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Optimization and Kinetics Study of Solvent Pretreatment of South African Corn Cob for Succinic Acid Production

by

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(389767)

A dissertation submitted to the Faculty of Engineering and the Built Environment, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Engineering.

October 2017

DECLARATION

I declare that this dissertation is my own unaided work. It is being submitted to the Degree of Master of Science to the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination to any other University.

_____

(Signature of Candidate)

_____ day of _____, _____

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ABSTRACT

Increasing concerns over environmental and geo-political issues on resources' sustainability have driven the industries to shift their efforts to produce chemicals from renewable biomass. Amongst the lignocellulosic biomass, corncob contains cellulose, hemicellulose and lignin that are built in a compact structure which makes it difficult to access. Pre-treatment is then applied to make the content to be accessible to enzymatic hydrolysis which breaks down the polysaccharides to monomers. The sugar monomers can be converted to a wide range of bio-products such as biofuels and bio-chemicals. The objective of the study was to determine, evaluate and optimize the best solvent system to pre-treat corn cob. In addition, the study evaluated the effect of pre-treatment parameters on the yield of cellulose and hemicellulose and attempt to develop a kinetic model to explain the dissolution.

Lithium perchlorate, zinc chloride, phosphoric acid, sulphuric acid and sodium hydroxide were used during the pre-treatment, which was carried out at 70-80 ° C for 6 hours. Characterization of pre-treated samples showed a significant change in structure after pre-treatment indicating disruption in cell wall of the lignocellulosic material. FTIR revealed a reduction in phenolic group; indicating that the lignin content has been reduced. The XRD patterns show that crystallinity was considerably reduced; this was shown by an increase in calculated crystallinity index (CrI) after $LiClO_4$, $ZnCl_2$, H_3PO_4 and NaOH pre-treatment. The CrI of raw corncob (CrI= 32.7%) increased to 46.2 %, 42.3 %, 55.6 % and 53.4 % of $LiClO_4$, $ZnCl_2$, H_3PO_4 and NaOH, respectively. The crystallinity index increased for pre-treated material, indicating that the amorphous cellulose is dissolved in the liquor, as well as lignin and hemicellulose removal

This study has shown that $LiClO_4.2H_2O$ pretreatment agent is an efficient solvent system to pretreat corncob which consecutively increase the accessibility of cellulose and hemicellulose from the solid fractions. The accessibility was confirmed by an ease hydrolysis of cellulose & hemicellulose to glucose & xylose respectively. An increase of nearly four times compared to the untreated corncob. The effect of reaction operating parameters i.e. Reaction time, temperature and solvent concentration was carried out and then optimized by response surface methodology (RSM) using Minitab 16. The target was to maximize the yield of cellulose and hemicellulose. It was discovered that the increase in temperature and reaction time increase the accessibility of cellulose and hemicellulose until an equilibrium is reached at 3 & half hours and 176 °c. The pretreatment solvent concentration was discovered to have an effect on the accessibility but not as much as temperature and time. The best pretreatment

conditions to obtain high polysaccharides conversions to monomers were at 176°c for 3.5 hours using $LiClO_4.2H_2O$ for 10 g of corncob.

The results obtained from RSM were used to evaluate the temperatures profile, kinetic model for the corncob pretreatment as a function of temperature. The kinetics of pretreatment were studied by the amount of glucose, xylose and the lignin removed from the pretreated solids. The kinetic model of lignin removal and sugars accessibility was identified as a first-order reaction corresponding to the bulk phase for pretreatment time up to 24 hours. The rate constant results show that the kinetic rate increased with temperature. The activation energy for glucose, xylose and lignin were calculated to be 15.0 kJ/mol, 14.2 kJ/mol and 36.54 kJ/mol, respectively.

Publications from the Study

Listed below are the publications emanated from the study (see Appendix D for copy)

Journal Article

Mudzanani K, Kalombo L, Daramola M O. Aqueous Pretreatment of Corncob Lignocellulosic Biomass for Bio-commodities Production: Characterization, Quantification & Optimization, *South African Journal of Chemical Engineering* (submitted)

Oral and Poster Presentations at Conferences

Mudzanani K, Daramola M O., 2016. Optimization and kinetics study of Green solvent pretreatment of Non-edible lignocellulosic biomass for bioenergy, biofuels & biochemical production. *Annual National Renewable and Sustainable Energy Postgraduate Symposium ,REPS 2016,* 04-06 September University of Fort Hare, Alice Eastern Cape **(Poster).**

Mudzanani K, Daramola M O., 2016. Comparative study for Aqueous Pretreatment of the South African Corncob towards Succinic Acid Production, *3rd National Conference on Global Climate Change, GCC 2016,* 5-8th December. Durban. Kwazulu Natal **(Poster).**

Mudzanani K, Daramola M O., 2016. Aqueous Pretreatment of Lignocellulosic Corncob: Characterization and Quantification *Environment, Materials and Green Technology Conference 2016, CREW 2016,* 24-25th November Sebokeng Gauteng (**oral presentation**)

Award

Master's Best Poster, Bioenergy Platinum Prize (see appendix), Annual National Renewable and Sustainable Energy Postgraduate Symposium, REPS 2016, 04-06 September University of Fort Hare, Alice Eastern Cape.

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List of Symbols and Nomenclature

Symbol	Description
β	beta
α	alpha
σ	Adsorption capacity
μ	microns
Θ	theta
i	index

Nomenclature

Abbreviation / Acronyms	Description
LCB	Lignocellulosic Biomass
bSA	bio-based succinic acid
MHS	Molten Hydrate Salts
AFEX	Ammonia Fiber Explosion
LHW	Liquid hot water
BDO	1,4-Butanediol
THF	Tetrahydrofuran
GW	Giga Watts
OAc	Acetyl groups
ASA	Accessible Surface Area
DP	Degree of Polymerization
SE	Steam explosion
SC-CO_2	Supercritical carbon dioxide
DI	De-ionized water
N-U	NaOH/Urea System
N-T	NaOH/Thiourea System
N-U-T	NaOH/Urea/Thiourea System

ASL	Acid Soluble Lignin
RSM	Response Surface Methodology
CrI	Crystallinity Index
k	Rate constant
Ea	Activation energy

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CHAPTER 1: Introduction

1.1. Background and Motivation

The increasing concerns over the depletion of fossil resources and its associated geo-political issues have driven the entire world to move towards environmental & resources sustainability consciousness. The African continent faces many challenges and suffers from some serious environmental problems including; climate change, deforestation, water pollution, coal mining & nuclear waste (Bounaim, 2013). South Africa is currently contributing to the global greenhouse gas emissions reductions, in the form of its intended nationally determined contribution, which includes implementation of green technology in the industries (SACNSP, 2015). The advancement of green technology becomes a primary concern and has attracted continuing research efforts into greener production of bio-products. The term "green technology" encompasses a continuously evolving group of methods and materials, from techniques for generating energy and non-toxic cleaning products (Fargo, 2013).

Lignocellulosic biomass, the most abundant renewable resource on earth has been regarded as the significant feedstock material for the process. Lignocellulosic biomass refers to plant based materials that are composed of cellulose, hemicellulose and lignin such as corncob, bagasse, wheat & rice straws, wood chips and etc. Biomass products are abundant and renewable, about 220 billion tons are produced per annum worldwide (Nagoor Gunny and Arbain, 2013). Compared to current practices, plant biomass is a natural resource that captures carbon dioxide for its own growth, emits no harmful gases and therefore produces clean energy in processing and application (Binder and Raines, 2010; Wyman, 2013). In addition, biomass products are versatile such that they can be used to generate different kind of bio-commodities such as biofuels, bio-additives, biodegradable plastics, bio surfactants & bio-chemicals (Zheng & Lars Rehmann 2014; Kumar et al. 2009; Pointner et al. 2014; Sluiter et al. 2010).

Amongst these biomass wastes, corncob has been discovered to be a noble feedstock in bioprocessing. According to the National Agricultural Marketing Council (NAMC) of South Africa, 14.5 million tons of corncobs are reported as the 2nd highest produced agricultural wastes with the potential to grow each year (NAMC, 2012) . Because corncob are non-edible and will not compete with food security they give an additional motivation as a waste beneficiation material. Corncob is the core section of maize (Fig. 1.1) and is primarily waste

that is burnt as fuel causing environmental pollution. In order to achieve its value addition in new research areas, corn cob can be processed chemically to generate new end products with greater values at very low processing cost.

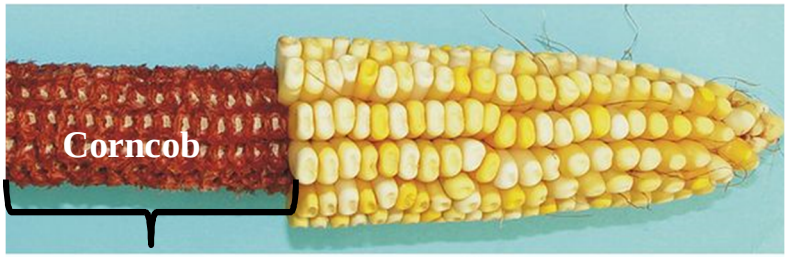


Figure 1.1: Position of corncob in a maize (Ashour *et al.*, 2013)

Because lignocellulosic biomass (LCB) is generally recalcitrant to enzymatic hydrolysis, pretreatment is necessary to enhance its enzyme digestibility. Biomass is recalcitrance since it resists biological deconstruction by simple methods. Pretreatment process removes the lignin and other complex materials and therefore makes cellulose accessible for hydrolysis (Zhang *et al.*, 2015; Yan *et al.*, 2016). Generally plant biomass contains 40–50% cellulose 20–40% hemicellulose, 20–30% lignin by weight (Ragauskas *et al.*, 2014). Composition of the South African corncob ranges from 28-41.5, 30-45, & 6.7-13 % of cellulose, hemicellulose and lignin, respectively (Anukam et al., 2017; Awosusi et al. 2017). Similar to other biomass it is therefore recalcitrance to bioprocessing since the recalcitrance is directly related to the inherent properties of the biomass source. Properties such as lignin content, cellulose accessibility to cellulase and cellulose crystallinity determine the overall digestibility of the biomass (Godin *et al.*, 2013; Zheng *et al.*, 2014).

As the primary component of lignocellulosic biomass, cellulose is the most abundant organic compound on earth and has the potential to be a renewable source for energy and chemicals. The challenge for scientists is to access these polymers and convert them into fuels and other value added products. Luckily, chemistry provides an alternative means to hydrolyze the lignocellulosic biomass. It was demonstrated by Braconnot in the 1819s, when linen was dissolved in concentrated H_2SO_4 and heated to convert it to fermentable sugar (Binder and Raines, 2010). The acid disrupts the intra- and inter-chain hydrogen bonds network, de-crystallize cellulose and make it accessible to reactants attack. The acid will in turn catalyze hydrolysis and break the glycosidic bonds; strong acids cleave cellulose and hemicellulose into sugars monomers (Bardone et al., 2014; Dagnino et al., 2013; Zhang et al., 2015). The idea is to achieve the same phenomena on South African corncob but using greener solvent such as molten hydrate salts.

1.2. Research Problem

Tons of corn cobs wastes are discarded annually in South Africa after the corns or maize grain is processed to various food products. After the processing, the residue ends up in landfills, open dumps or drainage systems. These practises are the foundation of pollution to the environment which include contamination of surface & ground water and land pollution. It is therefore necessary to convert these wastes to useful end-products rather than allowing them to degrade the environment (Nahman, Wise and de Lange, 2009; Bounaim, 2013; Mu and Wang, 2016)

Corn cob contains sufficient amount of cellulosic material, which is the best source of fermentable sugars (Ashour *et al.*, 2013). In order to produce these fermentable sugars from biomass, the pretreatment is usually the initial process wherein the accessibility of the cellulose polymers is improved (Ragauskas *et al.*, 2014; Zhang *et al.*, 2015). Enzymatic cellulose hydrolysis is then applied to ferment the sugars by means of cellulase enzyme obtained from enzyme manufacturers or synthetized onsite. These fermentable sugars can later be converted into desired products (Qing et al. , 2010).

The end objective of pretreatment method is to break down and interrupt the lignin & crystalline structure of cellulose, such that the cellulose is easily accessible to hydrolysis by acids or enzymes (Bensah and Mensah, 2013) . However, the pretreatment does not only result in the interference of the lignin seal to increase enzyme access to cellulose but also escalate surface area and porosity of pre-treated substrates, resulting in increased hydrolysis rate. Consequently this increase the biological step and by default catalyses the speed of the entire bio-commodities production (Salvachúa *et al.*, 2016).

Pretreatment process is the most expensive section in biomass-to-bioproduct conversion; however it is important and necessary for increased efficiency & thus decreasing the costs of the entire bio-processing route (Yang and Wyman, 2006). Furthermore, bio commodities production process may not be efficient or sometimes may not occur without the application of pre-treatment method (Salvachúa *et al.*, 2016). In addition, the production of many of these organic acids via hydrolysis and fermentation of lignocellulosic biomass is still not economically attractive (Sannigrahi and Ragauskas, 2013)

Green technology application in manufacturing industries has become the major focus of countries around the world. In-line with this, lignocellulosic biomass is often advocated as

significant starting material for a greener production. However, due to the use of acid or alkaline during biomass pre-treatment the process cannot be considered as green (Kumar et al. 2009). Ionic liquids are considered as the most common green pre-treatment solvent gaining interest due to their minimal waste, non-volatility and low toxicity (Nagoor-gunny and Arbain, 2013). Because of this properties and the fact that they are not readily available from cheap industrial synthesizing process, ionic liquids are expensive (Liu et al., 2012). An attempt has been made to synthesize the material in lab scale but this deemed to be unsuccessful since the performance of the synthesized material was not as good as pristine ionic liquids. In addition, the generated material's toxicity was not quantified (Ratti, 2014).

The aim of the study is to establish the green solvent that will disrupts the intra- and inter-chain hydrogen bonds network, de-crystallize cellulose. This includes making the cellulose accessible to reactants, break the glycosidic bonds & cleave cellulose and hemicellulose into sugars monomers. Eventually the sugar monomers can be used to produce bio-products such succinic acid.

1.2. Research Questions

Through the course of this research, attempt was made to answer the following question:

1.2.1. Which solvent system (single or coupled) resulted into the most efficient pre-treatment method for the South African corn cob?
1.2.2. What is the effect of pretreatment parameters (operating variable) on the yield of cellulose from South African corn cob?
1.2.3. What are the kinetics governing this pre-treatment process?

1.3. Research Objectives

The overall aim of this study was to improve the economical use of corncob as bio-processing feedstock. This was achieved by developing an efficient pretreatment route owing to identification of the most efficient pretreatment solvent which was succeeded by the optimization of the operating parameters. The detailed objectives of the dissertation were;

1.3.1. To investigate and conduct a study of non-derivatizing solvents (solvent system) applied as a pre-treatment method to the dissolution of the South African corn cob to identify the most efficient solvent.

1.3.2. To determine the effects of reaction operating parameters such as reaction time and temperature, on the performance of the pretreatment process. Also to optimize the reaction conditions of the pretreatment process to maximize the yield of cellulose, hemicellulose

1.3.3. To investigate the efficacy of pretreatment of lignocellulosic biomass by examining the impact of solvent treatment on subsequent step.

1.3.4. Study the kinetics of the pretreatment method towards developing a conceptual process design for the dissolution process.

1.4. Scope of Study

The research focuses on aqueous pretreatment of corncob using solvents with the aim to discover and develop a novel solvent system and successively model the kinetics of chemical reactions that occur during the process. Even though the overall goal of the study was to produce succinic acid, it should be noted that the study document in this dissertation did not cover the succinic acid production. However having covered the optimization and kinetic study of pretreatment in this study, subsequent studies can look into the conversion of the monomers obtained from pretreatment for succinic acid production

In this study, pretreatment of corncob was carried out by dilute acid, dilute alkaline and molten salts. The efficiency of the thermochemical pretreatment of corncob was assessed by analysis to provide comparative data on glucose and xylose yields of the pre-treated material. The composition of pre-treated solids on each substrate was also measured to determine the extent glucose release could be related to these features.

The pre-treating agents are categorized as alkaline, acids, organic solvents, molten hydrate salts and ionic liquids including ammonia (AFEX) and carbon dioxide. However some of these chemicals will be disregarded in this research due to the absence of an environmental friendly factor in the process of extracting cellulose from its primitive resources, cost of the chemical, unfavourable operating conditions (high temperatures or pressures), and modifying cellulose properties.

The pretreatment of corncob was carried out using molten hydrate salts such as zinc chloride ($ZnCl_2.3H_2O$), lithium containing salt hydrates ($LiClO_4.nH_2O$), lithium chloride ($LiCl.nH_2O$), alkaline with urea & thiourea and acids i.e. phosphoric, sulphuric and citric acid. The study

omitted the use of ionic liquids because they are too expensive, ammonia due to environmental concern (Liu *et al.*, 2012; Weerachanchai and Lee, 2013; Ratti, 2014).

1.5. Dissertation Layout

The layout of the dissertation followed an order of;

Chapter 1

This chapter gives background information as well as motivation for the proposed research, furthermore the scope of the project and research objectives are explicitly and briefly stated.

Chapter 2

This chapter gives a review of literature to put things into perspective and provide background information on lignocellulosic biomass (corncob) pretreatment and bio-production process.

Chapter 3

In this chapter procedures for corncob pretreatment under different aqueous media are described in complete detail. A description of the equipment and instrumentation used is also provided.

Chapter 4

The results obtained in the experiments are presented and also discussed in this chapter.

Chapter 5

Presented in this chapter are the conclusions and recommendations of the research.

CHAPTER 2: Literature Review

The characteristics of lignocellulosic biomass that advocate its desirability as a feedstock materials for bioprocessing are high cellulose & hemicellulose content, energy density, moisture content and easy particle size reduction (Kumar, Negi and Upadhyaya, 2010). Since biomasses are renewable materials, their application on biofuels production is deemed to be sustainable. There is substantial evidence that proves that to produce the desired product with a high concentration, the non-edible material must have a significant cellulose and hemicellulose content (Oke *et al.*, 2016). After removal of lignin from lignocellulosic biomass (LCB) material, the cellulose and hemicellulose can be converted to monomeric sugars (Samuel, 2011). These sugars can then be fermented to bio-product using glucose and xylose fermenting yeast cells (Binder and Raines, 2010).

Because lignocellulosic biomass is generally recalcitrant to enzymatic hydrolysis, pretreatment is necessary to enhance its enzyme digestibility (Wyman, 2013). Pretreatment is the first step in any biochemical conversion process for the production of bio-commodity from LCB. This is carried out to eliminate the lignin and produce fermentable sugars by hydrolysis (Yoo, 2012). Conventional techniques have several limitations which can be addressed by using them in tandem with non-conventional methods for biomass pretreatment (Kumar *et al*., 2010).

The goal of pretreatment is to make the cellulose accessible to hydrolysis for conversion to bio-commodities. Various pretreatment techniques change the physical and chemical structure of the lignocellulosic biomass and improve hydrolysis rates. During the past few years a large number of pretreatment methods have been developed, including alkali treatment, ammonia explosion, and others. Many methods have been shown to result in high sugar yields, above 90% of the theoretical yield for lignocellulosic biomasses such as woods, grasses and corn (Kumar *et al.*, 2009; Ayeni *et al.*, 2012, 2013; Ravindran and Jaiswal, 2016)

As a renewable raw material, corncobs are agricultural wastes which are currently used as substrate for biofuels and biochemical production to fulfil the increasing demand. Corncobs contains sufficient amount of cellulosic material, which can be hydrolysed to glucose by the action of the cellulase (Ali and Arshad, 2014). Along with cellulose, hemicellulose is converted to xylose. Both xylose and glucose are fermentable sugars that can be converted

into bioproducts. It is for this reason that the hydrolysates of corncobs are considered for biochemical production.

2.1. Lignocellulosic biomass: Importance

Lignocellulose is a complex matrix, comprising many different polysaccharides, phenolic polymers and proteins (Lee *et al.*, 2014). Cellulose, the major component of cell walls of land plants, is a glucan polysaccharide containing large reservoirs of energy that provide real potential for conversion into biofuels. Lignocellulosic biomass consists of a variety of materials with distinctive physical and chemical characteristics. It is the non-starch based fibrous part of plant material (Of *et al.*, 2012; Zafar, 2014).

First generation biofuels, from food crops are lacking in their ability to achieve targets for oil-product substitution, climate change mitigation, and economic growth. Their sustainable production is still under review, as is the possibility of creating undue competition for land and water used for food and fibre production. The cumulative impacts of these concerns have increased the interest in developing bio-commodities produced from non-food biomass. Feedstocks from lignocellulosic materials, the second-generation biofuels could avoid many of the concerns facing first-generation biofuels and potentially offer greater cost reduction potential in the longer term

Amongst other benefits of using non-edible lignocellulosic biomass as a feedstock for bio-fuels and bio-chemicals production is the fact that it does not compete with food security and feed supply, availability over a wide range of locations and climates and an added bonus of low production costs (Zafar, 2015). Plant biomass is attracting increasing attention as a sustainable resource for large-scale production of renewable fuels and chemicals. However, in order to successfully compete with petroleum, it is vital that biomass conversion processes are designed to minimize costs and maximize yields. Advances in pretreatment technology are critical in order to develop high-yielding, cost-competitive routes to renewable fuels and chemicals.

As the only foreseeable sustainable source of food, organic materials, and fuels that are liquid at atmospheric pressure, plant biomass is a central and essential component of a sustainable world. Whereas biomass can be converted to high-performance liquid fuels, other large-scale sustainable energy sources are most readily converted to electricity and heat. Due to energy density considerations, it is reasonable to expect that organic fuels will meet a significant

fraction of transportation energy demand for the indefinite future. Biofuels are by far the most promising sustainable source of organic fuels and are likely to be a nondiscretionary part of a sustainable transportation sector – especially for aviation and heavy-duty vehicles. It is very unlikely that anyone alive today will ride in a battery-powered jet (Lynd & Laser, 2013).

It has been reported that the choice of bioprocessing feedstock represents the most important factor impacting key bioenergy performance metrics such as: scale of sustainable production, productivity, land availability, fossil fuel displacement, feedstock cost, conversion cost, and environmental impact (Ragauskas *et al.*, 2014; Oke *et al.*, 2016). Regarding productivity, recurring lignocellulosic crops generally outperform annual row crops, which makes sense given that plants grow faster when their composition is optimized for photosynthesis rather than for producing components that are easy to digest or process (e.g., starch, sugar, oils) (Godin *et al.*, 2013).

Cellulosic crops can also be grown on marginal land unsuitable for annual row crop production, reducing potential competition with food production. Biofuels production from cellulosic feedstocks offers greater potential for displacing fossil fuels, this material contain a significant fraction of energy-rich lignin that can be used to fuel the conversion process. In contrast, processes involving annual row crops typically require external fossil-energy inputs. Lignocellulosic feedstocks also appear to have a cost advantage relative to row crops and sugarcane. Corn, for example, is currently priced above $5/bushel, equivalent to $12/GJ, and soy oil at above $0.50/lb, or $30/GJ (Maryana et al. 2014; Sluiter et al. 2010; Godin et al. 2013).

Both commodities are likely to remain at these levels or higher for the foreseeable future. By comparison, cellulosic energy crops are likely to be valued at $60–$100/dry ton or $4–$7/GJ. Finally, cellulosic biofuels offer potentially greater environmental benefits relative to biofuels made from annual crops, including lower net greenhouse gas emissions, improved water use efficiency and water quality, reduced soil erosion, enhanced soil fertility, and more positive biodiversity attributes (Kahn et al, 2011). In fact, many view the use of perennial cellulosic species as essential to achieving sustainable agriculture for reasons beyond bioenergy production. For example, in their detailed discussion, Kahn et al. (2011) state “Perennial crops would increase soil organic matter, reduce pollution, and stabilize soils against erosion. They would help fields, forests, and rangelands retain water, thereby reducing flooding and

helping aquifers recharge. Perennials would also sequester large quantities of CO_2, helping to slow climate change."

The types and amounts of biomass available for conversion to biomass-derived fuels, whether agricultural residues, forest residues, or purpose grown energy crops such as switchgrass, willow or poplar is dependent on geography and climate. The three main classes of herbaceous, hardwood and softwood feedstocks have unique aspects of the cell-wall anatomy, macromolecular architecture, and polymer chemistry. These differences extend down to each species within these broader categories, and such differences in cell-wall composition and architecture contribute to the phenomena of biomass recalcitrance at multiple scales (Pointner *et al.*, 2014).

2.2. Biomass Recalcitrance

There are two primary barriers to realizing the potential of lignocellulosic biomass for biofuels production on a large scale: (1) the recalcitrance of cellulosic biomass and (2) land-use concerns, especially those regarding competition with food production. The land use concern is not a bigger issue since this research focuses on second-generation non-edible waste crops, corncob.

Lignocellulose biomass including corn cobs are recalcitrance to hydrolysis because of its rigidity and compact structure within plant cellular wall. This is due to the components within each cell in the biomass; they build a three-dimensional network as a defensive wall (Zhao et al., 2012). The factors that affect the accessibility cellulose can be categorized in direct and indirect factors. In which the direct factors are surface area, and the indirect factors are the factors related to chemical compositions such as cellulose, hemicelluloses, lignin and acetyl group, biomass structure such as particle & pore size, volume & specific surface area, and factors related to the cellulose structure such as degree of polymerization and cellulose crystallinity, (Zhao et al., 2012). Just like any other lignocellulose biomass, corncobs have the building blocks of plant cell walls. Plant biomass mainly consists of lignin, hemicellulose, cellulose and a slight amount of acetyl (OAc) group & ash (Zhang et al, 2013).

2.2.1. Structure of Lignocellulosic Biomass

Plant cell walls represent an enormous source of complex polysaccharides that can be broken down to monosaccharides for potential conversion into biofuels and chemicals. The

framework of plant cell walls is cellulose; this is a highly ordered, water-excluding natural crystalline polymer of glucose molecules joined by (1-4) glycosidic bonds, with its chains connected by many intra- or inter- chain hydrogen bonds. Outside the framework, cellulose microfibrils and hemicellulose are intimately interlocked with one another and often with lignin, both covalently and non-covalently. The hydrophobic association of cell wall polysaccharides and lignin termed as the lignin-carbohydrate complex (LCC) is an important part of plant cell wall defence and has been recognized as the main barrier for economic deconstruction of cell wall polysaccharides. Such collective resistance, which plants and plant materials pose to deconstruction by microbes and enzymes, is defined as "biomass recalcitrance". Although the aspect/s most responsible for biomass recalcitrance to conversion are not clear, a better understanding of cell wall polysaccharides compositions and structures would greatly facilitate advanced process designs that achieve more effective breaking of such defences with lower cost as well as aid in production of less recalcitrance plants using genetic tools. For example, through comparison of glucuronoxylan (GX) structures in poplar wood, it was found that transgenic reduction of GX in secondary cell wall reduced recalcitrance of wood to cellulase digestion (Lee, Hamid and Zain, 2014).

Unfortunately, direct characterization of cell wall polysaccharides is difficult because of the heterogeneous and complex nature of cell walls. Thus, using either enzymes or chemicals to breakdown cell wall polysaccharides followed by characterizing the corresponding oligosaccharides and monomers has been an effective way to study cell wall polysaccharides structures and their possible roles in biomass recalcitrance.

Effective structural studies normally contain two parts. First, optimized enzymatic or chemical treatment methods are applied to extract certain types of polysaccharides from the insoluble cell wall in which they are held. For example, heteroxylans are typically extracted by 4% KOH whereas heteroglucans may require 24% KOH (Vainio, 2007; Sluiter *et al.*, 2012). The isolated polysaccharides or fragments are then purified and broken down into oligosaccharides for detailed characterization. Important structural information about hemicellulose polysaccharides can be determined, such as the glycosyl residue composition, the glycosyl linkage composition, the sequence of glycosyl residues in both the backbone and side chains, and non-carbohydrate substituents through characterizing hemicellulose oligosaccharides.

Lignin content is especially high in softwoods and in some hardwoods such as eucalyptus and poplar. In addition to the contents of the main components, the composition of hemicellulose and lignin varies considerably between hardwoods, softwoods and grasses. Pectins, proteins, minerals, and various lipophilic compounds (or extractives) are also present as minor constituents in lignocellulosic biomass.

Cell walls are built up of several layers: middle lamella, primary and secondary cell walls, and the warty layer (Sjöström, 1993). These layers differ from one another with respect to their ultrastructure as well as their chemical composition. The highly lignified middle lamella is located between cells and it binds them together. The thin primary cell walls consist of cellulose microfibrils that are embedded in a matrix consisting of hemicellulose, lignin and pectins (Harris and Stone, 2008). The primary cell wall is supported by the thicker secondary cell wall. Secondary cell wall consists of several sublayers: S1, S2, and S3 that are composed of cellulose, hemicellulose and lignin (Harris and Stone, 2008; Sjöström, 1993). The secondary cell wall layers differ in respect to orientation of the cellulose microfibril network.

2.2.2. Composition of Plant Biomass

Lignocellulosic biomass is biomass which is principally composed of cellulose, hemicellulose and lignin. The cell wall of lignocellulosic biomass is a composite material of crystalline cellulose fibrils bound by non-crystalline hemicellulose and surrounded by a matrix of hemicellulose and lignin (Ramos, 2003; Wyman, 1994). The composition of biomass is different depending on the species and environmental conditions (Cen et al., 2001). The compositions of biomass and interaction of these components in the cell wall affect the hydrolysis of carbohydrates; therefore, understanding each component is important to efficiently utilize lignocellulosic biomass.

Cellulose

Cellulose is one of the most abundant materials in the natural world, the primary carbohydrate comprising the cell wall. It is a linear polymer of glucose composed of glucopyranose units coupled to each other by β-(1,4)-glycosidic bonds. Cellulose in the cell wall forms long, oriented microfibrils, which may coalesce into larger and longer fibrils as indicated in Figure 2. The cellulose microfibrils are hydrophobic and can be highly crystalline, features that contribute greatly to the recalcitrance of biomass. For example, the

100 crystal face of a microfibril is more hydrophobic than the other faces and selectively binds the cellulose binding domain.

Cellulose, polymer of glucose, is the main component of lignocellulosic biomass and can be hydrolyzed to monomeric glucose for biofuels conversion. Cellulose is a linear polymer of glucose units linked with β-(1,4)-glycosidic bonds, and the degree of polymerization (DP) of this component is up to 15,000 (Bodîrlău et al., 2007). Intra- and inter-molecular hydrogen bonding in the cellulose structure forms the crystalline regions which hinder enzymes access during hydrolysis (Moore and Hatfield, 1991). Cellulose I which is the natural form of cellulose has parallel glucan chains and strong hydrogen bonds.(Mu and Wang, 2016)

In the enzymatic hydrolysis of LCB, the crystallinity of cellulose can also limit the rate in which the process will occur. Many literatures have reported that cellulose comprises of amorphous and crystalline regions (Zhao et al, 2012). Cellulose occurs as micro fibrils in crystalline regions which are para-crystalline assemblies of numerous dozen (1, 4) β-D-glucan molecular chains hydrogen bonded to each other alongside this chains (Laureano-Perez, 2005). The crystalline section of cellulose is more recalcitrance to enzyme hydrolysis and microbial attack when compared to the amorphous section because of these hydrogen bonds. Other literatures have reported that crystallinity is undesirable to the digestibility of cellulose, particularly to initial rate of hydrolysis, whereas reconstructing the crystalline hydrogen bonds network might improves the depolymerization rate (Zhao et al, 2012).

The Degree of Polymerization (DP) is characterized by the amount of the glucose component in the cellulose molecule chain. The cellulose enzymatic hydrolysis is essentially depolymerization method of cellulose chain such that the lower DP provide more pathway of cellulase or enzymes to cellulose by creating more binding sites, and therefore the hydrolysis rate increases. However, the final hydrolysis rate is not only affected by the initial DP since DP is not an independent factor. These imply that fluctuating DP will constantly go together with a change in crystallinity or substrates porosity (Zhao et al, 2012).

It has been reported in literature that biomass recalcitrance doesn't actually originate from particular individual physical structural factor (Zhao *et al.*, 2016). The cell wall of every plant is built in a cross-linked form such that the chemical compositions construct a rigid and compact three-dimensional structure. Consequently, all the interactive effects will obviously contribute to the LCB recalcitrance based on the property of each constituent (Zhao et al, 2012).

Hemicellulose

Hemicellulose is the second largest polysaccharides in the lignocellulosic biomass. It supports cellulose microfibrils by hydrogen bonding and also binds to lignin by covalent bonds. Unlike cellulose which is homogeneous polysaccharide, hemicellulose is heterogeneous polysaccharides composed of polymer of pentoses (xylose and arabinose), hexoses (glucose, galactose and mannose) and sugar acids such as D-glucuronic acid, 4-Omethyl- D-glucuronic acid and D-galacturonic acid, and its DP is generally around 200 (Saha, 2003; Sjöström, 1993).

Hemicelluloses are a class of polysaccharides that have variable compositions and structures depending on the plant source. For example, hemicelluloses isolated from herbaceous grass species, such as switchgrass, are composed of glucuronoarabinoxylans which are complex, branched polysaccharides composed mainly of pentose (five carbon) sugars. On the other hand, hemicelluloses in softwood species, such as pines, are predominantly composed of galactoglucomannans, which have a backbone of b-1,4 linked D-mannopyranose and D-glucopyranose units. Hemicelluloses are amorphous, branched, single-chain polysaccharides and are not particularly recalcitrant to conversion. Hemicellulose chains are thought to interact with more than one cellulose fibril so that they form non-covalent cross-links between cellulose bundles.

The composition and structure of hemicellulose vary depending on the species. Moreover, considerable differences of hemicellulose composition exist between the stem, branches, roots and barks (Sjöström, 1993). Xylan is the main component of hemicellulose in grasses and hardwood, while glucomannan is the dominant hemicellulose component in softwood (McMillan, 1993). Hemicellulose is amorphous and hydrophilic; therefore, it can be easily hydrolyzed by chemical treatments and enzymatic hydrolysis (Moore and Hatfield, 1994).

Lignin

The final main structural polymer, lignin, is a complex three-dimensional polyphenolic polymer that partially encases the plant cell-wall polysaccharides and cellulose microfibrils in lignified (i.e., secondary) plant cell walls. Lignin is generally not found in the primary wall of newly formed cells. Lignin provides mechanical and elastic support, facilitates water and nutrient transport, provides a chemical barrier to microbial pathogens, and is also understood to be a key contributor to recalcitrance.

Lignin is a complex aromatic polymer present abundantly in nature and is an integral component of plant cell walls. Softwoods contain approximately 27–29% of lignin, whereas hardwoods, grasses and cereals usually have lower amounts. The exact structure of lignin in the native form is still unclear. The structure of isolated lignins is always modified to a certain extent. Lignin is a three dimensional heteropolymer of methoxylated phenyl propane units, for which the precursors, monolignols, are p-coumaryl, coniferyl and sinapyl alcohol. Monolignols differ in respect to methoxylation. The corresponding aromatic constituents of these alcohols in the polymer are called 4-hydroxyphenyl (H), guaiacyl (4-hydroxy- 3-methoxyphenyl, G) and syringyl (4-hydroxy-3,5-dimethoxyphenyl, S) units. The composition of lignin varies widely with species. Softwood lignin is dominated by G units, whereas hardwood lignin is a mixture of G and S units (Fengel and Wegener, 1989; Sjöström, 1993). Lignin from grasses typically contains all three types of monolignols with different ratios (Buranov and Mazza, 2008). Different types of linkages connect the phenylpropane units. The most common is the β-O-4 linkage making up more than half of the lignin linkages in soft- and hardwoods (Dorrestijn et al., 2000; Sjöström, 1993). The amount, composition, and chemical bonds of lignin vary between plants, in different plant parts and cell types or even within a single cell wall (Campbell and Sederoff, 1996). In lignocellulosic biomass lignin is cross-linked with carbohydrates by ether or ester linkages via e.g. arabinoseferulic acid or glucuronic acid (Takahashi and Koshijima, 1988).

