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Understanding Substrate Features Influenced by Pretreatments that Limit Biomass Deconstruction by Enzymes

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Chemical and Environmental Engineering

by

Xiadi Gao

March 2013

Dissertation Committee:

Dr. Charles E. Wyman, Chairperson
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The Dissertation of Xiadi Gao is approved:

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At last, I will always remember the love and support from my parents, Mr. Qixian Gao and Mrs. Yanhua Ji; my husband, Hongjia Li; and all other family members. I would like to dedicate this thesis to them.

ABSTRACT OF THE DISSERTATION

Understanding Substrate Features Influenced by Pretreatments that Limit Biomass Deconstruction by Enzymes

by

Xiadi Gao

Doctor of Philosophy, Graduate Program in Chemical and Environmental Engineering
University of California, Riverside, March 2013
Dr. Charles E. Wyman, Chairperson

Conflict between dwindling reserves of fossil energy and growing consumption presents significant challenges to our society. To shift society's dependence away from petroleum to renewable energy, biorefineries are needed that employ advances in genetics, biotechnology, process chemistry, and engineering to convert biomass to valuable fuels and other products. Lignocellulosic biomass is the only sufficiently abundant source of renewable carbon that can support large-scale sustainable and economic production of transportation fuels and industrial chemicals. However, lignocellulosic biomass is naturally resistant to microbial or enzymatic deconstruction, a characteristic collectively

termed “biomass recalcitrance,” and overcoming this resistance is the main hurdle to realizing low costs. A better understanding of relationships among biomass recalcitrance, pretreatment, and enzymatic hydrolysis can accelerate this quest.

Against this background, this thesis seeks to understand the influence of pretreatment on substrate features and how such features affect subsequent enzymatic hydrolysis. First, application of a high throughput pretreatment and co-hydrolysis system (HTPH) was extended to dilute acid pretreatment to facilitate development of a novel two-stage pretreatment strategy that sought to achieve high cellulose digestibility and total sugar yields while keeping hemicellulose degradation low. Along with this, the feasibility of recycling liquid for low temperature pretreatment was demonstrated. Inspired by the HTPH screening results, a new method was developed for simple and rapid quantification of the hemicellulose sugar content in biomass. Finally, compositional and structural features of solids produced by application of two-stage pretreatment to switchgrass and three leading pretreatment technologies to corn stover were compared to provide new insights into control of biomass recalcitrance, as well as how pretreatments with much different deconstruction patterns impact enzymatic hydrolysis.

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Chapter 1 Introduction

1.1 Biorefinery

Modern industrial and transportation systems all rely heavily on fossil energy. However, the conflict between dwindling reserves of fossil energy and growing consumption raise significant challenges for our society. Additionally, heavy reliance on petroleum creates a series of environmental issues, such as greenhouse gas emissions and resulting global climate change. Therefore, the task of developing promising sustainable energy sources with low carbon emissions should not be delayed [1-4]. To shift society's dependence away from petroleum to renewable biomass, a different manufacturing concept is needed: biorefineries that employ advances in genetics, biotechnology, process chemistry, and engineering to converting renewable biomass to valuable fuels and products (Figure 1.1) [2, 5].

Compared to other sustainable energy resources, such as solar, wind, hydro, and nuclear power, biomass is the only abundant source of renewable carbon that has great promise for providing both sustainable transportation energy and industrial chemicals on a meaningful and economic scale [1, 6]. Biofuels, such as ethanol derived from lignocellulosic biomass, show great potential in providing an alternative source of transportation fuels for US. Unlike starch based biomass for fuel production, utilization and conversion of lignocellulosic biomass - non-food, low cost and abundant feedstocks such as agricultural and forestry residues and woody and herbaceous crops reduces competition between food and energy [7]. It is estimated that the current combined lignocellulosic biomass resource from forest and agricultural lands ranges from 138 to nearly 258 million dry tons at prices from $40 to $60 per dry ton. And the supply is

projected as increasing to 187 to 602 million dry tons by 2022 at the same prices. By 2022, based on an assumed yield of 85 gallons ethanol per dry ton, a combination of forest and agricultural residues and energy crops could provide 20 billion gallon per year of cellulosic biofuels [8]. Other than transportation fuels, value added sugar-derived chemicals and materials from biomass could also be made by biorefinery processes [9].

The U.S. Department of Energy has set a goal to replace 30% of liquid petroleum-derived transportation fuels with biofuels and 25% of industrial organic chemicals with biomass-derived chemicals by 2025. In addition to supporting commercialization through policies, research and development is vital to increase the impact, efficiency, and sustainability of biorefineries.

