processing steps. The compositions of different lignocellulosic materials are given in Table 1. Generally, the lignin content of softwoods is higher than hardwoods due to differences in their chemical composition.

2.4.1 Cellulose

Cellulose is the main constituent of plant cell walls comprising about 50% of wood as seen in Table 1. Cellulose is closely associated with hemicelluloses and lignin and the isolation of cellulose requires intensive chemical treatments. Cellulose consists of D-glucopyranose monomer units bound by ß-1-4-glycosidic linkages. The successive

glucose residues are rotated by 180^0 relative to each other, and thus the repeating unit of the cellulose chain is the cellobiose unit. The average degree of polymerization (DP) of plant cellulose varies between 7000 and 15000 glucose units, depending on the source (Mosier *et al.*, 2005).

The functional groups in the cellulose chain are the hydroxyl groups. These OH-groups are able to interact with each other or with O-, N-, and S-groups, forming hydrogen bonds. H-bonds also exist between OH-groups of cellulose and water molecules. These hydroxyl groups make the surface of cellulose largely hydrophilic. The cellulose chain has OH-groups at both ends. The C1-end has reducing properties. The cellulose chain is stabilized by strong hydrogen bonds along the direction of the chain. In native cellulose found in plant sources, cellulose chains are packed together to form highly crystalline microfibrils in which the individual cellulose crystal contains tens of glucan chains in a parallel orientation. Seven crystal polymorphs have been identified for cellulose, which are designated as Iα Iß, II, III_I, III_{II}, IV_I, and IV_{II} (Hayn *et al.*, 1993). In nature, cellulose Iα, and Iß, are the most abundant crystal forms. In addition to highly crystalline regions, native cellulose contains less-ordered amorphous regions. In wood fibers, the winding direction of cellulose microfibrils varies in different cell wall layers. Several reviews have surveyed cellulose structure and it is still the subject of intense study (Brown *et al.*, 2000).

Table 1. Chemical composition of various lignocellulosic materials (percent of dry weight)

Lignocellulose	Cellulose	Xylan	Mannan	Galactan	Arabinan	Lignin	Extractives	Ref.
Spruce wood	41.9	6.1	14.3	na	1.2	27.1	9.6	Hayn *et al.*, 1993
Pine wood	37.7	4.6	7.0	na	na	27.5	10.8	Hayn *et al.*, 1993
Birch wood	38.2	18.5	1.2	na	na	22.8	4.8	Wiselogel *et al.*, 1996
Poplar wood	49.9	17.4	4.7	1.2	1.8	18.1	na	Wiselogel *et al.*, 1996
Corn stover	36.4	18.0	0.6	1.0	3.0	16.6	7.3	Wiselogel *et al.*, 1996
Wheat straw	38.2	21.2	0.3	0.7	2.5	23.4	13.0	Wiselogel *et al.*, 1996
Switchgrass	31.0	20.4	0.3	0.9	2.8	17.6	17.0	Wiselogel *et al.*, 1996

na not available

Source: Nigam, 2011

2.4.2 Hemicelluloses

Hemicelluloses are generally classified according to the main sugar residue in the backbone, e.g. xylans, mannans, galactans and glucans, with xylans and mannans being the main groups of hemicelluloses. Hemicelluloses are often reported to be chemically associated with or cross-linked to other polysaccharides, proteins or lignin. Xylans appear to be the major interface between lignin and other carbohydrates . Hemicelluloses are more soluble than cellulose, and they can be isolated from wood by extraction. However, alkali extractions deacetylate the hemicelluloses completely . The average degree of polymerization of hemicelluloses varies between 70 and 200 depending on the wood species (Hayn *et al.*, 1993).

Hemicelluloses in hardwoods and annual plants is mainly xylan (15-30%), whereas softwood hemicelluloses consist of galactoglucomannans (15-20%) and xylans (7-10%). Hardwood xylan is composed of ß-D-xylophyranosyl units, which contain 4-O-methylglucuronic acid is linekd to the xlan backbone by O-(1 2) glycosidic bonds and acetic acid is esterified at the carbon 2 and/or 3 hydroxyl group. The molar ratio of xylose: glucuronic acid:acetyl residues is about 10:1:7. Softwood xylans are arabino 4-O-methlylglucuronxylans, which are not acetylated, but the xylan backbone is substituted at carbon 2 and 3 with 4_O-methyl-α-D-glucuronic acid and α-L-arabinofuranosyl residues respectively (Wiselogel *et al.*, 1996).

Softwood galactoglucomannan has a backbone of ß-1-4-linked ß-D-glucopyranosyl and ß-D-mannophyranosyl units, which are partially substituted by α-D-galactopyranosyl and acetyl groups. Two types of galactoglucomannans can be separated; water and alkali soluble fractions, with ratios of mannose:glucose:

galactose:acetlyl residues 3:1:1:1:0.24 for the water soluble fraction, and 3 :1 : 0.1 : 0.24 for the alkali soluble fraction (Hayn *et al*,1993).

2.4.3 Lignin

Lignin is a complex hydrophobic, cross-linked aromatic polymer.ln nature, lignin is mostly found as an integral part of the plant cell wall, embedded in a carbohydrate polymer matrix of cellulose and hemicelluloses. lsolation of native lignin is complicated, when at all possible (Wiselogel *et al.*, 1996).

Lignin are polymers of phenylpropene units: guaiacyl (G) units from the precursor trans-coniferyl-alcohol, syringyl (S) units from trans-sinaphy-alcohol, and p-hydroxyphenyl (H) units from the precursor trans-p-coumaryl alcohol. The exact composition of lignin varies widely with species. In addition to classification as softwood, hardwood and grass lignins, lignins can be divided into two major groups: guaiacyl lignins and guaiacyl-syringyl lignin (Hayn *et al*,1993). Guaiacyl lignins are predominantly polymerization products of coniferyl alcohol while guaiacyl-syringyl lignins are composed of varying parts of the aromatic nuclei guaiacyl and syringyl, together with small amounts of p-hydroxyphenyl units. (Wiselogel *et al.*, 1996).

Softwood contains mainly guaiacyl units while hard wood contains also syringyl units. For spuce (*Picea abies*) a ratio G:S:H=94:1:5 has been reported (Erickson *et al.*, 1973), and for pine (*Pinus taeda)* G:S:H=86:2:13.(Glasser, 1981). It has been found that softwood is more resistant to lignin removal by alkaline extraction than hardwood (Ramos *et al.,* 1992). It has been suggested that guaiacyl lignin restricts fibre swelling and thus the enzymatic accessibility more than syringyl lignin.it was observe that the

residual substrate remained after extensive hydrolysis of steam pretreated aspen and eucalyptus was mainly composed of vessel elements.vessel elements are known to have a greater guaiacyl to syringyl ratio than other cells found in hardwood (Ramos *et al.*,1992).The more resistant structure of guaiacyl lignin has also been observed in degradation studies of (synthetic) lignins by the lignin-degrading fungus *Phanerochaete chrysosporium* (Faix *et al.*, 1985).

Recent observations have given indications that all lignin is not homogeneous in structure.Lignin seems to consist of amorphous regions and structured forms such as oblong particles and globules (Novikova *et al.*,2002).Lignin in higher plant cell wall is not amorphous. Phenyl rings of softwood lignin have been shown to be aligned preferentially in the plane of the cell wall. There are also indications that both the chemical and three-dimensional structute of lignin is strongly influenced by the polysaccharide matrix (Houtman and Atalla, 1995). Molecular dynamic simulations have suggested that the hydroxyl and methoxyl groups in lignin precursors and oligomers may interact with cellulose microfibrils despite the fact that lignin is hydrophobic in character (Houtman and Atalla, 1995).