Lignin is a polymer of phenylpropane units like guaiacyl, syringyl and phydroxyphenyl units. These precursors are derived from three aromatic alcohols (coniferyl, sinapyl and p-coumaryl alcohols) and form three dimensional phenolic polymers by crosslinkage.

In general, softwood lignin is mainly composed of guaiacyl with small quantity of phydroxyphenyl units, and hardwood lignin consists of guaiacyl and syringyl units with a small amount of p-hydroxyphenyl units (Ramos, 2003; Whetten et al., 1998). Grass lignin is composed of all three types of units (p-hydroxyphenyl, guaiacyl and syringyl units) interconnected by aryl ether bonds (β-O-4 linkages) and/or resistant C-C bonds (Zhang et al., 2011). Lignin is hydrophobic and highly branched polymer by the oxidative polymerization (Higuchi, 1985). It fills the space between the cellulose, hemicellulose and pectin in the cell wall (Medie et al., 2012). Chemical bonds have been reported between lignin and carbohydrates (hemicellulose and cellulose). Lignin-carbohydrate complex (LCC) is the covalently bonded aggregates. The linkages are formed by ester, ether and glycosidic types of bonds (Sjöström, 1993). This structure gives physical strength or rigidity to plant tissue and

prevents the collapse of the water-conducting elements (Ramos, 2003). It also protects plants from attack by microorganisms (Moore and Hatfield, 1994; Sarkanen and Ludwig, 1971).

Although the aforementioned three components are the main components of lignocellulosic biomass, a minor fraction of various components which are soluble in neutral organic solvents or water exists in the biomass. These components are called extractives. Extractives include chlorophyll, waxes, fats, resin acids, terpenoids, phenolic substances and gums (NREL, 2008a; Sjöström, 1993). In addition, biomass also includes structural or extractable inorganic materials which are called ash. Extractable ash can be removed by washing or extracting, while structural ash is bound in the physical structure of the biomass. In general, extractable ash is considered as soil remaining in biomass (NREL, 2008b).

Extractives are a complex mixture of compounds which can include sugars, terpenoid compounds, and monolignols. A major theme of biomass recalcitrance that adds to the complexity is that many of the cell-wall components such as lignin, hemicellulose, and proteins can cross-link with each other to create a complex matrix that is resistant to chemical or biological attack.

2.3. Pretreatment

Lignocellulosic biomass is attractive feedstock because of affordable price and high polysaccharide content. However, it is not easy to use lignocellulosic resources for ethanol and other chemicals production (Fujii et al., 2009). Due to the compact structure of lignocellulose, it is difficult for enzyme to penetrate into the fiber. In addition, the crystalline structure is unfavourable for enzymatic hydrolysis. Thus specific pretreatment are required to overcome the barriers, increase surface area and make it is more accessible for enzyme molecules. An ideal pretreatment can increase the digestibility of cellulose and hemicellulose, remove lignin and avoid producing inhibitors for enzyme or microorganisms. However, the pretreatment process is energy intensive; therefore, the cost is still the limitation and challenge for most pretreatment.

In particular, several factors including lignin, hemicellulose, and crystallinity of cellulose, lignin-carbohydrate complex, and degree of polymerization, ash content, pore size and surface area are the native recalcitrance of lignocellulosic biomass to hinder biological conversion. The native form of lignocellulosic biomass can be partially digestible by enzymatic hydrolysis (Lee et al., 1999). For this reason, pretreatment is necessary to overcome the recalcitrance and convert biomass more efficiently.

Various pretreatment methods have been developed to change the physical and chemical structures and break down the hemicellulose and lignin shield efficiently. However, pretreatment is still one of the most expensive stages within lignocellulosic biomass conversion process. Developing effective pretreatment methods and finding optimal operating conditions can contribute to overcoming the cost barriers of biomass utilization.

Minimizing energy, chemical and water inputs, preserving cellulose and hemicellulose fractions, avoiding size reduction and limiting formation of inhibitors are key issues to develop cost-effective pretreatment methods (National Research Council, 1999; Zheng et al.,

2009).

In general, pretreatment technologies enhance the enzymatic hydrolysis by removing lignin and hemicellulose, swelling pores in the biomass structure, increasing surface area, breaking and regenerating cellulose structure. These pretreatment methods including dilute acid pretreatment (Jacobsen and Wyman, 2000; Kim et al., 2001; Schell et al., 2003), hydrothermal pretreatment (Garrote et al., 2002; Petchpradab et al., 2009), ammonia pretreatment (Kim and Lee, 2007; Li and Kim, 2011; Murnen et al., 2007), lime pretreatment (Kim and Holtzapple, 2005), microwave pretreatment (Hu and Wen, 2008), ionic liquid pretreatment (Zhu, 2008), ultrasonic pretreatment (Montalbo-Lomboy et al., 2010) and microbial pretreatment (Wan and Li, 2011) and each pretreatment has different impacts on compositional and structural feathers of biomass.

Acid pretreatment hydrolyze the hemicellulose fraction and the accessibility of cellulose is improved (Grohmann et al., 1985; Torget et al., 1990). In addition, it is a relatively inexpensive method. However, this process produces a large amount of byproducts which can be inhibitors for biological conversion. Hydrothermal pretreatment releases acidic components such as acetic acid and formic acid in hemicellulose with high temperature water. The acetyl group works as a catalyst to break down hemicellulose (Fernandez-Bolanos et al., 1999).

Ammonia pretreatment mainly break down the lignin structure with some of hemicellulose and change the crystalline structure of cellulose (Dale et al., 1996; Yoon et al., 1995; Kim and Lee, 1996; Kim et al., 2002). It is a strong swelling reagent and easy to recover because of its high volatility. Lime pretreatment removes lignin, acetyl group and uronic acid (Chang and Holtzapple, 2000). Ionic liquid pretreatment also targets lignin removal. However, ionic liquid recovery is still an issue (Shill et al., 2010).

Microwave pretreatment and ultrasonic pretreatment contribute to improving the enzymatic hydrolysis with ammonia or acids. However, input energy for this pretreatment is relatively large. Biological pretreatment breaks lignin by fungi with low energy and no chemical inputs; however, this pretreatment is slow, and controlling the treatment conditions is difficult (Chandra et al., 2007; Zheng et al., 2009)

2.3.1. Fundamental of Biomass Pretreatment

Biomass pretreatment involves the use of physical processes (e.g., size reduction, steaming/boiling, ultrasonication, and popping), chemical methods (e.g., acids, bases, salts, and solvents), physicochemical processes (e.g., liquid hot water and ammonium fibre explosion or AFEX), biological methods (e.g. brown-rot/ white-rot fungi & bacteria), and several combinations thereof to fractionate the lignocellulose into its components (Bensah & Mensah, 2013).

The fundamental goal of biomass pre-treatment is to prepare the feedstock material for further processing. Pretreatment is a crucial step in biomass conversion processes since it can affect the technical and economical requirements of the downstream operations. Pretreatment processes might be energy demanding and costly. It is possible that the cost of pre-treating the biomass to exceed the cost of the energy content in it and consequently, cheap and simple pretreatment methods are preferred. In this section physical, physicochemical and chemical pretreatment are discussed (Ching, 2014).

Pre-treatment is the single most crucial step used in biofuels production from lignocellulosic material, this is because it determines the efficiency of the steps that follow. The drive to pre-treatment method is the removal of lignin, increase surface area of the cellulose & hemicellulose, and enhance porosity of substrate (Samuel, 2011). Typical cellulose is well protected by means of a matrix pattern of lignin and hemicellulose (Figure 2.1). This needs to be broken down to expose cellulose and hemicellulose for enzyme hydrolysis.

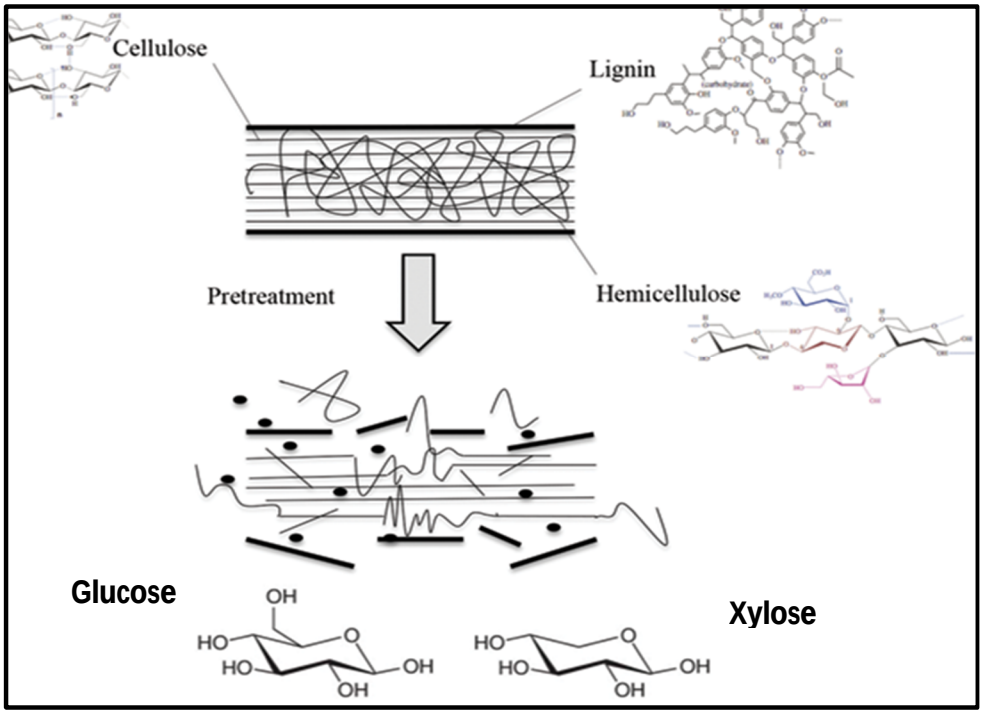


Figure 2.1: Diagram demonstrating effects of pre-treatment on lignocellulosic biomass. Removal of lignin in lignocellulosic substrate exposes cellulose and hemicellulose (Mosier, 2005).

Pre-treatment is the initial step of the bio-succinic acid production process from LCB. Therefore, the efficiency and quality of pre-treatment stage directly affect all the succeeding steps, especially the fermentation & enzymatic hydrolysis steps. A higher pre-treatment efficiency is identified by specific critical factors. It has been reported by Kumar et al., 2009 that pretreatment method (solvents) efficiency is determined by the following criteria.

In theory, the ideal pretreatment process produces a disrupted, hydrated substrate that is easily hydrolyzed but avoids the formation of sugar degradation products and fermentation inhibitors.

Assessment of biomass pretreatment agent's efficiency depends on its ability to;

1. Overcome LCB recalcitrant by the disruption of lignin, breakdown the crystalline structure on cellulose and accessibility of hemicellulose
2. Achieve maximum sugar yield or rather easily converted solid/chemicals
3. Circumvent carbohydrates degradation & preserve the utility of hemicellulose fraction
4. Circumvent toxic by-products formation
5. Permit the recovery of lignin
6. Operate at low cost i.e. requires low heat & energy (operate at low temperatures) , produce minimal wastes amount and need reasonable reactor size/volume,
7. Ability to scale to commercial production.

2.3.2. Methods for biomass pretreatment

Different pretreatment and bioconversion strategies have been developed to overcome biomass recalcitrance, and their success has been shown to vary with the biomass feedstock. For example, dilute acid pretreatment have been shown to be very effective for the pretreatment of herbaceous materials but fail at the same thermochemical severity to be an effective pretreatment for woody feedstocks.

A. Physical pretreatment

In the physical pretreatment of biomass the chemical composition and characteristics of the biomass are not subjected to any change. The main physical pre-treatments are achieved by mechanical means.

Size reduction

Biomass size reduction is performed to augment specific surface area available in the biomass so that it could ease the handling of material and enhance the heat & mass transfer characteristics of feedstock, e.g. to improve the drying or combustion properties. With size reduction of LCB material, the degree of polymerization & the cellulose crystallinity are reduced. The feedstock can be logs and forestry residues (Ching, 2014).

Size reduction can be done as a step for further treatment like densification or hydrolysis, or it can be done after the biomass feedstock has been subjected to a process. Wood chips can be fed into a boiler for direct combustion. The machinery employed in size reduction can be classified according to grinding mechanism (Naimi et al., 2006). However, this process comes with a disadvantage of high power consumption which is not practical since it is even greater than the inherent energy of biomass (Kumar et al, 2009).

Drying

In solvent pre-treatment process wet biomass is produced and the conversion process requires dry biomass, some of the energy released by combustion is utilized in evaporation of the water content of the feedstock. As a consequence lower efficiencies are achieved. Employing dry biomass has other advantages such as higher flame temperatures resulting in complete combustions and lower CO emissions (Ching, 2014). In combustion the necessity of excess air is reduced due to high temperatures, thus reducing the equipment size as less capacity in equipment such as fans is needed. In solvent pretreatment, the volume and the reaction is reduced if biomass is dried before the actual solvent pretreatment

The drying equipment can be broadly classified depending on how the heat is transferred to the biomass into direct and indirect dryers (Amos, 1998). In direct dryers air or superheated steam is in direct contact with the wet biomass, the air or superheated steam lose sensible heat to evaporate the moisture from the material, however for the case of the superheated steam the temperature never goes low enough to make the steam condensate. In indirect dryers the biomass is not in contact with the wet biomass and the vapour produced in the dryer side of the equipment is removed by mechanical means. It is possible to recover heat from the vapour produced during the drying operations. Nevertheless, the drying uses heat as utility and therefore this process is cannot be regarded as environmental and economical sustainable.

B. Chemical and physicochemical pretreatment

In chemical and physicochemical pretreatment the chemical composition of the bulk of the biomass is altered by chemical or physical means, because in general biomass is recalcitrance to bioprocessing. Its recalcitrance is produced by the crystallinity and polymerization of cellulose, lignin content, porosity, hemicellulose encasing cellulose and fiber strength (Agbor, 2011).

It is necessary to disintegrate the biomass fiber structure to its components, as the cellulose contained in lignocellulose is packed in polymer arrangements insoluble in water and resistant to depolymerization, which makes it inaccessible to further conversion in other processes (Ching 2014). With pretreatment operations the 3 constituents of LCB: lignin, hemicellulose & cellulose can be separated, making the cellulose and hemicellulose accessible for digestibility so as to generate fuels and chemicals. The typical goals of chemical & physicochemical pretreatment are: production of highly digestible solids, circumvent the sugars degradation, reduce the development of inhibitors and capability to recover of lignin for further use (Brodeur, 2011). As mentioned before the costs of the pretreatment operations should be considered as it will affect the overall economic performance of the plant.

Dilute Acid pre-treatment

In acid pre-treatment, a diluted acid is used to dissolve and hydrolyze the lignocellulosic material; the most common acid employed for this operation is sulfuric acid (H_2SO_4). During acid pretreatment the hemicellulose is dissolved and hydrolyzed to its monomers mainly xylan, making the cellulose available for further processing (Wang, 2010), common feed

stocks are corn stover and switch grass. Temperature conditions to carry out the reaction range from 140 °C to 215 °C and the time of reaction (residence time) in the reactor is ranged from 30 seconds to 10 minutes dependent on the reaction conditions (Timang et al, 2015). The reaction is mainly dependent on the temperature and solid/liquid fraction concentration of the feedstock, with a slight dependency on the acid concentration (López-Arenas et al, 2010). However, the process comes with the disadvantage that the acid might be an inhibitor for further fermentation of the substrate and as in any other acid reaction the equipment materials are more expensive.

Alkaline pretreatment

Alkaline pretreatment methods employ bases to increase the digestibility of cellulose by increasing the surface area of biomass since when biomass is exposed to bases it swells. This swelling also causes a reduction in DP and crystallinity of cellulose. The lignin structure is also altered by bases; they break the linkage between lignin and other biomass fractions, making them more accessible (Agbor, 2011). The alkaline pre-treatment can be sub-divided in three groups: Calcium hydroxide, Potassium or Sodium pre-treatments and ammonia pre-treatments (Carvelheiro, Duarte & Girio, 2008). The conditions for the process vary depending on the type of bases chosen but in general alkaline pretreatment are less severe than others. The process is carried out by soaking and mixing in an alkaline solution at a range of temperature during a distinct course of time. The process can be carried out at ambient temperature sacrificing processing time (Brodeur, 2011).

Liquid Hot Water

Liquid Hot Water (LHW) pretreatment of biomass is done in water at a higher temperature that ranges from 160 °C to 240 °C, but kept in liquid state under high pressures (Ching, 2014). Thereby the hemicellulose and part of the cellulose & lignin are dissolved, promoting the separation of the LCB matrix and enhancing the digestibility of cellulose (Ching, 2014). Fermentation inhibitors, like furfural and formic acid, are formed when the process takes place at high temperatures thus an adequate temperature selection is crucial for the process (Brodeur, 2011). After the biomass is pre-treated two phases will be formed, a liquid phase comprising of about 20–22% cellulose, 35–70% lignin and almost all of the hemicellulose. A solid phase will be composed of cellulose and lignin that can be utilized to generate chemicals such as ethanol (Mosier, 2005). The process can take place in different reactor configurations, in a co-current process the biomass and the hot liquid water are contacted in a

plug-flow reactor during 15-20 minutes, heat is provided using heat exchangers. In a flow through configuration the hot-water passes through the biomass contained in a jacketed reactor vessel, the reactor pressure is between 2.5- 3 MPa. In a counter current configuration, the biomass is passed through the reactor in the opposite direction than the water (Mosier, 2005). This can also be considered as "green solvent". However this is still unattractive since it requires heat as utility.

Steam explosion

Steam Explosion (SE) is a physico-chemical process wherein the accessibility to the cellulose fibers is improved by improving its digestibility. In SE the hemicellulose is also removed from the biomass material. In this process the biomass is pre-treated under high pressure produced by injecting saturated steam in a reactor together with the biomass, the temperature of steam is around 160–250 °C and the pressure is between 0,6 to 4,9 MPa. These conditions are maintained for several minutes inside the reactor before the steam is suddenly released causing the rupture ("explosion") of the rigid biomass fibres (Nova & Ramos, 2003).

During the pretreatment some of the hemicellulose is hydrolyzed resultant to the release of xylose and glucose monomers that are fermentation inhibitors, however longer residence times and lower temperature (10 min, 190 °C) are favourable if fermentation is the next step since at these conditions there is a lower monomer release (Öhgren et al., 2007). Catalysts such as H_2SO_4, CO_2 or SO_2 can be used. A major advantage of SE is the limited use of chemicals and the resulting sugars are not excessively diluted (Nova & Ramos, 2011). Steam explosion is widely used in paper and pulp industry and to pre-treat the lignocellulosic biomass prior to fermentation to produce ethanol, however, recently also being studied to produce wood pellets with improved properties. Steam treated biomass pellets show a maximum low heating value of 18.5 to 20 MJ/kg (Padban, 2014), higher energy density and more resistance to impacts and abrasion. However steam explosion can only breakdown a percentage of xylan fraction in the hemicellulose; accomplish an unfinished lignin disruption -carbohydrate matrix & production of inhibitory compounds that hinder micro-organisms effect on biomass (Kumar et al, 2009).

Organosolv

Organosolv is a pre-treatment process in which the lignin of LCB is extracted using an organic solvent. Different organic solvents such as ethanol and methanol can be used. After extraction the remaining celluloses are in a solid state and present increased enzymatic

digestibility. The amount of lignin and hemicellulose contained in the cellulose varies. The hemicelluloses in the feedstock are dissolved together with the lignin, the solvent is then recovered and the remaining liquid is diluted by water for precipitating the lignin. The hemicelluloses are recovered from the aqueous solution. General advantages of the process are (Zhao et al., 2009); Organic solvent is easier to recover and lignin and cellulose can be isolated as a solid, hemicellulose remains as a syrup. However, this process is comparatively costly due to the use of organic solvents. The process should be performed in tight and control conditions to avoid volatiles being released. Solvents should be reused after being recovered from the process.

Ammonia Fibre explosion

In the Ammonia Fibre Explosion (AFEX), LCB is pretreated under high temperature and pressure conditions with liquid ammonia (Teymouri et al., 2005). However, pressure is suddenly reduced after few seconds. In a typical AFEX, approximately 1.5 kilograms of ammonia is carried out for every 1 kilograms of dry biomass at temperature of 90 °C for each 30 minutes run. This will reduces the lignin content, eliminates nearly all hemicellulose as the cellulose is de-crystallizing (Teymouri et al., 2005). The cost of the pre-treatment process is determined by total ammonia cost including its recovery even though ammonia volatility makes it easily recoverable.

CO_2 explosion

Carbon dioxide explosion is comparable to AFEX and steam pretreatment method; they operate on extreme conditions. In CO_2 explosion, high pressure CO_2 is liberated by an explosive de-compression after it has been injected in the batch reactor. As reported before, CO_2 will reacts to form carbonic acid which is basically CO_2 in water and therefore improve the rate enzymatic hydrolysis. However, sugar yields in CO_2 explosion are significantly low compared to those achieved from AFEX or steam (Sun and Cheng, 2002).

The carbonic-acid formation offers the same benefits as that of an acid catalyst besides the use of any acid similar to sulfuric acid. Literatures have specified that the joint operating costs and capital of sulfuric acid system are comparable to that of a carbonic acid-based system. In addition the system is highly sensitive to solids concentration and reactor pressure (Jayawardhana and Van Walsum, 2004). Carbonic-acid is a potential reagent to endorse hydrolysis at the absence of mineral acids in the bio-processing plant (Van Walsum, 2001).

The partial pressure of CO_2 (pCO_2) in water gives indication of carbonic acid pH, hence the release of the reactor pressure neutralizes the pH.

The alternative to CO_2 Explosion pretreatment is the extraction with supercritical CO_2. It is known that CO_2 becomes supercritical under temperature and pressure of Tc=304 K and Pc=73 bar respectively, which are moderately mild conditions. Super-critical Carbon dioxide ($SC\text{-}CO_2$) has gained more attention after it was discovered that it can be applied as a biomass pre-treatment solvent. Lately, $SC\text{-}CO_2$ extraction is amongst the commonly recognized technique for LCB pre-treatment route.

Until now, many pretreatment technologies have been established, developed and others are still developing to overcome the lignocellulosic biomass recalcitrant and subsequently to improve biomass digestibility, such as acid, alkali, steam explosion, organosolv, biological pretreatment, and others. Recently, Ionic Liquids (IL), which are regarded as green solvents, have attracted much attention due to their interesting features being low vapour pressure, non-flammability, thermal and chemical stability, and phase behaviour (Ly et al, 2015; Wang, 2012; Liu, 2012)

Green solvents (ionic liquids)

Green solvents are ionic liquids that melt down at temperatures below 100 °C. They have high thermal stability, high electrical conductivity and negligible vapour pressures. In the pretreatment process they don´t produce any toxic by-product and they are recoverable (Ching, 2014). These liquids are composed by different combination of ions which make their properties tenable. Since there is a wide variety of ionic liquids that can be synthesized efforts to identify ionic liquids capable of dissolving cellulose, lignin or other components of lignocellulose have increased in recent times (Mora-Pale, 2011). Some of these liquids are 1-butyl-3-metyl-imidazalium-chloride [Bmim][Cl-] to dissolves cellulose or 1-Ethyl-3-methylimidazolium-acetate [Emim][Ac] for dissolving lignin (Fitz-Patrick et al, 2010). Liquid solid extractions at mild temperatures are used to extract the lignocellulosic component of interest. After the liquid is used to dissolve the lignocellulosic fraction this can be recovered with another solvent like water or acetone, the regeneration rates for the ionic liquids are >99%.

Table 1: Summary of different approaches used for Lignocellulosic Biomass (LCB) Pre-treatment, (Adapted from Kumar, 2009)

Pre-treatment Technique	Advantages	Disadvantages or Restrictions
Mechanical comminution	reduce crystallinity of cellulose	Energy consumption frequently greater than inherent biomass energy
Acid Pretreatment	Disruption of lignin structure Convert hemicellulose to xylose and other sugar monomers;	Development of toxic materials High cost; possibility of corrosion formation on operating equipment;
Ionic Liquids	high thermal stability, high electrical conductivity and negligible vapor pressures	Very expensive
Alkaline Pre-treatment	Removal of lignin & hemicelluloses Accessible Surface Area (ASA) is increased	Salts formed cannot be recovered and it is fused with biomass pre-treated Time consuming (reaction time is long)
AFEX	Increases ASA Removal of hemicellulose & lignin to a certain extent; Do not form inhibitors in the downstream process.	Limited to LCB with low lignin content. Thus, it is not proficient in lignin with great biomass content.
CO_2 Explosion(CE)	Increase ASA; Cost effective; Do not form inhibitory compounds	Cannot alter the structure of hemicelluloses or lignin entirely.
Organosolv	Hydrolyzes hemicellulose & lignin	High cost and time consuming since solvents must be drained out of the reactor, vaporized, condensed & the recycled
Hot Water pretreatment	All hemicellulose is dissolved	part of the cellulose and lignin are dissolved,
Ultrasonic	Double the enzymatic hydrolysis reaction rate.	Only pure cellulose has been used.

2.3.3. Efficacy of pretreatment

There are a number of key features for the effective pretreatment of lignocellulosic biomass. The pretreatment process should have a low capital and operational cost. It should be effective on a wide range and loading of lignocellulosic material and should result in the recovery of most the lignocellulosic components in a useable form in separate fractions. The need for preparation/handling or preconditioning steps prior to pretreatment such as size reduction should be minimized. It should produce no or limited amounts of sugar and lignin degradation products that inhibit the growth of fermentative microorganisms or the action of hydrolytic enzymes, and it should have a low energy demand or be performed in a manner that energy invested could be used for other purposes such as secondary heating (Chandra et al. 2007) .

Other features such as regeneration/cost of catalyst, generation of higher-value lignin co-products, and obtaining hemicellulose sugars in the liquid phase to reduce the need for the use of hemicellulases in subsequent enzymatic hydrolysis forms the basis of comparison of different pretreatment options (Galbe and Zacchi 2007; Mosier et al. 2005). All these features are considered in order that pretreatment results balance against their impact cost on downstream processing steps and the trade-off with operational cost, capital cost and biomass cost (Lynd et al. 1996; Mosier et al. 2005).

Many pretreatment methods have been studied, and are still in development. It is difficult to evaluate and compare pretreatment technologies because they involve upstream and downstream processing cost, capital investment, chemical recycling and waste treatment systems (Jeoh et al. 2007). However, as an integrated part of an industrial system or biorefinery, mass balance analysis can be used to validate the pretreatment efficacy of a process with any given feedstock. This necessitates thorough economic analysis to determine the best pretreatment method suitable for a particular local feedstock for an industrial process especially with regards to co-location with existing plants where inexpensive power, steam or default treatment is available (Aden et al. 2002).

2.3.4. Physical and chemical features of pretreated biomass

In a similar manner they have a huge impact on the pretreatment process, physical and chemical features of pre-treated biomass influence on biological conversion. These features include surface area or porosity; hemicellulose and lignin contents; particle size; and cellulose crystallinity, type, and chain length (Wyman, 2013). However, all these physical and chemical features are aspects of two principal factors that control conversion. The first one is enzyme accessibility to cellulose substrates; and the second is enzyme effectiveness once adsorbed on substrate's surface. Because enzymes such as β-glucosidase and β-xylosidase (for cellulolytic and xylanolytic catalytic homogenous reactions respectively) do not appear to play a direct role in controlling accessibility to their substrates. Nevertheless, they can affect their effectiveness.

Kumar and Wyman (2013) categorize enzyme accessibility into macro- and micro-accessibility. This contributed towards assessing the impacts of physical and chemical features on cellulose accessibility and enzyme effectiveness once adsorbed. They stated that macro-accessible of pre-treated lignocellulosic biomass can therefore be pictured as having high degree of macro-accessibility compared to untreated biomass. This is due to removal of obstacles such as lignin and hemicelluloses that prevent cellulase enzymes from reaching the cellulose surface. The hemicellulose and lignin sheath in cellulosic biomass interferes with cellulase reaching cellulose, therefore limiting the ability for enzymes to attach to cellulose. However, above all physiochemical conditions such as solids concentration and pH should be optimized to not impede accessibility (Wyman, 2013).

The second stage of accessibility, micro-accessibility is encountered once enzymes reach the cellulose surface. This can be pictured in terms of how readily the enzymes can reach cellulose binding/catalytic sites buried within the compact, semi-crystalline cellulose structure. A clear illustration of micro accessibility is the amorphous cellulose region, it can be agreed that the region represent the highest degree of macro- and micro-accessibility in that cellulase enzymes should be able to reach all cellulose chains as opposed to the crystalline region, refer to Figure 2.2.

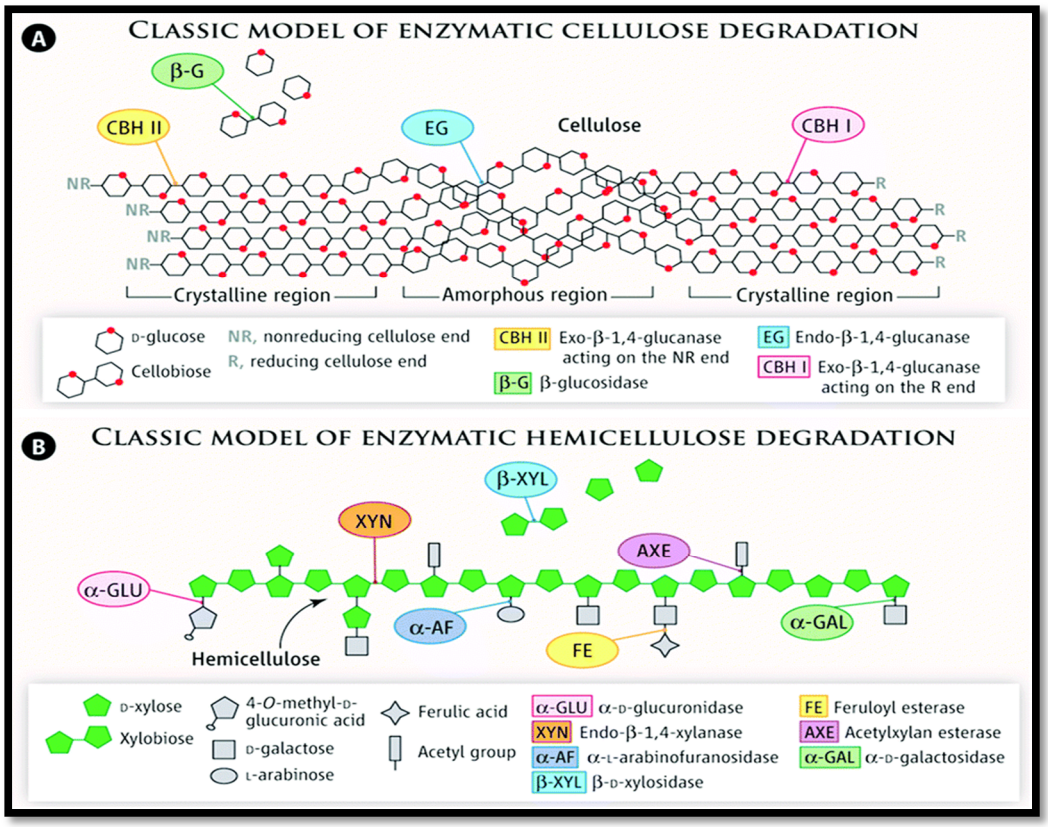


Figure 2.2: A simplified schematic of cellulose hydrolysis by a typical non-complexed cellulase system mediated by cellobiohydrolases (CBH I and CBH II) and endoglucanases (EG I to EG V) adsorption, cellobiose and cellodextrin production, and their catalysis to glucose by b-glucosidase. Also shown is one of the cellulase components with different domains (Wyman, 2013).

Enzymatic conversion also known saccharification or hydrolysis of structural carbohydrates is a two steps heterogeneous reaction which involves enzyme adsorption on the substrate surface and hydrolysis of the polymers to form shorter chained molecules (Wyman, 2013). As illustrated in Figure 2.2, enzymatic hydrolysis requires enzymes to adsorb onto a cellulose surface with specific or common binding sites. The accessibility of cellulose binding sites to cellulase can be expressed in terms of the maximum adsorption capacity σ_{max} in mg or mg/g biomass) of cellulase (or its individual components) per gram of biomass or glucan and can be estimated by fitting multiple points of cellulase adsorption data to a non-linear Langmuir model.

Compared to the hydrolysis step, enzyme adsorption is fast and usually completed within a few hours, 2 hours compared to 72–120 hours for hydrolysis. Most carbohydrases in the fungi family that includes cellulases and hemicellulases are composed of two distinct domains connected by a peptide linker: a cellulose or carbohydrate binding domain and a catalytic domain schematically shown in Figure 3. Although adsorption is largely mediated by CBMs, catalytic domains also participate in adsorption.

Employing harsh pretreatment conditions can reduce macro barriers to enzymes reaching cellulose and improve micro accessibility of cellulose to enzymes through changes in its crystal structure and degree of polymerization and result in better conversion to sugars. But such conditions also degrade xylooligosaccharides and xylose into byproducts, such as furfural resulting in sugar losses and formation of inhibitors to enzymes and microbes for sugars fermentation. Thus, pathway optimization is needed to achieve the highest sugar recovery for economical processing. For that reason, qualitative and quantitative measurements of xylooligosaccharides have to be carried out.

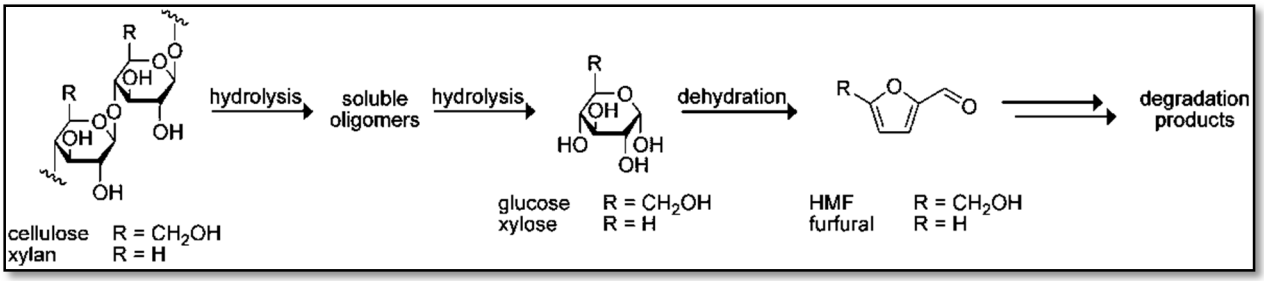


Figure 2.3: Hydrolysis reactions of cellulose and xylan. Chemical hydrolysis of cellulose and hemicellulose into monomeric sugars proceeds through oligomers and is accompanied by side reactions that form furans and other degradation products Adapted from Binder, 2010).

By disrupting its network of intra- and inter-chain hydrogen bonds, strong acids decrystallize cellulose and make it accessible to reagents; and by catalysing the hydrolysis of glyosidic bonds, strong acids cleave cellulose and hemicellulose into sugars, See Figure 2.3.

2.4. Overview of Bio-processing of Lignocellulosic Biomass

The production of biofuels or (bio) chemicals via bioconversion pathways typically consists of three discrete processing steps: chemical pretreatment, enzymatic hydrolysis, and microbial conversion. The role of the first two steps is to generate soluble carbohydrates, whereas the goal of the final step is the conversion of these carbohydrates to fuels or chemicals.