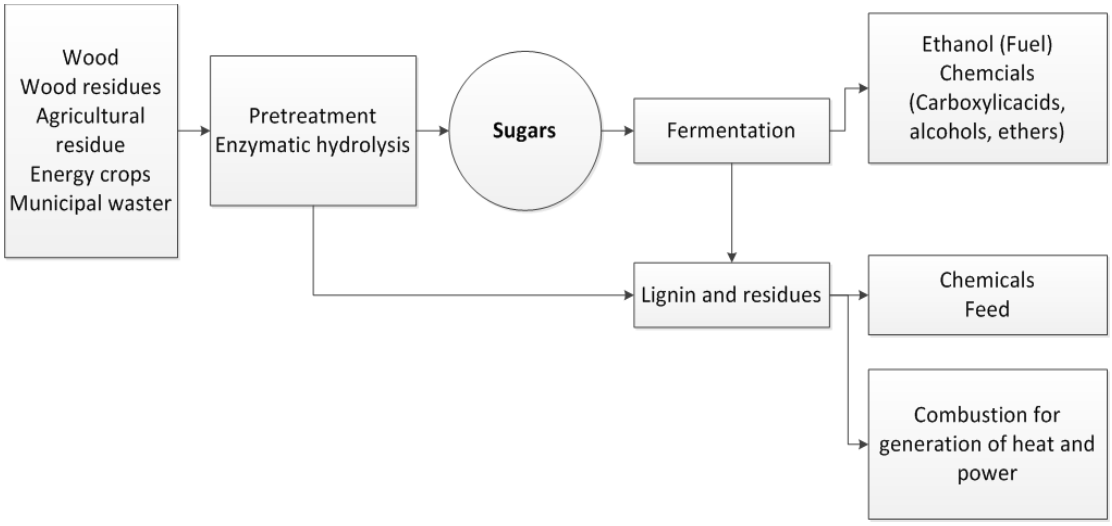

Figure 1.1 An integrated biorefinery for producing fuels, chemicals, and energy from lignocellulosic biomass [10]

1.2 Plant cell wall structure and biomass recalcitrance

As shown in Figure 1.1, a key demand biological conversion is breakdown of complex carbohydrates in lignocellulosic biomass into fermentable sugars. However,

lignocellulosic biomass is naturally resistant to microbial or enzymatic deconstruction, a characteristic collectively termed “biomass recalcitrance” [11], and overcoming this resistance is the main hurdle to realizing low cost conversion. Overcoming recalcitrance is essential to economically feasible and environmentally sustainable biomass conversion.

Improved insights into plant cell wall structure and its role in recalcitrance would be very valuable in developing ways to deconstruct biomass efficiently. However, our knowledge in this field is still limited as plant cell walls are a complex system at both the molecular and nanoscale levels [1]. There are three major components of secondary plant cell wall: cellulose (35-50%), hemicellulose (20-35%) and lignin (10-20%) [12]. Cellulose consists of a linear chain of hundreds to thousands of β-1, 4 linked glucose units. Hemicellulose is a shorter, branched chain typically dominated by xylose with some other sugars such as mannose, galactose, glucose, and arabinose, depending on the type of plant. Additionally, lignin is an amorphous, crossed-linked, three dimensional aromatic polymer. The organization of cell walls can be described as consisting of cellulosic microfibrils and structural proteins inserted into and reinforcing a multicomponent matrix, composed of stereo-irregular heteropolysaccharides (also known as hemicellulose). The matrix polysaccharides also covalently interact with non-carbohydrate cell wall components, such as lignins and proteins [13]. The interactions between wall polymers, both non-covalent and covalent, result in a very rigid structure that impedes extraction of matrix polysaccharide from cell walls for utilization [13].

1.3 Biological conversion of lignocellulosic biomass

Over the past decades, multiple conversion technology routes have been developed to overcome biomass recalcitrance and convert lignocellulosic biomass to fuels and chemicals, with the dominate objective being to make the processes cost-competitive [1]. In a leading approach to biochemical conversion, biological catalysts, such as enzymes and microbial cells along with heat and chemicals are applied to convert biomass into sugars for fermentation to ethanol or other products [1].

There are three major unit operations for biochemical conversion of lignocellulosic biomass: pretreatment, enzymatic hydrolysis, and fermentation (Figure 1.1). Biomass pretreatment is essential to achieving high yields at low cost [7, 14], and a variety of different pretreatment methods have been applied with the overall goal to break down the physical barrier of cell walls and make cellulose more easily accessible and digestible by enzymes[15]. In addition, pretreatment strongly influences other major operations in the overall conversion scheme, such as choice of feedstock, hydrolysis rate, enzyme loading and formulation, product recovery, and co-product potential [7, 14]. During subsequent enzymatic hydrolysis of pretreated material, cellulose and hemicelluloses are converted into fermentable sugars by enzymes[2, 5, 7, 11]. In this step, endoglucanases, exoglucanases, and β-glucosidase as well as supplementary enzymes such as xylanases are generally applied to catalyze such heterogeneous reactions effectively and efficiently [16-20]. Although near theoretical yields are possible, the high doses of enzymes that are required with current technology are expensive and the major obstacle to commercial use [2, 10]. The monomeric sugars obtained can then be

converted to fuels and chemicals via fermentations or other reactions [21, 22]. For ethanol production, as an example, monomeric pentoses and hexoses are fermented to ethanol by yeast or other organisms. Many naturally occurring microorganisms can readily ferment hexoses to ethanol, but fermentation of pentose to ethanol with high yields requires use of genetically engineered organisms [23]. Ethanol from the resulting broth is recovered by distillation. And the solid residue, which contains mainly lignin, can be used as fuel to produce process heat and power for both the process and export [24].

1.4 Thesis objectives and organization

Despite numerous studies to understand biomass recalcitrance, a comprehensive understanding is still lacking about physicochemical features that hinder biological conversion of lignocellulosic biomass because of the complexity of plant cell wall structure, the versatility of pretreatment technologies, and the interdependence of structural and compositional changes that result from pretreatment. A bettering understanding of biomass recalcitrance and its relationship with different pretreatment strategy will provide meaningful information and guidance for overcoming biomass recalcitrance in an economic manner. Thus, this thesis seeks to understand the influence of several leading pretreatment technologies on substrate features and how such features affect subsequent enzymatic hydrolysis.

