

CONVERSION OF LIGNOCELLULOSIC MATERIAL INTO FERMENTABLE SUGARS

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Dedication

To my parents

wife

and my beloved children

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Abstract

Lignocellulosic biomass to biofuel conversion is a promising technology to provide a unique and sustainable resource for environmentally safe organic fuels and chemicals. Most of global energy use projections predict that biomass will be a more important component of primary energy supply in the future, and that woody crops will be the primary source of biomass.

Short-rotation willow wood crops (*Salix* sp.) are considered a promising source of bioenergy, willow wood has several characteristics that make it ideal for woody crop systems, including high yields obtained in a few years, ease of vegetative propagation, a broad genetic base, a short breeding cycle and the ability to resprout after multiple harvests.

Unfortunately, enzymatic hydrolysis of lignocellulosic biomass has been shown to be a limiting factor in the conversion of biomass to chemicals and fuels. This limitation is due to inherent structural features (i.e., acetyl content, lignin content, crystallinity, surface area, particle size and pore volume) of biomass. These structural features are barriers which prevent complete hydrolysis, therefore, pretreatment techniques are necessary to render biomass highly digestible.

In oxidative short-term lime pretreatment, willow wood is mixed with an excess of calcium hydroxide (0.4 g $\text{Ca}(\text{OH})_2$ /g raw biomass) and water loading (15 g/g raw biomass). Lime pretreatment is carried out for various periods of time (1, 2, 3.5, 5, 6) hours, temperatures at (100, 113, 130, 147, 160°C) and pressures of oxygen as oxidative agent (6, 9, 13.5, 17.8, 21 bar (absolute)).

The optimization depended on the maximum overall yields of glucan (g glucan hydrolyzed/100 g original glucan) and xylan (g xylan hydrolyzed/100 g original xylan) by using a cellulase loading of 0.1 g enzyme /g glucan in raw biomass, at substrate concentration 50 g/L during 72 h of enzymatic hydrolysis. The optimal conditions for oxidative short-term lime pretreatment of willow wood were as follow: (1) 1.33 h, 147°C, 17.8 bar absolute, 0.26 g $\text{Ca}(\text{OH})_2$ /g raw biomass and (2) 1.25 h, 155°C, and 21 bar absolute, 0.26 $\text{Ca}(\text{OH})_2$ /g raw biomass.

In these two cases, the reactivity was nearly identical, thus the selected condition depends on the economic trade off between pressure, temperature. The optimal particle size was less than 3 mm. Enzymatic hydrolysis under these recommended conditions were as follow: 96.00 g glucan/100 g of glucan in raw biomass, and 65.00 g xylan/100 g xylan in raw biomass.

Nomenclature

Abbreviations

<i>AC</i>	Anhydro correction, it is 0.9 for glucose and 0.88 for xylose	
<i>AFEX</i>	Ammonia Fiber Explosion	
<i>ARP</i>	Ammonia Recycled Percolation	
<i>BG</i>	β -glucosidase enzyme	
<i>CBH</i>	cellobiohydrolases enzyme	
<i>C_HPLC</i>	Concentration of the sugar as given by HPLC	[g/L]
<i>cm</i>	centimeter	
<i>CP</i>	constant pressure	
<i>DI</i>	deionized water	
<i>DP</i>	degree of polymerization	[unit]
<i>D_{anhydro}</i>	glucan or xylane hydrolyzed to sugar	[g]
<i>EC</i>	exoglucanases enzyme	
<i>EG</i>	endoglucanases enzyme	
<i>g</i>	gram	
<i>h</i>	hour	
<i>HMF</i>	5-hydroxymethyl furfural	
<i>HPLC</i>	High-performance liquid chromatography	
<i>IPCC</i>	The Intergovernmental panel on Climate Change	
<i>K</i>	number of variables	
<i>K_a</i>	acid dissociation constant	
<i>mL</i>	milliliter	
<i>mm</i>	millimeter	
<i>min</i>	minute	
<i>N</i>	number of experiments	
<i>ND</i>	Not determined)	

<i>NREL</i>	National Renewable Energy Laboratory	
<i>pKa</i>	$\text{PKa} = -\log_{10} Ka$	
<i>ppm</i>	par per million	
<i>psi</i>	pounds per square inch	
<i>rpm</i>	Revolutions per minute	
<i>RPM – Monosaccharide</i>	Rezex Pd Monosaccharide	
<i>SSF</i>	simultaneous saccharification fermentation	
<i>SRC</i>	short-rotation coppice	
<i>Std.Dev</i>	Standard deviation	
<i>SRS</i>	Sugar Recovery Standards	
<i>1t</i>	1 t = 1000	[kg])
<i>UV</i>	Ultra Violet	
<i>V</i>	volume	[L]
<i>X₁</i>	temperature	[°C]
<i>X₂</i>	pressure	[bar]
<i>X₃</i>	time	[hour]
<i>VP</i>	varying pressure	[bar]
<i>Y_G</i>	Pretreatment yield of glucan	
<i>Y_g</i>	hydrolysis yield of glucan	
<i>Y_T^G</i>	Overall yield of glucan	
<i>Y_L</i>	Pretreatment yield of lignin	
<i>Y_{Lf0}</i>	yield of lignin for fast-region at time zero	
<i>Y_{Ls0}</i>	yield of lignin for slow-region at time zero	
<i>Y_P</i>	total solids pretreatment yield	[gram]
<i>yr</i>	year	
<i>Y_T^S</i>	Overall yield of total sugar (cellulose and hemicellulose)	
<i>Y_X</i>	Pretreatment yield of xylan (hemicellulose)	
<i>Y_x</i>	hydrolysis yield of xylan (hemicellulose)	
<i>Y_T^X</i>	Overall yield of xylan	
<i>W</i>	Dry biomass	[g]
Latin Letters		
Greek symbols		
α	\sqrt{K}	

$\mu\text{ m}$ micrometer $[10^{-6} \times m]$

Introduction

The Intergovernmental panel on Climate Change (IPCC) reports have shown that global surface temperatures have increased by 0.3-0.6°C since the late nineteenth century [14]. There were being suggestions that the increase in global temperature strongly correlates with atmospheric CO₂ concentration which has increased 24% (from 290 to 360 ppm) since 1860, and also found that average temperature will increase 1.5-4.5°C if atmospheric CO₂ concentration doubles [15]. This rapid increase in atmospheric CO₂ results mainly from fossil fuel combustion, which currently generates about 2.2×10^{10} t of CO₂ annually [16]. An additionally the depletion of global fossil fuels and increasing energy demand lead to increase price of fossil fuels.

In order to reduce net CO₂ emissions and global warming, fossil fuel combustion must decrease, and the use of alternative environmentally friendly energy must increase.

Lignocellulosic biomass gives an unique and sustainable resource for environmentally safe organic fuels and chemicals. However, the digestibility of lignocellulosic biomass is low owing to structural features such as lignin content, acetyl groups and crystallinity. Hence, lignocellulosic biomass must be pretreated to enhance its digestibility before conversion to biofuel.

1.1 Biomass conversion to biofuel

Lignocellulosic biomass is the most widely available source of carbohydrates, however, this feedstock is not readily digestible. To overcome this difficulty, lignocellulose structure must be modified through pretreatment [17].

There are three major components of lignocellulosic biomass, cellulose, hemicellulose and lignin. Cellulose and hemicellulose are not directly available for bioconversion because of their intimate association with lignin [18].

For degrading biomass to sugars, there are two basic approaches to convert biomass to sugars: enzymatic hydrolysis and dilute acid hydrolysis. Compared to dilute acid hydrolysis, the enzymatic approach is promising because it can achieve high sugar yields and eliminate the need for large quantities of chemicals, and the formation of inhibitory by-products during dilute acid hydrolysis [19].

To increase the enzymatic digestibility of lignocellulosic biomass, we treat the biomass mechanically (reducing size ≤ 4 mm, ball milling) and chemically (e.g., acid/alkali treatment). The treated biomass will become more readily available to enzymatically hydrolysis of their sugars by cellulase and hemicellulase enzymes and the resulting sugars are fermented to ethanol or other biofuel by microorganisms via fermentation process [20].

Alternatively, biomass can be converted to mixed acids by a mixed-culture fermentation using the MixAlco process as shown in Figure 1.1. The latter process converts lignocellulosic biomass directly into carboxylate salts using rumen or marine microorganisms. The carboxylate salts are thermally converted to ketones and then hydrogenated to produce mixed ($C_2 - C_{13}$) alcohols [20].

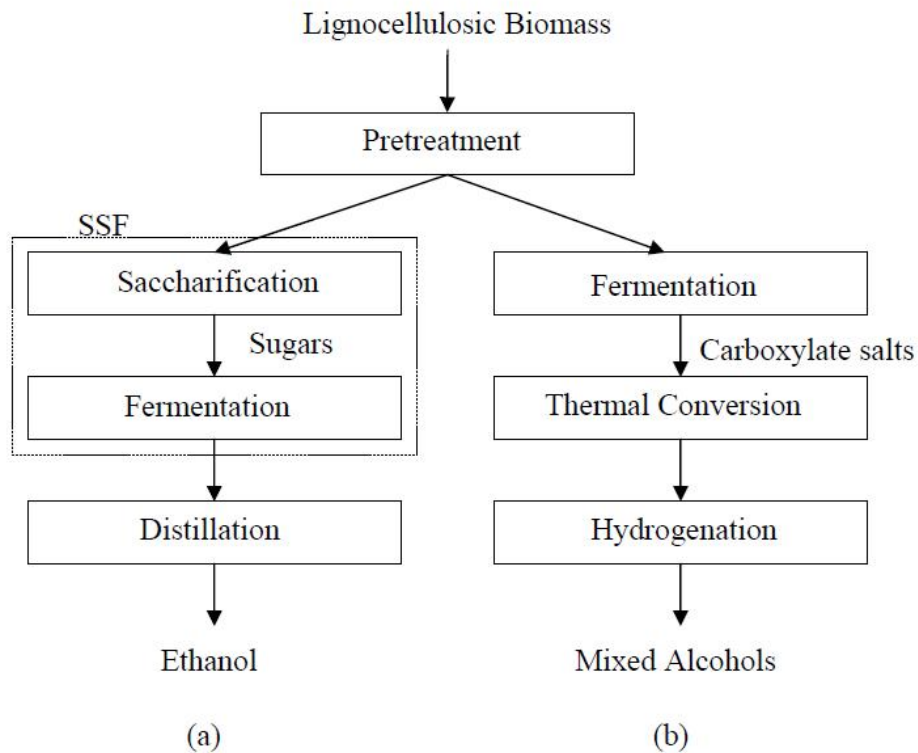


Figure 1.1: Schematic diagram of biomass conversion to ethanol and MixoAlcohol [?]

1.2 Poplar and willow wood as feedstock

Biomass comes from a number of different sources involving forests, agricultural crops, various residue streams and dedicated woody or herbaceous crops. Biomass currently provides about 10.5% of the global primary energy supply, although some is not being produced and used sustainably [21].

Most of global energy use projections predict that biomass will be a more important component of primary energy sources in the future and that woody crops will be the primary source of biomass [22]. In addition to combustion and gasification conversion pathways for power and heat production, woody crops represent a uniform, locally available feedstock for the production of liquid fuels, chemicals and other advanced materials currently made from petroleum products [20].

There has been increasing interest in developing dedicated woody crops grown on short rotation. More specifically short-rotation coppice (SRC) describes any high-yielding woody species managed in a coppice system. Typically these crops are harvested on a 3-5 yr rotation and remain viable for 15-30 yr. Dedicated SRC energy crops, such as poplar (*Populus* spp.) and willow (*Salix* spp.) are grown commercially for heat and power generation as a consequence of their rapid growth rate and favourable energy ratio. Provided local markets exist, SRC offers growers the chance to diversify into nonfood crops [23].

Short-rotation willow (*Salix* sp.) is considered a promising source of bioenergy, i.e. for the production of heat and power. An alternative, perhaps in a longer perspective, it is possible to convert the carbohydrate fraction of *Salix* into a liquid fuel, ethanol and to use the solid residue, mainly lignin, for heat and power generation [24].

Willow wood has several characteristics that make it ideal for woody crop systems, including high yields obtained in a few years, ease of vegetative propagation, a broad genetic base, a short breeding cycle and the ability to resprout after multiple harvests. There are about 450 species of willow worldwide, ranging from prostrate, dwarf species to trees that are over 40 m high. The willows used in woody crop systems are primarily drawn from the subgenus *Caprisalix* (Vetrix), which includes over 125 species worldwide. While they share many characteristics, their growth habits, life history and resistance to pests and diseases vary. This diversity is important in the successful development of woody crops [25].

1.3 Structure of lignocellulosic biomass

Lignocellulose is the three-dimensional polymeric composite formed by plants as structural material. It consists of variable amounts of cellulose, hemicellulose and lignin. Cellulose is a linear polysaccharide of glucose residues connected by β -1,4 linkages. The cellulose degree of polymerization (DP) is about (10,000), although chemical pulping reduces this greatly. Cellulose molecules form intra- and inter-molecular hydrogen bonds that result in highly ordered crystalline as shown in Figure 1.2. Cellulose is relatively inert to chemical treatment and insoluble in most solvents [2].

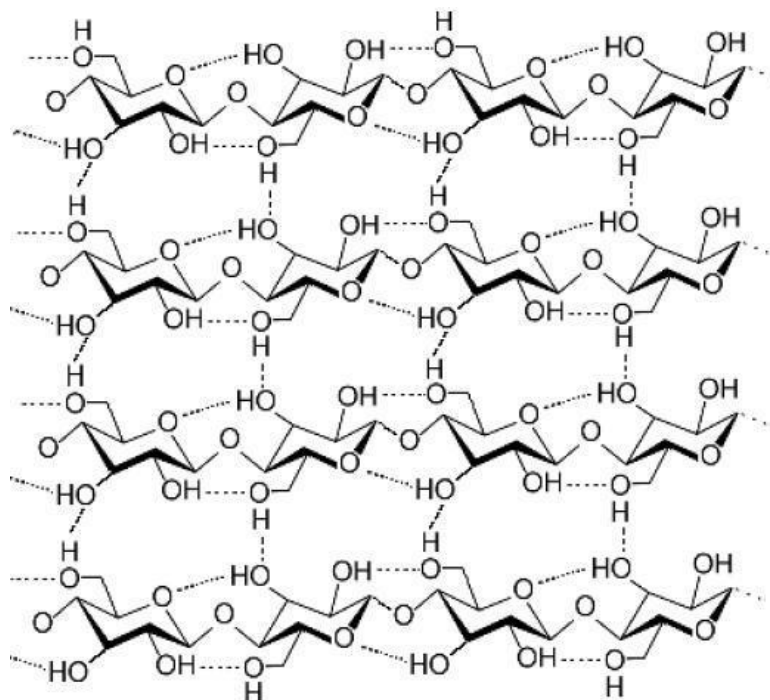


Figure 1.2: Cellulose structure [1]

Hemicellulose is a short, highly branched chain of heteropolysaccharides (DP 100-200) built from hexoses (D-glucose, D-mannose, and D-galactose), pentoses (D-xylose, L-arabinose, and D-arabinose), and deoxyhexoses (L-rhamnose or 6-deoxy-L-mannose and rare L-fucose or 6-deoxy-L-galactose). Small amounts of uronic acids (4-O-methyl-D-glucuronic acid, D-galacturonic acid and D-glucuronic acid) are also present (Figure 1.3) [26]. The monosaccharides released upon hemicellulose hydrolysis include a large fraction of pentoses. The chemical and thermal stability of hemicellulose is lower than cellulose due to its lack of crystallinity and lower DP [2].

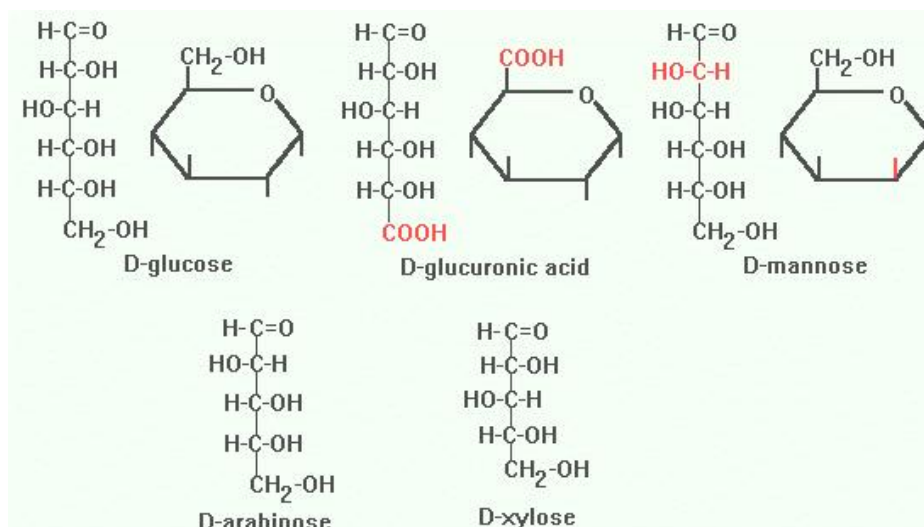


Figure 1.3: Hemicellulose component [1]

Lignin is a phenylpropane-based polymer and is the largest non-carbohydrate fraction of lignocellulose. It is constructed of three monomers: coniferyl alcohol, sinapyl alcohol, and coumaryl alcohol (Figure 1.4). Each has an aromatic ring with different substituents. Unlike cellulose, lignin can't be depolymerized to its original monomers. Lignin and hemicellulose form a sheath that surrounds the cellulosic portion of the biomass [27].

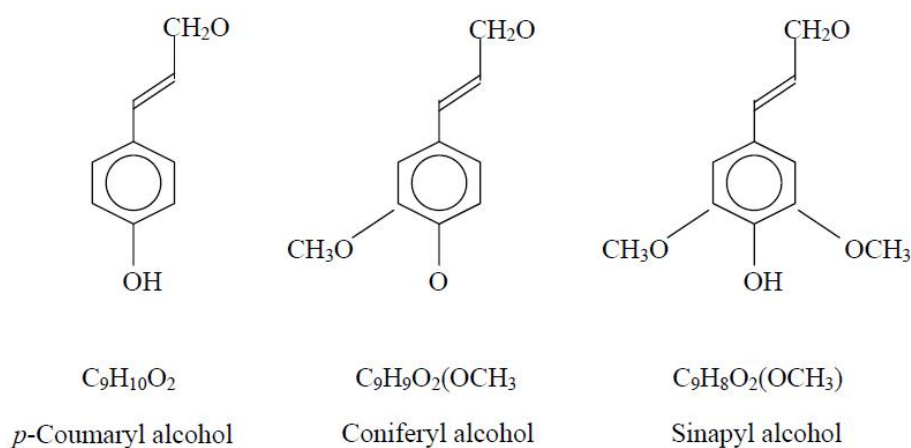


Figure 1.4: Building blocks of lignin [2]

1.4 Effects of structural features on biomass digestibility

The enzymatic hydrolysis of lignocellulose is affected by many factors. The limiting factors have been traditionally divided into two groups: The first is related to lignocellulose structural features and the second related to the mechanism and interactions of the cellulolytic enzymes, however, the heterogeneous nature of lignocellulose and the multiplicity of enzymes make it difficult to fully understand the interactions between enzyme and lignocellulose, furthermore, the interactions change during hydrolysis progress, therefore it is apparent that the rate and extent of biomass hydrolysis are inextricably linked to biomass structural features.

Generally, structural features can be categorized into two groups: physical and chemical. Physical structural features include cellulose crystallinity, degree of cellulose polymerization, pore volume, accessible surface area and particle size. Chemical structural features include the contents of lignin, hemicellulose and acetyl groups [5]. more detail of structural features in the following items

- Lignin content

Lignin plays a significant role in the rate and extent of lignocellulose hydrolysis. Literature results have all shown that cellulose digestibility enhances with increasing lignin removal [13, 28–30]. The major inhibitory role of lignin has been attributed to nonspecific adsorption of enzyme to lignin [31, 32], and enzyme inaccessibility to cellulose due to steric hindrance [33].

- Acetyl content

Xylan backbones in native plant cell walls are extensively acetylated [26]. Several studies showed that the removal of acetyl groups from hemicellulose reduced the steric hindrance of enzymes and greatly enhanced cellulose and xylan digestibility [34, 35]. The acetate groups interfere with enzyme recognition thereby slowing the hydrolysis rate [5].

- Crystallinity

It is broadly accepted that highly crystalline cellulose is less accessible to cellulase attack than amorphous cellulose, therefore, crystallinity affects the efficiency of enzyme contact with cellulose [13]. Although a negative relationship between crystallinity and digestibility has been reported, some researchers proposed that the effect of reduced crystallinity on hydrolysis rate might be a consequence of increased surface area [29], or decreased particle size [36], because ball milling tends to decrease the particle size and crystallinity of biomass and increase the specific surface area simultaneously [37]. Also there are several investigations showing that further reduction of particle size below 40 mesh (0.4 mm) did not

enhance the hydrolysis rate [30, 38]. Decrease in both crystallinity and specific surface area were observed when cellulose was ball milled for 96 h, whereas the extent of hydrolysis still increased significantly [39].

- Accessible surface area

Accessible surface area of lignocellulosic biomass is a crucial factor that affects digestibility. There is a positive correlation between accessible surface area and biomass digestibility [28, 39]. However, accessible surface area was not considered as a dependent factor that affects cellulose digestibility [40], because it may correlate with cellulose crystallinity or lignin removal [41]. In addition, accessible surface area is intimately coupled with pore volume.

Although these structural features are divided into two groups (physical and chemical), interactions exist among the structural features in the two groups. For example, lignin removal changes the percentage of cellulose and hemicellulose, pore volume and accessible surface area. Table 1.1 summarizes the relationship between structural features and biomass digestibility [5].

Table 1.1: Summary of relationship between structural features and digestibility [5]

Structural Features		Relationship between structural features and digestibility	Reference
Physical	Surface Area	Positive	[36]
	Crystallinity	Negative	[37]
		No correlation	[36]
	Degree of polymerization	Negative	[42]
		No correlation	[41]
	Pore volume	Positive	[36]
Chemical	Particle size	No correlation	[30]
	Lignin	Negative	[30]
	Hemicellulose	Negative	[43]
	Acetyl group	Negative	[43]

Among all of these structural features, lignin content, acetyl content and crystallinity, are key features affecting biomass digestibility because they are characteristic factors of the three main components of lignocellulose (lignin, hemicellulose, and cellulose). Furthermore, these parameters can be independently manipulated in pretreatment processes and are easy to measure. This does not rule out other factors affecting biomass digestibility. For example, several researchers correlated the hydrolysis rate to pore volume [44], degree of polymerization [42], and particle size [30, 41].

1.5 Pretreatments

Many structural feature and compositional factors prevent the enzymatic access to cellulose fibers in lignocellulosic biomass. In the pretreatment step we aimed to find a balance between opening the fibers to increase water access and enzyme accessibility while minimizing sugar loss and inhibitor generation to ensure high yields and a substrate suitable for enzymatic hydrolysis and fermentation. The different methods of pretreatments cause physical and/or chemical changes in the plant biomass in order to break the lignin seal and disrupt the crystalline structure of cellulose (Figure1.5).

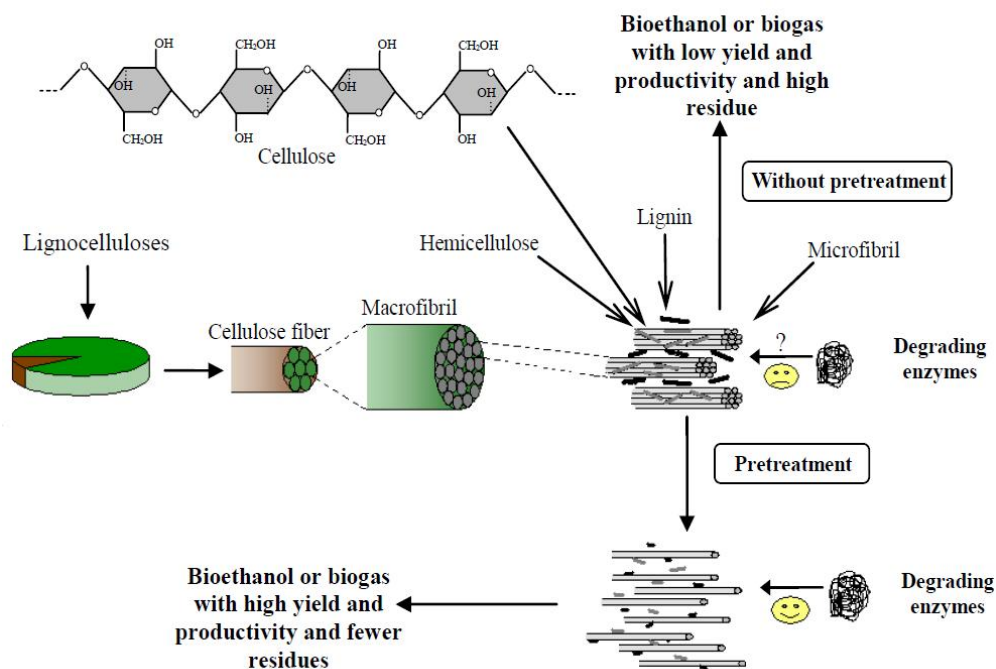


Figure 1.5: Effect of pretreatment on accessibility of degrading enzymes [3]

Pretreatment has been viewed as one of the most expensive processing steps in cellulosic biomass-to-fermentable sugars conversion with costs as high as \$0.3/gallon ethanol produced. Pretreatment also has great potential for improvement of efficiency and lowering of cost through research and development [4].

Currently available pretreatment methods are biological, chemical (acids or bases) and physical (mechanical size reduction, comminution, steam explosion, vibratory ball milling, compression milling and hydrothermolysis) [45].

During biological pretreatment microorganisms will be used to treat the lignocellulose and enhance enzymatic hydrolysis.

The applied microorganisms usually degrade lignin and hemicellulose but only a very little part of cellulose, since cellulose is more resistant than the other parts of ligno-

cellulose to the biological attack, but the treatment rate is very low in most biological pretreatment processes [45].

Some physical pretreatments (size reduction or comminution) are needed to make material handling easier for subsequent steps and increase the surface area of the lignocellulosic material to be pretreated by other means. Primary size reduction employs hammer mills to produce particles that can pass through 3-mm screen openings [46]. High energy requirements and long times requirements are some of the drawbacks of mechanical pretreatments. Furthermore, recent research suggests that more important than the mechanical disruption are the chemical changes that occur during pretreatment [4].

Chemical treatments are usually designed to pretreat lignocellulosic biomass by removing lignin as major effect in base-pretreatment, while the acid-pretreatment is designed to remove hemicellulose as a major effect. Both of them increase the pore size and surface area. There are well known methods used in pretreatment have a potential cost effect (Table 1.2).

Table 1.2: *Effect of various pretreatment methods on the chemical composition and chemical/physical structure of lignocellulosic biomass [4] (●:Major effect, ○:Minor effect, ND:Not determined)*

Pretreatment metod	Increases accessible surface area	Decrystalizes cellulose	Removes hemicellulose	Removes lignin	Alters lignin structure
Uncatalyzed steam explosion	●		●		○
Liquid hot water	●	ND	●		○
pH controlled hot water	●	ND	●		ND
Flow-through liquid hot water	●	ND	●	○	○
Dilute acid	●		●		●
Flow-through acid	●		●	○	●
AFEX	●	●	○	●	●
ARP	●	●	○	●	●
Lime	●	ND	○	●	●

1.5.1 Uncatalyzed steam explosion

Uncatalyzed steam explosion is applied commercially to hydrolyze hemicellulose for manufacture of fiberboard and other products by the Masonite process. Wood chips are conveyed into large vessels and high-pressure steam is applied for a few minutes without addition of chemicals. At a set time, some steam is rapidly vented from the

reactor to reduce the pressure, and the contents are discharged into a large vessel to flash cool the biomass [47,48].

- Mode of action:

The major chemical and physical changes to lignocellulosic biomass by steam explosion are often attributed to the removal of hemicellulose. This improves the accessibility of the enzymes to the cellulose fibers. Reduction in biomass particle size and increased pore volume after explosive decompression is less important in improving the digestibility of steam exploded lignocellulosic biomass [4].

1.5.2 Liquid hot water pretreatments

Water pretreatments use pressure to maintain the water in the liquid state at elevated temperatures [49–51]. Flow-through processes pass water maintained in the liquid state at elevated temperatures through cellulose. This type of pretreatment has been termed hydrothermolysis [50], aqueous or steam/aqueous fractionation [52], uncatalyzed solvolysis [53], and aquasolv [54].

Solvolysis by hot compressed liquid water contacts water with biomass for up to 15 min at temperatures of 200–230°C. There are three types of liquid hot water reactor configurations: co-current, counter-current and flow through, illustrated in Figure 1.6 [53].

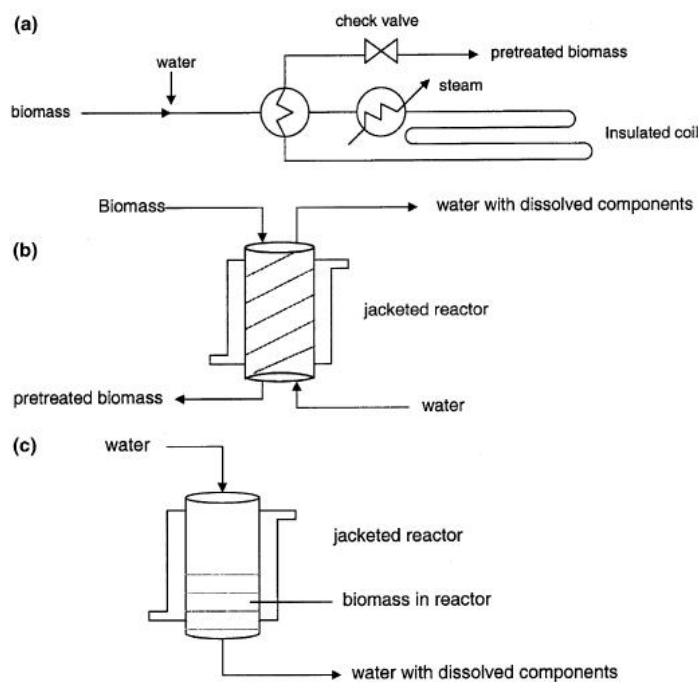


Figure 1.6: Schematic illustrations of (a) co-current at, (b) counter-current, and (c) flow-through pretreatment method:. [4]

- Mode of action:

It is likely that structural and chemical changes occur to the lignin in the non flow-through methods. However, the lack of effective analytical methods for evaluating lignin structure and chemical composition has hindered developing an understanding this phenomenon.