Similar to cellulose, hemicellulose polysaccharide chains can be broken into oligosaccharides and then further hydrolysed to monosaccharides, especially during low to neutral pH pretreatment, which in turn can react to degradation products as depicted by Equation (1) below:

$$Polysaccharides_{(solids)} \rightarrow Hemicellulose/Cellulose\ Oligosaccharides_{(aq)} \rightarrow Monosaccharides_{(aq)} \rightarrow Degradation\ products_{(aq)} \quad (1)$$

Biological conversion of cellulosic biomass to fuels and chemicals offers the high yields to products vital to economic success and the potential for very low costs. Enzymatic hydrolysis that converts lignocellulosic biomass to fermentable sugars may be the most complex step in this process due to substrate-related and enzyme-related effects and their interactions. Although enzymatic hydrolysis offers the potential for higher yields, higher selectivity, lower energy costs and milder operating conditions than chemical processes, the mechanism of enzymatic hydrolysis and the relationship between the substrate structure and function of various glycosyl hydrolase components is not well understood. Consequently, limited success has been realized in maximizing sugar yields at very low cost (Yang et al, 2014). The sugar monomers obtained from the enzymatic hydrolysis are then fed to the fermentation process.

Fermentation is an anaerobic process that breaks down the glucose, a sugar monomer into organic materials. It is a series of biochemical reactions that with an end goal of producing biofuel with sufficient alcohol concentration to fuel automobiles by converting the sugars to bioethanol. Itelima et al, (2013) obtained maximum ethanol yield of 10.08% (v/v) and Pimental & Patzek, (2005) showed that 95% of pure ethanol is attainable from corn cob. Literature reports that the produced bio based fuels perform in a similar manner as petroleum derived fuels even though their properties are not the same.

Succinic acid, also known as amber acid or butanedioic acid can be produced by various microorganisms and has a tremendous potential as a building block for many industrially important commodity chemicals in food, chemical and pharmaceutical industries (Salvachúa *et al.*, 2016). Recent works showed that succinic acid production by fermentation from renewable resources could be more cost-effective than the petroleum-based process. As it has been explained before, fermentation can be applied as a pathway to break down the glucose to succinate, acetate and ethanol when the cells are transferred to oxygen-deprived conditions. Literature reports that 52 g/L of succinic acid can be produced, when given a feed of 72 % glucose (Vanswani, 2010).

Succinic acid is currently expensive; its price has varied in the range of $5 to $9 per kg. A detailed techno-economic analysis for bio-based succinic acid production was done and it showed that the market potential can increase significantly. The succinic market could expand to 180,000-tons by 2015 largely due to the introduction of bio-based succinic acid (Vaswani, 2010). Additionally, there is a growing interest towards discovering a more economical and environmentally cleaner way for its production. As a result, succinic acid

bio-production has a tremendous potential for commercialization and replacing the current petroleum-based process.

Economically, lignocellulosic biomass has an advantage over other agriculturally important biofuels feedstocks such as corn starch, soybeans, and sugar cane, because it can be produced quickly and at significantly lower cost than food crops. Lignocellulosic biomass is an important component of the major food crops; it is the non-edible portion of the plant, which is currently underutilized, but could be used for biocommodities production. In short, lignocellulosic biomass holds the key to supplying society's basic needs for sustainable production of this biocommodities including succinic acid without impacting the nation's food supply (Zafar, 2014).

2.5. Effect of pretreatment operating parameters

The effectiveness of pretreatment is dependent on the physical structure and chemical composition of the substrate as well as the treatment conditions (Chen *et al.,* 2013). The main operating conditions known yet are concentration of the pre-treating agent, pH, pretreatment time & temperature, solid-to-liquid ratio and employed models reactors (Djioleu and Carrier, 2016). The effect of these operating conditions has been investigated by several researchers.

Yesim *et al.,* (2013) studied the effects of temperature and concentration of the pre-treating agent in the alkaline peroxide oxidation pre-treatment process of sunflower stalks. The results showed an increase in extensive lignin degradation (up to 60%). The pretreatment time and solid to liquid ratio are also important parameters to maximize the cellulose and hemicellulose accessibility. As time increases the accessibility also increases until the pretreatment reaction equilibrium is reached. This is also the case with solid to liquid ratio. Thus an optimal conditions in pretreatment step such as pretreatment time, temperature and the solid to liquid ratio needs to be investigated. The pretreatment rate can be improved by optimizing these conditions. An aspect of this study was to optimize the pretreatment conditions, to maximize sugar accessibility, thus enhancing the pretreatment process.

2.6. Parametric Optimization using Response Surface Methodology

Response surface methodology (RSM) is a combination of statistical and mathematical techniques for construction of the empirical model. The objective is to optimize the response (output variable) which is influenced by several independent variables (input variables) by careful design of experiments. The experiments are carried out to evaluate changes in the input variables in order to identify possible explanations for changes in the output response (Gunst, 1996; Kraber, 2014). Factorial designs are good preliminary experiments, this the design that is often used to find the "vital few" significant factors out of a large group of potential factors also known as a screening experiment. However, once those vital factors are determined, it is recommended to map the response surface (Minitab, 2016).

In order to improve the process, an understanding of how certain factors influence the response is vital. It will be easier to find out what adjustments can be made in factor settings, while staying near the optimal response. A decent approximation of any mathematical function can be made via an infinite series of powers of X, such as that proposed by Taylor, (Minitab, 2016) For RSM, this takes the form:

$$Y = \alpha_0 + \alpha_1 X_1 + \alpha_2 X_2 + \alpha_3 X_3 + \alpha_{1,1} X_1^2 + \alpha_{2,2} X_2^2 + \alpha_{3,3} X_3^2 + \alpha_{1,2} X_1 X_2 + \alpha_{1,3} X_1 X_3 + \alpha_{2,3} X_2 X_3 \quad (2)$$

This model is an equation that describes the surface in that specific region. The Y represent the predicted response, $X_{1,\,2,\,3}$ are the coded independent variable, α_0 is the intercept, $\alpha_{1,2,3}$ are the linear coefficient, $\alpha_{1,1}$ $\alpha_{2,2}$ & $\alpha_{3,3}$ are the quadratic coefficient, and $\alpha_{1,2}$, $\alpha_{1,3}$ & $\alpha_{2,3}$ are the interactive coefficient (Xiao *et al.*, 2015). These values are obtained using a central composite design (CCD), which will then generate the Analysis of variance (ANOVA) and contour & response surface plots. The response surface maps will therefore assist in finding the factor settings that optimize the response by providing the maximum & minimum problem or hitting a specific target. Basically, the response surface plots obtained is a model that describes the relationship between the vital factors and the response (Xiao *et al.*, 2015; Minitab, 2016).

2.7. Dissolution Kinetic for lignocellulosic biomass

Kinetic modelling plays an important role in the design, development, and operation of many chemical processes. Li et al. 2013, identified pre-treatment reaction kinetics as an important tool for the economics of a process since reactor volume, and hence the capital equipment requirement, is said to be proportional to the residence time in the reactor (Li et al., 2013). Kinetic data are also important in the design and evaluation of processes to understand the accessibility of the polysaccharide (hemicellulose and cellulose) from the biomass after pre-treatment. During pre-treatment polysaccharides are made accessible so that they be decomposed to oligomers and monomers. This makes it easier to hydrolyse cellulosic materials to glucose for fermentation into ethanol or a variety of other chemical intermediates.

Predictive modelling of monosaccharides yield during pre-treatment is attractive for further development of technological processes and equipment. Several kinetic studies of corncob pre-treatment with lime, hydrogen peroxide, sulphuric, formic and phosphorus acid have been reported in the literature (Fuentes *et al.*, 2011; Dong et al., 2015; Ayeni *et al.*, 2016). Significant discrepancies in values of the kinetics parameters obtained by various authors can be explained by differences in the applicable form of raw materials, the type and concentration of the pre-treating agent, pre-treatment conditions, temperature, solid-to-liquid ratio and employed models and reactors (Djioleu and Carrier, 2016).

As mentioned before, the conditions of pre-treatment and variations in composition & structure of biomass affect reaction rates, it is necessary to know the kinetic parameters that determine optimal conditions of treatment (Djioleu and Carrier, 2016). Reaction rate Kc of the monomers can be modelled according to the Arrhenius equation. For hydrothermal pre-treatment without adding any chemicals, a constant molar hydrogen-ion concentration can be assumed (Lei et al., 2013).

Activation energy associated with the dissolution process can be determined from the logarithmic curve of reaction rates and temperature. Activation energy give information of how sensitive the pretreatment process is to temperature (Carrier *et al.*, 2016). It also articulate the minimum amount of energy the required to start a dissolution process (Ayeni *et al.*, 2016). The goal of this study is to approximate evaluation of kinetics parameters of corncob pre-treatment using the best pre-treating agent.

CHAPTER 3: Materials and Method

3.1. Materials

3.1.1. Substrate Collection & Preparations

The corn cobs (red and white) were collected from domestic farming homes in Mukula Village, Limpopo South Africa. The dried samples were stored in capped plastic bottles tightly and were used shortly after collection.

The corncob samples in all the experiments were crushed using grinder to small size and were passed through 2000 µm (No. 10), 1000 µm (No.18), 850 µm (No.20) and 500 µm (No.35) mesh sieves (USA Standard Sieve specifications). The samples were classified in the size range of 2000-1000, 1000-850 and 500 µm. Based on the milling process the size classification of 1000-850µm was excellent sample class to use. This is because it resulted in more than 50% of the sieved corncob material and was easier to handle when performing experiments. Samples were dried in a conventional oven to dry matter content. This was carried out to avoid degradation of material during storage and for accuracy during weighing (dry weight) of material. Drying for 3 hours at 105 °C was the correct temperature & time combination to vaporize the water without negatively impacting the compositional quality of the biomass.

3.1.2. Chemicals

The solvents used were chosen in terms of availability, cost and mild-low operating conditions. The solvents were classified as;

1. Molten Hydrates Salts (MHS)
2. Dilute Acids
3. Dilute Base
4. Others

The complete list is provided on the appendix A. The solvents were prepared guided by the analytical grade as provided by Sigma Aldrich.

3.2. Experimental Methods

3.2.1. Compositional analysis of raw corncob

Composition of corncob was examined in terms of cellulose, hemicellulose and lignin contents using the NREL procedure titled "Determination of Structural Carbohydrates and Lignin in Biomass Laboratory Analytical Procedure (LAP)". This procedure uses a two-step acid hydrolysis to fractionate the biomass into forms that are more easily quantified. All monomer sugars after acid hydrolysis were analyzed by HPLC (Agilent HPLC 1200 Series) and then used to calculate polysaccharides composition. Since the lignin fractionates into acid insoluble material and acid soluble material, it was measured as acid soluble lignin with UV-Vis spectroscopy.

To conduct an experiment, 300 mg of the sample was weighed in duplicate. 3.00 ± 0.01 mL of 72% sulfuric acid was added to each pressure tube. Magnetic stirring rod was used to mix until the sample is thoroughly mixed. The pressure tubes were placed in a water bath set at 30 °C and incubate the sample for 60 min. The acid was diluted to a 4% concentration by adding 84.00 ± 0.04 mL deionized water using an automatic burette.

Set of sugar recovery standards was prepared by weighing out the required amounts of each sugar, to the nearest 0.1 mg, then add 10.0 mL deionized water and 348 µL of 72% sulfuric acid. Both the sealed samples and sugar recovery standards were autoclaved for one hour at 121°C. To prepare the sample for HPLC analysis, a small aliquot of samples liquor were passed through a 0.45 µm filter into an autosampler vials. The calibration standards and samples were analyzed by HPLC using a Biorad Aminex HPX-87H column equipped with the appropriate guard column.

For all the dissolution trials the same total batch volume was used for preparing the corn cob dopes. Maintaining the content of particles at a constant value of the total batch volume, the solvent concentration could be tuned using different molar ratios of salt and water. This procedure was repeated for all the dissolution trials conducted with different ratios of salt and water in the solvent system. The pH for the solvent was measured before and after pretreatment.

The flowchart that shows the outline of experiments as it is listed on the research objectives is shown in Figure 3.1.

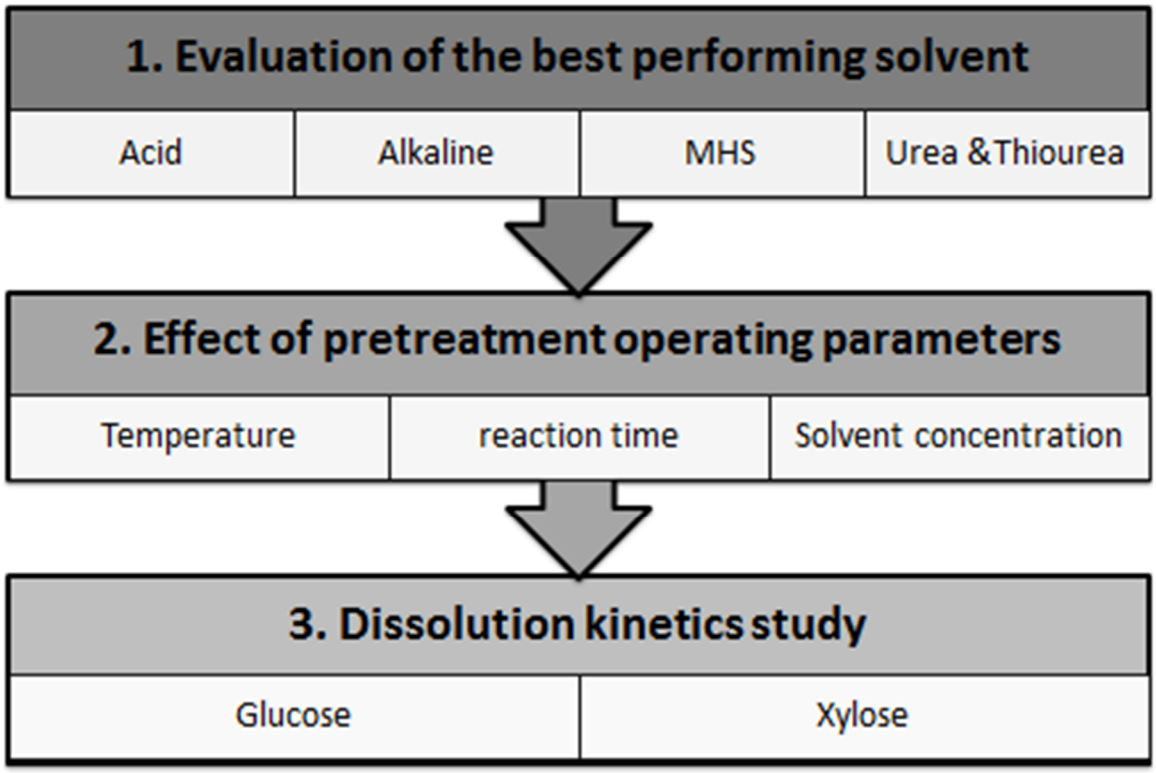


Figure 3.1: Methodology flowchart from research objectives

3.2.2. Evaluation of the best performing pretreatment solvent

To evaluate the best solvent, the same molarity was used for different solvents, see appendix A. Three concentration sets of solvents were prepared as 1.0, 2.0 and 2.5 M. The 10 grams of corncob particles were measured on a weighing scale, then added to the 100 ml solvent and the pre-treatment experiments were performed in a semi-closed flask on a magnetic stove-stirrer. The magnetic stirrer-stove used was equipped with temperature probe to measure & maintained operating temperature. The experiments were carried out for 3.5 hours at ~ 80°C for all MHS, Urea and Thiourea dissolution. After each experiment, the mixtures were separated using vacuum filter system in which the solid fractions and the filtrates were separated by 47 mm filter papers. The solid fractions were cleaned by extensive washing with deionized water until neutral pH. The sample was then washed with distilled water and dried 50 °C overnight, the material was kept for further characterization. Please note that the data for liquid fractions from pre-treatment which were analysed with HPLC are not reported since they were too low and undetectable by an instrument, refer to appendix B.

In acid pre-treatment, 0.1 M NaOH was used for neutralization of solid fractions. It should be noted that sulfuric acid pre-treated solid material was badly burned and could not be taken further to XRD and FTIR characterization. As a result only phosphoric and citric acid was reported for this part of the study.

Glycerol pre-treatment was additional step carried out to identify if it could be added as preliminary step before pre-treatment. Therefore the resulting fraction was only solid material which was taken through characterization and immediate conclusions were made. To do this, 100 g of corncob sample to be pre-treated was mixed with 10 ml of glycerol using a blender which was used to stir and to also blend the mixture.

For alkaline pre-treatment, 1.0 M HCl was used to neutralize the material. After pre-treatment the mixture was filtered, and the filtrates were pre-cooled in the salt-ice bath at 0°C to - 2°C for approximately 2 hours. Both the solid and liquid fractions were stored inside the refrigerator until analysis. The general dissolution procedure for all the different solvent systems was similar and essentially consisted of the following steps:

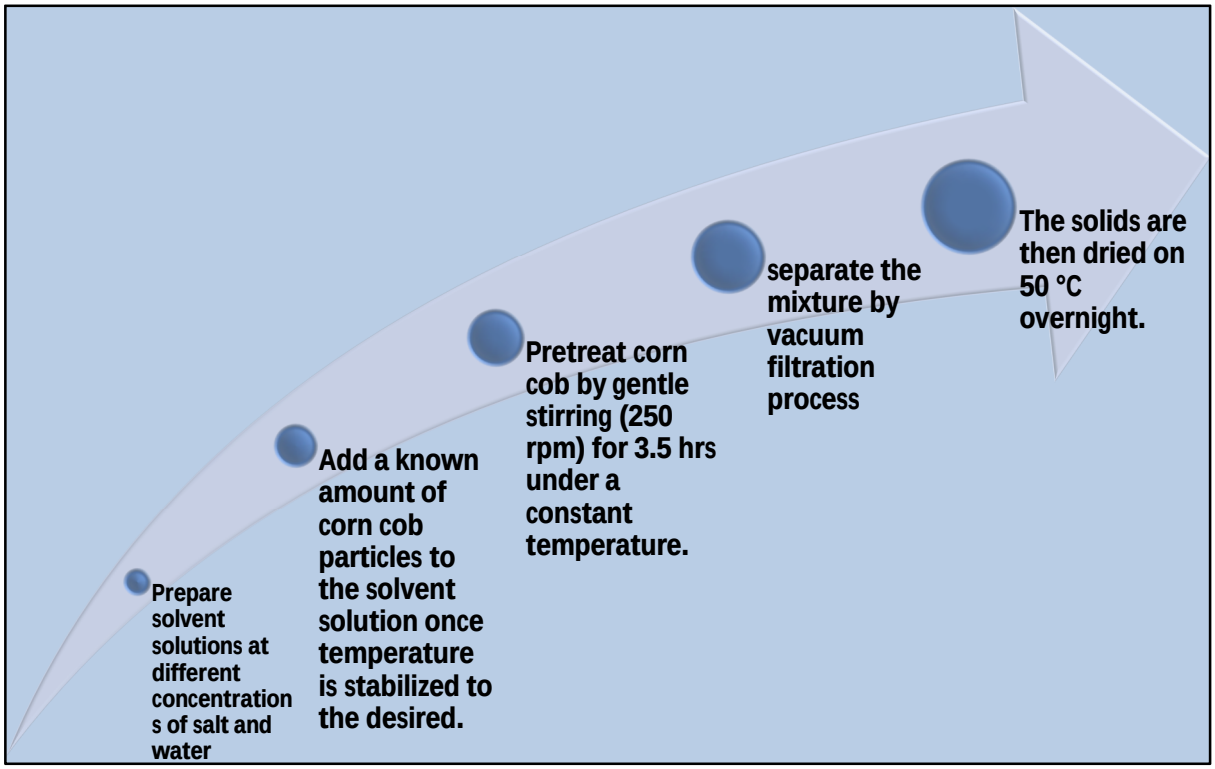


Figure 3.2: Dissolution procedure for all the different solvent systems

3.2.3. Effect of operating parameters using RSM

To evaluate the effect of operating parameters, the initial step was to identify and select the influential process parameters that have an impact on the output. The most influential independent variables, according to literature were identified as pre-treatment temperature, solvent concentration and residence time (Vargas *et al.*, 2016). These parameters were coded and used to determine the dependent variables glucose & xylose yield, which were the output variables.

Response surface methodology (RSM) was used as a technique for optimizing the pre-treatment conditions of biomass. A second order mathematical model was developed using RSM to predict the surface response in turning operation for known values temperature, reaction time and solvent concentration. These process parameters were optimized using genetic algorithm to obtain minimum surface response.

The experiments were conducted using 2^3 central composite designs with two star points and three replicates.The outputs of these experiments were converted into 3 variable contour plots to demonstrate the impact of the responses. An analysis of variance (ANOVA) was conducted on the data generated to determine the significance of the factors as linear, squared and interacting factors. MINITAB 17 was used to determine the linear, square and interaction coefficients for a quadratic equation.

Determination of the upper and lower limits for each parameter

A three-level, three-factor factorial central composite design (CCD) at the centre points leading to 16 runs was employed for pre-treatment experiments. A CCD design is one type of response surface design. It is a factorial design (2^k or 2^{k-p}) with 2k additional points. The variables were coded according to the following Equation (2) (Ayeni *et al.*, 2012; Dagnino *et al.*, 2013):

$$x_i = \frac{X_i - X_0}{\Delta X_i} \qquad i = 1, 2, \ldots \ldots \ldots k \tag{2}$$

Where x_i and X_i are the dimensionless and the actual values of the independent variable i, X_0 is the actual value of the independent variable at the centre point, and ΔX_i is the step change of X_i corresponding to a unit variation of the dimensionless value.

Table 2: Variables and experimental design levels for response surface

Variable (unit), Symbol	Range and level				
Code	-1.682	-1	0	1	1.682
Temperature (°C), X_1	25	70	115	160	180
Time (hours), X_2	0.0318	0.1	3.55	7	11.8
Solvent Concentration (%), X_3	0.15	0.5	2.75	5	8.4

Development of experimental design matrix and conducting of the experiments

The experiments were carried out as described in section 3.2.1. Corncob was dried, grounded and placed into different screw capped laboratory bottles. For optimization, it is recommended to evaluate over a large range of operating parameter which will then reinforce the validation & conclusion of the results. Thus the experiments were carried over a wide range of temperature, reaction time and concentration as shown in Table 3, as suggested on the literature. The reaction temperatures were operated at 25 - 180 °C. The temperature was kept within ±2 °C of the set point values with constant stirring of 220 rpm. The reaction times were operated between 0.032 hr (2 min) – 11.8 hrs (Satimanont et al., 2012). Effect of the concentration of the solvent was also determined at different values of pretreatment agent to water ratio for n= 0.15-8.4.

The pretreated residues were washed by distilled water, filtered, and then dried in an oven as before. Both the solid and liquid fractions were kept for further analysis. It should be noted that the solvent which was used for this part of experiment, was obtained from Section 3.2.2.

Development of the mathematical model

The generic quadratic model for investigating the influence of operating variables is expressed as Eq. (3):

$$y_i = b_0 + \sum b_i x_i + \sum b_{ii} x_i^2 + \sum b_{ij} x_i x_j \qquad i = 1, 2, \dots k; j = 1, 2, \dots \dots k, i \neq j \qquad (3)$$

Where y_i is the predicted response, b_0 is the interception coefficient, b_i is the linear regression coefficient, b_{ii} is the quadratic regression coefficient term, and b_{ij} is the interaction regression coefficient term.

The model was obtained from Minitab and it was used to obtain response surface and contour plots based on experimental data acquired from experiments using Response Surface Methodology. The next step was the analysis of the effect of input parameters on the response (sugar yield) using the plots. ANOVA was performed to estimate the statistical significance of the model.

Model validation

The proposed mathematical model was then validated by comparing the predicted roughness values with the observed values. The mathematical model was optimized to determine optimum values of input parameters to achieve maximum glucose and xylose yield using Response Optimizer tool in Minitab.

3.2.4. Dissolution kinetic study

The pretreatment reagent was dissolved in deionized water to prepare pretreating agent solutions. Corncob was mixed with the prepared solvent at ratio of 1:10 of corncob to solvent. It was then incubated on solvent for a maximum of 10 hours at stirring rate of 250 rpm. The samples were taken after every 10 minutes for the first hour and 1 hours until the end of the experiment. The samples obtained were filtered to separate the liquid and the solid fractions as shown in Figure 3.3. Please note that for glucose and xylose, the data for liquid fractions from pretreatment which were analyzed with HPLC are not reported since they were too low and undetectable by an instrument.

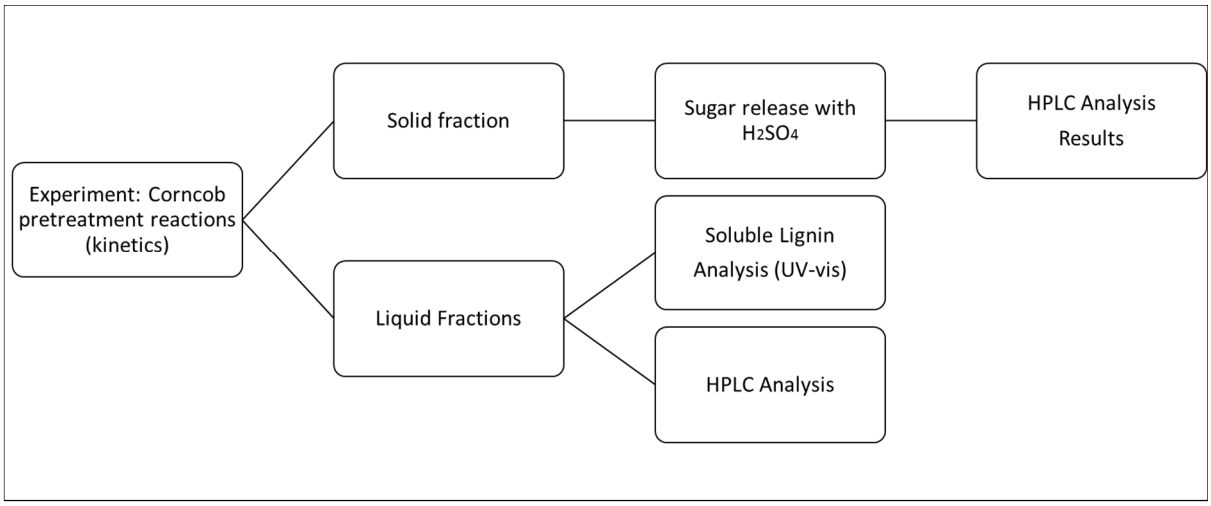


Figure 3.3: Flow diagram for kinetic methods analysis

Kinetic data for the pretreatment were evaluated at different temperatures; 120 °C, 140 °C, 160 °C 180 °C. The flasks were equipped with thermal jacket to make sure high temperatures were attained and maintained until the end of the experiment. The kinetic model for the corncob pretreatment as a function of temperature was determined. For each of these temperatures, the composition of pre-treated corncob was determined at different reaction times. The kinetics of pretreatment was studied using the amount of glucose, xylose and the lignin removed from the pre-treated solids. As it is illustrated in Figure 3.4, the glucose and xylose were analysed with HPLC and the soluble lignin was analysed with UV-vis.

The reaction was considered to be irreversible and to follow this reaction mechanism

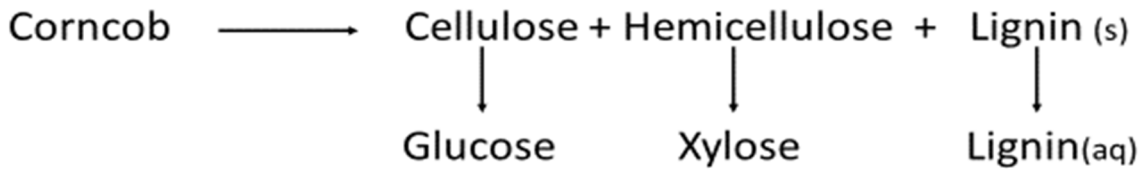


Figure 3.4: Dissolution and Hydrolysis mechanism

Such that the reaction rate, could be expressed as:

$$\frac{dC}{dt} = -kC \quad (4)$$

Where C is either glucose, xylose or lignin yield, k = chemical reaction rate constant, t is time Equation (4) was rearranged and integrated at ranges between C_i and C_f which are the initial and final concentrations of the sugars (xylose & glucose), initial & final dissolution times (t_i = 0 and t_f), respectively. As a result, Equation (5) is a linear relationship:

$$ln\left(\frac{C_i}{C_f}\right) = -kt + z \quad (5)$$

Plotting $ln\left(\frac{C_i}{C_f}\right)$ against reaction time, the rate constant (k) was obtained from the slope of the linear plot. The temperature dependence of the rate constant is characterized by the value of the energy of activation (Ayeni *et al.*, 2013). The energy of activation is the minimum energy that must be possessed by reacting molecules before the reaction can occur (Carrier *et al.*, 2016). Reaction constant is related to temperature by the Arrhenius law which can be written as:

$$k = A\,exp\left(-\frac{E_a}{RT}\right) \quad (6)$$

Where A = Arrhenius constant; E_a = activation energy; R = gas constant = 8.314 kJ/mole·K; T = absolute temperature (K). The activation energy (Ea) of dissolution was calculated from the logarithmic form of the Arrhenius equation:

$$ln\,k = ln\,A - Ea/RT \quad (7)$$

Plotting ln k against 1/T gives a linear plot in which the slope is equivalent to Ea/R. ln A was obtained from the y-intercept which was used to calculate the Arrhenius constant, A.

3.3. Analytical Methods

The pretreatment of lignocelluloses results in changes in the different properties of these materials. In a recent review the details of compositional, imaging and crystallinity analyses of lignocelluloses were reviewed and critically discussed. Changes in the cellulose degree of polymerization, accessibility, and enzyme adsorption/desorption by pretreatment are also among the effective parameters. This chapter deals with the measurement techniques in relation to bioconversions and this is summarized in Figure 3.5.

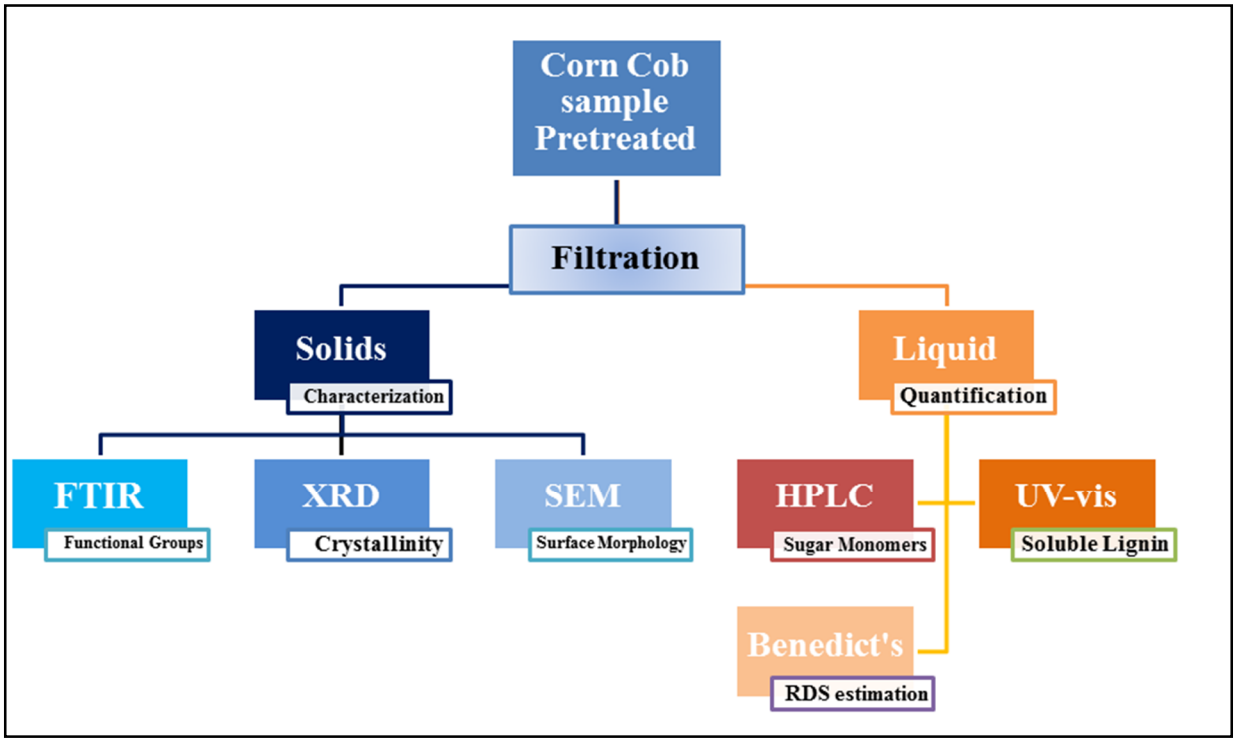


Figure 3.5: Schematic diagram for corncob methods of analysis

3.3.1. Quantitative analysis preparations

Following the National Renewable Energy Laboratory (NREL, 2008) procedure, 300 mg of the dried pretreated samples were weighed into labelled pressure tubes. 3 ml of 72% H_2SO_4 was added to each pressure tube and stirred for 2 minutes to ensure uniform hydrolysis. After 60 minutes of hydrolysis, samples were then diluted to a 4% acid concentration by adding 84 ml of deionized water. The same set of sugar recovery standards (SRS), D-(+) glucose and D-(+) xylose used during the liquid samples analysis were used in this procedure. The prepared samples together with the SRS were sterilized in the autoclave for 1hour at 121 °C.

Calibration Verification (CV) standards, i.e. glucose and xylose, were prepared in order to verify the quality and stability of the calibration curves. To prepare the samples for the HPLC analysis, they were filtered through 0.45 µm syringe filter into an auto sampler vial. Each sample prepared was duplicated, sealed and labelled on the vial. The CVs and samples were analyzed using the carbohydrate and the fermentation columns in order to compare the results. The mobile phase used for the carbohydrate column was 70% acetonitrile and dilute H_2SO_4 solution was used as the mobile phase for the fermentation column.

3.3.2. Liquid fraction Analysis: HPLC

Monomeric sugars (glucose and xylose) were analyzed with High Performance Liquid Chromatography (HPLC) system (model 2695). HPLC system equipped with a refractive index detector (Model 6040 XR, Spectra-Physics, USA) was used to analyze glucose, xylose, and arabinose. An organic acid column (Aminex HPX- 87H column, Bio-Rad Lab, USA) was applied with 0.005 M sulfuric acid solution as a mobile phase at the total flow rate of 0.6 ml/min and the column temperature was maintained at 60 °C. HPLC (Agilent Technologies-1200 Series) was used to analyse the presence and concentration of sugars in the hydrolysate, to measure the ethanol produced and monitor the periodic utilisation of fermentable sugars, and finally to measure the concentration of the fermentation product and distillate.

The samples were filtered by passing through the Whatman's filter paper (45 µm) to remove the suspended solid particles from the hydrolyzate. Stock solution was prepared by dissolving 0.1 g glucose in 100 ml distilled water. Dilution of different concentration 100 µg/ml, 200 µg/ml, 400 µg/ml and 800 µg/ml were made by pipetting out this 0.1 % stock solution into 5 different test tubes. Final volume to each test tube was made up to 10 ml with the addition of distilled water. The concentration of monomeric sugars such as glucose (µg/ml) was plotted against respective absorbance values at 540 nm to get standard curve for glucose

3.3.3. UV-Vis Analysis for solubilized Lignin

To analyze the sample for solubilized lignin, UV-Visible spectrophotometer was used. The instrument was set to pick up absorbance at 205 nm wavelength. Diluted sulfuric acid was used to run a background (blank) for absorbance correctness. The sample was diluted with deionized water to bring the absorbance into the range of 0.1 – 1.0. The sample were analyze in duplicate to ensure a replication of 0.05 on absorbance units. The amount of acid soluble lignin was calculated using Beer Lambert's Law which states that the absorbance of an analyte is proportional to its concentration and the path length through the sample (Lee *et al.*, 2013)

3.3.4. Fourier Transform Infrared (FTIR) Spectroscopy

Lignocellulosic feedstocks, cellulose-rich fractions and hemicelluloses were ground into fine powders and dried in the oven overnight. The samples were structurally characterised on Thermo Nicolet Nexus 870 Fourier-Transformer Infrared (FT-IR) spectroscopy (FT-IR) device with the Golden Gate ATR measuring system.

