Studies of Cellulosic Ethanol Production from Lignocellulose

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ABSTRACT

At present, the world's transportation sector is being principally supplied by fossil fuels. However, energy consumption in this sector is drastically increasing and there are concerns with supply, cost, and environmental issues with the continuing use of fossil fuels. Utilizing non-petroleum ethanol in the transportation sector reduces the dependence on oil, and allows for cleaner burning of gasoline.

Lignocellulose materials are structurally composed of five types of polymeric sugars, glucan, galactan, mannan, arabinan, and xylan. NREL has developed a quantitative saccharification (QS) method for determining carbohydrate composition. We proposed a new protocol based on the NREL 2006 Laboratory Analytical Procedure "Determination of Structural Carbohydrates and Lignin in Biomass" (Sluiter et al. 2006a) with a slight modification, in which xylose concentration was determined after the secondary hydrolysis by using 1% sulfuric acid rather than 4% sulfuric acid. We found that the current NREL protocol led to a statistically significant overestimation of acid-labile xylan content ranging from 4 to 8 percent.

Lignocellulosic biomass is naturally recalcitrant to enzymatic hydrolysis, and must be pretreated before it can be effectively used for bioethanol production. One such pretreatment is a fractionation process that separates lignin and hemicellulose from the

cellulose and converts crystalline cellulose microfibrils to amorphous cellulose. Here we evaluated the feasibility of lignocellulose fractionation applicable to the hurds of industrial hemp. Hurds are the remaining material of the stalk after all leaves, seeds, and fiber have been stripped from the plant. After optimizing acid concentration, reaction time and temperature, the pretreated cellulosic samples were hydrolyzed to more than 96% after 24 hours of hydrolysis (enzyme loading conditions of 15 FPU/g glucan Spezyme CP and 60 IU/g glucan Novozyme 188) at the optimal pretreatment condition ($\geq$ 84% H_3PO_4, $\geq$ 50 °C and $\geq$ 1 hour). The overall glucose and xylose yields were 89% (94% pretreatment; 96% digestibility) and 61%, respectively. All data suggest the technical feasibility of building a biorefinery based on the hurds of industrial hemp as a feedstock and a new lignocellulose fractionation technology for producing cellulosic ethanol. The choice of feedstock and processing technology gives high sugar yields, low processing costs, low cost feedstock, and low capital investment.

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1. INTRODUCTION AND REVIEW OF LITERATURE

Global energy consumption has been steadily increasing with the growing population and rising living standards over the course of the twentieth century. As developed and developing countries require more energy in the next few decades, the demand of energy will increase. Already, there have been several energy crises in many parts of the world, as is evident with increasing energy prices and the "energy crises" of the United States in the late 1970s. The use of convenient fossil fuels results in several problems, such as carbon dioxide emissions, which are believed to strongly influence climate change. Alternative energy sources must be sought to fulfill the growing energy demands of the global society, to reduce the dependence of countries like the United States on foreign petroleum, and to ensure economic stability and decrease trade deficits.

In the U.S., the transportation sector accounts for ~28% of the total energy consumption, and ~70% of crude oil is used for transportation. Currently the principle source for transportation energy is the combustion of liquid fossil fuels through internal combustion engines. One of the most promising alternative renewable liquid transportation fuels is ethanol produced from biomass.

Ethanol, a liquid fuel carrier, has a number of advantages over petroleum-based gasoline, and is already beginning to be integrated into the transportation sector. Ethanol has a high heat of vaporization, low flame temperature, high gas volume change, and high octane rating, all of which make it a favorable fuel. Although ethanol only has

approximately 70% the energy content of gasoline on a per volume basis, ethanol has a 12 to 14 octane number advantage over gasoline. In theory, ethanol's higher octane rating could allow for an increase in compression ratio in an internal combustion engine from 9 in most modern automobiles, to 12 or 13, resulting in a 6% to 10% increase in theoretical thermal efficiency in Otto-cycle gasoline engines (Hardenberg and Schaefer 1981; Lynd 1996). In 2006, nearly 5 billion gallons of bioethanol was produced annually and blended into gasoline as a fuel additive to reduce nitric oxide emissions and replace MTBE use (Renewable Fuels Association 2007). Bioethanol is an attractive alternative to petroleum because the net release of carbon dioxide is nearly zero based on a life cycle analysis, thus resulting in significantly lower greenhouse gas emissions. Additionally, ethanol can be produced from domestic sources of readily available agricultural and forestry biomass, thus reducing foreign dependence on petroleum.

Currently, almost all fuel ethanol blended into gasoline for sale in the United States is derived from starch-based feedstock -- corn kernels. The price of corn grain has risen by > 70% during the past 2 years, and these increases are squeezing the profit margin of corn biorefineries. The profitability of biorefineries has to rely on federal subsides. Additionally, since corn is valuable food and feed in the United States, the high prices of corn kernels have raised a great deal of concerns about food supplies worldwide. For example, the Chinese Central Government banned the construction of new corn-ethanol plants and suspended corn ethanol production due to high corn prices in 2007.

The largest renewable sugar source for ethanol production is the carbohydrates locked in lignocellulosic biomass. The DOE and USDA estimate that there is 1.3 billion tons of available renewable cellulosic biomass per year without change to our food supply (DOE and USDA 2005). Lignocellulosic feedstocks include forestry and agricultural residues, municipal solid waste, and woody and grassy crops.

The sugars (cellulose and hemicellulose) in lignocellulose plant cell walls cannot easily be fermented into ethanol because they are plant cell wall structural building blocks, whereas starch is naturally used as an energy reserve, and therefore easily hydrolyzed. Effective conversion of recalcitrant lignocellulose to cellulosic ethanol requires five sequential steps: (1) lignocellulose size reduction, (2) pretreatment/fractionation, (3) enzymatic hydrolysis, (4) ethanol fermentation, and (5) ethanol recovery. However, due to high processing costs, great capital investment in the conversion plant and a narrow margin between product value and feedstock cost (Zhang et al. 2006b), cellulosic ethanol produced from lignocellulose biomass is not produced commercially,.

1.1 LIGNOCELLULOSE STRUCTURE

The composition of polymeric carbohydrates in a feedstock varies with the species of plant materials. In general, 30-50% of the dry weight of the material is cellulose, 20-35% hemicellulose, and 10-20% lignin. Additionally, there is also a small amount of compounds such as, minerals, pectin, acetic acid, and proteins.

The major components of plant cell walls are cellulose and hemicelluloses, which together with lignin and proteins form a complex and rigid structure (Figure. 1-1). The walls of plant cells of different plant types differ greatly in appearance and properties.

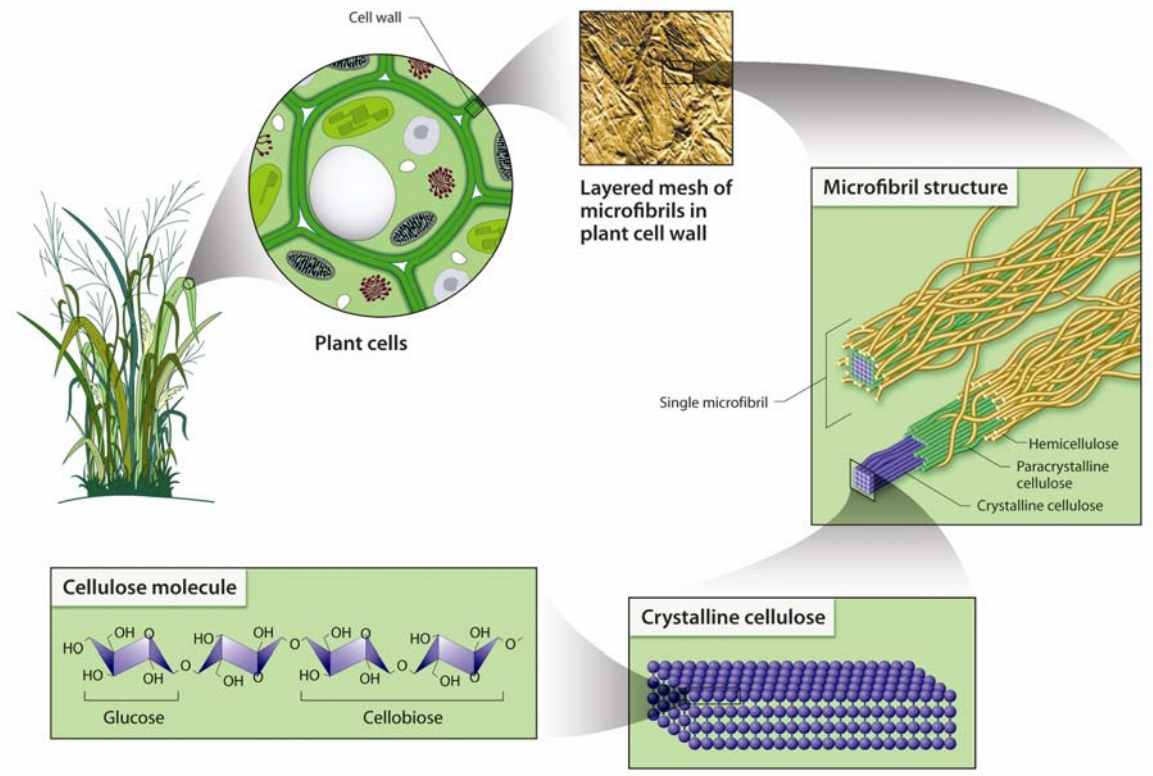

Figure 1-1. Schematic structure of plant cell walls; the lignocellulose components and structure (U.S. Department of Energy Genome Programs 2007a).

Cellulose is a linear biopolymer of anhydroglucopyranose connected by β-1,4-glycosidic bonds. Coupling of adjacent cellulose chains by orderly hydrogen bonds and Van der Waal's forces leads to a parallel alignment and a crystalline structure, resulting in low accessibility (Figure1-1). The evidence from cell wall biophysics, biosynthesis, genomics, and atomic force microscope (AFM) images suggests that elementary cellulose

fibrils are synthesized by the cellulose synthase complex (CelS) locus that contains 36-glucan chains and they have both crystalline and subcrystalline structures (Ding and Himmel 2006). A number of elementary fibrils coalesce into much larger microfibrils; these microbrils can then form macrofibrils. Hemicelluloses are situated between the lignin and the collection of cellulose microfibrils underneath. Consistent with their structural chemistry and side-group substitutions, the xylan seems not only to be interweaved and ester-linked at various points with the overlaying "sheath" of lignin, but also to produce a coat around underlying strands of cellulose *via* hydrogen bonds. The xylan layer with its covalent linkage to lignin and its non-covalent interaction with cellulose may be important in maintaining the integrity of the plant cell wall in situ and in helping protect the fibers against degradation by enzymes (Beg et al. 2001). At least two types of covalent cross-links have been identified between hemicellulose and lignin: i) diferulic acid bridges, and ii) ester linkage between lignin and glucuronic acid attached to xylans (de Vries and Visser 2001).

Hemicellulose was originally defined as the fraction isolated or extracted from plant materials using dilute alkali. Main hemicellulose chains contain either xylose or mannose, to which may be linked short side-chains composed of sugars such as arabinose and glucuronic acid. Xylans are the most common hemicellulosic polysaccharide in cell walls of land plants. They consist of a β-1,4-linked D-xylose backbone, and can be substituted by different side groups such as L-arabinose, D-galactose, acetyl, ferulyl, p-coumaroyl, and glucuronic acid residuals (Beg et al. 2001; de Vries and Visser 2001). Hardwood xylan consists of at least 70 *O*-acetyl-4-*O*-methylglucuronoxylan unites linked

by β-1,4-glycosidic bonds with an average degree of polymerization (DP) between 150 and 200. Hardwood xylan is often referred to as glucuronoxylans due to the large amount of D-glucuronic acid attached to the backbone. Hardwood xylan is highly acetylated (e.g. birchwood xylan contains more than 1 mol of acetic acid per 2 mol of xylose), which is preferentially at the C-3 position, as opposed to the C-2 position. The presence of these acetyl groups is responsible for the partial solubility of xylan in water. These acetyl groups are readily removed using alkali extraction (Beg et al. 2001). Softwood xylan consists of arabino-4-*O*-methylglucuronoxylan with the DP between 70 and 130, shorter than hardwood xylans and less branched (Beg et al. 2001; Singh et al. 2003). It is not acetylated but the 4-O-methylglucoronic acid and the L-arabinofuranose residues are attached to the C-2 and C-3 positions, respectively, of relevant xylopyranose backbone units (Singh et al. 2003).

Lignin is a polyphenolic biopolymer formed by non-enzymatic polymerization of delocalized phenoxy radicals derived from the monomers, i.e., substituted *p*-hydroxycinnamyl alcohols (Figure 1-2). Lignin is a noncrystalline solid polymer with a density of ~1.3-1.4 g/cm^3. The bonds among monomeric units include ether and biphenyl linkages, which are resistant to hydrolytic attack (Broda et al. 1996). The biological role of lignin is to hold cellulose and hemicellulose in the plant cell walls together and to prevent water loss from plant vascular systems because of its hydrophobic properties. Lignin has a high energy density (24~26 MJ/kg), thus 1 kg is equivalent to 0.6 kg of petroleum.

The complicated structure of lignocellulose produces its resistance to biological and chemical degradation. For example, in nature, lignocellulose biodegradation is slow, requiring that a number of hydrolysis enzymes work together, including cellulases (endoglucanase, cellobiohydrolases, and beta-glucosidase), hemicellulases, and lignin-degrading enzymes.