Liquid hot water pretreatments are both helped and hindered by the cleavage of O-acetyl and uronic acid substitutions from hemicellulose to generate acetic and other organic acids. The release of these acids helps to catalyze formation and removal of oligosaccharides. However, the polysaccharides and especially hemicellulose, may be further hydrolyzed to monomeric sugars which are subsequently partially degraded to aldehydes if acid is used. These aldehydes, principally furfural from pentoses and 5-hydroxymethyl furfural from hexose, are inhibitory to microbial fermentation [55].

The pKa of water is affected by temperature such that the pH of pure water at 200°C is nearly 5.0 [56]. Water has an unusually high dielectric constant that enables ionic substances to dissociate. Water is able to dissolve all of the hemicellulose. One half to two thirds of the lignin also dissolves from most biomass materials when these materials are treated at 220°C for 2 min. Hot water cleaves hemiacetal linkages and liberates acids during biomass hydrolysis. This facilitates the breakage of such ether linkages in biomass [57]. Softwoods are less susceptible to solubilization for reasons that are not well understood. The control of pH during pretreatment of Avicel and other types of lignocellulose using potassium hydroxide to prevent the pH of the liquid hot water from falling below 4 limits and/or controls the chemical reactions occurring during pretreatment [58]. The base differs in function from chemicals added as catalysts in chemical pretreatment methods with its role to maintain the pH constant above 5 and below 7 in order to minimize hydrolysis to monosaccharides [56].

1.5.3 Acid pretreatment

Acid pretreatment has received considerable research attention over the years. Dilute sulfuric acid has been added to cellulosic materials for some years to commercially manufacture furfural [59]. Dilute sulfuric acid is mixed with biomass to hydrolyze hemicellulose to xylose and other sugars and then continue to break xylose down to form furfural. The furfural is recovered by distillation. The volatile fraction contains the furfural which is purified and sold. The acid is mixed or contacted with the biomass,

and the mixture is held at temperatures of 160-220°C for periods ranging from minutes to seconds [4].

Addition of sulfuric acid has been initially applied to remove hemicellulose either in combination with breakdown of cellulose to glucose or prior to acid hydrolysis of cellulose. Hemicellulose is removed when sulfuric acid is added and this enhances digestibility of cellulose in the residual solids [60]. The most widely used and tested approaches are based on dilute sulfuric acid. However, nitric acid, hydrochloric acid and phosphoric acid have also been tested [4].

- Mode of action:

Acid hydrolysis releases oligomers and monosaccharides and has historically been modeled as a homogeneous reaction in which acid catalyzes breakdown of cellulose to glucose followed by breakdown of the glucose released to form HMF and other degradation products [61]. This reflects the approximately equal reactivity of glycosidic bonds in these polymers with respect to hydrolysis. Various researchers adopted Saeman's kinetics to describe the hydrolysis of hemicellulose and formation of furfural and other decomposition products [62].

Oligomers are generally ignored in many models since they are viewed as being too short-lived to be important [63]. Others have shown oligomers to be present in batch hydrolysis systems and to be a significant fraction of the product for very dilute acid and water only flow-through systems [64]. Kinetic models have been modified to include hemicellulose hydrolysis to oligomers followed by their breakdown to sugars where the sugars degrade to furfural and other compounds for batch, percolation, and flow-through dilute acid-catalyzed systems. Modeling of hemicellulose hydrolysis as a biphasic reaction incorporates fast and slow hydrolyzing solid hemicellulose fractions [65]. Improvements have also been realized by adding additional acid to compensate for the capacity of minerals in the substrate to neutralize some of the acid and by calculating the hydrogen ion concentration from the pH [66]. The influence of pH on rate has been factored into kinetic models to predict that the sugar yields will be highest in a pH range of 2.0-2.5 [67].

1.5.4 Alkaline pretreatments

Alkali pretreatment processes utilize lower temperatures and pressures compared to other pretreatment technologies. Alkali pretreatment may be carried out at ambient conditions, but pretreatment time is measured in terms of hours or days rather than minutes or seconds [4].

Compared with acid processes, alkaline processes have less sugar degradation and many of the caustic salts can be recovered and/or regenerated. Sodium, potassium, calcium, and ammonium hydroxide are suitable alkaline pretreatment agents. Of these four, sodium hydroxide has been the most studied [68, 69]. However, calcium hydroxide (lime) has been shown to be an effective pretreatment agent [70] and it is the least expensive per kilogram of hydroxide. Furthermore, it is possible to recover calcium from an aqueous reaction system as insoluble calcium carbonate by neutralizing with inexpensive carbon dioxide; the calcium hydroxide can subsequently be regenerated using established lime kiln technology [71].

The following list shows the most important methods of alkaline pretreatment are introduced:

1. Ammonia Fiber Explosion (AFEX)

In AFEX pretreatment, biomass is treated with liquid anhydrous ammonia at moderate temperatures (60-100°C) and high pressure (17-20.5 bar) for 5 minutes, then the pressure is rapidly released. In this process, the combined chemical and physical effects of lignin solubilization, hemicellulose hydrolysis, cellulose decrystallization and increased surface area enable nearly complete enzymatic conversion of cellulose and hemicellulose to fermentable sugars [4]. In this process, nearly all of the ammonia can be recovered and reused whereas the remaining serves as nitrogen source for microbes in downstream processes [72]. It is also characterized by high yields and no need of neutralization after pretreatment. However, this method works only moderately well on hardwoods, and is not attractive for softwoods [73].

2. Ammonia Recycled Percolation (ARP)

An ammonia solution (15%) is fed to a column reactor packed with biomass at temperatures of 160 to 180°C and fluid velocity of 1 mL/(cm²·min) with residence times of 14 minutes. Aqueous ammonia reacts primarily with lignin (but not cellulose), which causes depolymerization of lignin and cleaves lignin-carbohydrate linkages. A large and adjustable degree of delignification has been reported in tests with hardwood [74].

- Mode of action:

The ammonia freeze explosion pretreatment simultaneously reduces lignin content and removes some hemicellulose while decrystallizing cellulose. Thus it affects both micro- and macro-accessibility of the cellulases to the cellulose. Liquid ammonia causes cellulose swelling and a phase change in the crystal structure from cellulose I to cellulose III. It is believed that ammonolysis of glucuronic cross-links make the carbohydrate more accessible [75]. The

cost of ammonia and especially of ammonia recovery drives the cost of this pretreatment [76].

3. Lime pretreatment

Calcium hydroxide ($\text{Ca}(\text{OH})_2$), water and an oxidizing agent (air or O_2) are mixed with the biomass at temperatures ranging from 40 to 160°C for a period ranging from hours to weeks. Two types of lime treatment that show high total sugar yields and they are currently used: short term and long term.

Short-term lime pretreatment involves boiling the biomass with a different lime loading, temperatures and with or without oxygen at different pressure [38, 77]. Long-term pretreatment involves using lower lime loading and temperatures (40-55°C) for 4-6 weeks in the presence of air [10].

- Mode of action:

The major effect is the removal of lignin from the biomass, thus improving the reactivity of the remaining polysaccharides. In addition, this pretreatment removes acetyl and the various uronic acid substitutions on hemicellulose that lower the accessibility of the enzyme to the hemicellulose and cellulose surface [78].

By comparison, alkaline pretreatments are less expensive and only consume low-to-moderate energy. Sodium hydroxide (NaOH), ammonia (NH_3) and lime ($\text{Ca}(\text{OH})_2$) are the most commonly used alkalies [13].

Table 1.3 compares these alkalis and shows that lime is a good pretreatment agent because it is very inexpensive (0.06/kg \$) [79], is safe, and can be recovered by carbonating wash water with CO_2 [77]. Unfortunately, because lime is a weak base and has a low solubility, it appears to be less effective than other alkalis. However, under appropriate pretreatment conditions, lime substantially enhances the digestibility of moderate-lignin biomass (e.g., switchgrass, bagasse, and wheat straw) by removing 30-43% of lignin and all acetyl groups. For high-lignin biomass, lime alone does not remove enough lignin to significantly enhance the digestibility; an oxidant must be added [13].

Table 1.3: Comparison of three common alkali pretreatment agents [13]

Alkali	Price (\$/kg)	Operating pressure	Health hazard	Recoverability
NaOH	0.68	Low	Moderate	Hard
NH_3	0.13	High	Low	Easy
$\text{Ca}(\text{OH})_2$	0.06	Low	High	Moderate

1.6 Hydrolysis of carbohydrate

Hydrolysis of carbohydrates include breaking the polymer of cellulose and himecellulose to their monomers. Hydrolysis of cellulose gives glucose, whereas hydrolysis of hemicellulose results pentoses and hexoses.

There are two basic methods to degrade the biomass to sugars: enzymatic hydrolysis and dilute acid hydrolysis. Compared to dilute acid hydrolysis, enzymatic approach is promising because it can achieve high sugar yields and eliminate the need for large quantities of chemicals and the formation of inhibitory by-products during dilute acid hydrolysis.

During the enzymatic hydrolysis we need the enzyme cellulase which is responsible to catalyze cellulose degradation to glucose and the enzyme hemicellulase as additive to improve the hydrolysis process. Cellulase is actually a complex mixture of several enzymes including endoglucanase, exoglucanase and β -glucosidase (Figure 1.7). Endoglucanase randomly attacks internal bonds in the cellulose chain and acts mainly on the amorphous cellulose. Exoglucanase (cellobiohydrolase) hydrolyzes from the chain ends and produces predominately cellobiose, and it can degrade crystalline cellulose. Cellobiose is cleaved to form two glucose molecules by β -glucosidase (cellobiase).

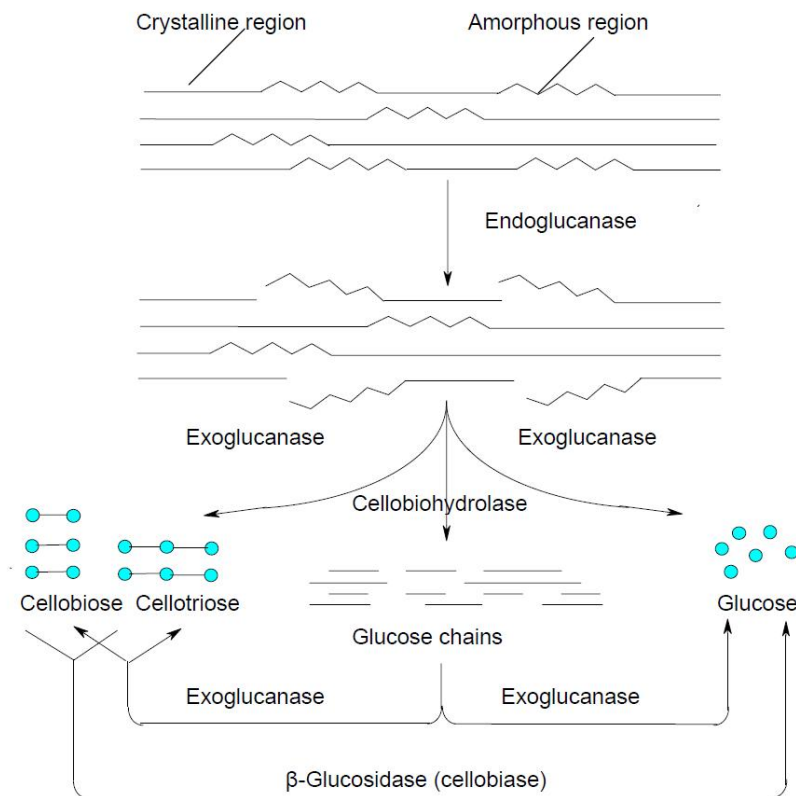


Figure 1.7: Mode of cellulolytic enzyme action [5]

1.7 Objectives

The main purposes of this study can be summarized as follows:

1. Compositional analysis of willow wood by analyzing the biomass sugars by using HPLC method.
2. Study the effect the different conditions of short-term lime pretreatment for pre-treated willow wood on the enzymatic hydrolysis process.
3. Optimize the pretreatment conditions(i.e., pretreatment time, pressure of oxygen pressure, temperature,lime loading, water loading, and particle size).
4. Deriving empirical model for kinetic degradation of lignin and carbohydrate.
5. Optimize of enzyme loading and substrate concentration of enzymatic hydrolysis process.

Compositional Analysis of Feedstock

2.1 Preparation, storage and handling

Feedstock for this study is willow wood type Tordis or its scientific name is called ((*Salix viminalis* x *Salix schwerinii*) x *Salix viminalis*). From a private plantation. Approximately 5 bundles of tree stems of 2-3 inch diameter were harvested in January 2010 from private land in Saxony-Anhalt, Germany.

As soon we got them we took a part of them for debarking, drying and chipping and then chips were milled by wood mill to pass a 8.0 mm round screen. Then the milled feedstock was taken to reduce particle size pass a 2 mm round screen by using laboratory mill.

The biomass was collected after comminution process, sieved to pass 20 mesh (0.850 mm) and 80 mesh (0.180 mm). Stack the sieves in the following order, starting at the bottom: the bottom pan 80, 20-mesh sieve and lid of stack. The purpose is converting a variety of biomass samples into a uniform material suitable for compositional analysis in a reproducible way.

Finally the samples are air-dried to a moisture content less than or equal to 10%. Neither the particles retained on the 20 mesh nor the ones passing the 80 mesh were used, just we used those less than 20 mesh more than 80 mesh [80]. Because out this rang of particle size, there is a big deviation in carbohydrate and lignin contents. This procedure is based on the NREL standard procedure (Preparation of Samples for Compositional Analysis).

With respect of enzymatic hydrolysis the effect of particle size on enzymatic hydrolysis sugar yields after lime pretreatment is not important, as long as the particle size is smaller than 4 mesh (4.760 mm) [81].

Once we finished preparation of samples, the particles of biomass was re-packaged into polyethylene bags (either completely filled or tightly wrapped to reduce evaporation

into the headspace), and stored frozen at -20°C . When needed, the biomass is directly used. More informations about this section explained in Appendix A.

2.2 Compositional analysis of willow wood feedstock

It is well known that the composition of willow wood and other types of tree can vary greatly with the particular season, the place where the crop is grown, the part of the tree from which the sample is taken and the variety of willow trees.

For the assessment, the whole stem of each tree was de-barked, dried, chiped and then milled to be eventually in one batch. Table 2.1 shows the composition of the willow wood that is used in this study. We measured the composition of biomass and pretreated biomass in our institute (EVUR) and Department of Brewing Science as well, at Berlin Institute of Technology. The analysis of each components is briefly explained below. A more detailed description of each of the experimental procedures is included in the corresponding appendixes.

Table 2.1: *Composition of raw willow wood feedstock*

Components (g/100 g biomass)	Washed willow wood				Average	Std.Dev
Glucane	40.70	40.90	41.10	40.09	41.00	0.14
Xylane	17.70	16.60	15.60	16.70	16.65	0.13
Mannan	4.00	3.00	5.00	5.83	4.45	1.25
Galactan	2.00	1.90	2.00	2.00	2.00	0.05
Lignin	25.70	26.00	25.80	25.9	25.85	0.111
Extractive	5.80	6.20	5.90	6.00	6.00	0.15
Ash	0.8	0.8	0.86	0.82	0.82	0.033
Acetyl	2.8	2.8	2.6	2.75	2.72	0.06
Total	98.6	98,5	99.66	99.00	99.50	

2.2.1 Extractives

It is necessary to remove non-structural material from biomass prior to analysis to prevent interference with later analytical steps. This procedure uses a two-step extraction process to remove water soluble and ethanol soluble material. Water soluble materials may include inorganic material, non-structural sugars, and nitrogenous material. Ethanol soluble material includes chlorophyll, waxes and other minor components. Some biomass may require both extraction steps, while other biomass may only require exhaustive ethanol extraction (Appendix B).

2.2.2 Acetyl content

Aliphatic groups in wood and herbaceous feedstocks are acetyl and formyl groups which can be combined as O-acyl groups with the polysaccharide portion. A number of different approaches can be used to analyze for acetyl (Appendix C).

2.2.3 Ash content

Ash content as expressed as the percentage of residue remaining after dry oxidation (oxidation at 550 to 600°C). The procedure used to measure ash is based on NREL Standard Procedure (Determination of Ash in Biomass) (Appendix D).

2.2.4 Determination of structural carbohydrates and lignin

Carbohydrates and lignin make up a major portion of biomass samples. These constituents must be measured as part of a comprehensive biomass analysis. Carbohydrates can be structural or non-structural. Structural carbohydrates are bound in the matrix of the biomass, while non-structural carbohydrates can be removed using extraction or washing steps. Lignin is a complex phenolic polymer.

The method used is based on NREL Standard Procedure (Determination of Structural Carbohydrates and Lignin in Biomass) and it is similar to ASTM E1758-01 (Standard method for the Determination of Carbohydrates by HPLC) (Appendix E).

This procedure uses a two-step acid hydrolysis to fractionate the biomass into forms that are more easily quantified. The lignin fractionates into acid-soluble material and acid-insoluble material (Klason lignin). The acid soluble lignin is quantified by UV-visible spectroscopy and the insoluble lignin is determined by gravimetric analysis.

During hydrolysis, the carbohydrates are hydrolyzed into monomeric forms, which are soluble in the hydrolysis liquid, then they are measured by HPLC device.

High-performance liquid chromatography (HPLC) is a form of liquid chromatography used to separate compounds that are dissolved in solution. HPLC instruments consist of a reservoir of mobile phase, a pump, an injector, a separation column, and a detector. Compounds are separated by injecting a plug of the sample mixture onto the column. The different components in the mixture pass through the column at different rates due to differences in their partitioning behavior between the mobile liquid phase and the stationary phase [82].

HPLC separations of carbohydrates depend on differences in conformation, configuration and bonding mode. Carbohydrates exhibit a significant degree of chemical and

physical similarity, thus they are more difficult to analyze than most other classes of compounds. For this reason, no single HPLC column or method is capable of separating all carbohydrates. Here some types of carbohydrates columns as follow:

- Potassium (K) form resin column is useful for separating raffinose, sucrose, glucose, fructose and betaine.
- Lead (Pb) form resin column provides the highest resolution and best selectivity for monosaccharides including excellent separation of xylose, galactose and mannose (we used it in our test).
- Calcium (Ca) form resin column provide excellent separations of monosaccharides and sugar alcohols. Di-, tri-, and oligosaccharides are separated by class. This column often is used to separate sugars in high fructose corn syrup.
- Hydrogen (H) columns are ideal for separating carbohydrates, alcohols, and organic acids present in the same sample: fermentation products, fruit juices, etc.
- C-611-form resin columns contain a unique ion exchange resin containing two divalent cations, rather than one. This provides different selectivities for separating monosaccharides and sugar alcohols. Di-, tri-, and oligosaccharides are separated by class. Galactose and mannose are well separated.
- Silver (Ag) form resin columns provide rapid oligosaccharide separations. Glycerol and ethanol are well resolved.
- LC-NH₂ columns separate monosaccharides, disaccharides, and some trisaccharides. Sugar retention decreases as the proportion of water:acetonitrile in the mobile phase is increased. Sugars generally will be eluted in order of increasing molecular weight.

2.3 Calibration curve

In order to analyze the carbohydrates in biomass by HPLC method, we need to prepare the calibration curve before the test. Calibration curve is based on a series of calibration standards containing the compounds that are to be quantified. The range of the concentration of the calibration standards for willow wood is suggested as 0.1, 0.5, 1, 2, 3, 4 mg/mL for D-cellobiose, D-(+)glucose, D-(+)xylose, D-(+)Galactose, and D-(+)mannose [83]. More information in appendix I.

2.4 Result and Conclusion

The results of compositional analysis for willow wood samples had an error of 1 % for the summative mass closure. they were very close to the expected values and compatible with the previous studies [84,85].

The major components of raw willow wood were glucan, xylan and lignin. The weight percent of each component is listed in Table 2.1. The ratios of glucan to xylan were 41/16.5 (2.48). Other hemicelluloses such as mannan, and galactan were present in small amounts (less than 4.45 and 2 %, respectively). Lignin content was approximately 25.85 %.

To determine the reproducibility of compositional analysis of raw willow wood samples, we depended on coefficient of variation and standard deviation . Generally coefficient of variation was less than 5 %. Among carbohydrates, the highest standard deviation and coefficient of variation were obtained for mannan sugar (1.25 and 28 %respectively,). This can be attributed for two reasons. The first one, the retention time of mannose and arabinose is very close for each other in the Rezex RPM-Monosaccharide (RPM Pb²) column of HPLC device (16.39, 16.47 min, respectively) and both of mannose and arabinose seems to appear together in a single peak. As it is well known in woody biomass that the arabinan component is smaller than mannan component, so we considered this peak belong to mannose alone. The second reason that RPM-Monosaccharide column (RPM Pb²) does not have sufficient sensitivity to measure small concentrations of mannose [84,85]. Also we noticed this phenomena during the test of samples and calibration curve at concentration less than 0.5 g/L, where the peak of mannose appears as wavy. Figure 2.1 shows chromatograms for standard sugar and real sugar of willow wood.

Due to destruction of sugars during dilute acid hydrolysis to other compounds like furfural (degrading of xylose) and HMF (degrading of cellulose). Accordingly, and in order to correct that losses, we should made a set of sugars that is called (Sugar Recovery Standards (SRS)). SRS sugar concentrations should be chosen to be closely resemble the concentrations of sugars in the samples of test as much as possible (Appendix E).

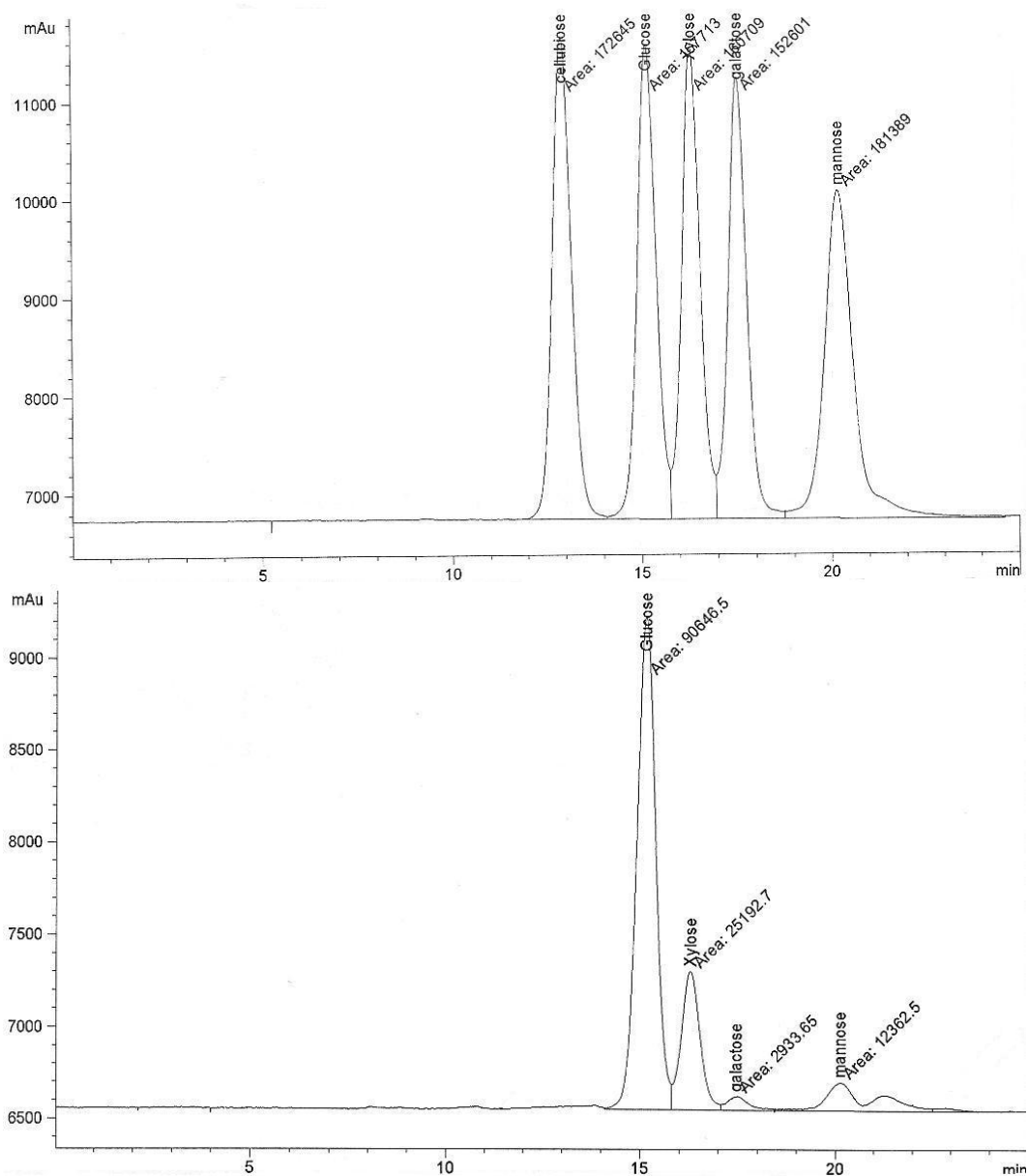


Figure 2.1: In the upper figure is shown a chromatogram of standard sugar (4 g/L), and in the lower figure is shown a chromatogram of wood sugar

Oxidative Short-term Lime Pretreatment

3.1 Introduction

Oxidative short-term lime pretreatment is an effective method for improving lignocellulose digestibility, partial delignification of biomass is obtained allowing for some biomass swelling, increased internal surface area, removes acetyl and the various uronic acid substitutions on hemicellulose and larger median pore volume, all of which enhance enzyme accessibility to carbohydrate polymers.

Previous studies have shown that pretreatment time, temperature and pressure of oxygen have greater impacts on enzymatic hydrolysis than water loading, lime loading and biomass particle size, therefore, the pretreatment condition studies were conducted to hold the low-impact variables constant while systematically varying the high-impact variables. After the high-impact variables were determined, the low-impact variables were then investigated [13,86]. Depending on this basis the experimental conditions for this study were selected as follows.

3.1.1 Oxidizing agent

We used compressed oxygen as oxidizing agent because oxygen is more cost-effective than other traditional chemical and also because it is environmentally safe.

In our experiments we couldn't use air instead of pure oxygen because the percentage of oxygen in air up to 21%.

Oxygen was supplied to the reactor in either one of two modes: varying pressure (VP) in which a single charge of oxygen was added to the reactor at the beginning of the pretreatment process, and constant pressure (CP) in which oxygen was continuously provided during pretreatment at the desired pressure. CP was attained by using flexible tubing (1/8-inch stainless steel) connected to an oxygen tank (Figures 3.2 and 3.3).

It was found that the glucan yields were always lower in VP than in CP and required higher temperatures and longer pretreatments [86], thus the CP mode was used in our experiments. The pressure of oxygen has been studied in our study was between 6 to 21 bar (absolute) depending on the previous study [13,86].

3.1.2 Temperature

Woody biomass is more recalcitrant to pretreatment than other types of biomass. Consequently it is necessary to attack the lignin more severely and at the same time to try to avoid the degradation of carbohydrates as much as possible.

Generally, at 110°C lignin removal is insufficient and at 180°C carbohydrate do not preserve well [87]. Temperatures were chosen in this study between 100°C and 160°C.

3.1.3 Pretreatment time

In oxidative short-term lime pretreatment of poplar wood in a former study, wood is submitted to 2-10 hour at different pressure (8-28 bar) of lime pretreatment. That study showed important delignification with good preservation of carbohydrates content. The optimum sugar yields were found at about: (1) 2 hours, 140°C, 21.7 bar (absolute) and (2) 2 hours, 160°C and 14.8 bar (absolute). The periods of pretreatment time chosen for our investigation were 1-6 hour [88].

3.1.4 Lime loading and water loading

To determine how much lime is consumed, we began with excess lime loading of 0.4 g Ca(OH)_2 /g dry biomass. Because it usually the lime consumption is between 0.1-0.3 g Ca(OH)_2 /g dry biomass [86, 88, 89]. After completing of the pretreatment, back-titration with HCl was used to determine the excess lime [86,88] and to calculate the actual lime consumption [88].

To determine the desired loading of water, the water loading must be provided in a sufficient quantity to make this mixture (water, wood particle and lime) slurry, as well it (the water) will function as a medium, in which the lime can be dispersed. In this way, the water will maintain a uniform temperature, because its good heat transfer coefficient. Excessive water will have no effect on the pretreatment but its presence will demand more energy.

In this study we found the minimum water loading which make the mixture homogeneous was (15 g H_2O /g dry biomass) (Table 3.2). Therefore, we supposed it as optimum loading.

3.2 Experimental design methodology

A scientific approach to plan the experiments is a necessity for efficient conduct of experiments.

The process of planning of our experiments is carried out using a statistical design, and then the corresponding data should be collected and analyzed by appropriate statistical methods to achieve valid and objective conclusions.

Experimental design is used to find a useful relationship between experimental variables and observed response (dependent variables). The experimental variables are usually called factors (independent variables), the particular value of these variables is called the level of factor or (coded level). The combination of the factors that used in a particular experimental run is called (treatment) and the change response produced by a change in the level of factor is known as the effects of that factor. When the effect of one factor is different from another, the two factors are said to interact.

The experimental design technique consists of two parts:

1. Planning the experiments according to a specified plan.
2. Achieving the regression analysis for a specified set of runs in the plan.

These two points are closely related to each other, since the method of analysis depends directly on the design of the employed experiments.

The advantages of experimental design are:

- Numbers of trials is significantly reduced.
- Important decision variables which control and improve the performance of the product or the process can be identified.
- Optimal setting of the parameters can be found out.
- Qualitative estimation of parameters can be made.
- Experimental error can be estimated.

In the present work, the response surface methodology type (Central Composite Design) have been used to plan the experiments and subsequent analysis of the collected data.

3.2.1 Box -Wilson Design (Central Composite Design)

Box-Wilson composite design is a general series of experiments that have been developed to efficiently serve as a base for deriving the mathematical model to estimate the coefficients of a quadratic model. It is commonly called a (Central Composite Design) (CCD), and it contains an embedded factorial or fractional factorial design with center points that is augmented with a group of (star points) that allow estimation of curvature. That mean the central composite design (CCD) has three levels of design points:

- Factorial points

The two-level factorial part of the design consists of all possible combinations of the $+1$ and -1 levels of the factors. For the two factor case there are four design points: $(-1, -1)$ $(+1, -1)$ $(-1, +1)$ $(+1, +1)$. As shown in Figure 3.1

- Star or axial points

The star points or (axial points) have all of the factors set to 0, the midpoint, except one factor, which has the value $+/- \alpha$. For a two factor problem, the star points are: $(-\alpha, 0)$ $(+\alpha, 0)$ $(0, -\alpha)$ $(0, +\alpha)$ (Figure 3.1). The value for Alpha (α) is calculated in each design for both rotatability and orthogonality of blocks. The experimenter can choose between these values or enter a different one. The default value is set to the rotatable value.