Chapter 2 begins with a review of literature relevant to this thesis. Structural features of lignocellulosic biomass and major processes for biological conversion of biomass are reviewed first. Then existing pretreatment strategies and resulting effects on

biomass physiochemical features are summarized, with emphasis on dilute acid and hydrothermal pretreatments as well as some insights into AFEX and ionic liquid technologies that are covered in this thesis. After that, changes in physiochemical features from pretreatment that affect the enzymatic hydrolysis of pretreated biomass are reviewed.

Due to structural and chemical differences between glucan and xylan, conditions that maximize sugar yields from pretreatment or enzymatic hydrolysis alone are usually different from conditions that maximize the yields of both these sugars from the two steps combined. To overcome resulting sugar losses, a novel two-stage pretreatment strategy was developed based on first using dilute sulfuric acid at low temperatures to maximize recovery of hemicellulosic sugars followed by hydrothermal pretreatment of the washed solids at higher temperature to increase cellulose digestibility to economically viable levels. *Chapter 3-7* report on results from application of this approach and new insights it provided into factors controlling recalcitrance. In *Chapter 3*, a new one-step neutralization and buffering method is described to enable application of our novel high throughput pretreatment and co-hydrolysis system (HTPH) to dilute acid thermochemical pretreatment. After that, *Chapter 4* discusses application of this new dilute acid HTPH system to screen for promising combinations of low temperature first stage dilute acid pretreatment conditions coupled with hydrothermal pretreatment at higher second temperatures with the goal of achieving high glucan digestibility and total sugar yields from combined pretreatment and enzymatic hydrolysis steps while minimizing xylan degradation. Then larger conventional laboratory equipment was applied to validate the

conditions identified with the HTPH system and provide more comprehensive comparisons of performance of the two-stage system to that possible by conventional one-stage pretreatment. *Chapter 5* is focused on characterizations of composition and structural features (hemicellulose residue, surface accessibility, and surface distribution of cellulose) of the biomass substrate after the first and second stage pretreatments. Correlations among enzymatic digestibility and compositional and structural properties were developed to shed light on what features were major contributions to the reduction of biomass recalcitrance after pretreatment and how these features were controlled by pretreatment conditions. *Chapter 6* investigated the possibility of recycling liquid from the first stage low temperature dilute acid pretreatment in the two-stage pretreatment strategy to provide supporting information for Chapter 4. Inspired by HTPH screening results from Chapter 4, *Chapter 7* shows how hemicellulose sugars in biomass can be rapidly quantified by a one-step acid hydrolysis method to provide an alternative faster method for analysis of hemicellulose sugar content in biomass.

Chapter 8 and Chapter 9 present our work in a collaboration project among three DOE Biomass Research Centers (BRCs). *Chapter 8* reports how application of common sources of enzymes and biomass (corn stover) by the three BRCs to their pretreatment technologies of dilute acid (DA), ammonia fiber expansion (AFEX), and ionic liquid (IL) provided new insights into how biomass pretreatments with much different deconstruction patterns impact enzymatic hydrolysis. These studies examined how the three pretreatments impact composition following pretreatment, sugar release patterns in enzymatic hydrolysis at different enzyme loadings, substrate accessibility to enzymes,

and oligomers released during enzymatic hydrolysis. The results were then integrated to understand how different pretreatments change substrate features and their role in achieving high yields. *Chapter 9* provides information on the two of the most used batch laboratory reactors for dilute acid or hydrothermal treatment, small tubes and well mized Parr reactors, that can provide valuable tools for defining combinations of acid concentrations, temperatures, and times that maximize yields of targeted reactive intermediates from cellulosic biomass. Finally, *Chapter 10* summarizes key conclusions and major findings of this overall thesis as well as suggests future research directions based on results from this thesis.

1.5 Acknowledgements

We gratefully acknowledge support for this research by the Office of Biological and Environmental Research in the DOE Office of Science through the BioEnergy Science Center (BESC). The author is also grateful to the Center for Environmental Research and Technology of the Bourns College of Engineering (CE-CERT) at the University of California, Riverside for providing key equipment and facilities. Gratitude is also extended to the Ford Motor Company for funding the Chair in Environmental Engineering at the Center for Environmental Research and Technology of the Bourns College of Engineering at UCR, which augments support for many projects such as this one.

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Chapter 2 Literature Review

2.1 Introduction

To shift society's heavy dependence away from petroleum to renewable biomass resource, a new manufacturing concept is brought up: biorefinery, which employs advances in genetics, biotechnology, process chemistry and engineering to convert renewable biomass to valuable fuels and products [1, 2]. Lignocellulosic biomass, referring to non-food, low cost and abundant feedstocks, such as agriculture and forestry residues and woody and herbaceous crops, is the only abundant source of renewable carbon[3]. It shows great promising for providing both sustainable transportation fuels and industrial chemicals on a meaningful scale economically and reduces competition between food and energy [4]. Recent discussions around lignocellulosic based biorefinery suggest that biological platform, the strategy of which is basically liberation and fermentation of sugars from biomass feedstock, is one of the technological platforms that may be suitable for the biomass-to-biofuel production [5]. As it can be seen in Figure 2.1, a key issue for such strategy is efficient conversion of carbohydrates from lignocellulosic feedstocks into fermentable sugars, so it is also known as "sugar platform".