The chemical structure of native lignin is essentially changed under high temperature and acidic conditions, such as conditions during steam pretreatment. At reaction temperatures higher than 200^0C, lignin has been shown to be agglomerated into smaller particles and separated from cellulose. (Hayn *et al.*, 1993).

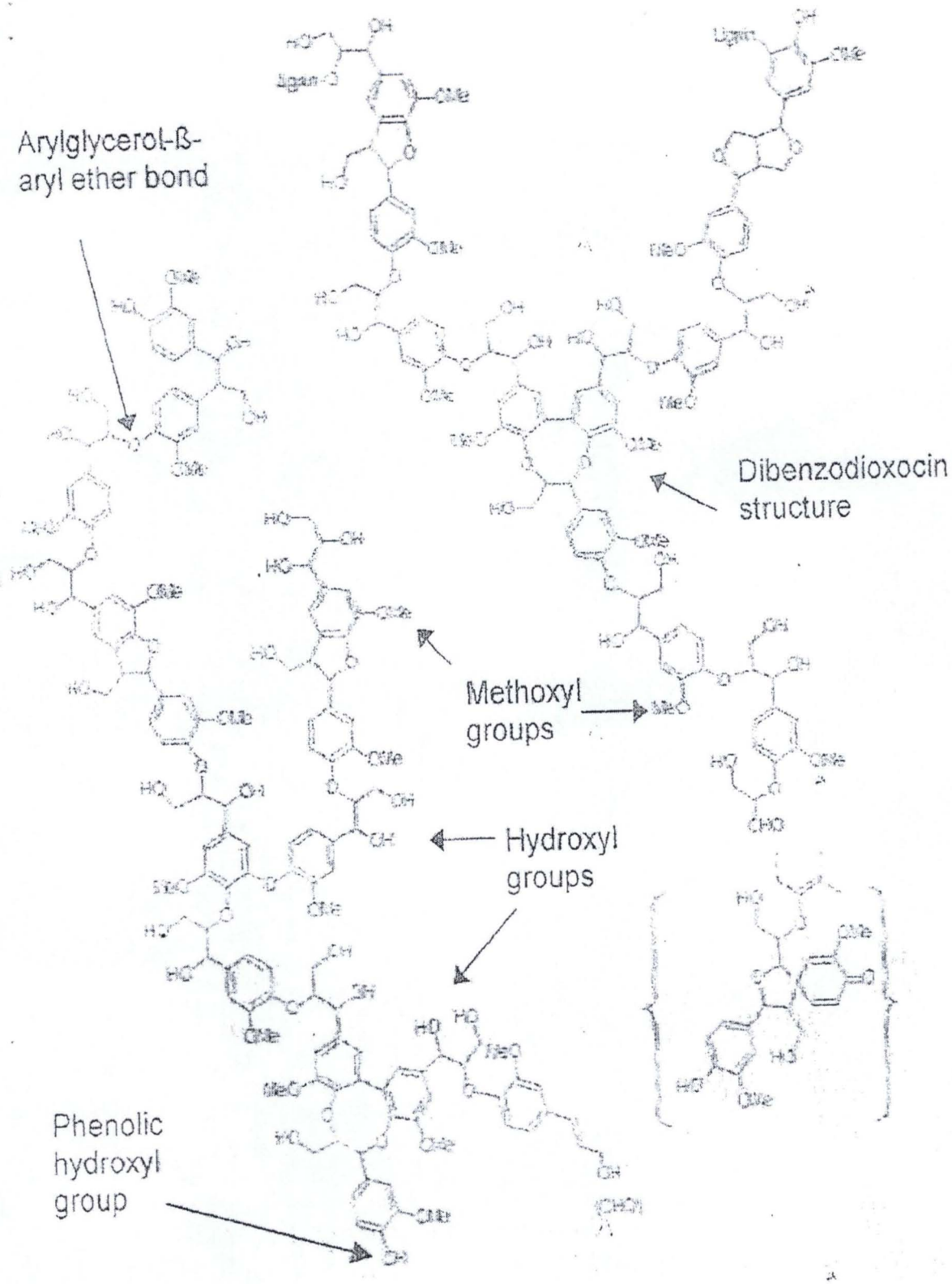

Figure 2. The Structure of softwood lignin (Brunow, 1998). The most common structures and functional groups in the lignin molecule are pointed out.

2.5 Industrial Enzymes

Enzymes are proteins that catalyze and control the rate of chemical reactions. In enzymatic reactions, the molecule at the beginning of the process is called substrate and the enzymes convert the substrate into different molecules known as the products (Galbe *et al.*, 2002).

Industrially, enzymes are defined as any of the numerous protein that are produced by living cells which catalyzes various biochemical reactions.

2.5.1 Advantages of Industrial Enzymes

i They are specific in their actions and therefore less likely to produce unwanted products.

ii They are biodegradable and so can cause less environmental pollution.

iii They work in mild conditions at low temperatures, neutral pH and normal atmospheric pressure.

iv They are energy saving.

Despite the advantages of industrial enzymes, they still have disadvantages.

Note: Work conditions must be stringently controlled else the enzymes may be denatured.

2.5.2 Types of Industrial Enzymes

2.5.2.1 Carbohydrases

Carbohydrases are a group of enzymes that either promotes the synthesis or hydrolysis of carbohydrates into disaccharide molecules.

Examples of carbohydrases are Maltases, Sucrases, Amylases and Lactases (Galbe *et al.*, 2002).

2.5.2.2 Proteases

Proteases are enzymes that breakdown peptide bonds between amino acids of proteins. The process is called proteolytic cleavage. Proteases are classified into two Exo-proteases (they cleave off single amino acids from either end of the protein chain) and Endo-proteases (they attack peptide bonds on the interior of the protein chain).

Industrially, proteases are used

i For laundry detergents where they help remove protein based stains from clothing.

ii In cheese making.

iii For modifying food ingredients.

iv As meat tenderizers and flavor developer.

Examples of proteases are neutral acid and alkaline proteases,bromalain, rennin, trypsin and thermolysin.

2.5.2.3 Lipases

Lipases are water soluble enzymes that catalyzes the hydrolysis of ester bonds in water soluble substrate (Svendsen, 2000). It performs essential role in digestion, transportation and processing of dietary lipids in all living organisms. Most lipases act at specific positions in the glycerol back bone of the lipid substrate (A1, A2 or A3). Myriad of other lipases occur especially the phospholipases and sphingomyelinases . Industrially, lipases from fungi and bacteria serve important roles in human practices (yoghurt and cheese fermentation, exportation as biocatalysts). For instance, lipases are used in baking and laundry detergents (Dossat *et al.*, 2002)

Example of lipases are lysozyme, penicillum acyclase, amidases, phosphatases, phospholipases and triglyceridases.

2.5.2.4 Lyases

Lyase is a class of enzymes that catalyses the cleavage of C-C,C-O,C-N and other bonds by means other than hydrolysis or oxidation. They often form new bonds after the reactions. Typical example is in the reaction shown below;

$$ATP \longrightarrow c\ AMP + PPi$$

Their reactions differ from other enzymatic reactions in that a substrates is required for the reaction in one direction and two others for the reaction in the opposite direction.

Examples of enzymes in the class are Fumarases, Histadases, Aspartic B-decaboxylases and acetolactate decarboxylase.