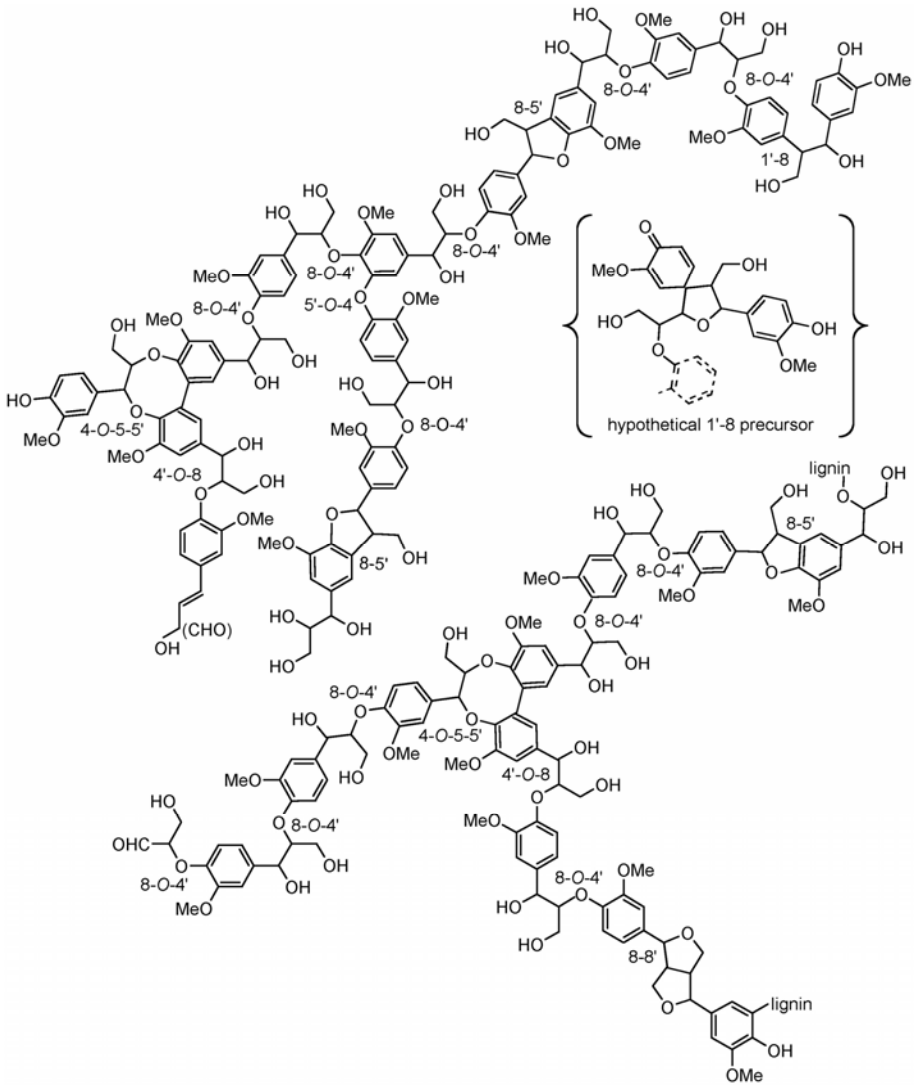

Figure 1-2. Structural view of lignin biopolymer (U.S. Department of Energy Genome Programs. 2007b).

1.2 LIGNOCELLULOSE PRETREATMENT TECHNOLOGY

Effectively overcoming the recalcitrance structure of lignocellulose and releasing the locked polysaccharides is one of the most important and urgent R&D priorities for the emerging cellulosic ethanol and bio-based chemical industries (Biomass Research and Development Technical Advisory Committee 2002; Office of Energy Efficiency and Renewable Energy and Office of Science 2006). Lignocellulose pretreatment is among the most costly steps and has a major influence on the costs of both prior operation (e.g., lignocellulose particle size reduction) and subsequent operations (e.g., enzymatic hydrolysis and fermentation) (Wooley et al. 1999; Wyman et al. 2005b). Typically, substrate factors that have been shown to affect digestibility of cellulose are the crystallinity of cellulose, degree of polymerization of the cellulose, particle size/specific surface area, lignin distribution, and accessible surface area. To increase efficiency of hydrolysis of the polysaccharide sugars into fermentable sugars, a pretreatment process is required for conversion of lignocellulosic biomass.

All lignocellulose treatments can be divided into four main categories:

1. Physical methods, including dry milling (chipping, ball milling and comminuting), wet milling, irradiation, microwave, and swelling reagents ($ZnCl_2$) (Awafo et al. 1995; Chang et al. 1981; Hsu 1996; Kumakura and Kaetsu 1983; Kumakura et al. 1982; Lam et al. 2000; McMillian 1994; Millett et al. 1976; Sun and Cheng 2002; Tolbert et al. 1986);

2. Chemical methods, including dilute acids (dilute H_2SO_4, H_3PO_4, HCl, acetic acid, formic acid/HCl) (Brennan and Wyman 2004; Chung et al. 2005; Fan et al. 1982; Grethlein and Converse 1991; Jacobsen and Wyman 2000; Kim et al. 2002; Kim and Lee 2002; Lee et al. 1997; Lloyd and Wyman 2003; Lloyd and Wyman 2004; Lloyd and Wyman 2005; McMillian 1994; Nagle et al. 2002; Nguyen et al. 2000; Schell et al. 2003; Song and Lee 1984; Teixeira et al. 2000 ; Tucker et al. 2004; Um et al. 2003; Vlasenko et al. 1997; Weil J 1994; Zhu et al. 2004; Zhu et al. 2005), alkalis (NaOH, lime, ammonia, amine, etc.) (Chang et al. 2001; Coward-Kelly et al. 2005; Ferrer et al. 2002a; Ferrer et al. 2002b; Foster et al. 2001; Galbe and Zacchi 2002; Horvath et al. 2005; Kim et al. 2000; Kim and Holtzapple 2005a; Kim and Holtzapple 2005b; Kim and Holtzapple 2005c; Kurakake et al. 2001; Lynd et al. 2002; Mosier et al. 2005b; Sulbaran-de-Ferrer et al. 2003; Wyman et al. 2005a; Wyman et al. 2005b), organosolv (Chum et al. 1988; Holtzapple and Humphrey 1984; Mutje et al. 2005; Pan et al. 2005a), oxidizing agents (O_3, NO, H_2O_2, $NaClO_2$) (Ahring et al. 1996; Charles et al. 2003; Fan et al. 1982; Schmidt and Thomsen 1998; Vidal and Molinier 1988; Weil J 1994; Yang et al. 2002), cellulose solvents (Wood and Saddler 1988) (e.g., cadoxen (Ladisch et al. 1978), DMAc/LiCl (Striegel 1997; Valjamae et al. 2003), concentrated H_2SO_4 (Fengel and Wegener 1984), etc.);

3. Physio-chemical methods, including steam explosion with or without catalysts (Ballesteros et al. 2000a; Ballesteros et al. 2000b; Bura et al. 2003a; Bura et al. 2003b; Bura et al. 2002; Converse and Grethlein 1987; Emmel et al. 2003; Grethlein and Converse 1991; Kobayashi et al. 2004; Laser et al. 2002; Martin et al. 2002;

Negro et al. 2003; Ohgren et al. 2005; Oliva et al. 2003; Palmarola-Adrados et al. 2004; Sassner P 2005 ; Soderstrom et al. 2002; Soderstrom et al. 2003; Tanaka et al. 1990; Tengborg et al. 2001; Vlasenko et al. 1997), CO_2 explosion (van Walsum and Shi 2004; Zheng et al. 1998), ammonia fiber explosion or expansion (AFEX) (Alizadeh et al. 2005; Dale et al. 1996; Ferrer et al. 2000; Gollapalli et al. 2002; Tengerdy and Nagy 1988; Teymouri et al. 2005; Vlasenko et al. 1997), hot water (Ballesteros et al. 2000b; Laser et al. 2002; Li et al. 2003; Mosier et al. 2005a; Negro et al. 2003; van Walsum and Shi 2004), hot water with flow-through (Liu and Wyman 2004; Liu and Wyman 2005), supercritical fluid extractions (CO_2, CO_2/H_2O, CO_2/SO_2, NH_3, H_2O) (Kim and Hong 2001; McMillian 1994; van Walsum and Shi 2004), and lignocellulose fractionation (Zhang et al. 2007b); and

4. Biological methods (white rod fungi) (Akin et al. 1995; Breen and Singleton 1999; Crawford 1978; Fan et al. 1987; Hatakka 1983; Kerem and Hadar 1995; Leonowicz et al. 1999; Rahmawati et al. 2005; Valaskova and Baldrian 2005).

Recently, a Biomass Refining Consortium for Applied Fundamentals and Innovation (CAFI) has been founded, composed of Auburn University, Dartmouth College, Michigan State University, the National Renewable Energy Laboratory (NREL), Purdue University and Texas A&M University. It was supported by the U.S. Department of Agriculture Initiative for Future Agriculture and Food Systems (IFAFS) Program and the Office of the Biomass Program of the U.S. Department of Energy and undertook the first coordinated project to develop comparative information on the performance of leading

pretreatment options on corn stover. The pretreatment choices were narrowed down to a few methods: dilute acid (Grethlein and Converse 1991; Kim and Lee 2002; Lloyd and Wyman 2003; Lloyd and Wyman 2005; Nguyen et al. 2000; Schell et al. 2003; Tucker et al. 2004; Um et al. 2003), flow-through pretreatment (Liu and Wyman 2004; Liu and Wyman 2005; Yang and Wyman 2004), ammonia fiber explosion (Alizadeh et al. 2005; Dale et al. 1996; Gollapalli et al. 2002; Teymouri et al. 2005), ammonia recycle percolation (ARP) (Kim et al. 2000; Kim et al. 2003; Kim and Lee 2005; Kim and Lee 2006), and lime pretreatment (Chang et al. 2001; Coward-Kelly et al. 2005; Kim and Holtzapple 2005a; Kim and Holtzapple 2005b; Kim and Holtzapple 2005c) in Table 1-1.

Table 1-1. Technologies and representative reaction conditions for corn stover, modified based on the review (Wyman et al., Biores. Tech. 2005, 96: 1959-1966) plus lignocellulose fractionation.

Pretreatment technology	**Chemicals used**	**Temperature, (°C)**	**Pressure, atm absolute**	**Reaction times, min**
Dilute sulfuric acid	0.5–3.0% sulfuric acid	130–200	3–15	2–30
pH-controlled water pretreatment	water or stillage	160–190	6–14	10–30
AFEX	100% anhydrous ammonia	70–90	15–20	< 5
ARP	10–15 wt.% ammonia	150–170	9–17	10–20
Lime	0.05–0.15 g $Ca(OH)_2$/g biomass	70–130	1–6	60-360
Lignocellulose Fractionation (here)	Concentrated H_3PO_4 & acetone	50	1	~30

A project of CAFI-II is being conducted to deal with a hardwood feedstock -- hybrid poplar. Additionally, two other methods have been intensively investigated in Europe and Canada: steam explosion with or without SO_2 impregnation (Ballesteros et al. 2000b; Bura et al. 2003a; Bura et al. 2003b; Bura et al. 2002; Converse and Grethlein 1987; Emmel et al. 2003; Grethlein and Converse 1991; Laser et al. 2002; Martin et al. 2002; Palmarola-Adrados et al. 2004; Soderstrom et al. 2002; Soderstrom et al. 2003; Tengborg et al. 2001) and organosolv (Chum et al. 1988; Holtzapple and Humphrey 1984; Mutje et al. 2005; Pan et al. 2005a).

All leading pretreatments share one or several common shortcomings:

1. Severe pretreatment conditions (except AFEX and lime) (see Table 1-1), result in sugar degradation and inhibitor formation;
2. Low or modest cellulose digestibility because of the presence of residual lignin and hemicellulose and the remaining crystalline cellulose;
3. High cellulase loading;
4. Low hydrolysis rates;
5. Large utility consumption;
6. Huge capital investment for high pressure and temperature reactors; and
7. Less co-utilization of other lignocellulose components (except organosolv).

Therefore, there is an urgent need to find new pretreatment technologies for overcoming the recalcitrance of lignocellulose to achieve attractive investor returns, and/or increase

revenues (Lynd et al. 1999; Wyman 1999). Development of a lignocellulose pretreatment, featuring modest reaction conditions, is highly desired because it can not only decrease utility consumption and initial capital investment but also reduce sugar degradation and inhibitor formation (McMillan 1994).

Although biomass recalcitrance has been attributed to up to seven factors (Himmel et al. 2007), the two main root causes of the recalcitrance of lignocellulose to cellulase enzymatic hydrolysis are hypothesized to be 1) the low accessibility of (micro-) crystalline cellulose fibers, which prevents cellulases from working efficiently (Zhang et al. 2006a; Zhang and Lynd 2006), and 2) the presence of lignin (mainly) and hemicellulose on the surface of cellulose, which prevents cellulase from accessing the substrate efficiently (Pan et al. 2005b; Zhang et al. 2007b).

A new lignocellulose pre-treatment featuring modest reaction conditions (50°C and atmospheric pressure) has recently been invented to fractionate lignocellulose to amorphous cellulose, hemicellulose, lignin, and acetic acid by using a non-volatile cellulose solvent (concentrated phosphoric acid), a highly volatile organic solvent (acetone), and water (Zhang et al. 2007b). The high sugar yields after enzymatic hydrolysis were attributed to the lack of sugar degradation during the fractionation and the high enzymatic cellulose digestibility (~97% in 24 h) during the hydrolysis step at the enzyme loading of 15 filter paper units of cellulase and 60 IU of beta-glucosidase per gram of glucan. Isolation of high-value lignocellulose components (lignin, acetic acid,

and hemicellulose) would greatly increase potential revenues of a lignocellulose biorefinery.