- Center points

Center points, as implied by the name, are points with all levels set to coded level 0 - the midpoint of each factor range: $(0, 0)$. Center points are usually repeated 4-6 times to get a good estimate of experimental error (pure error). (Figure 3.1)

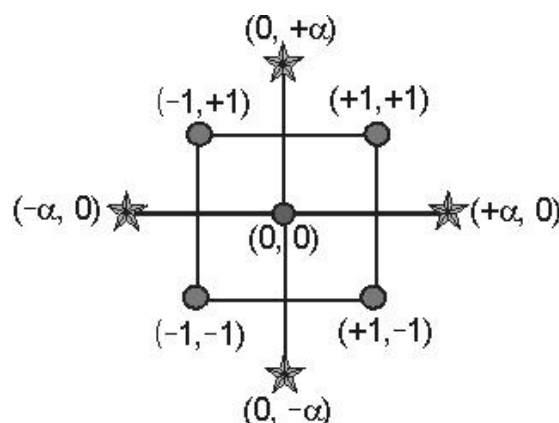


Figure 3.1: Generation of a central composite design for two factors

The relationship between the coded levels and the corresponding real process variable is according to:

$$X_{coded} = \frac{X_{actual} - X_{center}}{\frac{X_{center} - X_{minimum}}{\sqrt{K}}} \quad (3.1)$$

$$X_{center} = \frac{X_{maximum} - X_{minimum}}{2} \quad (3.2)$$

and K is the number of variables in the system.

$$\alpha = \sqrt{k} \quad (3.3)$$

Finally, the number of experiments need to estimate according to the following equation

$$N = 2^k + 2K + 1 \quad (3.4)$$

where N=number of experiments

3.2.2 Experimental design for oxidative short-term lime pretreatment

In this study, the experimental design depends upon the relation between the observed responses (yields of glucan, xylan and lignin) and the independent factors (temperature, pressure and time) at five levels(1,-1,0, α ,- α). The specified values of these five levels for each factor depend on the number of included factors in the model.

Table 3.1: The relationship between coded level and corresponding real variable

NO.Exp	Coded variables			Real variables		
	X ₁ (Temp.)	X ₂ (Press.)	X ₃ (Time)	X ₁ (Temp.°C)	X ₂ (Press.bar)	X ₃ (Time.h)
1	1	1	1	147	17.8	5.0
2	-1	1	1	113	17.8	5.0
3	1	-1	1	147	9.2	5.0
4	1	1	-1	147	17.8	2.0
5	-1	-1	1	113	9.2	5.0
6	-1	1	-1	113	17.8	2.0
7	1	-1	-1	147	9.2	2.0
8	-1	-1	-1	113	9.2	2.0
9	α	0	0	160	13.5	3.5
10	0	α	0	130	21.0	3.5
11	0	0	α	130	13.5	6.0
12	- α	0	0	100	13.5	3.5
13	0	- α	0	130	6.0	3.5
14	0	0	- α	130	13.5	1.0
15	0	0	0	130	13.5	3.5
16	0	0	0	130	13.5	13.5
17	0	0	0	130	13.5	13.5
18	0	0	0	130	13.5	13.5

Table 3.1 shows the data of experimental design:

- N=18 experiments.
- Eight factorial points $(-1,-1,-1), (1,-1,-1), (-1,1,-1), (-1,-1,1), (1,1,-1), (1,-1,1), (-1,1,1), (1,1,1)$.
- Six star points $(-\sqrt{\alpha},0,0), (\sqrt{\alpha},0,0), (0,\sqrt{\alpha},0), (0,-\sqrt{\alpha},0), (0,0,\sqrt{\alpha}), (0,0,-\sqrt{\alpha})$.
- Four center points $(0,0,0)$.

Polynomial form will often be satisfactory to express the relationship between the response(Y) and the important variables (X_1, X_2, \dots, X_k) . The two most common forms of Y are the first order polynomial:

$$Y = B_0 + \sum_{i=1}^k BX_i + \epsilon \quad (3.5)$$

and the second order polynomial:

$$Y = B_0 + \sum_{i=1}^k BX_i + \sum_{i=1}^k B_{ii}X_i^2 + \sum_{i,k=1}^k B_{ik}X_iX_k + \epsilon \quad (3.6)$$

- Where ϵ is a random error component.

If the response is well-modeled by a linear function of the independent variables, then the approximating function is the first order model, but if there is a curvature in the system, then the polynomial is of higher degree such as the second-order model.

3.3 Experimental setup

3.3.1 Scheme of lime pretreatment

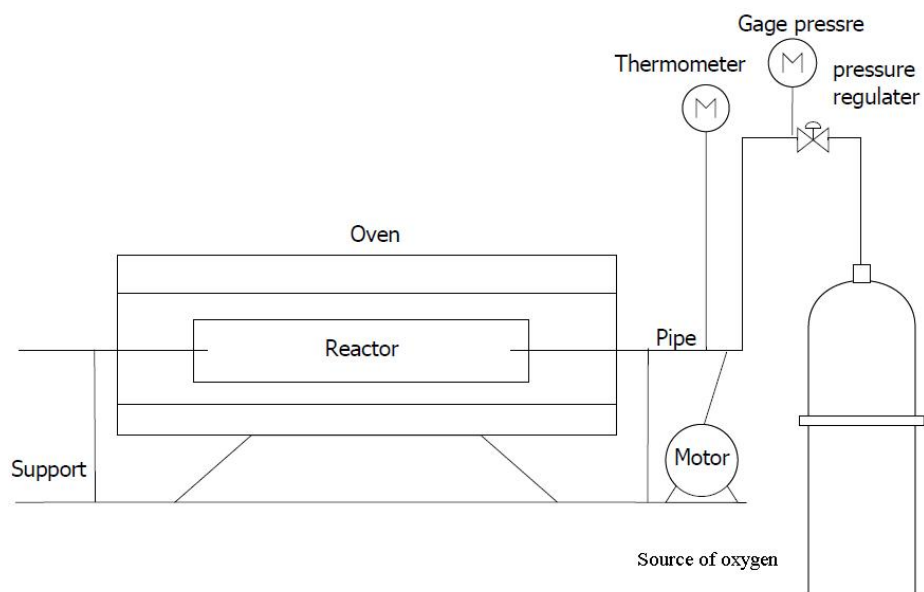


Figure 3.2: Schematic process of lime pretreatment



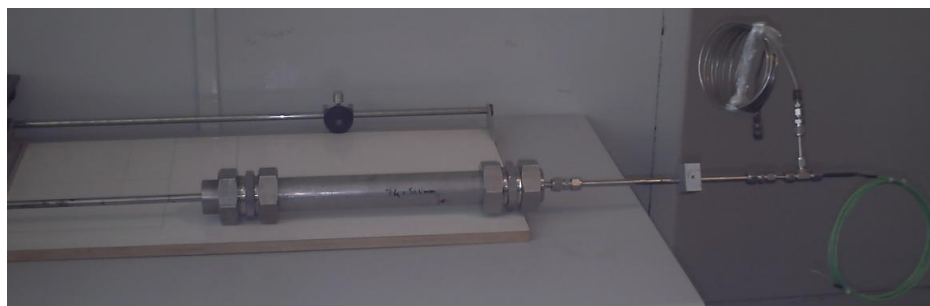
Figure 3.3: Photograph of reactor system to pretreat the biomass with lime

Table 3.2: *Experimental condition for pretreatment*

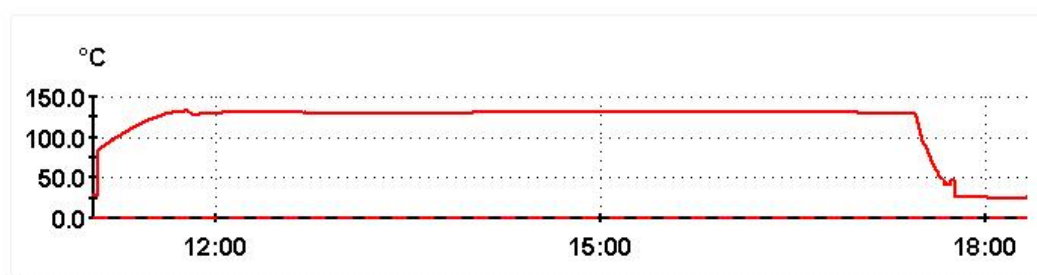
Variables	Conditions
Oxygen pressure	6-21 bar (absolute)
Temperature	100-160 °C
Time	1-6 hour
Lime loading	0.4 g $\text{Ca}(\text{OH})_2$ /g dry biomass
Water loading	15 g H_2O /g dry biomass

3.3.2 Experimental setup and operation

Short-term lime pretreatment process was performed in a system of a reactor constructed from 30-cm long, 38-mm inside diameter, 304 stainless steel pipe with a 285-mL volume. The reactor was supplied at both of ends by fittings made of stainless steel (Figure 3.4).

**Figure 3.4:** *Photograph of lime pretreatment reactor*

To get the temperature profile of reaction progress inside reactor, we used a bimetal stem thermometer (Type K) inserted inside the reactor through the pipe at the end of the reactor and fixed by stainless steel fitting thus we can record the temperature by, USB Thermocouple Data Logger every 5 sec (Figure 3.5).

**Figure 3.5:** *Temperature profile of reactors for a target pretreatment temperature 130°C*

Oxygen used in the experiments was as a compressed oxygen and pressurize to reactor at constant pressure (CP) in which oxygen was continuously provided during pretreatment

at the desired pressure. CP was attained by using flexible tubing (1/8-inch stainless steel) connected to an oxygen tank (Figure 3.3).

Raw biomass (11.4 g dry weight) and excess of calcium hydroxide (0.4 g/g dry biomass) were placed in a beaker size 250 mL. The mixture was thoroughly mixed with deionized water (15 g/g dry biomass), then put the mixture inside the reactor. The mixing process during the reaction progress have done by using a system of motion which consist of electrical motor and set of levers which can move the reactor toward left and right around 50 rpm.

After tightly capping, the reactor was preheated to 75°C by water bath, then placed in the preheated oven. The pretreatment temperature was maintained by inserting the reactor in a temperature-controlled tube-oven at desired temperature. Time of experiments begin when the temperature of the reactor reaches the desired temperature. Therefore we need some time between 30 to 40 minutes to reach to desired temperature in most of the experiments.

At the end of the pretreatment, the reactor was cooled by pressurized air, depressurized slowly by the valve that is connected to the reactor, remove the reactor from the oven, cool it down in cold water and open it. The pretreated biomass was transferred to a convenient container using about 250 mL of deionized water(DI). The slurry was then neutralized by titrating with 5.0-N HCl to measure unreacted lime. After that the solids were extensively washed with clear DI water and filtered using a vacuum filtration apparatus with a Whatman glass fiber filter paper (particle retention = 2 μ m). More detail about neutralization see (Appendix F). Once filtered, the biomass to be analyzed for composition was air-dried at room temperature. The weight of the dry biomass and its moisture content were recorded to account for the pretreatment yield of solids. The biomass was stored at -20°C until used for analysis or enzyme hydrolysis.

Chemical Reactions During the Oxidative Lime Pretreatment

Oxygen delignification process can be conducted under the concepts of the chemistry of oxygen, lignin and carbohydrates, kinetics of delignification and carbohydrate degradation, and finally mass transfer of oxygen from the gas to the fiber phase.

4.1 Oxygen chemistry

The reaction of oxygen requires the release of electrons, which is promoted by ionizing functional groups in a strongly alkaline media. At high pH, oxygen is reduced by one electron transfer to several oxygen species (radicals), as shown in Figure 4.1.

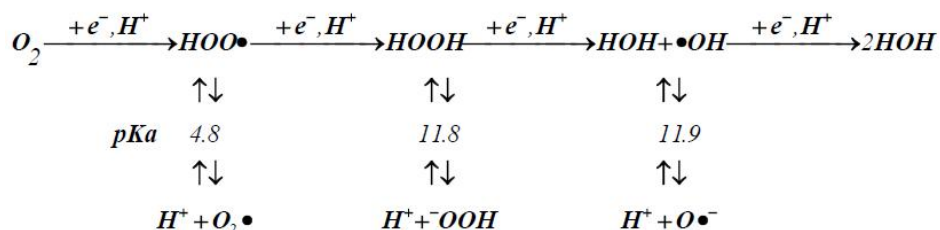


Figure 4.1: Oxygen chemistry in aqueous solution [6]

Oxygen is a molecule with the triplet state as its normal configuration (low energy). It contains two electrons that are unpaired, therefore, each of these electrons has an affinity for other electrons of opposite spin, and thus oxygen behaves as a free radical. Consequently, oxygen has a strong tendency to react with organic substances, and radical chain reactions are initiated which liberate hydroperoxy radicals $HOO\bullet$.

At alkaline conditions, the hydroperoxy radical is converted into the superoxide anion radical. By subsequent one electron reduction reactions, the hydroperoxy radical is transformed to hydrogen peroxide, subsequently the hydroxyl radical and finally water [6].

4.2 Carbohydrate chemistry

Carbohydrates in the presence of alkali and oxygen undergo both oxidation and alkaline degradation to produce a complex mixture of products [90–92].

Hydroxy-carboxylic acids, such as glucoisosaccharinic and xylosaccharinic acids, are formed from the degradation of cellulose and hemicellulose respectively (Figure 4.2 and 4.3). The peeling reaction (or endwise depolymerization reaction) is a β -elimination that begins at the reducing end of the molecule and proceeds along the chain liberating saccharinate molecules [93]. The formation of low-molecular-mass fragments, such as glycolic and lactic acids increases at more severe reaction conditions, i.e., high alkaline concentration or high-temperature conditions [94].

Cellulose is relatively more stable to alkaline wet oxidation (alkali, water, oxygen, high temperature and pressure) than xylan. Degradation reactions of cellulose and hemicellulose are limited by the formation of D-glucometasaccharinate and D-xylometasaccharinate, respectively, which terminate or 'cap' the reactions [7]

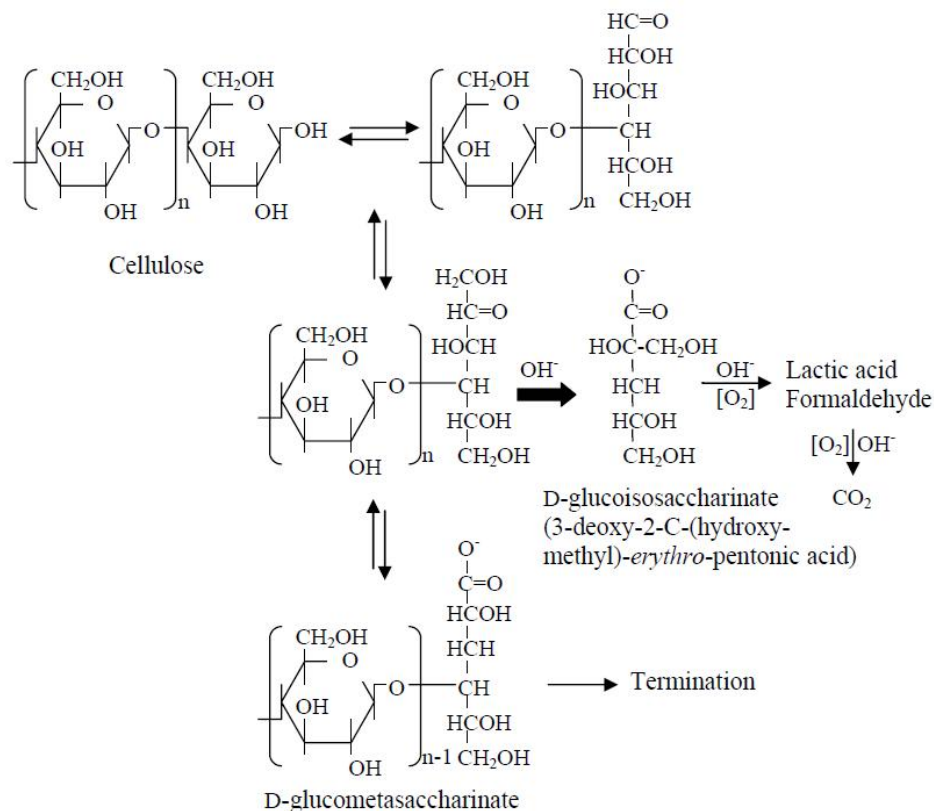


Figure 4.2: Cellulose degradation in alkaline conditions [7]

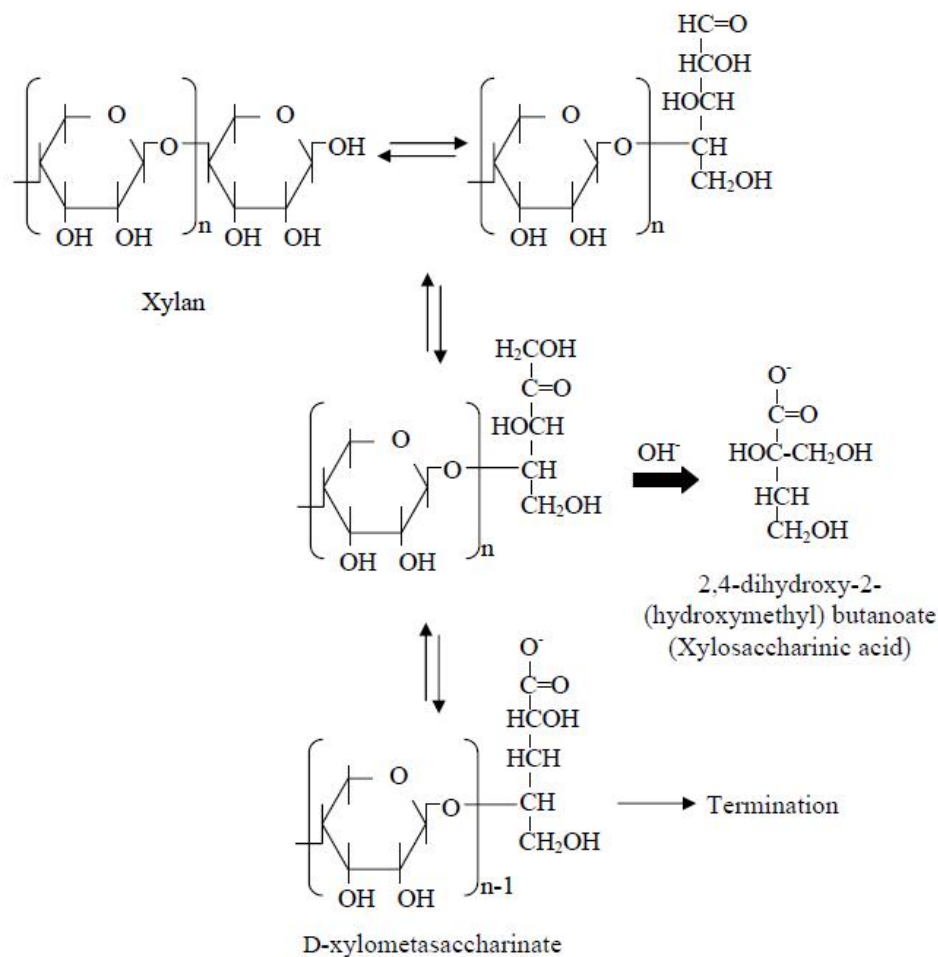


Figure 4.3: Xylan degradation in alkaline conditions [7]

4.3 Lignin chemistry

A large amount of research has been done to learn about the mechanisms of lignin removal during oxygen delignification. Many researchers [8, 95, 96] used model compounds to study the reaction of lignin during oxygen alkaline treatment. Figure 4.4 shows how a phenolic hydroxyl group in lignin reacts with alkali to generate a phenolate ion, which is considered to be the initiation of the lignin reactions. The anion then reacts with oxygen to form a reactive intermediate called a hydroperoxide. The primary reaction of oxygen with lignin under alkaline conditions proceeds via a resonance stabilized phenoxy radical.

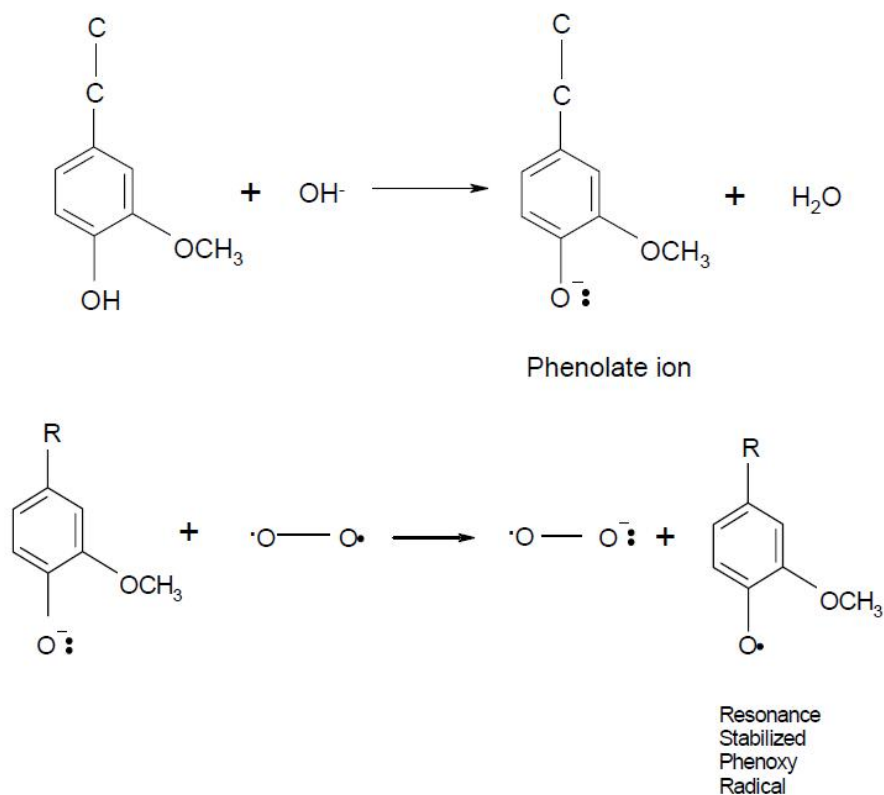


Figure 4.4: Initial attack of oxygen on phenolic nuclei [8]

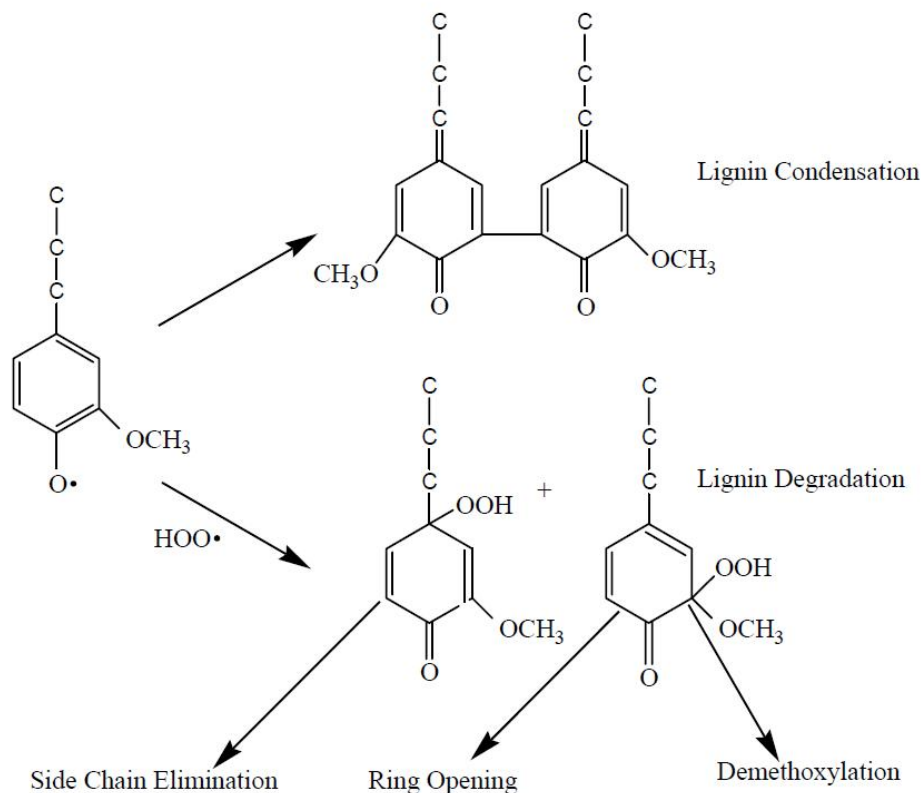


Figure 4.5: Possible reactions of lignin via phenoxyradical [8]

The resonance stabilized intermediates then undergo reaction with themselves (lignin condensation) or with oxygen species such as hydroxyl ($\text{HO}\cdot$), hydroperoxy ($\text{HOO}\cdot$) and superoxide ($\text{O}_2\cdot^-$) radicals to form organic acids, carbon dioxide and other small molecular weight organic products via side chain elimination (Figure 4.5).

Alkaline depolymerization of lignin mostly depends on the cleavage of two types of aryl ether bonds, phenolic α -aryl groups ($\text{C}_{aliph}\text{-O-C}_{arom}$) are removed by cleaving ether linkages, and β -aryl groups ($\text{C}_{arom}\text{-O-C}_{arom}$) are removed by a neighboring group participating in the type of reaction shown in Figure 4.6. In addition to these type of bonds, carbon to-carbon bonds are also found in lignin structure. [97]. Carbon-carbon bonds are cleaved by the aldol type of reaction shown in Figure 4.7 [98,99].

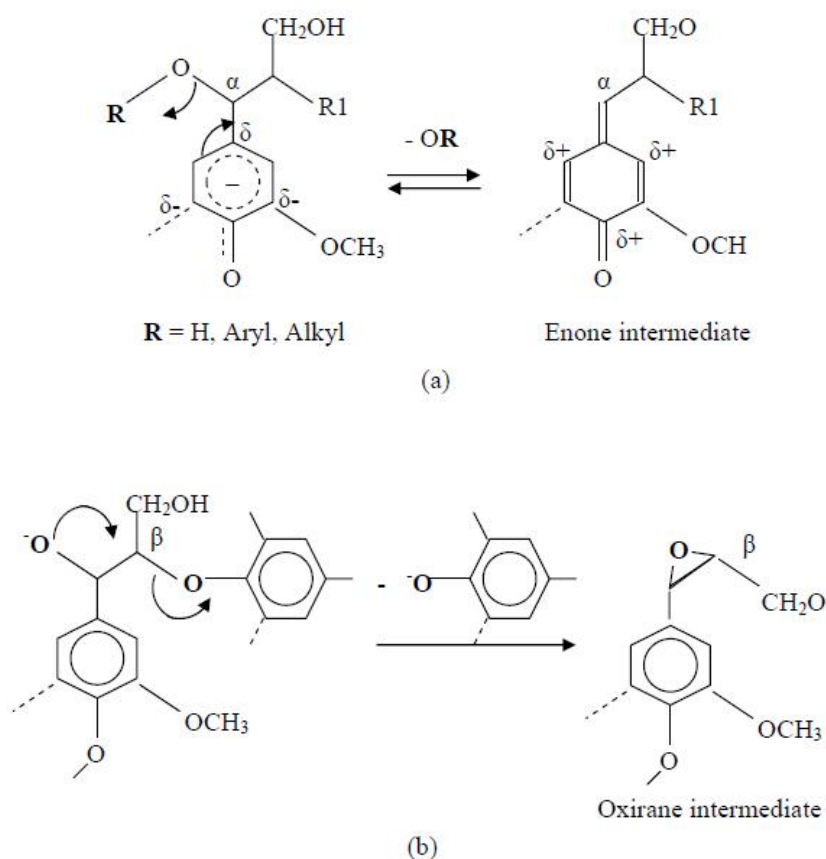


Figure 4.6: Cleavage of (a) α -aryl and (b) β -aryl ether linkages in alkaline conditions. [7]

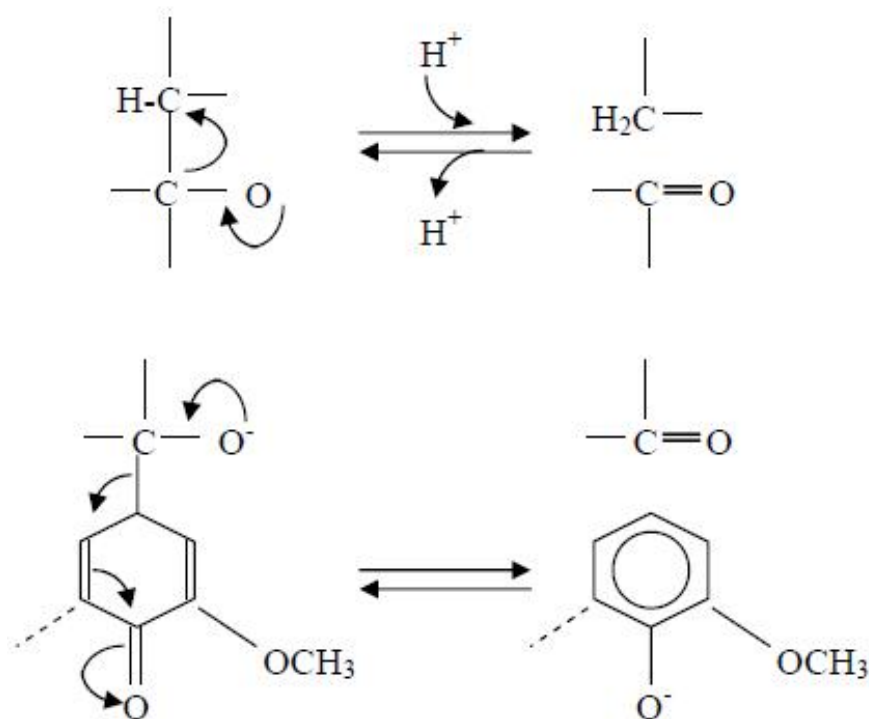


Figure 4.7: Alkaline cleavage of carbon-carbon bonds. [7]

4.4 Mass transfer

Oxygen delignification involves three phases: solid (fiber), liquid (aqueous alkali solution), and gas (oxygen). Therefore, mass transfer between the multiple phases must be considered. Oxygen must cross the gas-liquid interface, diffuse through the liquid film surrounding the fiber, and finally diffuse into the fiber wall before oxygen delignification reactions occur. The rate of oxygen transport is important to the rate of the overall process [8, 100]

4.5 Mathematical model for oxygen delignification

A kinetic model is important as a key factor to design, optimize, and control the pretreatment process. Large amounts of data have been generated on delignification kinetics applied to oxygen bleaching [101–103]. There are several models published in the literature in which mass transfer phenomena and chemical reactions are combined in to a simple power law. The models account for different degradation rates that occur with lignin and carbohydrates moieties of differing reactivity. Some of these models follow: single equation, high order on lignin. This model was not interesting because it contradicts studies on the mechanisms of oxygen delignification that show first-order

kinetics in residual lignin [9]. Additionally, in some instances, high-order lignin models gave abnormal reaction rates [104].