2.5.2.5 Transferases

Transferases are enzymes that catalyze the transfer of a functional group (example is a methyl or phosphate group) from molecule (donor) to another (acceptor). One important example of those used industrially is cyclodxtringlycerol transferases (Sterling, 2006).

2.5.2.6 Nuclease

Nucleases are class of enzymes that catalyses the hydrolysis of nucleic acids (DNA, RNA) in all organisms. Nucleases have possible application in science, commerce and industry in that they can be exploited to make new products. For example nucleases are used for diagnosis and treatment of diseases when their structure and properties are well understood.

Molecular cloning makes possible the understanding of application of nucleases and also leads to identification and characterization of gene responsible for diseases. It

also enhances the possible alleviation of diseases gene therapy and development of designer drugs (Osagie *et al.*, 2002).

2.5.3 Sources of Industrial Enzymes

Biologically active enzymes may be extracted from any living organism. A wide range of source (Table 2.0) are used for commercial enzymes production; from Actinoplanes to Zymomonas, spinach to snake venom.

At present times, microbes are preferred to plants and animals as sources of enzymes. Reasons are because;

i Microbes are generally cheaper to produce

ii Their enzymes content are more predictable and controllable.

iii Plants and animal tissues contain more potentially harmful materials than microbes. Some of these harmful materials are phenolic compounds (from plants), endogenous enzymes inhibitors and proteases (Omojasola *et al.*, 2008).

Most of the enzymes used industrially have different origins. There origin could be plant, animal or microbial. Table 2.0 shows the various enzymes, their sources and EC numbers.

TABLE 2.0 Some Important Industrial Enzymes and their Sources

ENZYME	EC NUMBERS	SOURCES
1) ANIMAL ENZYMES		
Renner	3.4.23.4	Abomasums
Lipase	3.1.1.3	Pancreas
Catalase	1.11.1.6	Liver
Trypsin	3.4.21.4	Pancreas
2) PLANT ENZYMES		
Actinidin	3.4.22.14	Kiwi Fruit
& - Amlase	3.2.1.1	Malted barley
B-amylase	3.2.1.2	Malted barley
Bromelain	3.4.22.4	Pineapple latex
b-glucanase	3.2.1.6	Malted barley
Ficin	3.4.22.3	Fig latex
Lipoxygenase	1.13.11.12	Soya beans
3) BACTERIAL ENZYMES		
Pullulanase	3.2.1.41	Klebsiella
Asparaginase	3.5.1.1	Escherichia coli
Pencillin amidase	3.5.1.11	Bacillus
Protease	3.4.21.14	Bacillus
4) FUNGAL ENZYMES		
&-Amylase	3.2.1.1	Aspergillus
Celullase	3.2.1.4	Trichoderma
Lactase	3.2.1.23	Aspergillus
Lipase	3.1.1.3	Rhizopus
Rennet	3.4.23.6	Mucor miehei
Raffinase	3.2.1.22	Mortierella
5). YEAST ENZYMES		
Invertase	3.2.1.26	Sacchromyces
Lactase	3.2.1.23	Kluyveromcyes
Blipase	3.1.1.3	Candida
Raffinase	3.2.1.22	sacchromyces

Source: Omojasola *et al.*, 2008

2.5.4 Application of Industrial Enzymes.

Enzymes are used industrially when specific catalyst are required. However, enzymes in general are limited in the number of reactions they have evolved to catalyst. They are not stable in organic solvents and at high temperatures (Osagie *et al.*, 1998).

TABLE 2.1 Industrial Enzymes and their Common Uses

Application	Enzymes used	Uses
Baking Industry	Fungal Alpha Amylase	Catalyse breakdown of starch in flour to sugars used in production of white bread, buns and rolls. They are used by biscuit manufactures to lower the protein level of flour
Baby foods	Trypsin	To predigest baby foods
Brewing industry	Enzymes from barley, released during mashing stage of beer production.	They degrade starch and proteins to produce simple sugar, amino acids and peptides that are used by yeast for fermentation.
	Amylase, Glucanases, proteases	Split polysaccharides and proteins in mall
	Acetolactate decaboxylase (ALDC)	Avoid the formation of diacatyl
	Amyloglucosidase and pullubanases	Low calorie beer and adjustment of fermentability.
Paper industry	Amylases, Xylanases, cellulases	Degrade starch to lower viscosity aiding sizing and coating paper. Xylanases reduce bleach required for decolorizing; cellulase smooth fibers, enhance water drainage and promote ink removal; lipases reduce pitch and lignin – degrading enzymes remove lignin to soften paper.
Starch	Amylases	Coverts starch into glucose and various syrups
	Amyloglucosidases and glucoamlases	
	Glucose isomerase	Converts glucose into fructose in production of high fructose syrups from starchy materials.
Biofuel industry	Cellulases	Used to breakdown cellulose into sugars that can be fermented.
	Ligninases	Used on lignin waste.

Source: Osagie *et al.*, 2008

These enzymes are in one way or the other involved in the hydrolysis of lignocellulosic materials into the end products – reducing sugars.

2.5.5 Cellulases

Cellulase is responsible for hydrolysis of cellulose. It is composed of a complex mixture of enzymes proteins with different specificities to hydrolysis glycosidic and can be of diverse sources.

Cellulase can be of plant and microbial origin; plants such as Asha. The activity of cellulase can be tested at pH range 8-12. Cellulase are of three classes

2.5.5.1 Endoglucanases

Endoglucanses are often called carboxymethylcellulase (CM – cellulases). They are proposed to initiate attack randomly at multiple sites in the amorphorus region of the cellulose fibre. Endoglucanases opens up sites in the cellulose fibre for subsequent attack by the cellobiohydrolases (Vikari *et al.*, 2001)

2.5.5.2 Cellobiohydrolases

It is often called exoglucanase. It is a major component of the fungal cellulose system. Cellobiohydrolases accounts for 40-70% of the total cellulose proteins.It can hydrolyse highly crystalline cellulose removing mono-and dimmers from the end of the glucose chains.

2.5.5.3 Beta (B) Glycosidase

B-glucosidases hydrolyse glucose dimmers and in some cases cello-oligosaccharides to glucose. Addition of B-glycosidases is needed to prevent inhibition by cellobiose

2.5.6 Hemicellulases

These are enzymes that hydrolyses hemicelluloses.ln wood,the two most common hemicelluloses are xylans and glucomannans and for total enzymatic hydrolysis of hemicellulosic polysaccharides,several synergistically acting enzymes are needed (Vikari *et al*,2001).Endo-xylanases (EC 3.2.1.8) and Endo-mannanases (EC 3.2.1.78) attack the main chain of xylan and glucomannan respectively.Enzymes needed for further hydrolysis of the short oligomeric compounds produced by endo-enzymes from hemicelluloses are β-xylosidase, β-mannosidase and β-glucosidase.side groups which are attached to oligosaccharide the action of xylanase and mannanase are removed by α-arabinosidase and D-galactosidase.Acetyl groups bound to hemicelluloses are removed by esterases (Vikari *et al.*, 2001).

2.6 Pretreatment of Cellulosic Materials

It is generally known that cellulose is resistant to enzymatic hydrolysis due to high crystalline structure and lignin contents which block cellulolytic enzymes activities. The blockage results in slow and incomplete hydrolysis (Scriban *et al.*, 2005).

Pretreatment of cellulosic biomass materials is referred to as the solubilization and separation of one or more of the major component of biomass (hemicelluloses, cellulose, lignin and extractives), making the solid biomass more accessible to further chemical or biological treatment.