1.3 Research Goals

In this study, my research plan includes two tasks:

1. Developing a more accurate quantitative saccharification (QS) method for acid-labile carbohydrate composition in lignocellulose;
2. Evaluating the feasibility of lignocellulose fractionation applicable to a special lignocellulosic feedstock.

The goal of Task 1 is to decrease the inherent theoretical error of the quantitative saccharification with slight modifications (Chapter 2); the goal of Task 2 is to demonstrate the potential of lignocellulose fractionation for other lignocellulosic materials that cannot be addressed by the most popular technology – steam explosion (Chapter 3).

2. MORE ACCURATE DETERMINATION OF ACID-LABILE SUGARS IN LIGNOCELLULOSE BY QUANTITATIVE SACCHARIFICATION

Abstract. Quantitative saccharification is a widely used method for determining carbohydrate composition in lignocellulosic materials. The NREL methods (Ruiz and Ehrman 1996; Sluiter et al. 2006) involve a primary hydrolysis (high sulfuric acid concentration at low temperature) breaking down polysaccharides into oligosaccharides, followed by a secondary hydrolysis (dilute acid stage at high temperature) converting all the oligosaccharides to monosaccharides that can be easily quantified. The monomeric sugars, after neutralization, are measured by HPLC. Since some saccharides are degraded during the harsh hydrolysis processes, a sugar control set of monomeric sugars is run in parallel and the mono-saccharide correction coefficients (representing sugar degradation) are used to reflect those for polysaccharides. This could not be a valid assumption because the polysaccharide and monosaccharide sugars have different degradation rates. For acid-labile polysaccharides, such as xylans and arabinans, the lower value of correction coefficients for xylose and arabinose could result in an overestimation of the total sugars in the samples. We propose a new protocol based on the NREL 2006 protocol (Sluiter et al. 2006) with slight modifications, in which xylose concentration at the secondary hydrolysis by using 1% sulfuric acid rather than 4% sulfuric acid is determined. The less acidic reaction can significantly decrease xylose degradation ~3.8 fold. We found that the current protocol led to a statistically significant overestimation of acid-labile xylan contents for Douglas fir by 9.1%, hybrid poplar by 8.1%, switchgrass by 4.2%, corn stover by 8.5%, and wheat straw by 8.3%.

2.1 INTRODUCTION

Lignocellulosic material, the main product of photosynthesis, is the most abundant renewable biological resource. Lignocellulose is a natural composite, consisting of three main polymeric components – cellulose, hemicellulose and lignin, as well as other minor components, such as, minerals and protein (Zhang and Lynd 2004). Cellulose is a linear polymer consisting of D-anhydroglucopyranose connected by β-1,4-glycosidic bonds. Distinct from cellulose, hemicellulose is a polymeric carbohydrate with some branching, composed of pentoses and hexoses. The complete separation of hemicellulose and cellulose with full carbohydrate recoveries is still a nearly impossible task because of the complicated linkage among cellulose, hemicellulose, and lignin.

It is still a technical challenge to quantitatively measure polymeric saccharides in lignocellulosic materials directly, but it is easy to convert polymeric carbohydrates (polysaccharides) in lignocellulose to monomeric sugars, which can be quantitatively measured by a number of sugar assays, HPLC, or GC. In 1945, Saeman and his coworkers (Saeman et al. 1945) at the U.S. Department of Agriculture Forest Products Laboratories developed an analytical protocol, called "Quantitative Saccharification (QS) of Wood and Cellulose". The method involved a primary hydrolysis with 72% w/w sulfuric acid at 30^{o}C for 45 minutes, which converted polysaccharides to oligosaccharides, followed by a secondary hydrolysis (4% w/w sulfuric acid) at 121^{o}C for 1 hour, which converted oligosaccharides to monomeric sugars. The total reducing sugars after neutralization by $CaCO_3$ were measured by the Somogyi method (Shaffer and Somogyi 1933; Zhang et al. 2006b).

Quantitative saccharification is a widely used method for determination of carbohydrate contents in lignocellulosic materials. Accurate determination of carbohydrate composition is important for a variety of fields, including emerging biofuels and biobased products industries, paper and pulping industries, as well as agriculture and forest product industries (Bjarnestad and Dahlman 2002; Schultz et al. 1985).

With the invention and wide applications of HPLC, researchers at the National Renewable Energy Laboratory (NREL) published the first QS (Method 1) for lignocellulosic materials in 1996 (Figure 2-1). The basic idea of the NREL (LAP-002) Determination of Carbohydrates in Biomass by High performance Liquid Chromatography was similar to that of Saeman *et al.* (1945). The major modifications were i) using 3 mL of 72% sulfuric acid for the primary hydrolysis for 2 hours rather than 45 minutes, and ii) measuring monomeric saccharides by HPLC with a carbohydrate column (e.g., Bio-Rad Aminex HPX-87P). The acid hydrolysis conditions are harsh, resulting in some carbohydrate degradation. Therefore, the amounts of the real polysaccharides after the conversion via the correction coefficients ($\eta_{Pi}^{1,total}$) are calculated as below,

$$P_i = \frac{87(C_i)}{\eta_{Pi}^{1,total}} \frac{MW_{Pi}}{MW_{Mi}} \qquad [1]$$

in which

i = Sugar type for polysaccharides or monomeric sugars:

G, glucose or glucan,

M, mannose or mannan,

Gal, galactose or galactan,

X, xylose or xylan,

A, arabinose or arabinan

P = Polysaccharide:

O for oligosaccharides,

M for monomeric sugar,

D for degraded sugars

C_i = Monomeric sugar concentration measured by HPLC (mg/mL)

$\eta_{Pi}^{1,total}$ = Overall correction coefficient for polysaccharides ($\eta_{Pi}^{1,1} * \eta_{Pi}^{1,2}$)

$\eta_{Pi}^{1,1}$ = Correction coefficient for polysaccharides to oligosaccharides during the primary hydrolysis

$\eta_{Pi}^{1,2}$ = Correction coefficient for oligosaccharides to monosaccharides during the secondary hydrolysis

MW = Molecular weights for hexose and pentose polysaccharides (162 g/mol or 132 g/mol), respectively; for monomeric hexose and pentose (180 g/mol or 150 g/mol), respectively.

However it is nearly impossible to estimate the $\eta_{Pi}^{1,total}$ value. The NREL suggested that measurement of $\eta_{Mi}^{1,total}$ value for each monomeric sugar as the control set. Here $\eta_{Mi}^{1,total}$ was measured by

$$\eta_{Mi}^{1,total} = \eta_{Mi}^{1,1}(\eta_{Mi}^{1,2}) = \frac{C_{Mi}^{1,1}}{C_{Mi}^{0}}(\frac{C_{Mi}^{1,2}}{C_{Mi}^{1,1}}) = \frac{C_{Mi}^{1,2}}{C_{Mi}^{0}} \quad [2]$$

in which

$\eta_{Mi}^{1,1}$ = Correction coefficient for monosaccharide (M_1) in the primary hydrolysis

$\eta_{Mi}^{1,2}$ = Correction coefficient for monosaccharide (M_2) in the primary hydrolysis

C_{Mi}^{0} = Initial monomeric sugar concentration (mg/g)

$C_{Mi}^{1,1}$ = Monomeric sugar concentration after the primary hydrolysis (mg/g),

$C_{Mi}^{1,2}$ = Monomeric sugar concentration after the primary and secondary hydrolysis (mg/g).

The above correction method could suffer from theoretical error because $\eta_{Pi}^{1,total}$ is much less than, $\eta_{Mi}^{1,total}$. If the difference between $\eta_{Pi}^{1,total}$ and $\eta_{Mi}^{1,total}$ is significant it could result in theoretical error.

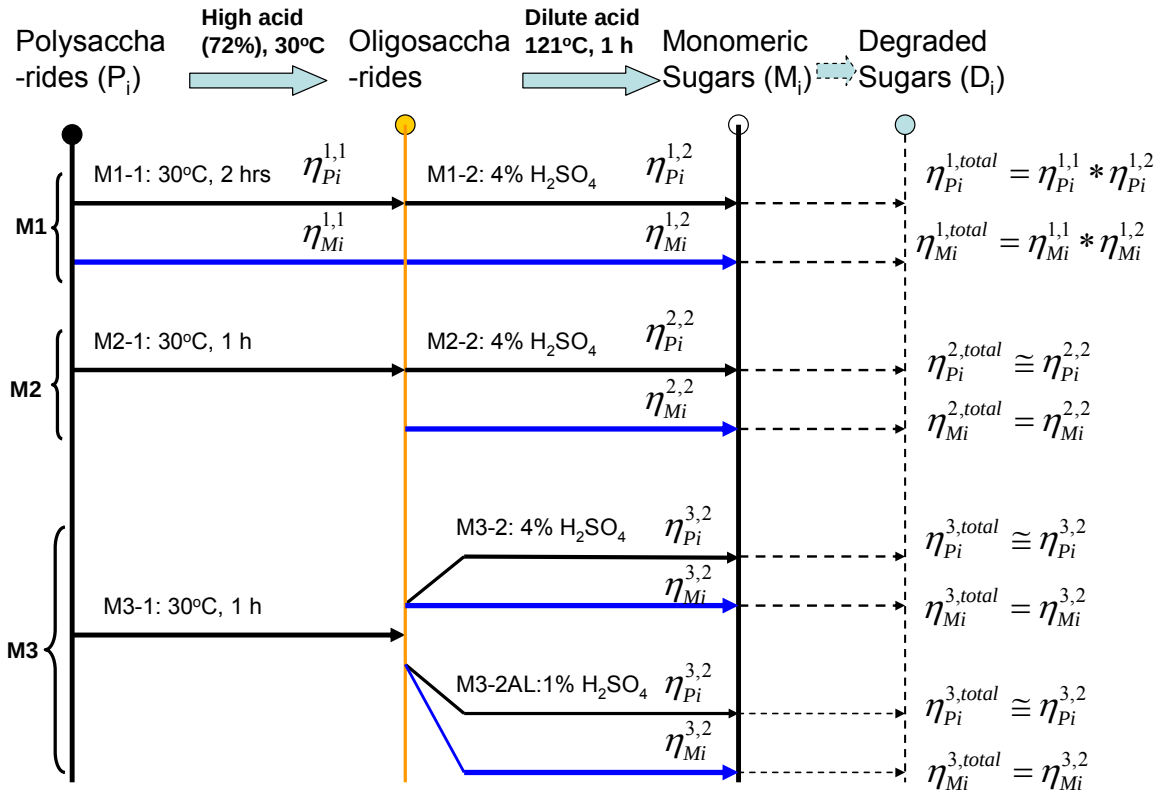

Figure 2-1 Comparison of three quantitative saccharification methods for determination of carbohydrates in lignocellulose and the proposed modified Methods good for more accurate determination of acid-labile carbohydrate contents.

Recently, researchers at NREL (Sluiter et al. 2006a) released an updated analytical protocol (LAP-002, Version 2006) with the modifications (Figure 2-1). They suggested i) to shorten the primary hydrolysis time from 2 hours to 1 hour for decreasing sugar degradation and ii) to measure the correction coefficients of the controls -- monomeric sugars during the secondary hydrolysis rather than those for both primary and secondary hydrolysis because during the primary hydrolysis stage only a small fractionation of

polysaccharides were converted to monomeric sugars and even much smaller monomeric sugars were degraded (i.e., $\eta_{Pi}^{2,1} = 1$) at the shorter primary hydrolysis. Therefore, $\eta_{Pi}^{2,total} = \eta_{Pi}^{2,2}$.

Regardless of the method (Method 1 or Method 2), the sugar degradation rates of monomeric sugars ($\eta_{Mi}^{1,total}$ or $\eta_{Mi}^{2,2}$) were greater than those of polysaccharides ($\eta_{Pi}^{1,total}$ or $\eta_{Oi}^{2,2}$), especially for acid-labile sugars – xylan and arabinan (Lloyd and Wyman 2003; Lloyd and Wyman 2005). Lower values of correction coefficients of acid labile polysaccharides might lead to over-estimation of acid-labile carbohydrate contents. In this study, we investigated the degradation rates of monomeric sugars at different acid concentration at the primary and secondary hydrolysis. We propose to slightly modify NREL procedure LAP-002 (Method 2) for more accurate determination of carbohydrate concentration, especially for acid-labile carbohydrates. The analysis suggested that NREL LAP-002 (2006 protocol) leads to a statistically significant over-estimation of acid-labile xylan contents as compared to our method (Method 3), and the degree of over-estimation depends on the plant species.

2.2 METHODS AND MATERIALS

Chemicals and Materials. All chemicals were reagent grade and purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Atlanta, GA) unless otherwise noted. Corn stover, switchgrass, wheat straw, hybrid poplar, and Douglas fir samples were graciously provided by James McMillan at the National Renewable Energy Laboratory

(NREL, Golden, CO). All lignocellulosic materials were knife-milled and screened. The lignocellulose particles smaller than 40 mesh screen and larger than 60 mesh screen were used for quantitative saccharification determination of structural carbohydrates. The lignocellulose samples were dried in a convection oven at 105 ± 3 °C for 4 hours or longer until a constant weight was achieved. The samples were then removed from the oven and allowed to cool to room temperature in a desiccator with silica gel dessicant.

Sugar Assays. Monomeric sugars were measured by Shimadzu HPLC (Kyoto, Japan) equipped with Bio-Rad Aminex HPX-87P column and the corresponding guard column. Ten μL injection volume was used; 18.2 MΩ deionized water was used a mobile phase at a rate of 0.6 mL/min; the column temperature was 80 °C; a refractive index detector was used as the detector; the run time was 35 minutes.