Despite intensive research on biomass utilization, great challenges remain on releasing sugars from natural resistant lignocellulosic biomass with high yield and low cost [6]. Pretreatment combined with enzymatic hydrolysis are major steps to achieve fermentable sugars from biomass [7, 8]. Pretreatment alters physical and chemical characteristic of biomass and greatly impacts the downstream operations, including enzymatic hydrolysis and fermentation. Numerous features of biomass have been

postulated to explain how such physicochemical features in hindering the biological conversion, and how these properties are affected by pretreatment and impact the following enzymatic digestion [8-11]. A bettering understanding of relationship among biomass recalcitrance, pretreatment strategy and enzymatic hydrolysis will provide meaningful guidance for overcoming biomass recalcitrance in an economic manner.

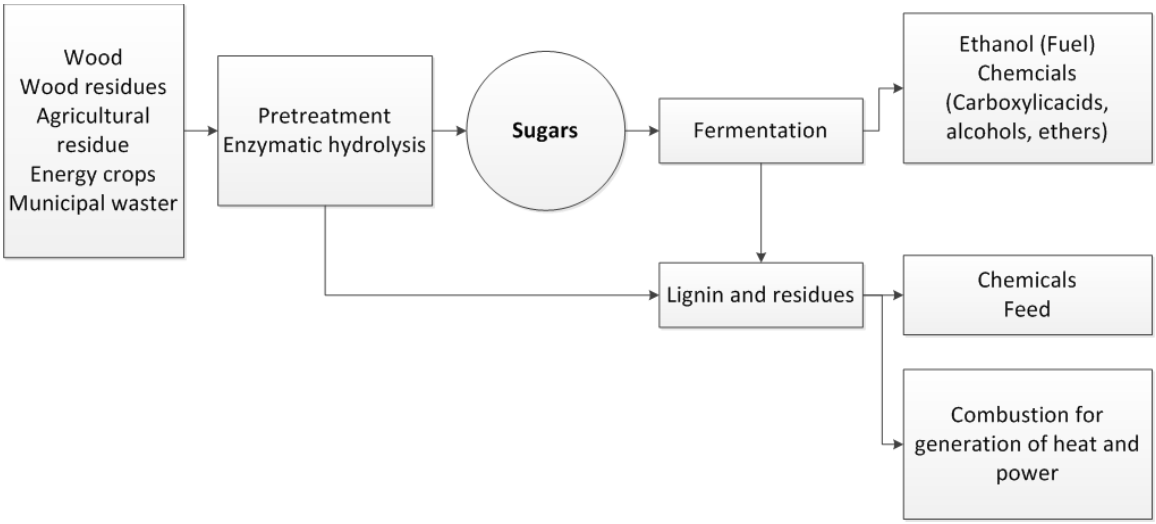

Figure 2.1 An integrated biorefinery producing fuel, chemical and energy from lignocellulosic biomass [8]

2.2 Biomass recalcitrance and plant cell wall structure

As can be seen in Figure 2.1, a key for successful "sugar-platform" biorefinery is efficient conversion of carbohydrates from lignocellulosic biomass into fermentable sugars. However, the natural resistance of biomass to microbial or enzymatic deconstruction, which is collectively known as "biomass recalcitrance", is considered as the main hurdle to low cost of lignocellulosic conversion [12]. It is evident that biomass recalcitrance is a multi-scale phenomenon that scales several orders of magnitudes and includes structural, molecular and chemical features [13, 14].

From the perspective of bioconversion of lignocellulosic biomass, the most important element is the plant cell wall since there stores a great amount of fermentable sugar. There are three major components of plant cell wall: cellulose (35-50%), hemicellulose (20-35%) and lignin (10-20%) [15]. There are both primary and secondary cell wall and the secondary cell wall accounts for a larger mass fraction than primary cell wall in mature plants[16].

Cellulose is the most stable of all of the cell wall polysaccharides. It consists of a linear chain of hundreds to thousands of β-1, 4 linked glucopyranosyl with cellobiose as repeating units. Cellulose fibers are bundles of microfibrils that stabilized by hydrogen bonds between hydroxyl groups on liner cellulose chains. However, the microfibrils are not absolutely uniform in all regions, in some regions they are highly crystalline, while in others they can be amorphous [17].

Unlike cellulose, hemicellulose is not chemically homogeneous, and the chemical nature depends on the source. It is a shorter, branched chain typically dominated by 1,4-linked β-xylose units with some other sugars such as mannose, galactose, glucose, and arabinose, depending on the type of plant. Compared to cellulose, hemicellulose is amorphous and hydrophilic, therefore, more vulnerable to thermochemical pretreatment and more easily removed from cell wall [16].

Additionally, lignin is another important component of plant cell wall. However, the structure of lignin is unclear yet. What is known about lignin is that, differ from the polysaccharides in cell wall, lignin forms a three-dimensional structure with complex, crossed-linked and hydrophobic aromatic polymers, which provides the mechanical

strength and a water-impermeable barrier to the secondary cell wall. And lignin is primarily composed of three subunits, p-hydroxyphenyl (H-unit), guaiacyl (G-unit), and syringyl (S-unit), the ratio of which can vary between different plant [16].

The organization of cell wall can be described as consisting of cellulosic microfibrils and structural proteins embed in multi-component matrix of stereo-irregular heteropolysaccharides (mostly hemicellulose), mainly through non-covalent bond. The matrix polysaccharides also covalently interact with non-carbohydrate cell wall components, such as lignins and proteins [18]. Although these matrix interactions vary with plant cell type and with maturity, they greatly determine structural feature limiting the rate an extent of utilization of biomass materials [18, 19].