Sum of an infinite number of parallel first-order reactions and rate constants that are interpreted as a function distribution. This model introduces large complexity to calculations, but its ability to fit the data is not considerably improved compared to simpler approaches [105].

Currently, delignification kinetic models have been obtained for poplar wood lime treated at temperatures between 110°C and 180°C, under pressure of oxygen between 7.9 and 21.7 bar (absolute), for period of time between 1 and 10 hours [87].

The following models will represent the proposal kinetic model in that study:

Model 1: Two parallel, first-order reactions, and

Model 2: Three parallel, first-order reactions. To accurately represent delignification and carbohydrate degradation processes with this approach, it is necessary to consider differing reactivities [106, 107]. For each biomass component (lignin, cellulose, and hemicellulose).

Model 1 uses two parallel simultaneous reactions (fast and slow) for lignin, illustrated in Figure 4.8. Similarly, Model 2 uses three parallel simultaneous reactions (fast, medium, and slow), which are often considered in Kraft delignification [106, 108, 109]. Additionally, Models 1 and 2 use first-order kinetics on lignin because previous studies have reported evidence for this reaction order [9, 101]. In the previous studies it was usually obtain kinetic parameters are using Kappa number, a widely used estimate of lignin content (Note: Kappa number is 6.7 times larger than the lignin content) [102]. But in recently study lignin yield used instead of Kappa number, which was also used in kinetic modeling of lime pretreatment by Kim and Holtzapple [87, 110].

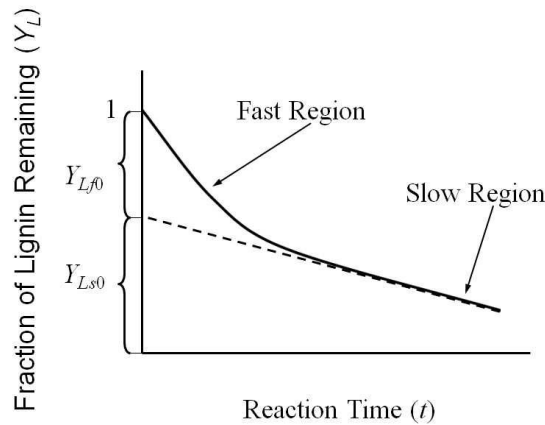


Figure 4.8: Delignification of lgnocellulosic biomass in two simultaneous stages: fast (Y_{Lf0}) and slow (Y_{Ls0}). [9]

Where: Y_{Lf0} is yield of lignin for fast-region at time zero, Y_{Ls0} is yield of lignin for slow-region at time zero.

The generalized Eq.4.1 defines lignin, glucan and xylan yields.

$$Y_i \equiv \frac{C_i \cdot Y_P}{C_0} \quad (4.1)$$

Where:

i = lignin L, glucan G, and xylan X

Y_i = pretreatment yield of component i at time t (kg residual component i/kg component i in raw biomass)

C_0 = component i content at time zero (kg component i in raw biomass/kg raw biomass)

C_i = component i content at time t (kg residual component i/kg residual biomass)

Y_P = total solids pretreatment yield at time t (kg residual biomass/kg raw biomass).

Models 1 and 2 describe each biomass component (lignin, glucan and xylan) as the sum of fast f, medium m (for Model 2 only), and slow s moieties

$$Y_i = \sum_j Y_{ij} \quad (4.2)$$

where: i = lignin L, glucan G, or xylan X; j = f and s (Model 1) and f, m, and s (Model 2); Y_{ij} = yield of component i at time t (kg residual component i/kg initial component i).

At time zero,

$$Y_i = \sum_j Y_{ij0} = 1 \quad (4.3)$$

Because an excess of lime is employed in all experiments, and lime is sparingly soluble, hydroxide concentration $[\text{OH}^-]$ is always constant, i.e., it is not a variable in the models. As a result, the models must only describe the effects of oxygen pressure, time, temperature, and the amount of component i in the residual biomass

$$\frac{dY_i}{dt} = \sum_j K_{ij} P_{O_2}^{(\beta_{ij})} Y_{ij} \quad (4.4)$$

$$K_{ij} = a_{ij} \exp\left(-\frac{E_{ij}}{RT}\right) \quad (4.5)$$

Where: K_{ij} = rate constant $((\text{min} \cdot \text{bar}^{(\beta_{ij})})^{-1})$;

a_{ij} = frequency factor $((\text{min} \cdot \text{bar}^{(\beta_{ij})})^{-1})$; E_{ij} = activation energy (kJ/mol); R = ideal

gas constant (8.314×10^{-3} kJ/(mol·K)); T = absolute temperature (K); P_{O_2} = oxygen pressure (bar, absolute) ; β_{ij} = exponent (dimensionless).

The integral form of Eq. 4.4 is

$$Y_i = \sum_j Y_{ij0} \exp(-K_{ij} P_{O_2}^{\beta_{ij}} t) \quad (4.6)$$

where: Y_{ij0} = yield of component ij at time zero (kg residual component ij/kg initial component i).

The last equation represent nonlinear kinetic equations of lignocellulosic components .

4.6 Limitations in oxygen delignification

Hydroxyl radicals formed during oxygen delignification can react with both lignin and polysaccharides. Reaction of hydroxyl radicals with lignin leads to hydroxylation of aromatic rings and thus making lignin more reactive for further attack by oxygen. However, the random chain cleavage of cellulose and hemicellulose by hydroxyl radicals limits the extent of delignification which can be achieved without serious polysaccharides degradation. Oxygen is a rather weak reactive oxidant because it requires high temperature and/or the ionization of the reacting functional groups to get a reasonable reaction rate. For this reason why oxygen delignification is performed at alkali conditions, and fairly high temperature and high pressure. However, the temperature increase is limited because severe carbohydrate degradation takes place when the temperature is very high [111].

Limitation in the mass transfer of oxygen from the gas phase to the liquid phase is also a quite important issue in oxygen delignification [9].

4.7 Lime consumption

In lime pretreatment, $Ca(OH)_2$ was used as alkali agent to pretreat woody biomass, and the lime consumption depended on the conditions such as temperature and oxygen pressure. During the pretreatment, OH^- reacted with many different functional groups in lignocellulosic biomass, e.g., phenolics and ethers in lignin, acetyls and the end groups of cellulose and hemicellulose, and oxygen molecules.

Ca^{+2} , is deposited in the cellulose matrix in the form of calcium carbonate, where Ca^{+2} can react with carbon dioxide to form calcium carbonate, which gradually deposits in the lignocellulosic matrix.

Carbon dioxide may be generated from delignification and degradation of cellulose and hemicellulose. In general, for higher temperatures, pressures and pretreatment times more lime was consumed. Lime consumption will be determined by titration for different conditions by neutralization with 5-N HCl (Appendix F).

General Definitions of Process Yields

Pretreatment yields, hydrolysis yields, and overall yields will be discussed in this chapter before representing the results. An illustration of the general definitions of these yields is presented in the end of this chapter in Figure 5.1.

5.1 Pretreatment yields

Pretreatment yields specify how much of the component (i.e., lignin, glucan or xylan) in the raw biomass was found in the pretreated biomass. Thus they assess pretreatment. Pretreatment yield is also named recovery yield of total mass or yield of total solid. Pretreatment yields can be defined as follows:

Pretreatment yield:

$$Y_P = \frac{(g) \text{ pretrated biomass}}{(100 g) \text{ raw biomass}} \quad (5.1)$$

Pretreatment yield of lignin:

$$Y_L = \frac{(g) \text{ pretrated lignin remaining after pretreatment}}{(100 g) \text{ lignin in raw biomass}} \quad (5.2)$$

Pretreatment yield of glucan (cellulose):

$$Y_G = \frac{(g) \text{ pretrated glucan remaining after pretreatment}}{(100 g) \text{ glucan in raw biomass}} \quad (5.3)$$

Pretreatment yield of xylan (hemicellulose):

$$Y_X = \frac{(g) \text{ pretrated xylan remaining after pretreatment}}{(100 g) \text{ xylan in raw biomass}} \quad (5.4)$$

5.2 Hydrolysis yields

Hydrolysis yields specify how much glucan or xylan present in the pretreated biomass was found after saccharification (i.e., enzymatic hydrolysis), thus they assess saccharification. Hydrolysis yields also can be defined as follows:

Hydrolysis yield of glucan (cellulose):

$$Y_g = \frac{(g) \text{ pretrated glucan hydrolyzed}}{(100 g) \text{ glucan in treated biomass}} \quad (5.5)$$

Hydrolysis yield of xylan (hemicellulose):

$$Y_x = \frac{(g) \text{ pretrated xylan hydrolyzed}}{(100 g) \text{ xylan in treated biomass}} \quad (5.6)$$

5.3 Overall yields

Overall yields indicate how much glucan or xylan present in the untreated biomass were found after pretreatment and enzymatic hydrolysis, thus they assess the combined effect of the two operation. Overall yields also can be defined as follows:

Overall yield of glucan (cellulose):

$$Y_G^T = \frac{g \text{ pretrated glucan obtained after pretreatment and enzymatic hydrolysis}}{100 g \text{ glucan in raw biomass}} \quad (5.7)$$

Overall yield of xylan (hemicellulose):

$$Y_X^T = \frac{g \text{ pretrated xylan obtained after pretreatment and enzymatic hydrolysis}}{100 g \text{ xylan in raw biomass}} \quad (5.8)$$

Overall yield of total sugar(cellulose and hemicellulose):

$$Y_S^T = \frac{(g \text{ pretrated glucan hydrolyzed} + g \text{ pretrated xylan hydrolyzed})}{100 g \text{ raw biomass}} \quad (5.9)$$

We want to mention here that there was relationship between all type of yield as follow:

$$Y_G^T = Y_G \times Y_g \quad (5.10)$$

$$Y_X^T = Y_X \times Y_x \quad (5.11)$$

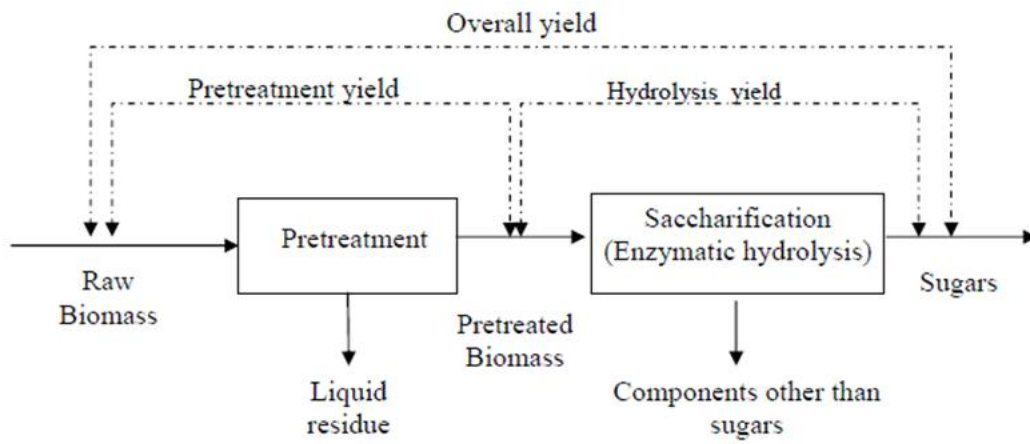


Figure 5.1: Schematic definitions of pretreatment yield, hydrolysis yield and overall yield [10]

Optimization of Lime Pretreatment

The aims of this research involve the study of effect of temperature, oxygen pressure and pretreatment time on the (pretreatment yield and the content of the treated biomass). On the other hand this study aims to determine which lime pretreatment conditions cause the greatest increasing in pretreated willow wood digestibility at the same time with highest pretreatment yield (total sold mass recovery).

After finishing of pretreated process, the content of the reactor was transferred to a 1-L beaker using about 250 mL of deionized water (DI) . Then the slurry was neutralized by titrating with 5.0-N HCl to measure the unreacted lime. The solid recovery was extensively washed with clear DI water and filtered using a vacuum filtration apparatus with a Whatman 934/AH glass fiber filter paper (particle retention = 2 μm , Fisher Scientific Co., Pittsburgh, PA). Once filtered, the only biomass that need to be analyzed for composition was air-dried at room temperature. The weight of the dry biomass and its moisture content were recorded to account for the pretreatment yield of solids. The biomass was stored at -20°C until using for analysis or enzyme hydrolysis.

6.1 Recovery yield of total mass (Y_P)

Mass balances was performed to determine how much biomass was solubilized by the lime pretreatment. Recovery yield of total mass (also named pretreatment yield or yield of total solid).

Table 6.1 shows the results of recovery yield of total mass. Generally, the tendency of more solubilization is for high temperatures, long pretreatment times and high pressure. Also the rate of solubilization increases along time at all temperatures and especially when increasing the oxygen pressure.

Table 6.1: Pretreatment yield of total mass with pretreatment conditions

pretreatment conditions				pretreatment yield	
	temperature, °C	pressure, bar	time, hour	recovery total mass, g	Y_P
1	147	17.8	5.0	6.090	0.535
2	113	17.8	5.0	7.700	0.675
3	147	9.2	5.0	6.450	0.565
4	147	17.8	2.0	6.810	0.597
5	113	9.2	5.0	8.160	0.715
6	113	17.8	2.0	8.550	0.750
7	147	9.2	2.0	7.420	0.650
8	113	9.2	2.0	8.900	0.780
9	160	13.5	3.5	5.740	0.504
10	130	21.0	3.5	7.110	0.623
11	130	13.5	6.0	6.750	0.592
12	100	13.5	3.5	9.210	0.807
13	130	6.0	3.5	7.750	0.679
14	130	13.5	1.0	8.350	0.732
15	130	13.5	3.5	7.540	0.661
16	130	13.5	3.5	7.560	0.663
17	130	13.5	3.5	7.530	0.660
18	130	13.5	3.5	7.550	0.662

6.2 Pretreatment yields of cellulose (Y_G) and hemicellulose (Y_X)

The pretreatment yields of interest includes glucan pretreatment yields (i.e., glucan remaining in the solids after pretreatment), xylan pretreatment yields (i.e., xylan remaining in the solids after pretreatment) and lignin pretreatment yields (i.e., lignin remaining in the solids after pretreatment).

Table 6.2 shows pretreatment yield of glucan and xylan. For all conditions, glucan yields were above 90 g glucan recovered/100 g glucan in raw biomass. In the case of xylan, the degradation was much faster than glucan. In general, less carbohydrates were recovered when the solids underwent aggressive pretreatments (higher temperatures, higher pressures and longer time). Also glucan was much more stable than xylan.

Table 6.2: Pretreatment yield of glucan and xylan with pretreatment conditions

pretreatment conditions				pretreatment yield			
	temperature, °C	pressure, bar	time, h	glucan recovered, g	glucan yield	xylan recovered, g	xylan yield
1	147	17.8	5.0	4.324	0.927	0.800	0.422
2	113	17.8	5.0	4.395	0.942	1.051	0.555
3	147	9.2	5.0	4.386	0.941	0.825	0.436
4	147	17.8	2.0	4.392	0.942	0.951	0.502
5	113	9.2	5.0	4.433	0.950	1.057	0.558
6	113	17.8	2.0	4.543	0.974	1.154	0.610
7	147	9.2	2.0	4.526	0.970	1.007	0.532
8	113	9.2	2.0	4.628	0.992	1.291	0.682
9	160	13.5	3.5	4.218	0.904	0.796	0.420
10	130	21.0	3.5	4.331	0.929	0.959	0.314
11	130	13.5	6.0	4.325	0.927	0.923	0.487
12	100	13.5	3.5	4.605	0.987	1.473	0.778
13	130	6.0	3.5	4.382	0.987	1.123	0.593
14	130	13.5	1.0	4.580	0.982	1.169	0.617
15	130	13.5	3.5	4.411	0.946	1.025	0.541
16	130	13.5	3.5	4.417	0.947	1.008	0.532
17	130	13.5	3.5	4.405	0.945	1.024	0.541
18	130	13.5	3.5	4.416	0.947	1.025	0.541

6.3 Delignification

In lime pretreatment, the rate of lignin degradation is much more than carbohydrate. Like the carbohydrate, lignin degradation rate increases in severe conditions. The effect of temperature and pressure on delignification were very important, where more lignin was removed at higher temperatures and pressure.

It is well known that lignin associate with hemicellulose in the cell wall thus, hemicellulose degradation will increase with lignin removal. Lignin yields Y_L obtained for all pretreatment conditions are summarized in Table 7.3.

Table 6.3: Pretreatment yield of lignin with pretreatment conditions

pretreatment conditions				lignin yield	
	temperature, °C	pressure, bar	time, hour	lignin recovered, g	yield
1	147	17.8	5.0	0.610	0.206
2	113	17.8	5.0	1.850	0.627
3	147	9.2	5.0	0.846	0.286
4	147	17.8	2.0	1.030	0.349
5	113	9.2	5.0	2.122	0.719
6	113	17.8	2.0	2.134	0.723
7	147	9.2	2.0	1.360	0.460
8	113	9.2	2.0	2.229	0.755
9	160	13.5	3.5	0.570	0.193
10	130	21.0	3.5	1.422	0.482
11	130	13.5	6.0	1.147	0.388
12	100	13.5	3.5	2.302	0.780
13	130	6.0	3.5	1.705	0.578
14	130	13.5	1.0	1.920	0.651
15	130	13.5	3.5	1.508	0.511
16	130	13.5	3.5	1.464	0.496
17	130	13.5	3.5	1.486	0.503
18	130	13.5	3.5	1.480	0.502

6.4 Material balances

Material balances were performed on willow wood to determine how much biomass was solubilized by oxidative lime pretreatment. Untreated and pretreated willow wood were repeatedly washed with fresh distilled water until the decanted water become colorless. The total dry weight for each sample was measured before and after the pretreatment-and-wash. Using the dry weight measurement for biomass composition was describe in chapter two, for determination of sugar yield and material balance. Raw biomass composition (ash content, lignin, xylan, and glucan) and pretreated-and-washed willow wood were determined using the standard analysis procedures provided by NREL [112].

Table 6.4: Summary of material balance from various pretreatments of willow wood

No. Of Exp.	Y_p^a %	$1-Y_p^b$ %	Lignin		Glucan		Xylan		Other	
			Pret ^c .	Loss ^d	Pret ^e .	Loss ^f	Pret ^g .	Loss ^h	Pret ^j .	Loss ⁱ
1	53.4	46.60	10.00	79.40	71.00	7.30	13.50	58.00	5.50	81.50
2	67.5	32.50	24.00	37.40	56.68	3.00	13.63	48.80	5.60	76.08
3	56.5	43.5	11.00	71.40	68.00	5.90	12.80	56.4	6.03	78.66
4	59.7	40.3	15.10	49.80	64.50	5.80	14.00	49.22	6.47	75.70
5	71.5	28.5	26.00	28.10	54.33	5.00	12.96	44.20	6.71	78.66
6	75.0	25.0	42.96	27.70	53.14	3.60	13.50	39.00	8.41	60.59
7	64.0	36.0	18.32	52.00	62.00	3.00	13.80	46.80	7.20	71.14
8	78.0	22.0	25.00	24.50	62.00	1.00	14.50	31.80	8.50	58.75
9	50.3	49.7	8.70	0.82	74.00	9.60	13.50	58.00	4.72	85.13
10	62.3	37.7	20.00	51.80	61.02	7.10	13.50	68.60	5.60	78.16
11	59.2	48.8	17.00	61.20	63.50	7.30	13.68	51.30	5.00	81.78
12	80.7	19.3	25.00	22.00	50.00	1.30	16.00	32.20	9.91	49.91
13	67.9	32.1	22.00	42.20	56.00	1.30	14.00	40.70	7.05	69.90
14	73.2	36.8	23.00	34.90	55.10	1.80	14.00	38.30	8.20	62.79
15	66.1	43.9	20.00	48.90	58.50	5.40	13.60	45.90	7.21	70.16
16	66.3	43.7	19.90	51.40	58.50	5.30	13.70	46.80	7.20	70.10
17	66.0	44.0	20.00	49.70	58.24	5.50	13.78	45.90	7.34	69.66
18	66.20	43.8	20.00	49.80	58.12	5.30	13.86	45.90	7.33	70.10

$$Y_p^a(\text{total sold yield}) = \frac{\text{total sold recovery}}{11.4}$$

$$1-Y_p^b = \text{total weight lost}$$

$$\text{Pret}^c(\text{composition of lignin in pretreated biomass}) = \frac{\text{lignin recovered}}{\text{total solid recovery}}$$

$$\text{Loss}^d = 1 - Y_L$$

$$\text{Pret}^e(\text{composition of glucan in pretreated biomass}) = \frac{\text{glucan recovered}}{\text{total solid recovery}}$$

$$\text{Loss}^f = 1 - Y_G$$

$$\text{Pret}^g(\text{composition of xylan in pretreated biomass}) = \frac{\text{xylan recovered}}{\text{total solid recovery}}$$

$$\text{Loss}^h = 1 - Y_X$$

$$\text{Pret}^j(\text{composition of ash, mannan and acetyl in pretreated biomass}) = 1 - (\text{Pret}^c - \text{Pret}^e - \text{Pret}^g)$$

$$\text{Loss}^i = 1 - \frac{\text{Pret}^j \times \text{total sold recovery}}{\text{other recovered}}$$

Composition of dry biomass, total mass(11.40 g), mass of glucan(4.66 g), mass of xylan(1.89 g), mass of lignin(2.95 g), other compounds include(extractive, ash content, mannan, acetyl group and galactan)(1.315 g)

6.5 Applications of statistical method (Box-Wilson Design) to find optimum conditions

Box-Wilson experimental design was used in the optimization of oxidative of lime pretreatment, at temperature ($X_1, ^\circ\text{C}$), pressure (X_2 , bar(absolute), and time (X_3 , hour) were chosen as independent factors in experimental design.

Yield of lignin, glugan and xylan represent the dependent output variable. For convenience, the independent variables in the model are utilized in their coded form. The variables $X_{i \text{ actual}}$ were coded as $X_{i \text{ coded}}$ according to the equations 3.1 and 3.2. Box-Wilson experimental plan with six star points ($\alpha = 1.732$) and four replicates at the center point with a total number of experiments (18) employed. The data at the point (0,0,0) is needed to measure the error involved in the experiment. The coded values for the independent variables and the corresponding real values are given in Table 3.1 and 6.5. X_1 is temperature (100-160 $^\circ\text{C}$), X_2 is pressure (6-21 bar) and X_3 is pretreatment time (1-6 h). Table 6.5 shows experimental design with their responses.

Table 6.5: The relationship between coded level and corresponding real variable, with real response of pretreatment yields, according to central composite design

Codede Values				Real Values			Pretreatment yield		
Exp.No	X_1	X_2	X_3	$X_1, ^\circ\text{C}$	X_2 , bar	X_3 , h	Lgnin yield	Glucan yield	Xylan yield
1	1	1	1	147	17.8	5.0	0.206	0.927	0.420
2	-1	1	1	113	17.8	5.0	0.627	0.942	0.555
3	1	-1	1	147	9.2	5.0	0.286	0.941	0.436
4	1	1	-1	147	17.8	2.0	0.349	0.942	0.502
5	-1	-1	1	113	9.2	5.0	0.719	0.950	0.558
6	-1	1	-1	113	17.8	2.0	0.723	0.974	0.610
7	1	-1	-1	147	9.2	2.0	0.460	0.970	0.532
8	-1	-1	-1	113	9.2	2.0	0.755	0.992	0.682
9	α	0	0	160	13.5	3.5	0.193	0.904	0.420
10	0	α	0	130	21.0	3.5	0.482	0.929	0.314
11	0	0	α	130	13.5	6.0	0.388	0.927	0.487
12	- α	0	0	100	13.5	3.5	0.780	0.987	0.778
13	0	- α	0	130	6.0	3.5	0.578	0.987	0.593
14	0	0	- α	130	13.5	1.0	0.651	0.982	0.617
15	0	0	0	130	13.5	3.5	0.511	0.946	0.541
16	0	0	0	130	13.5	13.5	0.496	0.947	0.532
17	0	0	0	130	13.5	13.5	0.503	0.945	0.541
18	0	0	0	130	13.5	13.5	0.502	0.947	0.541

6.6 Results and discussion

Experimental design technique is used for the empirical study of the relationship between a measured objective on one hand and a number of operating conditions on the other hand. This method is used to find out how a particular objective is affected by a given set of operating conditions over some specified region of interest and to determine the values of operating conditions which will yield a maximum for the specific objective as a result of optimization. The major advantage of applying an experimental design is to reduce the number of experiments that have to be carried out to get maximum information [113,114]. Their benefit is enhanced in the study of industrial applications because most physical situations can usually be approximated by a quadratic function over a reasonable range of factors. Therefore the model of regression fitted as second order polynomial.

$$Y = B_0 + \sum_{i=1}^k B_i X_i + \sum_{i \leq k} \sum B_{ik} X_i X_k + \epsilon \quad (6.1)$$

Where Y is the predicted response, subscripts i, and k vary from 1 to the number of variables, B₀ is the intercept term, B_is are linear coefficients, B_{ik}s are quadratic coefficients, ϵ is a random error component.

Parameters of this equation were evaluated from experimental results of specific experiments that designed to determine their value with the NLREG (Nonlinear Regression Analysis Software) by using STATISTICA-program software. The resultant functional relationship in terms of real values with pretreatment yields of different component are:

$$\begin{aligned} Y_L = & +1.72070 - 6.884E-003 \times X_1 - 0.011937 \times X_2 + 8.540E-004 \times X_3 \\ & - 4.433E-005 \times X_1 \times X_2 - 1.224E-005 \times X_1 \times X_3 - 5.212E-006 \times X_2 \times X_3 \\ & + 3.862E-007 \times X_1^2 + 3.795E-004 \times X_2^2 + 2.387E-007 \times X_3^2. (R = 0.98) \end{aligned} \quad (6.2)$$

$$\begin{aligned} Y_G = & +1.23308 - 2.326E-003 \times X_1 + 5.563E-003 \times X_2 - 8.232E-004 \times X_3 \\ & - 3.266E-005 \times X_1 \times X_2 + 2.639E-006 \times X_1 \times X_3 + 1.269E-006 \times X_2 \times X_3 \\ & + 5.192E-006 \times X_1^2 - 1.213E-004 \times X_2^2 + 6.708E-007 \times X_3^2. (R = 0.87) \end{aligned} \quad (6.3)$$

$$\begin{aligned} Y_X = & +2.461 - 0.023 \times X_1 + 0.0177 \times X_2 - 1.047E-003 \times X_3 \\ & + 5.6170E-005 \times X_1 \times X_2 + 5.847E-008 \times X_1 \times X_3 + 2.723E-005 \times X_2 \times X_3 \\ & + 6.841E-005 \times X_1^2 - 1.5082E-003 \times X_2^2 + 4.809E-007 \times X_3^2. (R = 0.89) \end{aligned} \quad (6.4)$$

Where Y_L lignin yield, Y_G glucane yield and Y_X xylan yield respectively.

These equations include all the terms regardless of their significance. It can be seen that the model is in a good fit with high value of regression coefficient (R) . All of the statistical models of different components are statistically significant because they have P-values lower than 0.05. These equations are valid for temperatures between 112 and 148°C, pressure values ranging from 9 to 18 bar (absolute) and the pretreatment time between 2 and 5 hours (according with this type of central composite design).

Table 6.6 represents the results at each point of the predicted values and real values of different yield of components. The predicted values are displayed in Table 6.6 along with the observed values. Comparison of these values indicates that the calculated yield values were in accordance with experimental yields.

Table 6.6: Comparison of experimental and predicted yield for different components

Exp.No	Real Y_L	Predicted Y_L	Real Y_G	Predicted Y_G	Real Y_X	Predicted Y_X
1	0.206	0.218	0.922	0.915	0.420	0.410
2	0.626	0.601	0.936	0.941	0.554	0.572
3	0.290	0.302	0.940	0.933	0.430	0.425
4	0.350	0.387	0.943	0.938	0.503	0.482
5	0.691	0.672	0.950	0.950	0.580	0.593
6	0.723	0.700	0.972	0.980	0.620	0.639
7	0.445	0.463	0.971	0.959	0.532	0.534
8	0.754	0.758	0.993	0.991	0.682	0.697
9	0.193	0.205	0.921	0.934	0.42	0.433
10	0.468	0.460	0.930	0.927	0.492	0.484
11	0.389	0.402	0.986	0.988	0.477	0.462
12	0.780	0.802	0.988	0.977	0.770	0.730
13	0.601	0.589	0.940	0.952	0.573	0.554
14	0.610	0.615	0.982	0.986	0.617	0.609
15	0.503	0.503	0.947	0.946	0.541	0.540
16	0.496	0.503	0.946	0.946	0.532	0.540
17	0.511	0.503	0.946	0.946	0.541	0.540
18	0.501	0.503	0.945	0.946	0.542	0.5402

We desire by the optimal pretreatment conditions, most of carbohydrate glucan should be remain in the lignocellulosic fiber matrix, while the lignin should be removed as much as possible. Accordingly, and in order to determine the optimal conditions for enzymatic hydrolysis, we need to know the mass balances at different lime pretreatments. Also selectivity data need to be considered between lignin removal and carboydrate degradation. In addition, the optimal condition for lime pretreatment should be de-

terminated by comparing the pretreatment yields, as well as the enzymatic hydrolysis of polysaccharides to monomeric sugars in the saccharification for each condition. This issue will be discussed in the next section (Enzymatic Hydrolysis).

To study the effect the independent variables (temperature, pressure, time) on the yields (degradation rate) of different components of glucan, xylan and lignin. We calculated the predicted values of different yields empirically by using equations 6.2, 6.3, 6.4.