Most pretreatment are aimed at the removal of hemicelluloses or lignin. Several physical or chemical (or their combination), pretreatments methods are possible. Such methods include ball milling, dilute acid pretreatment, steam pretreatment, ammonia

fibre explosion, alkaline pretreatment and organo solvent procedures (Vikari *et al.*, 2001).

2.6.1 Pretreatment Methods

Various pretreatment methods have been studied. It can be physical,chemical or biological. The purpose of these methods is to provide better access to the cellulose for enzymes or acid digestions.

2.6.1.1 Physical Method

Physical pretreatment method is divided into two.This is done by drying and fine drying of the materials and Phyrolysis (*Oznur, 2002*).

2.6.1.2 Chemical Method

Chemical pretreatment methods are classified into two: alkaline and acidic pretreatment.

2.6.1.2.1 Alkaline Pretreatment

Alkaline pretreatment of cellulose materials require delignification of the materials. Example of alkaline pretreatments are discussed below.

2.6.1.2.1.1 Cold Caustic Soda Pulping

It is a chemi-mechanical process that has been known for a long time. However, the process reduces hemicelluloses contents in the product and increases both the glucan

and lignin contents . The recovering of the treatment has not been explored.(Brown *et al.*, 2000)

2.6.1.2.1.2 Ethylenediamine and Monoethylamine

Ethylenediamine and Monoethylamine in alkaline solution and other amines delignify pure biomass with high hemicelluloses content. Treatments such as ammonia recycled percolation will degrade some of the hemicellulose and alter the crystallinity and accessibility of the cellulose to digestive enzymes.

2.6.1.2.2 Acid Methods

Much is known about the dilute acid pre-hydrolysis for making cellulose accessible for further degradation. Examples of acid pretreatment include: Acetic Acid and Formic Acid Pulping Processes

Acetic acid is an additional product recovered during formic acid recycling. In some configuration, acetic acid uses additional mineral acids that aid degradation of lignin components. The hemicelluloses is increasingly degraded with higher temperatures, longer cook times, and greater loading of acetic acid (Baig *et al.*, 2004). Gaseous source of acids has also been used. Example of such acids is Sulphur dioxide. Sulphur dioxide can be introduced into chips, leading to the formation of sulphuric acids.

2.6.1.3 Steam Pretreatment

Acid-cataylsed steam pretreatment is the most extensively studied. Others are supercritical carbon dioxide treatment, oxygen delignification of Kraft pulp or reject knots (Chang *et al.*, 2001) and two stage-dilute acid pretreatment.

The most investigated acid-catalyst for steam pretreatment are SO_2 and H_2SO_4. After steam pretreatment, the lignocellulosic material contains mainly cellulose and lignin, while most of the hemicelluloses is dissolved (Brown *et al.*, 2000).

In the steam pretreatment, the recovery of a maximal amount of sugars from both hemicelluloses and cellulose has been shown to require two-step pretreatment procedure.

The highest derived hemicelluloses and cellulose sugars in a one-step pretreatment have been 66% and 67% respectively (Ronald *et al.*, 2008).

2.6.1.4 Wet Oxidation Pretreatment

This method operates with water and oxygen (or air) at an elevated temperature and pressure.

The method has been applied for treatment of polluted soil and waste water. Recently also, it is used for pretreatment of wheat flour and hard wood.

The wet oxidation method has been reported to produce less furfural and 5-Hydromethyl furfural which are potential inhibitors of the fermentation step, when compared with steam pretreatment.

It is an alternative way of introducing acid that does not involve impregnation of the chips. The extent of treatment can be varied with time or temperature, providing flexibility in the treatment. Sulphur dioxide makes the sugar more accessible to digestion. With sulphur dioxide and steam explosion, there is better recovering of the sugars when the chip size is increased. This is in contrast to alkaline treatment where the extraction is much better with smaller materials. Wet oxidation dissolves mainly hemicelluloses (Vikari *et al.*, 2005).

2.6.1.5 Solvent Methods

Dimethyl sulfoxide (DMSO) is a solvent that has been used in the study of hemicelluloses structure. The solvent extracts the hemicelluloses and does not cleave the acetyl ester allowing for the determination of structure of the hemicelluloses .Dimethyl sulfoxide is used to extract hemicelluloses from pulp. Other solvents used include alcohols, phenols, esters, and combination of solvents with acidic and alkaline conditions. The aim of solvent treatment process is to remove lignin from the lignocellulosic materials.

2.7 Significance of Pretreatment

The high order molecular packing of cellulose in its crystalline regions limits the heterogeneous chemical reactions to the external surface of crystallinities. Further, lignin the natural cement, acts as a constraint to microbial/enzymatic attack. Hence, biodegradation of native untreated cellulose is very slow. This low rate and extent of conversion inhibit the development of an economically feasible hydrolytic process, to increase the susceptibility of cellulosic material, structural modification by various pretreatment schemes are essential (Klinke *et al.*, 2003). Due to the protection of lignin sheath and the crystalline structure, the cellulose is normally not degradable. (Change *et al.*, 1998). The separation of lignocellulosic component is possible only when hydrogen bonds are broken between the constituents and the hemicelluloses-lignin ester cross linkings are broken. Pretreatment is one of the most important steps in the process of converting renewable lignocellulosic biomass into useful products. If the pretreatment is not efficient enough the resultant residue is not easily hydrolysable by cellulose enzyme and if it is more severe, result is the production of toxic compounds which inhibit the microbal metabolis (Galbe *et al.*, 2002). It would be

desirable to test innovative pretreatment process to enhance cellulose hydrolysis). Many researchers have studies the effect of different pretreatment methods upon various lignocellulosic wastes such as wheat bran, wheat straw.

2.8 Bioconversion of Lignocellulosic Materials

The term bioconversion is used in several ways. In this study bioconversion will be considered as the use of biological processes to transform biomass materials from one form to another (Hayn *et al.*, 1993). It may include; the use of enzymes, microbes, other biological agents or the agents in combination.

There has been huge effect to develop pretreatment processes to convert biomass to be compatible with reducing with reducing sugar production. One of such is the use of single organism that produces enzymes. An example is fungi. Among the filamentous fungi are white-rot fungi belonging to *basidomycetes*, *Phanerochaete chysosporin*, and *Phanerochaete radiate* and *Phanerochaete floridensis* (Chang *et al.*, 2005). The organism secrete cellulases, hemicellulase and ligninase enzymes enzymes which hydrolyses cellulosic materials. The enzymes/the organism secrete bioconvert lignocellulosic materials into sugars, ethanol, carbon dioxide and biomass.

Trichodema, Penincillum and Aspergillus species are also involved in bioconcersion of biomass. One of the advantages of bioconversion of lignocellulosic is the opportunity to create a biorefinery, produce value added products plus fuel ethanol. Bioconversion of lignocellulosic wastes could make a significant contribution to the production of organic chemicals. Over 75% organic chemicals are produced from purely base chemicals. They are ethylene, propylene, benzene, toluene and xylene. Theses chemicals are used to synthesize other organic compounds (Nigam 2001).