2.3 Pretreatment

In the biological platform of biorefinery, fermentable sugars from biomass are achieved by pretreatment and enzymatic hydrolysis for following utilization. The overall goal of pretreatment is to break down the physical barrier of cell wall and makes cellulose ready to digest by enzymes [20]. In addition, pretreatment strongly influences on other major operations through overall conversion scheme, such as choice of feedstock, hydrolysis rate, enzyme loading and formulation, product recovery and co-product potential [4, 7]. There are varieties of different pretreatment options, but only those employ chemicals currently offer the high yields and low costs vital to economic success, such as dilute acid, ammonia fiber expansion (AFEX), steam explosion, ammonia recycle percolation (ARP), lime, soaking in aqueous ammonia (SAA), and hot

water controlled pH[7]. Recently, ionic liquid has also received attentions [21, 22]. In the following, pretreatment technologies that involved in the thesis will be reviewed briefly.

2.3.1 Proton catalyzed pretreatment

Both dilute acid and hydrothermal pretreatment can be considered as proton catalyzed pretreatment. The difference is in dilute acid pretreatment, additive acidic chemicals provide protons, while for hydrothermal pretreatment, protons come from the dissociation of water as temperature increases. As a result, the hydrothermal pretreatment usually requires higher temperature to provoke the alternation of biomass [23, 24]. Sulfuric acid is the most commonly used acid for dilute acid pretreatment for its low cost and low volatility [25-27].

Both dilute acid and hydrothermal pretreatment remove hemicellulose and recover its component sugars, partially disrupts and dislocates lignin while increasing cellulose digestibility for subsequent biological conversions such as enzymatic hydrolysis with cell free enzymes [25-28]. The major difference among these two methods is that for hydrothermal pretreatment, the resulting liquid contains relative large amount of xylooligomers while for dilute acid, most of hemicellulose is hydrolyzed to monomers [29, 30].

2.3.2 Two-stage pretreatment

Maximum utilization of both cellulose and hemicellulose is essential for efficient conversion of lignocellulosic materials to fuels and other value-added products [31]. However, due to structural and chemical differences between cellulose and hemicellulose,

the hemicellulose is more readily hydrolyzed than cellulose [32]. In fact, conditions that maximize sugar recovery from hemicellulose are usually less severe than conditions that give high yields of glucose from cellulose, either in chemical or enzymatic hydrolysis of cellulose [32, 33]. Therefore, to address the contradiction between the relatively mild conditions appropriate to achieve high xylose yields and the more severe conditions for high glucose release or glucan digestion, a stage-wise strategy has been applied to increase overall yields of fermentable sugars from biomass and may help to alleviate unwanted byproducts. In such strategy, the hemicellulose is first hydrolyzed by a relatively mild pretreatment then a harsher condition is applied to hydrolyze cellulose to glucose or to increase the enzymatic digestibility of the glucan-enriched residues [34]. Figure 2.2 shows a representative scheme of the two-stage pretreatment.

Most of the developed two-stage pretreatment strategies use dilute acid at high temperatures and short retention times, but the temperature at Stage 1 for depolymerized hemicellulose is lower than Stage 2 for treat of remaining cellulose[33, 35-41]. A few pretreatments applied just water in both stages but different temperatures [33, 41] or sulfuric acid combined with ethanol at temperature below 100 ℃ for pretreatment [39]. Such two-stage strategy has been applied on versatile feedstocks, such as soft and hard woods (Eucalyptus, ponderosa pine, white fir, spruce), grasses (coastal Bermuda), agricultural residues (corn stover, wheat straw) and benefits virtually high recovery of sugars from lignocellulosic materials.

Besides dilute acid or hot water two-stage pretreatment system, other strategies were also explored to provide a sustainable medium for the two-stage pretreatment of

prehydrolysis of hemicellulose before hydrolyzing cellulose or increasing its digestibility. for example, biphasic CO_2-H_2O mixtures [42], or two-step steam pretreatment with SO_2 impregnation[43]. For the two-step steam pretreatment with SO_2 impregnation, it was found that high conversion of cellulose can be obtained either from second stage at a high severity or from a low severity second stage pretreatment combined with enzymatic hydrolysis, which resulted an nearly constant overall sugar yields over a wide range of severity[43]. A stage-wise hot water/ dilute acid and aqueous ammonia pretreatment of corn stover was used to separate both hemicellulose and lignin to improve the cellulose digestibility [44-46].

Though with the potential advantages of two-stage process, such as high yield , high purity and concentrated products [34], the separating of the solubilized hemicellulose sugars with a filtering and washing to avoid their degradation during the second high-temperature stage adds significant amounts of water to the process and filtering can significantly add to economic and energetic costs[47, 48]. Increase the pentose utilization by produce value-added product or success of pentose fermentation is essential in offset the cost [31, 49].