Figures (6.1 A-6.9 A) represent the yields of different components, that are in relate to the time and oxygen pressure at 3 different levels of certain temperatures,

In order to demonstrate fas region reaction and slow region reaction during progress of degradation for each component we fixed the temperature at the same certain temperatures, and at each level we choose three different values of oxygen pressure (9, 14, 17 bar) (Figures 6.1 B-6.9 B).

Our choice for values of pressure and temperature depend on maximum, meddle and lowest values of valid region in imperial equations.

During oxidative short-term lime pretreatment, some portions of carbohydrate (glucan and xylan) were removed by action of oxygen and hydroxide ions (peeling reaction) in addition to the delignification reaction. In lime pretreatment of willow wood lignin, cellulose and hemicellulose degradation represent the direct functions of oxygen pressure, pretreatment time and temperature. Under the alkaline conditions of short-term lime pretreatment, a strong correlation between lignin and xylan degradation has been demonstrated. Cellulose degradation was minimal, thus lime pretreatment selectively removes lignin and hemicellulose as described below.

- Lignin degradation

Delignification depends highly on temperature and the presence of oxygen. Thus higher temperatures, oxygen pressures and longer pretreatment time result in a much higher lignin degradation

According to empirical model Eq.6.2 (Figures 6.7 B, 6.8 B, 6.9 B). Higher and lower Y_{Lf0} are observed for higher and lower temperature and pressure 0.50 and 0.18, respectively. Therefore lignin component degradation was even more drastic than others components. Changes of slope of Y_{Lf0} from high (mostly fast-degrading lignin) to low (mostly slow-degrading lignin) were observed approximately at 2h for all figures. This may be suppose that the fast region reaction continues during the first two hours of pretreatment time while the slow region starts after that. Because Y_{Lf0} is related to the fraction of easy-to-degrade lignin, these results indicate a strong effect of temperature and pressure on the rate of

lignin degradation. As well as the temperature has effects in the internal energy available for reaction whereas oxygen pressure relates to solubility.

Figures 6.7 A, 6.8 A, 6.9 A represent the curves of lignin yields that were derived from statistical program, which based on the empirical model (Eq.6.2) corresponding to varying pressure of oxygen and pretreatment while temperature was held at (113, 130, 147°C). The color gradient of these Figures which begins with red indicates the highest lignin yield (undesirable), and ends with dark blue represents the lowest lignin yield (desirable). In these figures we observe the relationship between the oxygen pressure and pretreatment time on the lignin yield. It can also be observed that increasing temperature leads to shifts of lignin yield toward more removal.

The highest recorded delignification was obtained for the pretreatment at 147°C, 17.8 bars and 5 hours. The yield was 19.3 g of lignin remaining/100 g lignin in the raw biomass. Whereas a very poor delignification response is achieved for the pretreatment at 100.0°C, 13.5 bar and 3.5 hours, the lignin yield was 78 g of lignin remaining/100 g lignin in the raw biomass.

- Glucan degradation

Glucan pretreatment yields usually were above 90 g glucan recovered/100 g glucan in raw biomass, for all our conditions as shown in the Figures (6.1 B, 6.2 B, 6.3 B). The difference between higher and lower Y_{Lf0} is approximately 0.07 while for lignin degradation is 0.32. the fast region reaction remained around during the first 4 h of pretreatment time and the slow region reaction start after that. This indicates that glucan degradation / lignin degradation will increase after 2h (time of fast region reaction for lignin) of the progress of pretreatment.

Figures 6.1 A, 6.2 A, 6.3 A, show the relationship between the oxygen pressure and time pretreatment on the glucan yield by holding the temperature at (113, 130, 147°C). The color gradient, unlike lignin degradation, these Figures which begins with red indicates the highest lignin yield (desirable), and ends with dark blue represents the lowest lignin yield (undesirable), the further degradation within progress of pretreatment at increasing temperature and/or pressure.

The highest observed glucan degradation is recorded for the pretreatment at 160°C and 13.5 bar 3.5 hours (90.47 g glucan degraded/g glucan in raw biomass) while the lowest glucan degradation is at 113°C, 9.2 bar and 2 hours, and its yield is 99.3 g of glucan remaining/100 g glucan in the raw biomass.

Generally, glucan degradation was very small in comparison with lignin and hemicellulose. Because as soon the pretreatment starts, some rapid peeling degradation of glucan occurs. Fast stopping reactions follow and degradation of remaining glucan depends on the generation of newly accessible reducing-end groups [17].

- Xylan degradation

In the case of xylan, the degradation was much faster than glucan. Figures 6.4 B, 6.5 B, 6.6 B shows The difference between higher and lower Y_{Lf0} was approximately 0.27 while for lignin degradation was approximately 0.32. The small difference between lignin and xylan degradation proves the intimate relationship between lignin and xylan components. But changing of slope of Y_{Lf0} from high (mostly fast-degrading) to low (mostly slow-degrading) were observed approximately at 120 min, it is almost like time of glucan degradation. Also Figures 6.1 A, 6.2 A, 6.3 A, show the relationship between the oxygen pressure and time pretreatment on the glucan yield by holding the temperature at (113, 130, 147°C). And the color gradient similar to glucan degradation, red color (desirable) and blue color (undesirable).

At 113°C, 31.5 bar and 3.5 hours the yield was above 75 g xylan recovered/100 g xylan in the raw biomass, whereas the highest xylan degradations were observed for the pretreatment at 160°C, 13.5 bar and 3.5 hours, and 147°C, 17.8 bars and 5 hours, the yields were 42 g of lignin remaining/100 g lignin in the raw biomass. Hemicellulose degradation is important, because the contribution of this carbohydrate polymer to the total carbohydrate yield is potentially significant. The average yield of xylan degradation for all our conditions is approximately close to the average yield of lignin degradation (0.536 and 0.511, respectively). Similar to lignin and cellulose degradation, xylan degradation is triggered by higher temperatures and pressures. Furthermore, xylan degradation is much more significant than glucan degradation and behaves very similar to lignin degradation. This is because of covalent bonds between hemicellulose and lignin in the cell wall [115].

Generally, Lignin content and crystallinity have major effects on biomass enzymatic digestibility, whereas acetyl content has a minor impact [5,116]. Therefore, pretreatments that can significantly remove lignin or reduce crystallinity are particularly effective by taking into consideration the balance between the lignin removal and carbohydrate degradation.

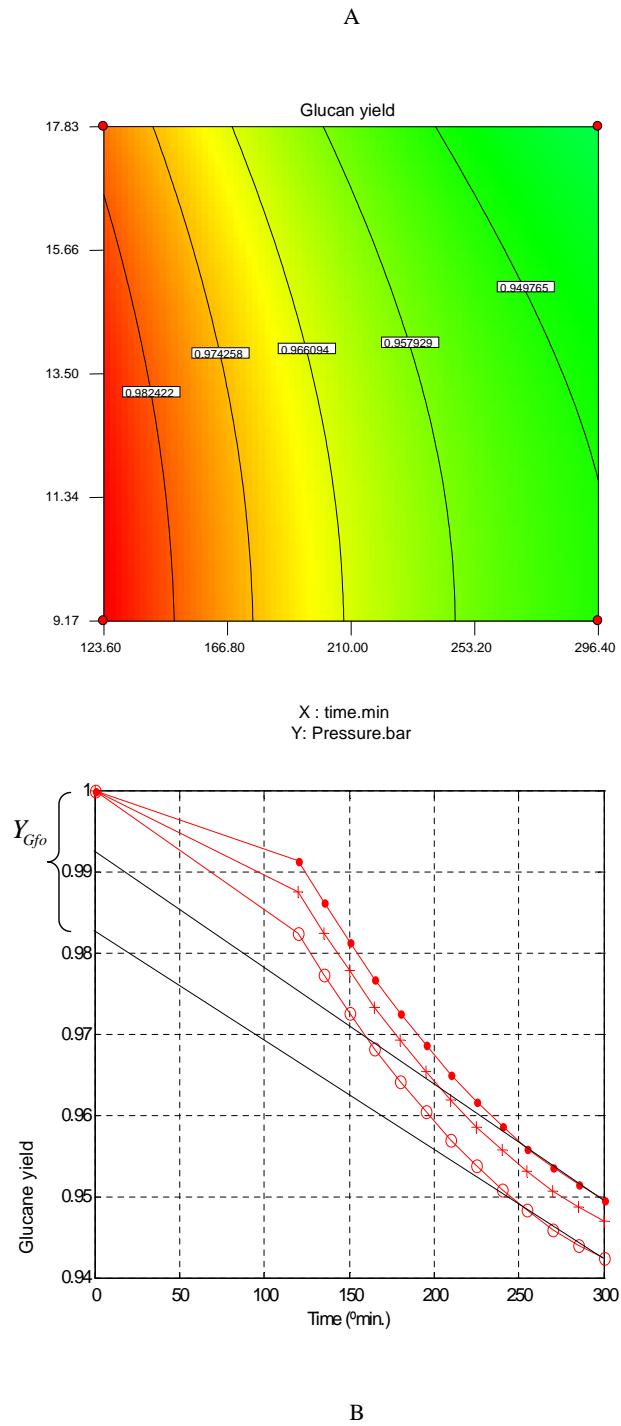


Figure 6.1: Glucan pretreatment yield in two different ways at 113°C , (A) according to statistical model (B) at (\bullet 9, \times 14, \circ 17 bar)

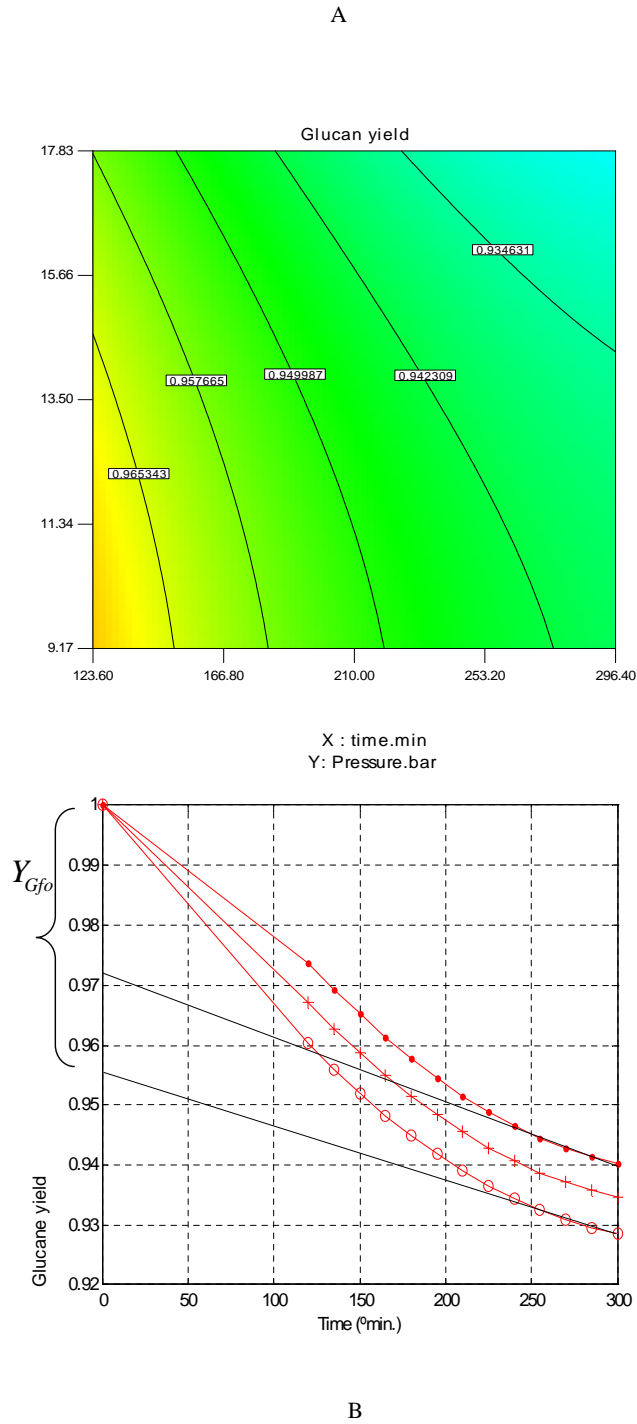


Figure 6.2: Glucan pretreatment yield in tow different ways at 130°C , (A) according to statistical model (B) at (\bullet 9, \times 14, \circ 17 bar)

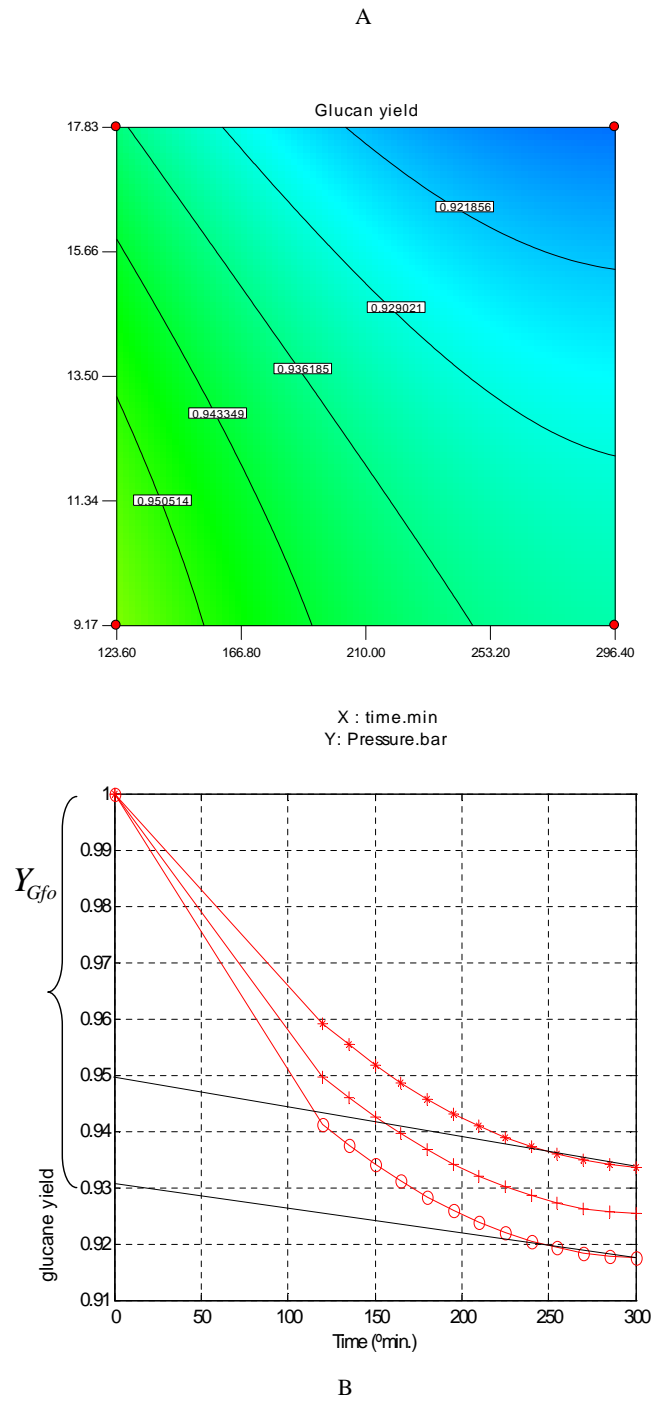


Figure 6.3: Glucan pretreatment yield in tow different ways at 14°C , (A) according to statistical model (B) (B) at (\bullet 9, \times 14, \circ 17 bar)

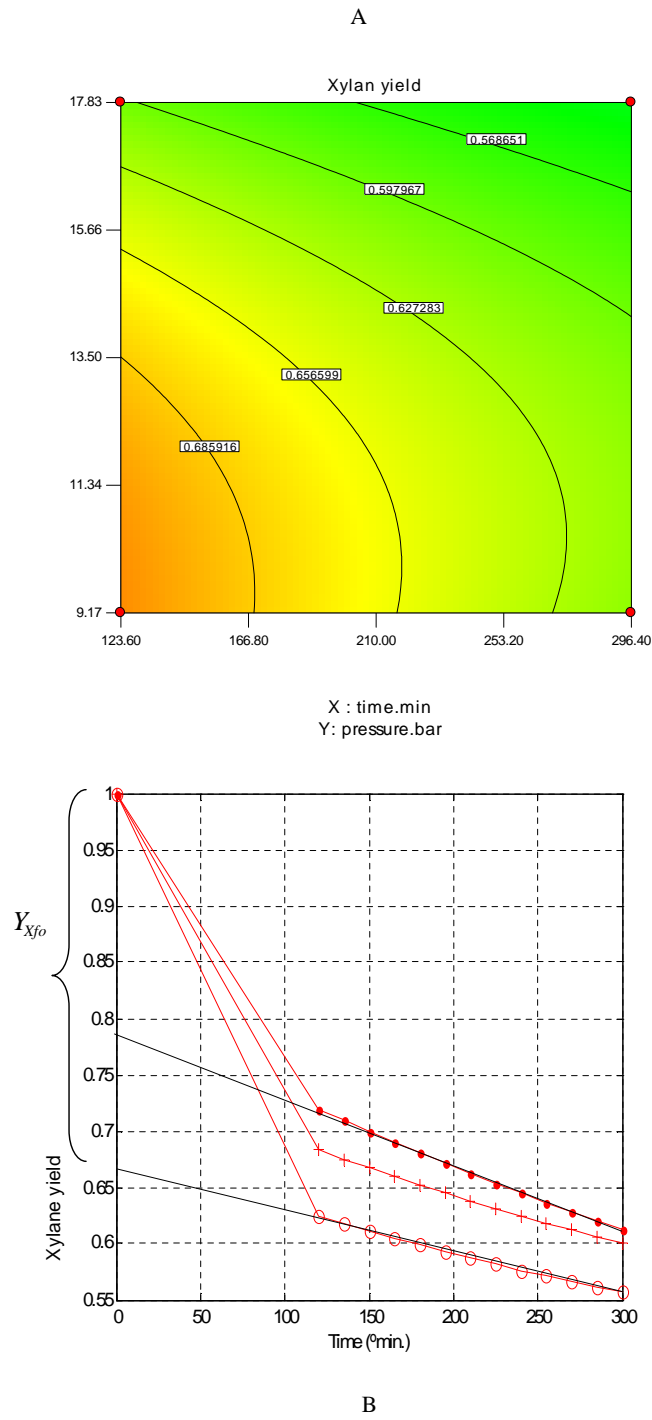


Figure 6.4: Xylan pretreatment yield in tow different ways at 113°C , (A) according to statistical model (B) (B) at (\bullet 9, \times 14, \circ 17 bar)

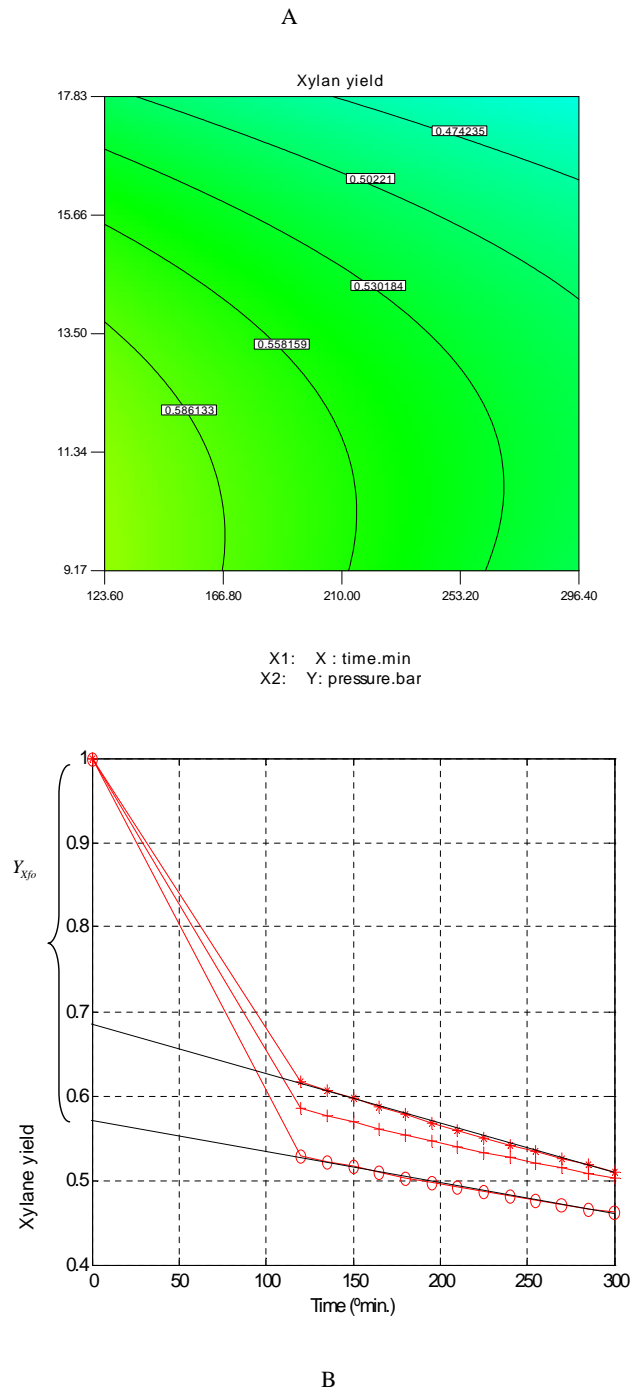


Figure 6.5: Xylan pretreatment yield in tow different ways at 130°C , (A) according to statistical model (B) (B) at (\bullet 9, \times 14, \circ 17 bar)

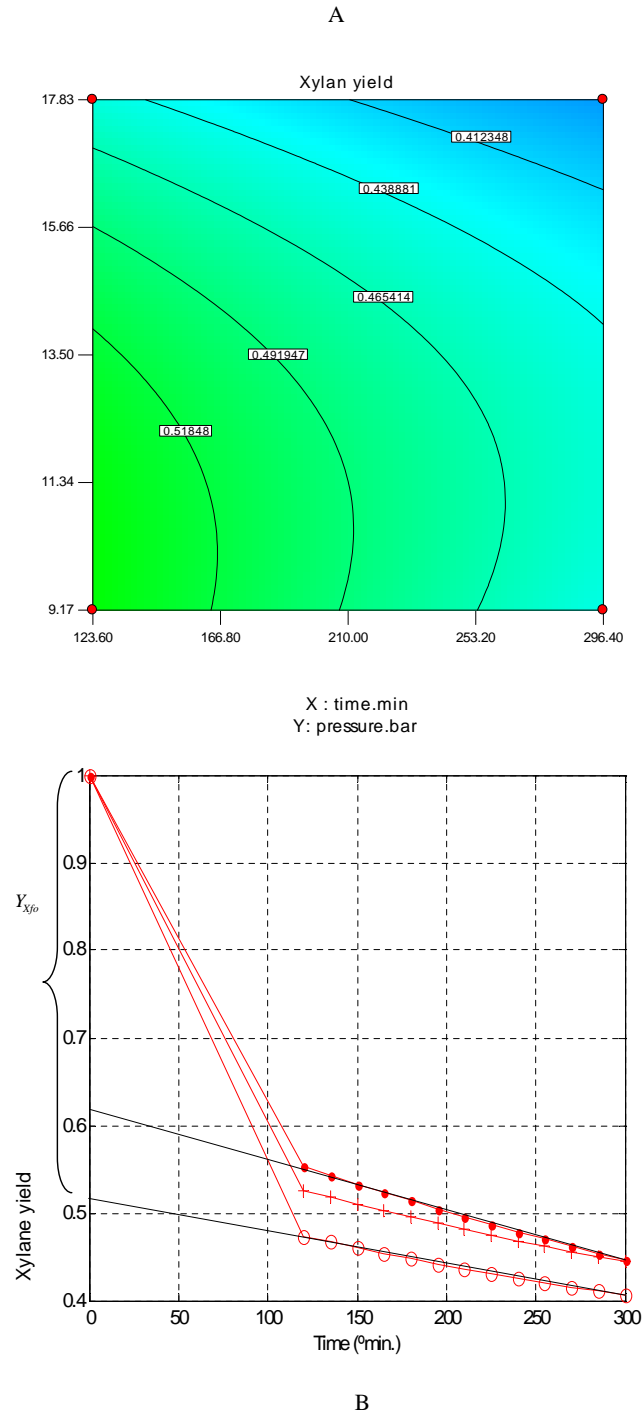


Figure 6.6: Xylan pretreatment yield in tow different ways at 147°C , (A) according to statistical model (B) (B) at (\bullet 9, \times 14, \circ 17 bar)

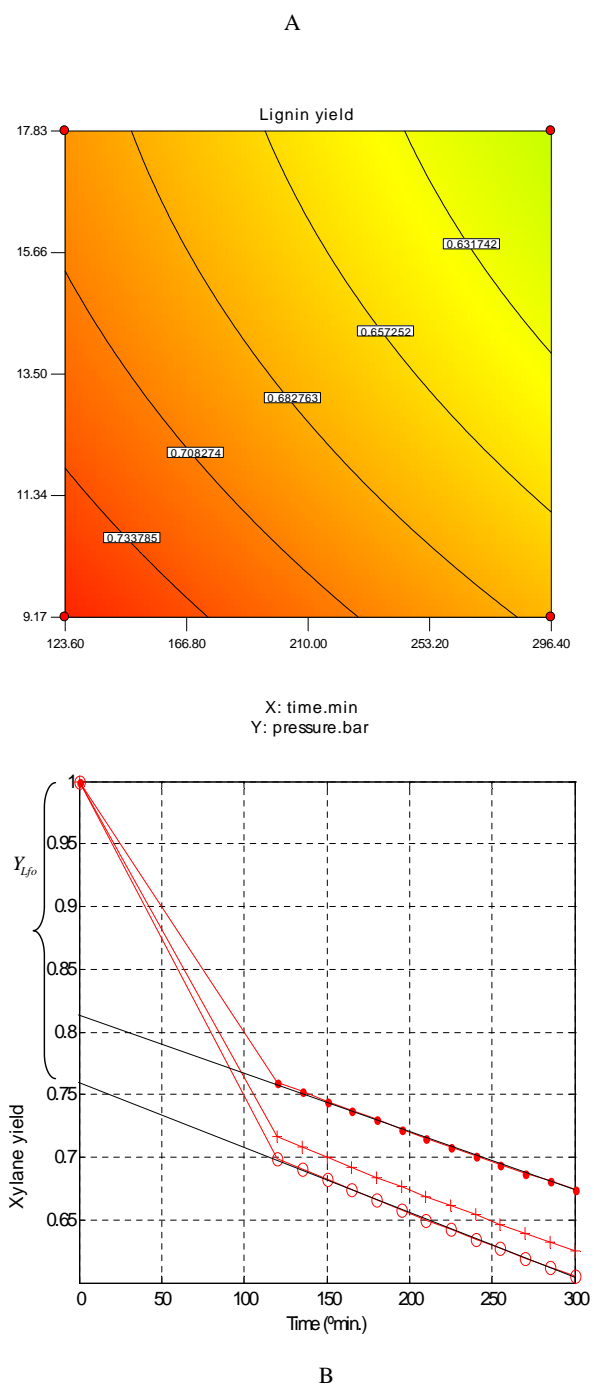


Figure 6.7: Legnin pretreatment yield in tow different ways at 113°C , (A) according to statistical model (B) (B) at (● 9, × 14, ○ 17 bar)

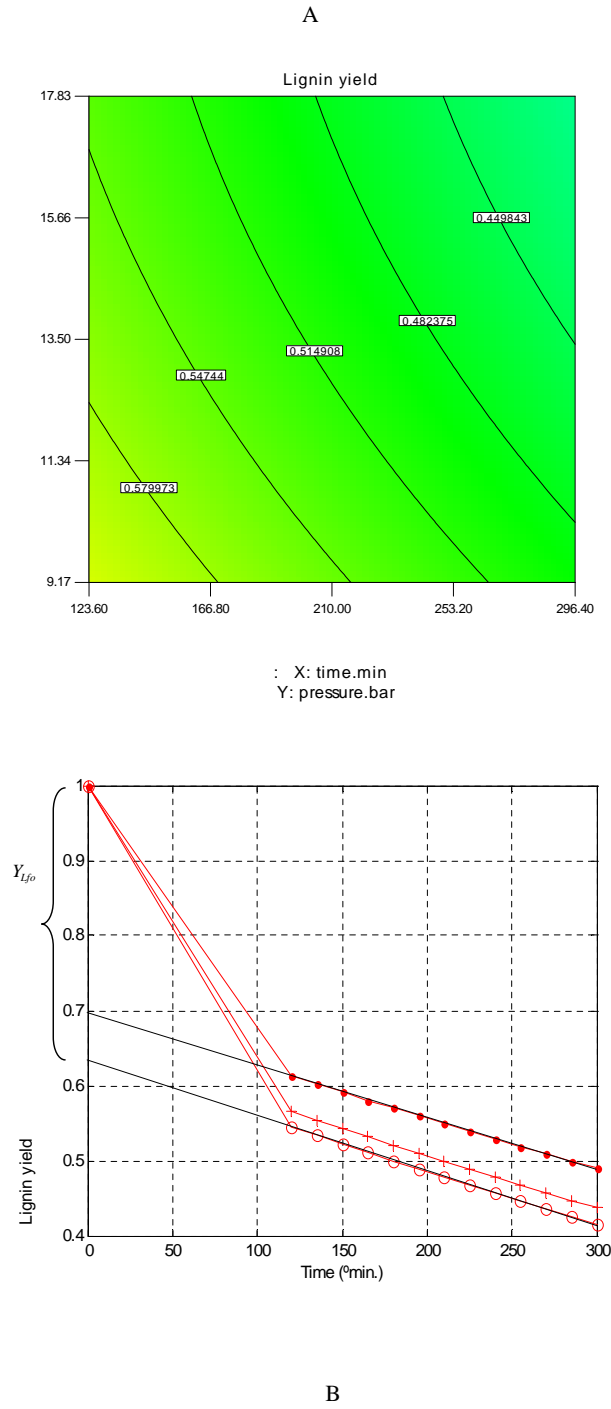
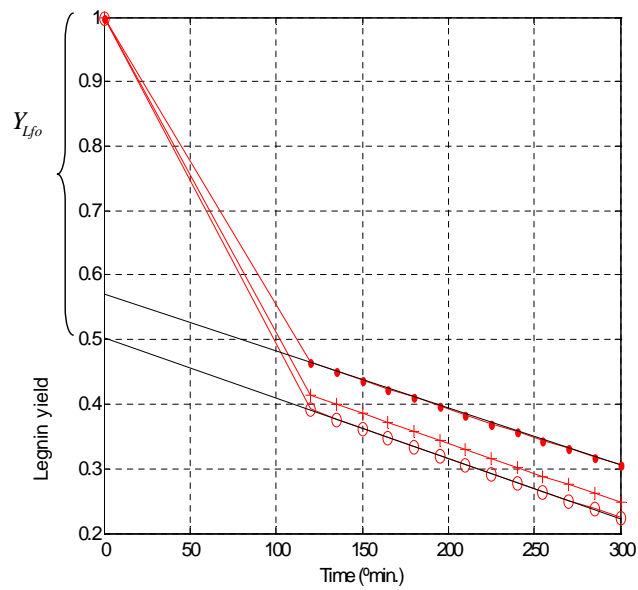
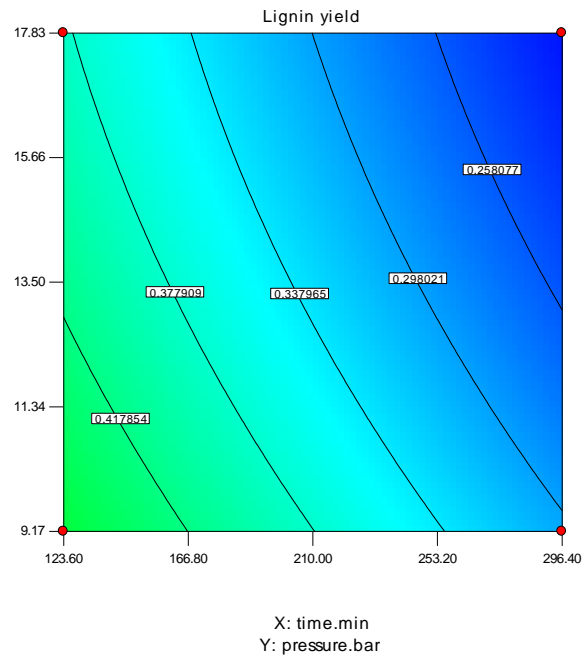


Figure 6.8: Legnin pretreatment yield in tow different ways at 130°C , (A) according to statistical model (B) at (B) at (\bullet 9, \times 14, \circ 17 bar)

A



B

Figure 6.9: Lignin pretreatment yield in tow different ways at 147°C , (A) according to statistical model (B) at (B) at (\bullet 9, \times 14, \circ 17 bar)

6.7 Conclusion

Lignin removal or delignification, sugar degradation, extractive and deacetylation represent the major contributors to the solubilization of biomass during pretreatment. However, the level of those solubility of willow wood during oxidative pretreatment is different from one condition to other. In general the solubility is increased with severe conditions of temperature, pressured oxygen and pretreatment time. The effect of these results on the overall effectiveness of the oxidative lime pretreatment of willow wood must be determined by measuring enzymatic hydrolysis yields.