TABLE 2.2 Lignocellulosic Content of Common Agricultural Residues and Wastes

Lignocellulosic materials	Cellulose (%)	Hemicellulose	Lignin (%)	Sources
Nut shells	25-30	25-30	30-40	Betts *et al.*, 1991
Corns cobs	45	35	15	Betts *et al.*, 2002
Paper	85-99	0	0-15	Okasnen *et al.*, 2002
Wheat straw	32.1	24	18	Okasnen *et al.*, 2002
Leaves	15-20	80-85	0	Okasnen *et al.*, 2002
Fresh bagasse	33.4	30	18.9	Beauchemin *et al.*, 2001
Grass (average for grasses)	25-40	25-50	10-30	Beauchemin *et al.*, 2001

Source: Nigam, 2011

2.9 Hydrolysis of Cellulosic Biomass Materials

After pre-treatment processes, there are processes to hydrolyze the cellulosic biomass to reducing sugars and subsequent fermentation of the sugars to bioethsanol. hydrolysis is also known as saccharification. It breaks down the hydrogen bonds in the hemicelluloses and cellulosic materials to enzymes (Klinke *et al.*, 2003).

2.9.1 Types of Cellulosic Hydrolysis

Hydrolytic methods are divided mainly into two. They are chemical and enzymatic hydrolysis.

The chemical pre-treatment of cellulosic biomass is necessary before enzymatic hydrolysis in order to remove the hemicelluloses and cellulose molecules. Enzymatic hydrolysis is accomplished by cellulosic enzymes (Chang *et al.*, 2005). Different kinds of "cellulases" can be used to cleave celluloses and hemicelluloses.

2.9.2 Factors Limiting Enzymes Hydrolysis

Various factors limit enzymatic hydrolysis. They are highlighted below;

i Structures of lignocelluloses set barriers for chemical and enzymatic degradation.

ii Gradual drop in the reaction rate is generally observed. This is due to end-product inhibition, depletion of degradable parts, enzymes inaction and unproductive binding of entrapment of cellulose in the small pores of cellulose.

iii Effect of raw materials. This can be observed in non-wood biomasses which are more refractory than hard and soft wood biomass materials.

iv Removal procedures. Hemicelluloses are altered by many lignin removal procedures

v Effects of pore size. Hydrolysis yield had been found to correlate with pore size which is dependent on swelling of the materials. Drying of the lignocellulosic substrate and the subsequent collapse of the cell wall capillaries and decrease in pore size decreases the effectiveness of enzymatic hydrolysis.

vi Lignin content and distribution have impact on enzyme hydrolysis. Partial removal of lignin especially the oxygen delignification process using NaOH has shown decrease in hydrolysis rate and yield in the case of steam pretreatment (Chang *et al.*, 2005).

CHAPTER THREE

MATERIAL AND METHODS

3.1 Materials

The plantain stems were collected from a major wholesaler in Uwelu market in Benin City, Edo State and were screened in the department of Botany at the University of Benin to ensure they were of the same species *Musa paradisiaca.*

The snail were bought from Kada market in Akure and were screened at the department of Zoology, Federal University of Technology Akure (FUTA) to ensure they were of the same family. All chemicals and regents used were bought from Akure Lagos and were confirmed to be of standard grade in the department of Biochemistry, FUTA were the work was done.

3.2 Methods

3.2.1 Drying and Storage of Samples

The plantain stems collected were washed and sun dried. Upon drying, it was cut into smaller pieces to expose its tissues for proper drying. The dried smaller portions were ground into powder and stored in a dry closed container which was then referred to as biomass. For the snail, the shell was removed and the gut was isolated and used for the experiment.

3.2.2 Optimized Pretreatment of Biomass

The dried biomass (powder plantain stem) were pretreated successively with 1N and 5N HCL and $Ca(OH)_2$ respectively for 10, 20 and 30mins.

3.2.3. Estimation of Cellulose

Acetic reagent (3ml) is added to 1 gram of the sample in a test tube and mixed with a vortex mixer. The tube was placed in a water bath at 100^0C for 30 minutes, cooled and then centrifuged for 15-20 minutes. The supernatant liquid was discarded. The residue was washed with distilled water. Then 10 ml of 67% sulphuric acid is added and allowed to stand for 1hour.The resulting solution (1ml) was diluted to 100ml. To 1ml of this diluted solution, 10 ml of chilled anthrone reagent was added and mixed well. The tubes was heated in boiling water bath for 10mintes, cooled and the intensity of colour was measured spectrophotometically at 630 nm in UV/VIS – Double Beam Biospectrophotometer using blank as reference Similar procedures were followed for the other concentration containing known quantity of cellulose.

3.2.4 Estimation of Hemicelluloses

To 1gm of the powdered sample in a refluxing flask, 10ml of cold Neutral Detergent Solution was added followed by 2ml of Decahydronaphthalene and 0.5g of Sodium sulphite was added. The mixture was heated to boiling point in a water bath and is refluxed for about 60min. The contents were filtered through sintered glass crucible (G-2_ by suction and washed with hot water. Finally two washings was done with acetone. Then the residue was transferred to a crucible, dried at 100^0C for 8hr. The crucible was cooled in a desiccators and weighed (Goering and Vansoest, 1970). Calculation: Hemicellulose = Neutral detergent fibre (NDF) - Acid detergent fibre (ADF).

3.2.5 Estimation of Lignins

To 1g of powdered sample in a round bottom flask,100ml of acid detergent solution was added. The mixture was then boiled to 5-10min and cooled to avoid foaming as boiling begins. It was refluxed for 1hr after the onset of boiling. To maintain the even level the boiling rate was controlled. The container was removed, swirled and filtered the contents through a preweighed sintered glass crucible and washed with hot water twice. Finally the residue is washed with acetone until the filtrate became colorless. Dried at 100^0C for overnight and weighed after cooling in a desiccators (Goering and Vansoest, 1975). Calculation; the ADF content expressed in percentage as i.e., W/S x 100, where W was the weight of the fiber and S is was the weight of the sample.

3.2.6 Estimation of Total Carbohydrate

The total carbohydrate moiety in the sample was estimated by anthrone method of Gerhardt *et al.*, 1994. It is a simple calometric method with relative insensitivity to interference from other cellular component.

Firstly, the sample (50g) was hydrolysed with 50ml chilled Sulphuric acid (75%) and dehydrated with heat (100 C).The resulting compounds were reacted with 5ml freshly prepared anthrone to give a coloured compound and the amount of total carbohydrate in the sample was then estimated via reading the absorbance of the resulting solution against that of glucose standard curve.

3.2.7 Extraction of Crude Enzyme from Snail

The isolated gut of the snail was weighed and crushed in 0.55m citrate buffer (pH 5.0) at ratio 1:20 (w/v) and the mixture was centrifuged at 200rpm for 15mins. The supernatant of the resulting mixture was obtained and assayed for its enzyme activity.

3.2.8 Cellulase Activity Assay of the Snail

Cellulase activity was assayed using Akinsoye method (Akinsoye *et al.*, 1995). 1ml of 10% carboxy methyl cellulose (cmc) was pipetted into a test tube and 1ml of the crude enzyme was added and incubated at 50^0C for 30mins. Reaction was stopped by adding 3ml of Dinitrosalicycic acid (DNSA) and boiled at temperature of 50^0C for 3mins. Absorbance was read at 540mm and the enzyme activity was defined as the mg glucose released per gram per second of reaction conductivity.

3.2.9 Hydrolysis of Pre-treated Biomass

Citrate buffer (pH5.0) of 25ml was measured into a conical flask and 1g of pretreated biomass was added to it. The resulting mixture (9ml) was pipetted into a test tube and 1ml of the snail enzyme was added and boiled in a water bath at a temperature of 50^0c for 30mins. Reaction was stopped by adding 3ml of DNSA reagent and the amount of glucose released was recorded.