Surface chemical analysis was conducted to determine changes of functional groups in pre-treated biomass. Prior to analysis, each sample was homogenized by vortexing and pressed against the diamond surface by an anvil. Spectra were obtained using the triangular apodization in resolution of 4 cm^{-1} with 30 two scans for each sample from 4000 to 600 cm^{-1}. The absorbance frequency range of FT-IR considered was 4000-400 cm^{-1}. For each run 64 scans were run on background and sample, and the spectra were recorded in the Omnic ® 7 software. Similar absorbance frequency range of FT-IR as with solid state was used and similar number of scans.

3.3.5. X-ray Diffraction (XRD) Analysis

Cellulose Crystallinity of is one of the main factors influencing enzymatic hydrolysis. Cellulose crystallinity has long been thought to play an important role in enzymatic hydrolysis. The concept that cellulose structure is divided into two regions, an amorphous region that is easy for enzymes to digest and a crystalline region that is difficult to digest, is extremely appealing The pre-treated whole biomass slurries, including hydrolysate and solid residues, and Avicel slurries, and untreated biomass and Avicel were analyzed by XRD. Following the pretreatment, the samples were freeze-dried, and then stored at room temperature prior to XRD analysis. The samples were measured using a PanAlytical X'Pert

MPD powder diffractometer with a vertical θ−θ goniometer (190 mm radius) and post-diffraction monochromator. The X-ray source was a ceramic X-ray tube with Cu anode operated at 40 kV and 50 mA (2.0 kW). X-ray diffraction patterns of freeze-dried whole slurries were recorded at room temperature.

The X-ray diffraction (XRD) measurement was conducted by a D8 ADVANCE diffractometer (Bruker, German) equipped with a Cu K ceramic X-ray tube. Copper radiation with a wavelength of 0.15406 nm was generated at 40 kV voltage value and 40 mA current values. Scans scope was from 5◦ to 60◦ with step size of 0.03 at 4 s per step.

The crystalline indices of cellulose samples were calculated from the X-ray diffraction patterns by the following equation (8):

$$C_rI = \frac{I_{002} - I_{amp}}{I_{002}} \times 100\% \quad (8)$$

I_{002} is the crystalline region intensity for the crystalline portion of biomass (i.e., cellulose) at about 2θ = 22.5° and I amorphous was the peak for the amorphous portion (i.e., cellulose, hemicellulose, and lignin) at about 2θ = 18.3° - 19°.

3.3.6. Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) is a high resolution imaging system with an extraordinary depth of field. It indicates topographical, structural, and elemental data at low magnifications up to 200,000x (Chandra *et al.*, 2008). SEM in this study was used for surface morphological view of the material to establish if the corncob material is disrupted enough to increase the accessibility of cellulose & hemicellulose due to the pre-treatment processes.

The dried samples were mounted on the stubs using a carbon tape before being coated with Gold-Palladium. The samples were characterised using a field emission scanning electron microscopy (FE-SEM) (Carl-Ziess Sigma, Germany) operating with accelerating voltage of 15 kV. The micrographs were generated at different magnifications (250–1000x) by a computer program. The data was collected over a selected area of the surface of the sample and a two-dimensional image was generated that displayed spatial variations in properties.

CHAPTER 4: Results & Discussion

4.1. Characterization of Corncob Biomass

4.1.1. Cellulose Crystallinity Index

X-ray diffraction (XRD) was used to comprehend the interactions of cellulose presence in the corncob pre-treated with all specified aqueous solvents. The characteristic XRD pattern generated provide a unique "identification" of the cellulose crystals in each sample (Zhang et al, 2013). With an aid of this XRD pattern, identification of changes and quantification in the crystallinity index (CrI) of the corncob before and after the pre-treatment step were obtained. Since the experiments were designed in such a way that it accommodate for all characterisation process the triplicates samples were used to validate the accuracy of the method. Further instrument verification on the stability was obtained by creating two sets of scans of the brass interference ($2\Theta = 42.2°$) on each of two different days & compare the outcome (Zhang et al, 2013).

The proposed method for empirical measurements to allow rapid comparison of cellulose samples was the XRD peak-height method. This method makes use of the two heights measurement in the X-ray diffractogram produced to accurately measure the crystallinity of cellulose (Li et al, 2015). CrI can be calculated from the ratio of the height of the 002 peak (I_{002}) and the height of the minimum (I_{am}) (Park et al., 2010). The XRD peak-height method is widely used due to the fact that it is rapid, user friendly and very simple to understand so as to interpret the data obtained. It is highly recommended for comparing the relative differences between samples i.e. before and after pre-treatment. However the measurements obtained are limited to a qualitative & semi-quantitative evaluation of the amounts of amorphous and crystalline cellulosic components in a sample (Charilaos, 2012).

It is difficult to extract the absolute value of cellulose CrI of biomass samples from the 1-D powder data without additional aid from computer simulation. For further quantitative CrI analysis, the peak deconvolution method was used to help in validating the calculations obtained in the peak-height method. The peak deconvolution method requires software to separate amorphous and crystalline contributions to the diffraction spectrum using a curve-fitting process (Zhang et al, 2015). Which in this case was the built-in Gaussian function in Origin Pro 8 to fit peaks as it is elaborated in Zhang et al., 2015.

The XRD curves of corncob samples are given along with the crystallinity index. The crystallinity indices for both crystalline and amorphous peaks were calculated using the peak-height empirical method as in Equation (8), and the indices are presented in Table 4. Lignocellulosic biomass crystallinity index (CrI) is defined as the mass fraction of crystalline cellulose in the biomass (Chunxiao et al, 2016), and it can be calculated from the X-ray diffraction patterns by:

$$C_rI = \frac{I_{002} - I_{amp}}{I_{002}} \times 100\% \quad (8)$$

As mentioned before, CrI was calculated from the ratio of the height of the 002 peak (I_{002}) and the height of the minimum (I_{am}). These are the intensity read from XRD pattern corresponding to the crystalline peak, I_{002} at maximum 2θ angle is between 22° & 23°, whereas the intensity of the amorphous materials I_{am} at 2θ angle is between 18°& 19°.

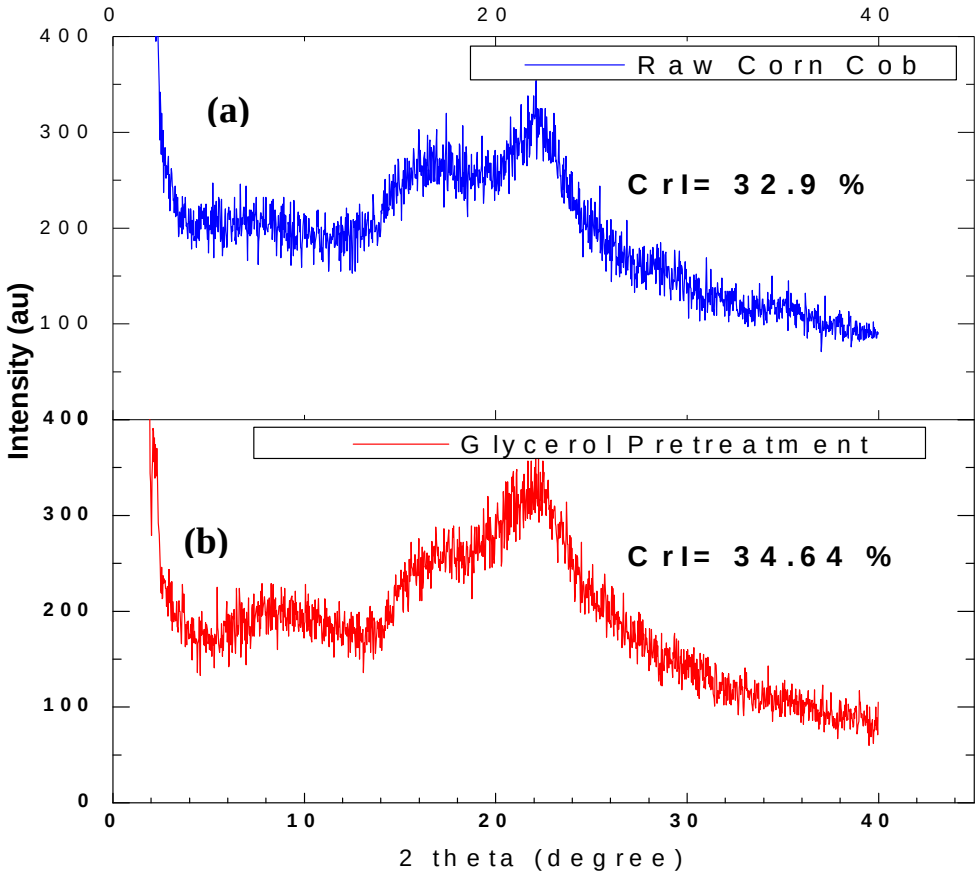


Figure 4.1: The X-ray diffractograms of the corncob before and after glycerol blending (a) raw corncob (b) Glycerol blended corncob

For glycerol pre-treatment, it was expected to see a change in corncob structural composition since it has been reported that blending of the lignocellulosic biomass with glycerol ($C_3H_8O_3$) reduces the H-bonding intermolecular forces, (Nur Ami et al, 2015). Glycerol interacts with component molecules and replaces the hydrogen bonding within molecules, the crystallization is interrupted and the intensity of the hydrogen bonding is reduced in the way.

However, the hydrogen bonding is very complex as shown in Figure 4.1 and there is no specific analytical instrumentation to quantify the changes. The idea was that the XRD analysis of the glycerol blended corncob sample will give a good examination of chemical changes which will lead to an evaluation of biomass H-bonding properties. If this is so, it will be highly recommended to blend the corncob before pre-treatment. The results revealed that glycerol blending did not change the structure of the biomass, let alone the crystalline unit cell as all the peak shapes were identical.

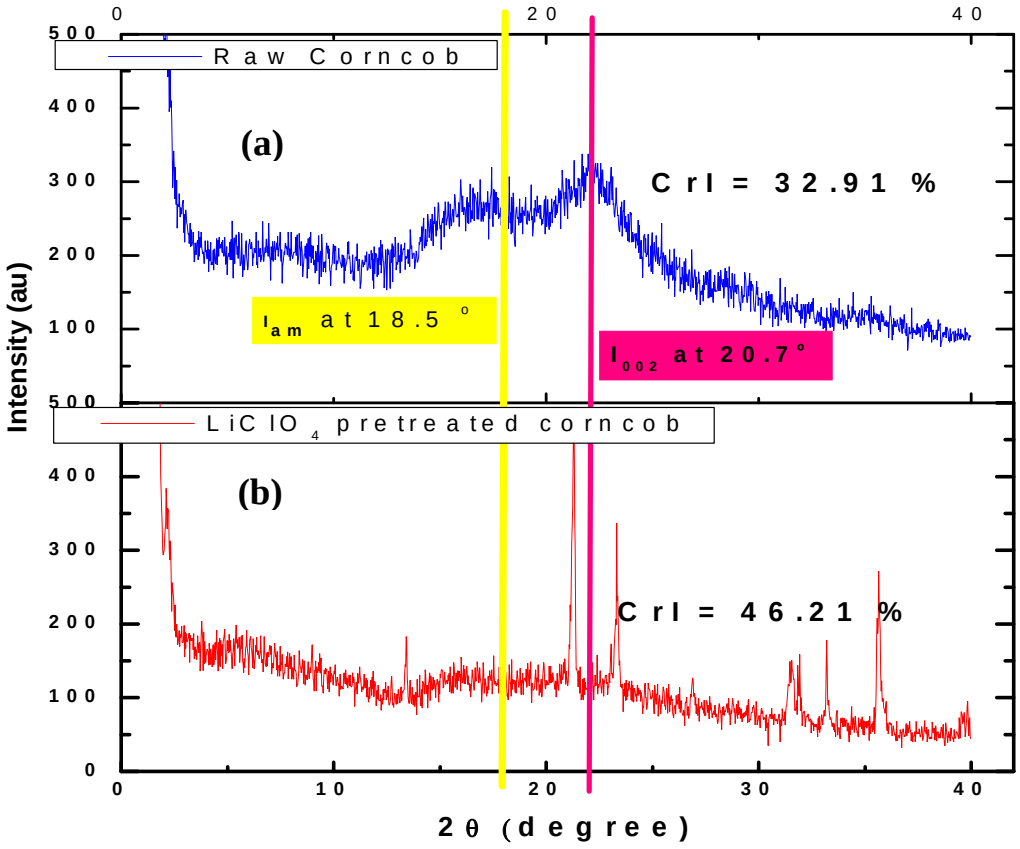


Figure 4.2: The X-ray diffractograms of the corncob before and after lithium perchlorate pretreatment (a) raw corncob (b) Lithium perchlorate pretreated corn cob

The X-ray diffractograms of the corncob before and after lithium perchlorate ($LiClO_4$) pre-treatment are given in Figures 4.2. The results revealed that pre-treatment with $LiClO_4$ changed the structure of the corncob crystalline region since the peak shapes are not identical. According to Roncero et al. (2005), the intensity of the crystalline peak at maximum 2θ angle is between 22° and 23°, whereas the intensity of the amorphous materials at 2θ angle is between 18° and 19°. It can be observed from the diffractograms of raw corncob that there are two peaks appearing in the native sample at $2\theta \approx 22°$ and at $2\theta \approx 18°$, which is suggestive of the corncob samples' crystallinity.

Many studies reports that pre-treatment of lignocellulosic biomass result in the reduction of the cellulose crystallinity in the crystalline region. The XRD data is obtained from the solid residue, thus the CrI should increase after pre-treatment due to the removal of amorphous hemicellulose. It should be noted that cellulose CrI is obtained by dividing biomass crystallinity with cellulose content in the biomass, which reflects real changes in crystalline during pre-treatment. Consequently this modification in cellulose crystallinity structure enhances the enzymatic hydrolysis (Haldar Sen and Gayen, 2016).

The raw corncob has a biomass CrI of 32.91%, which increases to 46.21% for $LiClO_4$ pre-treated samples. This indicates the high possibility of lithium perchlorate pre-treatment disrupting the native cellulose crystalline structure (Haldar et al., 2016). More work is needed to understand the mechanism of cellulose decrystallization during $LiClO_4$ pre-treatment.

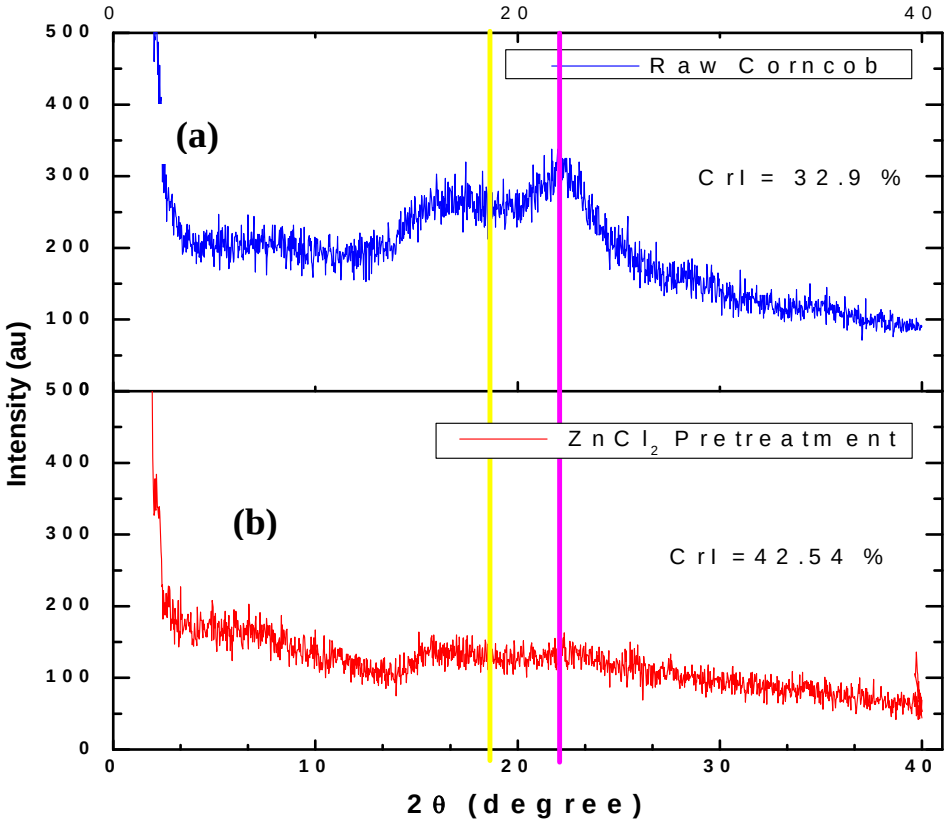


Figure 4.3: The X-ray diffractograms of the corncob before and after zinc chloride pretreatment (a) raw corncob (b) zinc chloride pretreated corn cob

In the same way as Figure 4.2, Figure 4.3 shows XRD patterns of the corncob particles at 2θ peaks which demonstrate information of both I_{am} and I_{002} structures. The peak at ~18° corresponds to the diffraction pattern of the amorphous whereas the peak at ~21° is representing the crystalline structure. However, due to the overlapping signals at the amorphous peaks the CrI calculations was extracted from the Excel data of XRD results. The peak at 22° disappeared after $ZnCl_2$ pre-treatment, indicating a noticeable modification in the chemical structure of corncob.

For the corncob treated by zinc chloride, diffraction angles around 14.9 and 22.6º decreased much, this indicated that an amorphous structure with great change. During the process, the hemicellulose and other materials in the corncob were removed, the relative content of cellulose increased. So the crystalline polymorphism was not destroyed, however the crystalline structure of corncob was transformed into amorphous after treatment by zinc chloride. (Zheng et al, 2013)

The $ZnCl_2$ pre-treated corncob has a biomass CrI increased to 42.54% for samples. Just as the $LiClO_4$ pre-treatment, this indicates the high possibility of $ZnCl_2$ pre-treatment disrupting the native cellulose crystalline structure. Crystallinity decreased for continued degradation both in crystalline and non-crystalline regions. Increased numbers of chain scission reactions increased the amorphous character of cellulose, which reduced the total amount of cellulose crystalline regions (Zheng et al, 2013). Furthermore, hemicellulose hydrolyzed and formed acetic acid, which plays a role in the degradation of micro-fibril (Sribala et al, 2016). Additionally, no free hydroxyl groups were involved in the reaction to generate hydrogen bonds.

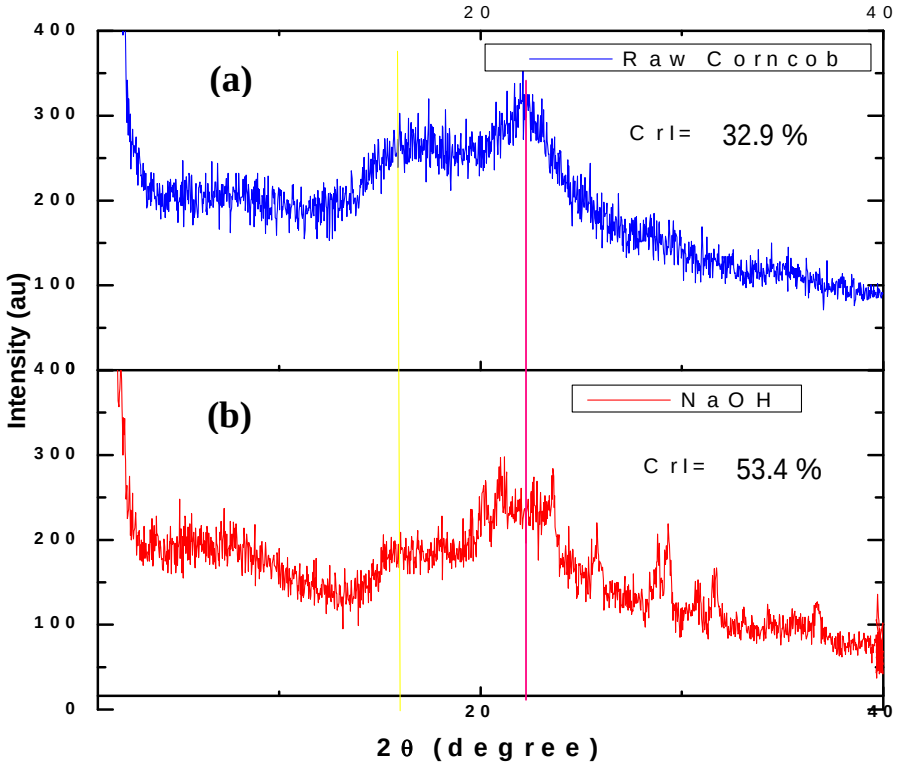


Figure 4.4: The X-ray diffractograms of the corncob before and after sodium hydroxide pretreatment (a) raw corncob (b) NaOH pretreated corn cob

After pre-treatment with NaOH 1% for 4.5 hours, the peak declined in crystallinity index, Figure 4.4. This is due to reaction with NaOH which then altered the conformation and morphology of cellulose fibres. Wyman et al, 2014 reports that during the alkaline pre-treatment process, the cellulose chain expands due to diffusion of base into the crystalline cellulose. Furthermore, the cellulose chain will undergo re-arrangements that result in damage to the structure of crystalline cellulose. Damage in the crystalline structure to non-

crystalline cellulose led to an increase amorphous cellulose, so that the crystallinity index decreased (Zhang, 2015).

The cellulose crystallinity value of untreated sample was 32.94 % while the pre-treated sample was up to 53.41 %. The crystallinity index in pre-treated corncobs with NaOH increased due to lignin and hemicellulose removal, which was good evidence that the amorphous portion of the corncobs was removed with the liquid fractions and the crystalline portion remained on the solids residue (Boonsombuti, Luengnaruemitchai and Wongkasemjit, 2013)

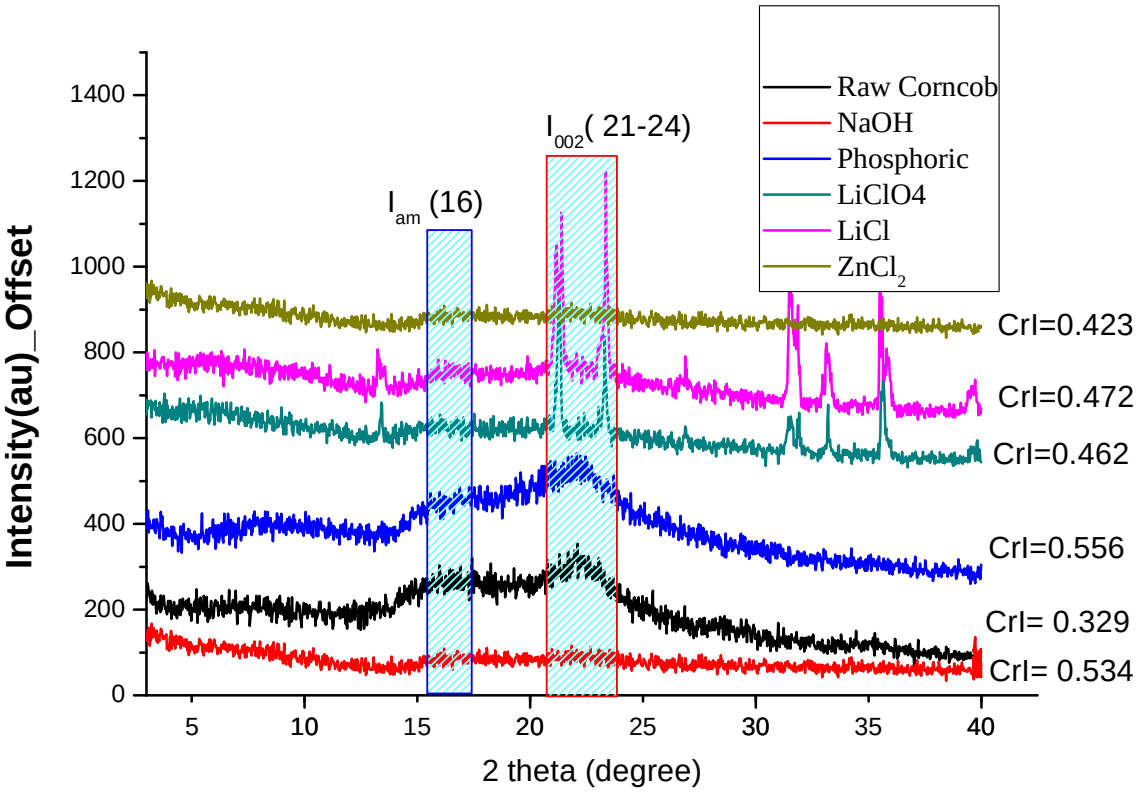


Figure 4.5: The X-ray diffractograms of the corncob before and after aqueous pretreatment.

The corncob samples displayed an XRD pattern with a secondary peak between 15.7° and 17.1° scattering angles, and a primary peak at 21.5-24° corresponding to cellulose (French, 2014). The effectiveness of pretreatment technique was examined through the changes in crystallinity of the cellulose, which was calculated from the maximum diffraction intensity at primary peak position in the XRD patterns (Figure 4.5). A change in pattern among

samples was observed, which was remarkably at this peak. The primary peak is defined as the crystalline cellulose structure (Sribala et al, 2016).

The calculated crystallinity index, shown in Figure 4.5 exhibits a significant increase in pretreated samples when compared to that of the untreated sample. The crystallinity of untreated corncob is 0.329 lower than that of other lignocellulosic biomass such as sugarcane bagasse, CrI= 0.5 or rice straw 0.57 (Boonsombuti, Luengnaruemitchai and Wongkasemjit, 2013) which implies that native corncob is a highly amorphous structure and it is necessary to treat it prior to enzymatic saccharification.

However, dilute phosphoric acid pretreatment significantly enhanced the CrI of the corncobs compared to the untreated corncob. The high CrI observed indicates an increase in hemicellulose removal and a high cellulose fraction, which would increase accessibility and probability that the cellulose would be hydrolysed in the liquid fraction. A small difference in CrI between NaOH and H_3PO_4 indicates a similar result in structural alternation. It should be noted that small changes in CrI from the calculation of peak height may not correlate to cellulose digestibility. This is because the presence of inhibitors in the resulting solution my hinder the cellulose digestibility by enzymes (Boonsombuti, Luengnaruemitchai and Wongkasemjit, 2013)

Compared to the lithium chloride pre-treated corncobs with CrI of 0.472, lithium perchlorate pretreatment yielded 0.462, of which is close suggesting that the cellulose content in both solutions should be roughly similar. The XRD pattern crystal structure of reaction residue after both pretreatment gained a sharper diffraction peak than raw material, which suggested that crystalline structure of cellulose remained excellently. An increase in crystallinity index from 0.329 for the original material to 0.472-LiCl & 0.462-$LiClO_4$ was observed, which indicated that the cellulosic crystal structure become neater after the removal of lignin and some cellulose existed in amorphous regions.

The XRD results in this study suggest that the increase of CrI is caused by progressively increased due to release of amorphous cellulose from the corncob residues by the pretreatment solvent. The pretreated cellulose transformed into a disordered structure upon displacement of solvent's molecules, leading to a drop in the biomass CrI (Zhang et al, 2013).

Celluloses having a high amorphous content are usually more easily digested by enzymes; it is unclear, based on studies published in the literature, whether CrI actually provides a clear indication of the digestibility of a cellulose sample. Cellulose accessibility should be affected

by crystallinity, but is also likely to be affected by several other parameters, such as lignin/hemicellulose contents and distribution, porosity, and particle size.

It can be concluded that the CrI is an important tool to predict the sugar yield after enzymatic hydrolysis due to its cellulose content exposure by XRD, however it cannot be used as the only tool to deduce that hence the HPLC analysis was used to support or disapprove this assumption.

4.1.2. Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectroscopy is a powerful tool for studying the chemical and conformational properties of polysaccharides. In addition to XRD studies, FTIR spectroscopy makes it possible to solve the problems of identification of polysaccharides, to confirm their purity, to carry out semi-quantitative functional analyses, to determine structure and to investigate complexing & intermolecular interactions (Kumar, 2011). Based on previous analysis of lignocellulosic feedstocks, it was anticipated that the choice of corncob would result in aliphatic carboxylic acids, furans and the complete spectrum of ketone, aldehyde, and acidic degradation products derived from all three lignin monomers being present in the sample (Chen, 2006). Indeed, the raw corncob spectroscopy is as it is predicted, as presented in Figure 4.6 along with corncob samples pretreated by a wide variety of compounds.

The main peaks of FTIR spectra of the raw material and aqueous pre-treated corncob particles can be seen in Figure 4.6. A broad absorption band in the range of 3120-3624 cm^{-1} can be attributed to the -OH stretching associated to polar groups linked through intra and intermolecular hydrogen bonding (Kumar, 2011). Reduction in the absorption intensity of this peak indicated that hydrogen bonds in cellulose were disrupted after pretreatment (Lijuan, 2015). These changes are more noticeable for H_3PO_4 acid and NaOH pretreated corncob sample. It is apparent that these aqueous pretreatment methods effectively break the hydrogen-bonding network in cellulose as the OH-stretching vibration region at 3200−3500 cm^{-1} is considerably broader (Schwanninger, 2004). In addition, other functional groups that represent strong hydrogen-bonding networks in cellulose also disappear, such as 1112 cm^{-1} for ring asymmetric stretching on H_3PO_4 pretreated sample and 1420 cm^{-1} for CH_2 bending vibrations for NaOH and LiCl (Dawei et al., 2013).

Lignins show a broad band at 3010–3460 cm^{-1}, attributed to the hydroxyl groups in phenolic and aliphatic structures, and the bands centred around 3200 cm^{-1}, predominantly arising from CH stretching in aromatic methoxyl groups and in methyl and methylene groups of side

chains (Fitigao, 2013). Raw corncob, urea and zinc chloride pretreated corncobs show strong bands in this region, with peaks at 3217 and 3347 cm^{-1}, arising from CH stretching in aliphatic methylene group that can originate from fatty acids present in the lignin preparations (Boeriu, 2004).

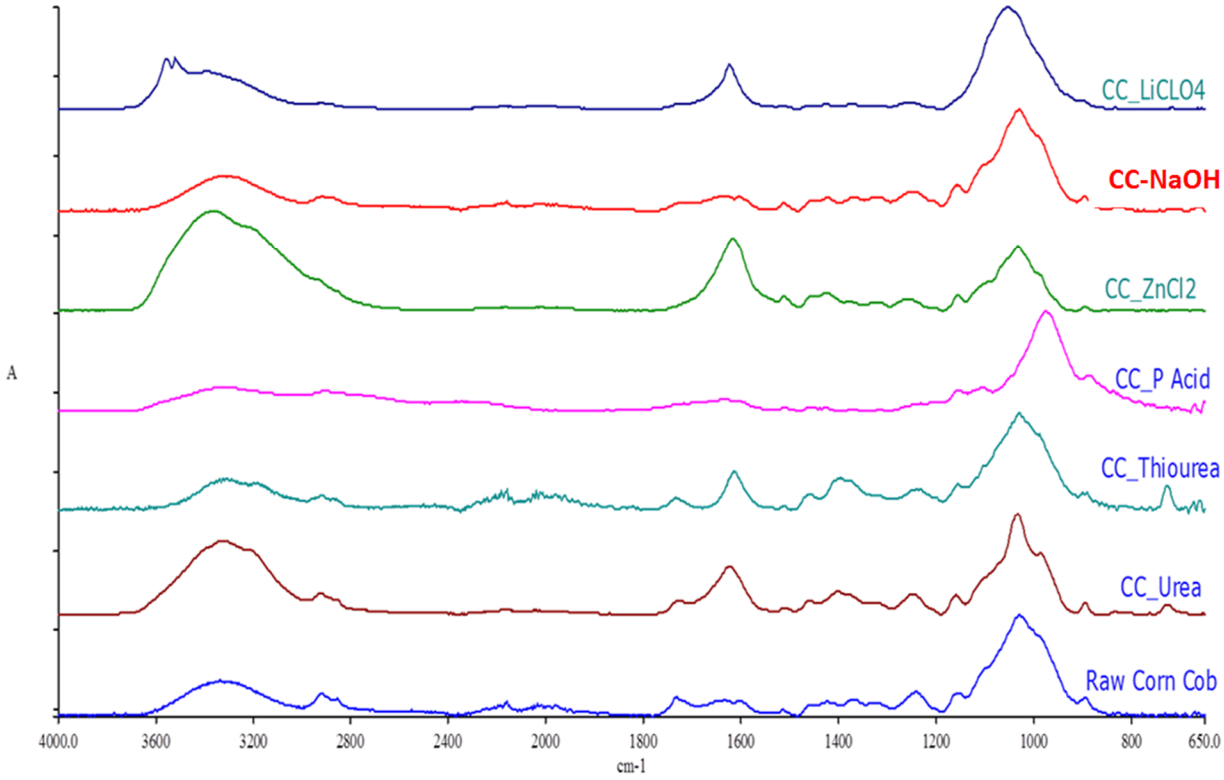


Figure 4.6: FTIR spectrum of raw corncob and aqueous pretreated corncob samples. For better visualization, the spectra were normalized.

Band around 1635 cm^{-1} and 1250 cm^{-1} in pretreated corncobs are mainly ascribed to the stretching vibration of the unconjugated C=O and C–O bonds of the acetyl ester units in xylan or hemicellulose (Li et al, 2015). The existence suggests that the pretreatment step could not break down the hemicellulose in the studied corncob i.e. $ZnCl_2$, $LiClO_4$, Urea and Thiourea. The decrease in the intensity of these bands was indicative of the dissolution of hemicelluloses from corncob and the deacetylation of hemicelluloses i.e. H_3PO_4, LiCl, NaOH. The analysis of FTIR data also shows that the pre-treated particles has most significant absorption peak at 1634 cm^{-1} which relates to C=O stretching of carboxylic group (Kumar, 2011) which has disappeared in NaOH, phosphoric acid and lithium chloride pre-treated corncob particles. The disappearance of peak in the sample indicates that the acetyl group of acetylated xylan (i.e. hemicelluloses) was removed almost completely.

The prominent peaks at 1046-1036 cm^{-1} are attributed to the C-C, C-O stretching vibration (Kumar, 2011) and C–OH bending vibration in both treated and untreated corncobs. A sharp band in this range, which is typically a β-glycosidic linkage between the sugar units in hemicelluloses, was detected in the region but this band shifted in H_3PO_4 treated corn cob particles, which may be suggestive of increased crystallinity in the solids residues. This confirms the XRD CrI results which deduce that the crystallinity of H_3PO_4 pretreated corncobs is increased for the native corncob sample.

The symmetric C–H stretching vibration band is found at 2900- 2913 cm^{-1} owing to CH_2 and CH_3 group (Chen, 2006). This band is observed on the original untreated biomass, Urea, Thiourea, LiCl and $ZnCl_2$ pretreated corncob samples. Furthermore on these samples, there is a band in the range 1160-1167 cm^{-1} which is a characteristic of glycosidic groups and attributed to C-O, C-O-C, stretching and C-OH bending vibration in arabinoxylan structure (Kumar, 2011).

