A COMPARATIVE STUDY OF THE VARIOUS PRETREATMENT TECHNIQUES FOR BIO-ETHANOL PRODUCTION FROM KIKUYU GRASS

University of Fort Hare
Together in Excellence

A dissertation submitted in fulfillment of the requirements for the Degree of

MASTER OF SCIENCE IN CHEMISTRY

By

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December 2013

Declaration

I declare that this thesis entitled "A comparative study of the various pre-treatment techniques for bio-ethanol production from Kikuyu grass" is the result of my own research. All non-original materials are duly acknowledged. The dissertation has not been submitted to any other University for a Masters or any other higher degree.

Signature : .....

Name : Zukiswa Coceka Mbande.....

Date : 01-12-2013.....

Dedication

This work is dedicated to my entire family, the Mbande's.

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Acknowledgements

Firstly I would like to thank the Lord God Almighty for enabling me to do this project.

I would like to thank my supervisor Prof Tichagwa for the support she gave me throughout this entire project. "Thank you for the guidance you have given me I will be eternally grateful." An appreciation also goes out to my co-supervisor, Dr S Mamphweli. "Thank you."

I would also like to thank the members of the Department of Chemistry: Dr Katwire (Head of Department) for his leadership and support. "You have made my transition to this new school a lot easier and I appreciate it". I would also like to thank Mr Manene for his guidance throughout the project for which I am grateful.. I am very thankful for the advice and help from other members of the Department of Chemistry.

My unalloyed gratitude goes to the following; Sasol Inzalo group for the financial support to enable me to carry out my Masters work. I am also grateful for financial assistance from Govan Mbeki Research and Development Centre (GMRDC)

I also extend my gratitude to my entire family for all they have done for me. "I would not be here if it wasn't for your sacrifices. Thank you."

Abstract

Bio-ethanol is by far the most widely used bio-fuel for transportation worldwide. Production of bio-ethanol from biomass is one way to reduce both consumption of crude oil and environmental pollution. There are mainly two processes involved in the conversion of lignocellulosic materials to ethanol production: hydrolysis of cellulose in the lignocellulosic biomass to produce reducing sugars and fermentation of the sugars to ethanol. However, pretreatment is an essential step before the hydrolysis of biomass and subsequent production of bio-ethanol. The current study was focused on four different chemical methods for the pretreatment of Kikuyu grass using mild temperatures. The temperature used was 100°C for various periods at 2% concentration. Pretreatments using Alkali, acid, oxidative means and were compared in their effect on lignin removal from Kikuyu grass. FT-IR, XRD and SEM analysis showed that pretreatment had affected the structural and morphological properties of the grass, which increased the accessibility of enzymes and further aided fermentation to occur more rapidly and with higher yields. Reducing sugars concentrations were determined by UV-Vis spectrophotometry using the 3,5 dinitrosalicylic acid (DNS) method. The greatest reducing sugars concentration was 4.21 g/L which was obtained with acid pretreatment at 2% acid concentration. Ethanol yields of 34.20 mg/mL, 30.30 mg/mL, 15.59 mg/mL and 12.10 mg/mL were obtained for H_2SO_4, NaOH, O_3 and H_2O_2 respectively.

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XRD: X-Ray Diffraction

SEM: Scanning Electron Microscope

FT-IR: Fourier Transmittance Infra Red

UV-Vis: Ultra-Violet-Visible

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Fossil fuels such as petroleum and natural gas currently serve the majority of the world's energy needs, As the world's population increases and more countries are being industrialized, it is clear that current supply of fossil fuels can no longer meet the ever increasing global energy demands, as illustrated in Figure 1.1 [Jin *et al*, 2012].

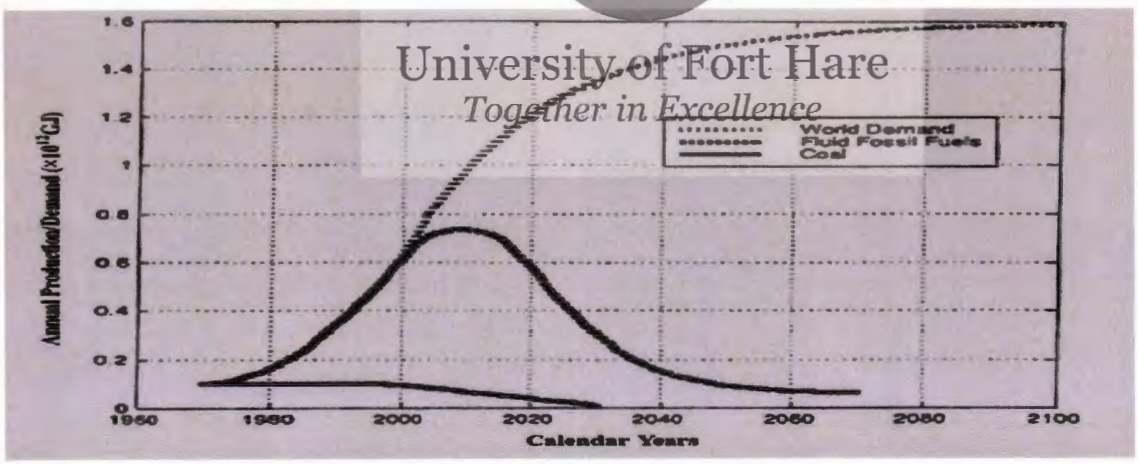


Figure 1.1: Estimated global energy demand and fossil fuel production [Jin *et al*, 2012]

This growth will affect the stability of ecosystems and global climate as well as global oil reserves [Balat 2011]. In addition, the combustion of these fuels is believed to be responsible for approximately three quarters of the anthropogenic emissions of carbon dioxide (CO_2), which is a major greenhouse gas contributor to global warming [Hayes,

2009]. The increase in the price of fuel, the finite nature of fossil fuels, increasing concerns regarding environmental impact especially with regards to greenhouse gas emissions, and health and safety considerations are forcing the search for new energy sources and alternative ways to power the world's motor vehicles [Balat, 2011].

Governments around the world have taken notice of these issues and have implemented minimum targets for the substitution of alternative fuels for conventional ones. For instance, the current European Sustainable Energy Policy states that 20% of the primary energy supply in 2020 should be delivered by renewable energy sources [Anna Sues, *et al* 2010]. According to the Intergrated Resource Plan approved in 2010 by the SA parliament, 40% of the country's energy should come from renewable resources. To achieve these goals the replacement of petroleum by renewable energy must be taken to an industrial scale. Such alternative fuels must be technically feasible, economically competitive, environmentally acceptable and readily available [Meher, 2006]. Numerous potential alternative fuels have been proposed, including bio-ethanol, bio-diesel, methanol, hydrogen, boron, natural gas, liquefied petroleum gas(LPG), Fischer–Tropsch fuel, p-series and solar fuel. [Balat, 2011] The alternative fuels from biomass have attracted much attention because of the following reasons: The first is that biomass is a renewable resource that is bio-degradable and could be sustainably developed in future [McKendry, 2002]. Secondly, biofuels and bioproducts produced from plant biomass could mitigate global warming. This is because the CO_2 released in burning equals the CO_2 taken up by the plant during photosynthesis and thus does not

increase the net CO_2 in the atmosphere. Lastly biofuel production along with bio-products can provide new income and employment opportunities in rural areas. [Naik,*et al* 2010] Table 1.1 summarises the major benefits of biofuels.

Table 1.1: Major benefits of biofuels [Balat, 2011]

Economic impacts:	Sustainabilty Fuel diversity, Increased number of rural manufacturing jobs Agricultural development, International Competitiveness, Reducing the dependency on petroleum
Environmental impacts:	Greenhouse gas reductions Reducing air pollution Improved land and Water use
Energy Security :	Reducing use of fossil fuels Ready availability, Domestic distribution

1.1.1 LIGNOCELLULOSIC BIOMASS

Currently bio-ethanol is produced from sugar and grains also known as first generation biomass for example cane, corn, and several other sugar or starch crops (sugar beets, wheat, potatoes). However these feedstocks compete directly with crops grown for food. Lignocellulosic materials (second generation) constitute the world's largest bio-ethanol renewable resource and is a plausible alternative to this fuel versus food crisis. However, the conversion of lignocellulosic biomass to ethanol is extremely challenging because the lignocellulosic-based feedstock is a recalcitrant material. Therefore a pre-treatment stage is necessary to alter the structural and chemical composition of lignocellulosic biomass to facilitate rapid hydrolysis of the carbohydrates to fermentable sugars. As such many pre-treatment processes have been developed to improve the accessibility of lignocelluloses for hydrolysis. Dilute and concentrated acid, alkali, oxidative, supercritical water and several other pretreatment methods have been used to delignify the biomass structure, decrease cellulose crystallinity and solubilize hemicelluloses. This will then permit hydrolysis to occur more easily which will in turn increase the ethanol yield.

1.1.2 RATIONALE OF THE STUDY

Ethanol has attracted worldwide attention because of its potential use as an alternative automotive fuel. Much effort has been made in bio-ethanol production using biomass (sugar cane and corn) however these feed-stocks are first generation and lead to so called 'fuel versus food problems'. As a result of such limitations associated with first

generation bio-fuels, widely available lignocellulosic biomass for the production of second generation biofuels can attempt to solve the problems associated with first generation biofuels. Bio-ethanol production from non-edible lignocellulosic materials such as grass attempts to solve this problem. Unlike food-based (starch or sugar) biomass, it has many advantages, such as low cost, abundant supplies, non-competition with food which is the major motivation for this research.

1.1.3 PROBLEM STATEMENT

There is growing interest from researches and the world at large on alternative fuels as a result of the energy crisis and environmental challenges associated with the extraction and use of fossil fuels. The lignocelluloses have attracted worldwide attention because they are by far the earth's most abundant, renewable organic material available for microbial or other conversions. Kikuyu grass is considered as an aggressive invader of pastures, crop lands and natural areas, especially moist coastal areas, streams and wetlands. It spreads rapidly by producing long rhizomes and runners. It also produces chemicals that kill other plants nearby and threatens the natural vegetation by smothering it.It however, contains an appreciable amount of cellulose (26.9%) which can be used for various applications. In this study bio-ethanol was produced from this type of grass. Production of food, fuels and chemicals from materials considered as 'waste' constitutes a valuable service in the self-sustaining society envisioned for the future and synthesis of bioethanol from grass is a possible method to achieve this goal [Ganguly *et al*, 2012]. Pretreatment is an important process in obtaining good yields of

bioethanol from biomass so, in this study the best pretreatment conditions are investigated to allow optimum yields of hydrolyzates.

1.1.4. AIMS AND OBJECTIVES

The major aim of this project is to prepare bio-ethanol from Kikuyu grass using hydrogen peroxide, sodium hydroxide, sulfuric acid and ozone as pre-treatment techniques.

The specific objectives are as follows:

(i) To study and identify the optimum pre-treatment techniques on fermentable sugar yield from grass

(ii) To study and optimize enzymatic hydrolysis of grass (Kikuyu) into fermentable sugars.

(iii)To convert the fermentable sugars into bio-ethanol

1.1.5 LAYOUT OF DISSERTATION

This dissertation contains five chapters.

Chapter 1 gives an overview of the study with an emphasis on the problem being addressed, the concepts that form the basis of this study, the motivation, aims and objectives as well as the scope of the dissertation.

Chapter 2 covers the literature review of lignocellulosic materials and biofuels. It gives a comprehensive account of the merits and demerits of various pretreatment techniques.

Chapter 3 outlines the experimental procedure on the pretreatment of Kikuyu grass. The chapter also describes characterization tools to monitor the effect of the different pretreatment methods.

Chapter 4 discusses the effects of different pretreatment methods, monitoring their crystallinity and production of reducing sugars.

Chapter 5 provides a general conclusion and highlights the extent to which the goals were achieved. In this chapter recommendations are presented on how to optimize the pretreatment techniques towards ethanol production.

REFERENCES

Alya,L., Ricke, S.C. (2012) Lignocellulosic biomass for bioethanol production: Current perspectives, potential issues and future prospects Progress in Energy and Combustion, Science, 38, 449-467.

Balat, M. (2011) Production of bioethanol from lignocellulosic materials via the biochemical pathway: A review, Energy Conversion and Management 52, 858–875.

Hayes, D.J. (2009) An examination of biorefining processes, catalysts and challenges, Catalysis Today, 145, 138–15.

Ganguly, A., Chatterjeea, P.K., Dey, A. (2012) Studies on ethanol production from water hyacinth – A review, Renewable Sustainable Energy Reveiws 16, 966–972.

Jin, H., Fei, Y., Yongwu, L. (2012) Application of Fischer–Tropsch Synthesis in Biomass to Liquid Conversion, Catalysts, 2, 303-326.

Meher, L.C., Sagar, D.V., Naik, S.N. (2006) Technical aspects of biodiesel production by transesterification—a review, Renewable and Sustainable Energy Reveiws; 10 248–68.

Naik, S.N., Vaibhav V., Prasant K. , Dalai, A. (2010) Production of first and second generation biofuels: A comprehensive review, Renewable and Sustainable Energy Reviews 14, 578–59

Sues, A., Juras˘c˘ı´k, M., Ptasinski, K. (2010) Exergetic evaluation of 5 biowastes-to-biofuels routes via gasification Energy 35, 996–1007

CHAPTER 2

LITERATURE REVIEW

2.1. INTRODUCTION

Bio-fuels have attracted a lot of attention as alternative sources of energy because of the current problemsassociated with the use of fossil fuels. Biofuels can be defined as any liquid fuel made from plant material that can be used as a substitute for petroleum derived fuel. Interest has been placed on bio-fuels such as bioethanol, biodiesel and syngas.

Bio-ethanol is produced from biomass feedstocks by fermentation process. These feedstocks can be classified as follows

(a) Sugar containing crops: Sugar cane, wheat, etc.

(b) Starch containing crops: Grain such as wheat, barely, rice, sweet sorgum, corn, etc. and root plants like potato, cassava.

(c) Cellulosic biomass: Wood and wood waste, cedar, pine, wood, etc. agricultural residues, fibers [Naik, 2010].

Based on food usage, chemical composition of feedstocks and the production pathway of biofuels, potential biomasses have been classified as generations.

2.1.1 First generation Bio-fuels

First generation bio-fuels refer to biofuels made from sugars, crops such as grain and corn, or seeds, and which use a relatively simple process for the production of fuel ethanol. These basic feedstock are generally harvested with a high carbohydrate or oil content and transformed into fuels such as biodiesel (bio-esters), alcohols and biogas (mixture of CH_4 and CO_4) [Srirangan *et al*, 2012].

Biodiesel is a vegetable oil that can be used for food or fuel obtained from a general synthesis as illustrated in the Equation 2.1.

Equation 2.1: Conversion of triglycerides to Biodiesel [Srivastava and Prasad 2000].

Bioalcohols: These are mostly produced by the action of micro-organisms and enzymes which ferment sugars or starch. Ethanol is the most common bioalcohol. Methanol, propanol and butanol, are less commonly produced. Hence bio-ethanol will be the focus of this study.

Biogas: This is the gas produced when bacteria convert organic matter to methane gas. This is a process of anaerobic digestion of organic material [Harold House, 2007].

Four ingredients are needed for biogas production, namely organic matter, bacteria, anaerobic conditions and heat.

2.1.2. Second Generation Bio-fuels

The first generation biofuels, i.e. corn-based and sugar based-ethanol are promising substitutes to gasoline production mainly in the transportation sector. However they are not sufficient to replace a considerable portion of the one trillion gallons of fossil fuels presently consumed worldwide each year. In addition the use of these fuels raises ethical concerns about using food products for fuel ethanol. Second-generation fuels are generally those made from non-edible lignocellulosic biomass [Margeot *et al*, 2009]. These liquid biofuels are generally produced by two fundamentally different approaches i.e. biological or thermochemical processing of agricultural lignocellulosic biomass, which are either non-edible residues of food crop production or non-edible whole plant biomass (e.g. grasses or trees specifically grown for production of energy [Nigam and Singh, 2011].

Lignocellulose-to-ethanol conversion is regarded as a sustainable technology to supplement corn-based ethanol production due to the abundance of lignocellulosic biomass and diverse raw materials available [Xu *et al*, 2011]. However there are some challenges regarding finding the appropriate pretreatment technique and fermentation technologies.

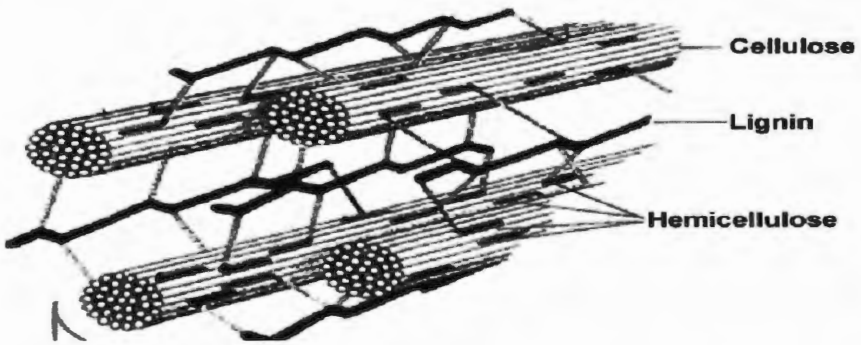


Figure 2.2: Structure of lignocelluloses [Yinghuai Z, 2005]

2.1.3. Third generation biofuels

Second-generation biofuels are derived from lignocellulosic agriculture and forest residues and from non-food crop feed-stocks. They address some of the problems associated with first generation biofuels; however, there is concern over competing land use or required land use changes [Brennan and Owende 2010]. Third-generation biofuels specifically derived from microalgae are considered to be a viable alternative energy resource that does not have the major drawbacks associated with first and second-generation biofuels. Algae are recognised as one of the oldest life-forms and are present in all existing earth ecosystems, representing a wide variety of species living in a wide range of environmental conditions [Mata, 2012].