3.2.10 Optimized Hydrolysis of Pre-treated Biomass

Same procedure was repeated in 3.2.9 but was optimized by boiling in a water bath at different temperature and time. The optimization time was 30mins, 1hr, 2hrs and 3hrs while the temperature were 30^0C, 40^0C, and 50^0C. The Absorbance was also read at 540nm.

3.2.11 Determination of Sugar

The sugar content was determined by the method of Dhaffer Samogyi sugar thiosulfate equivalent as described in AOAC (1990)

Principle

Sample (2.5g) was dissolved in 20ml distilled water and hydrolyzed in the presence of 20ml of 20ml 0.1M H_2SO_4. Five milliliter of the resulting solution was pipette into a test tube and then 5ml of Shaffer Somogyl carbonate was added and mixed well by swirling. The test tube was placed in boiling water bath and heated for the required minutes as stipulated in Table 3.1. After the test tube was removed carefully and without agitation to a running water cooling bath and allowed to cool for 4 minutes. The cap on the test tube was removed and 2ml KI and $K_2C_2O_4$ were gently added. The mixture was mixed thoroughly to ensure that Cu_2O was dissolved and then allowed to stand in cold water bath for 5 minutes with mixing done twice during the period. The remaining mixture was later titrated with 0.005M $Na_2S_20_3$ using starch indicator. The blank was equally run as described above and then the test solution titre value subtracted from that of blank. The titration was repeated until two concurring results were obtained. The amounts of sugar present were calculated based on the equation of Shaffer Somogyi sugar-thiosulfate equivalents given below.

Table 3.1 Shaffer – Somogyi Sugar-Thiosulfate Equivalent

(y = mg sugar in 5ml; x = ml of 0.005M $Na_2S_2O_3$+.

Sugar	Heating time	Equation
L-Arabinose	30	Y = 0.1234x + 0.060
Fructose	15	Y = 0.113x +0.079
D-galactose	30	Y = 0.1332x + 0.033
Glucose	15	Y = 0.1099x + 0.048
Lactose	25	Y = 0.2031x +0.030
Maltose	30	Y = 0.2199x + 0.072
D-mannose	35	Y = 0.1148x + 0.084
D-Ribose	25	Y = 0.1381x + 0.098
L-sorbose	15	Y = 0.1244x +0.116
D-Xylose	30	Y = 0.1130x + 0.044

Source: AOAC (1990).

3.2.12 Yeast Inocolum for Fermentation

A glucose fermenting yeast *Saccharomyces cerevisiae* was procured from a chemical store in Akure and the stock culture was maintained on malt-yeast glucose peptone (MYGP), Agar (3g/l malt, 3g/l yeast, 15g/l glucose, 10g/l peptone and 20g/l Agar. PH 6.0) slants and stored at 40^0C.

The yeast from the agar slant were suspended aseptically in 100ml liquid MYGP medium (PH5$^{.0}$) and incubated at 30^0C $\pm$ 1^0C for 24hrs with agitation at 150rpm. The suspension culture of the yeast was then used as inocula for fermentation.

3.2.13 Ethanol Production from the Pre-treated Plantain Stem

Separate Hydrolysis and Fermentation (SHF) was adopted. The hydrolysate of the pre-treated plantain stern (50g) obtained after 3hrs enzymatic hydrolysis was centrifuged and the supernatant filtered and sterilized and used as a sole carbon source in the fermentation medium. Yeast nutrients were added to have a basal medium composition of 5.0g/l yeast extract, 5.0g/l peptone, 5.0g/l NH_4 po4, 0.2g/l $MgSO_4$ and $7H_2O$.

Suspension (2% V/V) of S. cerevisiae (10^8cells /ml) was then inoculated to the broth to initiate the fermentation process and incubated at 30^0C for 72hrs with agitation at 150rpm for the first 24hrs.

3.2.14 Estimation of Ethanol Concentration / Analysis of Ethanol Distillate

At the end of the fermentation, the reaction broth was subjected to fractional distillation at 70^0C to collect the ethanolic solution. The concentration of ethanol was then determined spectrophotometrically by chemical oxidation with acidic dichromate solution (Caputi *et al.*, 1998).

This was achieved via a Gas chromatography techniques using Helium carrier at 5PSI and a flame ionization detector (FID).

The flame is ignited at the flame ionization detector port. The injector, detector and oven temperature were programmed. After reaching the stability, when the oven, detector and injector temperature are at the programmed temperature, a sample was injected from fermentation flask into injector post by using a micro-syringe (1-10**n**l). The oven temperature is held at 80^0C. The injector and detector temperature is maintained at 200^0C. The injected volume was 1uL and 0.760g/ml absolute ethanol

was used as the internal standard. The peak eluted was noted and the different constituents were detected.

Calculation

The 175ml of water mixed with the pretreated sample (5g) before fermentation was taken as the initial yield while the resulting mixture filtered out after the fermentation was said to be the final yield, while the acid medium had a final yield of 235ml,the basic medium had 228ml. The 235ml sample (final yield) was distilled to obtain 76% absolute ethanol (volume by volume). The % ethanol yield was estimated as

INITIAL YIELD / FINAL YIELD X 100. (Chang *et al.*, 2001).

The below standard GC peak table was adopted.

Component	Retention Time
Methane	0.850 – 1.000
Ethane	2.866 - 3.000
Propane	5.683 – 6.000
Butane	8.200 – 9.000
Pentane	10.283 – 11.000
Hexane	12.916 – 13.000
Benzene	2.083 – 2.500
Ethanol	4.000 – 5.500

Chang *et al.*, 2001.

CHAPTER FOUR

4.0 RESULTS AND CONCLUSION

4.1 Cellulase Activity of the Snail

The cellulase activity was assayed from the gut and haemoyph of the snail as shown in table 4.1. The cellulase activity in the heamolyph was significant higher ($P < 0.05$) than that of the snail gut.

TABLE 4.1 Celluase Activity in the Snail Gut & Heamolyph.

SAMPLE	CELLULOSE ACTIVITY in mg/g/sec
SNAIL HAEMOLYPH	$5.1 \times 10^{-3} \pm 2.0 \times 10^{-5a}$
SNAIL GUT	$3.5 \times 10^{-3} \pm 7.6 \times 10^{-5b}$

Each value is mean $\pm$ standard deviation of triplicate values.

Values with different letters (superscript) are statistically different at ($P < 0.05$)

The cellulose activity was higher in the snail haemolyph than the gut.

4.2 Lignocellulosic Content of Plantain Stem Before and After Pretreatment.

The result of lignocelluloses content of the plantain stem before and after pretreatment are shown in tables 4.2 and 4.3.

TABLE 4.2 Lignocellulosic Content of Plantain Stem Before pretreatment.

LIGNOCELLULOSIC COMPONENT	CONCENTRATION (MG/G)
LIGNIN	58 ± 0.02
HEMICELLULOSE	62 ± 0.02
TOTAL CARBOHYDRATE	84 ± 0.01
CELLULOSE	119 ± 0.03

Each value is mean ± standard deviation of triplicate values

As cellulose concentration increases there was a corresponding decrease in Lignin, hemicellulose and total carbohydrate concentration.