The increase in the intensity of C-O-C ester stretching band at 1167 cm^{-1} i.e. $LiClO_4$ pretreated corncob is a result of the esterification (Clave, 2004). Because corncobs are natural products consisting primarily of cellulose, hemicelluloses, and lignin, many reactive hydroxyl sites are available to form covalent bonding with various chemical reagents. Therefore, it is possible for esterification to occur by introducing a low pH molten hydrate salt to corncobs at 70 °C.

It is common for the spectra of lignin samples to result in weak bands at 1370–1375 cm^{-1} originating from phenolic OH (Boeriu, 2004). A slight increase is noticed in thiourea treated samples and this band completely disappeared in NaOH treated sample. In addition, the samples appears to have similar changes in aliphatic C-H in methyl groups and a strong vibration at 1215–1220 cm^{-1} that can be associated with phenolic compounds because of the C-C plus C-O plus C=O stretching. The aromatic C-H deformation at 1035 cm^{-1} appears as a complex vibration associated with the C-O, C-C stretching and C-OH bending in polysaccharides.

The FTIR band shifted to higher frequency (1167 cm^{-1}) for most pretreatment method, confirming that crystalline content increase in comparison to original sample. In addition, a sharp absorption peak appeared at 1115 cm^{-1} in the material is further indicative of high cellulosic content. A sharp band at 1638-1642 cm^{-1} was also detected and attributed to H-O-H stretching, which occurs mainly in the amorphous state, and crystalline spectra measured in

KBr which belongs to the absorbed water molecules associated with the cellulosic fibers (Kumar, 2011). The band at 1430 cm^{-1}, assigned to H-CH and -OCH in-plane bending vibrations in both crystalline and amorphous cellulose. In addition, an absorption band near 1375 cm^{-1} is detected owing to the C-H bending vibration present in cellulose and hemicellulose chemical structures (Wyman, 2013). These peaks match well with the spectra of cellulose from literature.

4.1.3. Comparison of the XRD Crystallinity Index and FTIR Absorbance Index

The peak area ratio between ~897 and ~1210 cm^{-1} on the FTIR curves is proportional to the ratio of the amount of amorphous cellulose and hemicellulose to that of lignin (Gaur et al., 2015; Zhang et al., 2015). This ratio can be calculated using Equation (9):

$$\text{FTIR AI} = \frac{A_{897}}{A_{1210}} \left[\frac{cm^{-1}}{cm^{-1}}\right] \quad (9)$$

The XRD CrI listed was calculated using, Equation (8). As shown in Table 4, the ratios obtained by two different methods are consistent with each other in terms of their variations with pretreatment conditions. This proves that the measured data of biomass compositions and cellulose crystallinity is fairly reliable. The values of the ratios cannot be compared since both XRD and FTIR gives qualitative information of cellulose and the crystallinity index are obtained in a complete different manner.

Table 3: Comparison of the XRD Crystallinity Index and FTIR Absorbance Index

Sample ID	XRD CrI	FTIR AI
Raw corncob	0.329	0.028
$LiClO_4$	0.462	0.304
$ZnCl_2$	0.423	0.932
H_3PO_4	0.556	0.526
NaOH	0.534	0.348
LiCl	0.472	0.271
Thiourea	0.394	0.121

4.1.4. Scanning electron microscopy: Surface Morphology of corncob

To better understand the significant enhancement of enzymatic digestibility of corncob after pretreatment, the morphological evidence of the untreated and pretreated samples was investigated by scanning electron microscopy (SEM) as presented in Figure 4.7. This figure demonstrates the clusters of corncob particles in different conditions with varying structures. The SEM image of untreated corncob samples in Figure 4.7 (A) exhibited rigid, compact fibrillary morphology with thick-walled fibre cells and fibres constituted by parallel stripes, limiting the cellulose accessibility shows a uniform cell wall surface. These cellulosic components are responsible for the cohesive forces within the cell wall that entail structural support to plants (Andry, 2015).

After the pretreatment with alkaline (1 % NaOH) at 70°C for 3.5 hrs, the structure was damaged, looked soft and the pores appeared, Figure 4.7 (B). Additionally, the cell structure size turn out to be bigger owing to the swelling mechanism of cellulose during alkaline pretreatment that will be discussed later in this chapter. The pretreated samples presented a more disorganized morphology characterized by the separation and greater exposure of fibres as well as loosening of the fibrous network, which may be due to the solubilisation of cell wall components (Li et al, 2015)

When the dilute acid (1% Phosphoric acid, 70°C for 3.5 hrs.) was used as pretreatment media, the structure became more porous, Figure 4.7 (C). The pH of the pretreatment media certainly has an effect on the external surface area of the corncob particles. At low pH the surface area of corncobs had many micropores which were favourable for cellulase to hydrolyze the cellulose into reducing sugar (Boonsombuti, Luengnaruemitchai and Wongkasemjit, 2013). It also indicated that acid pretreatment promoted the removal of lignin. The bundle structure of cellulose was perceived to be damaged, because of the exposure of cellulose in harsh reaction solvent. The results corresponded to those of the XRD measurement.

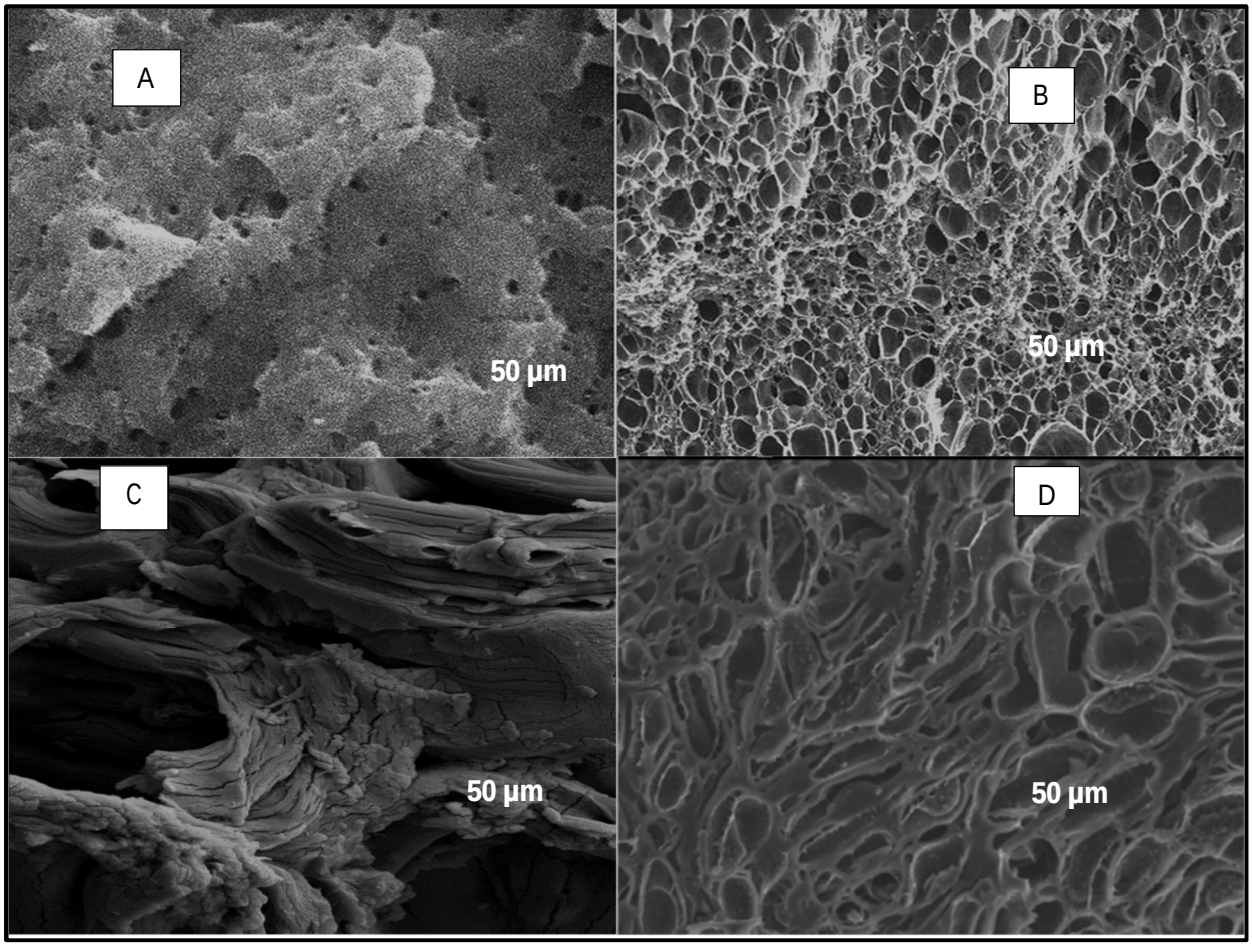


Figure 4.7: Scanning electron microscopy micrographs revealing the internal structure of corncob particles. (a) A section of untreated corncob (b) An alkaline (NaOH) pretreated corncob sample, (c) Dilute acid pretreated corncob (d) Molten hydrate salts pretreated corncob. Scale bars 50 µm.

The molten hydrate salts pretreated corncob had a rougher surface and more porous than raw corncob; refer to Figure 4.7 (D). These kinds of cracks are essential for enzymatic hydrolysis of cellulose because they can increase surface area and porosity of lignocellulosic biomass (Jiang, 2014). The particle sizes of the pretreated samples are greatly reduced when compared with the raw corncob, which is desirable since it increases the cellulose accessibility. However agglomerates are also formed in the $LiClO_4 \cdot H_2O$ pretreated sample because the hydrogen-bonding network in amorphous cellulose seems to promote the agglomeration of small particles. Compared to the smooth surface of raw material, the bundle structure of cellulose was still remained after treatment, which proved that the crystalline structure of cellulose was not damaged by treatment. However, the surface of reaction residues after treatment became rougher, which implied the dropping of lignin fraction from corncob residue.

4.2. Quantification of released sugars

In order to demonstrate efficacy of the investigated pretreatment technique, the High Performance Liquid Chromatography (HPLC) method was applied to quantify the sugar monomers on the corncob pretreatment liquor and the accessible sugar in the solid fraction. Separation of the sugar monomers was achieved by an Aminex HPX-87H column (BioRad, USA) at 60°C. The sugar monomers of interest were glucose, xylose and fructose including the oligosaccharides.

These studies were undertaken in order to determine the main properties involved in the dissolution process. Since the effect of solvent pretreatment on the chemical structure and composition of the corncob has been outlined, in this section the sugar releasing capability was quantified. For biomass pretreatment, two regimes are reported frequently in literature, either higher temperature & dilute concentration or low temperature & high concentration (Li et al, 2015). Both regimes have been tested to identify which one enhances sugar solubilisation and dehydration (Wang, 2012). At low temperature partial or incomplete dissolution were observed, whereas at high temperature complete dissolutions were guaranteed. However it upholds the potential undesirable decomposition of the pretreatment solvent species & product degradation. Thus corncob pretreatment was carried out at mild conditions, temperature of 70-80 °C, time 3.5 – 6 hours and concentration of 0-10% w/v or 0.1-2.0 M. For this part of the study, the experiment conditions were maintained in these parameters for all the different solvent pretreatment processes.

HPLC Calibration

Reference solution was prepared by dissolving glucose, xylose and fructose reagent in 100 ml distilled water. Dilutions of different concentrations ranging from 0.5 g/l, 1.0 g/l, 2.0 g/l, and 4.0 g/l to 24 g/l were made by pipetting out stock solution into volumetric flask. Final volume to each volumetric flask was made up to 100 ml with the addition of distilled water. The retention time was noted at 2.6, 3.448 and 7.667 min for fructose, glucose and xylose respectively. The concentration of the monomers (g/l) was plotted against respective peak area values at corresponding retention time to get standard calibration curve shown in Figure 4.9.

The amount of monomers in the pretreatment liquor was determined using 5 ml of sample in a HPLC vials. The area detected by HPLC was converted into g/l of monomer using the standard curve and the Beer Lambert law (Chi, 2012).

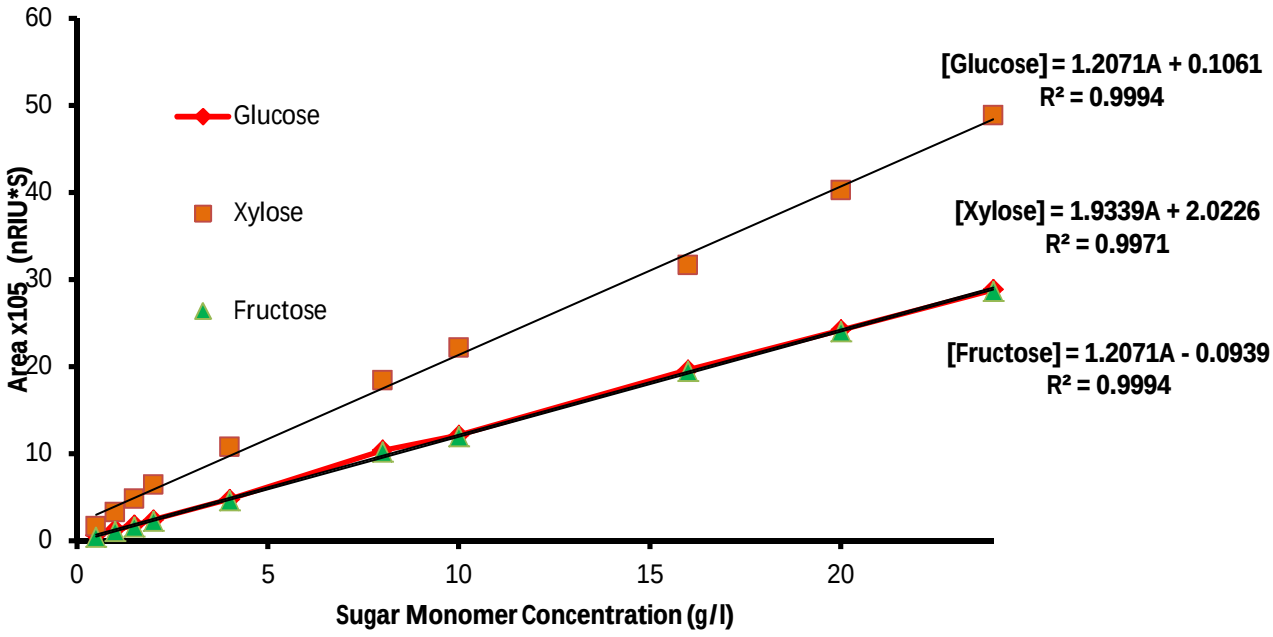


Figure 4.9: Standard calibration curve for estimation of glucose.

4.2.1. Comparison of total sugar concentration obtained from different pretreatment acid

A series of dilute acid pretreatment liquor was assayed on HPLC. As specified in Chapter 3; phosphoric, citric & sulphuric acid were used as the solvents for the pretreatment reaction. Being the most widely used in dilute acid pretreatment, sulphuric acid run was carried out to validate the maximum attained sugar monomers (Kootstra et al., 2009; Yang & Wyman, 2008). The use of these solvents significantly improved the sugar release comparative to the control with fewer degradation products formed. Figure 4.9 shows degradation products in the presence of different acid and control degradation without acid catalyst. The total amount of glucan & xylose dissolved in the solution are comparable in sulfuric and phosphoric acid pretreatment methods i.e H_2SO_4 is 38 g/l, H_3PO_4 is 36. 8 g/l. However, there is also an appreciable amount of fructose obtained from the sulphuric acid pretreatment and literature suggest that the presence of fructose suggest the presence of furfural, HMF and other by-products. Thus, sulphuric acid pretreatment has the potential to yield fewer more inhibitory degradation products that has the potential to limit subsequent step following pretreatment.

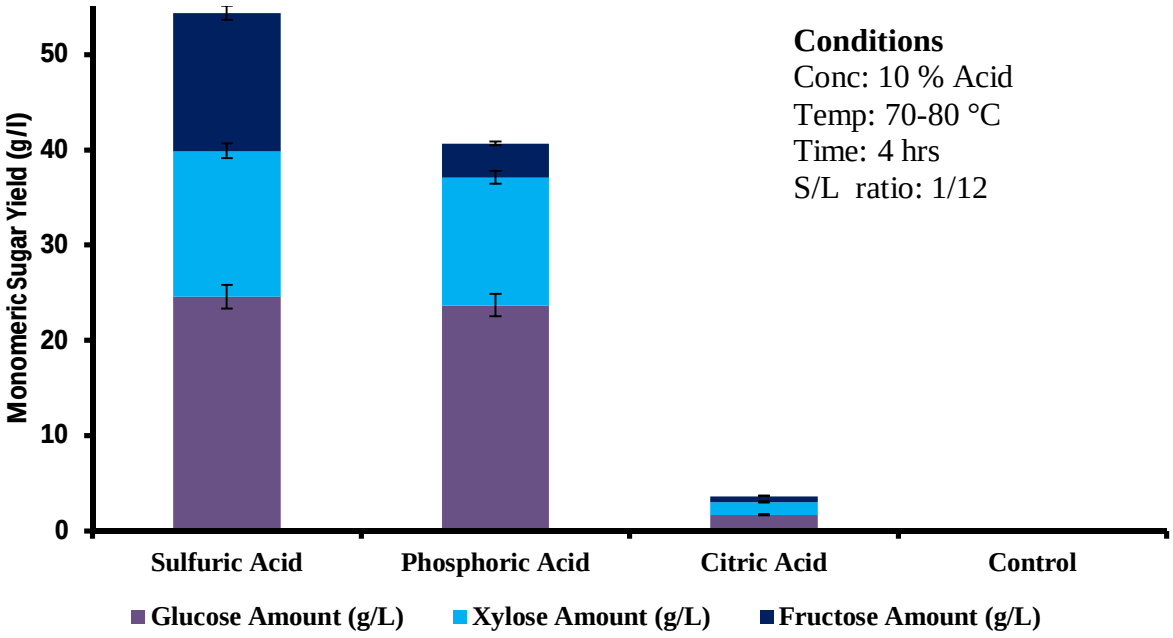


Figure 4.10: Monomeric sugar yield of corncob after diluting the sulfuric, citric and phosphoric acid pretreatment using 10% (w/w), temperature 70-80 °C, time 4 hrs, 10 g corncob solids and 120 ml liquid volume

Figure 4.10 shows that the total sugar monomers dissolved in the solution during sulphuric acid pretreatment can go to a maximum of 54 g/l, followed by phosphoric acid at 40.6 g/l and citric acid can only extract 4.3 g/l of sugar monomers under the specified conditions. It has been reported in literature that under harsh acidic conditions it is typical for processes such as enzymatic hydrolysis to occur during pretreatment stage.

Phosphoric acid is an inorganic weak acid that has a high potential to break the hydrogen bonding cleavage in the lignocellulosic biomass and thus enhance hydrolysis and degradation reactions. Due to its weak acid property, the disruption of lignin was limited hence some cellulose and hemicellulose could not be reached and as a result the sugar monomers were less compared to that of sulphuric acid. Though total sugar monomers formed under phosphoric acid are 40.6 g/l, it is very high compare to citric acid which is at 4.3 g/l total sugar. Thus citric acid was ruled out at this point and couldn't be investigated any further.

When comparing the physical structure of corncob biomass before and after pretreatment, it was apparent that sulphuric acid causes more damage to the physical, as much as chemical structure than phosphoric acid. It is shown on Figure 4.11 that the corncob granules become dark and muddy under H_2SO_4 however after H_3PO_4 pretreatment the corncob retains its physical structure which shows the potential to be used for further processes such as gasification and bioenergy production (Trajano & Wyman, 2013).

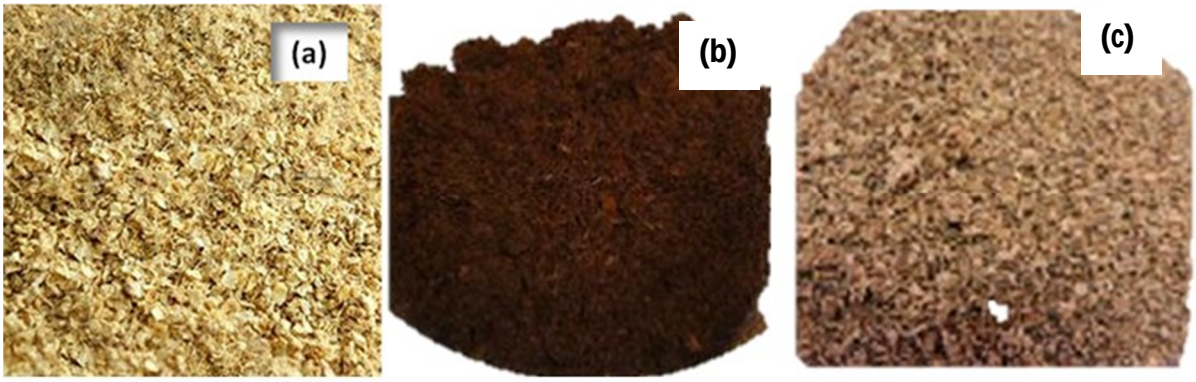


Figure 4.12: Physical structure of corncob (a) before pretreatment (b) sulphuric acid pre-treated (c) phosphoric acid pre-treated

Under severe pretreatment conditions, such as high temperature & acid concentration and long reaction time, the biomass material results in a dark brown granules or sometimes even black such as that of Figure 4.12b. This shows that the material is destroyed the physical structure of the lignocellulosic biomass (Fengel and Wegener, 1989).

The most optimal pretreatment regime in this case was selected based on the release and degradation of cell wall components. In order of abundance, pretreatment products are oligomeric xylose, monomeric xylose, acetic acid, oligomeric glucose, and monomeric glucose. In the spectrum of mild to harsh, two pretreatment stood out as having close-to-optimal sugar release.

4.2.2. Comparison of total sugar concentration obtained from alkaline pretreatment

Alkaline treatment can be used for removing lignin and thereby increasing the digestibility of cellulose. Compared to acid and hydrothermal processes, mild alkaline pretreatment lead to less solubilisation of hemicelluloses and less formation of inhibitory compounds and they can be operated at lower temperatures. Sodium hydroxide and potassium hydroxide are the most commonly used forms of alkali, but their cost is a serious limitation (Jonsson & Martin, 2016)

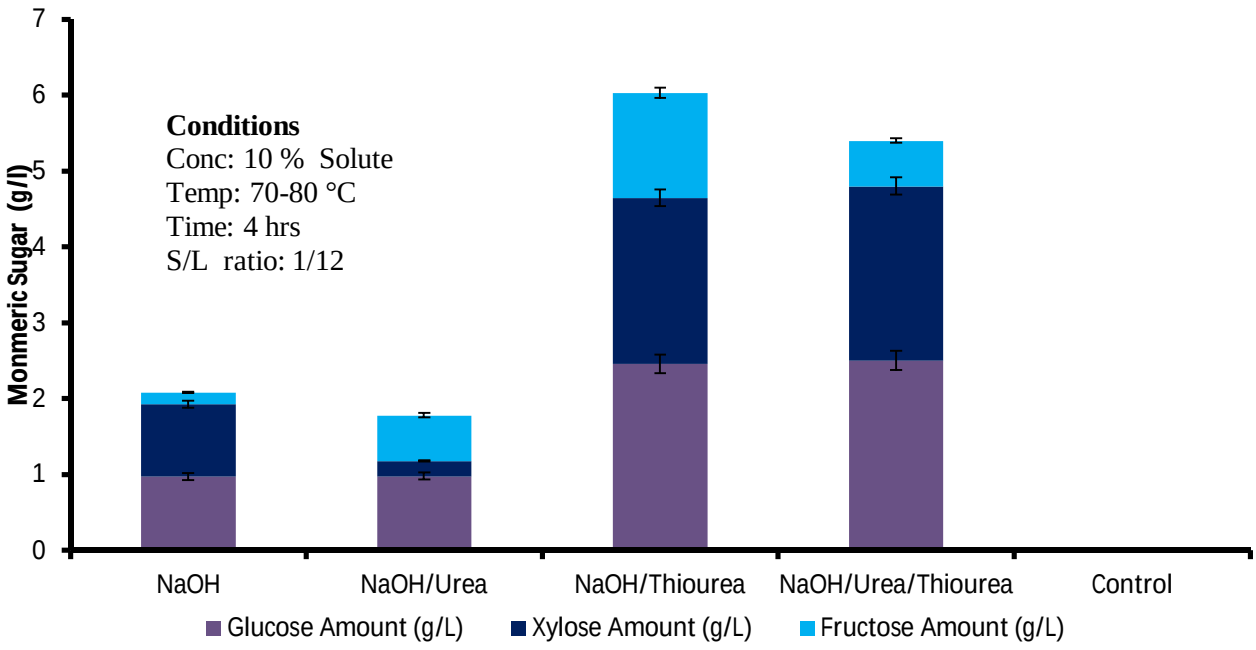


Figure 4.13: Monomeric sugar yield of corncob after dilute NaOH, urea and thiourea and combination pretreatment using 10% (w/v), temperature 70-80 °C, time 4 hrs, 10 g corncob solids and 120 ml liquid volume

The major strategy of alkaline pretreatment is to disrupt the lignin structure in biomass, thus improving the susceptibility of the remaining polysaccharides (cellulose and hemicellulose) for other treatment (Zhao et al., 2012). The optimum condition of alkaline hydrolysis occurs at milder conditions (below 140°C) and lower severity as compared to other pretreatment technologies; hence the pretreatment was carried out at 10% (w/v) concentration and 70°C temperature.

As shown in Figure 4.13, for pretreatment of corn cob biomass with alkaline alone the releases of monomeric sugars is low (<2.0 g/l), compared to the acid pretreated corn cob which is higher (>50 g/l), refer to Fig. 4.13. This is because it is known that for alkaline pretreatment the main mode of action is dissolution of lignin, and to a lesser extent hydrolysis of cellulose & hemicellulose to monomeric sugars (Maryana *et al.*, 2014). However, it is worthwhile investigating the content of monomeric sugars as the NaOH is coupled with less harsh pretreatment solvents which in this case are urea and thiourea. As expected, the sugar monomers were detected when NaOH is coupled with urea and even more when combined with thiourea. This illustrates the decomposition of cellulose and hemicellulose to yield glucose, xylose and fructose and hence NaOH/Thiourea blending has the potential of a good pretreatment regime under these alkaline conditions.

The mechanism of a typical alkaline pretreatment involves saponification of intermolecular ester bond, which crosslinks xylan and lignin (Firscher, 2014). The induced cleavage of ester linkages joining phenolic acids is explained better by the reaction with sodium hydroxide forming a carboxylic salt and an alcohol, Figure 4.14. The NaOH is known to act as the swelling agent to cellulose leading to an increase of internal surface area of the biomass (Wyman, 2013). Simultaneously, urea and thiourea as pretreatment agent are known to a decrease the degree of polymerization and crystallinity of cellulose destroying the structural linkages between lignin and carbohydrate and disrupting the lignin structure by breaking its glycosidic ether bond (Wyman, 2013). Collectively, all these lead to partial solvation of hemicellulose, lignin failing to act as protective shield to the cellulose after lignin solubilisation step, thus making the extracted cellulose more susceptible to a partial dissolution. Therefore the maximum detected sugar monomers amount to 6.1 g/l in NaOH/thiourea.

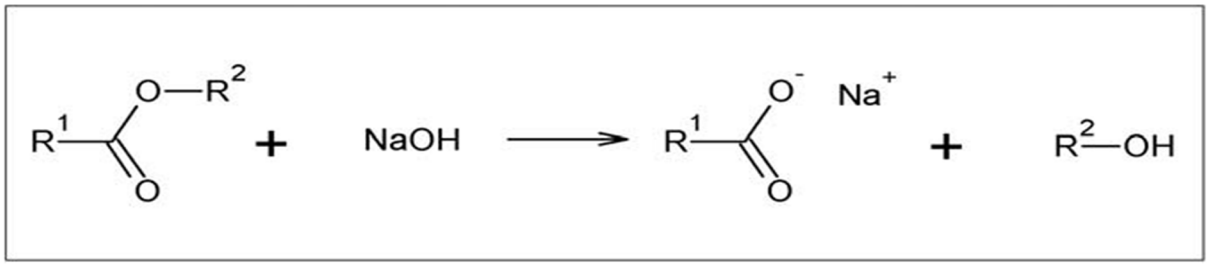


Figure 4.14: Saponification of ester bonds to yield a carboxylic salt and an alcohol Source: Pedersen & Meyer, 2010.

The HPLC results, in terms of sugar release from the pretreated solids, show that the glucose was the dominant soluble material in the products. However, the most accurate method of biomass sugar analysis is based on the Ion chromatography analysis which has the ability to detect even the derivatized sugars such as alditol acetates or trimethylsilanes. The derivatization method is time consuming which lead to the only alternative of high-performance liquid chromatography (HPLC) method and this cannot resolve most sugars found in biomass hydrolysates. But here, it was demonstrated that by careful manipulation of the HPLC mobile phase, biomass monomeric sugars (xylose, fructose, glucose, including mannose, arabinose and galactose) can be analyzed quantitatively and there is excellent baseline resolution of all the sugars. This method was demonstrated for standard sugars, pretreated corncob liquid and solid fractions.

4.2.3. Comparison of total sugar concentration obtained from different pretreatment molten hydrate salts

A multitude of pure molten salt hydrates as well as salt mixtures were investigated with respect to their interaction with polysaccharides especially cellulose. As a result it turned out to be reasonable to divide the salt hydrates into groups according to their optical visible effect on cellulose. A comparison of the dissolution efficiency for the different salts was made for each analytical trial.

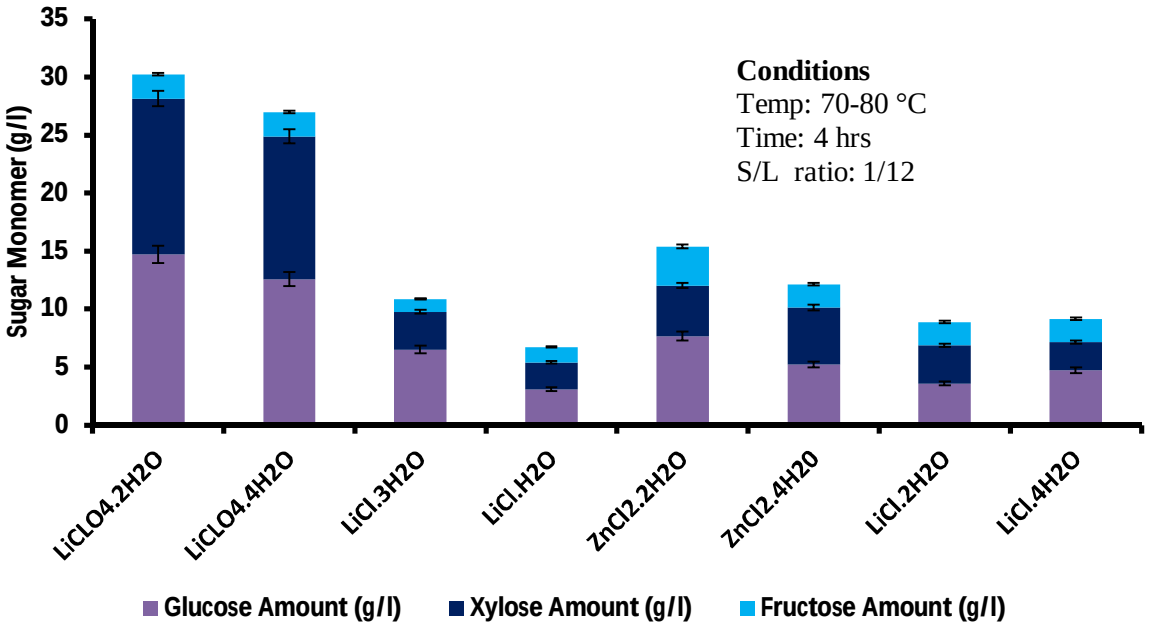


Figure 4.15: Effect of molten hydrates pretreatment process on glucose, xylose and fructose yield

The quantity of sugar monomers which were able to be dissolved from corncob were very different in composition. They varied in cation and anion and in their water content. It has been reported in literature that salts combining small hard cations with soft polarizable anions have the best dissolving power for cellulose (Fischer, 2004). But the good dissolving ability of lithium perchlorate dissolution observed from Figure 4.15 is inconsistent with this statement, and several other hydrated melts containing lithium with different anions do not show any dissolving ability to cellulose.

After a detailed investigation of the solvents the following characteristics that mainly determine the dissolution power towards cellulose were recognized:

- the pH of the solvent
- the water content of the melts,
- And the properties of the coordination sphere of the cations (Godin *et al.*, 2013)

This was obvious based on what was discussed before “The effect of low-pH during corncob pretreatment”. And it is well known that a change in the water amount correlates with the acidity, which increases with decreasing water in the solution. This led to additional consideration of the acidity of the solvent before and after the pretreatment. It was found that the solvents in question are comparable to those described for mineral acids. If a hydrated melt should be an effective dissolving agent for cellulose it must have a relative high acidity, low pH will tend to solubilise the sugar monomers higher (Fischer & Thummler, 2010).

The dependence of the dissolving power on the water content in different solvent systems could be shown for several systems. Molten $LiClO_4 \bullet 2H_2O$ has shown to be an excellent cellulose decomposition and monomer dissolution solvent, achieving a monomeric sugar yield of 30 g/l. An increase of the water amount up to a composition of $LiClO_4+4H_2O$ results in a solvent system slightly reducing its ability of monomer dissolution resulting to a decrease of ~3.0 g/l. However the $LiClO_4+4H_2O$ solvent system is still performing well compared to the LiCl solvent system in which the highest monomer dissolution was 10.7 g/l. This difference can only be explained by the structural conditions of the co-ordination sphere of the molecule cations.

The influence of the structural conditions of the co-ordination sphere of the cations could be explained by a comparison of the two solvents $LiClO_4 \bullet 2H_2O$ and $LiCl \bullet 2H_2O$. Although the salts have the same water content, there are differences in their structures. In the case of the perchlorate the water is completely bridged and bound to the cations. The anions do not affect the co-ordination sphere of the lithium ions. The cleavage of the water bridges results in “free” coordination sites. The replacement of water molecules by hydroxyl groups of the cellulose is possible.

On the other hand, the structure of the lithium chloride is characterized by water molecules not only bridged between the cations but also at interstitial positions. Because of the bound anions at the cations there is a water deficit at the lithium cations. The chloride ion cannot be easily replaced by hydroxyl groups because the water from the interstitial positions will prefer to saturate the co-ordination sphere of the cations (Fischer, 2004).

A significant increase of the swelling grade can be observed by decreasing the water amount of the system $LiCl \cdot nH_2O$ from n=4 to 1. The literature research suggested swelling rather than dissolution of cellulose (Fischer, 2004). The conditions which had been described in the literature confirmed that the molar ratios 1:2-1:5 ($LiCl \cdot H_2O$) only causes swelling of the

cellulose structure whereas the highest salt concentration 1:1 was suggested to give the most swelling. The swelling of cellulose in $LiCl{\cdot}3H_2O$ is strong enough to cause a transformation of the cellulose modification and 6.5 g/l of glucose was dissolved as a result, see Figure 4.15.

Kim et al, (2014) showed that fractionation of biomass using $ZnCl_2$ solution not only solubilizes the cellulose & hemicellulose fraction selectively, but also improves the enzymatic digestibility of the treated cellulose. So it was expected that the zinc chloride will perform better than lithium chloride and lithium perchlorate. On the contrary $ZnCl_2{\cdot}2H_2O$ & $ZnCl_2{\cdot}4H_2O$ dissolved only 16.1 and 12.6 g/l of total analyzed sugar respectively, refer to Figure 4.15. Many researchers reported that solubilized sugars can undergo severe undesired degradation under certain condition such as high temperature and low pH, particularly in case of batch reaction. This was taken into consideration since the temperature was maintained at 70- 80°C and the measured pH was between 5 and 6.