Enzymatic Hydrolysis

7.1 Introduction

The pretreatment and hydrolysis processes of lignocellulosic biomass are major contributors to the total production cost of ethanol from biomass (60%) when using an enzyme-based process [88]. These two processes are combined because of structural features of biomass. The rate and extent of hydrolysis depend significantly on enzyme loading and structural features resulting from pretreatment (Figure 7.1) [5].

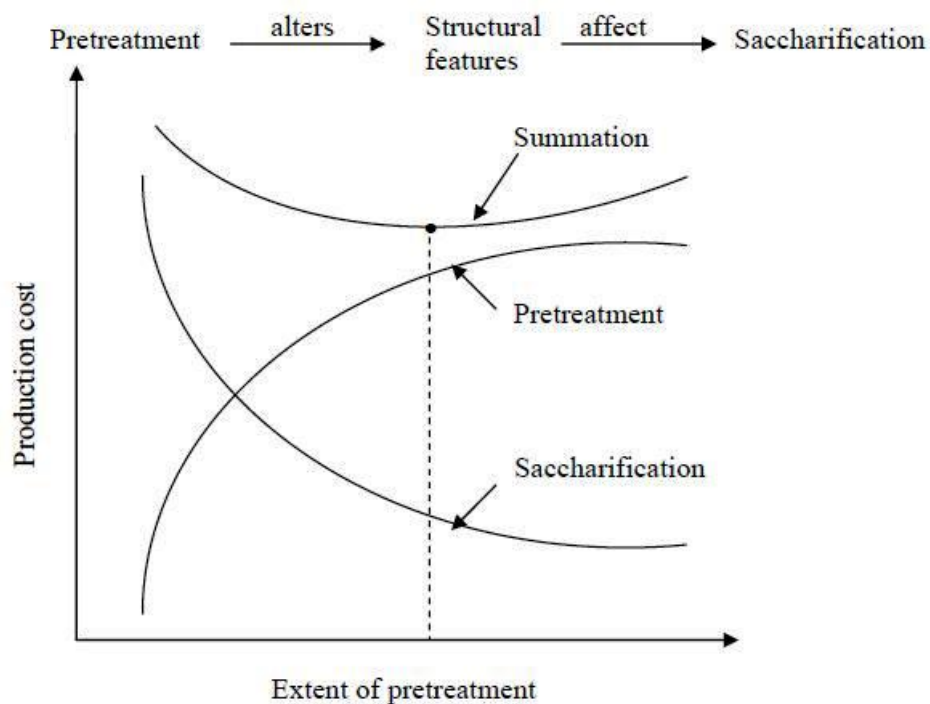


Figure 7.1: Relationship between pretreatment and production cost [5]

There are several biomass features are considered important in effecting enzymatic digestibility include: lignin content, the presence of acetyl groups, cellulose crystallinity,

degree of polymerization, surface area/pore volume of cellulose fiber and particle size. In the through pretreatment step, we try to change the key features favoring enzymatic hydrolysis. But many of researchers have reported conflicting results regarding the relationship between the structural features and enzymatic hydrolysis yields [5, 116, 117]. Therefore the effectiveness of a pretreatment is often reported in terms of enzymatic digestibility rather than in terms of the chemical composition and physical characteristics of the biomass after pretreatment. Accordingly, this study exploratory research of enzymatic hydrolysis of oxidative short-term lime pretreated willow wood as a tool to assess the pretreatment performance.

Enzymes play a crucial role in the conversion of lignocellulosic biomass into fuels and chemicals, but the high cost of these enzymes represents a significant barrier to commercialization. The cost is a function of the large amount of enzyme required to break down polymeric sugars in cellulose and hemicellulose to fermentable monomers.

Quantity and types of enzymes required for the saccharification of cellulose and hemicellulose are strongly dependent on the biomass being hydrolyzed and the type and severity of pretreatment. Enzymatic hydrolysis of cellulose is usually carried out by cellulase enzymes which are highly specific [118], and the products of the hydrolysis are usually reducing polymeric sugars to the glucose and xylose as main-product. Utility cost of enzymatic hydrolysis is low compared to acid or alkaline hydrolysis because enzyme hydrolysis is usually conducted at mild conditions at pH 4.8 and temperature 45-50°C, and also does not have a corrosion problem [119].

7.2 Cellulase enzymes

7.2.1 Enzyme Diversity

There are numerous organisms that rely on biomass degradation for their survival, often existing in the natural environment as a complex consortia of fungi, bacteria and protozoa, which are working synergistically to decay the plant cell wall. All of these organisms are potential sources of enzyme discovery, but current commercial products for biomass treatment are derived from fungi because these organisms produce a complex mix of enzymes at high productivity and catalytic efficiency and both of them are required for low-cost enzyme supply. Unlike most bacteria, which express complexes of many carbohydrate degrading activities arrayed on molecular scaffolds physically attached to the bacterial cell wall, fungal cellulases are typically secreted into the growth medium, allowing cost-efficient separation of the active enzymes in a liquid form suitable for delivery to a hydrolysis reactor [11]. One of the best known and most commercially utilized cellulolytic systems is from fungus *Trichoderma reesei* [120].

7.2.2 *Trichoderma reesei* cellulases

Commercial enzyme products currently available for biomass hydrolysis are produced by submerged fermentation of the saprophytic mesophilic fungus *T. reesei* [121,122]. Since its initial isolation, numerous mutants have been isolated that increase the productivity of the strain by over 20-fold [122,123].

Three enzymes form the core of the *T. reesei* cellulase system: exoglucanases (EC 3.2.1.91), comprised of two primary cellobiohydrolases CBH I and CBH II, that constitute roughly 60% and 20% of the secreted protein mix and are critical to the efficient hydrolysis of cellulose. The CBH I and II hydrolyze the cellulose chain processively from the reducing and non-reducing ends of cellulose chains, respectively, releasing the glucose disaccharide cellobiose. A number of endoglucanases (EG I-IV) (EC 3.2.1.4), that constitute roughly 15% of the secreted protein and hydrolyze β -1,4 linkages within the cellulose chains, creating new reducing and non-reducing ends that can then be attacked by the CBHs., and β -glucosidases (BG I and II) (EC 3.2.1.21), that constituting roughly 0.5% of the secreted protein mix, and hydrolyze cellobiose and some other short-chain cellodextrins into glucose as be shown in (Figure 7.2). In addition to the three major groups of cellulase enzymes, there are also a number of ancillary enzymes that attack hemicellulose, such as glucuronidase, acetylsterase, xylanase, b-xylosidase, galactomannanase and glucomannanase [11,119,124].

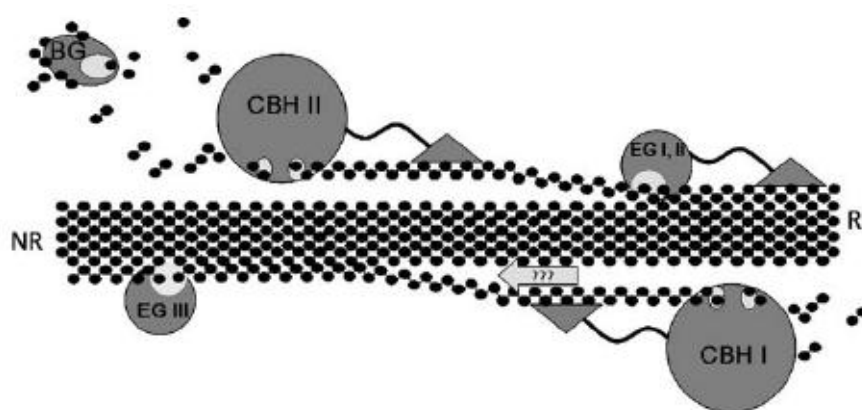


Figure 7.2: Schematic of the primary *T. reesei* enzymes involved in hydrolysis of cellulose. Cellulose is represented as stacked chains of black circles with reducing (R) and non-reducing (NR) ends indicated. [11]

7.2.3 Synergism

The primary factor in the high cost of enzymes for biomass hydrolysis is simply the amount of enzyme that must be used. Compared to starch hydrolysis, 40 to 100-fold more enzyme protein is required to produce an equivalent amount of ethanol (Novozymes data). It was recognized very early on that efficient cellulose hydrolysis requires a complex, interacting collection of enzymes during initial characterization of the *T. reesei* cellulase system [125]. To significantly reduce the amount of these enzymes requires either more efficient component enzymes are identified or that additional enzymes can be added that reduce the total enzyme loading. Cellulase enzyme systems exhibit higher collective activity than the sum of the activities of individual enzymes, this phenomenon known as synergism. Four forms of synergism have been reported: (i) endo-exo synergy between endoglucanases and exoglucanases, (ii) exo-exo synergy between exoglucanases processing from the reducing and non-reducing ends of cellulose chains, (iii) synergy between exoglucanases and β -glucosidases that remove cellobiose as end products of the first two enzymes, and (iv) intramolecular synergy between catalytic domains and CBMs. [126]

7.2.4 Supplemental enzymes

- β -glucosidase

The low-glucosidase activity of the enzyme system from *T. reesei* leads to incomplete hydrolysis of cellobiose in the reaction mixture and, as a result, to serious inhibition of the enzymes [127]. This problem can be overcome by addition of extra β -glucosidase, e.g., from the fungus *Aspergillus niger* [128]. The addition of β -glucosidase to a complex cellulase mix such as the Novozymes Celluclast 1.5 L dramatically improves the extent and, during the later stages of hydrolysis, the rate of cellulose saccharification. This is reflected in Figure 7.3, where the *T. reesei* strain used to produce Celluclast 1.5 L was compared to the same strain expressing *Aspergillus oryzae* BG (β -glucosidasein) hydrolysis assays. Due to relief of the product inhibition at high solids loadings (13.5%w/w in this example), the amount of total enzyme protein required to hydrolyze 80% of the cellulose to glucose was reduced by nearly two fold. At this solids loading, the beneficial effect of BG addition was saturated when it reached $\approx 5\%$ of the total enzyme protein, but higher solids would require higher BG levels or a more active BG [11].

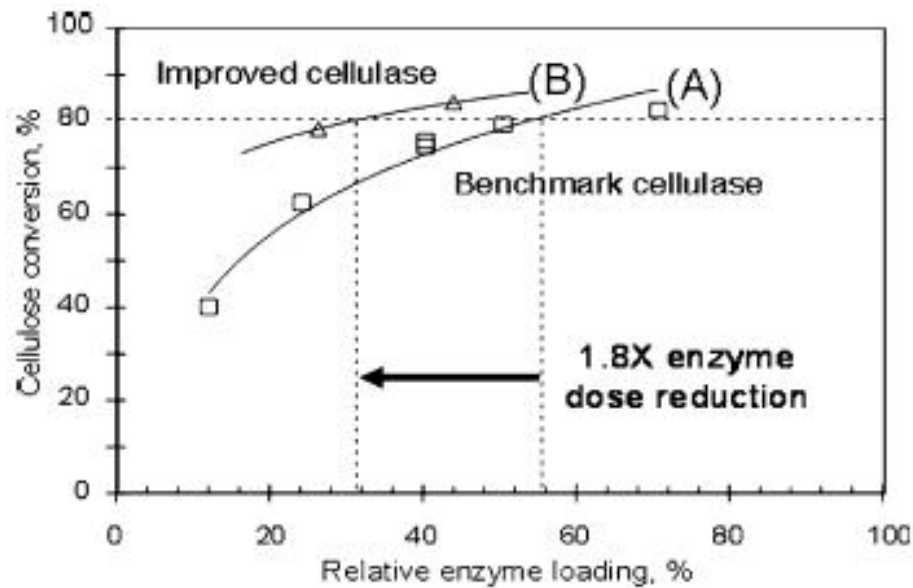


Figure 7.3: Improvement of hydrolyzing cellulases by increasing levels of β -glucosidase (BG) activity. Comparison of *T. reesei* cellulase preparations, with (B) and without (A) supplementation [12]

- Hemicellulases

Development of improved enzymes for the hydrolysis of the other major carbohydrate polymer present in lignocellulosic biomass is also of commercial interest, particularly to those utilizing neutral or alkaline pretreatments that leave much of the hemicellulose intact. Unlike cellulose, which is the most abundantly available component, hemicellulose is a heterosaccharide composed of different carbohydrate monomers which are linked to each other with several kinds of linkages and substitutions on a primary branch. Conversion of hemicellulose to their components monomers is necessary for its fermentation to ethanol. Many bacteria and fungi produce enzymes broadly called hemicellulase, which can effectively hydrolyze hemicellulose to its subsequent monomers [129].

Hemicellulases can be broadly classified into three categories: (i). Endo-acting hemicellulases. (ii). Exo-acting hemicellulases (iii). Accessory hemicellulases, which play a role in the hydrolysis of side chains and breaking hemicellulose-lignin bonds. The complexity of hemicellulose structure demands coordinated action of number of enzymes for its complete saccharification. In additions to the main function of degrade of hemicellulose to the their momomers, it was found a significant benefits could also be attributed to these enzymes component through improvement in cellulase enzyme performance. It is suggested that enzymes such as xylanase and pectinase stimulate cellulose hydrolysis by removing non-cellulosic polysaccharides that coat cellulose fibers [12].

- Glycosyl hydrolase Family 61 (GH61)

Complete cellulose hydrolysis occurs enzymatically through the synergistic action of 3 types of enzymes, namely, endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), and β -glucosidases (EC 3.2.1.21) [130]. Term of glycoside hydrolases (GHs) are catalyzes of the hydrolysis of the glycosidic linkage to release smaller sugars, extremely including the aforementioned enzymes, have been classified into more than 100 families [131]. The glycoside hydrolase family (GH61) is one of 16 families belong to endoglucanase [132]. It was found the GH61 family proteins were the major components responsible for the enhancement the activity of the *T. reesei* cellulases in synergism assays. For example, inclusion of these proteins at less than 5% of the total enzyme dose in some cases could reduce the required cellulase loading by as much as twofold [11].

7.2.5 Celluase activity

The common procedure to determine cellulase activity in terms of filter-paper units (FPU) per milliliter of original (undiluted) enzyme solution. FPU unit is the value of 2.0 mg of reducing sugar as glucose from 50 mg of filter paper (4% conversion) in 60 minutes. In our experiments of enzymatic hydrolysis we used commercialize cellulase enzymes for Novozyme Co. call (Cellic CTec2). In this type of enzyme, we could not determine cellulase activity such as filter-paper units because it contains proteins (GH61), none of the GH61 proteins were able to enhance the hydrolysis of pure cellulose in the form of filter paper [11], therefore Novozyme Co. recommend that it should use the concentration of enzyme as cellulase activity value as (g enzyme/g biomass), instead of filter-paper units.

7.2.6 Cellic CTec2-Enzymes for hydrolysis of lignocellulosic materials

(Cellic CTec2) are state of the art enzymes that are produced from Novozyme company and have been proven effective on a wide variety of pretreated lignocellulosic materials for the conversion of the carbohydrates in these materials to simple sugars prior to fermentation. Table 7.1 describe (Cellic CTec2) in the following way (Novozyme Application sheet):

Tech.specifications/ description	Cellulase complex for degradation of cellulose to sugars. A blend of aggressive cellulases with high level of β -glucosidases- and hemicellulase .
Features	High conversion yield . Effective at high solids concentrations. Inhibitor tolerant. Compatible with multiple feedstocks and pretreatments. High concentration and stabilit.
Benefits	Up to 50 %lower enzyme dosage Lower operating and capital costs from- optimization of process, e.g., higher total solids loading and higher- ethanol titer. Enabling low ethanol cost. Increased process flexibility .

Table 7.1: A description of Cellic and their features

7.3 Experimental design for enzymatic hydrolysis

Various theoretical, empirical, and hybrid models have been developed by researchers to predict the enzymatic hydrolysis of biomass [116, 133, 134]. Because cellulose is a highly complex substrate, its hydrolysis involves two distinct stages: enzyme substrate complex formation and cellulose hydrolysis. Enzyme-substrate complex formation consists of two major steps including mass transfer of enzyme from the bulk aqueous phase to the cellulose surface and formation of the enzyme-substrate complex following enzyme adsorption. Cellulose hydrolysis consists of three major steps including transfer of reactant molecules to the active site of the enzyme-substrate complex, reaction promoted by the enzyme, and transfer of soluble products to the bulk aqueous phase. The mechanism of complex-heterogeneous reaction that is involved in cellulose hydrolysis and the intricate morphology of biomass makes it difficult to model enzymatic hydrolysis [134, 135]. therefore there are many of researchers have reported conflicting results regarding the relationship between the structural features and enzymatic hydrolysis yields. Some of attempts have been made to develop mathematical models that relate the chemical composition and physical characteristics of biomass with its enzymatic yields [5, 116, 117]. Unfortunately, during these studies have been conducted since the 1950s, there is not an uniform result available

Therefore, we derived empirical model from statistical method (Box-Wilson design). It can be connected between condition of oxidative short-term lime pretreatment and enzymatic hydrolysis (Table 7.2).

For assessment our results we focused on the residual lignin content and total solid recovery as major variables because the lignin content is considered as more important than other structure feature. This is because most of acetyl groups will be removed and it has minimal effect, and cellulose crystallinity appears to have much less effective than lignin content on 72-h of enzymatic hydrolysis [116], As well as the total solid recovery represents the amount of biomass which we can convert to sugars.

Our plan for detecting the optimum points of enzymatic hydrolysis process includes:

1. To investigate the effect of lime pretreatment conditions on the enzymatic hydrolysis and to detect the optimum yield of total solid recovery, we conducted enzymatic hydrolysis experiments for all samples under recommended maximum enzyme loading 30.0%w/w(g enzyme/g glucan) and minimum substrate concentration(10 g/L).
2. In the next step and after determination the optimum points, we studied the effect of substrate concentrations of optimum points for lime pretreatment under substrate concentrations 10, 20 and 50 g/L at the same maximum enzyme loading 30.0%w/w(g enzyme/g glucan).
3. After determination the best substrate concentration which was 50 g/L, we studied the effect of enzyme loading on the extent enzymatic hydrolysis. We found out that the best enzyme loading was 0.1 g enzyme/g glucane, after we were taking different enzyme loading (0.15, 0.10, 0.05)g enzyme/g glucan.
4. In the final stage, we found the optimal points of particle size and lime loading which represented low-impact variables for lime pretreatment process.

The enzymatic hydrolysis process of lignocellulose is affected by many factors as hydrolysis temperature, time, pH, enzyme loading, substrate concentration, product concentration and biomass structural features. The optimal temperature and pH for (Cellic CTec2 enzymes) lie between (45-50°C) and pH (5.0-5.5) (Figure 7.4). Therefore the temperature was kept constant at 50°C±1 for the pH profile, and the pH was kept at 5.0±0.2 for the temperature profile during the 72-hour of enzymatic hydrolysis reaction. (Novozyme application sheet).

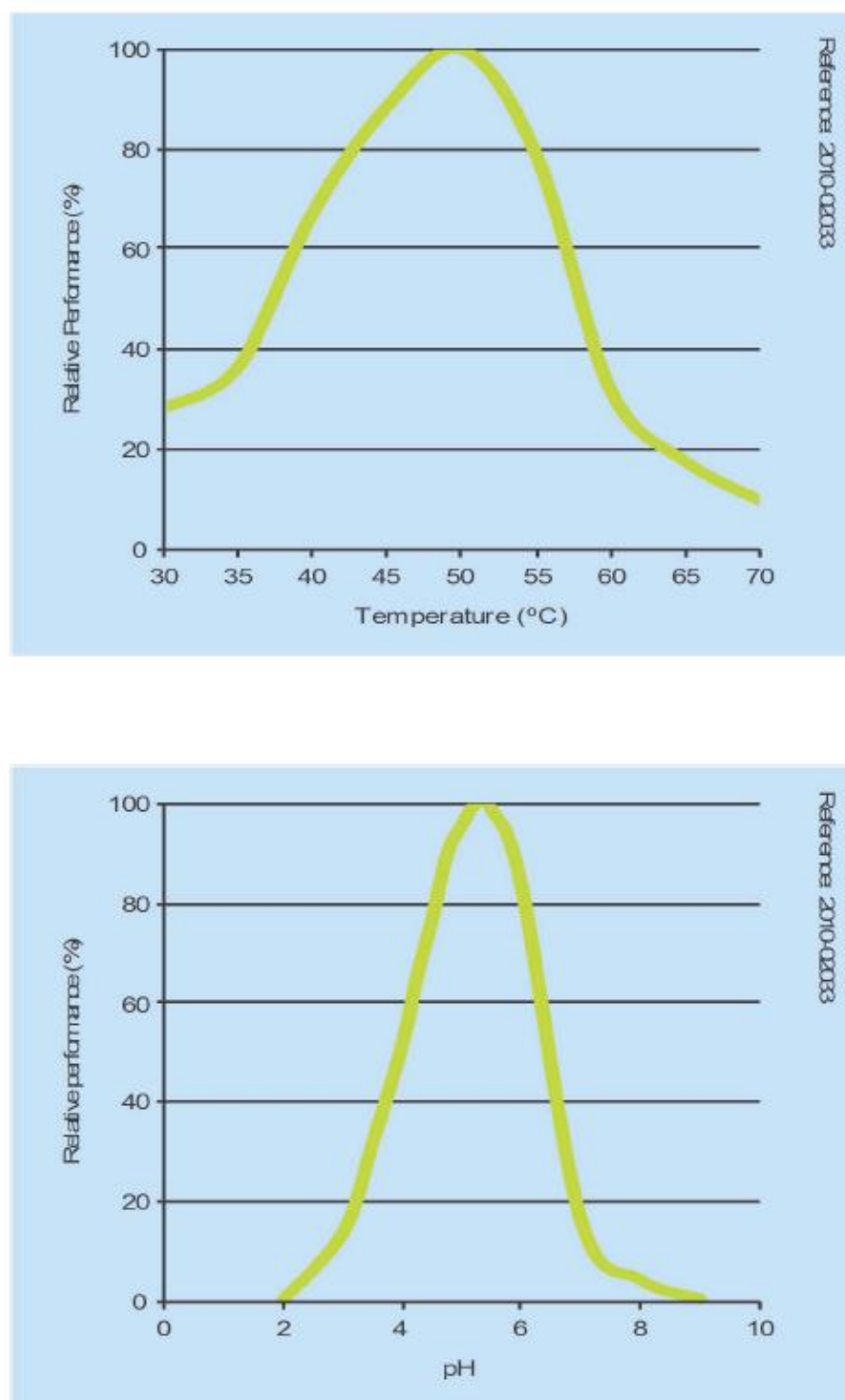


Figure 7.4: Temperature curve and pH curve(Novozyme application sheet)

7.4 Materials and Methods

In all conditions of oxidative short-term lime and at the end of this process the pre-treated biomass was neutralized (Appendix F), wash, and stored wet in the freezer at -20°C . The substrates used in enzymatic hydrolysis are raw biomass (untreated) and pretreated-neutralized-washed willow wood.

The materials used in enzymatic hydrolysis are: (Cellic CTec2 Cellulase) was provided by Novozyme, Deionized water, sodium citrate buffer 0.05-M (pH 5) and sodium azide 10.0 g/L to prevent microbial contamination; the final volume is 10 mL and the incubation period of enzymatic hydrolysis was 72 h. The details on this procedure is in Appendix G.

Enzymatic hydrolysis was performed in 20-mL Erlenmeyer flasks or scintillation vials (20-mL) in a static incubator (oven) set at $50^{\circ}\text{C} \pm 1$ which contains multiple place magnetic stirrer inside. After placing the 20-mL scintillation vials on the multiple place magnetic stirrer in the preheated incubator. The temperature was set at 50°C and the speed of magnetic stirrer at 200 rpm. The mixing process must be sufficient to keep solids in constant suspension for a period of 72 h, where most of glucan or xylane is hydrolyzed to their sugars from the solid pretreated biomass. The samples that subjected to enzymatic hydrolysis were withdrawn after 3 days and then boiled for 15 minute to denature the enzymes thus prevent further hydrolysis. After that the samples were centrifuged at 4000 rpm for 20 minutes. Then the samples were diluted by using mobile phase to get sugar concentration in the range of carbohydrate standard concentration. The samples are filtered through a $0.2\mu\text{m}$ filter and subjected to glucose analysis using HPLC method. The measured sugars of hydrolysis samples were used to indicate biomass digestibility. Enzymatic hydrolysis process was based on NREL standard method (Enzymatic saccharification of lignocellulosic biomass)(appendix G)

The required quantity of cellulase was calculated based on the amount of glucan in the raw biomass and the desired enzyme loading. The suggested enzyme trial dosage levels for initial investigation of a substrate are 30.0%w/w (g enzyme/g cellulose) and the amount of biomass to be weighed was calculated based on the moisture content and the glucan content to provide 0.1 g glucan for the reaction.

The concentration of glucan and xylan were measured after 72 h of hydrolysis by HPLC analysis using Phenomenex Rezex(RPM pd^+) column, and RI detector.

To calculate the concentration of the polymeric sugars from the corresponding monomeric sugars which were measured by HPLC device was used with an anhydro correction of 0.88 or (132/150) for C-5 sugars (xylose and arabinose) and a correction of 0.90 or (162/180) for C-6 sugars (glucose, galactose, and mannose) as follow:

$$\%Yield\ of\ glucan = \frac{C_{HPLC} \times AC \times V \times D}{W \times glucan\ content\%} \times 100 \quad (7.1)$$

$$\%Yield\ of\ xylan = \frac{C_{HPLC} \times AC \times V \times D}{W \times xylane\ content\%} \times 100 \quad (7.2)$$

Where C_{HPLC} is concentration of the sugar as given by HPLC in mg/mL, V is volume of biomass slurry (mL), D is dilution factor, W is weight of dry biomass (mg), AC is anhydro correction, it is 0.9 for glucose and mannose and 0.88 for xylose.

7.5 Overall yield and hydrolysis yield

We have already been defined the overall yields and hydrolysis yields in section 5.2 as follow: Hydrolysis yield of glucan (cellulose):

$$Y_g = \frac{g\ pretrated\ glucan\ hydrolyzed}{100\ g\ glucan\ in\ treated\ biomass}$$

Hydrolysis yield of xylan (hemicellulose):

$$Y_x = \frac{g\ pretrated\ xylan\ hydrolyzed}{100\ g\ xylan\ in\ treated\ biomass}$$

Overall yield of glucan (cellulose):

$$Y_G^T = \frac{g\ pretrated\ glucan\ obtained\ after\ pretreatment\ and\ enzymatic\ hydrolysis}{100\ g\ glucan\ in\ raw\ biomass}$$

Overall yield of xylan (hemicellulose):

$$Y_X^T = \frac{g\ pretrated\ xylan\ obtained\ after\ pretreatment\ and\ enzymatic\ hydrolysis}{100\ g\ xylan\ in\ raw\ biomass}$$

.