TABLE 4.3 Lignocelluosic Content of Plantain Stem After Pretreatment

NORMALITY	TIME	LIGNIN		HEMICELLULOSE		CELLULOSE		TOTAL CARBOHYDRATE	
N	Mins	$Ca(OH)_2$	HCl	$Ca(OH)_2$	HCl	$Ca(OH)_2$	HCl	$Ca(OH)_2$	HCl
1	10	28 ± 0.02^{b}	28 ± 0.02^{b}	11 ± 0.03^{b}	14 ± 0.02^{a}	143 ± 0.02^{a}	116 ± 0.01^{a}	16 ± 0.02^{a}	15 ± 0.01^{b}
1	20	13 ± 0.03^{b}	19 ± 0.02^{b}	2 ± 0.01^{b}	25 ± 0.01^{b}	195 ± 0.01^{a}	132 ± 0.02^{b}	21 ± 0.01^{a}	17 ± 0.02^{b}
1	30	8 ± 0.02^{b}	14 ± 0.02^{a}	1 ± 0.03^{b}	4 ± 0.02^{a}	217 ± 0.02^{a}	159 ± 0.01^{b}	22 ± 0.05^{a}	18 ± 0.01^{b}
5	10	14 ± 0.02^{b}	21 ± 0.05^{a}	3 ± 0.01^{b}	29 ± 0.01^{a}	127 ± 0.01^{a}	95 ± 0.04^{b}	11 ± 0.01^{a}	12 ± 0.05^{a}
5	20	17 ± 0.04^{a}	16 ± 0.02^{a}	5 ± 0.05^{b}	21 ± 0.01^{a}	168 ± 0.05^{a}	155 ± 0.01^{b}	16 ± 0.04^{a}	14 ± 0.04^{b}
5	30	24 ± 0.02^{b}	$26^{a}\pm0.02^{a}$	11 ± 0.02^{a}	7 ± 0.01^{b}	203 ± 0.01^{b}	209 ± 0.01^{a}	17 ± 0.01^{b}	21 ± 0.01^{a}

Each values are mean $\pm$ standard deviation of triplicate values

Values with different letters (superscript) are statistically different at ($p < 0.05$)

4.3 Sugar Content of Plantain Stem Before Pretreatment, After Pretreatment and After Hydrolysis

Results showing the sugar content before pretreatment is shown in table 4.4 while table 4.5 and 4.6 respectively shows the sugar content after pretreatment and after hydrolysis.

TABLE 4.4 Sugar Content of Plantain Stem Before Pretreatment

SUGAR	CONCENTRATION mg/100g
Glucose	63 ± 0.02
Xylose	40 ± 0.01
Arabinose	47 ± 0.03
Mannose	38 ± 0.02
Galactose	43 ± 0.04

Each values are mean ± standard deviation of triplicate values.

Values with different letters (superscript) are statistically different at ($P < 0.05$)

TABLE 4.5 Sugars Content of Plantain Stem After Pretreatment mg /100g

NORMALITY	TIME	GLUCOSE		XYLOSE		ARABINOSE		MANNOSE		GALACTOSE	
N	Mins	$Ca(OH)_2$	HCl	$Ca(OH)_2$	HCl	$Ca(OH)_2$	HCl	$Ca(OH)_2$	HCl	$Ca(OH)_2$	HCl
1	10	$163^{a}\pm 0.01$	$132^{b}\pm 0.01$	$121^{a}\pm 0.01$	$111^{b}\pm 0.01$	$127^{a}\pm 0.01$	$121^{b}\pm 0.01$	$121^{a}\pm 0.01$	$121^{a}\pm 0.001$	$111^{b}\pm 0.01$	$113^{b}\pm 0.01$
1	20	$171^{a}\pm 0.01$	$150^{b}\pm 0.01$	$132^{a}\pm 0.01$	$128^{b}\pm 0.01$	$139^{a}\pm 0.01$	$130^{b}\pm 0.01$	$132^{a}\pm 0.01$	$131^{b}\pm 0.001$	$121^{b}\pm 0.01$	$122^{a}\pm 0.01$
1	30	$197^{a}\pm 0.01$	$163^{b}\pm 0.01$	$146^{a}\pm 0.01$	$137^{b}\pm 0.01$	$142^{a}\pm 0.01$	$133^{b}\pm 0.01$	$148^{a}\pm 0.01$	$142^{b}\pm 0.001$	$142^{a}\pm 0.01$	$136^{b}\pm 0.01$
5	10	$118^{a}\pm 0.01$	$121^{a}\pm 0.01$	$120^{a}\pm 0.01$	$101^{b}\pm 0.01$	$108^{a}\pm 0.01$	$103^{b}\pm 0.01$	$129^{b}\pm 0.01$	$134^{a}\pm 0.001$	$112^{b}\pm 0.01$	$121^{a}\pm 0.01$
5	20	$138^{b}\pm 0.01$	$140^{a}\pm 0.01$	$132^{a}\pm 0.01$	$122^{a}\pm 0.01$	$122^{a}\pm 0.01$	$113^{b}\pm 0.01$	$134^{a}\pm 0.01$	$133^{b}\pm 0.001$	$128^{b}\pm 0.01$	$132^{a}\pm 0.01$
5	30	$149^{b}\pm 0.01$	$151^{a}\pm 0.01$	$141^{a}\pm 0.01$	$132^{b}\pm 0.01$	$135^{a}\pm 0.01$	$127^{b}\pm 0.01$	$141^{a}\pm 0.01$	$140^{a}\pm 0.001$	$142^{a}\pm 0.01$	$136^{b}\pm 0.01$

Each values are mean ± standard deviation of triplicate values.

Values with different letters (superscript) are statistically different at ($P < 0.05$)

TABLE 4.6 Sugar Content of Plantain Stem After Hydrolysis mg/100g

SUGAR MOLECULES	TIME (MINS)	30°C		40°C		50°C	
		1N Ca $(OH)_2$	5N HCl	1N Ca $(OH)_2$	5N HCl	1N Ca $(OH)_2$	5N HCl
Glucose	30	268 ±0.12^a	253 ±0.01^b	268 ± 0.11^a	253 ± 0.12^b	268 ± 0.01a	253 ± 0.02^b
	60	272 ± 0.01^a	263 ± 0.03^b	273 ± 0.10^a	263 ± 0.01^b	272 ± 0.02a	263 ± 0.04^b
	120	289 ± 0.17^b	271 ± 0.12^a	288 ± 0.01^a	271 ± 0.20^b	289 ± 0.03a	271 ± 0.21^b
	180	291 ± 0.22^a	283 ± 0.21^b	291 ± 0.02^a	283 ± 0.17^b	291 ± 0.01a	283 ± 0.22^b
Xylose	30	202 ± 0.01^a	211 ± 0.01^a	202 ± 0.21^b	211 ± 0.01^a	202 ± 0.11^b	211 ± 0.18^a
	60	217 ± 0.02^b	227 ± 0.12^a	217 ± 0.62^b	227 ± 0.03^a	217 ± 0.12^b	227 ± 0.21^a
	120	229 ± 0.01^a	218 ± 0.13^b	229 ± 0.17^a	218 ± 0.02^b	229 ± 0.21^a	218 ± 0.26^b
	180	243 ± 0.03^a	212 ± 0.01^b	243 ± 0.01^a	212 ± 0.01^b	243 ± 0.27^a	212± 0.07^b
Arabinose	30	221 ±0.01^b	227 ± 0.02^a	221 ± 0.02^b	228 ± 0.21^a	221 ± 0.11^b	228 ± 0.21^a
	60	237 ± 0.01^a	218 ± 0.12^b	237 ± 0.03^a	219 ± 0.16^b	237 ± 0.12^a	219 ± 0.01^b
	120	240 ± 0.03^b	249 ± 0.01^a	240 ± 0.01^b	249 ± 0.21^a	240 ± 0.23^b	249 ± 0.03^a
	180	250 ± 0.12^a	228 ± 0.03^b	250 ± 0.02^a	228 ± 0.02^b	250 ±0.13^a	228 ± 0.04^b
Mannose	30	219 ± 0.72^a	202 ± 0.03^b	219 ± 0.11^a	202 ± 0.20^b	219 ± 0.11^b	202 ± 0.02^b
	60	216 ± 0.56^b	218 ± 0.12^b	221 ± 0.17^a	220 ± 0.11^a	216 ± 0.21^b	220 ± 0.01^a
	120	233 ± 0.22^b	249 ± 0.01^a	233 ± 0.21^b	237 ±0.17a	233 ± 0.62^b	237 ± 0.02^a
	180	240 ± 0.11^a	228 ± 0.03^b	240 ± 0.11^a	218 ± 0.02^b	240 ± 0.57^a	218 ± 0.01^b
Galactose	30	233 ± 0.11^a	215 ± 0.12^b	233 ± 0.02^a	215 ± 0.12^b	233 ± 0.02^a	215 ± 0.12^b
	60	243 ± 021^a	242 ± 0.16^a	243 ± 0.07^a	242 ± 0.16^a	243 ± 0.07^a	242 ± 0.16^a
	120	252 ± 0.17^a	252 ±0.01^a	252 ± 0.08^a	252 ±0.01^a	252 ± 0.08^a	252 ±0.01^a
	180	253 ±0.52^a	252 ± 0.02^a	253 ± 0.22^a	252 ± 0.02^a	253 ± 0.22^a	252 ± 0.02^a