From the dissolution trials it could be concluded that the dissolution condition 1:2 molar ratio ($ZnCl_2$:H_2O) at 70^o C was the optimum condition. Since the $ZnCl_2$:H_2O solvent system has been concluded to have a high dissolution efficiency of cellulose only little residues of cellulose left in the solvent system. In order to obtain a wider background towards the possible dissolution mechanisms, different conditions including different temperatures were compared for both fresh and aged samples.

At this stage it is safe to conclude that molten salt hydrates are effective and efficient media for cellulose dissolution. During the investigations regarding the solubility of cellulose in molten salts, $LiClO_4{\bullet}nH_2O$ solvents system were found to be better pretreating agents than LiCl and $ZnCl_2$ solvents. The results shows that there is a need for further investigations using molten inorganic salts as a reaction medium for cellulose should be extended to other polysaccharides. It is known that zinc based hydrates are more preferred than lithium based hydrates salt, this is because zinc chloride is less corrosive than lithium perchlorate thus environmental friendly and economically feasible solvent systems especially in large volume.

4.2.4. The best performing solvent

Referring back to the first research objective and to answer the research questions, it is apparent that the best performing solvent in terms of sugar monomers dissolution is sulphuric acid followed by phosphoric acid. However it was noted from the SEM images that acid is very harsh such that it completely destroys the structure of the corncob and the residues needs

special disposal system that is expensive. In addition, the pretreatment liquor contains inhibitors that may limit the subsequent step i.e. enzymatic hydrolysis or fermentation. Because of the chemical properties associated with this acids and the concentration required, the application or scale-up in industry will not be feasible. The emerging industries consider the use of green solvent attractive and the use of acid is one of the methods they are moving away from, hence it was disregarded in this study.

This investigation revealed that lithium perchlorate, $LiClO_4 \cdot 2H_2O$ is a promising pretreatment method for producing high sugar yields. Following the acid pretreatment, achieving a significant higher yield of 30 g/l in total analysed sugar monomers was achieved. This was high compared to other pretreatment technique such as zinc chloride at 16 g/l and lithium chloride at 12.6 g/l. From this pretreatment, it was found that disrupting the corncob complex structures results in destruction of lignin which in turn increased the cellulose accessibility. Even though lithium perchlorate is not regarded as a green chemical, it is a low cost reagent and also environmental friendly to a certain extent (Nagoor Gunny and Arbain, 2013)

4.2.5. Effect of pH on pretreatment of corncob

A quantitative comparison of published data for biomass pretreatment illustrates that there is some correlation between the hydrolysis yields (glucose, fructose and xylose) and the pretreatment pH (Pedersen & Meyer, 2010). Under acidic pretreatment strategies cellulose and hemicelluloses are degraded via autohydrolysis reactions. This hydrolysis of polysaccharides forms monosaccharides which are readily fermentable. Especially, the acid catalyzed pretreatment strategies show removal of hemicellulose from the biomass and this removal in turn displays the cellulose to enzymatic attack.

The release of monosaccharides during acidic pretreatment reduces the need for enzymes to accomplish the saccharification process (Trajano & Wyman, 2013). Two of the primary advantages of low-pH reactions are the ready availability of catalysts and high product yields. However, the capital costs of reactors and associated equipment used for low-pH reactions are high due to the need for expensive, corrosion-resistant materials.

The high pH pretreatment agents are known for the ability to alter the lignin composition and thereby increase the digestibility of the biomass. Monomeric lignin compounds released during the lignin degradation comprise the third group of potential inhibitors i.e. phenolic compounds. These also inhibit the hydrolytic enzymes used in the glucose conversion by

denaturation and precipitation. The results show that at high pH low glucose yield is obtained as it shown in Figure 4.16. Even though the pretreatment is run at neutral pH, inhibitors can be produced during the process because of either high temperatures or residence time. Figure 4.16 also shows that pH has the effect on the efficiency of monomer dissolution, the ionic species of the solvent.

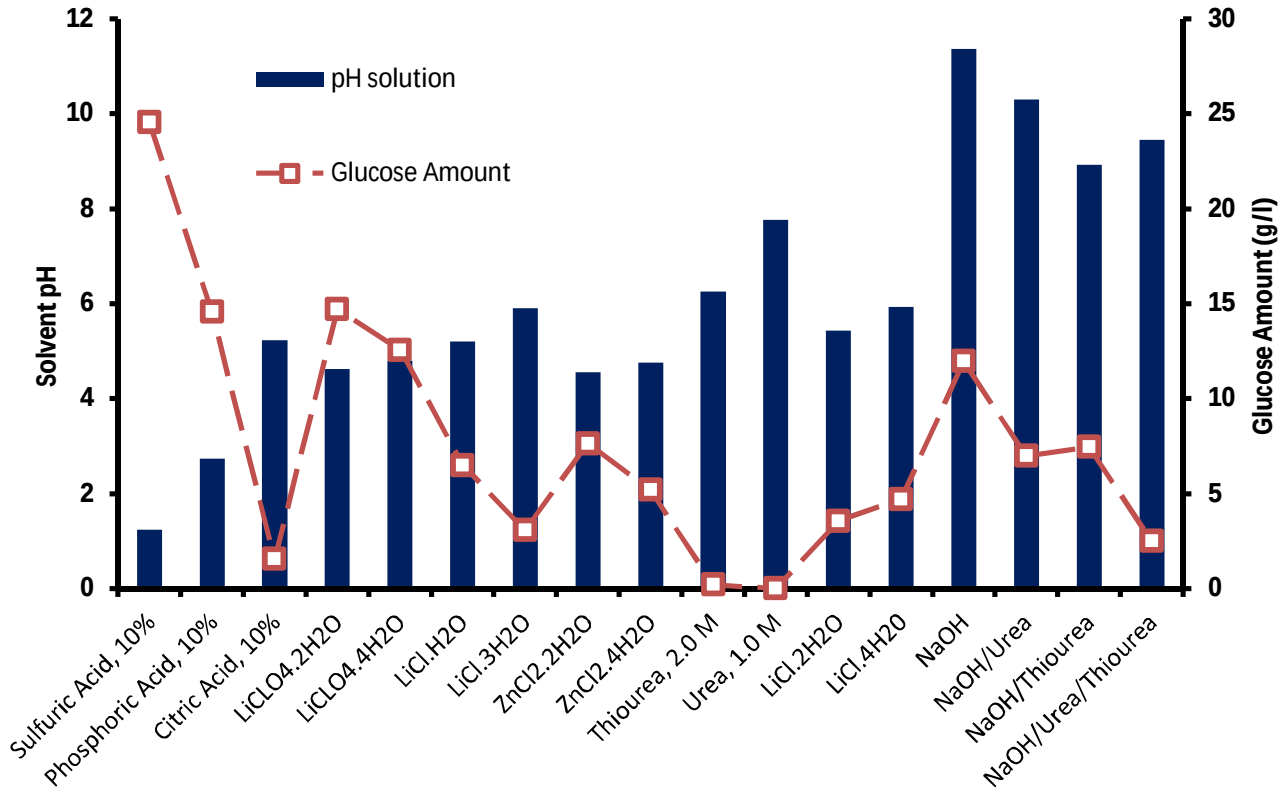


Figure 4.16: Effect of pH on corncob pretreatment using different

Figure 4.16 shows that pretreatment pH is the most significant factor affecting glucose yields after mild thermal pretreatments. The maximal glucose yields from the solid, pretreated corncob fraction were obtained after pretreatments at the low pH values i.e. pH 1.6, H_2SO_4 and pH 2.5 H_2PO_4.

4.3. Parameter optimization of aqueous pretreatment using response surface methodology

It was concluded in section 4.3 that lithium perchlorate, $LiClO_4 \cdot 2H_2O$ is a promising pretreatment agent since it increases the accessibility of cellulose and hemicellulose, thus increase the sugar monomer yield. This pretreatment agent achieved a yield of 30 g/l in total analysed sugar monomers. As mentioned on the objectives of this study (section 1.3), the best performing pretreatment agent is to be used for optimization of pretreatment operating parameters with an intention to obtain the maximum attainable sugar yield. Lithium perchlorate was then used in this section to optimize the pretreatment operating parameters.

4.3.1. Design of Experiment

Experiments have been carried out according to the experimental plan based on central composite rotatable second order design. Experimental design matrix consisting of experiment run order and coded values of the process parameters is shown in Table 5. It also includes the observed responses. The variables were studied at five different levels, namely, -1.6818 (lowest), -1 (low), 0 (middle), +1 (high), and +1.6818 (highest). The three variables chosen were designated as X_1 (Temperature), X_2 (Time), X_3 (% $LiClO_4$).

Table 4: Experimental design, input variable & corresponding results of glucose and xylose yield.

Temperature (°C)	Time (hrs)	Concentration (%)	Glucose (g/l)	Xylose (g/l)
160	7	0.5	10.63	9.14
115	11.8	2.75	12.65	10.88
70	7	0.5	3.45	2.97
180	3.55	2.75	12.40	10.66
115	3.55	2.75	8.42	7.24
70	0.1	5.00	5.34	4.59
115	3.55	2.75	8.30	7.14
70	0.1	0.50	7.98	6.86
25	3.55	2.75	0.95	0.82
70	7	5.00	10.37	8.91
115	3.55	2.75	8.10	6.97
115	3.55	8.4	12.74	10.96
115	3.55	2.75	7.87	6.77
115	3.55	2.75	8.01	6.89
160	0.1	5.00	11.93	10.26
160	7	5.00	12.94	11.13
115	3.55	0.15	0.54	0.46
115	11.8	2.75	1.30	1.12
115	3.55	2.75	8.00	6.88
160	0.1	0.5	5.00	4.30

A statistical 2^3 -central composite design (CCD) was used for the design of experiments. The CCD design was made up of 20 base runs. This includes; 8 cube points, 6 centre points in cube, 6 axial points, and 0 centre points in axial, 1 single base block, all in duplicates.

4.3.2. Development of the mathematical model

The second order regression model has been developed using Minitab software for all the three response variables. The model generated as a function of these variables on the predicted responses of glucose content and xylose yield is a second-order polynomial and is represented by the following equations:

$$Glucose\ \left(\frac{g}{l}\right) =$$

$$8.21 + 1.32\,X_1 - 1.58\,X_2 - 0.48\,X_3 + 0.96\,X_1^{\,2} + 0.67\,X_2^{\,2} - 0.576\,X_3^{\,2} + 0.63\,X_1X_2 + 0.30\,X_1X_3 - 0.42\,X_2X_3 \quad (7)$$

$$Xylose\ (g/l) =$$

$$7.06 + 1.13\,X_1 - 1.36\,X_2 - 0.41\,X_3 + 0.82\,X_1^{\,2} + 0.58\,X_2^{\,2} - 1.38\,X_3^{\,2} + 0.55\,X_1X_2 - 2.55\,X_1X_3 - 0.36\,X_2X_3 \quad (8)$$

ANOVA for equation (7) and (8) is presented in Table 7 & 8, respectively. The ANOVA of the regression model shows the coefficient of determination (R^2) of the xylose model was 0.842 (Table 6) and 0.822 for glucose in Table 7. This indicates that the model adequately represented the real relationship between the variables under consideration. An R^2 value of 0.842 & 0.822 means that 84.2%, 82.2% of the variability was explained by the model and the rest is as a result of chance. Table 6 & 7 presents results obtained after carrying out ANOVA. The ‘‘P-value” less than 0.05 indicate the model terms are significant. Values greater than 0.10 indicate the model terms are not significant. A model F-value of 9.00 and a very low probability value imply significant model fit. From the regression model of sugar concentration, the model terms X_1, X_2, X_3, X_1^2, X_2^2, X_3^2 were significant with a probability of 82%. The term X_1X_3, X_2X_3, X_1X_2 was not significant indicating that there was no interaction between temperature, pretreatment time and solvent concentration.

Table 5 ANOVA for response surface quadratic model for xylose yield.

Analysis of Variance Response Surface Regression: Xylose (g/l) versus Temperature (°C), Time (hr), and Concentration (%)

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	166.006	18.4451	3.19	0.043
Linear	3	157.593	52.5310	9.08	0.003
Temperature	1	57.618	57.6177	9.96	0.010
Time	1	37.232	37.2325	6.44	0.029
Concentration	1	62.743	62.7428	10.85	0.008
Square	3	52.391	67.1303	10.02	0.005
Temperature*Temperature	1	56.196	56.1959	10.03	0.008
Time*Time	1	79.009	79.0093	9.00	0.009
Concentration*Concentration	1	81.233	81.2333	12.04	0.005
2-Way Interaction	3	8.022	2.6741	0.46	0.715
Temperature*Time	1	3.486	3.4856	0.60	0.455
Temperature*Concentration	1	2.282	2.2818	0.39	0.544
Time*Concentration	1	2.255	2.2549	0.39	0.046
Error	10	57.825	5.7825		
Lack-of-Fit	5	57.669	11.5337	368.26	0.000
Pure Error	5	0.157	0.0313		
Total	19	223.831			

Model Summary

S	R-sq	R-sq(adj)
2.40469	84.17%	82.81%

Table 6 : ANOVA for response surface quadratic model for glucose yield.

Analysis of Variance: Response Surface Regression: Glucose (g/l versus Temperature (°C), Time (hr), And Concentration (%)

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	**9**	**224.454**	**24.9393**	**3.19**	**0.043**
Linear	**3**	**213.079**	**71.0262**	**9.08**	**0.003**
Temperature	**1**	**77.904**	**77.9038**	**9.96**	**0.010**
Time	**1**	**50.341**	**50.3414**	**6.44**	**0.029**
Concentration	**1**	**84.833**	**84.8335**	**10.85**	**0.008**
Square	**3**	**52.391**	**67.1303**	**10.02**	**0.005**
Temperature*Temperature	**1**	**56.196**	**56.1959**	**10.03**	**0.008**
Time*Time	**1**	**79.009**	**79.0093**	**9.00**	**0.009**
Concentration*Concentration	**1**	**81.233**	**81.2333**	**12.04**	**0.005**
2-Way Interaction	**3**	**10.847**	**3.6156**	**0.46**	**0.715**
Temperature*Time	**1**	**4.713**	**4.7129**	**0.60**	**0.455**
Temperature*Concentration	**1**	**3.085**	**3.0852**	**0.39**	**0.544**
Time*Concentration	**1**	**3.049**	**3.0489**	**0.39**	**0.546**
Error	**10**	**78.184**	**7.8184**		
Lack-of-Fit	**5**	**77.973**	**15.5945**	**368.26**	**0.000**
Pure Error	**5**	**0.212**	**0.0423**		
Total	**19**	**302.638**			

Model Summary

S	R-sq	R-sq(adj)
2.79615	**82.17%**	**80.91%**

The results of the response surface models for the xylose yield in the form of analysis of variance (ANOVA) were shown in Table 6. Using the designed experimental data and the second-order polynomial model for the yield of xylose in terms of coded factors was shown as the following Equation (8). The statistical treatment combinations of the test variables corresponding to all combinations revealed reliable regression coefficients. The p-values were used as a tool to check the significance of each of the variables as well as their interactive and quadratic effects (Xiao *et al.*, 2015). In general, smaller the value of p(< 0.05) and a high magnitude of t-value, the more significant is the corresponding coefficient term

(Ayeni *et al.*, 2013). As shown in Table 7 the p-values are less than 0.05 and the F-values are higher than 10.0, which are evident that the ANOVA of the response surface model demonstrated in both Equations (7) and (8) is highly significant.

4.3.3. Effect of operating pretreatment parameters

The impact of pretreatment input parameters on the glucose and xylose is analysed using the developed mathematical model. Figure 4.17 presents the main effect plots for operating parameters with response variables. The direct effect of each individual operating parameter on response is identified by keeping all input values at the middle level except the parameter under study. Literature suggest that if main effects plots for a particular operating parameter approaches the horizontal line, then the parameter has no significant effect. On the other hand, a parameter for which the line has the highest inclination will have the most significant effect (Kraber, 2014). It can be observed in Figure 4.17 that all pretreatment parameters have inclination plots.

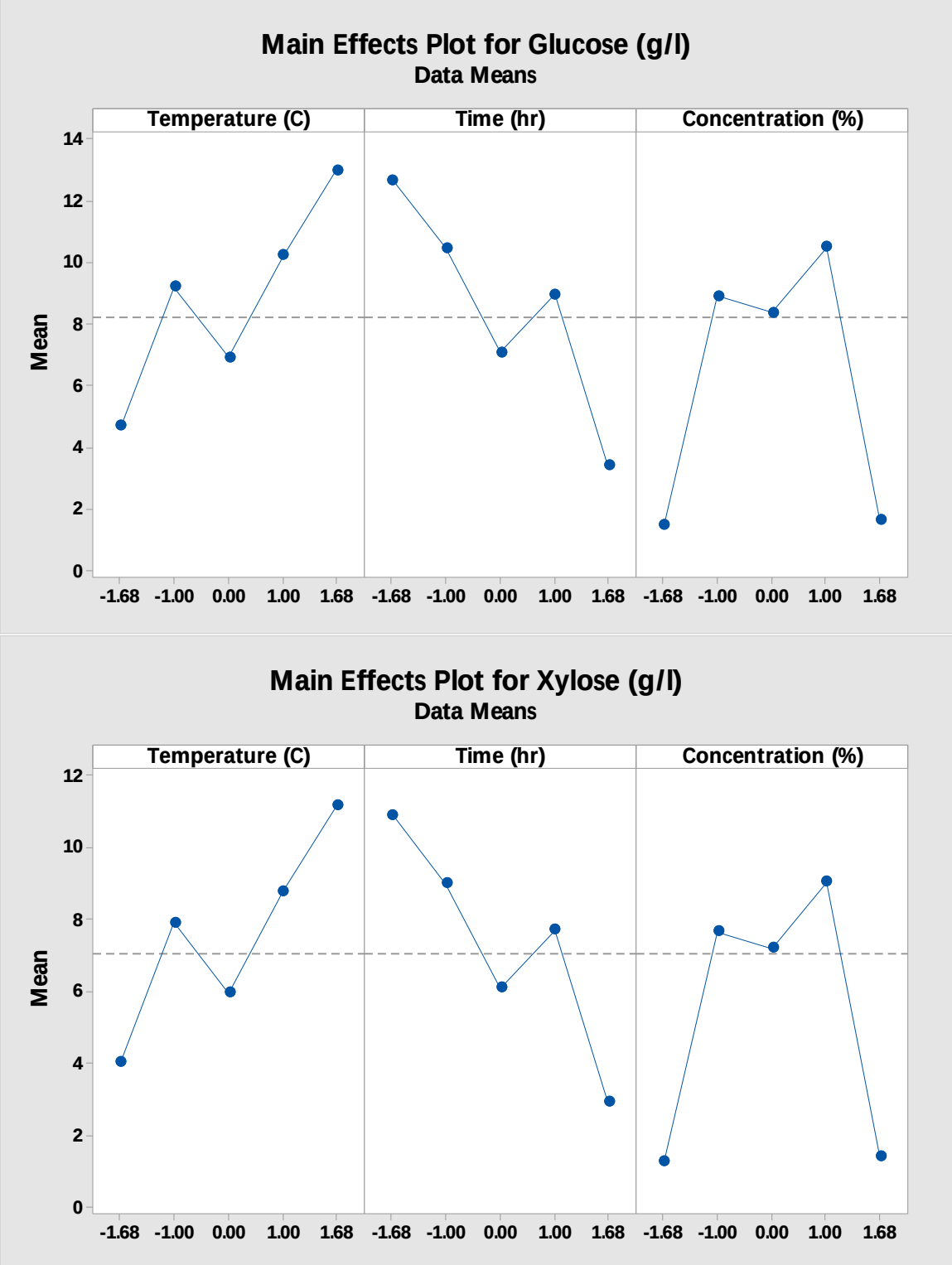


Figure 4.17 : Main Effect of Parameters, data means: (top) glucose yield (bottom) xylose yield

It is seen that the plots for both xylose and glucose form a similar trend, it can be deduced that operating parameter has the same effect on both response. It can be seen that with the increase of solvent concentration, both glucose and xylose yield increase gradually until it reaches maximum and starts to decrease. Increasing temperature tends to increase the yield.

This can be attributed to the fact that with increasing temperature and pretreatment time can disrupt crystalline structure in corn cobs and thus the sugar in the solid after pretreatment is more accessible and the released sugar concentration is high.

Interaction effect of parameters

Interaction effects are the impacts created by the combined effects of multiple input parameters on the response, other than the effects produced by them individually. The effects of interaction between the input parameters are shown in Figure 4.18. It is found that the interaction between temperature & time as well as temperature & concentration has a maximum impact on the response (glucose and xylose yield) compared to other interaction effects. Therefore the accessibility of sugars can be improved by maximising concentration and increasing temperature. Whereas increasing time is subjective since the increase become constant.

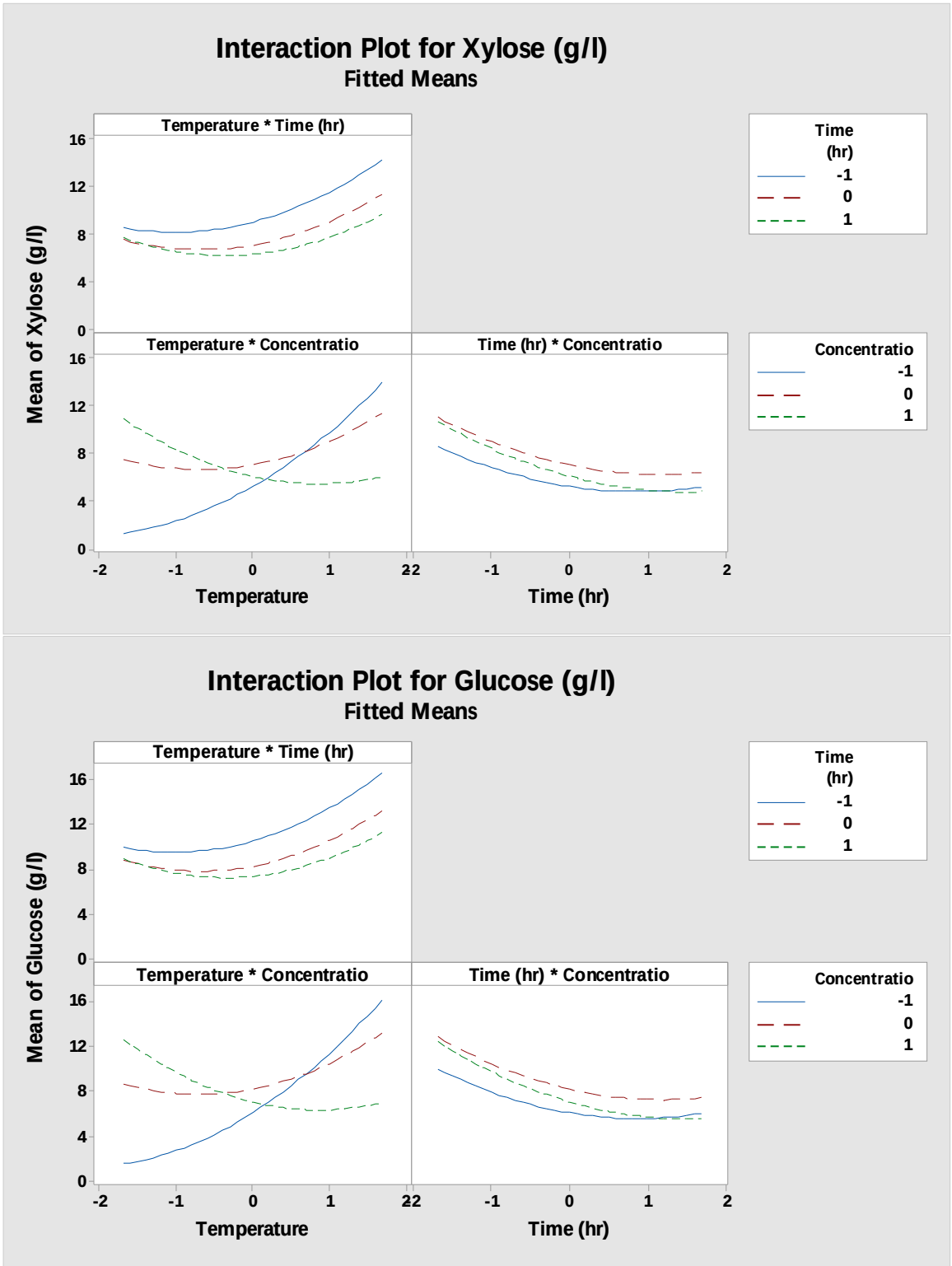


Figure 4.18: Interaction Effects of input Parameters

4.3.4. Contour and Surface plots

In order to optimise the variables that influence the pretreatment of corncob, response surface and contour plots were generated from the regression model. The three-dimensional (3D) plots were generated by keeping one variable constant at the centre point and varying the others within the experimental range. The resulting response surfaces showed the effect of acid concentration, temperature, and pretreatment time on the sugar concentration (xylose and glucose). The contour and the three-dimensional response surface plot described by the above-mentioned second order polynomial equation were generated for both xylose and glucose yields, Fig 4.19-4.21.These plots were obtained by holding the third variable at midpoint constant value. The shapes of response surfaces indicate the nature and extent of the interaction between different independent variables (Ayeni *et al.*, 2012), whereas the contour plots explore the potential relationship between three variables in a 2-D description with x- and y-factors as the predictors.

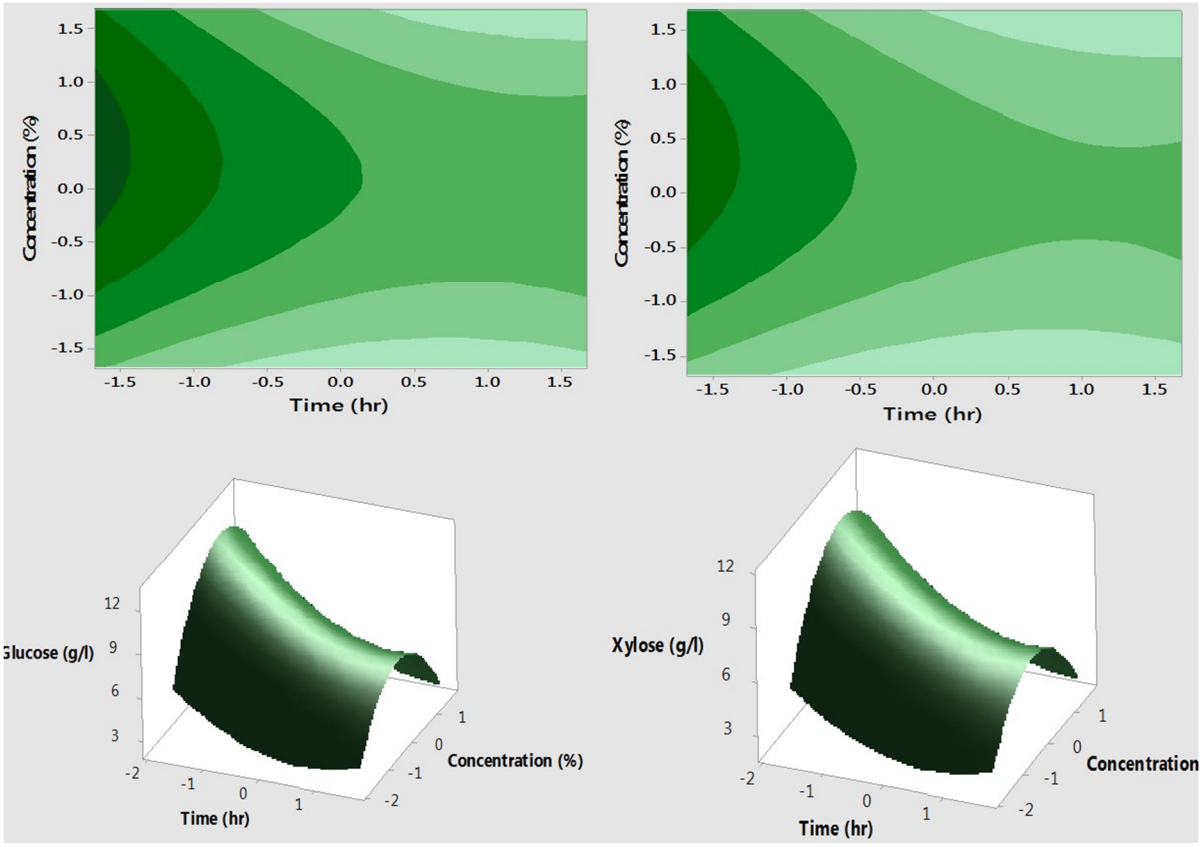


Figure 4.19: Temperature, Response contour plots and corresponding surface plots of the glucose and xylose yield from pretreated corncob

The contour plots shown in Figure 4.19 indicate how the reaction time (x) and $LiClO_4$ concentration (y) affect the contours of the temperature. The darker regions indicate higher quality. The contour levels reveal a peak in the zone of 3.5 hours (reaction time) and 1.5-2.0 % $LiClO_4$ for glucose, quality scores in this peak region are greater than 12. For xylose, the contour levels reveal a peak was highest at on two zones; higher concentration, shorter time and longer time, middle concentration. This means xylose yield increase with the increase of solvent concentration and an increase in reaction time solely however the increase in both parameters at once is not recommended since the interaction of time and concentration negatively affected the xylose yield, refer to Figure 4.19.

The surface plots shown in Figure 4.20 shows that the pretreatment time had the major increasing effect on both the glucose & xylose yield. This figure also shows that higher solvent concentration lead to a higher xylose yield, however this is only limited to the pretreatment at shorter reaction time. Additional, at lower solvent concentration, prolonging the reaction time above 7 hours increased the xylose and glucose yield while a higher solvent concentration had a negative effect on the xylose yield. Overall, the glucose yield was highest at 170 °C, 2.0% solvent concentration and the reaction time to more than 7.0 hours.

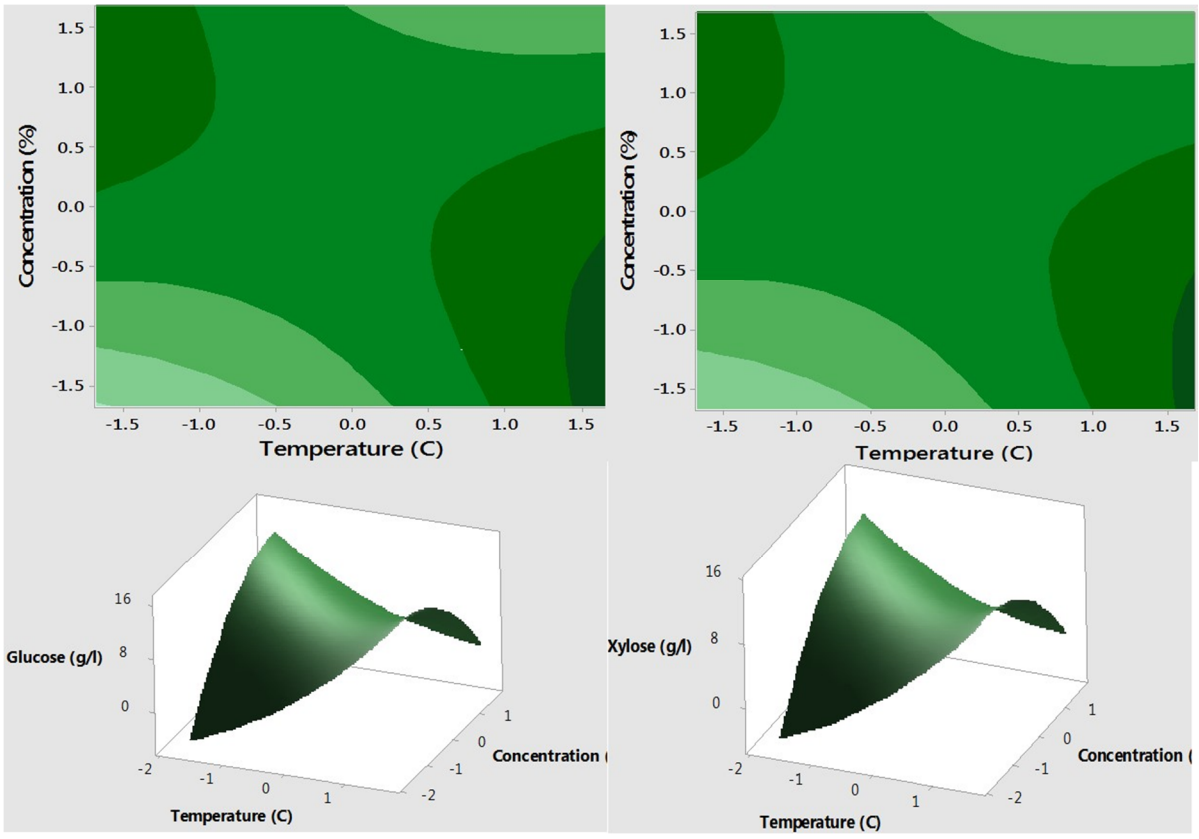


Figure 4.20: Reaction time, Response contour plots and corresponding surface plots of the glucose and xylose yield from pretreated corncob

The contour & response surface plots in Figure 4.20, present the temperature and solvent concentration response on both xylose and glucose yield. It can be observed that these parameters had effects on the response; the increase in temperature causes a significant increase in xylose and glucose yield. It can be observed that xylose yield above 175 °C, at 3.5 h, 1.95 % $LiClO_4$ is higher, and the response surface plots confirm this. The response surface showed that the highest glucose yield appeared at 123 °C, 3.5 h, and 0.24 % solvent concentration.

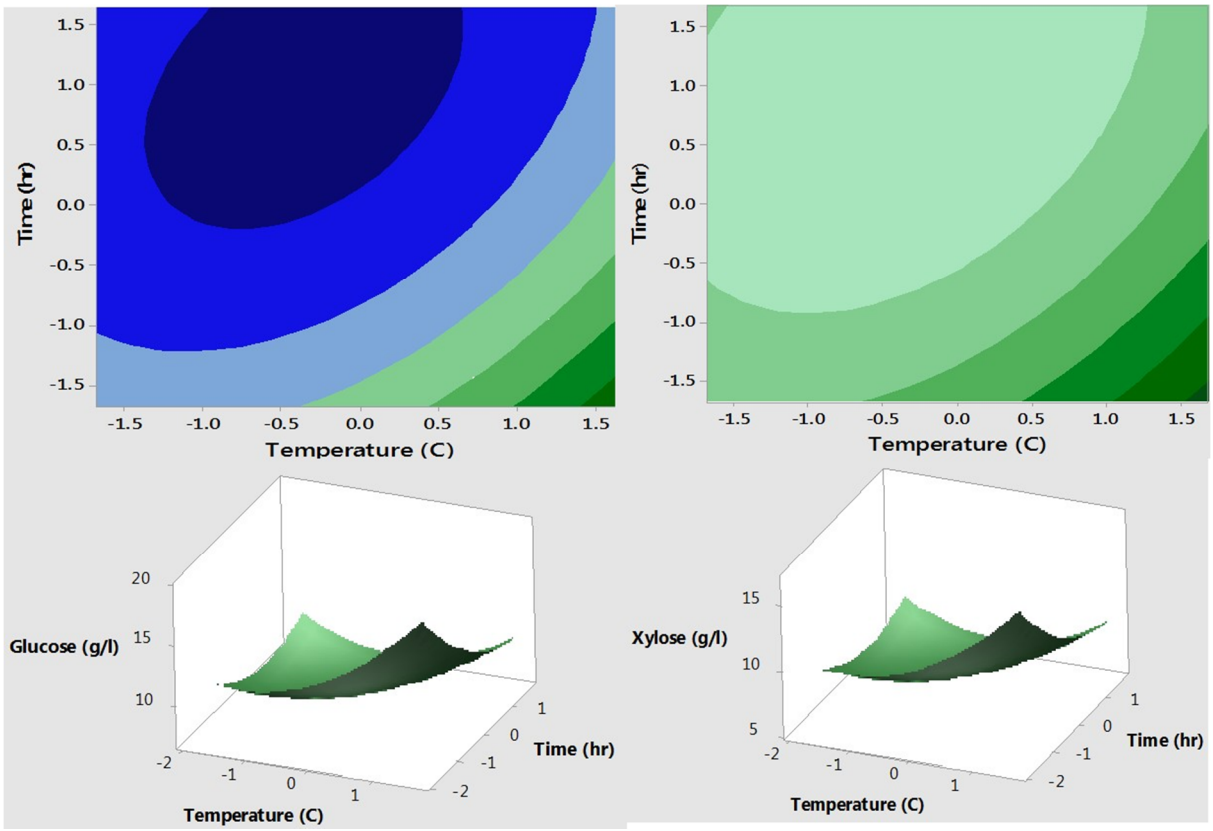


Figure 4.21: Concentration, Response contour plots and corresponding surface plots of the glucose and xylose yield from pretreated corncob

Temperature and reaction time also showed to be a crucial factor that affects the dissolution of xylose and glucose into pretreatment liquor directly, Figure 4.21. A lower solvent concentration, shorter time and high temperature is preferred to obtain an optimum sugar yield. The optimum solvent concentration, reaction time and temperature were found to be 2.0%, 3 hr 50 min and 176 °C, respectively. The corresponding glucose and xylose yield for these parameters were calculated to be 14.26 g/l and 12.4 g/l, respectively. Verification of the calculated optimum conditions for xylose yield was done by performing the experiment at optimized conditions.