Overall yield of total sugar (cellulose and hemicellulose):

$$Y_S^T = \frac{(g\ pretrated\ glucan\ hydrolyzed + g\ pretrated\ xylan\ hydrolyzed)}{100\ g\ raw\ biomass}$$

7.6 Results and discussion

Statistical method was used to find the relationship between overall total sugar yield (Y_S^T) and pretreatment conditions, in order to find the optimum point for oxidative short-term lime pretreatment conditions which gives the best overall yield of total sugar (Y_S^T). Table 7.2 shows the results of different types of yields:

Ex.No	$Y_G\%$	Y_g	Avar. Y_g	$Y_G^T\%$	Glucan(g)	$Y_X\%$	Y_x	$Y_X^T\%$	Xylan(g)	Y_S^T
1	92.00	0.897	0.903	83.076	3.873	43.317	0.840	36.386	0.688	0.400
1		0.910		0	0			0	0	0
2	93.27	0.942	0.950	88.606	4.131	55.467	0.955	52.971	1.002	0.45
2		0.955		0	0			0	0	0
3	94.00	0.955	0.940	89.770	4.185	43.613	0.976	42.566	0.805	0.437
3		0.937		0	0			0	0	0
4	94.34	0.999	0.994	93.700	4.371	50.185	0.992	49.784	0.943	0.466
4		0.988		0	0			0	0	0
5	99.40	0.815	0.820	81.200	3.800	68.200	0.909	62.000	1.173	0.436
5		0.825		0	0			0	0	0
6	97.20	0.905	0.914	88.841	4.141	60.961	0.920	56.084	1.061	0.456
6		0.922		0	0			0	0	0
7	97.20	0.955	0.950	92.340	4.305	53.200	0.950	50.536	0.956	0.461
7		0.945		0	0			0	0	0
8	95.12	0.970	0.972	92.456	4.310	55.867	0.990	55.308	1.047	0.47
8		0.973		0	0			0	0	0
9	90.47	0.965	0.971	87.435	4.076	40.623	0.990	40.217	0.761	0.424
9		0.977		0	0			0	0	0
10	93.00	0.991	0.990	92.070	4.292	49.181	0.990	48.690	0.868	0.452
10		0.990		0	0			0	0	0
11	92.77	0.970	0.972	90.172	4.204	48.779	0.945	46.096	0.872	0.445
11		0.975		0	0			0	0	0
12	98.80	0.784	0.779	77.000	3.590	78.610	0.909	0.714	1.353	0.434
12		0.774		0	0			0	0	0
13	94.00	0.930	0.925	86.025	4.010	59.323	0.920	48.577	1.033	0.442
13		0.920		0	0			0	0	0
14	98.87	0.904	0.918	87.714	4.090	61.800	0.956	59.087	1.118	0.457
14		0.931						0	0	0
15	94.65	0.950	0.950	90.200	4.205	52.720	0.953	50.242	0.951	0.448
15		0.950		0	0			0	0	0
16	94.75	0.939	0.938	89.065	4.152	53.249	0.942	50.160	0.950	0.445
16		0.937		0	0			0	0	0
17	94.50	0.949	0.951	90.000	4.196	54.094	0.950	51.390	0.973	0.452
17		0.953		0	0			0	0	0
18	94.70	0.950	0.950	90.000	4.196	54.147	0.940	50.898	0.963	0.45
18		0.950		0	0			0	0	0

Table 7.2: Different yields of overall process (Pretreatment and enzymatic hydrolysis)

where:

Y_G =Pretreatment yield of glucan (g glucan recovered/100 g glucan in raw biomass)

Y_g =Hydrolysis yield of glucan(g glucan hydrolyzed/ g glucan in treated biomass)

Y_G^T =Overall yield glucan (g glucan hydrolyzed/100 g glucan in raw biomass)

Y_X =Pretreatment yield of xylose(g xylan hydrolyzed/100 g xylan in raw biomass)

Y_x =Hydrolysis yield of xylan(g xylan hydrolyzed/ g xylane in treated biomass)

Y_X^T =Overall yield of xylane (g xylan hydrolyzed/100 g xylan in raw biomass)

Y_S^T =Overall yield of total sugar (glucan+ xylan)/ g raw biomass)

Glucan (g) =Quantity of glucan hydrolyzed after pretreatment and enzymatic hydrolysis of total raw biomass (11.40 g), Xylan (g) =Quantity of xylan hydrolyzed after pretreatment and enzymatic hydrolysis of total raw biomass (11.40 g)

Generally, for all samples that underwent to pretreatment conditions (pretreatment time, compressed oxygen, and temperature) are strongly affected through biomass enzymatic digestibility. During enzymatic hydrolysis, glucan and xylan converted to glucose and xylose, which were expressed as equivalent glucan and xylan, to calculate the yields from raw biomass. Since the enzymatic hydrolysis operation takes place in the separate stage, thus glucan and xylan enzymatic yields were obtained (i.e., yields based only on the enzymatic hydrolysis operation). Therefore, the recommended pretreatment conditions were chosen based on the glucan and xylan overall yield (i.e., yields after the combined operations of pretreatment and enzymatic hydrolysis). Table 7.3 shows the practical experiments with overall yield of total sugar as follow:

Table 7.3: Overall total sugar yield with pretreatment conditions

Pretreatment conditions				Total yield
	temperature, °C, X_1	pressure, bar, X_2	time, hour, X_3	Y_S^T
1	147	17.8	5.0	0.400
2	113	17.8	5.0	0.450
3	147	9.2	5.0	0.437
4	147	17.8	2.0	0.466
5	113	9.2	5.0	0.470
6	113	17.8	2.0	0.456
7	147	9.2	2.0	0.461
8	113	9.2	2.0	0.436
9	160	13.5	3.5	0.424
10	130	21.0	3.5	0.452
11	130	13.5	6.0	0.424
12	100	13.5	3.5	0.435
13	130	6.0	3.5	0.442
14	130	13.5	1.0	0.457
15	130	13.5	3.5	0.448
16	130	13.5	3.5	0.445
17	130	13.5	3.5	0.452
18	130	13.5	3.5	0.450

Figures 7.5 to 7.8 show statistical model for overall total sugar yields and their desirability. And the following equation represent the empirical equation or (polynomial equation) that was driven from statistical model :

$$\begin{aligned}
 Y_S^T = & +0.064316 + 2.51342E-003 \times X_1 + 0.012445 \times X_2 + 0.093164 \times X_3 \\
 & - 5.33365E-005 \times X_1 \times X_2 - 5.91400E-004 \times X_1 \times X_3 \\
 & - 1.64389E-003 \times X_2 \times X_3. (R = 0.83, p - value \subseteq 0.001)
 \end{aligned} \tag{7.8}$$

These figures show the effect of the independent variables (temperature and oxygen pressure) together on the overall yields of total sugar with keeping pretreatment time constant at three level (minimum, middle and maximum) of times (2.00, 3.5 and 5 h) Figures 7.5, 7.6, 7.7.

Figure 7.5 represent overall total sugar yields with its desirability at time 2.00 h. Increasing the temperature and oxygen pressure, leads to increase the yields of total sugar. If the temperature and oxygen pressure are increased together the overall yield of total sugar will increase faster as shown in this Figure 7.5. Color gradient toward red color represent the level of desirability of overall total sugar yields.

Figures 7.6 and 7.7 represent overall total sugar yields with its desirability at time 3.5 h and 5 h, respectively. Increasing the temperature decrease overall total sugar yields, while Increasing the oxygen pressure up to approximately 130°C will increase the yields of total sugar. at temperature higher 130°C, increase the oxygen pressure decrease overall total sugar yields as shown in the these Figures.

The highest value recorded of practical experiments of overall yield of glucan was 93.700 g glucan hydrolyzed/100 g glucan in raw biomass (equivalent to a hydrolysis yield of 99.00 g glucan hydrolyzed/100 g glucan in treated biomass) that has the overall total sugar yield 0.466 under 148°C, 17,8 bar and 2.06 hours. The experimentally calculated value of overall total sugar yield is almost the same as the predicted optimum value (0.468). This point represent the optimum point of overall precess.

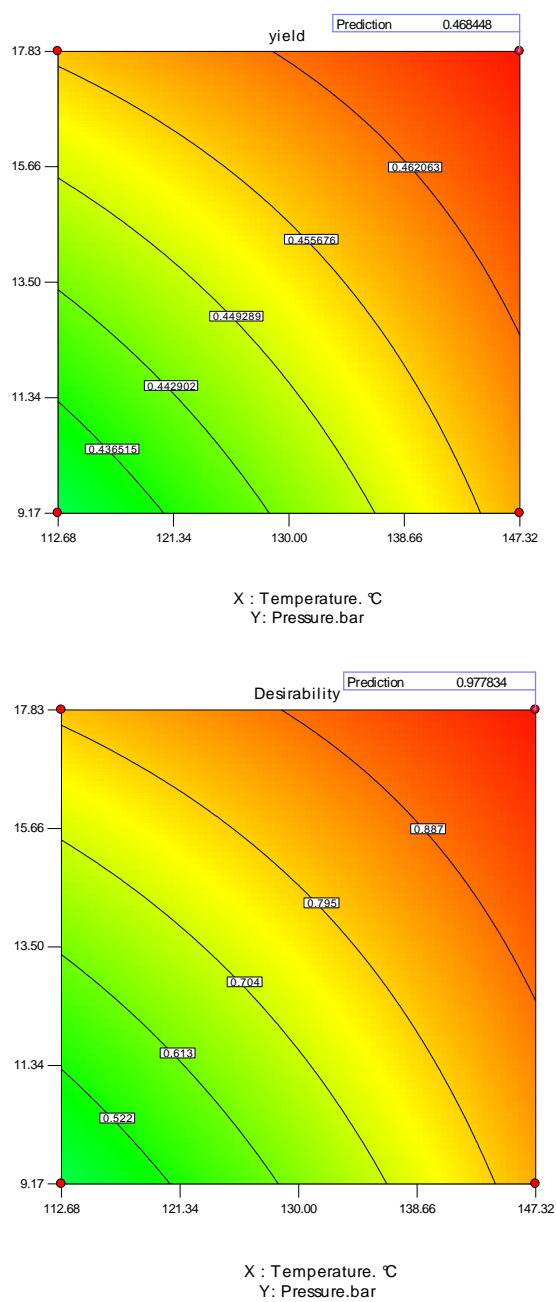
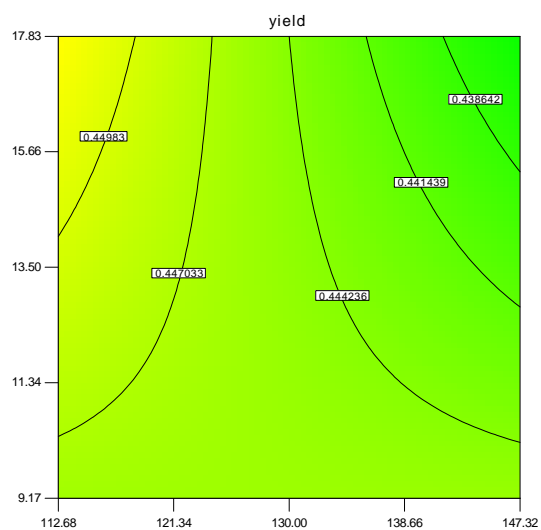
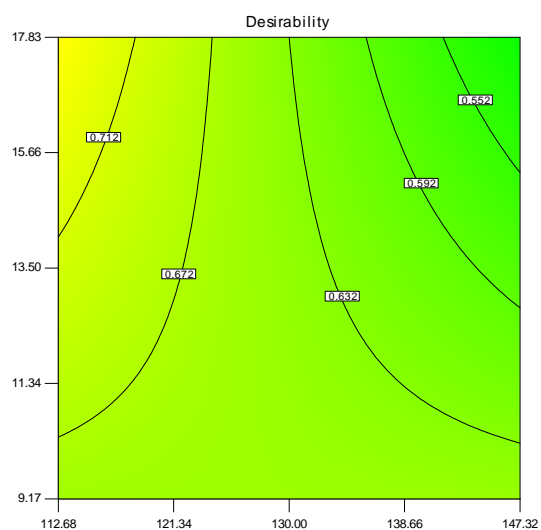


Figure 7.5: Overall total sugar yield and its desirability for pretreated willow wood at different conditions at time 2.06 h



X : Temperature. °C
Y: Pressure.bar



X : Temperature. °C
Y: Pressure.bar

Figure 7.6: Overall total sugar yield and its desirability for pretreated willow wood at different conditions at time 3.5 h

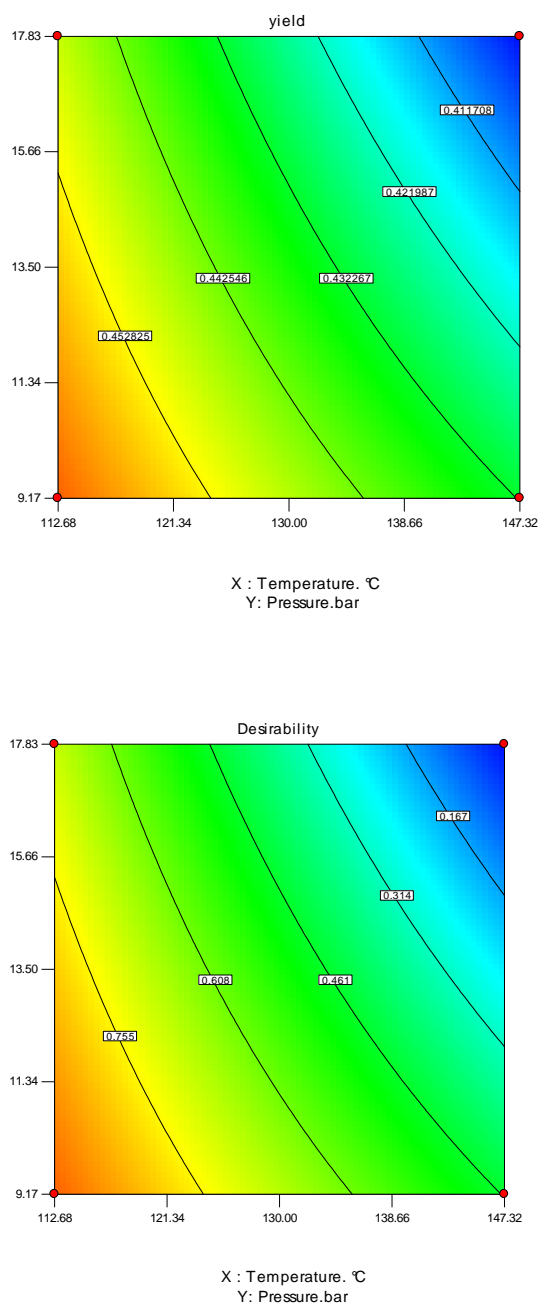


Figure 7.7: Overall total sugar yield and its desirability for pretreated willow wood at different conditions at time 5.h

Through the study of the relationship between the enzymatic hydrolysis and the residual lignin content, was noticed, that the enzymatic hydrolysis at 147.8°C, 17.8 bar and 5 hours was 90.30%, nevertheless the lignin content of this sample was lower than some samples but they have higher enzymatic hydrolysis. To realise this phenomena, we should return to enzyme activity. We have already mentioned, none of the GH61 proteins were able to enhance the hydrolysis of pure glucan in the form of filter paper. This lack of enhancement was also shown with other pure cellulose substrates such as avicel, phosphoric-acid swollen cellulose and carboxymethyl cellulose. Proteins GH61 are capable to enhance hydrolysis of acid pretreated corn stover and as it is well known that the samples which is conducted with acid pretreatment have large amount of lignin therefore, it was suggested that the cellulase-enhancing effect of such proteins is limited to substrates containing other cell wall-derived materials such as hemicellulose or lignin [11]. This mean that lignin content at 147.8°C, 17.8 bar and 5 hours was lower than lignin content which enhance cellulase activity, and at the same time this result will support this later suggestion.

Also we noticed at the end of conducting experiments of enzymatic hydrolysis for pretreated biomass under different pretreatment conditions, at 50°C for 3 days at enzyme loading 0.3 g enzyme/g glucan in biomass, we found out during the enzymatic hydrolysis that the highest residual concentration of lignin gives full hydrolyzed for glucan was approximately 0.15 g lignin remaining /g of pretreated biomass or lignin selectivity equal 0.15 residual lignin /g of pretreated biomass at 147.8°C, 17.8 bar and 2.06 hours. This point is very important because of going on more than this point will increase carbohydrate degradation without any benefit, and less than this point of lignin degradation will not get the full hydrolysis.

As shown in Figure 7.8, It is clear that the optimum point will be terminal at temperature 147.8°C, pressure 17.8 bar and time 2.06 hour, or in other mean this point lie on the circumference of circle.

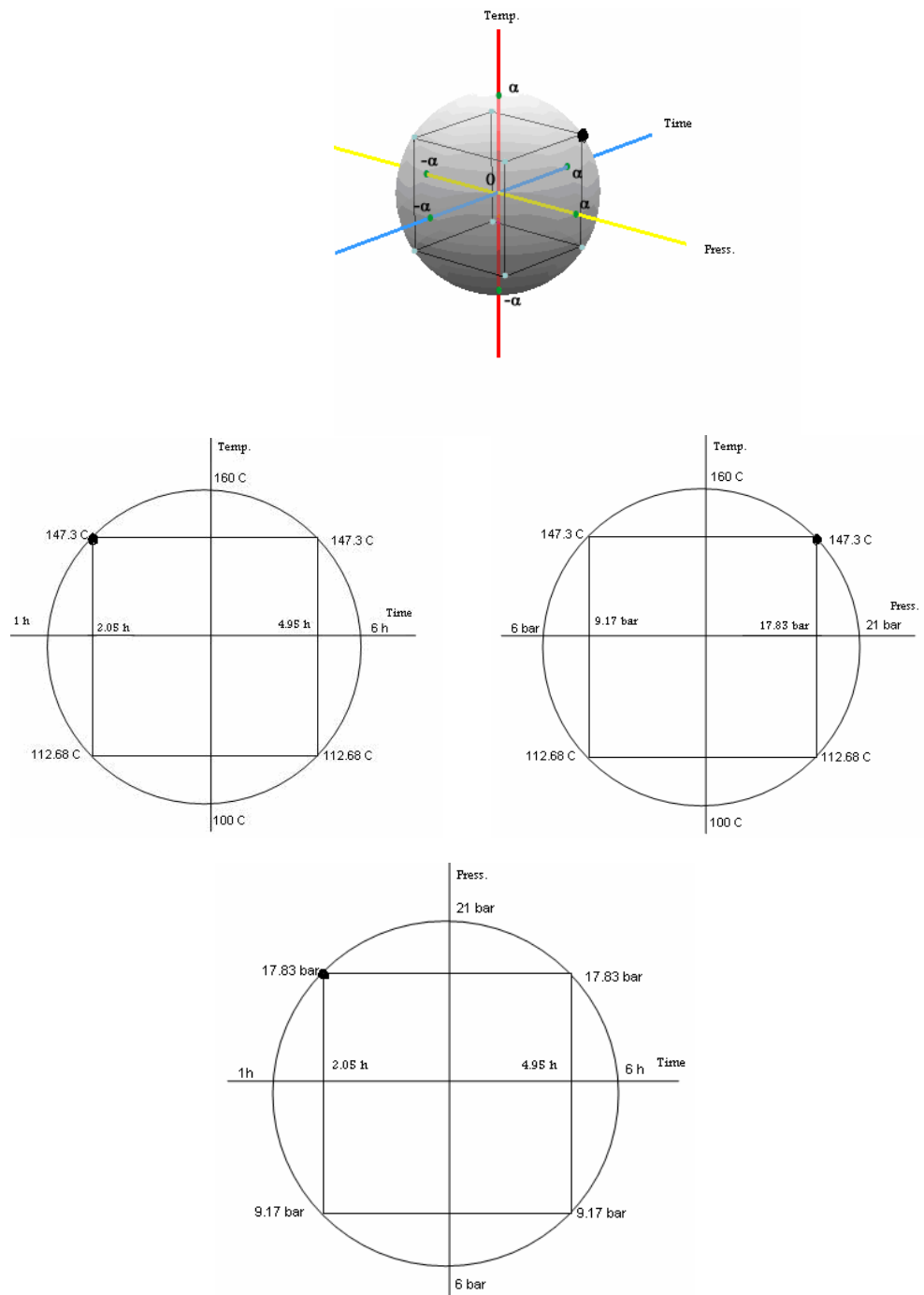


Figure 7.8: Points of a central composite circumscribed design with three input parameters at 3D and 2D

In Box-Wilson experimental design (Center Composite Design (CCD)), there are three type of CCDs. circumscribed (CCC), inscribed (CCI), and facedpictedured (CCF). Figure 7.9 illustrates these types of center composite design (CCD). The (CCC) explores the largest process space and the (CCI) explores the smallest process space. Both the (CCC) and (CCI) are rotatable designs, but the (CCF) is not. In the (CCC) design, the design points describe a circle circumscribed about the factorial square. For three factors, the CCC design points describe a sphere around the factorial cube.

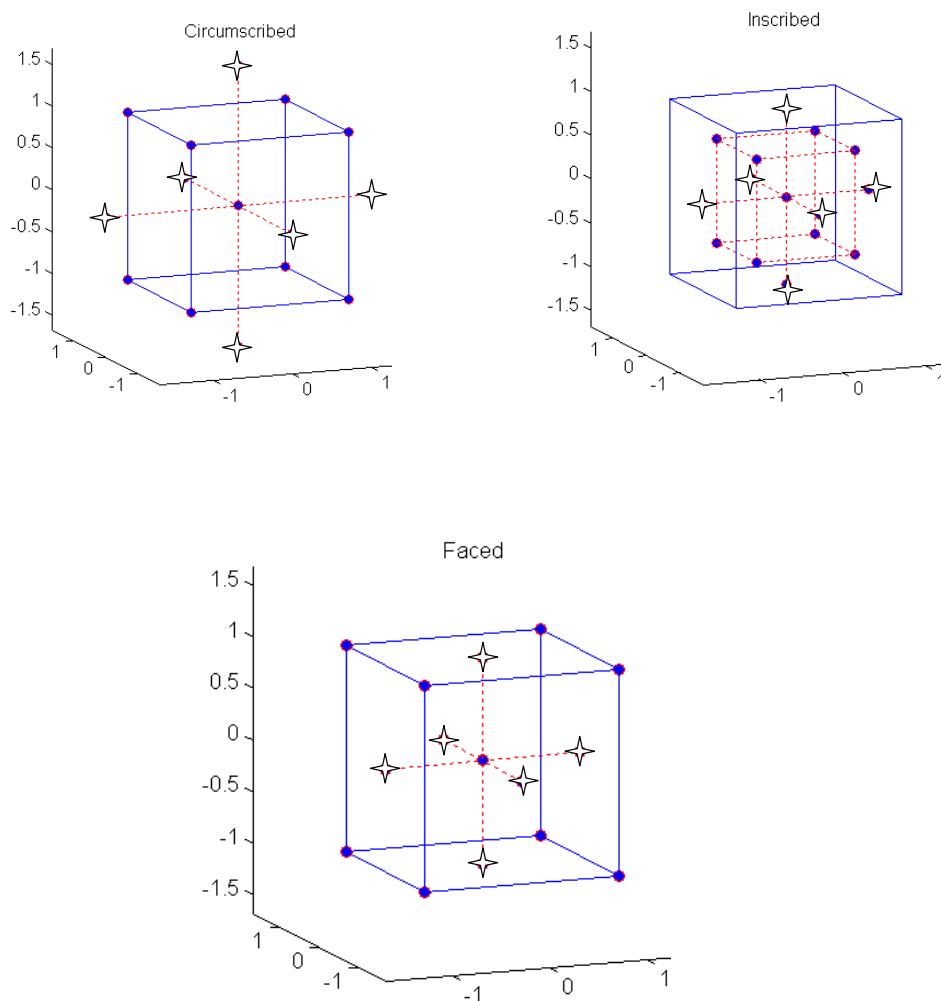


Figure 7.9: Types of a Central Composite design

In this study we used the center composite design type circumscribed (CCC). We notice this type doesn't cover whole the experimental region, where the star points represent extreme values (low and high) for each factor in the design. Therefore the optimum point is located on the edge of the Box-Wilson.

in order to complete whole region of my interested. We looked for around this point and within temperature range 147.8-160°C, pressure range 21-17.8 bar(absolute) and pretreatment time range 1-2 hour.

Extra experiments have been done in this region for points that have the potential to be a more optimal point with considering to reduced pretreatment time less than two hours and at the same time increasing the oxygen pressure and temperature Table 7.4.

Table 7.4: The yields for extra experiments

Pretreatment conditions				Yields				
EX.No	temperature,	pressure, bar	time,hour	$Y_G\%$	Y_g	$Y_X\%$	Y_x	Y_S^T
19	148	17.8	1.33	96.00	0.98	63.18	0.99	0.48
20	150	21.0	1	95.40	0.95	58.00	0.95	0.46
21	155	21	1.25	94.70	0.99	52.40	0.99	0.470
22	160	21	1	94.70	0.98	54.20	0.96	0.46

Table 7.4 shows that the best overall total sugar yield (Y_S^T) is (0.486) at 148°C, 17.8 and time 1.33 h (80 minutes), so this will be represent the optimum point of my work. As we noticed most of the values of overall total sugar yields in this table were approximately close. So in the next steps of my research, we will take an extra point with the optimum point for comparison. The extra point is at pressure 21 bar 155°C and time 1.25 h(75minutes).

In the next step we studied the effect of substrate concentration on the enzymatic hydrolysis process, to find the best high substrate concentration. We conducted these experiments at different concentrations (10, 20, 50 g/L) with the same concentration of enzyme loading (30.0%(g enzyme/g cellulose), for both points during 72 hours. Glucose, xylose and mannose were measured after hydrolyzing was finished by HPLC device.

After measuring, we found out that the highest concentration that can be obtained with the same digestion efficiency of 10 g/L, is 50 g/L. Also the substrate concentration (50 g/L) was compatible with other research, which conclude adding supplemental cellobiase enzyme [5]. Also we found that the concentration of substrate more 50 g/L will not be suitable for enzymatic hydrolysis because the mixture will be heterogeneous with a concentration more than 50 g/L.

In the my previous experiments we used excess enzyme loading to eliminate any interference related to amount of enzyme and then to determine the lowest enzyme loading

that gives same digestion efficiency of 30.0%w/w (g enzyme/g cellulose) at 50 g/L substrate concentration for both points at (148°C, 17,8 and time 1.33 h) and (155°C, 21 bar and time 1.25 h). We took three type of enzyme loading (15.0%, 10.0%, 5.0% w/w (g enzyme/g cellulose). Table 7.5 It shows that the lowest enzyme loading gives high digestion efficiency at (10.0%w/w (g enzyme/g cellulose) for both points as shown in this Table.

Table 7.5: *Enzymatic hydrolysis yield for both of optimum points at different enzyme loading*

Enzyme loading, g enzyme/g glucan	Y_g 155°C, 148°C	Y_x 155°C, 148°C
0.15	0.99, 0.98	0.99, 0.99
0.10	0.99, 0.98	0.98, 0.98
0.05	0.85, 0.85	0.80, 0.78

In the final stage of our research we studied the low-impact variables such as lime loading and particle size in order to reduce both of consumption of lime and energy comminution of biomass. This stage was divided into two parts. First part was studied consumption of lime during pretreatment process, while in the second part, the best particle size consume low energy was chosen (Table 7.6 and 7.7).

Table 7.6: *Different yields for both of optimum points to determine lime loading*

Lime loading (g lime/g raw dry biomass)	0.40	0.26	0.22
overall glucan yield at (148°C, 155°C), Y_G^T	0.950, 0.940	0.952, 0.955	0.921, 0.941
pretreatment yield at (148°C, 155°C), Y_P	0.649, 0.631	0.666, 0.649	0.684, 0.666
overall total sugar yield at (148°C, 155°C), Y_S^T	0.480, 0.470	0.547, 0.548	0.526/0.542
total solid recovery at (148°C, 155°C)	7.400, 7.200 g	7.600, 7.400 g	7.800, 7.600 g

Table 7.6 shows that the optimum points were at 155°C and 147°C at lime loading 0.26 (g lime/g raw dry biomass).

In the second part, we studied the effect of particle size of biomass. Previous studies showed that grinding to less than 10 mesh (2 mm) is sufficient for enzymatic hydrolysis of lime pretreatment [13]. In our research, was found that the best particle size lies between 2-3 mm, at 155°C, 21 bar and time 1.25h (Table 7.7)

Table 7.7: *Particle size with overall total sugar yield at 155°C*

particle size	0.2-0.8 mm	1.2 mm	2-3 mm	3-4 mm
overall glucan yield	0.955	0.950	0.950	0.864
overall total sugar yield (155°C)	0.548	0.540	0.540	0.485

The nearest research is relevant of my work it was for poplar wood and its results as follow: for poplar wood and thire results as follow. The recommended conditions for short-term lime pretreatment of poplar wood for (1) 2 h, 140°C, 21.7 bar absolute and (2) 2 h, 160°C, and 14.8 bar absolute. In these two cases, the reactivity was

nearly identical, thus the selected condition depends on the economic trade off between pressure and temperature. The overall yields were under these recommended conditions follow: (1) 95.5 g glucan/100 g of glucan in raw biomass and 73.1 g xylan/100 g xylan in raw biomass and (2) 94.2 g glucan/100 g glucan in raw biomass and 73.2 g xylan/100 g xylan in raw biomass [86]. And the recommended pretreatment conditions, the lime consumption was 0.234 and 0.198 g $\text{Ca}(\text{OH})_2$ consumed/g dry biomass, respectively. As regards the particle size, the best result for particle size was 2 mm [13,86].

7.7 Material Balances

Material balance was performed to determine how much biomass was solubilized by oxidative lime pretreatment. Also, the residual solid should be considered after enzymatic hydrolysis of the pretreated willow wood, because it contains the undigested cellulose and hemicellulose, and other residual solids, at recommended condition, i.e., 155°C, 21.0 bar(absolute), 1.25 h, 0.26 g of $\text{Ca}(\text{OH})_2$ /g of dry biomass, 15 mL of water/g of dry biomass. There was an error 2% for the summative mass closure of mass balances . Table 7.8 summarizes the solubility of each component before and after, pretreatment and enzymatic hydrolysis respectively.

Table 7.8: Mass balance for optimum point

Dry biomass			After pretreatment			After enzymatic hydrolysis		
Compositio	Raw %	Raw (g)	PS%	PS (g)	PL%	E.H	HL (g)	HS (g)
Glugane	41	4.662	60.00	4.441	4.74	0.98	4.35	0.10
Xylane	16.65	1.893	15.00	1.11	41.36	0.98	1.08	0.03
Mannan	4.45	0.547	4.00	0.269	50.84	0.98	0.29	0.00
Galagtan	2.0	0	0	0	0		0	0
Lignin	25.85	2.950	16.00	1.184	59.86		0.75	0.434
Extractive	6.0	1.00	2.0	0.148	78.36		0	0.148
Ash	0.82	0	1.50	0.11	-12.24		0	0.11
Acytel	2.72	0	1.20	0.088	71.14		0	0.088
Total	98.6	11.40	99.70	7.35/7.40	35.08		6.47	0.93/0.91

Where PS: solids obtained after pretreatment, PL%: percentage lost in the liquid after pretreatment; E.H: enzymatic hydrloysis yield, HL: liquor obtained after enzymatic hydrolysis, HS: solids obtained after enzymatic hydrolysis.

The theoretical overall total sugar yield is 62.30% (g total sugar in raw biomass/g of total raw biomass), whereas it was the best practical yield at optimal point 57.19 % (g total sugar in raw biomass/g of total raw biomass), so we can convert approximately 91.77 % of theoretical overall total sugar yield. Also we have approximately 0.12 of total raw biomass as solid lost and we can use it as source of heating in the pretreatment process and enzymatic hydrolysis.