QS Protocols

Three different methods for determination of carbohydrates in lignocellulose were preformed on five samples of lignocellulosic material, switchgrass, wheat straw, corn stover, hybrid poplar, and Douglas fir. Three hundred ± 10.0 mg of lignocellulose sample was weighted and put into a test tube. One set of the controls -- monomeric sugars, including glucose, xylose, galactose, arabinose, and mannose -- was prepared to estimate sugar losses during hydrolysis. To the sample, 3.00 ± 0.01 mL of 72% (w/w) sulfuric acid was added and well-mixed with a glass stir rod.

Method 1. The pressure tubes were placed in a water bath set at 30 ± 1 °C for 120 ± 1 minutes, and agitated at 120 rpm. Proper mixing was essential to ensure even acid-to-particle contact and uniform hydrolysis. After primary hydrolysis, the samples were diluted to 4% sulfuric acid to the serum bottles by adding 84 mL of distilled water. All pressure tubes were then capped and sealed. The sealed pressure tubes were autoclaved for 1 h at 121°C. The monomeric sugar control group was run from through both primary hydrolysis and secondary hydrolysis. The hydrolysis liquor to be analyzed was neutralized to a pH of ~6 by adding calcium carbonate. Upon reaching a pH of 6, the sample was allowed to settle and the supernatant decanted from the solids. The supernatant was then filtered through a 0.45 μm filter, and frozen. The sample was then thawed, and 1.0 mL of sample was transferred to an HPLC vial. The residual sugars measured by HPLC were measured for by calculating $\eta_{Mi}^{1,total} = \frac{C_{Mi}^{1,2}}{C_{Mi}^{0}}$. Here it was assumed that $\eta_{Mi}^{1,total} = \eta_{Pi}^{1,total}$. Therefore, polysaccharide contents in lignocellulose cannot be calculated by Equation (1).

Method 2. The pressure tubes were placed in a water bath set at 30 ± 1 °C for 60 ± 1 minutes and agitated at 120 rpm. Upon completion of the first hydrolysis, the tubes were placed into an ice-cold water bath. The samples were diluted to 4% sulfuric acid by adding 84 mL of distilled water. All pressure tubes were then capped and sealed. Another set of the controls using the same monomeric sugars were prepared as a control for Method 2 as above, but prepared in concentrations similar to those expected in the samples. The controls were acidified to 87 mL of 4% sulfuric acid. All samples and

controls were autoclaved for 1 hour at 121 °C. After completion of the autoclave cycle, samples were allowed to slowly cool to room temperature. The hydrolysis liquor to be analyzed was neutralized to a pH of ~6 by adding calcium carbonate. Upon reaching a pH of 6, the sample was allowed to settle and the supernatant decanted from the solids. The supernatant was then filtered through a 0.45 μm filter, and frozen. The sample was then thawed, and 1.0 mL of sample was transferred to an HPLC vial. The residual control sugars measured by HPLC were measured for by calculating $\eta_{Mi}^{2,total} = \eta_{Mi}^{2,2}$. Here it was assumed that $\eta_{Mi}^{2,total} = \eta_{Pi}^{2,total}$. Therefore, the polysaccharide contents in lignocellulose cannot be calculated by Equation (1).

Method 3. Solutions of monomeric sugars glucose, galactose, mannose, xylose, and arabinose were prepared in concentrations between 4.0 mg/ml and 0.1 mg/mL as sugar recovery standards (SRS), along with a calibration verification standard (CVS), prepared from sugars from a different lot than those used in the SRS. The samples after the primary hydrolysis (72% w/w sulfuric acid at 30^{o}C for 1 hour) were diluted to 4% by adding 84 mL of distilled water. After well mixing, one mL of the supernatant hydrolysate was withdrawn into a tube, and then diluted to 1% sulfuric acid by adding 3 mL of water. Concentrations of the controls (glucose, mannose, and galactose, 4% sulfuric acid; xylose and arabinose at 1% sulfuric acid) were prepared at levels similar to the expected sugars released in the samples during the secondary hydrolysis (121^{o}C for 1 hour). The hydrolysis liquor that was analyzed was neutralized to a pH of ~6 by adding calcium carbonate. Upon reaching a pH of 6, the sample was allowed to settle and the supernatant decanted from the solids. The supernatant was then filtered through a 0.45

μm filter, and frozen to remove any particulate. The sample was then thawed, and 1.0 mL of sample was transferred to an HPLC vial.

Lignin Determination. Lignocellulose samples were analyzed for acid-insoluble lignin by vacuum filtering the autoclaved hydrolysis solution (87 mL or 84 mL of hydrolysate) though a filtering crucible of known weight, and the filtrate captured in a filtering flask. An aliquot of 50 mL of sample was removed from the flask for determination of acid-soluble lignin and carbohydrates. The remaining solids in the pressure tube were quantitatively transferred into the filtering crucible with deionized water. The solids were then rinsed with 50 mL of fresh deionized water. The wet solids were weighed to the nearest 0.01 g for determination of acid-insoluble lignins and ash content. The crucible and acid-insoluble resins were dried in a convection oven at 105 ± 3°C for 12 hours, such that a constant weight was achieved. The samples were then removed from the oven and allowed to cool to room temperature in a desiccator. The samples were then re-weighed for determination of ash content. The crucibles and residue were then ashed in a muffle furnace at 575 ± 25°C for 24 hours. After this time, the crucibles were removed from the furnace and allowed to cool to room temperature in a desiccator. The acid-soluble lignin was measured by a UV-Visible spectrophotometer, with a background of deionized water. The absorbance of the hydrolysis liquor was measured at a wavelength of 240 nm.

Monomeric Sugar Degradation. Known amounts of the different sugars were dissolved in 4%, 3%, 2%, and 1% sulfuric acid, and sealed in pressure vials at 121°C for 1 hour or

longer to measure the sugar degradation during weak acid hydrolysis. After the reaction these samples were prepared and analyzed by HPLC.

Degradation of monomeric sugars during the strong acid hydrolysis phase was measured in two steps. A monomer sugar solution of known concentration was prepared in 72% ice-cold sulfuric acid. Three mL aliquots were transferred to the test tubes and placed in water baths set to 30°C, 40°C, 50°C, 60°C, and 70°C for a period of 1 hour or longer, at which point samples were immediately placed in another ice bath to stop degradation. Sugars can form sugar sulfate at high acid concentration and this molecule cannot be detected by HPLC. An extra step – acid dilution (1% dilute sulfuric acid, 121^{o}C, 1 hour) was needed to convert sugar sulfate to sugar and dilute sulfuric acid before HPLC assay. Sugar degradation at high acid concentration at low temperatures was estimated by the total sugar degradation minus the sugar degradation at the dilute acid conditions.

Preparation of Cellodextrins and Xylooligosaccharides. Cellodextrins were prepared from Avicel by mixed acid hydrolysis; the cellodextrin components were separated by size-exclusion chromatography as described before (Zhang and Lynd 2003). Xylooligosaccharides was hydrolyzed from Sigma xylans by hot water treatment (Li et al. 2003).

2.3 RESULTS

2.3.1. Monomeric sugar degradation at low acid concentration

Figure 2-2 shows the degradation rate constant as a function of acid concentration for five monomeric sugars (glucose, xylose, galactose, arabinose, and xylose) at various sulfuric acid concentrations from 1 to 4% w/w and 121^{o}C. The monomeric sugar degradation follows first order kinetics

$$r_{Mi} = \frac{dM_i}{dt} - k_{Mi} * M_i \qquad [3]$$

where,

k_{Mi} = Degradation rate constant for monomic sugar M_i (h^{-1})

At 4% wt/wt sulfuric acid concentration, glucose degraded at a rate of 0.02 h^{-1}, whereas xylose degraded at a rate of 0.14 h^{-1}. At 1% w/w sulfuric acid concentration, the xylose degradation constant decreased to 0.03 h^{-1}, and that of glucose decreased to 0.01/h. The degradation trend of monomeric sugars were xylose > arabinose > mannose > galactose > glucose, which was in a good agreement with Saeman's results (Saeman 1945). Five-carbon sugars, xylose and arabinose, are more acid-labile than six-carbon sugars such as glucose, and are much more subject to degradation with changes in acid concentration.

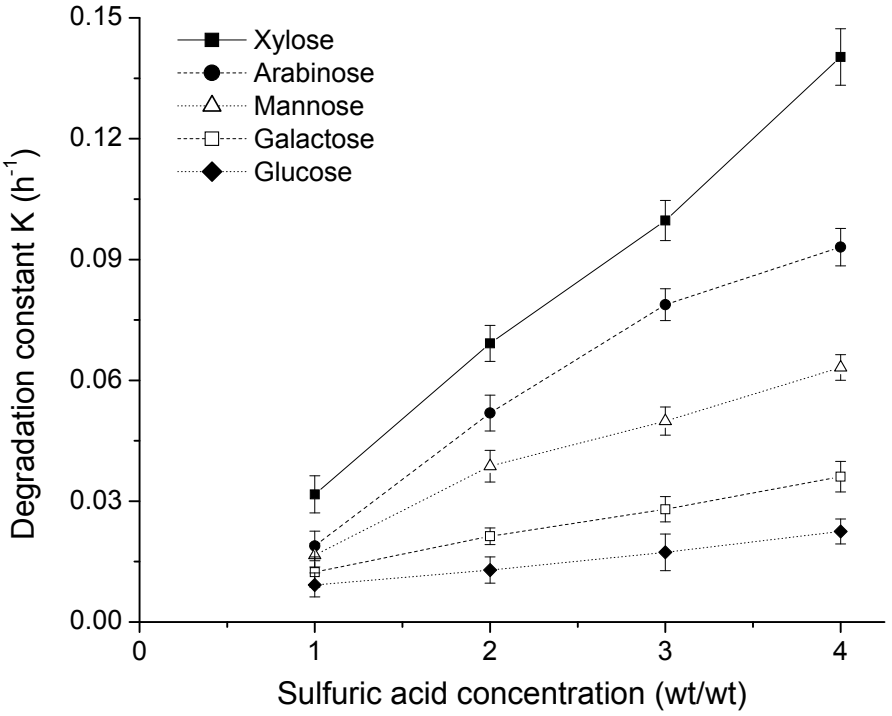

Figure 2-2. Monomer sugar degradation rate constants for different sulfuric acid concentrations at 121°C.

2.3.2. Monomeric sugar degradation at high acid concentration

Figure 2-3 shows the sugar degradation rates at high acid concentration (72% w/w) at different temperatures from 30 to 70°C. Clearly, the sugar degradation is very sensitive to temperature. At 30°C, actual degradation rate constants of monosaccharide sugars are small compared to those at dilute acid and high temperature. Comparison of sugar degradation rates for the primary and secondary hydrolysis clearly suggests that most of sugar degradation occurs at the secondary hydrolysis rate, although it was observed that much stronger dark colors for each sugar were formed in the primary hydrolysis step.

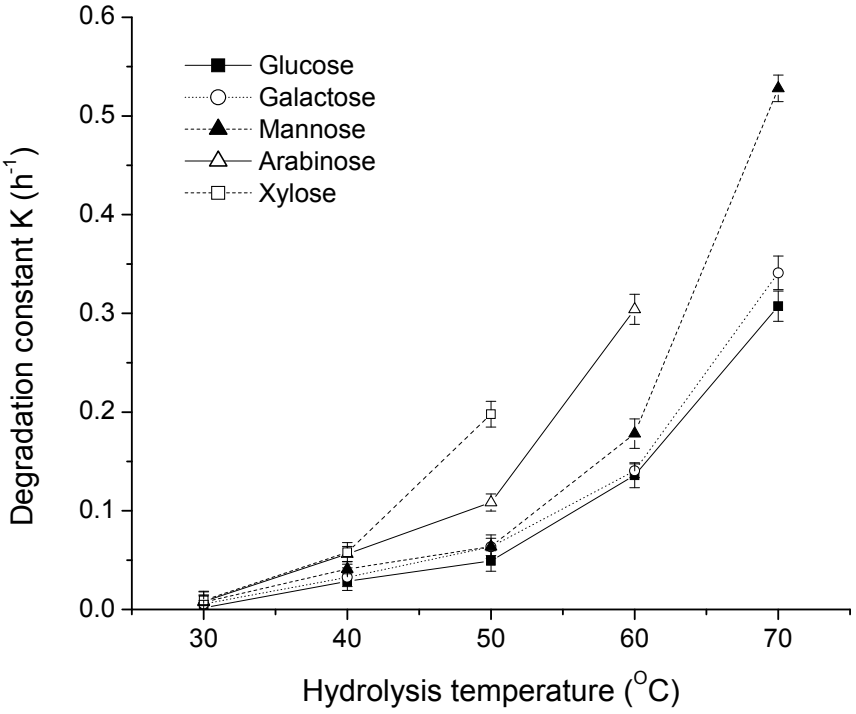

Figure 2-3. Monomer sugar degradation rate at various reaction temperatures in the presence of 72% sulfuric acid.

In Figure 2-3, data for xylose degradation constant above 50°C and arabinose degradation constant above 60°C was not able to be collected. During the experiment the xylose and arabinose degradation products polymerized, at which point it became impossible to quantitatively transfer all the remaining sugars from the sample vial. This results in a very high level of error using this data in conjunction with the Arrhenius equation to calculate the activation energy of xylose and arabinose, since the best fit line is drawn to only 3 or 4 data points, respectively.

The Arrhenius equation is the standard method of quantifying the temperature dependency of hydrolysis rates given by the form

$$k = k^0 e^{-E_a / RT} \quad [4]$$

where,

k = kinetic constant (h^{-1}),

k^0 = pre-exponential factor (h^{-1}),

E_a = activation energy (kJ/mol),

R = universal gas constant (kJ/mol/K), and

T = temperature (K).