4.4 Percentage Ethanol Yield from Pretreated Plantain Stem Hydrolysate

The percentage ethanol yield from the pretreated plantain stem hydrolysates are shown in table 4.7. The Ca $(OH)_2$ pretreated sample gave a higher ethanol yield than the acid pretreated sample.

Table 4.7 % Ethanol Yield from the Pretreated Plantain Stem Hydrolysate

SAMPLE	% ETHANOL YIELD BY VOLUME
HCl PRETREATED SAMPLE	74.5^{b}
Ca $(OH)_2$ PRETREATED SAMPLE	76.8^{a}

A higher bioethanol volume was obtained via Ca $(OH)_2$ pretreatment.Each values are in triplicate

4.5 Hydrocarbon Content of the Plantain Stem Ethanol

From the results in table 4.8, the ethanol from the Ca $(OH)_2$ pretreated sample had more hydrocarbon than the ethanol from the HCl pretreated sample.

Table 4.8 Hydrocarbon Content of the Plantain Stem Ethanol

HYDROCARBONS	ETHANOL FROM HCl PRETREATMENT		ETHANOL FROM Ca $(OH)_2$ PRETREATMENT	
(C - C_{10})	STATUS	GC RETENTION TIME	STATUS	GC RETENTION TIME
Methane	Nil	Nil	Present	0.85
Ethane	Present	3.00	Present	3.00
Propane	Present	5.75	Present	5.87
Benzene	Nil	Nil	Present	2.45
Ethanol	Present		Present	

The Gas Chromatography Retention Time -GC is the reaction peak at which the hydrocarbon is observed and read.

4.6 Discussion

In this study, cellulase activity was investigated in the haemolyph and gut of snail and the activity was found significantly higher ($P<0.05$) in the haemolyph than the gut.

Earlier work done by Krishna C.G 2009, on digestive enzyme and cellulolytic bacteria in the gut of giant land snail "*Achatina fulica*" shows that cellulase are more in the digestive glands while cellulolytic bacteria are more in the intestines and that, the haemolyph was a mixture of digestive and intestinal fluid and thus is expected to be of higher cellulase activity than the formers. In line with Krishna (2009), the higher cellulase activity in snail haemolyph was due to the combine cellulase activity of the digestive and intestinal fluid and hence was used in this study. On the basis of this finding, the cellulase extracted from snail haemolyph is suggested to be a better option in the hydrolysis of lignocelluloses of bioethanol.

The lignin, hemicelluloses, total carbohydrate and cellulose content before and after pretreatment were investigated. The cellulose content of the untreated plantain stem biomass was significantly higher ($P<0.05$) than the lignin, hemicelluloses and total carbohydrate content and this is due to fact that, cellulose constitutes 50-60% of plant cell walls (Hayn *et al.*, 1993).

Ronald *et al.*, (2008) reported a high cellulose content of 83±0.22 mg/100g sample in an untreated wheat straw as against the 119±0.03 mg/100g concentration of cellulose gotten from the untreated plantain stem in this study. The higher cellulose content of the plantain stem justifies it as a potential source for bioethanol production.

There was a significant increase ($P<0.05$) in the cellulose content after pretreatment with a corresponding decrease in the lignin, hemicellulose and total carbohydrate content in both $Ca(OH)_2$ and HCl pretreatment. However IN- $Ca(OH)_2$ at 30mins pretreatment gave the optimal yield of cellulose and thus taken as the optimal

pretreatment condition. This agreed with Ronald *et al*, 2008 where a higher cellulose content was recorded from lime-treated wheat straw. This justifies the higher content of cellulose gotten from 1N $Ca(OH)_2$ in this study, as it will help hydrolyse the un-wanted lignocelluloses and exposes the wanted lignocellulose (cellulose) for further hydrolysis to bioethanol. On the basis of this finding, 1N $Ca(OH)_2$ at 30 mins pretreatment is a better pretreatment option in the bioconversion of agro waste to ethanol.

The Glucose, Xylose, Arabinose, Mannose and Galactose content of a plantain stem before pretreatment, after pretreatment and after hydrolysis were investigated.

There was a significant increase in the Glucose, Xylose, Arabinose, Mannose and Galactose content after pretreatment. This agrees with earlier studies by Milala *et al.*, (2005) and do not only justifies plantain stem as a good source of Glucose but underscores the efficiency of the 1N $Ca(OH)_2$ at 30mins in further hydrolyzing the cellulose and other complex sugars to simple sugars. On the basis of this finding, In $Ca(OH)_2$ at 30mins was taken as the optimal pretreatment condition in determining the sugar content of the plantain stem.

After hydrolysis there was a significant difference between the hydrolytic time of the crude snail cellulase. The optimal hydrolytic condition of the snail cellulase was 180mins (3hrs). There was no significant difference in the incubation temperature at 30^0C, 40^0C and 50^0C that were used in this study, since they gave approximately same values. The Glucose, Xylose, Arabinose, Mannose and Galactose content after hydrolysis increased significantly at optimal hydrolytic condition of the snail cellulase. This increase underscore the efficiency of crude snail cellulase in further hydrolyzing the sugar molecules and thus exposing them for fermentation and subsequent ethanol fuel production. The finding agrees with work earlier done by

CHAPTETR FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The quest for alternative sources of fuel has persisted and the need to turn agro waste into useful product via chemical and enzymatic processes has become of keen interest (Klinke *et al.*, 2003).

Hence in this research, plantain stem - an agro waste rich in cellulose was pretreated and hydrolyzed with $Ca(OH)_2$/HCl and crude Snail Cellulase extract /Yeast respectively to bioethanol.

Conclusively, from the result gotten from this work, we can say plantain stem will be a potential source for the mass production of bioethanol in the near future.

5.2 Recommendations

It is recommended that the ethanol and its subsequent hydrocarbon be quantified by weight using higher GC-FID equipment while other agro waste can also be explored.

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