7.8 Repeatability

All the results reported in this study were obtained as the average of several measurements. At least duplicates were taken from the same batch of pretreated biomass and were analyzed to assure repeatability.

7.9 Conclusions

For high-lignin biomass, such as willow wood and poplar wood, oxidative short-term lime pretreatment was very effective for digestibility by enzymatic hydrolysis. Generally, oxidative short-term lime pretreatment showed less sugar degradation with more lignin removal.

Glucan was preserved much more than xylan because xylan degradation was more related to lignin removal and deacetylation. The most important pretreatment glucan and xylan yield which gives very high digestibility at 147°C, 17.8 bar and 1.33 h (80 minute), and 155°C, 21 bar, 1.25 h (75 minute) also they have lignin content lies between 15-16.5%.

A lower pretreatment yield of glucan and xylan were observed at 147.8°C, 17.8 bar and 5h (0.927 and 0.422, respectively) and 160°C, 13.5 bar, 3.5 h (0.904 and 0.420, respectively). is mean, the pretreatment temperature is more dominant than pressure and pretreatment time. At temperature 100°C, 13.5 bar and 3.5 h, the enzymatic hydrolysis of glucan was low (78%), because of poor delignification (22% lignin content)

A lonely criteria used in enzymatic hydrolysis is that the process is performing on 3-days to avoid the effect of glucan crystallinity. Accordingly, the lignin content was considered as the crucial factor.

This study found, that there are two of optimum points are: (1) 147.8°C, 1.33 h, 17.8 bar (absolute), 0.26 g Ca(OH)₂ and 15 g water/g raw biomass (2) 155°C, 1.25 h, 21.0 bar (absolute), 0.26 g Ca(OH)₂ and 15 g water/g raw biomass. The overall glucan and xylan yields(i.e., glucose and xylose recovered after both pretreatment and enzymatic hydrolysis, and expressed as equivalent glucan and xylan) that obtained in the first are: 0.96 g glucan hydrolyzed/100 g glucan in raw biomass, and 0.62 g xylan hydrolyzed/100 g xylan in raw biomass, in the second point are:0.94 g glucan hydrolyzed/g glucan in raw biomass and 0.52 g xylan hydrolyzed/100 g xylan in raw biomass, respectively.

Future Studies

When we were starting our research, we intended to complete converting pretreated willow wood biomass to biobutanol by using clostridium organisms, but because of time limitation, we could not carry out fermentation process.

A series of practical experiments including pretreatment, measurement of biomass content of raw biomass and pretreated biomass, and enzymatic hydrolysis which gave a good experience in this subject. Accordingly, some of recommendations can be suggested for future work:

1. Development of mathematical models for oxidative short-term lime pretreatment of willow wood depending on real experiments similar to Eq.4.6. These models account for different degradation rates that occur with lignin and carbohydrates moieties. And that models can also be used for accurately predict the measured quantities and account for the main process variables such as alkali concentration, temperature, oxygen pressure, fast region reaction, slow region reaction and activation energy for lignin and carbohydrates to find optimization .
2. We found that the measuring of biomass content takes a long time, therefore we recommend to choose other methods consume shorter time like spectroscopic methods.
3. Establishment of mathematical models for enzymatic hydrolysis between structural features (chemical and physical characteristics) of pretreated willow wood and progress of digestibility for different period of time in order to understand more about the mechanism of enzymatic reaction with taking into account the enzyme loadings, specially for this new type of cellulase enzyme. Also we try to increase the substrate concentrations above 50 g/L through by addition of biomass in the form of multiple small batches instead of one big to get rid of problem associated with high concentrations that have been mentioned earlier.

4. Complete the fermentation process for sugars produced from enzymatic hydrolysis.

Preparation of Samples for Compositional Analysis

This procedure is based on the NREL standard procedure (Preparation of Samples for Compositional Analysis). The purpose is to convert a variety of biomass samples into a uniform material suitable for compositional analysis in a reproducible way.

1. Dry

The biomass material is spread out on a long rectangular stainless steel pan. It is allowed to air-dry (conditioning air) in a hood (controlled air velocity 100 ft/min) prior to any milling. Do not pile the material deeper than 5 cm. Turn the material at least once per day to ensure even drying. After at least 4 days of drying, measure the solids content of the biomass sample following NREL (LAP Determination of Total Solids in Biomass) (Appendix H). If the moisture content is less than 10% and the subsequent measurements of the moisture content report a change in weight of less than 1% in 24 h, the biomass can be considered dried.

2. Mill

Feed the air-dried biomass into the knife mill and mill until the entire sample passes through the 2-mm screen in the bottom of the mill. Let the mill cool down between batches because the heat generated in the process may damage the sample.

3. Sieve

Stack the sieves in the following order, starting at the bottom: the bottom pan, 80, and 20-mesh sieve. Place the milled biomass in the 20-mesh sieve. The sample should be no more than 7 cm deep in the 20-mesh sieve. The milled sample may be processed in batches if necessary. Place the cover on the sieve stack and secure the stack in the sieve shaker. Shake the sieves for 15 ± 1 min. The fraction retained

on the 20-mesh sieve (+20 mesh fraction) should be milled and sieved again or stored separately to weigh. The fraction retained on the 80+mesh sieve (-20/+80 mesh fraction) should be retained for compositional analysis. The material in the bottom pan is the fines (-80 mesh) fraction. Retain this material for ash analysis. It is not used in any other pretreatment or analytical procedure.

Determination of Extractives in Biomass

It is necessary to remove non-structural material from biomass prior to analysis to prevent interference with later analytical steps. This procedure uses a two-step extraction process to remove water soluble and ethanol soluble material. Water soluble materials may include inorganic material, non-structural sugars, and nitrogenous material, among others. Inorganic material in the water soluble material may come from both the biomass and any soluble material that it is associated with the biomass, such as soil or fertilizer. No attempt is made to distinguish the source of the inorganic material. Ethanol soluble material includes chlorophyll, waxes, or other minor components. Some biomass may require both extraction steps, while other biomass may only require exhaustive ethanol extraction.

1. Preparation

Determine the moisture content of the sample NREL Standard Procedure (Determination of Total Solids and Moisture in Biomass) (Appendix H) and dry boiling flasks in a $105 (\pm 5)^{\circ}\text{C}$ drying oven for a minimum of 15 hours. After cooling in a desiccator, add boiling stones to the boiling flask, label it, and record its oven-dry weight (ODW) to the nearest 0.1 mg. Add 6-8 g of sample to a labeled cellulose extraction thimble (single thickness, Whatman) and record the weight to the nearest 0.1 mg. The height of the biomass in the thimble must not exceed the height of the Soxhlet siphon tube. Assemble the Soxhlet apparatus and insert the thimble into the Soxhlet tube.

2. Analyze the sample for water extractives

Add $190 (\pm 5)$ mL of HPLC grade water to the tared receiving flask. Place the receiving flask on the Soxhlet apparatus. Adjust the heating mantles to provide a minimum of 4-5 siphon cycles per hour. Reflux for 6-24 hours. The reflux time necessary will depend on the removal rate of components of interest, the temperature of the condensers, and the siphon rate. In some biomass, such as

corn stover, the reflux time is usually around eight hours, and any remaining water soluble material will be extracted during the ethanol extraction. When reflux time is complete, turn off the heating mantles and allow the glassware to cool to room temperature. If a successive ethanol extraction is to be performed, leave the thimble in the Soxhlet extractor, removing as much residual water from the Soxhlet tube as possible. If an ethanol extraction is not necessary, remove the thimble and transfer the extracted solids, as quantitatively as possible, onto cellulose filter paper in a Buchner funnel. Wash the solids with approximately 100 mL of fresh HPLC grade water. Allow the solids to dry using vacuum filtration or air dry.

3. Analyze the sample for ethanol extractives

Add 190 (± 5 mL) 190-proof ethyl alcohol to the tared ethanol receiving flask. Place the receiving flask on the Soxhlet apparatus. Adjust the heating mantles to provide a minimum of 6-10 siphon cycles per hour and reflux for 16-24 hours. When reflux time is complete, turn off the heating mantles and allow the glassware to cool to room temperature. Remove the thimble and transfer the extracted solids, as quantitatively as possible, onto cellulose filter paper in a Buchner funnel. Wash the solids with approximately 100 mL of fresh 190-proof ethanol. Allow the solids to dry using vacuum filtration or air dry. Combine any solvent from the Soxhlet tube with the solvent in the receiver flask.

4. Remove solvent from the extractives

Use a rotary evaporator equipped with a water bath set to 40 (± 5)°C for removing alcohol and set to 70 (± 5)°C for removing the water, and a vacuum source. The vacuum source should be sufficient to remove solvent without extreme bumping. Continue to remove solvent until all visible solvent is gone. Place the flask in a vacuum oven at 40 (± 2)°C for 24 hours. Cool to room temperature in a desiccator. Weigh the flask or tube and record the weight to the nearest 0.1 mg.

5. Calculate

Use the following equation to obtain the extractives content:

$$\%Extractives = \frac{WFR - WF}{ODW} \times 100$$

where

WFR = Weight of the flask plus residue

WF = Weight of the flask

ODW = Weight of the sample corrected by its moisture content (or dry weight)

Determination of Acetyl Groups in Biomass

Materials Anhydrous methanol (CH_3OH)

Sodium methoxide (CH_3ONa), 30% (w/w)

Sodium hydroxide (NaOH), 0.1 M

Hydrochloric acid (HCl), 0.1 M

Phenolphthalein indicator

Procedure

1. Determine the moisture content of the biomass.
2. Prepare 0.2 M sodium methoxide: dilute 19.5 mL of 30% (w/w) sodium methoxide in 500 mL anhydrous methanol.
3. Weigh 0.5 g dry biomass and transfer it in a 250-mL single-neck round-bottom flask (A). Attach the reaction flask (A) to a distillation apparatus as shown in Figure C-1.
4. Preheat the water bath to around 80°C.
5. Add 20 mL of 0.2 M sodium methoxide in the reaction flask (A) through the graduated separatory funnel (B) and add 40 mL of anhydrous methanol through funnel (B).
6. Collect the distillate in a 500-mL two-neck round-bottom flask (C), which is connected with Drierite Drying Column (D) containing desiccants. Immerse the flask (C) in ice bath.
7. When most of the liquid in the flask (A) has distilled, add 40 mL of anhydrous methanol in the reaction flask (A) through the funnel (B).
8. Repeat Step 7 twice (total 120 mL of anhydrous methanol is added).

9. When most of the liquid in the reaction flask (A) has distilled, add 25 mL of 0.1 M NaOH to the distillation flask (C) through the side neck. Immediately close the side neck with a glass stopper.
10. Remove the distillation flask (C) from the ice bath and place it in a hot water bath
11. Boil the flask (C) under reflux for 20 min.
12. Cool the flask (C) to room temperature.
13. Add 50 μ L of phenolphthalein indicator into the flask (C). Titrate the contents of the flask (C) with 0.1-N HCl until the color becomes colorless. Record the volume of HCl used.
14. Repeat Steps 9 to 13 for a blank determination with 120 mL of anhydrous methanol
15. The acetyl content in the biomass is estimated as follows:

$$\%Acetyl\ content = \frac{\Delta V \times N \times 0.043}{W} \times 100$$

where

ΔV = mL of HCl for blank - mL of HCl for sample

N = normality of HCl solution

W = dry weight of sample

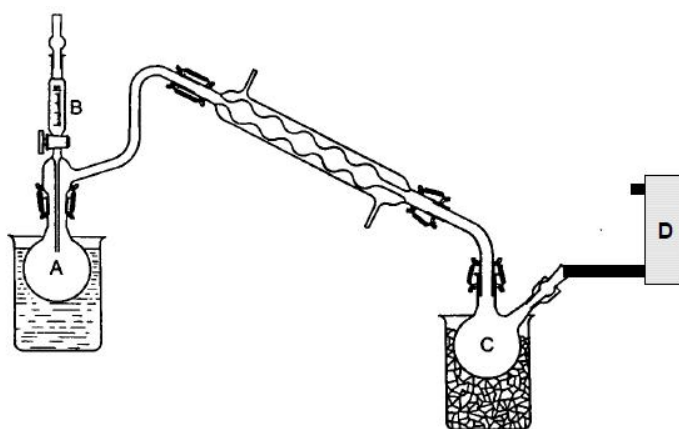


Figure C.1: Schematic diagram of distillation apparatus to determine acetyl groups in biomass (Modified from Whistler and Jeans, 1943).

Determination of Ash Content in Biomass

This procedure is based on the NREL standard procedure (Determination of Ash Biomass). The purpose is to measure the amount of inorganic material in biomass, either structural or extractable, as part of the total composition.

1. Preparation

Determine the moisture content of the samples using the NREL Standard Procedure (Determination of Total Solids and Moisture in Biomass) (Appendix H) at the time when the sample is weighed. Label the appropriate number of crucibles (ashing crucibles, 50- mL, porcelain) with a porcelain marker and place them in the muffle furnace at $575 (\pm 25) ^\circ\text{C}$ for a minimum of 4 hours. Remove the crucibles from the furnace directly into a desiccator. Cool for exactly 1 h. Weigh the crucibles to the nearest 0.1 mg and record this weight. Place the crucibles back into the muffle furnace at $575 (\pm 25) ^\circ\text{C}$ and dry to constant weight.

2. Ignite and ash

Weigh 0.5 to 2.0 g, to the nearest 0.1 mg, of the sample into the tared crucible. Record the sample weight. Using a burner and clay triangle with stand, place the crucible over the flame and let the sample burn until no more smoke or flame appears. Place the crucibles in the muffle furnace at $575 (\pm 25) ^\circ\text{C}$ by using a muffle furnace equipped with a ramping program.

Furnace Temperature Ramp Program:

Ramp from room temperature to 105°C

Hold at 105°C for 12 minutes

Ramp to 250°C at $10^\circ\text{C} / \text{minute}$

Hold at 250°C for 30 minutes

Ramp to 575°C at $20^\circ\text{C} / \text{minute}$

Hold at 575°C for 180 minutes Allow temperature to drop to 105°C

Hold at 105°C until samples are removed

When handling the crucible, protect the sample from drafts to avoid mechanical loss of sample. Carefully remove the crucible from the furnace directly into a desiccator and cool for exactly 1 h. Weigh the crucibles and ash to the nearest 0.1 mg and record the weight. At $575 (\pm 25)^{\circ}\text{C}$ ash to constant weight.

3. Calculate

Use the following equation to obtain the extractives content:

$$\%Ash = \frac{WCA - WC}{ODW} \times 100$$

where

WCA = Weight of the crucible plus ash

WC = Weight of the crucible

ODW = Weight of the sample corrected by its moisture content (or dry weight)

Determination of Structural Carbohydrates and Lignin in Biomass

This procedure is based on the NREL standard procedure (Determination of Structural Carbohydrates and Lignin in Biomass). The purpose is quantify the following components of biomass: cellobiose, glucose, xylose, galactose, mannose, lignin (insoluble lignin and soluble lignin). This procedure is suitable for samples that do not contain extractives

1. Sample preparation

Determine the moisture content of the sample according to NREL Standard Procedure (Determination of Total Solids and Moisture in Biomass) (Appendix H). The moisture content must be 10% or less, otherwise further air drying is necessary prior running this procedure. The particle size must be in the range 20/+80 mesh. Deviation to a larger or smaller particle size may result in bias in both the lignin and the carbohydrates content. It is also important to have the sample extractives free, running the procedure (Extractives in biomass) explained in Appendix B before this procedure.

2. Crucibles preparation

Filtrating crucibles (25-mL, porcelain, medium porosity ($0.2\mu\text{m}$)), are necessary in this procedure. An appropriate number of filtering crucibles must have been prepared at least one day before running this procedure. The preparation of the crucibles starts by ignition of the crucibles in a muffle furnace at $575 (\pm 25)^{\circ}\text{C}$ for a minimum of 4 hours. After ignition, the crucibles must be removed from the furnace directly into a desiccator. Let them cool for exactly 1 h and weigh them to the nearest 0.1 mg and record this weight. Place them back in the furnace and ash to constant weight defined as less than ± 0.3 mg change in the weight upon 1 h of reheating. The correct preparation of the crucibles and permanent

supervision of the calibration of the analytical balance during the weighing, are fundamental to obtain an accurate, consistent result.

3. Preparation of the samples for the calibration curve

The calibration curve samples may be prepared either in advance or after running this procedure, but they have to be ready for the analysis of carbohydrates in the HPLC. They are a series of sugar solutions of known concentration that are run in the HPLC to obtain the respective area. The results are then used to calculate an unknown concentration of sugars given an area. The range of the concentration of the calibration standards, for poplar wood is suggested as 0.1, 0.5, 1, 2, 4 mg/mL for D-cellobiose, D-(+)glucose, D-(+)xylose, D-(+)galactose, and D-(+)mannose. The samples for the sugar calibration curve may be prepared in a large batch that is stored frozen. Thaw and vortex frozen standards prior to use.

4. Concentrated acid hydrolysis

Weigh 0.3 (± 0.01) g of the sample and place it into a labeled 16 \times 100 mm test tube and record the weight to the nearest 0.1 mg. Run the NREL Standard Procedure (Determination of Total Solids in Biomass) (Appendix J) at the same time, to accurately measure the percent solids for correction. Add 3.00 (± 0.01 mL) of 72% sulfuric acid to each pressure tube. Place the pressure tube in a water bath set at 30 (± 3) $^{\circ}$ C and incubate the sample for 60 (± 5) minutes. Using a Teflon stir rod, stir the sample every 5 to 10 min without removing the sample from the bath.

5. Dilute acid hydrolysis

Once the time for the concentrated acid hydrolysis has elapsed, remove the tubes from the water bath and dilute the acid to a 4% concentration by adding 84.00 (± 0.04) mL deionized water with an automatic burette. Seal the bottles and place them in an autoclave. Autoclave the sealed samples and sugar recovery standards for 1 h at 121 $^{\circ}$ C. After that, allow the hydrolyzates to slowly cool to room temperature before removing the caps.

6. Acid insoluble lignin analysis

Vacuum filter the autoclaved hydrolysis solution through one of the prepared filtering crucibles. Capture the filtrate in a filtering flask. Transfer an aliquot, approximately 50 mL, into a sample storage bottle. This sample will be used to determine acid-soluble lignin as well as carbohydrates and acetyl content. Use a minimum of 50 mL of hot deionized water to quantitatively transfer all remaining solids out of the pressure bottle into the filtering crucible. Dry the crucible and acid insoluble residue at 105 (± 3) $^{\circ}$ C until a constant weight is achieved, minimum

overnight, better 24 hours or more. Remove the samples from the oven and cool in a desiccator. As accurately as possible, record the weight of the crucible and dry the residue to the nearest 0.1 mg. Place the crucibles and residue in the muffle furnace at $575 (\pm 25) ^\circ\text{C}$ for $24 (\pm 6)$ hours. Carefully remove the crucible from the furnace directly into a desiccator and cool for exactly 1 h. Weigh the crucibles and ash to the nearest 0.1 mg and record the weight. Place the crucibles back in the furnace and ash to a constant weight

7. Acid soluble lignin analysis

It must be performed within 6 h of hydrolysis on a UV-Visible spectrophotometer (background, deionized water) using the hydrolysis liquor aliquot obtained after vacuum filter the autoclaved hydrolysis solution. Measure the absorbance of the sample at 320 nm on a UV-Visible spectrophotometer. Using deionized water dilute the sample as necessary to bring the absorbance into the range of 0.2-1.0, recording the dilution. Record the absorbance to three decimal places.

8. Carbohydrates analysis

Transfer 20 mL of the hydrolysis liquor obtained after the filtering step to a 50-mL Erlenmeyer flask. Use Barium hydroxide to neutralize each sample to pH 5-6. Allow the sample to settle and decant off the supernatant. The pH of the liquid after settling will be approximately 7. Centrifuge the sample to eliminate the barium sulphate, and prepare the sample for HPLC analysis by passing the decanted liquid through a $0.2\text{-}\mu\text{m}$ filter into an autosampler vial. Seal and label the vial. Analyze the calibration standards and samples by HPLC using a Phenomenex Rezex, RPM-Pd⁺² column equipped with the appropriate guard column. HPLC conditions follow:

Injection volume: 20 μL

Mobile phase: HPLC grade water, $0.2\text{ }\mu\text{m}$ filtered and degassed

Flow rate: 0.6 mL/min

Column temperature: 79°C

Detector: refractive index

Run time: 25 minutes

Backwash the column overnight every time when the analysis is finished, according to the Biorad manual. The operating conditions for backwashing are as follows; Eluant $0.45\text{ }\mu\text{m}$ filtered DI water, flow rate 0.1 mL/min , column temperature ambient, column direction reverse.

If cellobiose and oligomeric sugars are detected in levels greater than 3 mg/mL, incomplete hydrolysis occurred and fresh samples should be hydrolyzed and analyzed. Peaks before cellobiose may indicate high levels of sugar degradations products in the previous sample, which indicates over hydrolysis. All samples

from batches showing evidence of over-hydrolysis should have fresh samples hydrolyzed and analyzed.

9. Calculations

Acid-insoluble lignin:

$$\%AIL = \frac{(WCR - WC) - (WCA - WC)}{ODW} \times 100$$

where:

%AIL = Percentage of acid insoluble lignin

WCR = Weight of crucible plus residue

WC = Weight of crucible

WCA = Weight of crucible plus ash

ODW = Dry weight of the sample (or weight corrected by moisture content)

Acid-soluble lignin

$$\%ASL = \frac{UV.87.D}{11.4.ODW} \times 100$$

where: %ASL = Percentage of acid insoluble lignin

UV = Average UV-Vis absorbance of the sample at 320 nm

D = Dilution factor

ODW = Dry weight of the sample (or weight corrected by moisture content)

The values 87 and 11.4 stand for volume of the filtrate and absorptivity of poplar wood at 320 nm, respectively

$$\%Total\ Lignin = \%AIL + \%ASL$$

Percentage of recovery of SRS

$$PR = \frac{SRS_A}{SRS_B}$$

where:

PR = percentage of recovery of SRS

SRSA = Concentration of sugar as measure by HPLC after dilute acid hydrolysis

SRSB = Concentration of sugar as measure by HPLC before dilute acid hydrolysis

Concentration of carbohydrates:

$$\%C_i = \frac{C_{HPLC} \cdot AC \cdot 87}{PR \cdot ODW \cdot 10}$$

where:

C_i = Concentration of Sugar i

C_{HPLC} = Concentration of Sugar i as given by HPLC

PR = Percentage of recovery of SRS

AC = Anhydro correction to calculate the concentration of polymeric sugars from the corresponding concentration of monomeric sugars. It is 0.90 for glucose and mannose and 0.88 for xylose.

ODW = Dry weight of the sample (or weight corrected by moisture content)

The values 87 and 10 stand for volume of the sample and conversion units factor, respectively.

Neutralization of Lime After Pretreatment

This procedure has a double purpose: determine the lime consumption during pretreatment and neutralize the sample to make it ready for analytical procedures that may be affected for pH.

1. Sample preparation

Once the pretreatment time is elapsed, let the reactor cool to room temperature, transfer its contents to a 1-L beaker or centrifuge bottle, using distilled water to rinse and move all the material as completely as possible. The volume of slurry in the bottle after this step is about 750 mL.

2. Procedure

Set up titration apparatus (buret, clamp, magnetic stirrer and a well-calibrated pH meter). Place a magnetic bar into the centrifuge bottle containing pretreated biomass slurry and place the bottle on the magnetic stirrer. Dip the pH probe inside of the bottle to measure the pH of the slurry. Fill 5-N HCl solution in the buret and clamp it over the bottle. Record the volume (V_i). Slowly drop the acid into the bottle up to the end point (pH 7.00). Provide enough time (1 h) to ensure the pH of the slurry is stabilized. Record the volume left in the buret (V_f).

3. Calculation Use the following equation to determine the lime consumption during pretreatment:

$$W_{Ca(OH)_2} = \frac{1 \text{ mol } Ca(OH)_2}{2 \text{ mol } HCl} \times \frac{N_{HCl} \cdot (V_i - V_f)}{1000} \times M_{Ca(OH)_2}$$

where:

$W_{Ca(OH)_2}$ = The amount of lime, $Ca(OH)_2$, unreacted (g)

N_{HCl} = Normality of HCl solution

$V_i - V_f$ = Total volume of HCl solution to titrate the biomass slurry (mL)

$M_{Ca(OH)_2}$ = Molecular weight of $Ca(OH)_2$, 74.092 g/mol

Enzymatic Hydrolysis

Enzymatic hydrolysis of pretreated biomass was performed in 20-mL scintillation vials at 50°C on the a multiple place magnetic stirrer at 200 rpm. The hydrolysis experiments were performed at 10-g/L solid concentration in 0.05-M citrate buffer (pH 4.8) supplemented with 0.01-g/mL sodium azide to prevent microbial contamination. Hydrolysis was initiated by adding appropriately diluted cellulase at 30.0%w/w (g enzyme/g cellulose). A series of experiments were conducted with strategic cellulase loadings based on biomass structural features. After the incubation periods 72 h, the reaction in the sealed scintillation vials was quenched in boiling water. Then sugar yields were measured at each time point. See the following complete hydrolysis procedure.

Materials

Citric acid monohydrate

Sodium hydroxide

Sodium azide

Cellulase enzyme Novozyme" Cellic CTec2"

Apparatus

Analytical balance, accurate to 0.1 mg

Convection drying oven, with temperature control of $105 \pm 3^\circ\text{C}$

200-rpm multiple place magnetic stirrer

Centrifuge machine

Adjustable pipettors, covering ranges of 0.02 to 5.00 mL

Heater

scintillation vials, 20-mL

Erlenmeyer flask, 50-mL

Centrifuge tubes, 15-mL

Syringe Filters Cellulose Acetate , 0.45 μm

Procedure

1. Determine the moisture content of the biomass. Appendix H.
2. Prepare 1 L of 1-M citrate buffer and 250 mL of 0.01-g/mL sodium azide.
3. Weigh out a biomass sample equal to the equivalent of 0.1 g of cellulose g dry biomass and appropriate of distilled water to complete 10-mL volume of mixture in 20-mL scintillation vials and label each flask with enzyme loading and incubation period..
4. Add 0.5 mL of 1-M citrate buffer and 0.3 mL of 0.01-g/mL sodium azide into the flasks to keep the pH constant and prevent the growth of microorganisms, respectively..
5. Measure the pH of the mixture and add glacial acetic acid or sodium hydroxide to adjust pH to 4.8-5, if necessary.
6. Place the rubber stopper on the top of the Erlenmeyer flasks and preheat the flasks at 50°C in a bath water for 1 h before adding enzymes.
7. Take out the heated flask from bath water and initiate the enzymatic hydrolysis by adding 0.5 mL of the appropriately diluted cellulase and 0.05 mL. The final volume is 10.0 mL
8. Cap the scintillation vials tightly using a clamp to seal the rubber stopper, so the stopper can stand the pressure during boiling. Place the flasks back into the multiple place magnetic stirrer.
9. After 72 h incubation, take out the flask and vigorously boil the whole flask for 15 min to denature enzymes..
10. Cool the boiled flasks in an ice-water bath for 10 min and transfer the mixture to centrifuge tube.
11. Centrifuge the mixture at 4,000 rpm for 20 min to separate the liquid and solid phases.
12. Transfer the liquid into a 10-mL scintillation vials and store it in the freezer for sugar analysis by HPLC later. Remember to vortex the sample after thawing and dilute the sample to get an appropriate concentration with calibration curve

Determination Of Moisture Content In Biomass

This procedure is based on the NREL standard procedure (Determination of Total Solids and Moisture in Biomass). The purpose is to measure the amount of water and other components volatilized at 105°C present in a biomass sample.

1. Procedure

Accurately weigh a predried aluminum foil weighing dish to the nearest 0.1 mg and record this weight (WD). Thoroughly mix the sample and then weigh 0.5 to 2 grams (± 0.1 mg) into the weighing dish. Record the weight of the sample plus the weighing dish (WWS). Place the sample into a convection oven at $105 \pm 3^\circ\text{C}$ for a minimum of four hours. Remove the sample from the oven and allow it to cool to room temperature in a desiccator. Weigh the dish containing the oven-dried sample to the nearest 0.1mg and record this weight. Place the sample back into a convection oven at $105 \pm 3^\circ\text{C}$ and dry to constant weight. Constant weight is defined as $\pm 0.1\%$ change in the weight percent solids upon one hour of re-heating the sample. Overnight drying is usually required for very wet or liquid samples. Remove the sample from the oven and place in a desiccator; cool to room temperature. Weigh the dish containing the oven-dried sample to the nearest 0.1 mg and record this weight (WDS). All the samples must be run in replicate (duplicates, at minimum).

2. Calculate

Use the following equation to obtain the extractives content:

$$\%Moisture = 1 - \frac{WDS - WD}{WWS - WD} \times 100$$

$$\%TotalSolids = \frac{WDS - WD}{WWS - WD} \times 100 \text{ or } \%TotalSolids = 1 - Moisturecontent$$

Calibration Curve Procedure

1. Prepare a series of calibration standards containing the compounds that are to be quantified, The range of the concentration of the calibration standards for willow wood is suggested as 0.1, 0.5, 1, 2, 3, 4 mg/mL for D-cellobiose, D-(+)glucose, D-(+)xylose, D-(+)galactose, and D-(+)mannose.
2. Prepare of an independent calibration verification standard (CVS) for each set of calibration standards, using sugars obtained from a source other than that used in preparing the calibration standards. The CVS must contain precisely known amounts of each sugar contained in the calibration standards, at a concentration that falls in the middle of the validated range of the calibration curve. The CVS is to be analyzed after each calibration curve and at regular intervals in the HPLC sequence, bracketing groups of samples. The CVS is used to verify the quality of the calibration curve(s) throughout the HPLC run.
3. Analyze the calibration standards, CVS, and samples by HPLC using a RPM-Monosaccharide column (RPM Pb⁺²) column equipped with the appropriate guard column. The following instrumental conditions are used for Analysing:
Sample volume: 20 μ L.
Eluant: 0.2 μ m filtered and degassed, deionized water.
Flow rate: 0.6 mL/min.
Column temperature: 79°C.
Detector: refractive index.
Run time: 25 minutes.
4. Plot (analyte) vs. Signal by software program. this would be between concentration analyte (g/L) on the x-axis and refractive index on the y-axis. The regression coefficient (R) of my Calibration curve is very high (0.999).
5. Use the signal for unknown samples to find the concentration of the analyte.

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