The measured degradation rate constants were fit to this equation to find the corresponding activation energy of the monomer sugars, shown in Figure 2-4. From the linearization attained by plotting the natural log of the degradation rate vs the inverse of the temperature, a best fit trendline was fitted to each of the curves. The slope of the trend line was set to the pre-exponential factor, k^0, and the activation energy, E_a was calculated. The measured activation energy of the monomer sugars fit well to previous literature of sugar degradation in sulfuric acid; measured activation energy of glucose was 107.13 kJ/gmol where Mosier *et al.* (2002) measured the activation energy of glucose to be 118 ± 37.5 kJ/gmol in sulfuric acid. Similarly, the activation energy of xylose was measured to be 124.63 kJ/gmol, close to the result (134 kJ/mol) of Bhandari *et al.* (1984).

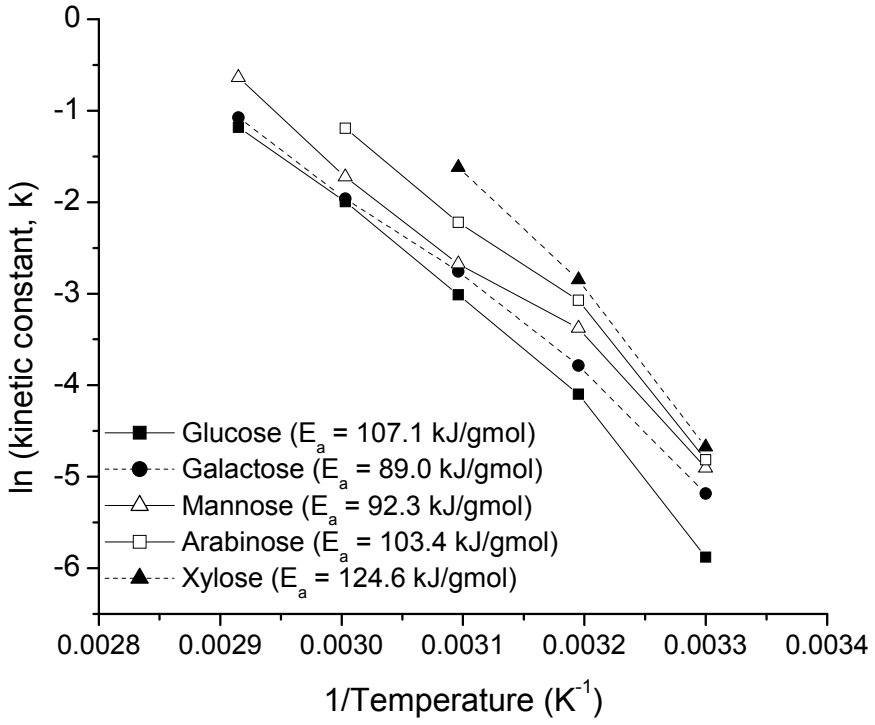

Figure 2-4 Arrhenius equation with degradation constants measured from strong acid hydrolysis and calculation of activation energy of monomeric sugars in sulfuric acid.

2.3.3. Oligosaccharide hydrolysis at low acid concentration

Previously, Zhang investigated the effects of acid concentrations (from 1 to 4 % sulfuric acid) and autoclave time on secondary hydrolysis of cellodextrins (cellooligosaccharides) and xylooligosaccharides with the degree of polymerization (DP) from 2 to > 10 (data not shown). For cellodextrins, complete hydrolysis of cellobiose requires at least 2% sulfuric acid and 60 minutes of autoclave time. Longer cellodextrins, e.g., G_{10} (DP = 10), which was prepared as described previously (Zhang and Lynd 2003), required higher acid concentrations (4% sulfuric acid) for > 40 minutes. Hydrolysis of xylooligosaccharides,

whose xylose units are linked by β-1,4-glycosidic acid required modest reaction conditions. It was found that even very long oligosaccharides were completely hydrolyzed to monomeric xylose under that condition (1% sulfuric acid and 60 minutes at 121^{o}C).

2.3.4. Overall degradation of monomeric sugars

We investigated the reaction condition effects on monomeric sugar degradation as below:

Condition 1 (primary hydrolysis by using 72% sulfuric acid and secondary hydrolysis by using 4% sulfuric acid),

Condition 2 (only secondary hydrolysis by using 4% sulfuric acid), and

Condition 3 (only secondary hydrolysis by using 1% sulfuric acid).

The sugar degradations for five monomeric sugars at different conditions are presented in Figure 2-4. Clearly, the majority of sugar degradation happened at the secondary hydrolysis process. Acid-labile sugars, such as xylose and arabinose, are more subject to acid concentration at the secondary hydrolysis. If we can decrease acid concentration from 4% to 1% at the secondary acid hydrolysis, it can significantly reduce the degradation of acid-labile polysaccharides (xylan and arabinan) so to obtain more accurate acid-labile carbohydrate concentrations because the difference $\left[\eta_{Mi}^{3,total} - \eta_{Pi}^{3,total}\right]$ is much smaller than the difference between $\left[\eta_{Mi}^{2,total} - \eta_{Pi}^{2,total}\right]$. Note: $\left[\eta_{Mi}^{total} - \eta_{Pi}^{total}\right] = 0$ means no difference in sugar degradation between the monomeric sugar and the polymeric sugar. Table 2-1 shows the correction coefficients for five monomeric sugars at different conditions.

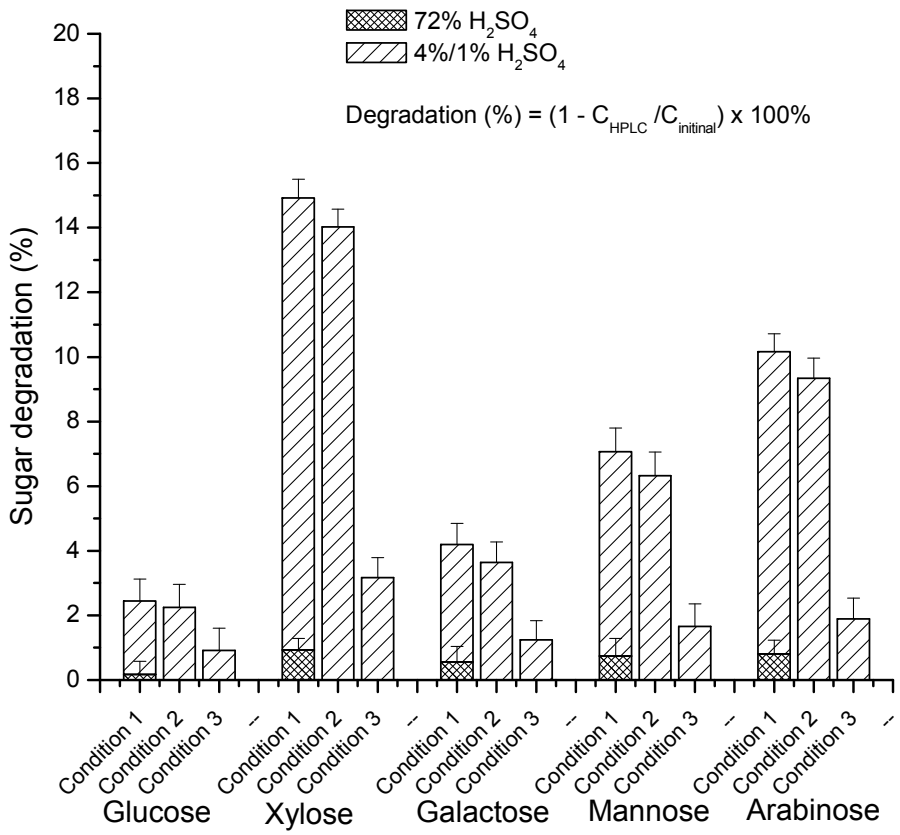

Figure 2-5. Comparison of monomeric sugar degradation between hydrolysis conditions in Condition 1, Condition 2, and Condition 3.

Table 2-1. Comparison of the correction coefficients (η_{Mi}^{total}) for various monomeric sugars at different conditions.

Sugar	Condition 1 (72% H_2SO_4, 2 hours, 30°C; 4% H_2SO_4, 1 hour, 121°C)	Condition 2 (4% H_2SO_4, 1 hour, 121°C)	Condition 3 (1% H_2SO_4, 1 hour, 121°C)
Glucose	0.974	0.978	0.991
Xylose	0.841	0.860	0.963
Galactose	0.953	0.964	0.988
Mannose	0.922	0.937	0.983
Arabinose	0.891	0.907	0.981

Table 2-2 presents the carbohydrate contents, total acid-soluble (AS) lignin, acid-insoluble (AI) lignin, and ash for switchgrass, corn stover, poplar, wheat straw, and Douglas fir. Glucan contents cannot be applicable at 1% H_2SO_4 hydrolysis because acid hydrolysis cannot convert all cello-oligo-saccharides to glucose, whereas other polysaccharides can be converted to monomeric units. Most values of sugar contents based on Method 1 are a little larger than those based on Method 2, but they are not significantly different as determined by Tukey-Kramer Honest Significant Difference shown in Table 2-3. The slightly lower deviated values for all carbohydrates based on Method 2 are expected because the value of $\eta_{Mi}^{2,total}$ is slightly higher than $\eta_{Mi}^{1,total}$.

We found more theoretical deviation for all hemicellulose contents based on Condition 3 as compared to those based on Condition 2. As shown in Table 2-3, statistical analysis clearly indicated that there are significant differences with $\alpha = 0.01$ level of significance

for acid labile xylans based on Condition 2 (Method 2) and Condition 3, suggesting that xylan contents based on Method 2 were overestimated, ranging between 4.2% overestimation and 9.1% overestimation of xylan sugars. They are 9.1% for Douglas fir, 8.1% for hybrid poplar, 4.2% for switchgrass, 8.5% for corn stover, and 8.3% for wheat straw. In general the herbaceous biomass samples were more consistent with estimations from current protocols, and the woody biomass samples deviated more. It could be attributed to the smaller difference in sugar degradation in polysaccharides and monosaccharide for herbaceous materials than that in woody materials. Testing indicated that although arabinan content measured by Method 3 was generally lower than that measured by existing protocol, it was not found to be significant.

We propose the modified QS for determining carbohydrate composition in lignocellulosic materials. The protocol involves a primary hydrolysis (72% w/w sulfuric acid, 30^{o}C, 1 hour), followed by a secondary hydrolysis (4% w/w sulfuric acid , 121^{o}C, 1 hour, for glucose, galactose and mannose) and another parallel secondary hydrolysis (1% w/w sulfuric acid , 121^{o}C, 1 hour, for xylose and arabinose). There is an increased need for the development of rapid, high-throughput analytical methods for the estimation of carbohydrates in lignocellulose, which will require a very accurate analytical method for calibration. In the above said cases, even a relatively minor overestimation of xylans of 8% will greatly affect the development of calibration methods, and will result in overall error in yields and throughput. It has been shown that by simply dividing the hydrolysis sample and optimizing hydrolysis conditions for various monomer sugars, a more accurate determination of the acid labile sugars can be found.

Table 2-2. Compositional analysis of lignocellulose feedstock by quantitative saccharification with 1996 NREL protocol (Method 1), 2006 revision to NREL protocol (Method 2), and the proposed revision to protocol for determination of acid-labile sugars (Method 3).

	Method	No	Glucan (mg/g)		Xylan (mg/g)		Galactan (mg/g)		Mannan (mg/g)		Arabinan (mg/g)		AS Lignin (mg/g)	AI Lignin (mg/g)	Ash (mg/g)
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	Mean	Mean
Switchgrass															
	Condition 1	5	336.1	± 1.2	218.4	± 1.3	10.4	± 0.7	7.7	± 0.3	37.6	± 0.6	72.4	118.5	63.2
	Condition 2	5	334.3	± 1.3	215.0	± 1.7	10.6	± 0.5	7.6	± 0.6	35.0	± 0.7	69.7	121.9	58.3
	Condition 3	5	NA		206.3	± 1.6	10.2	± 0.5	7.5	± 0.7	34.1	± 0.5	67.1	124.0	61.6
Corn stover															
	Condition 1	5	413.1	± 1.8	234.4	± 1.1	20.4	± 1.0	8.9	± 0.9	26.0	± 1.8	69.7	113.4	62.9
	Condition 2	5	409.0	± 1.9	230.6	± 1.1	18.8	± 0.9	9.6	± 0.9	23.6	± 1.4	66.0	121.8	65.2
	Condition 3	5	NA		212.6	± 1.6	17.8	± 0.9	8.6	± 1.3	23.1	± 1.6	70.2	115.1	64.6
Wheat straw															
	Condition 1	5	403.3	± 1.9	222.0	± 1.8	10.0	± 0.6	7.1	± 0.8	34.2	± 0.9	80.1	162.7	86.5
	Condition 2	5	398.5	± 2.1	215.0	± 1.3	9.8	± 0.7	7.9	± 0.8	29.2	± 0.8	78.3	167.4	82.1
	Condition 3	5	NA		198.5	± 1.5	10.3	± 0.9	7.2	± 0.5	28.5	± 1.0	81.6	164.2	95.8
Hybrid poplar															
	Condition 1	5	491.9	± 1.9	152.3	± 0.8	12.0	± 0.9	9.1	± 1.2	14.9	± 1.2	83.8	165.6	17.1
	Condition 2	5	490.7	± 1.8	149.1	± 1.3	10.5	± 0.7	9.4	± 1.3	14.5	± 1.3	79.4	171.7	19.3
	Condition 3	5	NA		137.9	± 1.6	10.8	± 1.1	9.5	± 1.4	14.2	± 1.0	77.0	173.4	18.5
Douglas fir															
	Condition 1	5	476.5	± 1.6	99.1	± 3.0	28.9	± 0.9	127.9	± 1.0	19.0	± 1.3	103.3	184.8	36.5
	Condition 2	5	471.6	± 1.4	94.4	± 1.4	27.7	± 1.6	125.2	± 1.9	18.2	± 1.1	97.6	180.2	37.9
	Condition 3	5	NA		86.5	± 1.5	28.4	± 1.4	120.7	± 1.4	17.8	± 1.1	93.1	187.0	39.4

NA: Not applicable.