These feedstocks have the potential to generate significant amount of biomass and are suitable agents for conversion to biodiesel as they synthesize TAGs (triglycerides) [Kothari *et al*, 2012]. The photosynthetic efficiency of these feed-stocks are much higher (6–8%, average) than that of terrestrial (1.8–2.2%, average). This makes third

generation biofuels able to solve a lot of energy problems because it has enhanced CO_2 fixation to afford a high biomass production [Aresta *et al*, 2005]. Also, the other advantage algae has over other biological sources is that algae has far lower space requirements than land-based plant production and several algal species can double their biomass in 1 day.

2.1.4. Bio-ethanol fuel

Bio-ethanol fuel is a liquid fuel used worldwide but particularly in Brazil and United States of America. It is produced by fermentation of sugars derived from corn, sugar beet sugar cane and any other sugar or starch that alcoholic beverage can be made from. In ethanol fermentation, glucose is evolved into ethanol and carbon dioxide. [Herna´ndez and Kafarov, 2009]

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \quad (2.2)$$

During combustion, ethanol reacts with oxygen to produce carbon dioxide, water, and heat: (other air pollutants are also produced when ethanol is burned in the atmosphere rather than in pure oxygen)

$$2C_2H_5OH + 6O_2 \rightarrow 4CO_2 + 6H_2O + \text{heat} \quad (2.3)$$

For photosynthesis, glucose is produced again from carbon dioxide and water.

$$6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6O_2 \quad (2.4)$$

2.2. LIGNOCELLULOSIC BIOMASS

Lignocellulosic materials have also been identified as potential feed-stocks, in view of their ready availability and low cost and no competition with food. Lignocellulosic materials have three main fractions: cellulose, hemicellulose and lignin. Fermentable fractions of these feed-stocks include cellulose and hemicellulose. The structure of these materials is highly complex, and native biomass is resistant to enzymatic hydrolysis. Cellulose is highly crystalline, water insoluble, and highly resistant to de-polymerization. For a lignocellulosic ethanol process to be economically competitive with starch or sugar based processes, all of the sugars present in the cellulose and hemicellulose have to be available to the fermenting organism [Hägerdal, 2006].

The success of using cellulosic sugars for bio-ethanol production depends on a number of factors namely the physical and chemical properties of the biomass, pretreatment techniques, effective micro-organisms, process integration, and optimization of the processing conditions [Corredor *et al*, 2009]

2.2.1. Cellulose

Cellulose, the major constituent of lignocellulosic biomass (33-51%), is a glucan whose D-anhydro-glucopyranoseunits are linked through (1→4)-glycosidic bonds, these links are linked through a β-configuration [Hayes, 2009]. Cellulose contains over 10,000 glucose units [Cheng and Timilsina, 2011]. Three hydroxyl groups, placed at the positions C2 and C3 (secondary hydroxyl groups) and C6 (primary hydroxyl groups) can form intra- and intermolecular hydrogen bonds. These hydrogen bonds allow the creation of highly ordered, three-dimensional crystal structures [Abdul *et al*, 2012]. This makes cellulose extremely difficult to hydrolyze.

2.2.2 Hemicellulose

Hemicelluloses are closely associated with cellulose in plant tissues and together with cellulose they are the most abundant carbonic material in plants. Hemicelluloses tend to be branched heteropolysaccharides that are mostly built up of the pentose D-xylose with smaller amounts of the pentose arabinose and the hexoses D-glucose, D-mannose and D-galactose, as well some uronic acids and acetylated derivatives [Nei, 2008]. They consist of about 100-200 sugar units. [Cheng and Timilsina, 2011] Different to cellulose, the hemicellulose structure does not present a high crystallinity, therefore, being more susceptible to the chemical hydrolysis under milder conditions.

2.2.3 Lignin

Lignin is a naturally occurring aromatic cross-linked polymer with molecular weight of more than 10,000 Daltons (Da). It is present in all lignocellulosic biomass; therefore, any ethanol production process will have lignin as a residue. Lignins are divided into two classes, namely ``guaiacyl lignins" and ``guaiacyl-syringyl lignins", differing in the substituents of the phenylpropanoid skeleton. Guaiacyl-lignins have a methoxy-group in the 3-carbon position, whereas syringyl-lignins have a methoxy-group in both the 3-carbon and 5-carbon positions [Palmqvist and Hahn-Hagerdal, 2000]. Lignin presents a highly complex structure, formed by polymerization of three different monomers: coumaric alcohol (I), coniferyl alcohol (II) and synapyl alcohol (III), which differ from one another by possessing different substituents in their aromatic ring [Nei, 2008].

Lignin effectively protects the plant against microbial attack and only a few organisms can degrade it. It restricts hydrolysis by shielding cellulose surfaces or by adsorbing and inactivating enzymes. Lignin holds the cellulose and hemicellulose fibers together [Cheng and Tirnilsina, 2011]. It is this close union between lignin and cellulose that prevents swelling of the fibres, thereby affecting enzyme accessibility to the cellulose. To solve this problem, several studies have shown that taking away lignin enhances cellulose hydrolysis (Wyman, 2005). Figure 2.3 shows the structure of cellulose, hemicellulose and lignin.

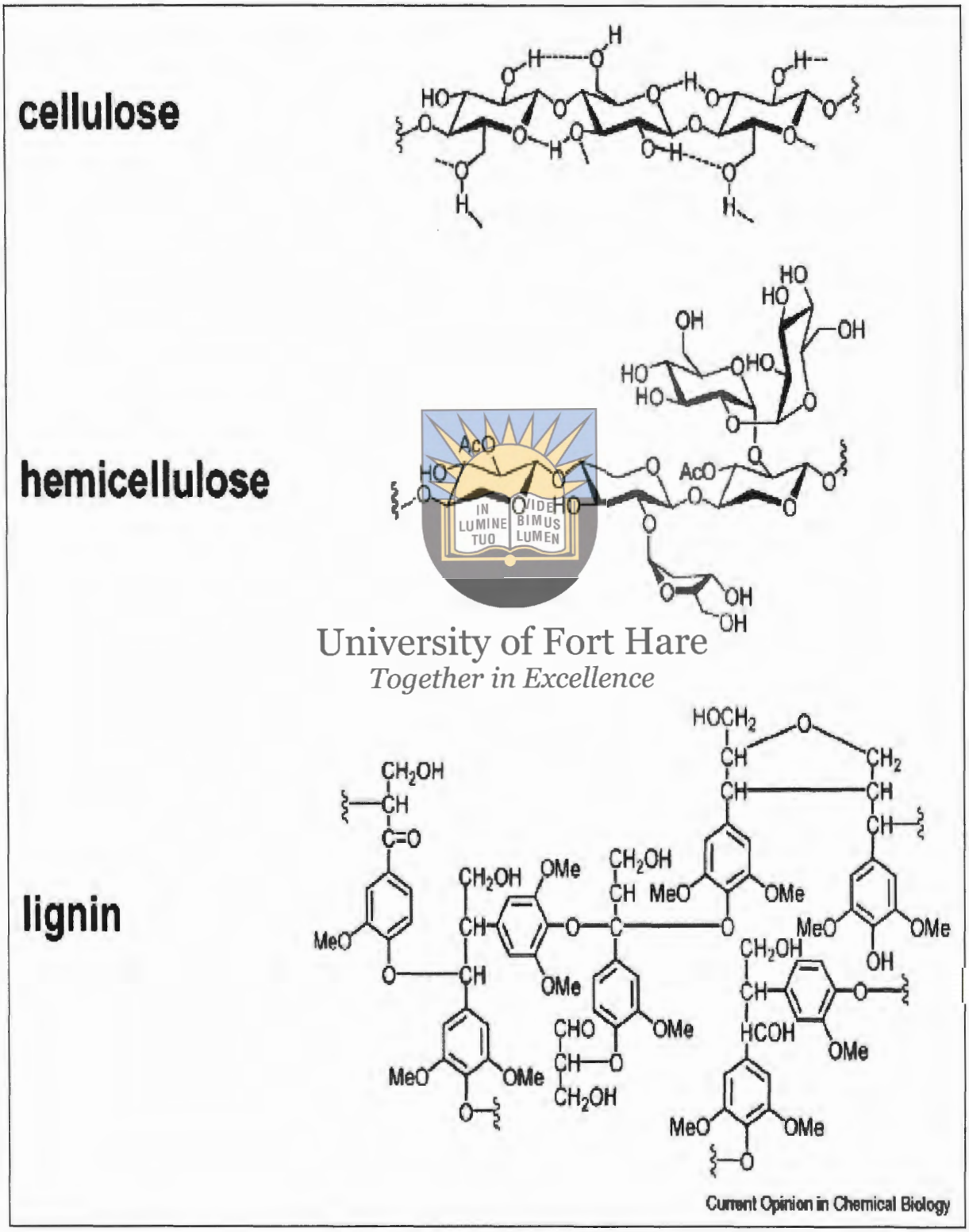


Figure 2.3 : Structure of cellulose, hemicellulose and lignin [Chang, 2007]

2.3. REACTIONS OF LIGNOCELLULOSE

The cellulose molecule contains three different kinds of anhydroglucose units, the reducing end with a free hemi-acetal (or aldehyde) group at C-1, the non-reducing end with free hydroxyl at C-4, and the internal rings joined at C-1 and C-4. As a result of the long chain length, the chemistry of the alcohol groups of the internal units predominates, on the condition that the chains are not cleaved by reaction conditions. Unlike simple alcohols, however, cellulose reactions are usually controlled by steric factors than would be expected on the basis of the inherent reactivity of the different hydroxyl groups.

There are potentially three hydroxyl groups available on each anhydroglucose ring, so derivatives are usually characterized in terms of a "degree of substitution", (DS), which is an average for the whole chain and can range between 0 and 3. In most cases, partial reaction to DS< 3 gives products that are essentially block copolymers, where virtually all the hydroxyls occurring in the less ordered regions may be derivatized, while those in the crystalline regions, remain un-reacted. Higher degrees of substitution, or reaction conditions which disrupt the crystalline regions, can be used to reduce interchain hydrogen bonding and force the chains apart. This can result in a cellulose derivative that is soluble in common solvents, and thus capable of extrusion to form filaments, or other structures[Malcolm, 1990].Malcolm,1990 reported some of the cellulose reactions by:

- **Esterification**, by reaction with acids or other acylating agents;

- **Etherification**, by the number of common alkylating agents;
- Acetal formation, the hydroxyls of cellulose react with aldehydes and hemiacetals to form acetals.
- **Thermal degradation**, which has been used by researchers to explore possibilities of franctioning cellulose in the production of bio-ethanol. Degradation at low temperatures is often predominantly thermo-oxidative and/or hydrolytic. At higher temperatures(>200^{0}C) water is lost, first from that absorbed the cellulose and then elimination from the cellulose hydroxyls. At even higher temperatures (>250^{0}C), several competing pyrolytic reactions begin to occur. These reactions can be grouped into three classifications:

- At lower temperatures: This is similar to aging reactions. Products are water, CO, CO_2 and a carbonaceous char.
- At higher temperatures: Another reactions begins, which results in depolmerization of the cellulose chain and formation of aahydroglucose derivatives, volatile organic materials and tars.
- At even higher temperatures: Random bond cleavage of cellulose and intermediate decomposition products result in the formation of a variety of low molecular weight compounds.
- Oxidative degradation, strong oxidizing agents and/or vigorous reaction conditions are needed to convert cellulose into CO_2 and H_2O. Under less vigorous reaction conditions, cellulose is capable of a variety of oxidation reactions, many of which are predictable by analogy to simle alcohols, trans-

glycols and acetals. In general oxidation of cellulosic hydroxyls forms the expected aldehyde and ketone, and carbonyl groups. However, unlike simple carbonyl analogs, the oxidation products of cellulose(termed oxycelluloses) are significantly less stable in the presence of alkali

2.4.KIKUYU GRASS

Kikuyu grass contains an appreciable quantity of starch as indicated by previous researchers. [Felipe *et al*, 2010] While starch will not contribute directly to the silage fermentation, as silage bacteria cannot ferment starch, hydrolysis of starch to sugars during wilting and prior to the establishment of anaerobic conditions in the silo could boost the supply of sugars available for fermentation, provided there is no significant loss due to respiration. Figure 2.4 represents a typical Kikuyu grass stem.

Figure 2.4: Photograph of a typical kikuyu grass stem [Glen, 2003].

Table 2.1 represents content of cellulose, hemicelluloses and lignin in kikuyu grass

Table 2.1 Cellulose, Hemicellulose and Lignin Content in Kikuyu Grass [Wyman, 2005].

Component	Mean
Hemicellulose	26.2
Cellulose	26.9
Lignin	5.88

2.5. LIGNOCELLULOSE PRETREATMENT

The effect of pre-treatment has been studied for a very long time. Pretreatment is an important tool for practical cellulose conversion processes and is crucial before enzymatic hydrolysis can take place, effectively. It is necessary in order to alter the structure of cellulosic biomass, thereby increasing its surface area which facilitates rapid and efficient hydrolysis of the polymer to fermentable sugars (Figure 2.5) [Chen *et al*, 2007]. An effective pretreatment method should meet the following requirements: [Kumar, 2009, Taherzadeh & Karimi, 2008].

(i)Improve the formation and availability of sugars through hydrolysis

The concentration of sugars from the coupled operation of pretreatment and enzymatic hydrolysis should be above 10% to ensure an adequate ethanol concentration and to keep recovery and other downstream cost manageable.

(ii)Avoid the degradation or loss of carbohydrates,

High yields close to 100% of fermentable cellulosic and hemicellulosic sugars should be achieved through pretreatment step

(iii)Avoid the formation of by-products that could be inhibitory to the fermentation process

The liquid hydrolyzate from pretreatment must be fermentable following a low-cost, high yield conditioning step. Harsh conditionsduring pretreatment lead to a partial hemicellulose degradation and generation of toxic compounds derived from sugardecomposition that could affect the proceeding hydrolysis and fermentation steps [Oliva *et al*, 2003]. The quantity of toxic compounds generated depends on the raw material and harshness of pretreatment. Degradation products from pretreatment of lignocelluloses materials can be divided into the following classes: carboxylic acids, furan derivatives, and phenolic compounds. Main furan derivates are furfural and 5-hydroxymethylfurfural (HMF) derived from pentoses and hexoses degradation, respectively [Palmqvist and Hahn-Hägerdal, 2000]. Weak acids are mostly acetic and formic and levulinic acids Phenolic compounds include alcohols, aldehydes, ketones and acids (Klinke *et al*, 2002).

(iv) Minimize energy consumption and operational costs

Pretreatment reactors should be low in cost through minimizing their volume, employing appropriate materials of construction for highly corrosive chemical environments, and keeping operating pressures reasonable

Various pretreatment options are available and can be classified as physical, chemical, physio-chemical and Biological pretreatment.

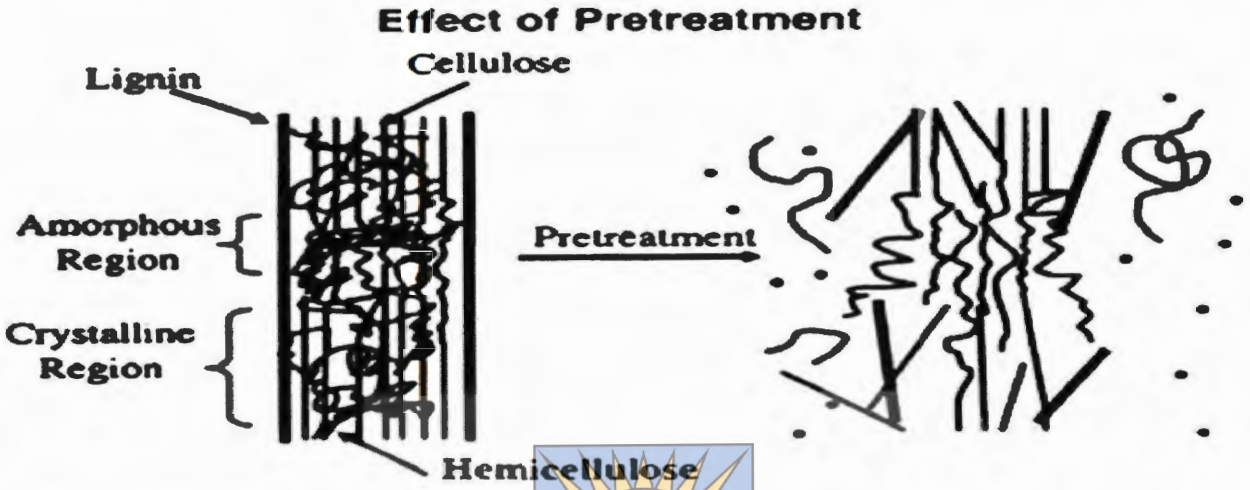


Figure 2.5 Simplified representation of pretreatment which shows the main goals of pretreatment. [Hsu *et al*, 1980]

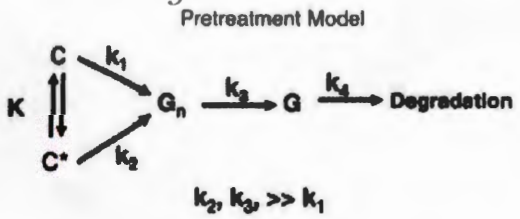


Figure 2.6: Schematic representation of pretreatment steps: Transformation between crystalline (C) amorphous cellulose (C*) is reversible. Both forms may lead oligosaccharides, which in turn form glucose. Glucose degradation can then occur to form fermentation inhibitors.