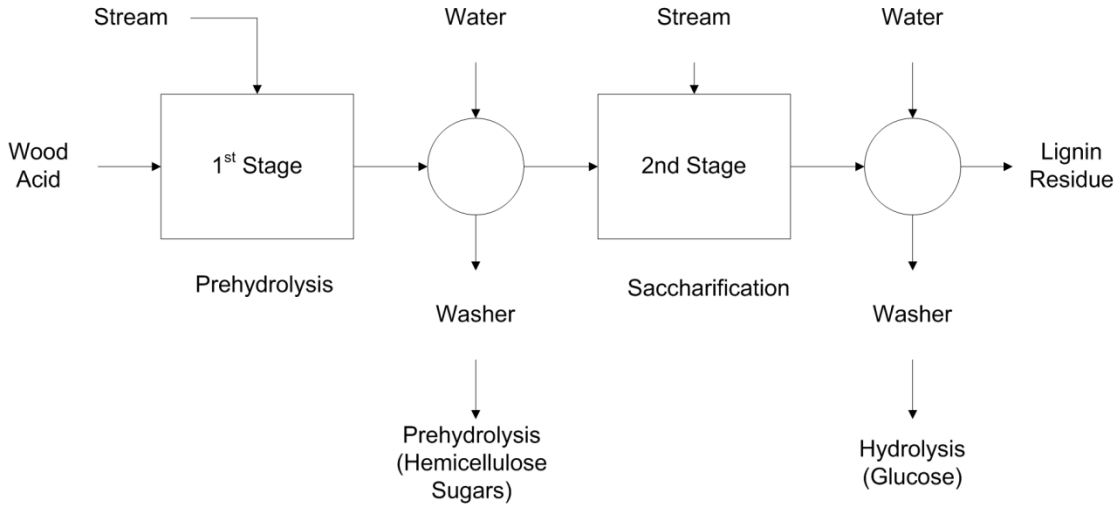

Figure 2.2 Representative scheme of the two-stage pretreatment [34]

2.3.3 Ammonia fiber expansion (AFEX)

The AFEX process pretreats biomass with anhydrous liquid ammonia at high pressure and moderate to high temperatures. Following pretreatment for a given time, the pressure is rapidly released resulting in biomass structure disruption and partial cellulose decrystallization, presumably resulting in enhanced cellulose digestibility [50-52]. AFEX achieves good cellulose digestibility from herbaceous and agricultural residues but its application on woody biomass is rarely seen. AFEX does not significantly change the compositions of biomass, but it is reported as creation of pore structure, and disruption of lignin-carbohydrates linkages for enhancement of cellulose digestibility [14, 53]. Most hemicellulose is oligomers for AFEX pretreated biomass and how to fully convert the oligomers is a primary challenge.

2.3.4 Ionic liquid pretreatment

Lately, the use of ionic liquids to dissolve biomass has received attention as promising pretreatment solvents [54]. Ionic liquid is a kind of salt, typically composed of large organic cations and small inorganic anions and exists as liquid at ambient temperatures [11]. The ionic liquid can simultaneously dissolve carbohydrate and lignin thus it is believed to be able to disrupt the intricate network of non-covalent interaction among cell wall polymers of cellulose, hemicellulose and lignin, thus improve the performance of enzymatic hydrolysis [22, 55, 56]. This regenerated cellulose from ionic liquid pretreatment can have a similar degree of polymerization as before but the degree of crystallinity reduced substantially [57]. Most data only show the utilization of pure cellulose while some demonstrate the possibility of ionic liquid on some lignocellulosic biomass [58, 59]. However, development of efficient method for recovering hemicellulose and lignin, recycling of ionic liquid, understanding its toxicity to enzymes and mricoorganisms are still needed [57].

2.3.5 Comparison of pretreatment methods

Comparison of different pretreatments using single source of biomass, enzymes and identical analytical methods in terms of sugar and ethanol yields, the characterization of the resulting solid and liquid stream, as well as economic assessment provides important guidance for selection of optimum pretreatment conditions[4, 60-62]. The advantages and challenges for dilute acid, hydrothermal, two-stage, AFEX and ionic liquid pretreatment are summarized in Table 2.1. Sugar release patterns, solids loadings

as well as the compatibility with the process, enzymes, and fermentative organisms should all be taken into consideration when choosing pretreatment technology [61].

Techno-economic analysis of leading pretreatment technologies suggests that overall ethanol yield, which is largely based on the overall sugar yield from pretreatment and enzymatic hydrolysis steps, is the single-most important factor in determining projected product value [63, 64]. Economic drivers are also influenced by solids concentration, enzyme loading and activity [65]. Limited differentiation was found between the projected economic performances of the leading pretreatment options, low cost pretreatment reactors are often counterbalanced by higher costs associated with pretreatment catalyst recovery or higher costs for ethanol due to lower monomer sugar yield [63, 65].

Table 2.1 Summary of advantages and disadvantages of leading pretreatments [11]

Pretreatment methods	**Advantages**	**Challenges**
Dilute acid	- Hemicellulose solubilization	- May generate inhibitors
Hydrothermal	- Less corrosive	- High water consumption - Generate oligomers
Two-stage	- Optimal fraction - High yield	- Extra operation cost caused by separation
AFEX	- Low formation of inhibitors	- Not efficient for biomass with high lignin content - Ammonia needs to be recycled properly
Ionic liquid	- Cause lignin and hemicellulose hydrolysis	- High cost - Solvent needs to be recycle properly

2.4 Enzymatic hydrolysis

For the biological platform of biorefinery, enzymatic hydrolysis is an irreplaceable step because it allows nearly theoretical yields after appropriate pretreatment [66]. In this step, pretreated material, cellulose and hemicelluloses are converted into fermentable sugar by synergic action of endoglucanases, exoglucanases and β-glucosidase as well as supplementary enzymes such as xylanase [17, 67-70]. Although near theoretical yields are possible, high dose of expensive enzymes is still a major obstacle to commercial use [1, 8, 71]. The hydrolysis of native cellulosic biomass is slow and incomplete, even for pretreated biomass, the rate of cellulose enzymatic hydrolysis decreases with conversion [72]. Tremendous researches have been directed to gain a better understanding on the enzymatic hydrolysis of lignocellulosic biomass from the perspective of both substrate and enzyme features as well as interactions between these two.