Table 2-3. The statistical t-values for the comparison of Method 1 *vs.* Method 2 by Tukey-Kramer HSD. Values greater than the threshold t-value of 2.67 ($\alpha = 0.01$ level of significance) denoted S. For this test there were no significant differences between methods.

t-value (Method 1 *vs.* Method 2)					
Feedstock	Glucan	Xylan	Galactan	Mannan	Arabinan
Switchgrass	0.90	1.54	0.22	0.12	2.11
Corn stover	1.65	2.05	1.14	0.47	1.03
Wheat straw	1.20	1.20	0.18	0.76	1.61
Hybrid poplar	0.48	2.09	1.15	0.17	0.27
Douglas fir	2.10	0.37	0.64	1.29	0.46

Table 2-4. The statistical t-values for the comparison of Method 2 *vs.* Method 3 by Tukey-Kramer HSD. Values greater than the threshold t-value of 2.67 ($\alpha = 0.01$ level of significance) denoted S. Glucans, Galactans, and Mannans were measured with Condition 2, whereas xylans and arabinans were measured by Condition 3.

t-value (Method 2 *vs.* Method 3)					
Feedstock	Glucans	Xylans	Galactans	Mannans	Arabinans
Switchgrass	NA	3.98 (S)	0.47	0.12	1.24
Corn stover	NA	5.79 (S)	0.73	0.68	0.91
Wheat straw	NA	3.72 (S)	0.46	0.64	0.17
Hybrid poplar	NA	3.75 (S)	0.22	0.04	0.78
Douglas fir	NA	3.34 (S)	0.38	1.13	0.88

2.4 CONCLUSIONS

Current NREL 2006 laboratory procedure for determination of structural carbohydrates in lignocellulose leads to an over-estimate acid-labile sugar (xylan) concentration due to differences in sugar degradation rates between polysaccharides and monosaccharides. A more accurate determination of degradation of both the polysaccharide and monosaccharide can be realized using milder reaction conditions for the acid-labile sugars, i.e., xylans, while still completely hydrolyzing xylan oligosaccharides to xylose and decreasing the formation of sugar degradation products, such as furfural. The proposed changes to the weak acid hydrolysis procedure add only a small amount of complication to testing, but these changes can significantly improve accuracy of xylose content determination.

3. EVALUATION OF LIGNOCELLULOSE FRACTIONATION APPLICABLE TO INDUSTRIAL HEMP HURDS

Abstract

Industrial hemp is a valuable plant for its fiber and oil. The hurds are a cellulose-containing residue that is not fully utilized. Here we evaluate the feasibility of lignocellulose fractionation applicable to the hurds of industrial hemp. After optimizing acid concentration, reaction time, and temperature, the pretreated cellulosic samples were hydrolyzed more than 96% after 24 hours of hydrolysis (enzyme loading conditions of 15 FPU/g glucan Spezyme CP, 60 IU/g glucan Novozyme 188) at an optimal pretreatment condition ($\geq$ 84% H_3PO_4, $\geq$ 50 °C, and $\geq$ 1 hour). The overall glucose and xylose yields were 89% (94% pretreatment; 96% digestibility) and 61%, respectively. All data suggest the technical feasibility of building a biorefinery based on the feedstock, the hurds of industrial hemp, and lignocellulose fractionation for producing cellulosic ethanol because of high sugar yields, low processing costs, nearly free feedstock, and low capital investment. In addition, we must emphasize the opportunities to produce other higher-value products, such as lactic acid and acetic acid, via fermentation starting with glucose.

3.1 INTRODUCTION

Effective release of the carbohydrates in lignocellulose is one of the major hurdles in profitable cellulosic ethanol production. As discussed in Chapter 1, there are several methods overcoming the recalcitrance of cellulose to utilize the carbohydrates for hydrolysis and fermentation into ethanol. All lignocellulose conversion technologies

utilize a pretreatment process in preparation for enzymatic digestion. The aim of this research is to evaluate the feasibility of applying lignocellulose fractionation technology, developed at Virginia Tech (Zhang et al. 2007b), to the hurds of industrial hemp and characterize the effects of pretreatment reaction conditions on the hydrolysis of cellulose (i.e., sugar yields for ethanol fermentation).

The hemp plant is grown for its fibers, seed, seed meal, and seed oil. Hemp is a distinct variety of the plant species *Cannabis sativa.* Hemp is different from the narcotic drug marijuana, as hemp contains virtually no delta-9 tetrahydrocannabinol, which is the active ingredient in marijuana. The primary value product of industrial hemp is fibers that are present around the hollow, woody core of the hemp stalk. These long, strong fibers, which grow the length of the hemp stalk, are considered bast fibers. Hemp fiber possesses properties similar to other bast fibers (flax, kenaf, jute, and ramie) and excels in fiber length, strength, durability, absorbency, anti-mildew, and anti-microbial properties. Hemp oil from the seed is the richest known source of polyunsaturated essential fatty acids (81% vol/vol), including gamma linoleic acid (GLA). Core fibers (hurds) are derived from the sturdy, wood-like hollow stalk of the hemp plant. It is usually regarded as a waste although it has some applications, such as animal bedding, garden mulch, or a component for light-weight concrete or plaster.

Bioethanol is anticipated to replace a significant fraction of gasoline. This replacement will increase national energy security, improve the rural economy, and decrease environmental pollution (Zhang and Lynd 2004). Lignocellulosic biomass is the most abundant feedstock source for fermentable sugars that will be used to produce most

ethanol. Current cellulosic ethanol production costs cannot compete with that from corn grain because of great capital investment, high processing costs, and low revenues from the sole cellulosic ethanol (Zhang et al. 2006b). Production of cellulosic ethanol from the hurds of industrial hemp will be very promising because of the removal of cellulosic wastes, the generation of more revenues, and the reduction of greenhouse gas emissions. Integration of a cellulosic bioethanol biorefinery with current industrial hemp factories will provide the following benefits: securing a low-cost feedstock for cellulosic ethanol production and a significant savings in feedstock collection and transportation (e.g., $50-$60/Mg of biomass). The hurds of industrial hemp contain a moderately high glucan and xylan content (~38 % glucan, ~23% xylan), and low lignin content (~10.4% lignin) (Table 3.1), but a routine pretreatment such as steam explosion cannot efficiently break up its recalcitrant structure.

3.2 METHODS AND MATERIALS

Chemicals and Materials. All chemicals were reagent grade and purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Atlanta, GA), unless otherwise noted. Industrial hemp stalks were provided by The Equator Group (Johor, Malaysia), and these stalks were then stripped of fiber to retain only the hurds. The hurds were knife-milled and screened and are hereafter referred to as "lignocellulosic material". The lignocellulose particles smaller than 40 mesh screen and larger than 60 mesh screen were used for fractionation. The lignocellulose was dried in a convection oven at 105 ± 3 °C for 4 hours or longer until a constant weight was achieved. The samples were then removed from the oven and allowed to cool to room temperature in a desiccator.

The structural composition of industrial hemp stalks (*C. sativa*) was determined based on the NREL protocol "LAP-002 Determination of Structural Carbohydrates and Lignin in Biomass" (Sluiter et al. 2006). The procedure for determination of structural carbohydrates involves a two-step acid hydrolysis to convert polymeric sugars to monomeric sugars, which can be detected by HPLC. Lignin can be divided into an acid-soluble and an acid-insoluble form. The acid-soluble lignin is measured by UV-Vis spectroscopy, and the acid-insoluble material by gravimetric analysis.

A nominal amount of 1.0 g of dry industrial hemp hurds sample was weighted into a 50 mL sample vial. This sample would then undergo fractionation by various acid concentrations, reaction time, and temperature. After samples were treated with a fractionation pretreatment, 30 mL of acetone was added to each sample to precipitate amorphous cellulose in the sample. Each sample was vortexed or inversely shaken to ensure adequate mixing. After mixing, samples were centrifuged at 3600 RPM for 5 minutes. The supernatant was decanted from the samples and 2 additional acetone washes preformed on the samples in a manner identical to the previous. After the acetone wash, 30 mL of room temperature deionized water was added to each sample as a secondary wash procedure. Samples were once again centrifuged at 3600 RPM for 5 minutes, and the supernatant decanted from the sample. Hemicellulose is soluble in water, and the temperature of the deionized water may affect solubility of those sugars. Care was taken to use water at the same temperature for each wash procedure, but the effect of the water temperature was not examined at this point. After 3 deionized water

washes, the pH of the samples was tested with litmus paper to ensure that the pH of the samples was near 6.

Of the pretreated samples, one replicate was freeze dried overnight and put through quantitative saccharification to determine sugar content of the pretreated sample. Some sugar will be lost from the sample during the wash procedure and we wanted to quantify this loss. The remaining samples were prepared for enzymatic hydrolysis. Samples were prepared to a glucan concentration of 10 g/L. Glucan concentration of the samples was determined by quantitative saccharification. 1 M citrate buffer (pH = 4.5) was added to the sample so that the buffer would be diluted to 0.05 M. 0.5 mL of enzyme solution was added to the sample at a loading of 15 FPU for Spezyme CP and 60 IU for Novozyme 188. The samples were hydrolyzed at 50 °C for a total of 72 hours. Samples were taken from the hydrolysis liquor at 1, 2, 4, 8, 12, 24, 48, and 72 hours by pipetting out 800 μL of sample and immediately centrifuging the sample at 10,000 RPM for 5 minutes. After centrifuging, 500 mL of supernatant was decanted and allowed to sit at room temperature for 10 minutes to allow all remaining cellobiose to convert to glucose. The sample was then mixed with 30 μL of 10% sulfuric acid, and filtered though a 0.20 μm filter to prepare the sample for HPLC analysis. After 72 h of hydrolysis, the remaining hydrolysate was transferred to a 50 mL sample vial and 10 mL of deionized water added to the sample. The sample was centrifuged at 3600 RPM for 5 minutes, and the supernatant decanted. The sample was then washed with an additional 20 mL of deionized water, and centrifuged. The remaining solid particle matter was freeze dried, and then analyzed by quantitative saccharification.

The glucose samples from hydrolysis were analyzed by HPLC for sugars on an Aminex-87H chromatography column with de-ashing guard column. Samples were analyzed using 0.01 M sulfuric acid as a mobile phase at a flow rate of 0.6 mL per minute and a column temperature of 65°C (Zhang et al. 2007b; Zhang et al. 2007c).

Glucan digestibility was calculated by the ratio of the soluble glucose equivalent in the supernatant after hydrolysis to the sum of the soluble glucose and the residual insoluble glucose equivalent ($G_r = S_{g,QS} * V_{QS}$) in the solid phase after hydrolysis,

$$X = \frac{S_{g,H} * V_H}{S_{g,H} * V_H + S_{g,QS} * V_{QS}} \times 100\% \qquad [5]$$

Where,

G_r, amount of glucose equivalent in the residual solid phase (g GE),

$S_{g,QS}$, glucose concentration in the quantitative saccharification solution for the residual solid samples after hydrolysis (g/L), and

V_{QS}, the volume of the quantitative saccharification solution (L, e.g., 0.087 L) (Sluiter et al. 2006).

3.3 RESULTS

The experiments were conducted in two separate steps – 1) lignocellulose fractionation by sequentially using cellulose solvent, organic solvent, and water, and 2) enzymatic cellulose hydrolysis, which release glucose from the pretreated biomass, so that the efficiency of the pretreatment can be evaluated.

3.3.1. Composition Changes Before and After Fractionation

Table 3-1 presents the changes in sugar and lignin composition before and after fractionation pretreatment, analyzed by quantitative saccharification (QS) (Sluiter et al. 2006b). On average, 6.5% of the structural glucans were lost during the pretreatment washing, whereas about 30% of hemicellulose was removed. The more important result is lignocellulose fractionation can remove large amounts of lignin (> 80%). The efficient removal of lignin from cellulose is vital to promoting enzymatic cellulose digestibility and reducing enzyme use (Mosier et al. 2005b; Yang and Wyman 2006; Zhang et al. 2007b).

Table 3-1. Carbohydrates and lignin composition of the hurds of industrial hemp before and after lignocellulose fractionation.

Feedstock	Sample Number	Glucan (mg/g)	Xylan (mg/g)	Galactan (mg/g)	Arabinan (mg/g)	Mannan (mg/g)	Lignins (mg/g)	
							Acid-Insol.	Acid-Sol.
Before pretreatment	5	384.1	231.6	26.3	47.4	14.4	99.5	38.9
After pretreatment	3	359.0	160.7	18.4	30.9	8.8	14.5	7.7

3.3.2. Enzymatic Cellulose Digestibility

We evaluated the enzymatic cellulose digestibility for the pretreated materials at different reaction conditions – acid concentration, reaction time, and reaction temperature. Enzymatic cellulose hydrolysis conditions were 15 filter paper units cellulase and 60

international units of beta-glucosidase per gram of glucan at 50^{o}C in 50 mM citric acid buffer (pH 4.8). The released glucose in the supernatant was measured by HPLC.

Acid Concentration Effects

Figure 3-1 presents the effect of phosphoric acid concentrations (81%, 84%, and 85.9%) on cellulose digestibility for the pretreated lignocellulose. Our previous results suggested that high phosphoric acid concentration was important to disrupt the linkage among cellulose, hemicellulose, and lignin as well as dissolve cellulose completely (Zhang et al. 2006a; Zhang et al. 2007b). Both samples pretreated by 84.0% and 85.9% phosphoric acid were hydrolyzed to achieve a maximum 94 % digestibility whereas the samples treated by lower acid concentration had a lower digestibility of 91.2%. The crucial acid concentration was approximately 84.0%.