2.5.1. PHYSICAL PRETREATMENT

Physical pretreatment is a preliminary step included in almost all pretreatment methods. Mechanical comminution involves milling, grinding or chipping which is basically done to reduce the size of the biomass to facilitate the breaking of cellulose crystallinity. Usually a small size results in most efficient hydrolysis, however such small sizes require additional energy.

2.5.2. PHYSIOCHEMICAL PRETREATMENT

This category includes methods that are a mixture of purely physical and chemical methods for the pretreatment of biomass.

2.5.2.1. Ammonia fibre explosion

Ammonia fiber explosion (AFEX) is a method that operates at elevated pressures using ammonia instead of water, in contrast to steam pretreatment [Galbe *et al*, 2012]. Four primary processing conditions are varied in order to optimize sugar yields these include ammonia loading, water loading, residence time, and temperature. These optimal conditions can change in severity based on the type and maturity of the biomass being pretreated [Bals *et al*, 2011]. It also does not liberate any sugars directly because of low hemicelluloses solubilization but opens up the structure of lignocellulosic biomass and increases polymers surface area and consequently, the enzymatic digestibility.

2.5.2.2. Steam explosion

Steam explosion is one of the most frequent methods for pretreatment of lignocellulosic materials, and has been studied by many scientists and engineers. In this method, chipped biomass is treated with high-pressure saturated steam and then the pressure is swiftly reduced, which makes the materials undergo an explosive decompression [Sun and Cheng, 2002]. Typically, the lignocellulosic material is treated at temperatures in range of 160-240° C for 1-20 min [Galbe *et al*, 2012]. The process causes hemicellulose degradation and lignin transformation due to high temperature, thus increasing the potential of cellulose hydrolysis [Sun and Cheng, 2002]. Limitations of steam pretreatment include destruction of a portion of the xylan fraction which decreases sugar recovery, incomplete disruption of the lignin carbohydrate matrix and formation of inhibitory compounds.

2.5.2.3 Treatment with supercritical water

It has been discovered that supercritical fluids (highly compressed gases or liquids at above the critical temperature and critical pressure) have the ability to dissolve materials not normally soluble in either liquid or gaseous phase of the solvent, thus promoting gasification or liquefaction reactions [Xu and Etcheverry, 2008]. Lignocellulose can be treated in supercritical water (≥ 374°C, 22.1MPa), and fractionated into the water-soluble, precipitates, methanol-soluble portion and supercritical water-insoluble residue. It was found that polysaccharides, oligosaccharides, glucose and glucose decomposed compounds could be obtained with

supercritical water treatment of cellulose, and the resultant polysaccharides, oligosaccharides and glucose can be converted to ethanol with simultaneous saccharification and fermentation [Ehara and Saka, 2002 and Nakata *et al*, 2006]. However, there are some drawbacks with using water as a solvent. These drawbacks include: Lower yields of the water-insoluble oil product (with higher heating value) compared with those of water-soluble product (with lower heating value) as well as higher oxygen content in the liquefied products, resulting in low-heating value for heating products.

2.5.3 CHEMICAL PRETREATMENT

Chemical pretreatment involves the use of different chemicals such as acid, alkaline, ozone and hydrogen peroxide to degrade lignin and disrupt the crystalline structure of cellulose.

2.5.3.1 Ozonolysis

The effect of ozone pretreatment has been found to be essentially limited to lignin degradation. Hemicellulose is slightly attacked, while cellulose is hardly affected. [Gerolova *et al*, 2011]

Moreover, ozonation reduces the formation of degradation products that might interfere with subsequent hydrolysis or fermentation stages. [García-Cubero *et al*, 2010] Ozonolysis is, however, a very expensive procedure due to the large amount of ozone required.

2.5.3.2. Acid pretreatment

Acid hydrolysis of cellulosic materials has been studied for a very long time. Concentrated acid such as HCl and H_2SO_4 has been used to treat lignocellulosic materials. Acid hydrolysis removes the hemicellulosic portion and some fraction of lignin, the remainder of the lignin remains intact to the cellulosic substrate [Kaya and Thomas, 2000]. Acid hydrolysis can be divided into two categories: Dilute acid and concentrated acid. Concentrated acid process operates at low temperature (40°C) and give higher sugar yields [Brethauer and Wyman, 2010] for example 90% of theoretical glucose yield. The acid concentration used in concentrated acid hydrolysis process is in the range of 10-30% [Iranmahboob, 2002]. This process provides a complete and rapid conversion of cellulose to glucose and hemicellulose to five-carbon sugars with little degradation [Balat, 2008].

Dilute acid hydrolysis is conducted at higher temperature (120-200°C) and high pressure (103-517 KPa) and have reaction times ranging from 30min- 2 hours by continues processes [Badger, 2002; Kim, 2000].

The alcoholic hydroxyl groups of cellulose are polar and can be substituted by nucleophilic groups in strongly acidic solution. [Kim *et al*, 1997] The molecular mechanism of acid catalyzed hydrolysis of cellulose (cleavage of β-1-4-glycosidic bond) follows the pattern as outlined in Figure 2.7 [Fengel and Wegener, 1984].

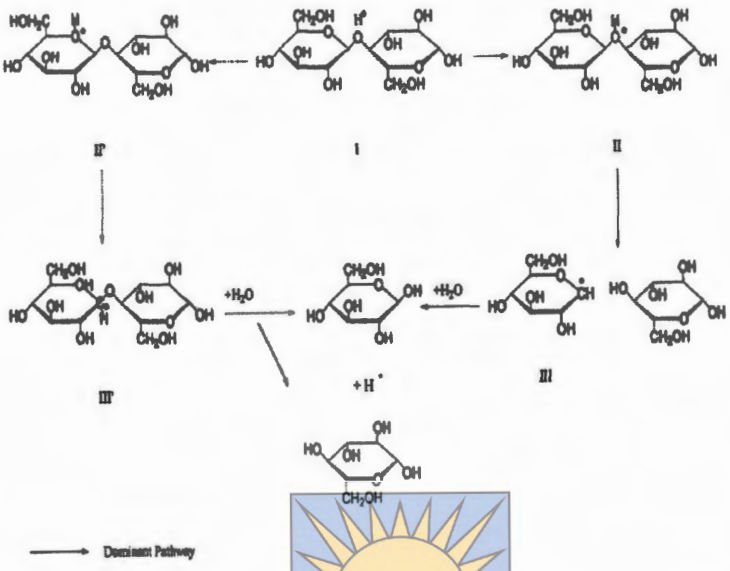


Fig 2.7: Mechanism of acid catalyzed hydrolysis of cellulose

Acid hydrolysis proceeds in three steps. The reaction starts with a proton from the acid interacting rapidly with the glycosidic oxygen linking two sugars units, forming a conjugate acid. The cleavage of the C-O bond and breakdown of the conjugate acid to the cyclic carbonium ion then takes place, which adopts a half-chair conformation. After rapid addition of water, free sugar and a proton are liberated [Shafidzah, 1963; Phillip *et al*, 1979]. The formation of the intermediate carbonium ion takes place more rapidly at the end than at the middle of the polysaccharide chain. In accordance with this, the yield of the monosaccharides after partial hydrolysis is higher than calculated on the basis of a random bond cleavage [Fengel and Wegener, 1984]

An obstacle with acid hydrolysis is the range of toxic compounds and by products which are generated during pretreatment and hydrolysis of lignocelluloses materials. Potential inhibitors that can be formed or released from hemicelluloses, lignin and cellulose during acid hydrolysis include: furfural, acetic acid, hydroxyl-methyl furfural, formic acid

and levulinic acid [Balat *et al*, 2008]. These by products interfere with fermentation, they would have to be removed before fermentation can occur.

2.5.3.3. Alkaline pretreatment

Bases can also be used for pretreatment of lignocellulosic materials and can be used at temperatures higher than 100°C [Chaudhary, 2012]. These bases include: NaOH, $Ca(OH)_2$, ammonia etc. The effect of alkaline pretreatment depends on the lignin content of the materials [Ganguly *et al*, 2012]. Alkali treatment increases cellulose and this can be attributed to decrease in lignin [Singh and Bishnoi, 2013]. Studies have shown that a strong alkali such as NaOH can create a high number of pores since a strong alkali results in a more severe pretreatment causing swelling which leads to an increase in internal surface area, a decrease in degree of polymerization, a decrease in crystallinity [Fan *et al*, 1987] The mechanism of alkaline hydrolysis involves the saponation of intermolecular ester bonds cross linking xylan hemicelluloses and other components such as lignin, and the porosity of the lignocellulosic materials increases with the removal of these crosslinks [Ganguly *et al*, 2012].

2.5.3.4. Oxidative pretreatment

Hydrogen peroxide removes the hemicelluloses and lignin in-order to increase the accessibility of enzymes [Singh and Bishnoi, 2013]. The pretreatment of cane bagasse with $(OH)_2$ greatly enhanced its susceptibility to enzymatic hydrolysis [Sun and Cheng, 2002] In $(OH)_2$ treatment several reactions can take place such as electrophilic

substitution, displacement of side chains, cleavage of alkyl aryl ether linkages and oxidative cleavage of aromatic nuclei [Hon and Shiraishi, 2001].

2.5.4 BIOLOGICAL PRETREATMENT

Unlike most of the pretreatment methods (i.e. chemical and physiochemical pre-treatment methods) that require high capital and operational cost, biological or microbiological is environmentally friendly and doesn't require any chemicals. Biological pretreatments simply employ microorganisms mainly brown, white and soft-rot fungi which degrade lignin and hemicelluloses and very little of cellulose, since cellulose is more resistant than the other components [Sánchez, 2009]. Lignin degradation by white-rot fungi, the most effective for biological pretreatment of lignocellulosic materials, occurs through the action of lignin-degrading enzymes such as peroxidases and laccases [Kumar *et al*, 2009]. In general, such processes offer advantages such as low-capital cost, low energy, no chemicals requirement, and mild environmental conditions. However, the main drawback to develop biological methods is the low hydrolysis rate obtained in most biological materials compared to other technologies [Sun and Cheng, 2002].

Table 2.2: Summary of selected pretreatment methods

Pretreatment Process	Pretreatment Method	Advantages	Limitations and Disadvantages
Physical pretreatment	Mechanical Comminution	Reduces cellulose crystallinity and increases biomass surface area	Energy required usually higher than inherent biomass energy
	Pyrolysis	Produces gas and liquid Products	High temperature; ash Production
Chemical pretreatment	Dilute acid	Hydrolyses hemicellulose to xylose and other sugars; alters lignin structure	High cost; corrosion of equipment; forms inhibitors
	Alkali	Removes hemicellulose and lignin; increases biomass surface area	Long residence times; irrecoverable salts formed and incorporated into biomass; not effective on softwoods
	Hydrogen peroxide	Solubilises lignin; does not produce inhibitors	Hydrogen peroxide decomposes at high temperature, causing a decrease in lignin and hemicelluloses solubilisation
	Organosolv	Hydrolyses lignin and hemicellulose	High cost; solvents need to be recovered and recycled
Physicochemical pretreatment	Steam pretreatment	Causes hemicellulose degradation and lignin transformation; short residence time; cost effective	Destroys a portion of the xylan fraction; incomplete destruction of the lignin carbohydrate matrix; formation of toxic compounds

2.6. FORMATION OF INHIBITORS

During hydrolysis especially acid hydrolysis, a range of toxic compounds (by-products) are generated during pretreatment. These by-products interfere with fermentation to a point beyond which the efficient utilization of sugars is reduced and product formation decreases [Mussatto and Roberto, 2004]. These toxic compounds must be removed before fermentation can occur. These inhibiting (toxic) compounds are divided into three groups based on origin: weak acids (for example acetic acid), furfural (furan derivatives) and phenolic compounds [Xiang *et al*, 2003; Palmqvist and Hahn-Hagardal 2000]. When hemicellulose is hydrolyzed, xylose, mannose, acetic acid, galactose, and glucose are liberated [Balat, 2011]. Under high temperatures and pressure xylose is further degraded to furfural. Similarly 5-hydroxymethyl furfural (HMF) is formed from hexose degradation. Cellulose is therefore hydrolyzed to glucose. (Figure 2.8)

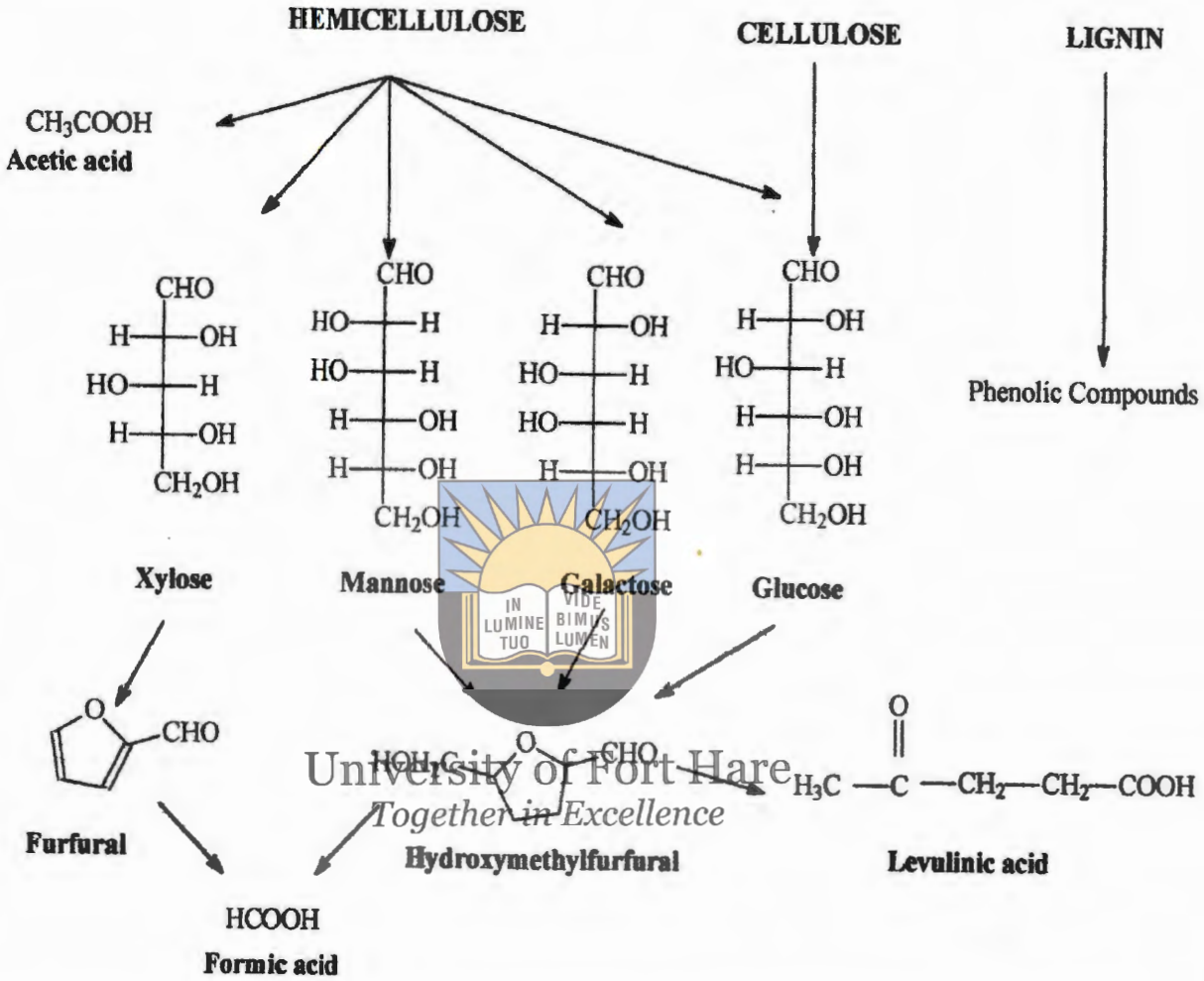


Figure 2.8: Possible reactions products that occur during the hydrolysis of lignocellulosic materials.