Enzyme factors considered as influence the cellulose hydrolysis include inhibition by endproduct, non-productive binding of enzymes and enzyme deactivation with time [9, 73-76]. A numbers of sugar monomer and oligomers have been proved to show inhibition effect on cellulose hydrolysis [77, 78]. Cellobiose and xylooligomers show greater inhibition than sugar monomers, and this explains the necessary of supplementary enzymes, such as β-glucosidase and xylanase to prevent or decrease such inhibition [79-81]. Furthermore, some degradation products formed during pretreatment are also believed to inhibit cellulase, such as aliphatic acid, aromatic acid and aldehydes [82, 83]. Non-productive binding to biomass component, such as lignin has also been purposed to

negatively affect the performance of enzymatic hydrolysis [84, 85]. Multiple substrate related features have also been purposed to contribute to biomass recalcitrance and affect the enzymatic hydrolysis, including crystallinity and degree of polymerization(DP) of cellulose, the substrate accessibility, lignin and hemicellulose structure , distribution and removal [9]. However, sometimes, conflicting results were obtained when try to understand what substrate related features play important role on enzymatic hydrolysis and what features do not. And it can be noticed that both enzyme and substrate related features that may impact the performance of enzymatic hydrolysis are affected by the pretreatment.

2.5 Biomass features affected by pretreatment and its impact on enzymatic hydrolysis

As describe previously, though various substrate features are recognized as affect the enzymatic digestibility of biomass, a clear elucidation is still not yet achieved. The conclusions are often dependent on the type of biomass, the pretreatment technologies and sometimes characterization techniques.

Table 2.2 Effect of various methods on the chemical composition and physiochemical structure of lignocellulosic biomass [20, 57]

	Increase accessible area	Decrystallize cellulose	Hemicellulose removal	Lignin removal	Alter lignin structure
Dilute acid	**	-	***	*	*
Hydrothermal	**	-	**	*	*
AFEX	**	*	-	-	**
Ionic liquid	***	**	*	**	**

As summarized in Table 2.2, the structure and composition changes resulting from pretreatment are always concurrent thus makes it uneasy to elucidate the relative

importance of different features on enzymatic hydrolysis. Both hemicellulose and lignin removal has been shown to positively relate to higher cellulose to glucose conversion [86]. It is believed that hemicellulose removal by dilute acid and/or hydrothermal pretreatment enhances cellulose digestibility by improving its accessibility [87] and/or reducing cellulase inhibition by xylooligomers, produced due to partial hydrolysis of xylan [78-80]. However, Joeh et al. also noticed that when using dilute acid pretreatment, xylan removal greater than 80% did not further improve enzymatic digestibility [87]. Some studies also reported that higher temperature was required for hemicellulose removal, indicating that hemicellulose is not the dominate factors impacting digestibility [26]. As for lignin, the removal or modification of lignin is purposed as not only decrease the hinder of cellulose accessibility due to lignin-carbohydrates linkages but also improve cellulase effectiveness by reduce unproductive binding , thus benefits the enzymatic hydrolysis[88-90]. However, some researches do not totally agree with the importance of lignin removal but suggest cellulose accessibility may be more important. Rollin et al compared the digestibility of switchgrass prepared by cellulose solvent- and organic solvent-based lignocellulose fractionation (COSLIF) and soaking in aqueous ammonia (SAA), suggesting that increasing cellulose accessibility was more important than removal of lignin[91]. Sinitsyn et al tested major physicochemical and structure parameters of biomass from mechanical, physical and chemical pretreatment and found that for bagasse, only specific surface areas was observed have a linear relationship with enzymatic reactivity [92]. Similar, when use milling, 1wt% NaOH, peracetic acid (1:1 v/v acetic anhydride and 35% hydrogen peroxide) and ethylene glycol pretreatment on

wheat straw, the specific surface area was found as the most influential of the structural features followed by the lignin content on enhancing enzymatic hydrolysis [93]. Such observation seems very logical enzymatic hydrolysis of cellulose is a heterogeneous reaction which requires successive adsorption of enzymes onto the biomass substrate [17, 68, 94].

Some studies exam and compare the effect from several factors on enzymatic digestibility of biomass. Chang and Holtzapple treated poplar wood with peracetic acid, KOH and ball milling with a broad spectrum of lignin contents, acetyl contents and crystallinity. The lignin content and crystallinity were found had greater impact on digestibility than acetyl content [88]. A model based on AFEX pretreatment of corn stover, suggested that the initial rate is most influenced by cellulose crystallinity while the lignin content most influence extent of 72h enzymatic hydrolysis[95].

It is also very interesting to notice that when examine across different pretreatment methods, including dilute acid, controlled pH, ammonia recirculation (ARP), ammonia fiber expansion (AFEX), SO_2 and lime pretreatment, the digestibility could not be consistently related to xylan, lignin, or acetyl group removal [96]. Kumar et al. characterized the physical and chemical properties of corn stover and poplar wood resulting from leading pretreatment technologies and claimed that removal of xylan and/or lignin served one purpose: disrupt carbohydrate-lignin linkages and enhance substrate accessibility, as well as reduce the inhibition caused by xylooligomers and unproductive binding of lignin to enzyme [89].