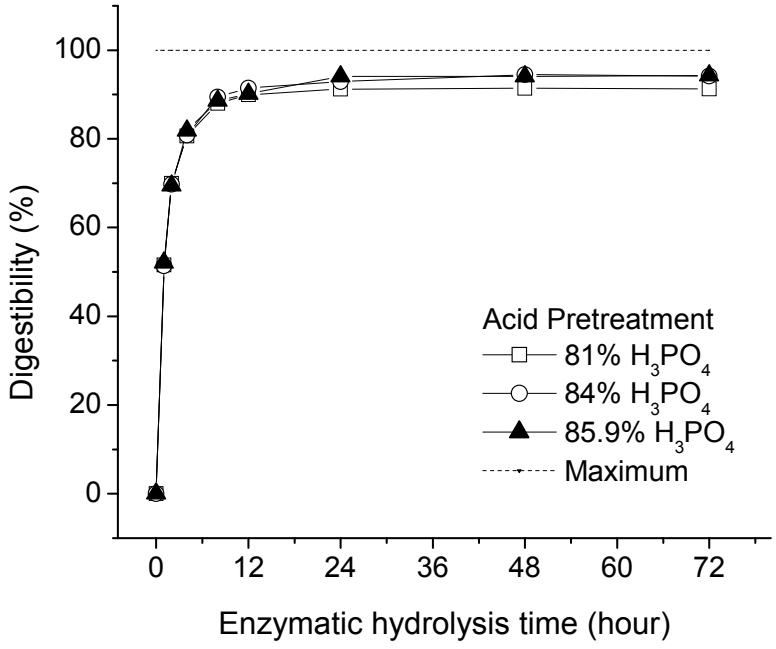

Figure 3-1. Effect of phosphoric acid concentration on cellulose digestibility of the pretreated lignocellulose. Experimental condition: 50^{o}C and 1 hour.

Reaction Time Effects

Figure 3-2 shows the effects of different reaction times from 30 minutes to 2 hours on the cellulose digestibility of the pretreated materials by 85.9 % (w/v) phosphoric acid. When the reaction time was longer than 1 hour, the digestibility of the pretreated samples was 93.6% after 24 hours of enzyme hydrolysis. But when the reaction time was shorter (e.g. 30 minutes), the digestibility after 24 hours was only 65 % of the theoretical yield. The reaction time was recommended to be an hour.

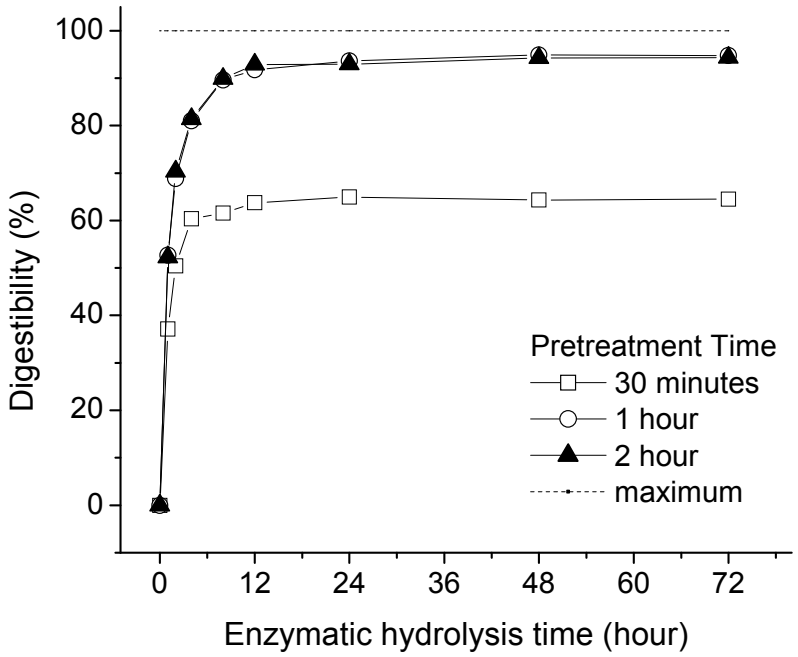

Figure 3-2. Effect of the pretreatment reaction time on cellulose digestibility of the pretreated lignocellulose. Experimental conditions: 85.9% phosphoric acid and 50^{o}C.

Reaction Temperature Effects

Figure 3-3 presents the effects of the pretreatment reaction temperatures on the digestibility of the pretreated materials. Both samples treated at 50°C and 60°C were able to reach the digestibility of 93.8 % and 94.8 %, respectively. The lower reaction temperature results in a little lower digestibility (90.9 %).

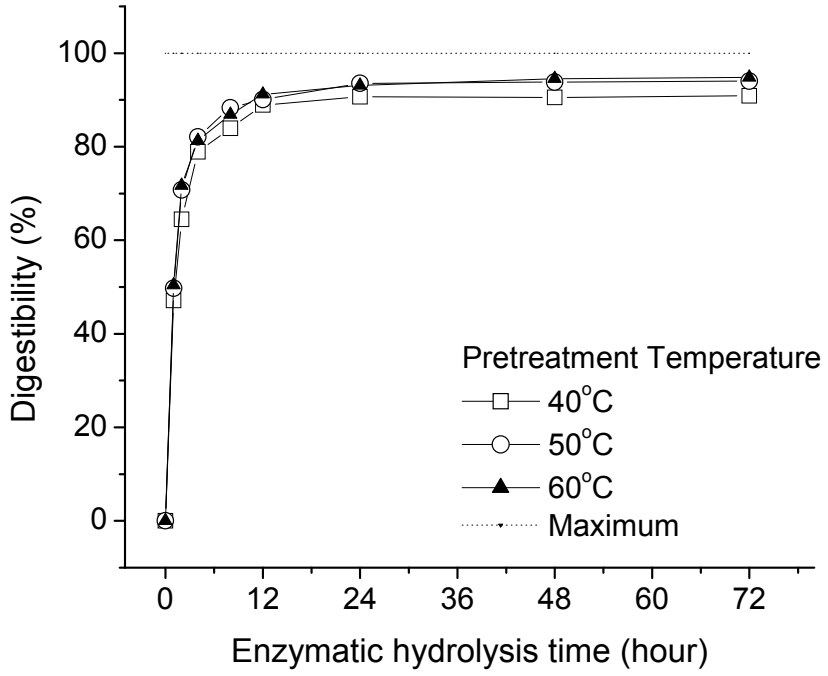

Figure 3-3. Effect of reaction temperature on cellulose digestibility for the pretreated lignocellulose. Experimental conditions: 85.9% phosphoric acid and reaction time = 1 hour.

3.3.3. Images of lignocellulosic structure after fractionation

After the optimal reaction conditions had been identified ($\geq$ 84% H_3PO_4, $\geq$ 50 °C and $\geq$ 1 hour) samples of lignocellulose treated by fractionation were examined by microscopy techniques. The first sample was treated with 84.0% H_3PO_4 at 50 °C for 1 hour, representing lignocellulosic material that had been treated with optimum fractionation conditions. The second sample was treated with 84.0 % H_3PO_4 at 50°C for 30 minutes. This sample was only partially treated by the fractionation pretreatment. The last sample was untreated, raw material. All samples were sputter coated with gold and imaged by a

Scanning Electron Microscope (SEM) at the Virginia-Maryland Regional College of Veterinary Medicine at the Virginia Tech main campus (Blacksburg, VA). The first column of Figure 3-4 are images of the untreated raw lignocellulosic material. SEM images clearly show the fibrous structure of the material. The second column of Figure 3-4, representing modestly-treated materials, illustrates that the overall structure of the lignocellulose cellulose is broken and large holes appear -- signs of partial disruption. The last column, representing well-treated lignocellulose samples, shows that the fibrous structure of the lignocellulose is completely disrupted. A comparison of the lignocellulose surface shows that the structures of the lignocellulose samples are drastically changed during the fractionation processes, depending on pretreatment severity. Crystalline cellulose microfibrils are disrupted, and a significant amount of hemicellulose is removed from the surface of the cellulose strands.

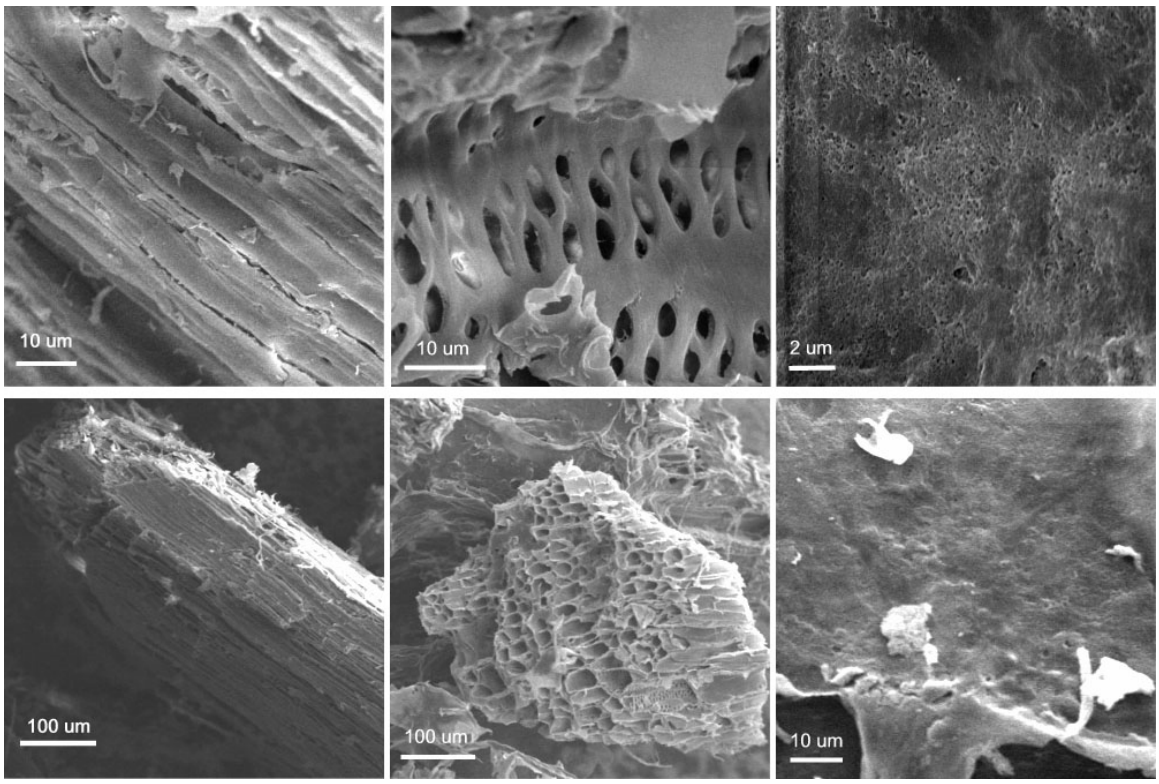

Figure 3-4. Scanning electron microscope images of lignocellulose samples before and after lignocellulose fractionation pretreatment, with modest and complete fractionation pretreatments.

Figure 3-5 also compares two images taken with the SEM, but are taken at relatively low resolutions to illustrate how the crystalline structure of cellulose is disrupted after fractionation pretreatment.

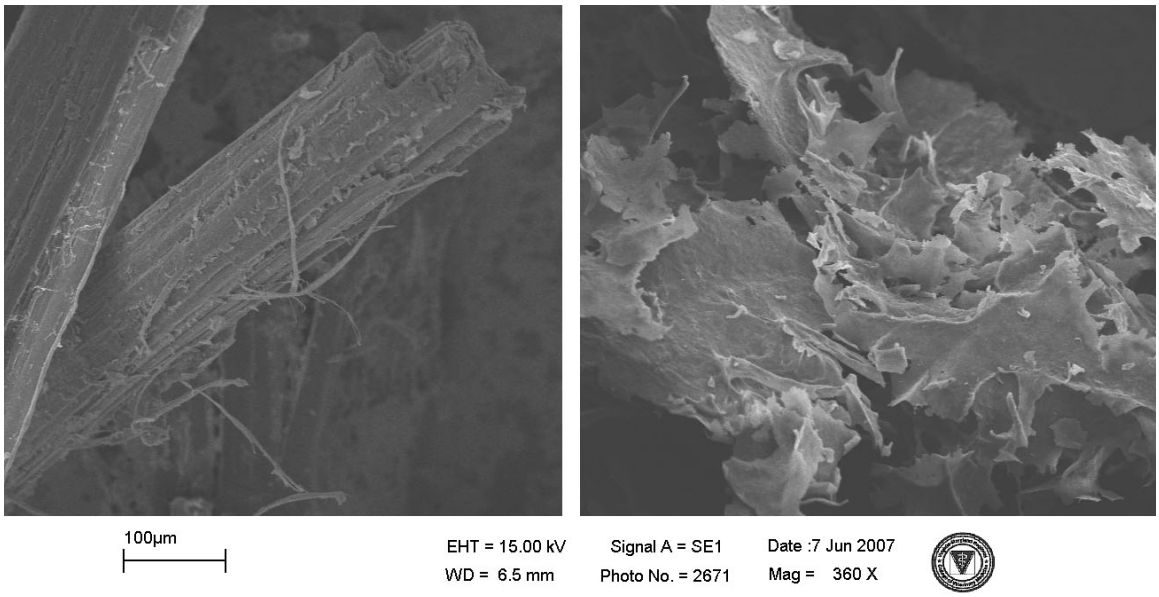

Figure 3-5. SEM images comparing untreated, raw lignocellulose sample (left) to completely fractionated lignocellulose material (right).