4.3.5. Model Verification

It is necessary to check the assumptions of ANOVA before drawing conclusions. The proposed mathematical model was validated using the ANOVA residual plots analysis. The comparison of the response values is represented in Figure 4.22. The normality plot of the residuals is used to check the normality of the treatment data. If the distribution of residuals is normal, the plot will resemble a straight line. The constant variance assumption is checked by the plot of residuals versus fitted values. If the plot of residual vs. fitted values (treatment) does not show any pattern, the constant variance assumption is satisfied. If the plot of residual vs. run order (time order of data collection) does not reveal any pattern, the independence assumption is satisfied (Kraber, 2014).

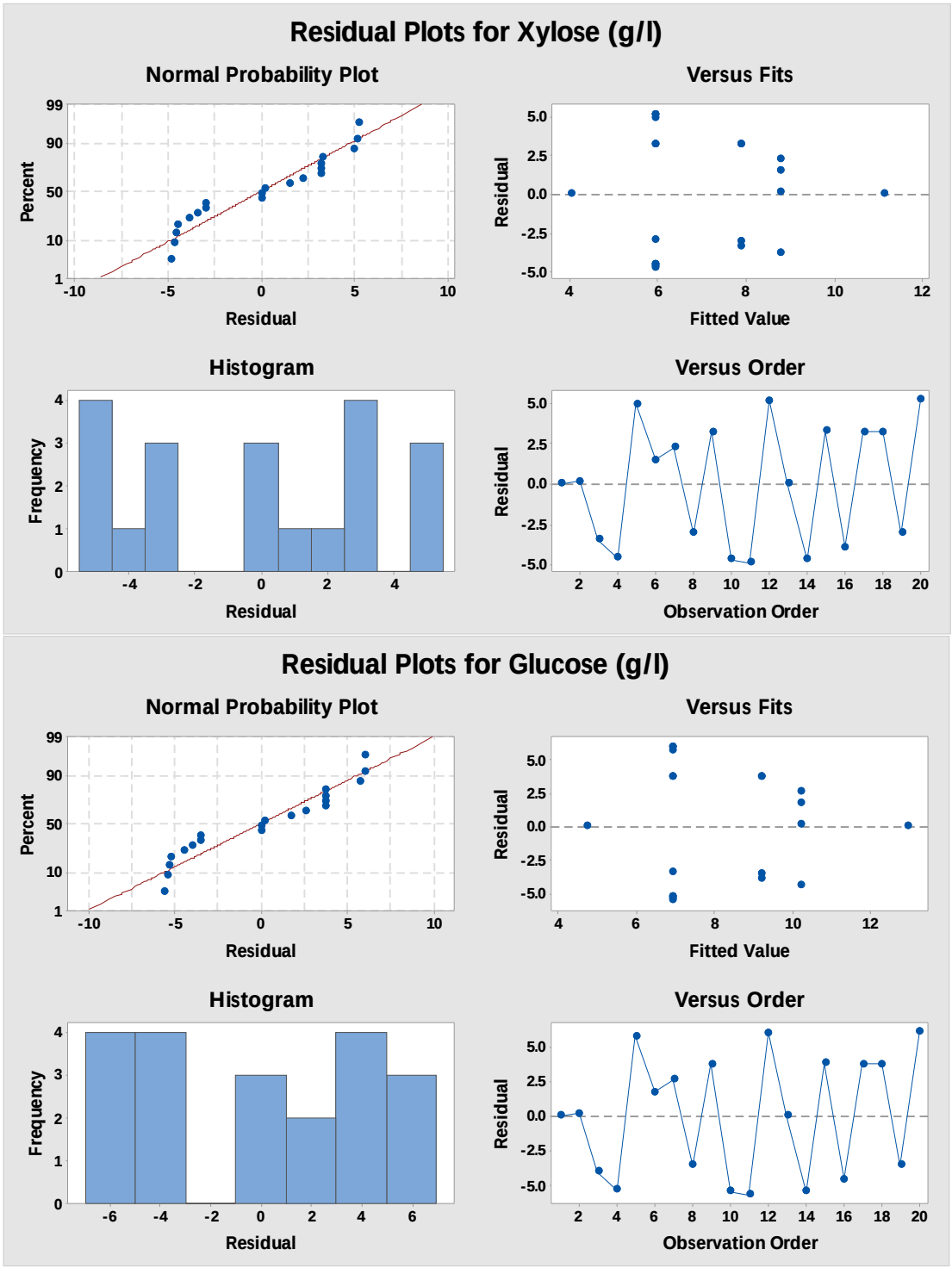


Figure 4.22: Validation of the ANOVA assumptions: normality, constant variance, and independence

The normality plot of the residuals above shows that the residuals follow a normal distribution. Both plot of residuals versus fitted values and plot of residuals versus run order do not show any pattern. Thus, both constant variance and independence assumptions are satisfied.

4.3.6. Model Optimization using Response Optimizer

The mathematical model has been optimized using Minitab response optimizer tool to determine optimum values of input parameters to achieve maximum glucose and xylose yield. For optimization, temperature (X_1) time (X_2) and concentration (X_3) values were determined by maximizing response using the improved second order model given in Equation (9) & (10):

$$Glucose\ (g/l) =$$
$$48.76 + 7.02\,X_1 - 8.6\,X_2 - 2.097\,X_3 + 5.19\,X_1^2 + 3.53\,X_2^2 - 8.31X_3^2 + 3.44\,X_1X_2 + 16.65\,X_1X_3 - 2.96\,X_2X_3 \quad (9)$$

$$Xylose\ (g/l) =$$
$$45.184 + 7.23\,X_1 - 8.6\,X_2 - 2.624\,X_3 + 5.25\,X_1^2 + 3.7X_2^2 - 8.83\,X_3^2 + 3.52\,X_1X_2 - 16.32\,X_1X_3 - 2.304\,X_2X_3 \quad (10)$$

Within ranges of operating parameters (constrain)

$$25\ ^\circ C \leq X_1 \leq 270\ ^\circ C$$

$$2\ min \leq X_2 \leq 11.8\ hrs$$

$$0.15\ \% \leq X_3 \leq 8.4\ \%$$

Table 8 shows multiple response optimization for xylose & glucose

Table 7: Multiple Response Optimization for Xylose (g/l), Glucose (g/l)

Variables				
Temperature (C)	176			
Time (hr)	3.52			
Concentration (%)	1.87			
Response	**Fit**	SE Fit	95% CI	95% PI
Xylose (g/l)	12.37	12.36	(2.26, 35.08)	(-0.05, 37.39)
Glucose (g/l)	14.26	14.56	(2.63, 40.79)	(-0.06, 43.48)

4.3.7. Summary

The response surface obtained in this study was in an unidentified nature, which according to literature suggests that the optimum conditions are not well defined. However the contour

plots become reinforcement since it indicates both the nature and extent of the interactions. The contour and response surface model predicted the optimum levels of significant variables; temperature, reaction time and solvent concentration were found to be 176 ℃, 3 hr 30 min and 1.87 % respectively. The maximum corresponding glucose and xylose content was 14.26 g/l and 12.4 g/l, respectively.

To confirm the predicted model, four replicate experiments were performed and the response activities of each were determined, See Table 9. Finally, the average maximum yield reached 14.24 g/l and 11.9 g/l of xylose and glucose yield, respectively, which is comparable to the predicted value. As a result, the projected models were considered to be accurate and reliable for predicting the dissolution of xylose and glucose.

Table 8: Mathematical models of sugar yields as a function of temperature, time and concentration with optimum sugar yields.

Mathematical Model	Optimal Pretreatment Parameter			Sugar Yield (g/L)	
	Temperature, X_1	Pretreatment time, X_2	Solvent concentration, X_3	Predicted	Experimental
$Glucose\,(g/l) = 48.76 + 7.02X_1 - 8.6X_2 - 2.097X_3 + 5.19X_1^2 + 3.53X_2^2 - 8.31X_3^2 + 3.44X_1X_2 + 16.65X_1X_3 - 2.96X_2X_3$	176 °C	3 hrs 30 min	1.87%	14.26	14.30
					14.26
					14.42
					13.96
				Average	**14.24**
$Xylose\,(g/l) = 45.184 + 7.23X_1 - 8.6X_2 - 2.624X_3 + 5.25X_1^2 + 3.7X_2^2 - 8.83X_3^2 + 3.52X_1X_2 - 16.32X_1X_3 - 2.304X_2X_3$	176 °C	3 hrs 30 min	1.87%	12.37	11.65
					11.94
					11.90
					12.10
				Average	**11.90**

4.4. Kinetics study

Kinetic modelling of pre-treatment chemical reactions was carried out to understand the chemistry, model and design of the system, and how it interacts with other physics that may come into play. Since the objective is to produce succinic acid, this research was focused on the hemicellulose and cellulose dissolution kinetics. However, an extra step was taken to quantify lignin, in this study only soluble lignin (SL) was quantified. To quantify the yield of hemicellulose and cellulose, the analysing technique used sulphuric acid to release the sugars after the pre-treatment stage. Thus, the models are represented in terms of xylose (hemicellulose monomer), glucose (cellulose monomers) concentrations.

4.4.1. Concentration profile

Figure 4.23 shows the concentration profile of glucose, xylose and soluble lignin accessibility at 176 ℃ in $LiClO_4 \cdot 2H_2O$. Glucose yield increases slightly slower than xylose until it reaches the equilibrium just after 7 hours. The xylose yield reached equilibrium after 3 hrs and started

to decrease after 5 hours, it was deduced that this could be due to further degradation of xylose forming its constituent sugars as time increased, hence the detected xylose decreased. Cellulose, which likely represents the amorphous region, dissolved and started depolymerisation to glucose after 30 min and reached equilibrium at 7 hours after 14.7 g/l glucose was achieved. It was observed that the acid-soluble lignin was in a small amount and it dissolves during pre-treatment, and it did not inhibit the dissolution of either cellulose or hemicellulose.

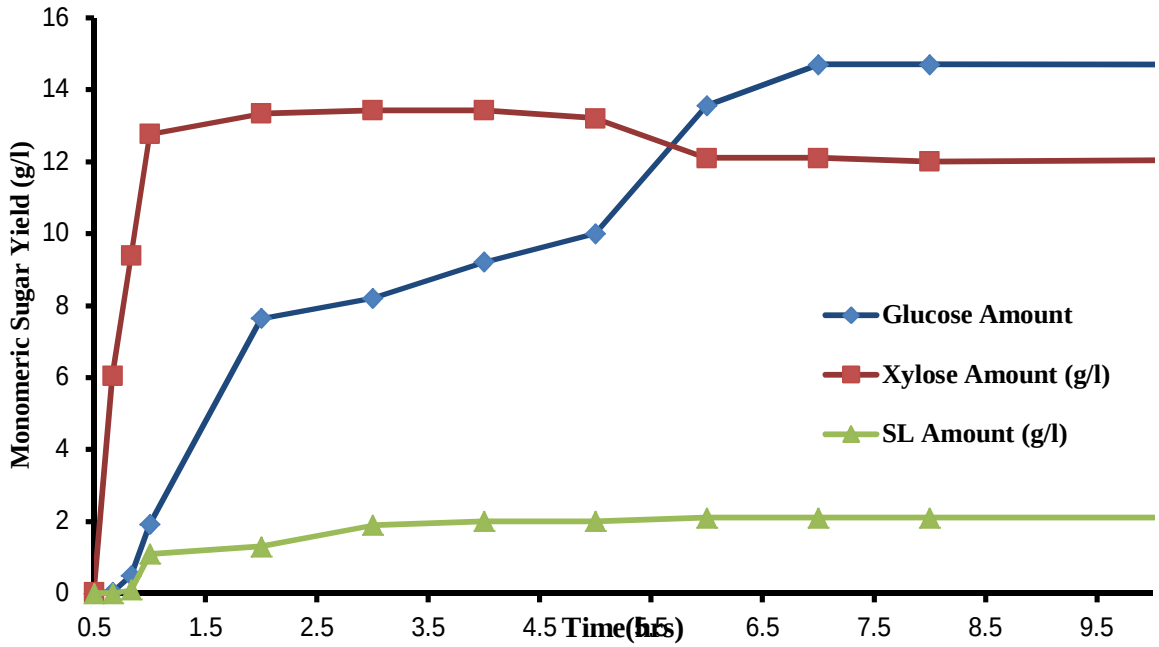


Figure 4.23: Monomeric sugar yields of total glucose, lignin and xylose at the optimal conditions

4.4.2. Determination of kinetic parameters

The data obtained was fitted into a kinetic model as it was stated in Section 3.2.5. The data was plotted as $ln\left(\frac{C_i}{C_f}\right)$ against reaction time, see Figure 4.24.

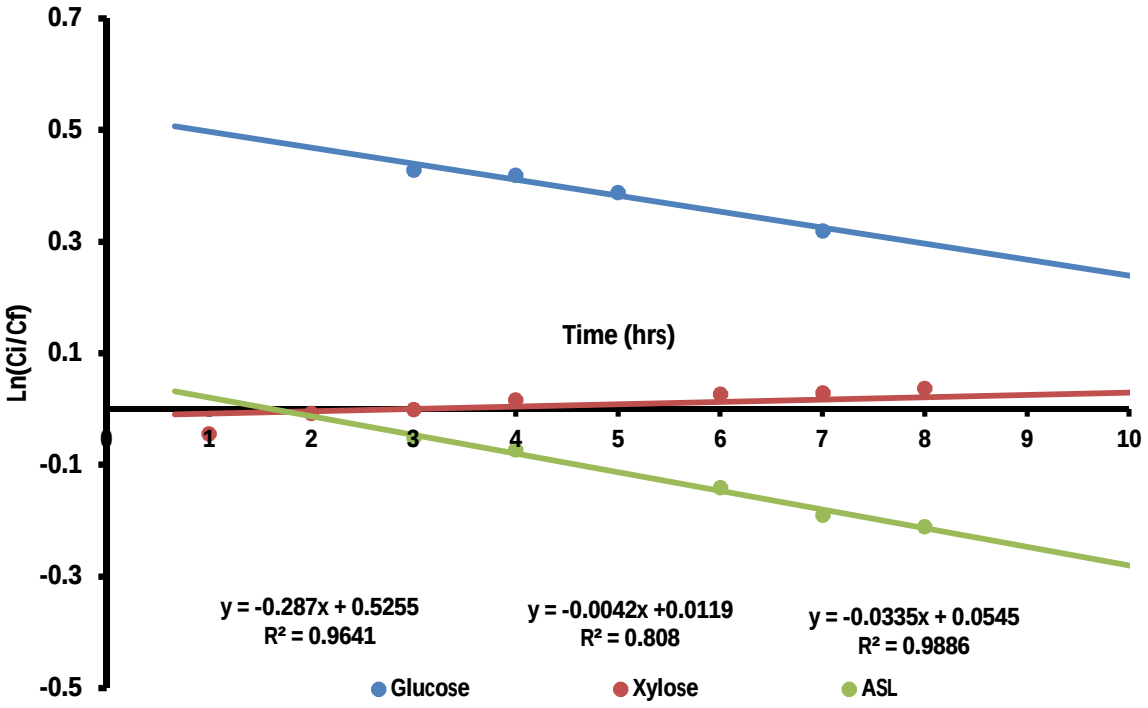


Figure 4.24: Kinetic model for glucose, xylose and SL dissolution at 120 °C: logarithm plots

Figure 4.24 shows corresponding kinetic models for lignin dissolution, xylose and glucose extracted using $LiClO_4 \cdot 4H_2O$ pretreatment agent at 120 °C. From the results it can be denoted that the model best described the kinetics of glucose dissolution (R^2=0.9641) and ASL (R^2= 0.9886) than it does for xylose (R^2= 0.808) dissolutions.

Equation (11) was used to obtain the rate constant (k) by means of linear regression coefficient.

$$ln\left(\frac{C_i}{C_f}\right) = -kt + z \qquad (11)$$

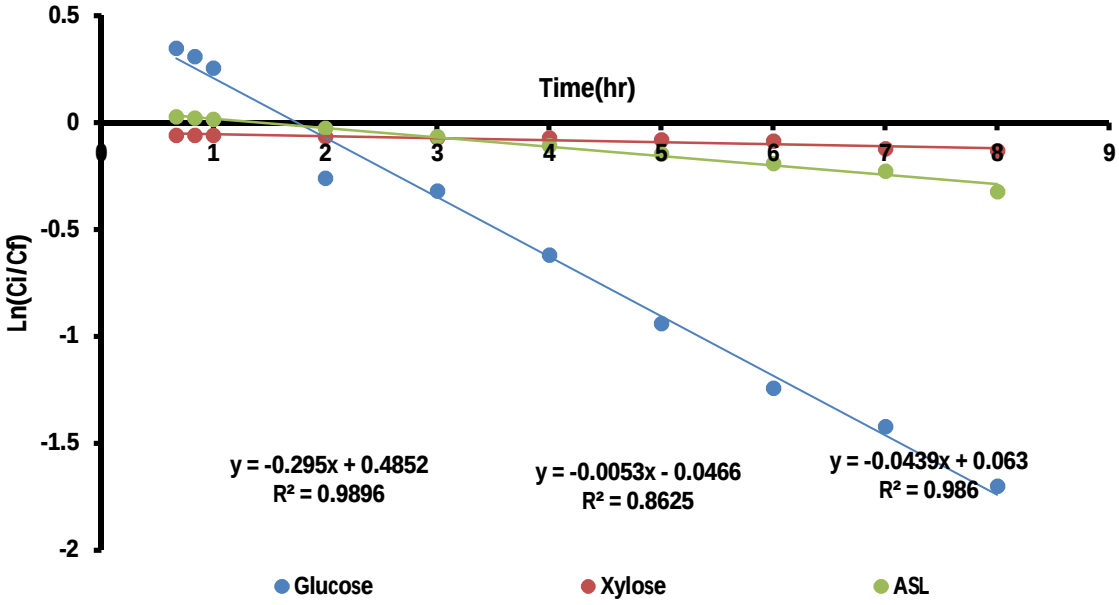


Figure 4.25: Kinetic model for glucose, xylose and SL dissolution at 140 °C: logarithm plots

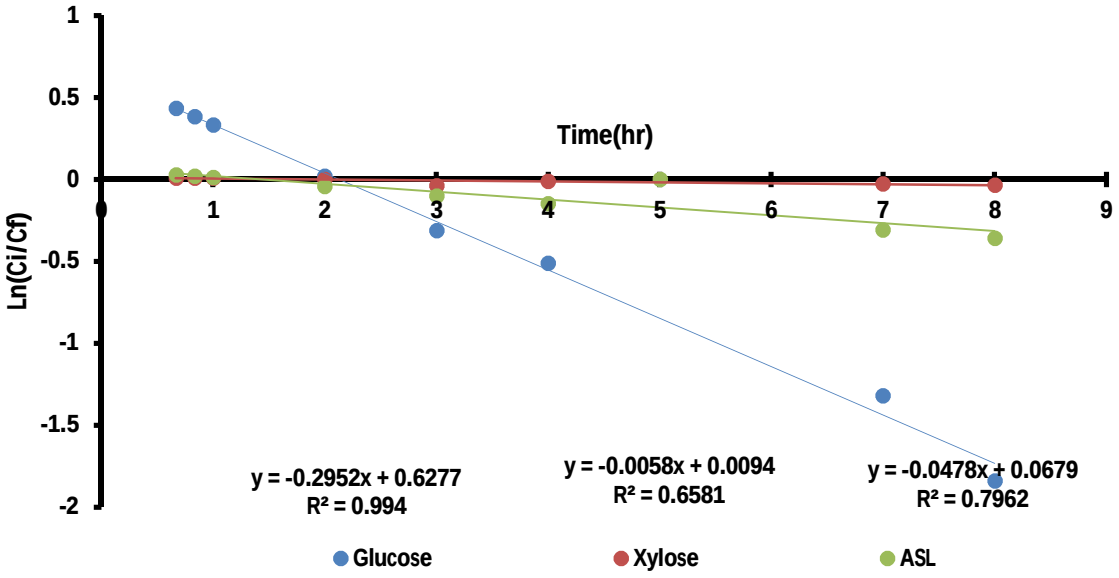


Figure 4.26: Kinetic model for glucose, xylose and SL dissolution at 160 °C: logarithm plots

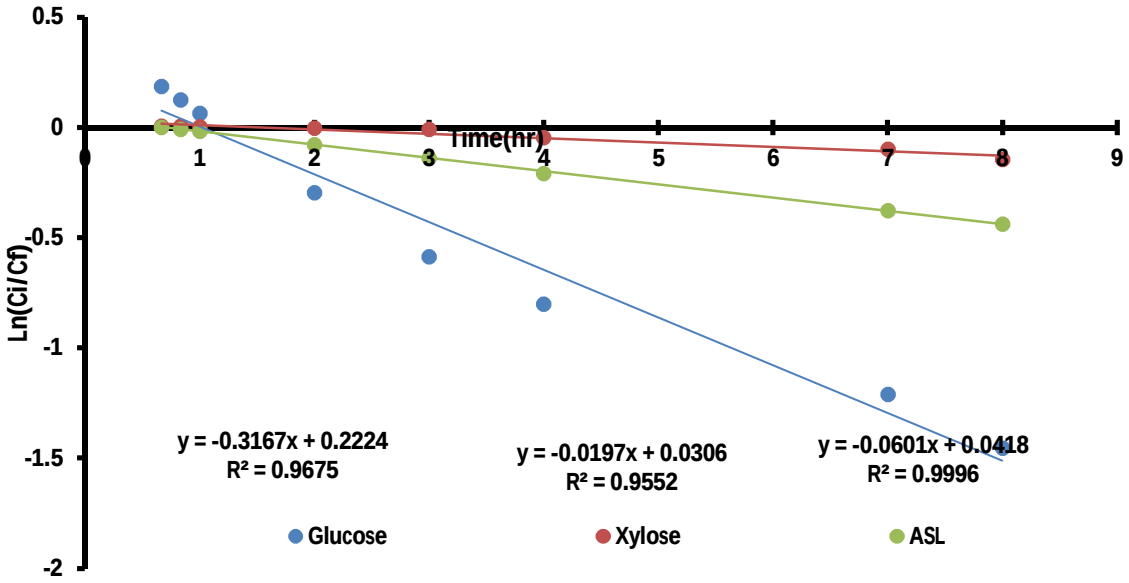


Figure 4.27: Kinetic model for glucose, xylose and SL dissolution at 180 °C: logarithm plots

The kinetic constants at 120°C, 140°C, 160°C, and 180°C are shown in Table 10. These results show that the kinetic constant increased as temperature increased. These values were obtained from the curves for ln (C_i/C_f) as a function of time (Eq. 11). Figure 4.25, 4.26 and 4.27 shows these curves for temperature 140 °C, 160°C and 180 °C respectively. It can be seen that the model represented the experimental data accurately. Although the model of Eq. 11 is semi-empirical, one of its important characteristics is that the resulting rate constants for each phase can be fitted to the well-known Arrhenius equation.

Table 9: Rate constant obtained from the logarithmic plots of dissolution amount as a function of time

	Rate constant, (hr^{-1})		
T (°C)	**Glucose**	**Xylose**	**SL**
120	0.287	0.004	0.534
140	0.295	0.005	0.540
160	0.295	0.006	0.548
180	0.360	0.020	0.560

The activation energy (Ea) of the lignin dissolution and sugar accessibility on the pretreated corncob was derived using Equation (12) from the slope of ln k against 1/T. The activation energy of dissolution was calculated from the logarithmic form of the Arrhenius equation

$$ln\ k\ = -\frac{Ea}{RT} + \ ln\ A \qquad (12)$$

Plotting ln k against 1/T, results in a linear relationship curve in which the obtained slope is equivalent to Ea/R. The Arrhenius constant (A) was calculated from the intercept, ln A.

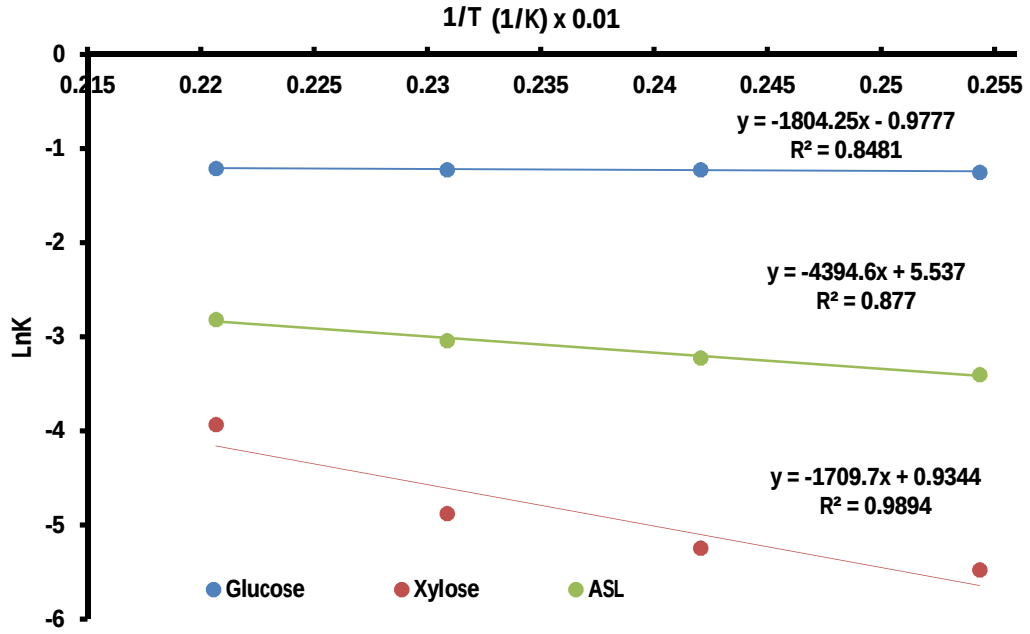


Figure 4.28: Kinetics of lignin content dissolution, glucose and xylose: Arrhenius plot for the treated biomass. Note: temperature in kelvin (K)

The activation energies were calculated from the linear equations shown in Figure 4.28. The resulting Ea were; 15.0 kJ/mol, 14.2 kJ/mol and 36.54 kJ/mol for glucose, xylose and lignin, respectively. It is apparent that the activation energy associated with lignin is higher than that of glucose and xylose. This is an indication that more energy is required to release lignin from corncob. However, Ea for xylose and glucose are low, this is also confirms that low activation energy and high temperature favor higher rate constants, and thus speed up the reaction. It should be noted that even a modest value of activation energy reduces the rate by a factor of 10^8 (Fuentes *et al.*, 2011).

4.4.3. Summary

The concentration profile of xylose, glucose and lignin dissolution shows that glucose yield in MHS $LiClO_4.2H_2O$ increases slightly slower than xylose until it reaches the equilibrium just after 7 hours. The xylose yield reached equilibrium after 3 hrs. It was also observed that the acid-soluble lignin was in a small amount and it dissolves during pre-treatment, and it did not inhibit the dissolution of either cellulose or hemicellulose.

The kinetic rate constants at 120°C, 140°C, 160°C, and 180°C are shown in Table 11 with their correspond R-squared values. These results show that the kinetic rate constant increased as the temperature increased. Chemical reactions are typically expected to proceed faster at higher temperatures and slower at lower temperatures. This is because thermal energy relates direction to motion at the molecular level. As the temperature rises, molecules move faster and collide more vigorously, greatly increasing the likelihood of bond cleavages and rearrangements.

Table 10 : Rate constant for glucose, xylose and lignin dissolution and their corresponding R-squared values

	Glucose		Xylose		ASL	
T (°C)	K_G	R^2	K_X	R^2	K_{ASL}	R^2
120	0.287	0.964	0.004	0.808	0.034	0.989
140	0.295	0.990	0.005	0.863	0.040	0.986
160	0.295	0.994	0.006	0.658	0.048	0.796
180	0.360	0.968	0.020	0.955	0.060	0.996

The apparent activation energies and regression coefficients (R^2) calculated for glucose, xylose and lignin dissolution are presented in Table 12.

Table 11: Calculated activation energies and R-squared value

	Ea (KJ/mol)	R^2
Glucose	15.002	0.848
Xylose	14.215	0.877
ASL	36.539	0.989

CHAPTER 5: Conclusions and Recommendations

To improve the economical use of corncob as a feedstock for bio-processing of lignocellulosic materials, an efficient pretreatment route was developed, optimized and the reactions associated with the process were examined. For characterization of corncob, it was found that the calculated crystallinity index of untreated corncob is 0.329 lower than that of other lignocellulosic biomass such as sugarcane bagasse, 0.5 or rice straw, 0.57 which implies that native corncob is a highly amorphous structure. The crystallinity index increased for pretreated material, indicating that the lignin and hemicellulose removal especially for the phosphoric acid and sodium hydroxide pretreated material. This is good evidence that the amorphous portion of the corncobs was removed with the liquid fractions and the crystalline portion remained on the solids residues. The FTIR and the SEM images were used to confirm the XRD interpretations.

5.1. Conclusions

Referring back to the first research objective and to answer the research questions, it is apparent that the best performing solvent in terms of sugar monomers dissolution is sulphuric acid followed by phosphoric acid. However, it was noted from the SEM images that acid is very harsh such that it completely destroys the structure of the corncob and the residues needs special disposal system that is expensive. In addition, the pretreatment liquor contains inhibitors that may limit the subsequent step i.e. enzymatic hydrolysis or fermentation. Because of the chemical properties associated with these acids and the concentration required, the application or an upscale in industry will not be feasible. The emerging industries consider the use of green solvent attractive and acid use is one of the methods they are moving away from, hence it was be disregarded in this study.

This investigation revealed that lithium perchlorate, $LiClO_4 \cdot 2H_2O$ is a promising pretreatment method for producing high sugar yields. Following the acid pretreatment, achieving a significant higher yield of 30 g/l in total analysed sugar monomers was achieved. This was high compared to other pre-treatment technique such as zinc chloride at 16 g/l and lithium chloride at 12.6 g/l. From this pre-treatment, it was found that disrupting the corncob complex structures results in destruction of lignin which in turn increased the cellulose accessibility. Even though lithium perchlorate is not regarded as a green chemical, it is a low-cost reagent and also environmental friendly to a certain extent. Furthermore, the pre-treatment kinetics was found to be is faster.

The response surfaces obtained in this study were in an unidentified nature, which according to literature suggest that the optimum conditions are not well defined. However the contour plots become reinforcement since it indicates both the nature and extent of the interactions. The contour and response surface model predicted the optimum levels of significant variables; solvent concentration, reaction time and temperature were found to be 1.87% solvent concentration, 3 hr 50 min and 176 °C temperature, respectively. The maximum corresponding glucose and xylose content was 14.26 g/l and 12.4 g/l, respectively.

To confirm the predicted model, four replicate experiments were performed and the response activities of each were determined. Finally, the average maximum yield reached 14.24 g/l and 11.9 g/l of xylose and glucose yield, respectively, which is comparable to the predicted value. As a result, the projected models were considered to be accurate and reliable for predicting the dissolution of xylose and glucose. The rate constant results showed that the reaction rate increased as the temperature increased. The resulting Ea were; 15.0 kJ/mol, 14.2 kJ/mol and 36.54 kJ/mol for glucose, xylose and lignin respectively. It is apparent that the activation energy associated with lignin is higher than that of glucose and xylose. This is an indication that more energy is required to release lignin from corncob.

5.2. Recommendations

For further studies on the feasibility of aqueous pre-treatment of South African corncob it is recommended that:

- Evaluation of the effect of $LiClO_4$ solvent on the subsequent step of bio-production process should be carried out
- Further studies are to be done on the optimization of the overall bio-production, this is because the verdict of the optimum pretreatment process was only limited to the $LiClO_4$ biomass pretreatment
- Pertinent adsorption capacity of biocatalyst & enzymes should be studied to further validate the efficacy of pretreatment process.
- To analyze the solid sample with Ion Chromatography to quantify the amount of xylooligosaccharides released pretreatment.

6. References

Ali, Z. and Arshad, M. (2014) 'Saccharification of corn cobs an agro-industrial waste by sulphuric acid for the production of monomeric sugars', (August).

Anukam, A. I. *et al.* (2017) 'Studies on Characterization of Corn Cob for Application in a Gasification Process for Energy Production', *Journal of Chemistry*, 2017(6478389), pp. 1–9.

Ashour, A. *et al.* (2013) 'Corncobs as a potential source of functional chemicals', *Molecules*, 18(11), pp. 13823–13830. doi: 10.3390/molecules181113823.

Awosusi, A. A. *et al.* (2017) 'Biocompositional and thermodecompositional analysis of South African agro-waste corncob and husk towards production of biocommodities', *Asia-Pacific Journal of Chemical Engineering*. doi: 10.1002/apj.2138.

Ayeni, A. O. *et al.* (2012) 'Optimization of pretreatment conditions using full factorial design and enzymatic convertibility of shea tree sawdust', *Biomass and Bioenergy*. Elsevier Ltd, 48, pp. 130–138. doi: 10.1016/j.biombioe.2012.10.021.

Ayeni, A. O. *et al.* (2013) 'Hydrogen peroxide and lime based oxidative pretreatment of wood waste to enhance enzymatic hydrolysis for a biorefinery : Process parameters optimization using response surface methodology', *Fuel*, 106, pp. 187–194. doi: 10.1016/j.fuel.2012.12.078.

Ayeni, A. O. *et al.* (2016) 'Effective alkaline peroxide oxidation pretreatment of shea tree sawdust for the production of biofuels: Kinetics of delignification and enzymatic conversion to sugar and subsequent production of ethanol by fermentation using saccharomyces cerevisiae', *Brazilian Journal of Chemical Engineering*, 33(1), pp. 33–45. doi: 10.1590/0104-6632.20160331s20140258.

Bardone, E. *et al.* (2014) 'Dilute-acid Hydrolysis of Cellulose to Glucose from Sugarcane Bagasse', *Chemical Engineering Transactions*, 38. doi: 10.3303/CET1438073.

Bensah, E. C. and Mensah, M. (2013) 'Chemical pretreatment methods for the production of cellulosic ethanol: Technologies and innovations', *International Journal of Chemical Engineering*, 2013. doi: 10.1155/2013/719607.

Binder, J. B. and Raines, R. T. (2010) 'Fermentable sugars by chemical hydrolysis of

biomass.', *Proceedings of the National Academy of Sciences of the United States of America*, 107(10), pp. 4516–21. doi: 10.1073/pnas.0912073107.