When compared with the fermentation of commercial sugars or detoxified hydrolyzates, the fermentation of non-detoxified hemicellulosic hydrolyzates is characterized by slow kinetics, with limited yield and productivity. This is due to the presence of toxic compounds as potent inhibitors of microbial metabolism. Therefore, the lignocellulosic substrates need to be pretreated and detoxified to attain the fermentation pH, thereby

becoming more suitable for microorganism metabolism [Roberto *et al*, 1991; Kuhad and Singh, 1993; Winkelhausen and Kuzmanova, 1998]. Various methods for detoxification of the hydrolyzates have been developed which include: biological, physical, and chemical detoxification techniques which have been proposed to transform inhibitors into inactive compounds or to reduce their concentration. Alkali treatment is considered one of the best detoxification methods [Sánchez and Cardona, 2008]. In this treatment method the pH of the hydrolyzate is increased to 9-10 with calcium hydroxide (overliming) and readjusted to 5.5 using sulphuric acid [Palmqvist and Hahn-Hagerdal, 2000]. By this method, furaldehydes and phenolic compounds are mainly removed leading to great improvement in fermentability, especially in the case of dilute-acid hydrolyzates [Persson *et al*, 2002]. $Ca(OH)_2$ has been reported to result in better fermentability than NaOH adjustment due to the precipitation of toxic compounds [Van Zyl *et al*, 1988] Other detoxification approaches include: treatment with enzymes peroxidase and laccase [Palmqvist and Hahn-Hagerdal, 2000] as well as overliming in combination of sulphite [Olsson *et al*, 1995]. The effectiveness of any detoxification method depends both on the type of hemicellulose hydrolyzate and on the species of microorganism employed, because each type of hydrolyzate has a different degree of toxicity, and each species of microorganism has a different degree of tolerance to inhibitors [Larsson *et al*, 1999].

2.7. ENZYMATIC HYDROLYSIS

A successful conversion of biomass into biofuel cannot be achieved by pre-treatment alone, the enzymatic saccharification of a pre-treated biomass into sugar is one of the significant steps involved in the conversion process [Puri *et al*, 2012]. Enzymatic hydrolysis of cellulose to glucose is carried out by cellulase enzymes which are highly specific catalysts [Beguin and Aubert, 1994]. Enzymatic hydrolysis is usually conducted at mild conditions (pH 4,8 and temperature 318-323K) and has no corrosion problems. Cellulases are usually a mixture of different enzymes [Sun and Cheng, 2002] which include: endocellulases which hydrolyze the cellulose polymer exposing reducing sugar and non-reducing ends of the linear polymer of glucose units "exoglucanases and cellobiohydrolases" which act on these ends to release "cellobiose" and cellooligosaccharides", and β-glucosidases which cleaves the cellobiose units to liberate glucose as end products [Coughlan and Ljungdahl, 1988].

Cellulase enzymes from fungas Trichoderma reesei (T. ressei) can hydrolyze biomass to sugars at near ambient temperatures, which results in little degradation [Brethauer and Wyman, 2010]. The critical problems in developing the bioprocess for cellulase production from T.reesei are [Lee, 1997]: (1) scale up of enzyme production due to oxygen transfer in mycelial broth; (2) poor mixing due to shear sensitivity of fungas; and (3) lower cell bound activity.

There are different factors that affect enzymatic hydrolysis of cellulose, these factors include: substrates, cellulose activity, reaction conditions (temperatures and pH) and a strong product inhibition [Balat, 2011].

The success of enzymatic hydrolysis of cellulose also depends on several structural features of cellulose. These include: molecular structure of cellulose, crystallinity of cellulose, surface area of cellulose fibre, degree of polymerization and associated lignin and other materials. To improve the yield and rate of enzymatic hydrolysis, research has been focused on optimizing the hydrolysis process and enhancing cellulase activity [Cantwell *et al*, 1988; Durand *et al*, 1988; Orpin, 1988]. During the enzymatic hydrolysis cellulose is degraded by the cellulases to reducing sugars that can be fermented by yeast or bacteria to ethanol [Sun and Cheng, 2002].

Table 2.3: Some of the main enzymes required to degrade lignocellulose to monomers. [Vanand Pletschke, 2012)

Lignin	**Laccase, Manganese peroxidase, Lignin peroxidase**
Pectin	**Pectin methyl esterase, pectate lyase, polygalacturonase, rhamnogalacturonan lyase**
Hemicellulose	**Endo-xylanase, acetyl xylan esterase, β-xylosidase, endomannanase, β-mannosidase, α-L-arabinofuranosidase, α-glucuronidase, ferulic acidesterase, α-galactosidase, p-coumaric acid esterase**
Cellulose	**Cellobiohydrolase, endoglucanase, β-glucosidase**

2.8 BIO-ETHANOL PRODUCTION

Bioethanol have been produced for a long time essentially for pharmaceutical, cosmetics, chemical and beverages industries by wet fermentation. The idea of ethyl alcohol as a fuel is not new. It received considerable discussion and publicity in the 1920's as a motor fuel. It was used as a fuel several countries during World War II. Interest was sparked again in the United States in the mid 1970's with the advent of the oil embargo and the rapidly escalating oil prices [Kvaalen *et al* 1984].Currently bio-ethanol for fuel purposes is produced mainly from sugar in Brazil and starch in the United States of America. The basic steps for such feedstocks are as follows: [Oner *et al*, 2005]:

- Enzymatic hydrolysis(use of amylase to break down the starch molecules into sugars) of the biomass
- Microbial(yeast) fermentation of the sugar solution
- Distillation and purification of the fermented solution

Bio-ethanol can also be produced from lignocellulosic materials such as switch grass, agricultural waste, water hyacinth etc. The basic steps for lignocellulosic materials are as follows [Huber *et al*, 2006]:

- Pretreatment phase, to disrupt the lignocellulosic material structure so that hydrolysis can occur more rapidly and with higher yields
- Cellulose hydrolysis, to break down the molecules into sugars
- Separation of the sugar solution from the residual materials in particular lignin and inhibitors during fermentation

- Detoxification of the hydrolyzate
- Microbial (yeast or fungus) fermentation of the sugar solution(hydrolyzate);
- Distillation and purification of the fermented solution to produce pure alcohol

Fermentation of lignocellulosic materials is more difficult than the well established processes of ethanol production. Ethanol fermentation can be carried out by 3 steps: separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) and simultaneous saccharification and co-fermentation (SSCF). Ethanol is then recovered by distillation.

2.8.1. Separate hydrolysis and fermentation (SHF)

In the separate hydrolysis and fermentation (SHF) process, cellulose is first hydrolyzed to glucose and then glucose is fermented to ethanol. The separation of hydrolysis and fermentation offers various processing advantages and opportunities. It enables enzymes to operate at higher temperature for increased performance and fermentation, organisms to operate at moderate temperatures, optimizing the utilization of sugars [Chandel *et al*, 2007]. The major drawback with SHF is that cellulolytic enzymes are end-product inhibited so that the rate of hydrolysis is progressively reduced when glucose and cellobiose accumulate [Tengborg *et al*, 2001].

2.8.2. Simultaneous saccharification and fermentation(SSF)

The SSF process, can attempt to solve the problems associated with SHF particularly end product inhibition; however the reaction conditions are compromised [Margeot *et*

al, 2009]. In addition, SSF has higher ethanol yields and less energetic consumption. In this case, the cellulases and microorganisms are added to the same process unit allowing that the glucose formed during the enzymatic hydrolysis of cellulose be immediately consumed by the microbial cells converting it into ethanol.[Sanchez and Cardona, 2008] A major disadvantage with using SSF is the need of employing more dilute media to reach suitable rheological properties which makes that final product concentration be low. Furthermore, this process operates at non-optimal conditions for hydrolysis and requires higher enzyme dosage, which positively influences on substrate conversion, but dramatically increases the process costs. [Sanchez and Cardona, 2008]

2.8.3. Simultaneous saccharification and co-fermentation (SSCF)

In SSCF, the enzymatic hydrolysis continuously releases hexose sugars, which increases the rate of glycolysis such that the pentose sugars are fermented faster and with higher yields [Ohgren *et al*, 2006]. This reduces the number of reactors involved by eliminating the separate hydrolysis reactor and avoids the problem of product inhibition associated with enzymes [Ojeda *et al*, 2011].

2.9 EFFECTIVE MICRO-ORGANISMS SUITABLE FOR BIOETHANOL PRODUCTION

For an economical process of ethanol production from lignocelluloses, an efficient and complete conversion of glucose and xylose should be achieved. The characteristics required for an industrially suitable micro-organism are summarized in Table 2.4.

Table 2.4: Characteristics required for an industrially suitable micro organism [Dien *et al*, 2003].

Trait	Requirement
Ethanol yield	>90% of theoretical
Ethanol tolerance	>40 g l^{-1} h^{-1}
Ethanol productivity	>1 g l^{-1} h^{-1}
Robust grower and simple growth and simple growth requirements	Inexpensive medium formulation
Able to grow in undiluted hydrolysis	Resistance to inhibitors
Culture growth conditions retard contaminants	Acidic pH or higher temperatures

The trait that has received the most attention is ethanol yield. This is primarily because feedstock typically account for greater than one third of the production cost, therefore maximizing ethanol yield is imperative. Obtaining a high ethanol yield means using strains that produce ethanol with few side effects, and metabolize all major sugars. These sugars include glucose, xylose, arabinose etc. However there is currently no single organism that can meet all the requirements listed in Table 2.4. Among the sugars listed below glucose and xylose are considered to be the main components. [Taniguchi 1997; Kordowska- wiater and targonski, 2002]. The main difficulty of using two microorganisms for the co-fermentation of these two sugars is the inability to provide optimal environmental conditions for the two strains simultaneously [Chandrakant, 1998]. Individually Sacchoromyces cerevisia and Zymomonus mobiles

have been reported for the glucose conversion while Pichia stipitus, Candida shehatae and pachysolen tannophilus have been reported for xylose conversion [Fu and Peiris, 2008].

2.9.1. Saccharomyces cerevisiae

Saccharomyces cerevisiae (S.cerevisia) is a model eukaryotic organism, often used in research because it is easy to manipulate and culture, and is widely used in industrial applications to manufacture enzymes and proteins for beer, wine and bread, [http:www.nature.com accessed on 4/10/2013] and it metabolizes glucose to ethanol during fermentation and is also known to produce higher alcohols and esters from amino acids [Schoondermark-Stolk *et al*, 2006; Sentheshanmuganathan, 1960; Yoshimoto *et al*, 2002]. S. cerevisiae is capable of very rapid rates of glycolysis and ethanol production under optimal conditions, However this high rate is maintained for only a brief period during batch fermentation and declines progressively as ethanol accumulates in the surrounding broth [Casey, 1986; Ingram, 1984; Moulin 1984].

2.9.2. *Escherichia coli* (*E-coli*)

E-coli has several advantages as a biocatalyst for ethanol production including the ability to ferment a wide spectrum of sugars, no requirements for complex growth factors, and prior industrial use. The major disadvantage associated with using *E-coli* cultures are narrow and neutral pH growth range (pH 6.0-8.0) less hardy cultures compared to yeast, and public perceptions regarding the danger of E-coli strains.

E-coli is able to ferment sugars to a mixture of ethanol and organic acids. Ethanol is produced from pyruvate using pyruvate formate lyase (PFL) [Dien, 2007]. This fermentation pathway is very unbalanced because one NaDH,H is generated for each pyruvate made from sugars and two $NaDH,H^+$ are required for converting pyruvate into ethanol. *E coli* balances it during fermentation by also producing acetic and succinic acid [Dien, 2007].

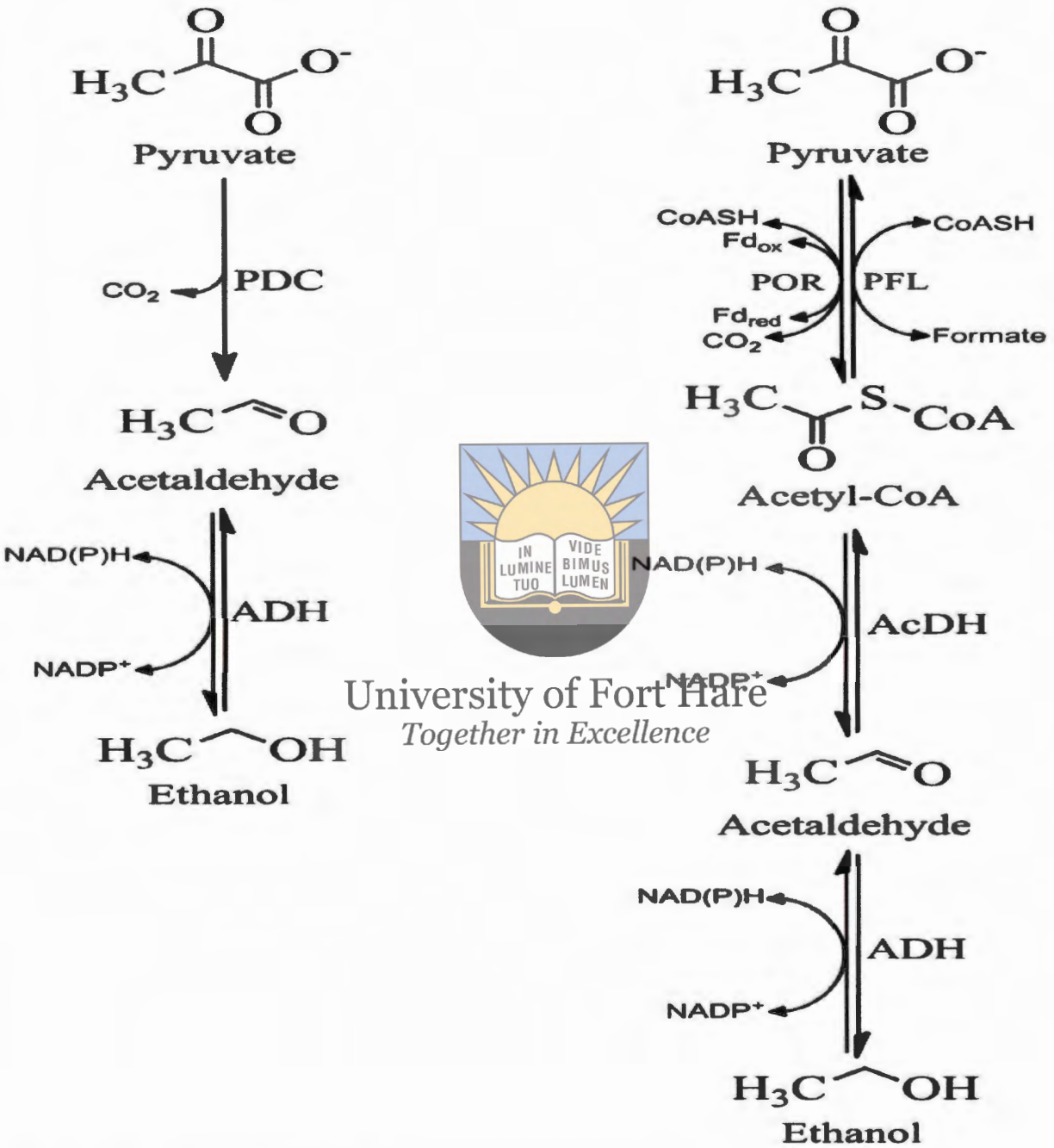


Figure 2.9 : Two pathways of ethanol production from pyruvate. POR; Pyruvate ferredoxin oxidoreductase; PFL; Pyruvate formate lyase, AcDH; Acetaldehyde dehydrogenase, ADH; Alcohol dehydrogenase, PDC; pyruvate decarboxylase; CoASH; coenzyme A, Fdox; oxidized ferredoxin, Fdred; reduced ferredoxin.

2.9.3. Zymomonas mobiles (Z-mobilus)

Zymomonas mobiles produces ethanol at high yields, however it is only able to ferment glucose and fructose. Z-mobilus appears to be homo-ethanol fermentative because they convert pyruvate to ethanol using pyruvate decarboxylase (PDC), however it only consumes one $NaDH,H^+$ for each ethanol produced [Dien, 2007].

2.10 SEPARATION PROCEDURES

2.10.1 Distillation

Distillation is a separation method mostly applied in chemistry, which is based on the volatility of the components of a liquid mixture. Distillation of ethanol-water mixture requires special attention unlike normal distillation. This is because the mixture ethanol-water, (boiling point 78.4 °C and 100 °C) depending on the ratio of ethanol to water [Young *et al*, 1922], is an azeotropic mixture.

2.10.2 Azeotropic distillation of alcohol

When mixtures of compounds are distilled, which produce vapors having the same composition as the liquid, the particular mixture undergoing distillation is called an azeotropic mixture. Boiling points of azeotropes are higher or lower than the boiling points of the components on the mixture and cannot be separated by normal distillation. A special distillation method must be applied as shown in figure 10.