The structural changes of lignocellulosic materials before and after the treatment were imaged by fluorescence microscopy. The first sample was composed of untreated lignocellulose. In Figure 3-6, the crystalline structure of the untreated sample is clearly visible under both normal light and under green fluorescent light. Lignin is auto-fluorescent under UV, therefore a suitable background was observed. The second set of images is the untreated lignocellulose samples that were mixed with 1 g/L of green fluorescent protein (GFP) with a cellulose binding module (CBM). The GFP-CBM samples are much more visible under UV, and the crystalline structure is still clearly visible. The last set of images were the well-pretreated sample, dyed by a high concentration of bovine serum albumin (20 g/L) followed by adsorption by a green fluorescent protein with CBM. In the last set of images in Figure 3-6, it is shown that the fibrous structure of the lignocellulose is greatly disrupted by fractionation.

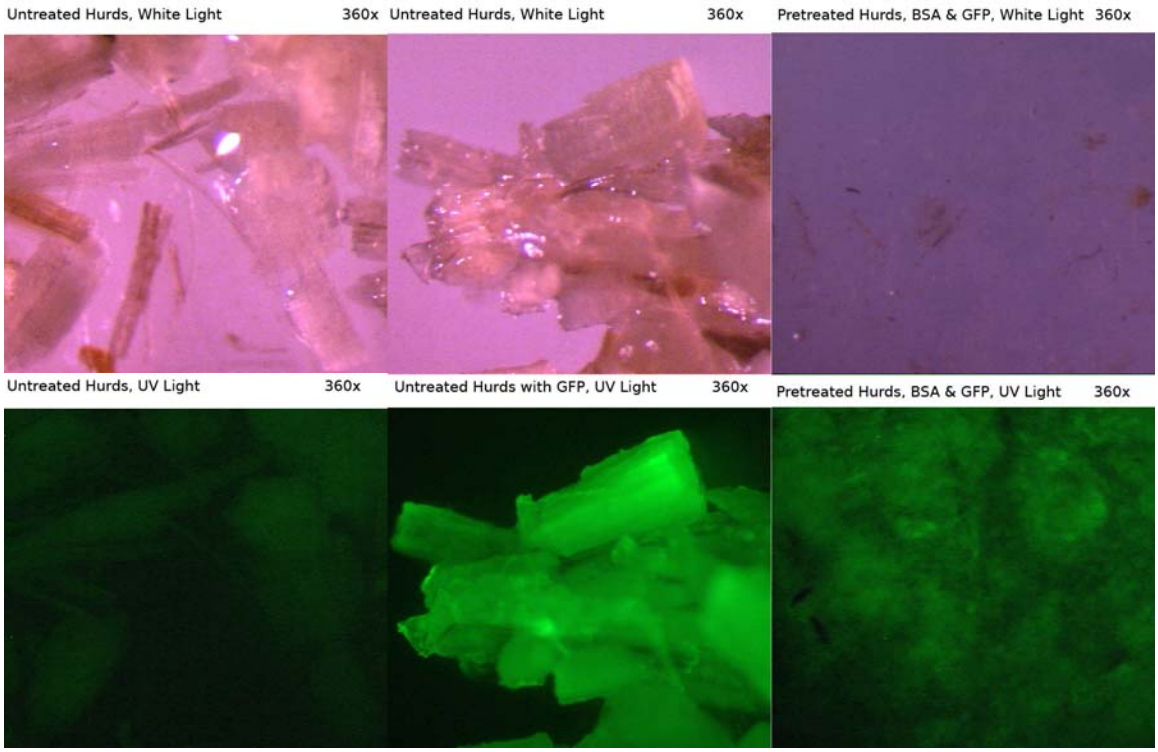

Figure 3-6. Fluorescence microscopy imaging of intact lignocellulose (left), intact lignocellulose mixed with 1 g/L GFP (center), and well-treated lignocellulose by 84.0% H_3PO_4 at 50 °C for 1 hour and mixed 20 g/L of BSA, then mixed with 1 g/L of GFP (right). The magnification is 360 fold.

3.3.4. Mass Balance for the Optimal Conditions

At optimum conditions (84.0% H_3PO_4, 50°C, 1 hour), cellulose digestibility was 93.3% after 12 hours, and 95.9% after 24 hours. The total glucose yield was then calculated as 89.0% (95.9% digestibility * 92.7% glucose recovery). Likewise, xylan digestibility was calculated to be 91.1% after 24 hours. To calculate the xylan digestibility, after enzymatic hydrolysis, the hydrolysis residue was put into a freeze dryer for 36 hours, and the pellets analyzed for carbohydrates by quantitative saccharification. The xylan content

found in the pellets was subtracted from the initial xylan content in the pretreated sample to estimate the digestibility of the xylan. The mass balance of carbohydrate and lignin at the optimal conditions is presented in Figure 3-7.

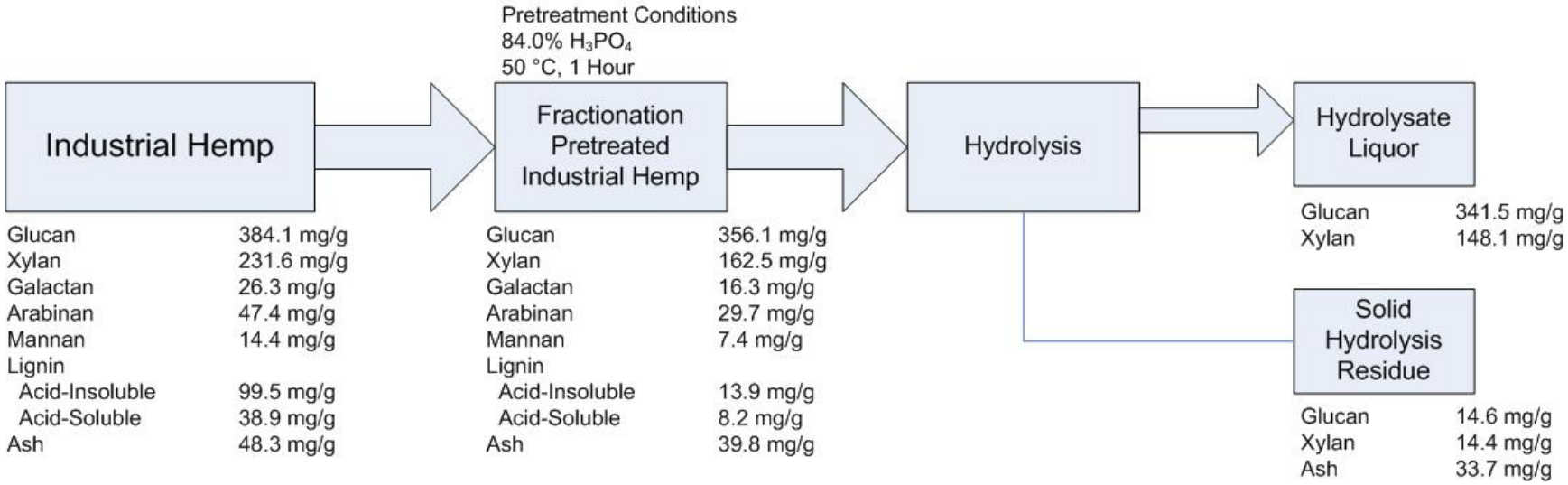

Figure 3-7. Mass balance of industrial hemp hurds after lignocellulose fractionation pretreatment and enzymatic hydrolysis. Hydrolysis conditions were 15 filter paper units (FPU) cellulase and 60 international units (IU) of β-glucosidase per gram of glucan at 50^{o}C in 50 mM citric acid buffer (pH 4.8)

3.4 CONCLUSIONS

Lignocellulose fractionation can effectively treat the recalcitrant hurds of industrial hemp. The glucose and xylose yields were 89% (94% pretreatment; 94.7, digestibility) and 61%, respectively, at an optimal pretreatment conditions are $\geq$ 84% H_3PO_4, $\geq$ 50 °C and $\geq$ 1 hour. In addition to cellulosic ethanol production, to the developed procedure will produce other products with higher selling prices such as lactic acid, acetic acid. Building a small size biorefinery based on lignocellulose fractionation technology and using the hurds of industrial hemp as a feedstock is promising because of high sugar yields, low processing costs, low-cost feedstock, and low capital investment.

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APPENDIX

Table 1A-1. Degradation rate constant of monomeric sugars at 4% - 1% wt/wt sulfuric acid concentrations, 121°C.

	Degradation Rate Constant, K (h^{-1})				
Acid Concentration	Xylose	Arabinose	Mannose	Galactose	Glucose
1%	0.1403	0.0931	0.0632	0.0361	0.0225
2%	0.0997	0.0788	0.0499	0.028	0.0173
3%	0.0692	0.0519	0.0387	0.0213	0.0129
4%	0.0317	0.0189	0.0166	0.0124	0.0092

Table 1A-2. Degradation rate constant of monomeric sugars at 30°C - 70°C in 72% wt/wt sulfuric acid.

	Degradation Rate Constant, K (h^{-1})				
Temperature (°C)	Glucose	Galactose	Mannose	Arabinose	Xylose
30	0.0018	0.0056	0.0074	0.0081	0.0093
40	0.0286	0.0327	0.0411	0.0563	0.058
50	0.0492	0.0635	0.0638	0.1084	0.1979
60	0.136	0.1406	0.1782	0.3041	
70	0.3073	0.3411	0.528		

Table 1A-3. Arrhenius equation data for determination of activation energy of monomeric sugars.

	1/Temperature (K^{-1})				
ln(K)	Glucose	Galactose	Mannose	Arabinose	Xylose
0.00330	-5.8781	-5.185	-4.9063	-4.8159	-4.6777
0.00319	-4.0984	-3.7854	-3.3785	-3.0726	-2.8473
0.00310	-3.0119	-2.7567	-2.6766	-2.2219	-1.62
0.00300	-1.9951	-1.9618	-1.7248	-1.1904	--
0.00292	-1.1799	-1.0756	-0.6387	--	--

Table 1A-4. Degradation of monomeric sugars during concentrated acid hydrolysis and dilute acid hydrolysis under test conditions:

Condition 1: 2 hour 72% H_2SO_4 30°C / 1 hour 4% H_2SO_4 121°C

Condition 2: 1 hour 72% H_2SO_4 30°C / 1 hour 4% H_2SO_4 121°C

Condition 1: 1 hour 72% H_2SO_4 30°C / 1 hour 1% H_2SO_4 121°C

	% Monomer Sugar Degraded		
		Concentrated Sulfuric Acid	Dilute Sulfuric Acid
Glucose	Condition 1	0.18	2.27
	Condition 2	0	2.25
	Condition 3	0	0.92
	--	--	--
Xylose	Condition 1	0.93	13.99
	Condition 2	0	14.03
	Condition 3	0	3.17
	--	--	--
Galactose	Condition 1	0.56	3.63
	Condition 2	0	3.64
	Condition 3	0	1.24
	--	--	--
Mannose	Condition 1	0.74	6.33
	Condition 2	0	6.32
	Condition 3	0	1.66
	--	--	--
Arabinose	Condition 1	0.81	9.35
	Condition 2	0	9.34
	Condition 3	0	1.89

Table 2A-1. The statistical t-values for the comparison of Method 1 *vs.* Method 3 by Tukey -Kramer HSD. Values greater than the threshold t-value of 2.67 (α = 0.01 level of significance) denoted S. Glucans, Galactans, and Mannans were measured with Condition 1, whereas xylans and arabinans were measured by Condition 3

t-value (Method 1 *vs.* Method 3)					
Feedstock	Glucose	Xylose	Galactose	Mannose	Arabinose
Switchgrass	NA	5.52 (S)	0.25	0.24	0.62
Corn Stover	NA	7.84 (S)	1.86	0.21	0.84
Wheat Straw	NA	4.92 (S)	0.27	0.12	1.94
Hybrid Poplar	NA	5.84 (S)	0.94	0.21	1.78
Douglas Fir	NA	4.27 (S)	0.27	2.42	0.51

Table 3A-1. Effect of acid concentration during pretreatment on digestibility of glucans in lignocellulose sample after enzymatic hydrolysis.

	% Digestibility		
Hydrolysis Time (h)	81% H_3PO_4 Pretreatment	84% H_3PO_4 Pretreatment	85.9% H_3PO_4 Pretreatment
0	0	0	0
1	51.59	51.35	52.12
2	69.98	69.76	69.45
4	80.63	80.87	81.86
8	87.99	89.42	88.61
12	89.97	91.49	90.16
24	91.18	92.91	94.11
48	91.4	94.46	94.06
72	91.24	94.18	94.27

Table 3A-2. Effect of reaction time of pretreatment on digestibility of glucans in lignocellulose sample after enzymatic hydrolysis.

	% Digestibility		
Hydrolysis Time (h)	30 Minute Pretreatment	1 hour Pretreatment	2 Hour Pretreatment
0	0	0	0
1	37.14	52.68	52.24
2	50.45	68.87	70.38
4	60.35	81	81.4
8	61.59	89.6	89.88
12	63.76	91.74	92.88
24	64.93	93.62	92.97
48	64.31	94.89	94.24
72	64.5	94.74	94.31

Table 3A-3. Effect of reaction temperature of pretreatment on digestibility of glucans in lignocellulose sample after enzymatic hydrolysis.

	% Digestibility		
Hydrolysis Time (h)	40°C Pretreatment	50°C Pretreatment	60°C Pretreatment
0	0	0	0
1	47.12	49.74	50.39
2	64.45	70.75	71.65
4	78.86	82.11	81.33
8	83.96	88.33	86.88
12	88.91	90.06	91.18
24	90.66	93.59	93.07
48	90.54	93.81	94.52
72	90.88	94	94.8