Boonsombuti, A., Luengnaruemitchai, A. and Wongkasemjit, S. (2013) 'Enhancement of enzymatic hydrolysis of corncob by microwave-assisted alkali pretreatment and its effect in morphology', *Cellulose*, 20(4), pp. 1957–1966. doi: 10.1007/s10570-013-9958-7.

Bounaim, N. (2013) 'Africa faces some serious environmental problems: Greenpeace', *SABC website*, (June 2013), pp. 5–7.

Carrier, M. *et al.* (2016) 'Using Apparent Activation Energy as a Reactivity Criterion for Biomass Pyrolysis', *Energy and Fuels*, 30(10), pp. 7834–7841. doi: 10.1021/acs.energyfuels.6b00794.

Chandra, R. *et al.* (2008) 'The characterization of pretreated lignocellulosic substrates prior to enzymatic hydrolysis, part 1: a modified Simons' staining technique.', *Biotechnology progress*. United States, 24(5), pp. 1178–1185. doi: 10.1002/btpr.33.

Chen, Y. *et al.* (2013) 'Understanding of alkaline pretreatment parameters for corn stover enzymatic saccharification.', *Biotechnology for Biofuels*, 6(1), p. 8. doi: 10.1186/1754-6834-6-8.

Dagnino, E. P. *et al.* (2013) 'Optimization of the acid pretreatment of rice hulls to obtain fermentable sugars for bioethanol production', *Industrial Crops and Products*, 42(1). doi: 10.1016/j.indcrop.2012.06.019.

Djioleu, A. and Carrier, D. J. (2016) 'Effects of Dilute Acid Pretreatment Parameters on Sugar Production during Biochemical Conversion of Switchgrass Using a Full Factorial Design', *ACS Sustainable Chemistry and Engineering*, 4(8), pp. 4124–4130. doi: 10.1021/acssuschemeng.6b00441.

Dong, L., Zhao, X. and Liu, D. (2015) 'Kinetic modeling of atmospheric formic acid pretreatment of wheat straw with " potential degree of reaction " models', *RSC Advances*. Royal Society of Chemistry, 5, pp. 20992–21000. doi: 10.1039/c4ra14634d.

Fargo, W. (2013) 'What Is Green Technology : Strategies', pp. 1–3.

Fuentes, L. L. G. *et al.* (2011) 'Kinetics of lime pretreatment of sugarcane bagasse to enhance

enzymatic hydrolysis', *Applied Biochemistry and Biotechnology*, 163(5), pp. 612–625. doi: 10.1007/s12010-010-9067-0.

Godin, B. *et al.* (2013) 'Chemical characteristics and biofuels potentials of various plant biomasses: Influence of the harvesting date', *Journal of the Science of Food and Agriculture*, 93(13). doi: 10.1002/jsfa.6159.

Goeij, S. De (2013) 'Quantitative analysis methods for sugars', *Amsterdam, Netherlands: Universiteit van Amsterdam*, p. 43p. Available at: https://esc.fnwi.uva.nl/thesis/centraal/files/f1341724672.pdf.

Gunst, R. F. (1996) 'Response surface methodology: process and product optimization using designed experiments', *Technometrics*, 38(3), pp. 284–286. doi: 10.2307/1270613.

Hsu, C. *et al.* (no date) 'Pretreatment and hydrolysis of cellulosic agricultural wastes with a cellulase- producing Streptomyces for bioethanol production'.

Kraber, B. S. (2014) 'Intro to Response Surface Methods Introduction to Response Surface Methods'.

Kumar, P. *et al.* (2009) 'Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production', *Industrial and Engineering Chemistry Research*, 48(8), pp. 3713–3729. doi: 10.1021/ie801542g.

Kumar, R. *et al.* (2009) 'Physical and chemical characterizations of corn stover and poplar solids resulting from leading pretreatment technologies', *Bioresource Technology*. Elsevier Ltd, 100(17), pp. 3948–3962. doi: 10.1016/j.biortech.2009.01.075.

Kumar, S., Negi, Y. S. and Upadhyaya, J. S. (2010) 'Studies on characterization of corn cob based nanoparticles', 1(3), pp. 246–253. doi: 10.5185/amlett.2010.9164.

Lee, H. V, Hamid, S. B. A. and Zain, S. K. (2014) 'Conversion of Lignocellulosic Biomass to Nanocellulose : Structure and Chemical Process', 2014.

Lee, R. A. *et al.* (2013) 'UV-Vis as quantification tool for solubilized lignin following a single-shot steam process', *Bioresource Technology*, 144, pp. 658–663. doi: 10.1016/j.biortech.2013.06.045.

Lei, H., Cybulska, I. and Julson, J. (2013) 'Hydrothermal Pretreatment of Lignocellulosic

Biomass and Kinetics', *Journal of Sustainable Bioenergy Systems*, 3(4), pp. 250–259. doi: 10.1533/9781845699611.1.3.

Liu, Q.-P. *et al.* (2012) 'Ionic liquids from renewable biomaterials: synthesis, characterization and application in the pretreatment of biomass', *Green Chem.*, 14(2), pp. 304–307. doi: 10.1039/C2GC16128A.

Maryana, R. *et al.* (2014) 'Alkaline Pretreatment on Sugarcane Bagasse for Bioethanol Production', 47, pp. 250–254. doi: 10.1016/j.egypro.2014.01.221.

Minitab (2016) *Getting Started with Minitab 17*, *Minitab Inc.*

Mu, B. and Wang, A. (2016) 'Adsorption of dyes onto palygorskite and its composites: A review', *Journal of Environmental Chemical Engineering*. Elsevier B.V., 4(1), pp. 1274–1294. doi: 10.1016/j.jece.2016.01.036.

Nagoor Gunny, A. A. and Arbain, D. (2013) 'Ionic Liquids: Green Solvent for Pretreatment of Lingnocellulosic Biomass', *Advanced Materials Research*, 701(May 2013), pp. 399–402. doi: 10.4028/www.scientific.net/AMR.701.399.

Nahman, A., Wise, R. and de Lange, W. (2009) 'Environmental and resource economics in South Africa: Status quo and lessons for developing countries', *South African Journal of Science*, 105(October), pp. 350–355.

NAMC (National Agricultural Marketing Council) (2012) 'Contribution Of Agricultural Industry Trusts To Agricultural Development And Transformation.', (March).

Of, S. *et al.* (2012) 'STUDY OF ENZYMATIC HYDROLYSIS OF PRETREATED', 7, pp. 4672–4682.

Oke, M. A. *et al.* (2016) 'Mixed Feedstock Approach to Lignocellulosic Ethanol Production — Prospects and Limitations', *BioEnergy Research*. BioEnergy Research. doi: 10.1007/s12155-016-9765-8.

Pointner, M. *et al.* (2014) 'Composition of corncobs as a substrate for fermentation of biofuels', *Agronomy Research*, 12(2).

Qing, Q., Yang, B. and Wyman, C. E. (2010) 'Xylooligomers are strong inhibitors of cellulose hydrolysis by enzymes', *Bioresource Technology*. Elsevier Ltd, 101(24), pp. 9624–

9630. doi: 10.1016/j.biortech.2010.06.137.

Ragauskas, A. J. *et al.* (2014) 'Lignin valorization: improving lignin processing in the biorefinery.', *Science (New York, N.Y.)*, 344(6185). doi: 10.1126/science.1246843.

Ratti, R. (2014) 'Ionic Liquids: Synthesis and Applications in Catalysis', *Advances in Chemistry*, 2014(3), pp. 1–16. doi: 10.1155/2014/729842.

Ravindran, R. and Jaiswal, A. K. (2016) 'Bioresource Technology A comprehensive review on pre-treatment strategy for lignocellulosic food industry waste : Challenges and opportunities', *Bioresource Technology*. Elsevier Ltd, 199, pp. 92–102. doi: 10.1016/j.biortech.2015.07.106.

SACNSP, S. A. C. for N. S. P. (2015) 'The Fossil Fuel Foundation', *Electricity, COP21, Energy Security, Emissions, Growth, Transport, Enviromental Impact - Can society find a sustainable solution? 3rd Energy Conference*, pp. 1–3.

Salvachúa, D. *et al.* (2016) 'Succinic acid production on xylose-enriched biorefinery streams by Actinobacillus succinogenes in batch fermentation.', *Biotechnology for biofuels*. BioMed Central, 9(1), p. 28. doi: 10.1186/s13068-016-0425-1.

Samuel, T.-M. (2011) *Bioethanol Fermentation of Corncob using Immobilised Yeast Cells*. University of the Witwatersrand.

Sannigrahi, P. and Ragauskas, A. J. (2013) 'Fundamentals of Biomass Pretreatment by Fractionation', in *Aqueous Pretreatment of Plant Biomass for Biological and Chemical Conversion to Fuels and Chemicals*. doi: 10.1002/9780470975831.ch10.

Satimanont, S., Apanee, L. and Sujitra, W. (2012) 'Effect of Temperature and Time on Dilute Acid Pretreatment of Corn Cobs', *International Journal of Chemical, Molecular, Nuclear, Materials and Metallurgical Engineering*, 6(4), pp. 316–320.

Sluiter, A. *et al.* (2012) 'Determination of Structural Carbohydrates and Lignin in Biomass Determination of Structural Carbohydrates and Lignin in Biomass', (April 2008).

Sluiter, J. B. *et al.* (2010) 'Compositional Analysis of Lignocellulosic Feedstocks. 1. Review and Description of Methods', *Journal of Agricultural and Food Chemistry*, 58(16), pp. 9043–9053. doi: 10.1021/jf1008023.

Vainio, U. (2007) *Characterisation of cellulose- and lignin-based materials using x-ray scattering methods METHODS*. University of Helsinki, Finland.

Vargas, F. *et al.* (2016) 'Biorefinery Scheme for Residual Biomass Using Autohydrolysis and Organosolv Stages for Oligomers and Bioethanol Production', *Energy and Fuels*, 30(10), pp. 8236–8245. doi: 10.1021/acs.energyfuels.6b00277.

Vaswani, S. (2010) 'Process Economics Program', (December).

Weerachanchai, P. and Lee, J. (2013) 'E ff ect of Organic Solvent in Ionic Liquid on Biomass Pretreatment'.

Wyman, C. E. (ed.) (2013) *Aqueous Pretreatment of Plant Biomass for Biological and Chemical Conversion to Fuels and Chemicals*. Chichester, UK: John Wiley & Sons, Ltd. doi: 10.1002/9780470975831.

Xiao, A. *et al.* (2015) 'Statistical optimization for tannase production by Aspergillus tubingensis in solid-state fermentation using tea stalks', *Electronic Journal of Biotechnology*, 18(3), pp. 143–147. doi: 10.1016/j.ejbt.2015.02.001.

Yan, L. *et al.* (2016) 'Physiochemical Characterization of Lignocellulosic Biomass Dissolution by Flowthrough Pretreatment', *ACS Sustainable Chemistry & Engineering*, 4(1), pp. 219–227. doi: 10.1021/acssuschemeng.5b01021.

Yang, B. & and Wyman, C. (2006) 'BSA Treatment to Enhance Enzymatic Hydrolysis of Cellulose in Lignin Containing Substrates', *Biotechnology and Bioengineering*, 94(4), pp. 610–617. doi: 10.1002/bit.

Yesim, Y. *et al.* (2013) 'Effect of Hydrogen Peroxide Concentration and Temperature on Alkali Pre-treatment of Sunflower Stalks for Fermentable Sugar', *ISESCO JOURNAL of Science and Technology*, 12(21), pp. 19–23. doi: 10.1155/2013/719607.19.

Yoo, C. G. (2012) 'Pretreatment and fractionation of lignocellulosic biomass for production of biofuel and value-added products', *Iowa State University*, p. 159. Available at: http://lib.dr.iastate.edu/cgi/viewcontent.cgi?article=3707&context=etd.

Zafar, S. (2014) 'Biofuels from Lignocellulosic Biomass', (December 2005), pp. 2–7. Available at: http://www.bioenergyconsult.com/what-is-lignocellulosic-biomass/.

Zhang, L. *et al.* (2015) 'Characterization of lignin derived from water-only and dilute acid flowthrough pretreatment of poplar wood at elevated temperatures', *Biotechnology for Biofuels*, 8(1), p. 203. doi: 10.1186/s13068-015-0377-x.

Zhao, C. *et al.* (2016) 'Enzymatic Hydrolysis and Physiochemical Characterization of Corn Leaf after H-AFEX Pretreatment', *Energy & Fuels*, p. acs.energyfuels.5b02817. doi: 10.1021/acs.energyfuels.5b02817.

Zhao, X., Zhang, L. and Liu, D. (2012) 'Biomass recalcitrance. Part I: the chemical compositions and physical structures affecting the enzymatic hydrolysis of lignocellulose Xuebing', *Biofuels, Bioproducts and Biorefining*, 6(3), pp. 246–256. doi: 10.1002/bbb.

Zheng, J. *et al.* (2014) 'Corn Cob: Substrate', *Green Chemistry*. Edited by C. E. Wyman. Chichester, UK: Royal Society of Chemistry, 1(3), p. 2050. doi: 10.1016/j.biotechadv.2011.05.005.

Zheng, J. and Lars Rehmann, S. (2014) *Mechanical Pretreatment of Corncobs for Bioethanol Production by a Twin-Screw Extruder Graduate Program in Chemical and Biochemical Engineering*. Available at: http://ir.lib.uwo.ca/etd (Accessed: 9 June 2016).

7. Appendices

Appendix A: Chemicals used as pretreatment agents

The solvents used were chosen in terms of availability, cost and mild-low operating conditions

5. Molten Hydrates Salts (MHS)
    a. 99.9 % Zinc Chlorides
    b. 99.0 % Lithium Chlorides
    c. 98.0 % Lithium Perchlorate
6. Dilute Acids
    a. 95-98% Sulfuric Acid: H_2SO_4
    b. 85 % Phosphoric Acid: H_3PO_4
    c. 99.99% Citric Acid: $C_6H_8O_7$
7. Dilute Base
    a. 50 % Sodium Hydroxide: NaOH
8. Others
    a. 99% Urea: CH_4N_2O
    b. 99 % Thiourea: CH_4N_2S
    c. 99.5 % Glycerol: $C_3H_8O_3$

Appendix B: Qualitative and Estimation of dissolved sugar

Qualitative analysis: Estimation of Reducing Sugar Using Benedict's Method

The Benedict's method was used as a qualitative method to give an indication of sugars present in the liquid fraction samples. The standard reducing sugar solution was prepared by dissolving 200 mg of glucose and xylose in 100 ml deionized water. Benedict's reagent was prepared using 100 mg of sodium citrate and 62.5 mg potassium thiocyanate which were dissolved in 300 ml of distilled water by warming gently and filtered. Copper sulphate is dissolved in 50 ml of water, and it is added with continuous stirring. 2.5 ml of 5% potassium ferricyanide is added and volume is made up to 500 ml with water.

5ml of Benedict's reagent was pippeted out into clear tubes. 5 ml of standard solution was added along with 600 mg of anhydrous sodium carbonate to provide the required alkalinity with a few porcelain bits and heated to boil on heating water bath. The same was done on the liquid sample solution. It should be noted that this methodology was used only as qualitative technique to give a quick estimation of sugar existence in the liquid sample. No quantitative results were obtained from this part of experiment

B.1. Qualitative analysis using Benedict's solution

Benedict qualitative test was performed to detect the presence of reducing sugar in the pretreatment liquor samples. The obtained data presented in Figure 4.8 shows that there is no reducing sugar or available in very low concentration in the liquid fractions to lend electrons that is to precipitate and produce the red colour. Based on this indication and material balance understanding, it was then assumed that the remaining sugar should be presence in the solid fraction. The thiourea and urea pretreatment liquors formed brown precipitates (far right) indicating the likelihood of reducing sugar presence in the solution. However this was overruled by literature which suggest that the brown precipitate was due to the evaporation which forms either an aldehyde or a ketone bondage and thus react to forms a complex which generates a brown colour precipitates (Goeij, 2013)

As shown in the XRD results and the SEM imaging it can then be projected that most of the reducing sugars should be accessible from the solid fractions. This was then concluded by the analysis of the released sugars from the solids which is reported in section 4.3 of this chapter.

As shown in Figure B.1, the pretreatment liquors contained none or very little concentration of reducing sugars. This was compared to see if they correspond with the results obtained from the HPLC analysis, presented in section 4.3.



Figure B.1: Qualitative analysis of Benedict's test

B.2. Quantification of released sugars

In order to demonstrate efficacy of the investigated pretreatment technique, the High Performance Liquid Chromatography (HPLC) method was applied to quantify the sugar monomers on the corncob pretreatment liquor and the accessible sugar in the solid fraction. Separation of the sugar monomers was achieved by an Aminex HPX-87H column (BioRad, USA) at 60°C. The sugar monomers of interest were glucose, xylose and fructose including the oligosaccharides.

These studies were undertaken in order to determine the main properties involved in the dissolution process. Since the effect of solvent pretreatment on the chemical structure and composition of the corncob has been outlined, in this section the sugar releasing capability was quantified. For biomass pretreatment, two regimes are reported frequently in literature, either higher temperature & dilute concentration or low temperature & high concentration (Li et al, 2015). Both regimes have been tested to identify which one enhances sugar solubilisation and dehydration (Wang, 2012). At low temperature partial or incomplete dissolution were observed, whereas at high temperature complete dissolutions were guaranteed. However it upholds the potential undesirable decomposition of the pretreatment

solvent species & product degradation. Thus corncob pretreatment was carried out at mild conditions, temperature of 70-80 °C, time 3.5 – 6 hours and concentration of 0-10% w/v or 0.1-2.0 M. For this part of the study, the experiment conditions were maintained in these parameters for all the different solvent pretreatment processes.

HPLC Calibration

Reference solution was prepared by dissolving glucose, xylose and fructose reagent in 100 ml distilled water. Dilutions of different concentrations ranging from 0.5 g/l, 1.0 g/l, 2.0 g/l, and 4.0 g/l to 24 g/l were made by pipetting out stock solution into volumetric flask. Final volume to each volumetric flask was made up to 100 ml with the addition of distilled water. The retention time was noted at 2.6, 3.448 and 7.667 min for fructose, glucose and xylose respectively. The concentration of the monomers (g/l) was plotted against respective peak area values at corresponding retention time to get standard calibration curve shown in Figure B.2

The amount of monomers in the pretreatment liquor was determined using 5 ml of sample in a HPLC vials. The area detected by HPLC was converted into g/l of monomer using the standard curve and the Beer Lambert law (Chi, 2012).

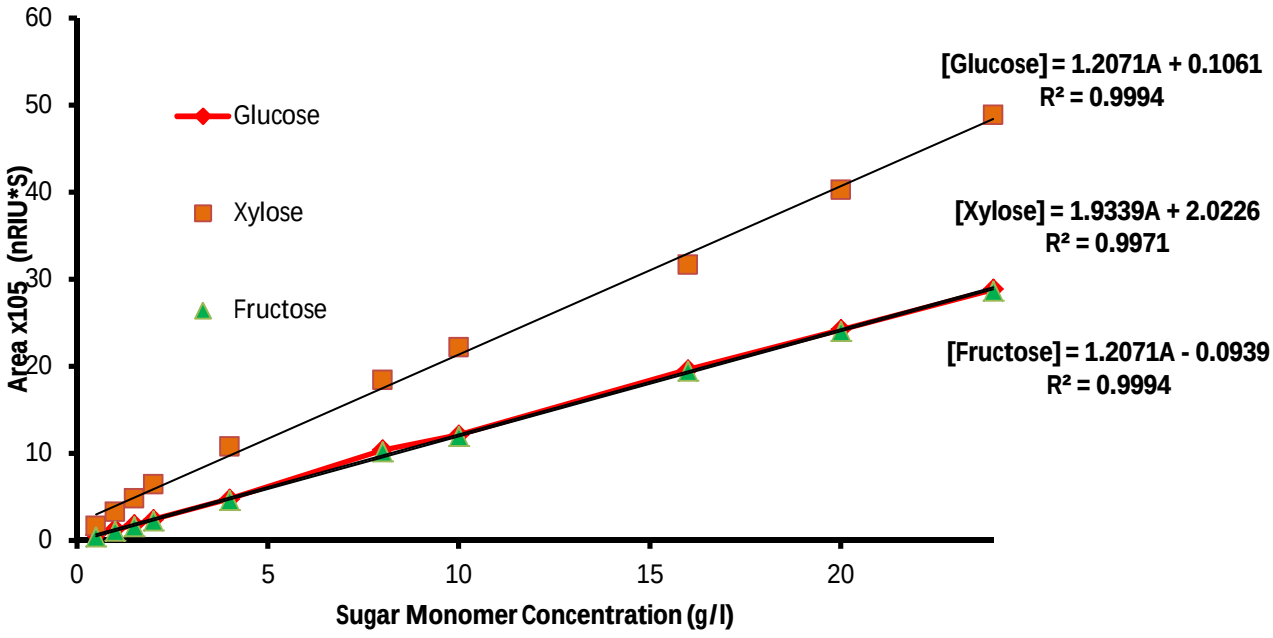


Figure B.2: Standard calibration curve for estimation of glucose.

Appendix C: Minitab Raw data

Central Composite Design

Design Summary

Factors:	3	Replicates:	1
Base runs:	20	Total runs:	20
Base blocks:	1	Total blocks:	1

α = 1.68179

Two-level factorial: Full factorial

Point Types

Cube points:	8
Center points in cube:	6
Axial points:	6
Center points in axial:	0

Response Surface Regression: Glucose (g/l) versus ... e, Concentration

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	224.454	24.9393	3.19	0.043
Linear	3	213.079	71.0262	9.08	0.003
Temperature	1	77.904	77.9038	9.96	0.010
Time	1	50.341	50.3414	6.44	0.029
Concentration	1	84.833	84.8335	10.85	0.008
Square	3	52.391	67.1303	10.02	0.005
Temperature*Temperature	1	56.196	56.1959	10.03	0.008
Time*Time	1	79.009	79.0093	9.00	0.009
Concentration*Concentration	1	81.233	81.2333	12.04	0.005
2-Way Interaction	3	10.847	3.6156	0.46	0.715
Temperature*Time	1	4.713	4.7129	0.60	0.455
Temperature*Concentration	1	3.085	3.0852	0.39	0.544
Time*Concentration	1	3.049	3.0489	0.39	0.546
Error	10	78.184	7.8184		
Lack-of-Fit	5	77.973	15.5945	368.26	0.000
Pure Error	5	0.212	0.0423		

Total	19	302.638

Model Summary

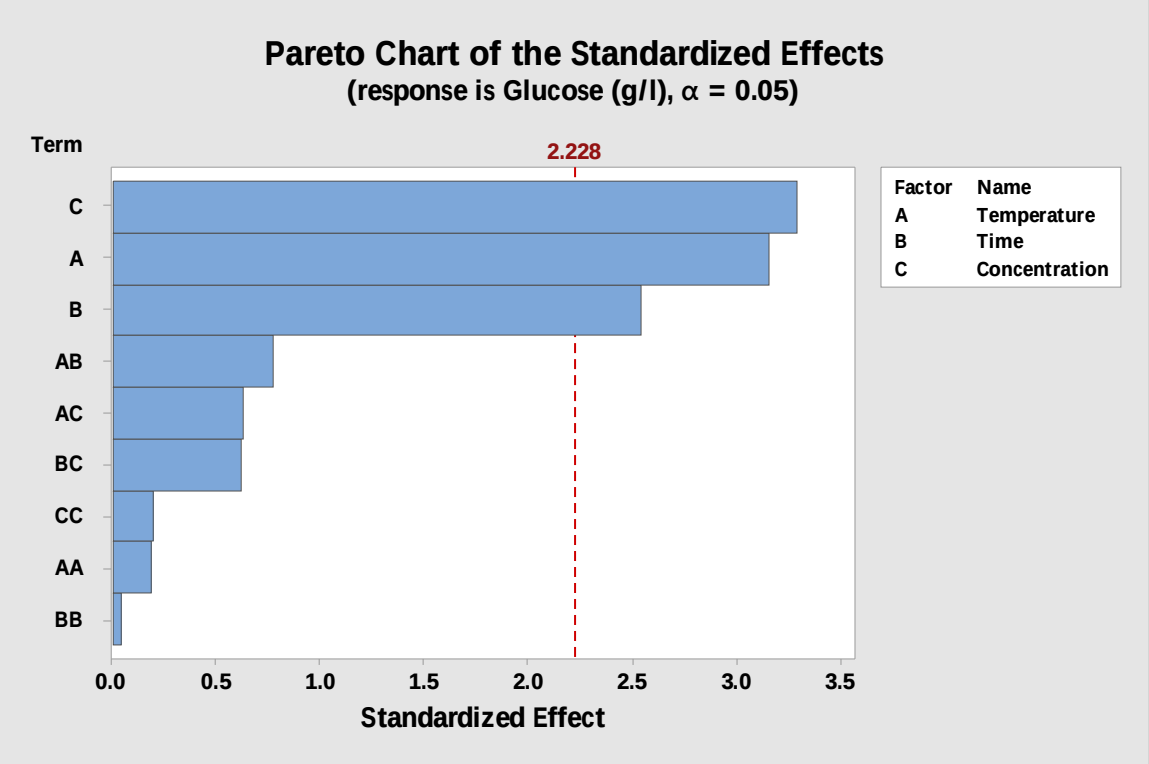
S	R-sq	R-sq(adj)	R-sq(pred)
2.79615	82.17%	80.91%	82.00%

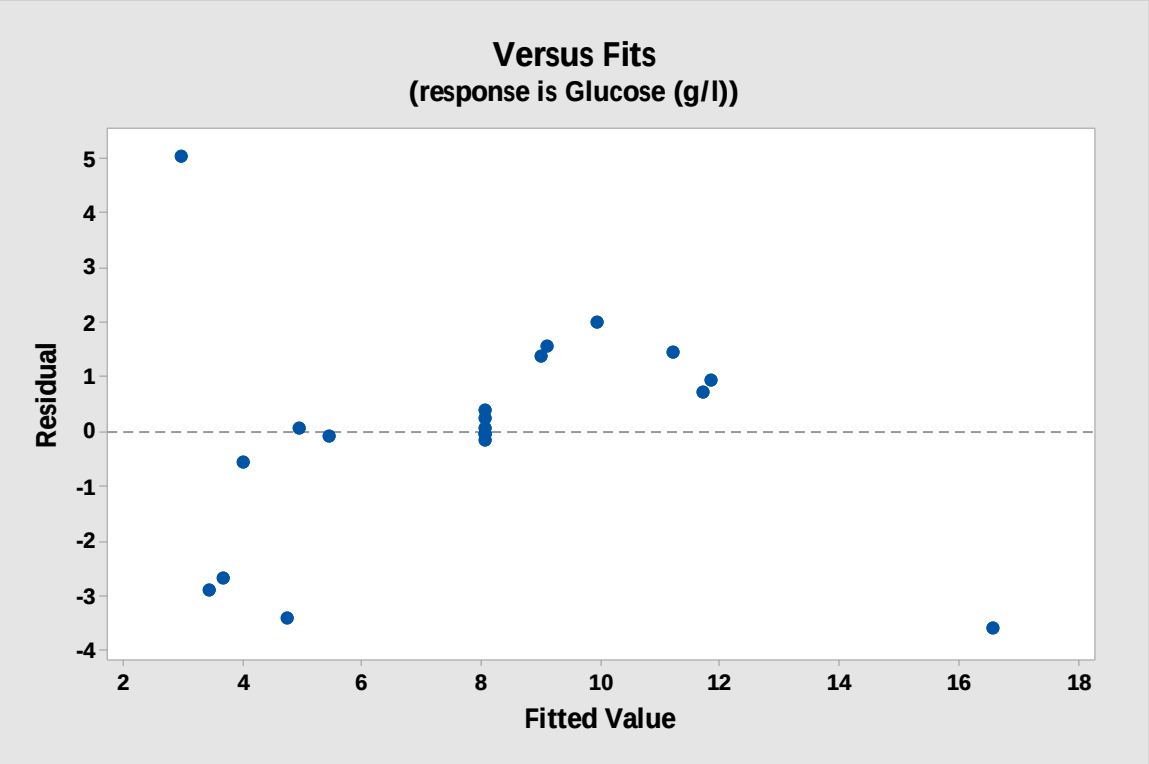
Coded Coefficients

Term	Coef	SE Coef	T-Value	P-Value
Constant	8.21	1.14	17.07	0.000
Temperature	1.32	0.757	13.16	0.010
Time	-1.58	0.757	12.54	0.029
Concentration	0.48	0.757	13.29	0.008
Temperature*Temperature	0.96	0.737	10.18	0.008
Time*Time	0.67	0.737	10.04	0.009
Concentration*Concentration	-1.61	0.737	10.20	0.005
Temperature*Time	-0.63	0.989	6.78	0.055
Temperature*Concentration	-2.96	0.989	6.63	0.044
Time*Concentration	-0.42	0.989	8.62	0.046

Regression Equation in Uncoded Units

Glucose (g/l) = 8.21 + 1.32 Temperature (C) - 1.58 Time (hr) + 0.48 Concentration (%)
+ 0.96 Temperature (C)*Temperature (C) + 0.67 Time (hr)*Time (hr)
- 1.61 Concentration (%)*Concentration (%) - 0.63 Temperature (C)*Time (hr)
- 2.96 Temperature (C)*Concentration (%) - 0.42 Time (hr)*Concentration (%)


Pareto Chart of the Standardized Effects
(response is Glucose (g/l), α = 0.05)
Term
2.228
C
A
B
AB
AC
BC
CC
AA
BB
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Standardized Effect
Factor Name
A Temperature
B Time
C Concentration


Versus Fits
(response is Glucose (g/l))
Residual
5
4
3
2
1
0
-1
-2
-3
-4
2
4
6
8
10
12
14
16
18
Fitted Value

Response Surface Regression: Xylose versus Temperature, Time, Concentration

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	166.006	18.4451	3.19	0.043
Linear	3	157.593	52.5310	9.08	0.003
Temperature	1	57.618	57.6177	9.96	0.010
Time	1	37.232	37.2325	6.44	0.029
Concentration	1	62.743	62.7428	10.85	0.008
Square	3	52.391	67.1303	10.02	0.005
Temperature*Temperature	1	56.196	56.1959	10.03	0.008
Time*Time	1	79.009	79.0093	9.00	0.009
Concentration*Concentration	1	81.233	81.2333	12.04	0.005
2-Way Interaction	3	8.022	2.6741	0.46	0.715
Temperature*Time	1	3.486	3.4856	0.60	0.455
Temperature*Concentration	1	2.282	2.2818	0.39	0.544
Time*Concentration	1	2.255	2.2549	0.39	0.546
Error	10	57.825	5.7825		
Lack-of-Fit	5	57.669	11.5337	368.26	0.000
Pure Error	5	0.157	0.0313		
Total	19	223.831			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2.40469	84.17%	82.81%	80.28%

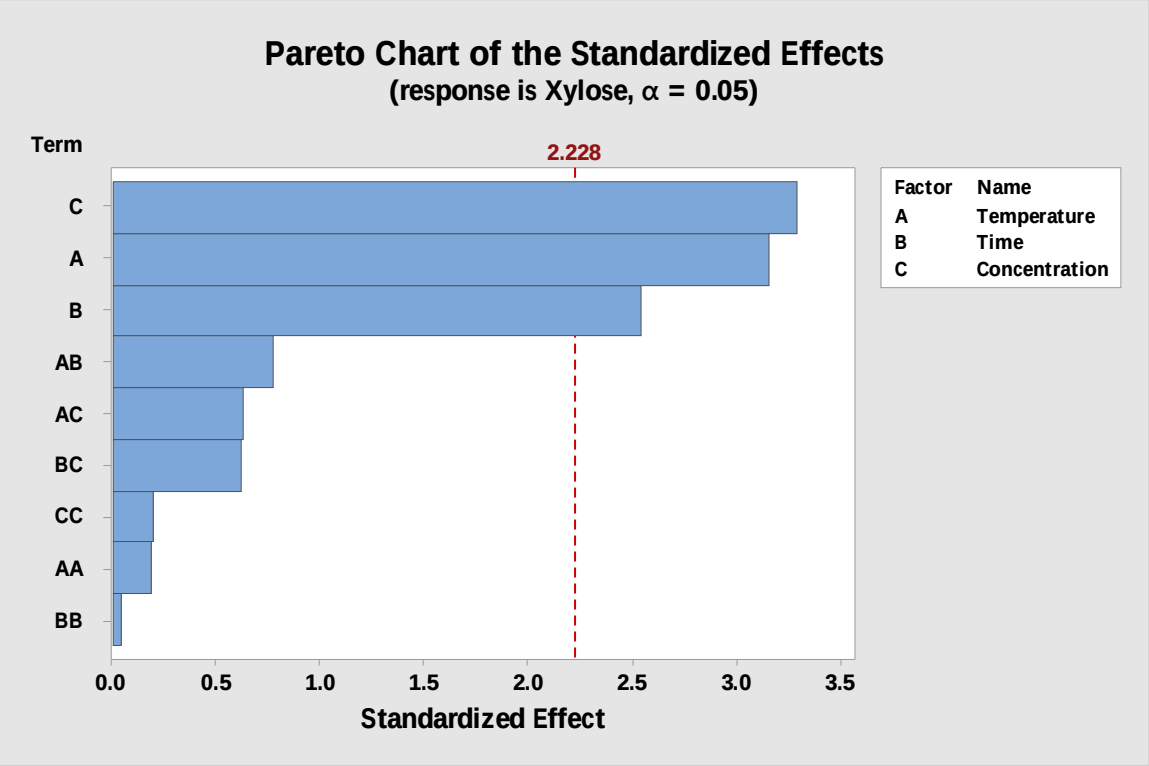
Coded Coefficients

Term	Coef	SE Coef	T-Value	P-Value
Constant	7.06	0.981	17.07	0.000
Temperature	1.13	0.651	13.16	0.010
Time	-1.36	0.651	12.54	0.029
Concentration	0.41	0.651	13.29	0.008

Temperature*Temperature	0.82	0.633	10.18	0.008
Time*Time	0.58	0.633	10.04	0.009
Concentration*Concentration	-1.38	0.633	10.20	0.005
Temperature*Time	-0.55	0.850	6.78	0.005
Temperature*Concentration	-2.55	0.850	4.63	0.044
Time*Concentration	-0.36	0.850	8.62	0.046

Regression Equation in Uncoded Units

Xylose (g/l) = 7.06 + 1.13 Temperature (C) - 1.36 Time (hr) + 0.41 Concentration (%)
+ 0.82 Temperature (C)*Temperature (C) + 0.58 Time (hr)*Time (hr)
- 1.38 Concentration (%)*Concentration (%) - 0.55 Temperature (C)*Time (hr)
- 2.55 Temperature (C)*Concentration (%) - 0.36 Time (hr)*Concentration (%)



Response Optimization: Xylose, Glucose (g/l)

Parameters

Response	Goal	Lower	Target	Upper	Weight	Importance
Xylose	Maximum	0.4644	11.1284		1	1
Glucose (g/l)	Maximum	0.5400	12.9400		1	1

Solution

Solution	Temperature (C)	Time (hr)	Concentration (%)	Xylose (g/l) Fit	Glucose (g/l) Fit	Composite Desirability
1	1.54179	0.45079	0.890671	11.6728	14.7125	1

Prediction for Xylose, Glucose (g/l)

Multiple Response Prediction

Variable	Setting
Temperature	1.545
Time	0.45
Concentration	0.89

Response	Fit	SE Fit	95% CI	95% PI
Xylose	12.37	12.28	(9.12, 32.65)	(7.96, 33.81)
Glucose (g/l)	14.26	14.14	(10.61, 37.96)	(9.25, 39.31)

Appendix D: Copy of Publication