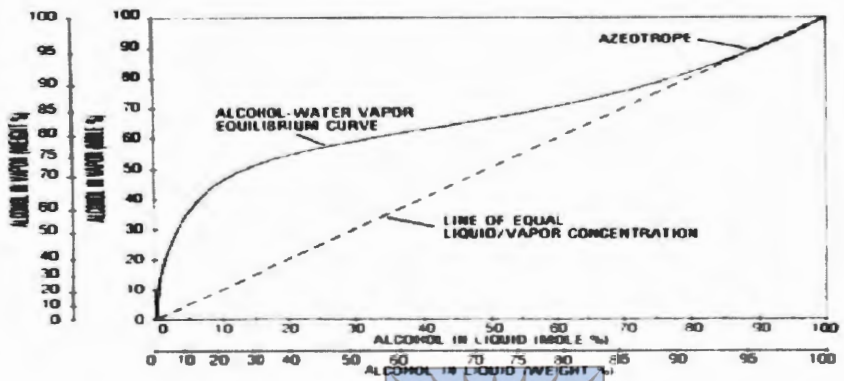


Figure 2.10: Separating curve of azeotrope mixture

Generally a third component or substance is introduced into the mixture to permit separation by distillation or some other separation scheme such as extractive distillation. However this third component must be carefully selected, so that the new azeotrope formed can be resolved by stratification, solvent extraction followed by distillation or distillation under a vacuum [Binning and Lee, 1960]. For the third substance to be introduced, salts are used as the separating agent in salt-distillation processes for separating azeotropic mixtures that cannot be easily purified using conventional distillation processes [Tan, 1987]. Experimentally it was found that the water/ethanol/butanol saturated with KCl, NaCl and NH_4Cl, vapor liquid equilibrium, shown that both NaCl and NH_4Cl have little or no preference in salting out the two organic solvent components while KCl shown preferential salting out of ethanol [Tan and Gan, 2005]. This is because as soon as any of the salt dissolves in the water the boiling point of the water will begin to rise. Ethanol produced by the various methods needs to be purified before use.

2.11. ETHANOL PURIFICATION

After distillation, ethanol can be further purified by many methods. These include drying with lime, addition of a carrier, molecular sieve of a membrane and pressure reduction to selectively absorb the water from the ethanol solution. Calcium oxide can also be used for drying because it has been found to be a good desiccant.

The ethanol-water azeotrope can also be broken by the addition of a small quantity of benzene or cyclohexane. Benzene, ethanol, and water form a tertiary azeotrope with a boiling point of 64.9° C. Since this azeotrope is more volatile than the ethanol-water azeotrope, it can be fractionally distilled out of the ethanol-water mixture, extracting essentially all of the water in the process.

A molecular sieve containing tiny pores of a precise and uniform size (aluminosilicate minerals, clays, porous glasses, microporous charcoal, activated carbons or Zeolite in pellet form) can also be used to selectively absorb the water from the ethanol solution. The Zeolite bed can be regenerated essentially an unlimited number of times by drying it with a blast of hot carbon dioxide.

Membranes can also be used to separate ethanol and water. The membrane can break the water–ethanol azeotrope because separation is not based on vapor-liquid equilibrium.

2.12. CONCLUSION

Biofuels seem to be a plausible alternative for the current fuel crises the economy is going through. In particular the negative environmental effects associated with the current combustion fuels. Lignocellulosic biomasses are abundantly available and easy to harvest.

Lignocellullosic biomasses are carbohydrate polymers tightly bound to lignin and other compounds by hydrogen and covalent bonds. These biomasses can be chemically or biochemically proceeded for fermentable sugar production, further used for alcoholic fermentation. The challenge is to break down the robustness or "recalcitrance" structure of lignocelluloses. However on-going researchers and specific approaches are being conducted to overcome this problem.

Ethanol production involves conversion of fermentable sugars such as glucose into pyruvic acid and the pyruvic acid into ethanol. Living cells (fungi or yeast) used in the fermentation process can only tolerate certain conditions of pH, temperature, concentrations of glucose, alcohol and nutrients. In the medium where yeasts ferments sugars, factors such as inhibition are responsible for cells death and cease the fermentation process.

During fermentation a mixture of more than two liquid products is formed, azeotropic distillation is used for separating bioethanol from the mixture. The process can produce higher alcoholic concentration if efficient drying methods such as calcium oxide or molecular sieves and membranes are used.

REFERENCES

Abdul Khalil, H.P.S.; Bhat, A.H.; Ireana Yusra. A.F. (2012) Green composites from sustainable cellulose nanofibrils: A review *Carbohydrate Polymers*, 87, 963– 979

Aggarwal, N.K., Nigam, P., Singh, D., Yadav, B.S. (2001) Process optimisation for the production of sugar for the bioethanol industry from sorghum a nonconventional source of starch, Bioresource technology, 1, 25-31

Alvira, P., Tomás-Pejó, E., Ballesteros, M., Negro, M.J. (2010) Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review Bioresource Technology,101, 4851–4861

Aresta, M.T., Dibenedetto, A., Barberio, G. (2005) Utilization of macro-algae for enhanced CO_2 fixation and biofuels production: Development of a computing software for an LCA study, Fuel Processing Technology, 86, 1679– 1693

Badger, P.C. In: Ja-nick J, Whipkey A, (2002) editors. Trends in New Crops and New Uses, Alexandria, VA: ASHS Press

Balat, M. (2011) Production of bioethanol from lignocellulosic materials via the biochemical pathway: A review, Energy Conversion and Management, 52, 858–875

Bals, B., Wedding, C., Balan, V., Sendich, E., Dale, B. (2011) Evaluating the impact of ammonia fiber expansion (AFEX) pretreatment conditions on the cost of ethanol production, Bioresource Technology, 102, 1277–1283

Barron, N., Brady, D., Love, G., Marchant, R., Nigam, P., McHale, L., McHale, A.P. (1996) Alginate immobilized thermotolerant yeast for conversion of cellulose to ethanol, In: Wijffels RH.

Buitelaar, R.M., Bucke, C., Tramper, J. (1994) Progress in biotechnology immobilized cells: basics & applications, Elsevier Science , 3, 79-83.

Beguin, P., Aubert, J.P. (1994) The biological degradation of cellulose, Journal of Microbiology , 13, 25–58

Binning, R.C. and Lee, R.J. (1960) Separation of azeotropic mixtures patent number 2953502

Brennan, L., Owende, P. (2010) Biofuels from microalgae a review of technologies for production, processing, and extractions of biofuels and co-products, Renewable Sustainable Energy Reveiws, 15, 57-77.

Brethauer, S., Charles E. Wyman (2010) Review: Continuous hydrolysis and fermentation for cellulosic ethanol production, Bioresource Technology 101, 4862–4874.

Cantwell, B.A., Sharp, P.M., Gormley, E., Mcconnell, D.J.(1988) Molecular cloning of bacillus b-glucanases. In: Aubert, J.P., Beguin, P., Millet, J. (Eds.), Biochemistry and Genetics of Cellulose.

Casey, G. P. and Ingledew, W.(1986) Ethanol tolerance in Yeasts, Critical reviews in biotechnology, 13, 219-290.

Chandel, A. K., Chan, E.S., Ravinder, M., Lakshmi, N., Venkateswar, R., Pogaku R. (2007) Economics and environmental impact of bioethanol production technologies: An appraisal Biotechnology and Molecular Biology Review, 2, 014-032.

Chandrakant, P., Bisaria, V.S. (1998) Simultaneous bioconversion of cellulose and hemicelluloses to ethanol, Critical reviews in biotechnology, 18, 295–331.

Chang, M. (2011) Harnessing energy from plant biomass Current Opinion in Chemical BiologyModel systems/Biopolymers 11, 677–684.

Chen, Y., Sharma-Shivappa, R., Chen, C. (2007) Ensiling agricultural residues for bioethanol production, Applied biochemistry and Biotechnology, 143, 80-92.

Cheng, J.J., Govinda, R., Timilsina, A. (2011) Status and barriers of advanced biofuel technologies: A review, Renewable Energy, 36, 3541-3549.

Chaudhary, G., Kumar, S., Sanjoy, G. (2012) Alkaline pretreatment methods followed by acid hydrolysis of Saccharum spontaneum for bioethanol production, Bioresource Technology, 124, 111–118

Corredor, D.Y., Salazar, J.M., Horn, K.L., Bean, S., Bean, B., Wang, D. (2009) Evaluation and characterization of forage sorghum as feedstock for fermentable sugar production, Applied biochemistry and biotechnology, 158, 164-179

Coughlan, M.P., Ljungdahl, L.G., (1988) Comparative biochemistry of fungal and bacterial cellulolytic enzyme system. In: Aubert, J.-P.,Beguin, P., Millet, J. (Eds.), Biochemistry and Genetics of Cellulose Degradation, 11–30.

Dien, B. S., Cotta, M. A. & Jeffries, T. W. (2003) Bacteria engineered for fuel ethanol production: current status, Applied Microbiology and Biotechnology, 63, 258-266.

Durand, H., Baron, M., Calmels, T., Tiraby, G.(1988) Classical and molecular genetics applied to Trichoderma reesei for the selection of improved cellulolytic industrial strains.In Aubert

Beguin, J.W., Millet, P. J. (Eds.), Biochemistry and Genetics of Cellulose Degradation. Academic Press, San Diego, CA, 135–151.

Ehara, K. and Saka, S. (2002). A comparative study on chemical conversion of cellulose between the batch-type and flow-type systems in supercritical water, Cellulase 9, 301-311

Fengel, D. and Wegener, G. (1984).Wood chemistry, Ultrastructure, Reactions Walter de Gruyter, Berlin, Germany

Fu, N., Peiris, P. (2008) Co-fermentation of an mixture of glucose and xylose to ethanol by Zymomonas mobilis and Pachysolen tannophilus. World Journal of Microbiology and Biotechnology, 24, 10-17.

Ingram, L. O. and Buttke, T.M.(1984) Effects of ethanol on micro-organisms. Advances in Microbial Physiology, 25, 253-300.

Ganguly, A., Chatterjeea, P.K., Dey, A.(2012) Studies on ethanol production from water hyacinth – A review, Renewable Sustainable Energy Reveiws, 16, 966–972

García-Cubero, T., Coca, M., Bolado, S., González-Benito, G. (2010) Chemical Oxidation with Ozone as Pre-treatment of Lignocellulosic Materials for Bioethanol Production, Chemical engineering transitions, 21, 101-103

Gerulova, K. and Blinova, L.(2011)Ozone pretreatment of wheat straw and its effect on reducing sugars in hydrolyzate faculty and materials science and Technology in Trnava Slovak University of Technology in Bratislava Number 31.

Hahn-Hägerdal, B., Galbe, M., Gorwa-Grauslund, M. F., Lidén, G. & Zacchi, G. (2006) Bio-ethanol - the fuel of tomorrow from the residues of today. Trends in Biotechnology 24, 549-556.

Harold House. (2007) Alternative energy sources – Biogas production Engineer, Dairy and Beef Housing and Equipment.

Hon, D.N.S., Shiraishi, N. (2001) Wood and Cellulosic Chemistry, 2nd ed. Dekker, New York.

Hsu, T.A., Ladisch, M.R. and Tsao, G.T. (1980) Alcohol from cellulose, Chemical Technology, 10, 315-319

Iranmahboob, J., Nadim, F., Monemi, S. (2002) Optimizing acid-hydrolysis: a critical step for production of ethanol from mixed wood chips. Biomass Bioenergy, 22, 01–4.

Hu, J., Yu, F. and Lu, Y. (2012) Application of Fischer–Tropsch Synthesis in Biomass to Liquid Conversion, Catalysts ,2, 303-326.

Kaiser, A. G., Piltz, J. W., Havilah, E.J. and Hamilton, J.F. Kikuyu Grass Compositionand Implications for Silage Production, FAO Electronic Conference on Tropical Silage, In:http://www.fao.org/AG/AGP/ag pc/g p/ SILAGE/PDF/4P4.pdf

Kim B.J., Hsieh, H.N., Labavitch, J.M. and Shoemaker, S.P. (1997)."Enzymatic hydrolysis of pre-treated straw". Bioresource Technology, 59, 109-119

Kim KH, Tucker MP, Nguyen Q.A. (2002) Biotechnol Prog 18(3):489, doi:10.1021/bp025503i.

Klinke, H.B., Ahring, B.K., Schmidt, A.S., Thomsen, A.B. (2002) Characterization of degradation products from alkaline wet oxidation of wheat straw. Bioresource Technology, 82, 15–26.

Kordowska-wiater, M., Targonski, Z. (2002) Ethanol fermentation on glucose/xylose mixture by co-cultivation of restricted glucose catabolite repressed mutants OfPichia stipitis with respiratory deficient mutants of Saccharomyces cerevisiae, Acta Microbiologica Polonica, 51:345–52.

Kothari, R., Pathak, V.V., Kumar,V. and Singh D.P. (2012) Experimental study for growth potential of unicellular alga Chlorella pyrenoidosa on dairy waste water: An integrated approach for treatment and biofuel production Bioresource Technology, 116, 466–470.

Kuhad, R.C., Singh, A.(1993) Lignocellulose biotechnology: Current and future prospects, Critical Reveiws in Biotechnology, 13:151-172.

Kumar, P., Barrett, D. M., Delwiche, M. J. and Stroeve, P. (2009) Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production, Industrial and Engineering Chemistry Research,48, 3713-3729.

Kvaalen, E., Wankat, P.C. and Mckenzie, B.A. (1984) Ethanol Distillation: Basic Principles, Equipment, Performance Relationships, and Safety, Purdue University. http://www.ces.purdue.edu/extmedia/AE/AE-117.HTML

Larsson, S., Reimann, A., Nilvebrant, N., Jonsson, L.J. (1999) Comparison of different methods for the detoxification of lignocelluloses hydrolysates of spruce, Applied Biochemistry and Biotechnology, 79, 91–103.

Lee, J. (1997) Biological conversion of lignocellulosic biomass to ethanol, Journal of Biotechnology, 56, 1–14

Margeot, A., Hahn-Hagerdal, B., Edlund, M., Slade, R. and Monot, F. (2009) New improvements for lignocellulosic ethanol, Current Opinion in Biotechnology, 3, 72–80.

Malcolm, P.S. (1990) Polymer Chemistry: An introduction. Second edition, Oxford University Press, 564-567

Mata, T. M., Martins, A. A., Caetano, N. S. (2010) Microalgae for biodiesel production and other applications: a review, Renew Sustain Energy Rev, 14, 7-32.

McKendry, P. (2002) Energy production from biomass (part 1): overview of biomass Bioresource Technology, 83, 37–46

Mohammad, J., Taherzadeh, K. and Keikhosro, K.(2008) Pretreatment of Lignocellulosic Wastes to Improve Ethanol and Biogas Production: A Review, International journal of molecular sciences ,9, 1621-1651

Mosier, N., Hendrickson, R., Ho, N., Sedlak, M., Ladisch, M. R. (2005) Optimization of pH controlled liquid hot water pretreatment of corn stover, Bioresource Technology, 96, 1986–1993

Moulin, G., Boze, H. and Galzy,P. (1984) Inhibition of alcoholic fermentation, Biotechnology and Bioengineering. 25, 365-382.

Mussatto, S.I.,Roberto, I. C.(2004) Alternatives for detoxification of diluted-acid lignocellulosic hydrolyzates for use in fermentative processes: a review, Bioresource Technology.,1, 1-10

Nakata T., Miyafuji, H. and Saka, S. (2006) Bioethanol from cellulose with supercritical water treatment followed by enzymatic hydrolysis, Applied Biochemistry and Biotechnology, 130, 476-485.

Nigam P.S., and Singh A., (2011) Production of liquid bio-fuels from renewable resources Progress in Energy and Combustion Science, 37, 2-6.

Ojeda, K.E., Sánchez, M., Halwagic, E.L., Kafarov, V. (2011) Energy analysis and process integration of bioethanol production from acid pre-treated biomass: Comparison of SHF, SSF and SSCF pathways Chemical Engineering Journal 176– 177

Oliva, J.M., Sáez, F., Ballesteros, I., Gónzalez, A., Negro, M.J., Manzanares, P., Ballesteros, M., (2003) Effect of lignocellulosic degradation compounds from steam explosion pretreatment on ethanol fermentation by thermotolerant yeast Kluyveromyces marxianus, Applied Microbiology and Biotechnology, 105, 141–154.

Olsson, L., Hahn-Hagerdal, B., Zacchi, G. (1995) Kinetics of ethanol production by recombinant Escerichia coli KO11, Biotechnology and Bioengineering, 45, 356-365.

Oner, E.T., Oliver, S.G. and Kirdar, B. (2005) Production of ethanol from Starch by Respiration-Deficient Recombinant Saccharomyces cerevisia, Applied and Enviromental Microbiology, 71, 6443-6445.

Palmqvist, E., Hahn-Hagerdal, B.(2000) Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition, Bioresource Technology, 74, 25-33.

Pereira, N., Peixoto, M.A., Couto Lídia, M., Melo, S.N. (2008) Biomass of lignocellulosic composition for fuel ethanol production within the context of biorefinery 1st. edition

Persson, P., Andersson, J., Gorton, L., Larsson, S., Nilvebrant, N.O., Jonsson, L.F. (2002), Effect of different forms of alkali treatment on specific fermentation inhibitors and on the fermentability of lignocelluloses hydrolysates for production of fuel ethanol, Journal of Agricultural and Food Chemistry , 50, 5318–5325.

Phillip, B., Jacopian, V., Loth, F., Hirte W. and Schulz, G. (1979), In hydrolysis of cellulose: Mechanisms of Enzymatic and Acid Catalysis, Advances in Chemistry Series 181, 127-143

Puri, M., Abraham, R.E, Barrow, C.J. (2012) Biofuel production: Prospects, challenges and feedstock in Australia Renewable and Sustainable Energy Reviews, 16, 6022–6031

Roberto, I.C., Felipe, M.G.A., Lacis, L.C., Silva, S.S., Mancilha, I.M., (1991) Utilization of sugar cane bagasse hemicellulosic hydrolysate by Candida guilliermondii for xylitol production. Bioresource Technology, 36, 271–275

Sánchez, O. J. & Cardona, C. A. (2008), Trends in biotechnological production of fuel ethanol from different feedstocks, Bioresource Technology ,99, 5270-5295

Schoondermark-Stolk, S. A., Jansen, M., Veurink, J. H., Verkleij, A. J., Verrips, C. T, Euverink, G., Boonstra, J., Dijkhuizen, L. (2006) Rapid identification of target genes for 3-methyl-1-butanol production in Saccharomyces cerevisiae. Applied Microbiology and Biotechnology, 70, 237-246.

Sentheshanmuganathan S (1960): Mechanism of the formation of higher alcohols from amino acids by Saccharomyces cerevisiae. Biochemistry Journal, 74:568-576

Shafidzah, F. (1963). TAPPI J.46, P 381-383

Singh, D., Dahiya, J.S., Nigam, P. (1995) Simultaneous raw starch hydrolysis and ethanol fermentation by glucoamylase from Rhizoctonia solani and Saccharomyces cerevisiae, Journal of Basic Microbiology,1, 17-21.

Srirangan, K., Akawi, L., Moo-Young, C., Chou, P. (2012) Towards sustainable production of clean energy carriers from biomass resources, Applied Energy, 100 172–186

Srivastava, A., Prasad, R. (2000) Triglycerides-based diesel fuels Renewable and Sustainable Energy Reviews, 4, 110-133

Sun, Y and Cheng, J. (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review Bioresource Technology ,83, 1–11

Sustainable biofuels: prospects and challenges. London: Royal Society; 2008

Tan, T.C. (1987) Model for predicting the effect of dissolved salt on the vapour liquid equilibrium of solvent mixtures Chemical Engineering Research and Design , 65, 355-366

Tan, T.C and Gan, S.H (2005) Vapour-Liquid Equilibrium of water/ethanol/1-Butanol/Salt: Prediction and Experimental Verification, Chemical Engineering research and design, 83, 1361-1371

Taniguchi, M., Itaya, T., Tohma, T., Fujii, M. (1997) Ethanol production from a mixture of glucose and xylose by a novel co-culture system with two fermentors and two microfiltration modules, Journal of Fermentation and Bioengineering, 84:59–64.

Taherzadeh, M. J. and Karimi, K. (2008)Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: A review, International Journal of Molecular Science, 9, 1621-1651.

Tengborg, C., Galbe, M., Zacchi, G.(2001) Influence of enzyme loading and physical parameters on the enzymatic hydrolysis of steampretreated softwood, Biotechnology Progress, 17, 110–117.

Van Zyl, C., Prior, B.A., du Preez, J.C. 1988Production of ethanol from sugar cane bagasse hemicellulose hydrolysate by Pichia stipites, Applied Biochemistry and Biotechnology, 17, 357-369.

Vásquez, A.F.L., Ortegón, G.A., Rey, R. (2010) Obtaining of Reducing Sugars from Kikuyu Grass (Pennisetum Clandestinum):Fecha de aceptación del artículo

Verma, G., Nigam, P., Singh, D., Chaudhary, K. (2000) Bioconversion of starch to ethanol in a single-step process by co-culture of amylolytic yeasts and Saccharomyces cerevisiae Bioresource Technology, 72, 1-6.

Winkelhausen, E., Kuzmanova, S. (1998) Microbial conversion of D-xylose to xylitol, Journal of Fermentation and Bioengineering, 86, 1–14.

Wyman, C.E., Dale, B.E., Elander, R.T., Holtzapple, M., Ladisch, M.R. and Lee, Y.Y. (2005) Coordinated development of leading biomass pretreatment technologies, Bioresource Technology, 96 1959-1966.

Xu, C. and Etcheverry, T. (2008) Hydro-liquefaction of woody biomass in sub-and supercritical ethanol with iron based catalysts, Fuel, 87, 335-345

Xu, J., Wang, Z. and Cheng, J.J. (2011) Bermuda grass as feedstock for biofuel production: A review, Bioresource Technology, 102, 7613–7620

Yoshimoto, H., Fukushige, T., Yonezawa, T., Sone, H. (2002) Genetic and physiological analysis of branched-chain alcohols and isoamyl acetate production in Saccharomyces cerevisiae, Applied Microbiology and Biotechnology, 59, 501-508.

Young, S. (1922) Distillation principles and processes, Mac Millan and Co. Limited, UK, 267-269

CHAPTER 3

EXPERIMENTAL PROCEDURES AND CHARACTERIZATION

3.1 INTRODUCTION

In this study, experimental procedures were carried out to isolate lignocelluloses from the soluble components of the biomass with hot water. The bio-polymer (residue) was broken down through a chemical route using sulfuric acid, sodium hydroxide, hydrogen peroxide and ozone to yield fermentable sugars. The sugar obtained was fermented under a nitrogen atmosphere with baking yeast to produce bio-ethanol.The materials and products of bio-ethanol production were analyzed at various stages, before and after pretreatment. Characterization methods included X-Ray Diffraction (XRD), Scanning Electron Microscope (SEM), Fourier transform Infra-Red (FT-IR) and UV-Vis (UV).

3.2 EXPERIMENTAL PROCEDURES

3.2.1 Materials

Grass (*Pennisetum clandestinum* or kikuyu grass) is the biomass that was used for hydrolysis and alcoholic fermentation in this study. The grass was collected from the University of Fort Hare grounds where it is available in abundance. This grass is generally known to tolerate low fertility and high temperatures. It can recover from drought conditions but has low tolerance for cold temperatures. The cellulose

percentage of Kikuyu grass increases with age [Gomide *et al*, (1969)]. Sulphuric acid, sodium hydroxide, hydrogen peroxide and ozone were used as pretreatment chemicals for hydrolysis.

3.2.2 Biomass Pretreatment

The grass was pretreated using various methods before hydrolysis to disrupt the complex structure of lignocelluloses which is the main constituent. The solid residues collected after pretreatment were used for enzymatic hydrolysis.

3.2.2.1Physical Pretreatment of Kikuyu grass

Grass was cut into pieces of about 1-2 cm, washed and air dried at ambient temperature. The plant material was then milled into powder using a domestic grinder to allow passage through a 425μm US standard sieve series in-order to achieve both size reduction and enhance penetration of the biomass particles by maximizing the internal surface area that will be directly exposed to the hydrolysis agent. Soluble substances were eliminated in boiling water (100°C) until the filtrate became colorless. The solicl residue was then dried to constant weight at 105°C overnight [Ruiz *et al*, 1996; Palmowski, 1999]. Finally the powder was stored in a glass bottle and kept in the dark at room temperature until it was used for chemical pretreatment. Figure 3.1 shows the physical pretreatment of Kikuyu grass.

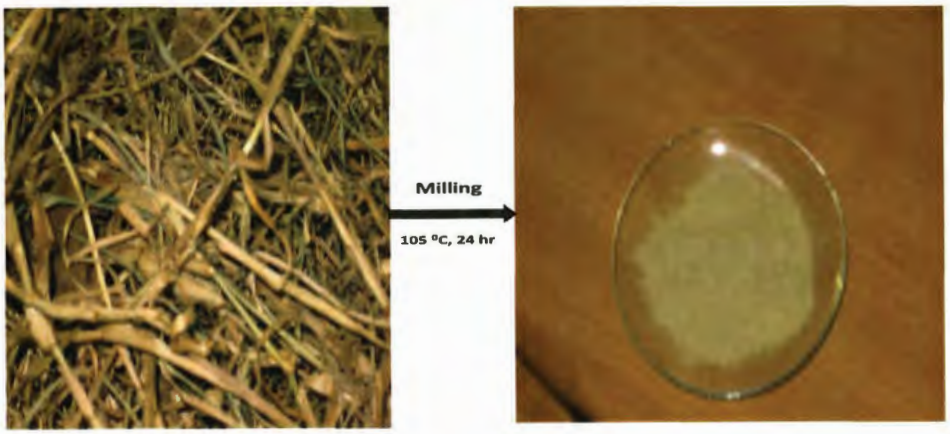


Figure 3.1: Physical treatment of Kikuyu grass

3.2.2.2 Chemical Pretreatment of Kikuyu grass

The hydrolysis of the grass was carried out as follows: 2 g of biomass were suspended in 25ml of 2%(w/v) H_2SO_4, H_2O_2 and NaOH. The solutions were stirred for one hour at room temperature and then heated under reflux in 250 ml flasks at 98°C at different time intervals of 2,4,6 and 24 hours).

3.2.2.3 Ozone pretreatment

The pretreatment of lignocellulosic material in ozone was adapted from a method reported by Gerulova and Blinova 2011 as follows: 2.5 g of Biomass was suspended in 20mL of distilled water in a boiling flask. This suspension was pretreated by bubbling with ozone for 2.0, 4.0, 6.0 and 24 hours at room temperature.

3.2.2.4 Enzymatic Hydrolysis

The enzymatic saccharification of lignocellulosic material in ozone, sulfuric acid, sodium hydroxide and hydrogen peroxide was adapted from Tomoko Sugimoto *et al* , 2009 as follows:

2 g, of pretreated biomass were treated with 0.2 g of Cellulase from Tricoderma reesei (ATCC 26921), in 100 ml of 0.1 mol sodium acetate buffer solution (pH 4.8) at 40° C for 72h with continuous shaking, this was performed twice for each sample.

3.3. FERMENTATION

3.3.1 Inoculation and fermentation

Fermentations were carried out in 250ml three necked flasks, which were oven sterilized at 105° C for 30 minutes. The fermentation process was adapted from those used by [Cheung and Anderson, 1997; Guilliermond, 2003; Xiros *et al*, 2008; Farfan *etal,* 2008; Han and Chen, 2008] as follows: Before adding the yeast into the hydrolyzate, the growth medium consisted of 7g/L baking yeast, 10g/L $(NH_4)_2HPO_4$; 0.3g/L $MgSO_4.7H_2O$ and 20g/L glucose. Incubation was done at 30° C to allow the culture to "bloom" (grow) for a total of one hour. The yeast culture was then added to the hydrolyzate, stirred and stoppered, and the yeast left to work under a nitrogen atmosphere. The substrates were comprised of hydrolyzates with pH adjusted to 4.8. After inoculation, the fermentation mixtures were incubated at 30° C in a water bath, and samples were collected after 48 hours for ethanol determination and subsequent distillation for further purification. Bio-ethanol was collected at 85° C during distillation.

Dry commercial baking yeast (with Sacchomyces cerevisia as active components cells) from Anchor yeast (Pty) Ltd was used for fermentation.

3.4 CHARACTERIZATION

3.4.1 Untreated and Treated Grass

The Kikuyu grass was analyzed to determine its degree of crystallinity using X-Ray diffraction. Samples of particle size less than 425 μm were scanned at a 2 theta range between 0 and 100° C, by positioning the sample holder on a quartz sample holder using a D8 Advance diffractometer in conjunction with a Cu Kα radiation source using an accelerating voltage of 40 KV with a cube current of 40mA. Biomass crystallinity as expressed by the Crystallinity Index (CrI) was determined according to a method by Segal *et al.* (1959) as follows:

$$CrI = 100 \times [I_{002} - Iamorphous/I_{002}] \quad \text{.....(3.1)}$$

where I_{002} is the intensity for the crystalline portion of biomass (i.e., cellulose) at about $2\theta = 22.5$ and Iamorphous is the peak for the amorphous portion (i.e., cellulose, hemicellulose, and lignin) at about $2\theta = 16.6$, and is assumed to correspond to the amorphous region [Kumar *et al*, 2009].

3.4.2 Scanning Electron Microscopy

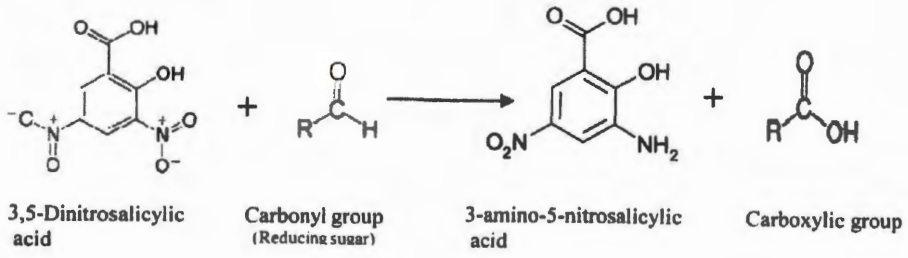
SEM images were taken for both the treated and untreated Kikuyu grass solids (dried powder of particle size< 425 μm) using a JEOL JSM 6390 LV microscope. Prior to acquiring images the samples were mounted on stubs with double sided tapes and were coated with Au-Pd using an Eloko .IB3 Ion coater. The representative images of the pretreated Kikuyu grass reported in this study were acquired with a 15KV accelerating voltage at 1000 magnification.

3.4.3 FT-IR spectroscopy

FT-IR was conducted using a Perkin Elmer System 2000, to investigate the functional groups present in the Kikuyu grass before and after pretreatment. FT-IR sample spectra were obtained using an average of 8 scans over a range of 600-4000cm^{-1} with a spectral resolution of 4.0cm^{-1}.

3.4.4 Determination of fermentable sugars

The content of reducing sugars obtained during pretreatment and hydrolysis processes were determined using the 3,5- Dinitrosalicylic acid method (DNS method) [Miller, (1959), Lindsay, (1973), Bailey, (1988)]. In this reaction, illustrated in Equation 3.2, the 3-amino group, NH_2 replaces the one nitro group, NO_2^- to give 3-amino-5-nitrosalicyclic acid.



Equation 3.2: Reaction of DNS with reducing sugar.

3.4.4.1 Materials and Instrument

-Dinitrosalicyclic acid

-Sodium sulfite from

-Sodium Hydroxide

-Potassium sodium tartrate solution

-T80/T80+ UV-VIS Spectrophotometer

3.4.4.2 Reagents Preparation

The DNS reagent 1% was prepared by making a solution with the following constituents:

-Dinitrosalycylic acid : 10g

-Sodium Sulfite: 0.5g

-Sodium hydroxide: 10 g

-Distilled water was added to make up to a litre.

Potassium sodium tartrate solution, 40% (w/v)

3.4.4.3 Experimental Procedure

DNS (3 mL) reagent was added to 3 mL of the sugar sample in a lightly capped test tube. To avoid the loss of liquid due to evaporation, the test tube was covered with a piece of paraffin film. The mixture was heated at 90° C for 5-15 minutes to develop a red-brown color. Potassium sodium tartrate (Rochelle salt) solution was added to the mixture to stabilize the colour. Absorbances were recorded on a spectrophotometer at 540 nm, after cooling to room temperature in a cold bath.

3.5 DISTILLATION AND PURIFICATION

3.5.1 Azeotropic Distillation

Ethanol forms an azeotropic mixture with water to give a constant boiling point. To separate the ethanol from the mixture, distillation was done according to the method described by Tan and Gan (2005), in a three necked flask; two thermometers were used to control the vapour (maintained at 84°C at the inlet of the condenser) and the mixture (maintained at 95°C) temperature. Potassium chloride (3.0 g) were added to saturate the mixture from fermentation, to eliminate the azeotropism and allow distillation.

3.6. DETERMINATION OF ETHANOL

3.6.1 Materials and Instrument

Reagents and the instrument used for ethanol determination wereas follows:

- 1.5-Diphenylcarbazide grade
- Potassium Dichromate
- Sulfuric Acid
- Absolute ethyl alcohol
- T80/T80+ UV-VIS Spectrometer

3.6.2 Reagents preparation

Solution of *s-Diphenylcarbazide* (1,5-Diphenyl carbazide): 95% ethanol as saturated with *s-diphenylcarbazide* and allowed to stand until the solid had settled.

For Potassium Dichromate-Sulfuric Acid Reagent: 1 g of dry potassium dichromate was transferred to a 100 mL volumetric flask, and concentrated acid was added to the mark. The above mixture was warmed slightly and shaken until the crystals dissolved. The contents of the flasks were brought to 20°C, sulphuric acid was added to the mark, and mixed thoroughly. The solution was stored in a volumetric flask.

Absolute ethyl alcohol: 10 mg of ethanol per mL (stock solution) as suggested by Williams and Resse (1950) was prepared as follows: 1g (1.266 mL) of ethanol was measured into a 100 mL volumetric flask and water was added to the mark.

3.6.3 Experimental Procedure

The experiment was carried out according to Williams and Reese (1950) as follows: Ethanol (10 mL), was pipetted into a test tube and 5 mL of dichromate added slowly using a micropipette as the mixture was constantly being shaken. The tube was immersed in a boiling water bath, heated for 2 minutes and cooled in cold water. The reaction mixture was transferred to a 2 L volumetric flask. The tube was rinsed several times with distilled water and the solution in the 2L flask made up to the mark with water at 20° C. The contents of the flask were thoroughly shaken. An aliquot of 10 mL was transferred with a pipette into a 100 mL volumetric flask and 3.3 mL of 6 N sulphuric acid and 1.0 mL of the *s-diphenylcarbazide* reagent was added. The solution was brought to the mark with distilled water at 20°C. This solution developed a violet colour after it had been thoroughly shaken, the absorbance was read within 10 minutes at 540 nm against an s-diphenylcarbazide- sulphuric acid solution being used as a reference.

3.5.4 Calculations

The concentration of chromium in a diluted solution is calculated by means of Bouguer-Beer law:

$$C=KE \qquad (3.3)$$

C= concentration of chromium, mg. Per litre, in the diluted solution

E= measured optical density at 540 nm.

K= a constant for known dichromate solutions

$$K=1000M/\varepsilon l \quad (3.4)$$

M=atomic weight of chromium

l= light path of absorption cell,cm.

ε= molecular extinction coefficient

The concentration of ethanol in an unknown alcohol solution was then calculated from the expression (back titration):

$$A=d(B-C)/aF \quad (3.5)$$

A=concentration of ethyl alcohol, mg/mL

B=concentration of chromium in diluted blank, mg/ml

C= concentration of chromium in diluted sample, mg/mL

d= dilution number (computed by multiplying total volume in litres of first dilution by the dilution factor

F= theoretical factor, 1.5053, computed from the equation below, relating the unknown ethanol concentration to mg of chromium reduced.

a= alcohol solution used, mL.

$$2K_2Cr_2O_7 + 8H_2SO_4 + 3CH_3CH_2OH \longrightarrow 2K_2SO_4 + 2Cr_2(SO_4)_3 + 3CH_3COOH + 11H_2O \quad (3.6)$$

REFERENCES

Bailey, M.J. (1988). A note on the use of dinitrosalicyclic acid for determining the products of enzymatic reactions, Applied Microbiology and Biotechnology, 29, 494-496

Cheung, S.W. and Anderson, B.C. (1997).Laboratory Investigation of ethanol production from municipal primary waste-water solids, Bioresource Technology, 59, 81-96

Farfan, M.C., Lappe, P., Saavedra, L.A., Mendez, A.M., Perez, L.B. (2008) Ethanol production from henequen (Agava fourcroydes Lem.) juice and molasses by a mixture of two yeasts, Bioresource Technology Volume 99, Issue 18, 9036-9039

Gomide, J.A., Noller, C.H., Mott, G.O., Conrad, J.H., Hill, D.L. (1969) Effect of Plant Age and Nitrogen Fertilization on the Chemical Composition and In Vitro Cellulose Digestibility of Tropical grasses, American Society of Agronomy Journal 61, 116-120

Guilliermond, A. (2003) Yeasts: Culture, Identification and Microbiology. Watchmaker Publishing, 108-118, Google books

Han, Y. and Chen, H. (2008) Characterization of β-glucosidase from corn stover and its application in simultaneous saccharification and fermentation, Bioresource Technology, 99, 6081-6087

Kumar, R., Mago, G., Balan, V., Wyman, C.E.(2009) Physical and chemical characterizations of corn stover and poplar solids resulting from leading pretreatment technologies, Bioresource Technology, 100, 3948–3962.

Lindsay, H. (1973) A colorimetric estimation of reducing sugars in potato with 3.5-dinitrosalicyclic acid, Potato research , 16, 176-179.

Miller, G.L. (1959) Use of dinitrosalicyclic acid reagent for determination of reducing sugar, Analytical Chemistry, 31, 426-428.

Palmowski, L. and Muller, J. (1999), Influence of the size reduction of organic waste on their anaerobic digestion In: II International Symposium on anaerobic digestion of solid waste. Barcelona 15-17 June, 137-144

Ruiz, R. and Ehrman, T. (1996), Dilute Acid Hydrolysis Procedure for determination of Total Sugars in the Liquid Fraction of Process Samples, Chemical Analysis and Testing Task Laboratory Analytical Procedures, LAP-014.

Segal, L., Creely, J.J., Martin, E., Conrad, C.M.(1959)An empirical method for estimating the degree of crystallinity of native cellulose using the X-ray Diffractometer, Textile Research, 29, 786–794.

Tan, T.C. and Gan, S.H. (2005). Vapour-Liquid Equilibrium of water/ethanol/1-Butanol/Salt: Prediction and Experimental Verification, Chemical Engineering research and design, 12,1361-1371

Tomoko, S., Kengo, M., Shuji, H., Satoshi, O., Takafumi, S., Kozo N. (2009) Ozone pretreatment of lignocellulosic materials for ethanol production: Improvement of enzymatic susceptibility of softwood. Holzforschung, 63, 537–543

Williams, M.B. and Reese, H.D. (1950) Colorimetric determination of Ethyl Alcohol, Analytical Chemistry 12, 1556-1561.

Yu, Z. and Zhang, H. (2004) Ethanol fermentation of acid-hydrolyzed cellulosic pyrolysate with Saccharomyces cerevisia, Bioresource Technolgy, 93, 199-204

CHAPTER 4

RESULTS AND DISCUSSION

4.1 ANALYSIS OF CRYSTALLINITY

Crystallinity is believed to be an important feature affecting enzymatic saccharification of cellulose [Converse, 1993; Mansfield et al, 1999; Zhang and Lynd, 2004]. Various pretreatments can change cellulose crystal structures by disrupting inter- and intra chain hydrogen bonding of cellulose fibrils [Mosier et al, 2005]. X-ray measurements of crystallinity index (CrI) are the best option to estimate the impact of various pretreatment methods on biomass cr[illegible] [Kumar *et al*, 2009] The X-ray diffraction profile of untreated and treated Kikuyu grass is shown in Figure 4.1.

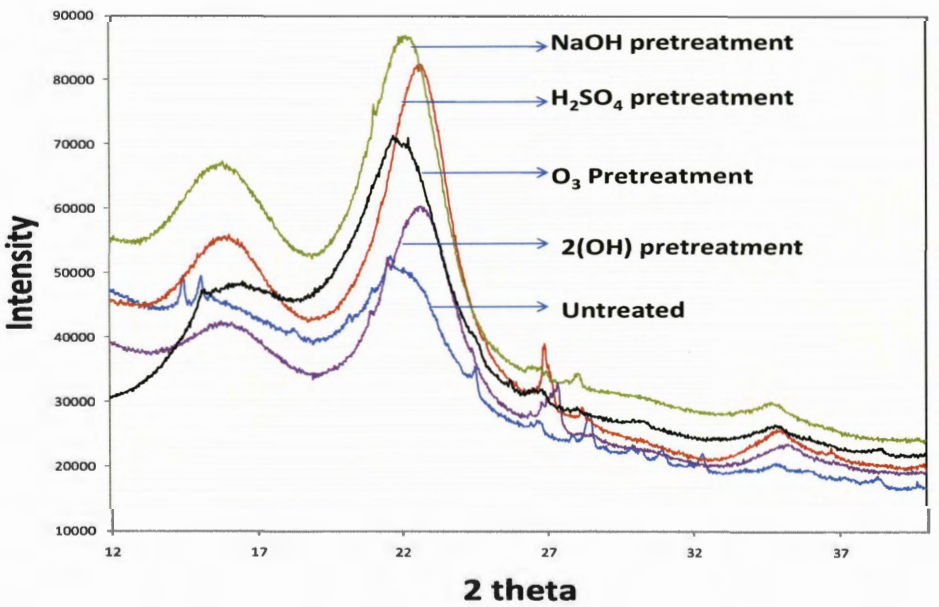


Figure 4.1: X-Ray Diffraction pattern of untreated and treated Kikuyu grass

The main peak position varies with different pretreatment methods: 22.88 for sulphuric acid, 22.32 for Hydrogen peroxide, 22.40 for sodium hydroxide and 21.51 for ozone. This peak is indicative of the distance between hydrogen bonded sheets in cellulose I [Cheng *et al*, 2011]. The crystallinity index (CrI) for all samples were calculated from the XRD data and the results are summarized in Table 4.1.

Table 4.1: Crystallinity index(CrI) for all samples

Pretreatment	**Crystallinity Index(CrI %)**
Untreated	30
Dilute Sulphuric Acid	33
Sodium Hydroxide	28
Hydrogen peroxide	27
Ozone	25

This increase in CrI after dilute acid pretreatment is consistent with the results reported by Kumar *et al*, (2009); and Li *et al*, (2012) suggesting that the amorphous cellulose breaks down more under acidic conditions but that this pretreatment process is unable to break apart the inter- and intra- chain hydrogen bondings in cellulose fibrils. The CrI calculated from remaining pretreated samples are slightly lower (25, 27, 28 CrI) than either the untreated or dilute acid pretreated samples. This decrease in cellulose crystallinity indicates that the recovered product is highly amorphous and therefore has

an increase in cellulose surface accessibility and would in theory enable more efficient cellulose hydrolysis.

Figure 4.2 shows the XRD spectra of untreated samples, the pretreated samples, and the remaining solids after enzymatic hydrolysis.

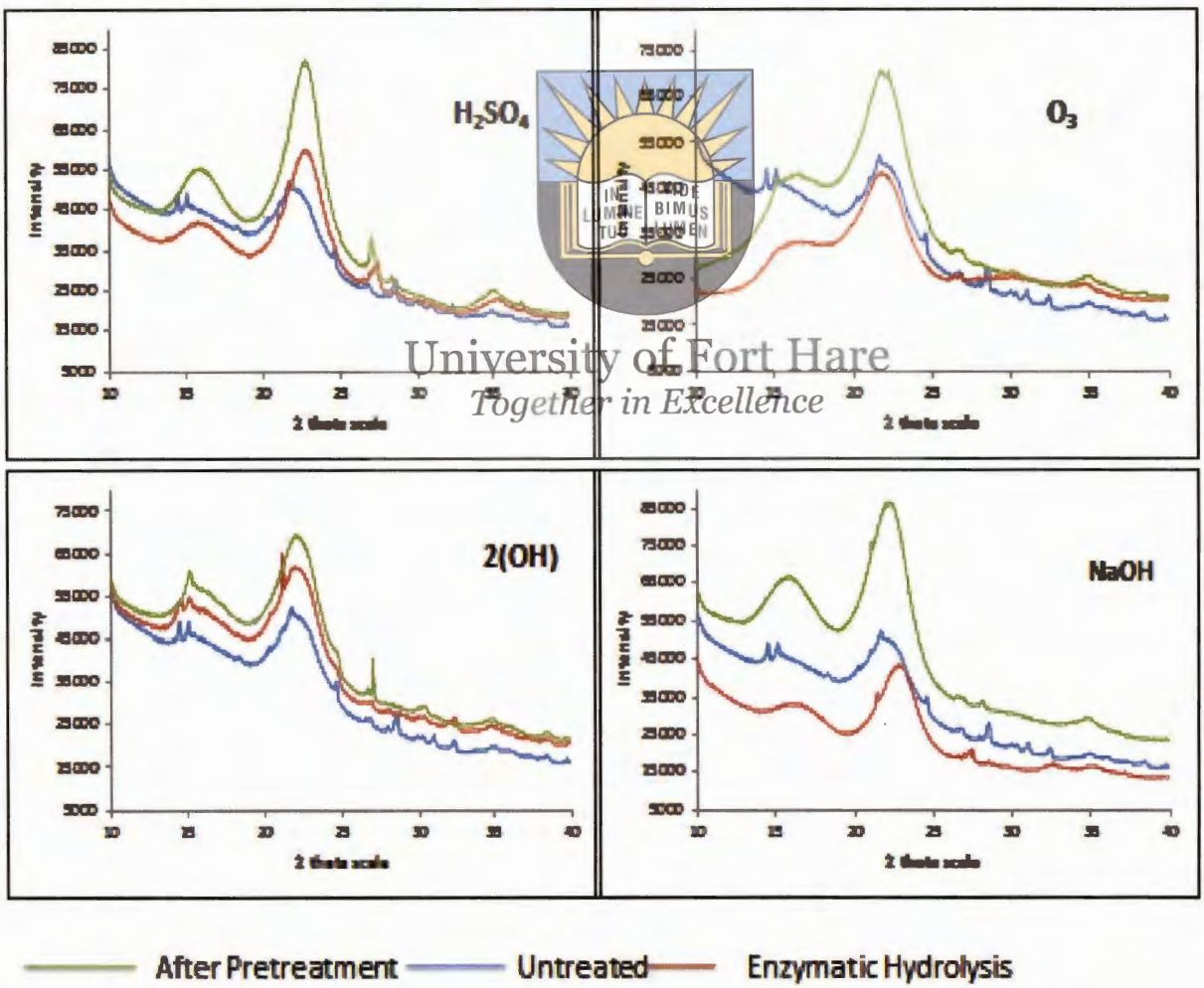


Fig. 4.2: X-ray Diffraction pattern of Kikuyu grass untreated, treated and after enzymatic hydrolysis

After pretreatment, the main peak relative to plane 002 is easily observed in all treated samples, showing that the amount of cellulose increased because of the removal of lignin and hemicellulose. This also confirms that pretreatment is effective in exposing cellulose to enzymatic attack. Furthermore, the crystalline peak is higher in intensity for all pretreated samples, suggesting that these samples have higher content of crystalline cellulose than amorphous cellulose after pretreatment. The XRD spectra of the solids after enzymatic hydrolysis showed a decrease in intensity of cellulose. Table 4.2 shows the Crystallinity Index of Kikuyu grass after pretreatment and enzymatic hydrolysis.

Table 4.2: CrI of Kikuyu grass after pretreatment and after Enzymatic hydrolysis

Pretreatment	CrI (after pretreatment)	CrI (enzymatic hydrolysis)
Dilute Sulphuric Acid	33	29
Sodium Hydroxide	28	23
Hydrogen peroxide	27	24
Ozone	25	21

The Crystallinity Index after Enzymatic Hydrolysis decreased, for all the different pretreatment techniques. This shows that enzymatic hydrolysis was able to decrease the crystallinity of cellulose and should permit fermentation to occur more easily. This suggests that enzymatic hydrolysis not only degrades but also slightly facilitates de-crystallization of cellulose.

4.2 FT-IR SPECTROSCOPY

FTIR spectroscopy was used to investigate the changes of cellulose structures during the various pretreatment methods. Figure 4.3 shows the FT-IR spectra of untreated grass with those obtained from the various pretreatment methods.

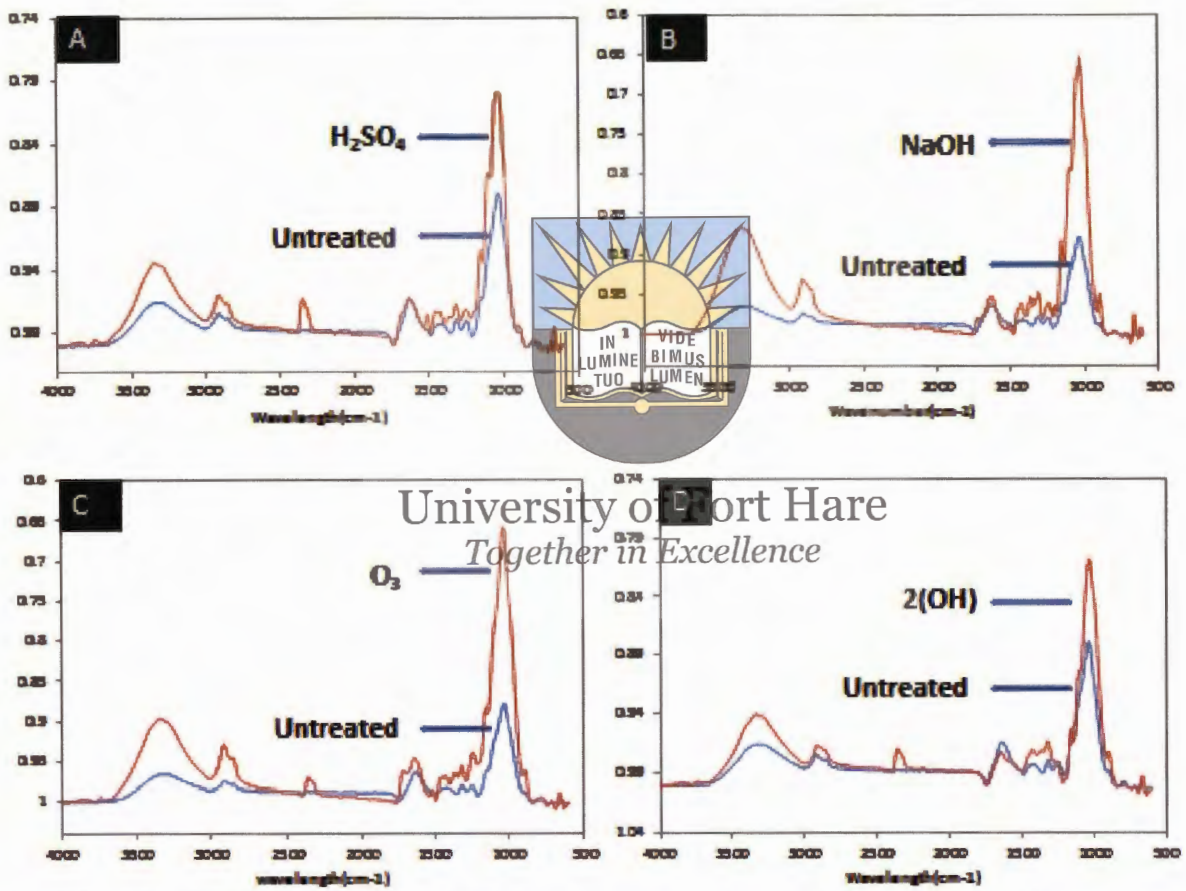


Fig 4.3: (A) -FT-IR spectra of H_2SO_4treated and untreated grass, (B)-FT-IR spectra of NaOHtreated and untreated grass, (C)-FT-IR spectra of O_3 treated and untreated grass, (D)-FT-IR spectra of 2(OH)treated and untreated grass

The profile of the FT-IR spectra was different for untreated and treated Kikuyu grass. This indicates that there were structural changes that occurred after pretreatment. A notable change in the FT-IR spectra is the broadening of the band at 3200-3400 cm^{-1}

which was associated with the O-H stretching of the hydrogen bonds [Hsu *et al*, 2010]. The other major change in the FT-IR spectra is the enhancement of absorption peaks at 1000-1100 cm^{-1}, this increase in intensity is attributed to the increase of cellulose content as a result of both lignin and hemicelluloses removal. [Li, 2010] There is an enhanced peak at 900 cm^{-1} after pretreatment which is referring to the removal of amouphous cellulose, these results are in agreement with the XRD spectra which showed reduced crystallinity after NaOH, Ozone and Hydrogen peroxide pretreatment methods. The peak of CH_2 stretching near 2900 cm^{-1} were distinguished for both untreated and treated Kikuyu grass. The most representative bands are shown in Table 4.3.

Table 4.3: Summary of the most representative bands.

Band Position	Assignment	Reference
3200-3400	O–H stretching	Wang *et al*, 2007
2900	C–H stretching (related to rupture of methyl/methylene group of cellulose)	Kumar *et al*, 2009
1700	Carboxylic acids/ester groups	Kumar *et al*, 2009
1593	Aromatic ring stretch (associated with lignin removal)	Kumar *et al*, 2009
1430	Symmetric CH2 bending and wagging	Cao and Tan , 2004
1280	Ester absorbance (related to removal of uronic acid)	Kumar *et al*, 2009
1240	C–O adsorption (resulting from acetyl groups cleavage)	Kumar *et al*, 2009
1000-1100	C-O stretch,	Li, 2010
900	Removal of lignin	Li, 2010

5.3. MORPHOLOGICAL STRUCTURES

Figure 4.4 shows the SEM micrographs for untreated and pretreated Kikuyu grass taken at 15KV and 1000 magnification mode.

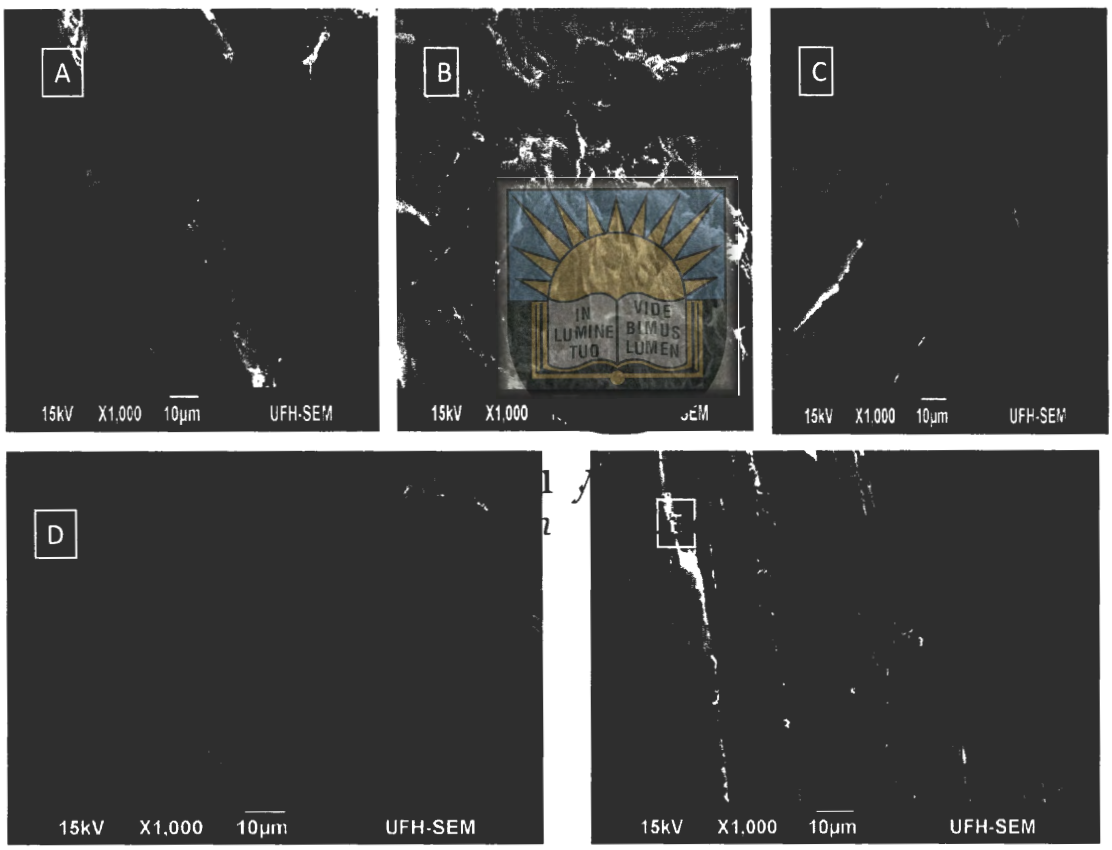


Figure 4.4: Scanning Electron microscope of (A)-untreated grass (B)-O_3 treated grass (C)-H_2O_2 treated grass (D)-H_2SO_4 treated grass and (E) NaOH treated grass.

These images taken at 24 hours of prtereatment showed that the pretreatment induced physical changes in the biomass. The untreated Kikuyu grass shows external fibres and layers covering the surface of the material, these layers could include cellulose,

hemicellulose and lignin and other waxes. After pretreatment the external fibres were completely removed, which suggests that the outer layer might include lignin and hemicelluloses which have been removed by these pretreatment methods. NaOH treated biomass showed a sieve like structure. This indicates that pretreatment removed external fibers which in turn increases surface area so that cellulose becomes more accessible to enzymes[Binod *et al.*, 2012] , which will in theory should increase yields in fermentation.

4.4. REDUCING SUGAR YIELDS

Table 4.4 shows total reducing sugar yields from all for pretreated methods and times.

TIME(hours)	REDUCING SUGAR YIELDS(g/L)			
	H_2SO_4	NaOH	O_3	2(OH)
2	2.63	1.09	1.08	0.5
4	1.85	2.82	1.02	1.3
6	2.01	1.46	1.53	1.01
24	4.21	3.90	1.98	1.59

Pretreatment time had a considerable effect on sugar yields. Sugar yields increased from 2 to 24 h, proving that new sugars are continuously produced as reaction time is elongated. This suggests that longer time is useful to hydrolyze and degrade the lignin layer. Hydrolysis results clearly showed that soluble sugars were released to a greater extent in acid pretreatment than all other pretreatment methods studied here. The

highest reducing sugar yields were 4.21 g/L for sulfuric acid, 3.90 g/L for sodium hydroxide, 1.98 g/L for ozone and 1.59 g/L for hydrogen peroxide. Table 4.5 shows the yield of reducing sugars (optimum conditions)

Table 4.5: Yield of reducing sugars (optimum conditions)

Pretreatment method	Pretreatment time	Duration of Incubation	Reducing sugar yields(g/L)
H_2SO_4	24	48	4.21
NaOH	24	48	3.90
O_3	24	48	1.98
2(OH)	24	48	1.59

4.4.1 Yield of Ethanol from Kikuyu grass

Ethanol yields were taken from hydrolyzates with highest reducing sugar concentrations. Table 4.6 shows the yield of ethanol from the various pretreatment methods.

Table 4.6: Yield of ethanol from the various pretreatment methods (optimum conditions)

Pretreatment method	Pretreatment time	Duration of Incubation	Ethanol yield(mg/mL)
H_2SO_4	24	48	34.2
NaOH	24	48	30.2
O_3	24	48	15.59
H_2O_2	24	48	13.1

The ethanol yields were 34.2 mg/mL for sulfuric acid, 30.2 mg/mL for sodium hydroxide, 15.59 mg/mL for ozone and 13.1 mg/mL for hydrogen peroxide. Ethanol yield increased with an increase in reducing sugar concentration. These results are in agreement with (Garcia-Aparicio *et al*, 2011) who stated that an increase in reducing sugar yield results in higher ethanol yields.

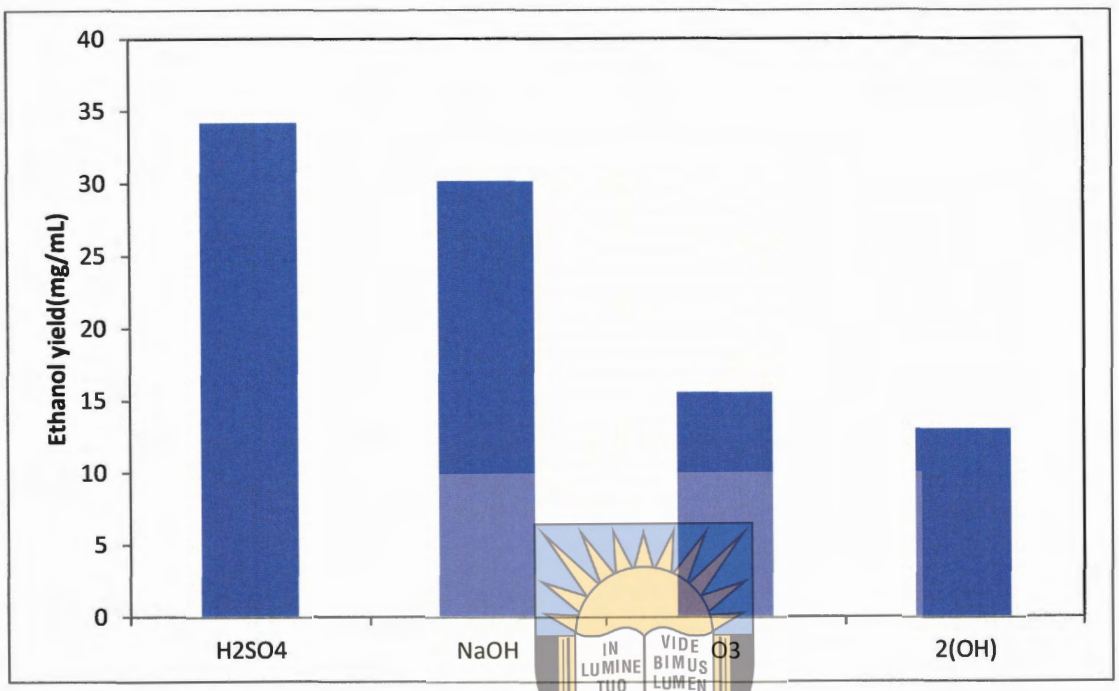


Fig.4.6: Bar graph showing ethanol yield obtained from the various pretreatment methods.

REFERENCES

Binod, P., Satyanagalakshmi, K., Sindhu, R., Janu, R.U., Sukumaran, R.K., Pandey A. (2012) Short duration microwave assisted pretreatment enhances the enzymatic saccharification and fermentable sugar yield from sugarcane bagasse Renewable Energy 37, 109-116

Cao Y and Tan H. (2004) Structural characterization of cellulose with enzymatic treatment, Journal of Molecular Structure, 70, 89-93.

Cheng, G, Varanasi, P, Li, C., Liu, H., Melnichenko, Y.L., Simmons, BL, Kent, M, and Singh., S. (2011) Transition of Cellulose Crystalline Structure and Surface Morphology of Biomass as a Function of Ionic Liquid Pretreatment and Its Relation to Enzymatic Hydrolysis pubs.acs.org/Biomac dx.doi.org/10.1021/bm101240z

Colom X, Carrillo F. (2002) Crystallinity changes in lyocell and viscose-type fibers by caustic treatment. European Polymer Journal, 38:22, 25-30

Converse, A.O., 1993. Substrate factors limiting enzymatic hydrolysis. In: Saddler, J.N. (Ed.), Wallingford, Oxon, UK. pp. 93–106.

Hsu T, Guo G, Chen W, Hwang WS. Effect of dilute acid pretreatment of rice straw on structural properties and enzymatic hydrolysis. Bioresource Technology 2010;101:49, 07-13.

Kumar, R., Mago, G., Balan, V., Wyman, C.E.(2009) Physical and chemical characterizations of corn stover and poplar solids resulting from leading pretreatment technologies, Bioresource Technology 100, 3948–3962.

Mansfield, S.D., Mooney, C., Saddler, J.N.(1999) Substrate and enzyme characteristics that limit cellulose hydrolysis, Biotechnology Progress, 15, 804– 816.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., Ladisch, M. (2005) Features of promising technologies for pretreatment of lignocellulosic Biomass, Bioresource Technology, 96, 673–686.

Oh S.Y., Yoo, D.I., Shin, Y., Seo, G. (2005) FTIR analysis of cellulose treated with sodium hydroxide and carbon dioxide, Carbohydrate Research, 4, 17-28.

Pandey, K.K. (2005) Study of the effect of photo-irradiation on the surface chemistry of wood, Polymer Degradation and Stability, 90, 9-20.

Wang L, Han G, Zhang Y. (2007) Comparative study of composition, structure and properties of Apocynum venetum fibers under different pretreatments. Carbohydrate Polymer, 39, 1-7.

Zhang, Y.H., Lynd, L.R. (2004)Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems, Biotechnol and Bioengineering, 88(7), 797–824.

CHAPTER 5

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

5.1. SUMMARY OF THE FINDINGS

The main aim of this research was to investigate the feasibility of bio-ethanol synthesis from Kikuyu grass using different pretreatment methods. It has been established that Sulfuric acid, Ozone, hydrogen peroxide and Sodium hydroxide can all be used to break down the lignocelluloses structure for fermentable sugars production which may later be converted to ethanol through fermentation. Despite the challenge associated with converting fermentable sugars to other chemicals, ethanol yields were obtained, from all the pretreatment methods. From this study, it can be demonstrated that hydrolyzates obtained by the chemical hydrolysis route with Sulfuric acid, Ozone, hydrogen peroxide and Sodium hydroxide can be fermented using baking yeast (S-cerevisia) to ethanol after the inhibitors (furfural etc..) have been removed.

The optimum conditions for bio-ethanol synthesis from Kikuyu grass were: 2% (w/v) Sulfuric acid, Ozone, hydrogen peroxide and Sodium hydroxide concentration after 24 hours under reflux at 105^0 C and 48 hours of incubation of the hydrolyzate using baking yeast. Kikuyu grass was collected as lawn cuttings from gardens around the University of Fort Hare. The yields obtained were: 34. mg/mL using sulfuric acid, 30.2 mg/mL

using sodium hydroxide, 15.59 mg/mL for Ozone and 13.1 mg/mL for hydrogen peroxide for every 2g of kikuyu grass used.

Through process modification, this study has demonstrated that grass, which is a non-food crop, can be used for bio-ethanol production instead of food crops and this should help reduce the concerns over food security.

5.2 SUMMARY OF THE CONTRIBUTIONS

This study has found that grass can be used for bioethanol production.Pretreatment of biomass is an extremely important step for efficient use of lignocellulosic materials as alternative fuels. Fundamental understanding and comparison of various pretreatment processes is essential. Hydrolysis results clearly showed that soluble sugars were released to a greater extent in acid pretreatment than all other pretreatment methods studied here. Pretreatment improved the recovery of fermentable sugars from Kikuyu grass with acid pretreatment and overall yield of 4.21g/mL fermentable sugars released.

5.3 CONCLUSIONS

Reducing sugar production from lignocellulosic material is a challenging task because of the hydrolysis and fermentation conditions surrounding the process. The reducing sugars obtained from the hydrolysis were analyzed by the DNS method. This involved the oxidation of the aldehyde or ketone functional group and the 3.5-dinitrosalicyclic acid (DNS) was reduced to 3-amino,5-nitrosalicyclic acid under alkaline conditions, the

product obtained was reddish-brown in colour and absorbed strongly in the UV/Vis region. The optimum pretreatment method was acid pretreatment which gave an overall ethanol yield of 4.21 g/mL.

5.4 RECOMMENDATIONS

For further research, this study suggests the investigation of different detoxification methods to remove inhibitors. It would also be recommended to upscale this work to check out viability of bioethanol production on a small community level once the process has been perfected because it is a relatively inexpensive and relatively economic way to produce bioethanol which uses a low cost renewable biomass source.