2.6 Closing Thoughts

How to release enormous amount of fermentable sugars that are stored in the plant cell wall of lignocellulosic biomass is very critical in successful biorefinery. Both pretreatment and enzymatic hydrolysis play important role in achieving fermentable sugars in a high yield and low cost manner. The review suggests that various pretreatment methods with much different deconstruction patterns impact the chemical and structural features of biomass and its biological conversion to sugars. Though many substrate related features have been assigned as affect the enzymatic hydrolysis, the lack of conclusive statement suggests that comprehensive understanding of the complex nature of plant cell wall structure, its interaction with different pretreatments and influence on enzymatic deconstruction is still needed. The key features attribute to more digestible solids varied for different pretreatment strategy. When elucidating the dominated substrate features for better digestibility, the interdependence of structural and compositional changes should be considered comprehensively. Understanding of influence of leading pretreatment technologies on substrate features and how such features affect the enzymatic hydrolysis will provide valuable guidance for overcoming biomass recalcitrance and economic compatible process design.

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Chapter 3 Application of High Throughput Pretreatment and Co-Hydrolysis System to Thermochemical Pretreatment. Part1: Dilute Acid

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3.1 Abstract

Because conventional approaches for evaluating sugar release from the coupled operations of pretreatment and enzymatic hydrolysis are extremely time and material intensive, high throughput (HT) pretreatment and enzymatic hydrolysis systems have become vital for screening large numbers of lignocellulosic biomass samples to identify feedstocks and/or processing conditions that significantly improve performance and lower costs. Because dilute acid pretreatment offers many important advantages in rendering biomass highly susceptible to subsequent enzymatic hydrolysis, a high throughput pretreatment and co-hydrolysis (HTPH) approach was extended to employ dilute acid as a tool to screen for enhanced performance. First, a single-step neutralization and buffering method was developed to allow effective enzymatic hydrolysis of the whole pretreated slurry. Switchgrass and poplar were then pretreated with 0.5 and 1% acid loadings at a 5% solids concentration, the resulting slurry conditioned with the buffering approach, and the entire mixture enzymatically hydrolyzed. The resulting sugar yields demonstrated that single-step neutralizing and buffering was capable of adjusting the pH as needed for enzymatic saccharification, as well as overcoming enzyme inhibition by compounds released in pretreatment. In addition, the effects of pretreatment conditions and biomass types on susceptibility of pretreated substrates to enzymatic conversion were clearly discernible, demonstrating the method to be a useful extension of HTPH systems.

Keywords: highthroughput, dilute acid, pretreatment, co-hydrolysis, biomass, yield

3.2 Introduction

Sustainable energy sources are needed to supplement petroleum use in light of limited reserves and growing energy demands, as well as to reduce the environmental impacts associated with production and combustion of these fossil fuels [1]. Lignocellulosic biomass, such as agriculture and forestry residues (e.g., corn stover and sawdust) and woody and herbaceous crops (e.g., poplar and switchgrass) [2], is recognized as a sustainable source of sugars that can be converted to biofuels and other biomaterials by a number of routes [3]. To produce biofuels economically, a large number of factors such as feedstock quality, conversion conditions, and catalyst loadings as well as their complex interactions must be better evaluated to identify combinations that can overcome the natural recalcitrance of biomass at the lowest cost [4, 5]. However, it is impractical to apply conventional testing for such purposes because it is slow, demands considerable labor, and requires larger sample sizes than may be available. Fortunately, high throughput pretreatment and enzymatic co-hydrolysis (HTPH) systems have been developed to considerably streamline these tests and allow evaluation of large number of combinations of variables effectively [6].

Several high throughput systems have been developed and applied to screen large biomass sample sets for sugar release from pretreatment and enzymatic hydrolysis, optimization of enzyme formulations, and more rapid biomass compositional analyses [6-10]. Such high throughput systems offer a number of important attributes in addition to the ability to process multiple samples quickly. For one, only milligram quantities of biomass are needed to complete a pretreatment and enzymatic hydrolysis reaction.

HTPH also lends itself to being highly automated and significantly reduce labor and time requirements [9]. In addition, some of the conditions employed are in fact more similar to those expected commercially than conventional approaches typically used for such tests, making the results more commercially relevant [7].

In most high throughput systems that target identifying favorable combinations of biomass types and pretreatment and enzymatic hydrolysis conditions, a "co-hydrolysis" method is applied [11]. In this approach, the entire pretreated slurry is directly subjected to enzymatic hydrolysis rather than separating the solid and liquid phases and washing the solids prior to enzyme addition. For application of co-hydrolysis to identification of the effects of hydrothermal pretreatment on substrate susceptibility to enzymes, low solids loadings (e.g., 1 to 2 wt%) and high enzyme loadings (e.g., 100 mg enzyme /g sugar in raw biomass) are generally used to minimize enzyme inhibition that could otherwise obscure differences in substrate digestibility [12-14].

Thermochemical pretreatments with dilute acid or base typically realize higher yields of sugars from hemicellulose and cellulose in the combined operations of pretreatment and subsequent enzymatic hydrolysis than possible with hydrothermal pretreatment [15]. Thus, it is desirable to be able to screen sugar yields from these pretreatments over a wide range of feedstocks and conditions. Dilute acid (2 wt% sulfuric acid) and base (0.025wt% NaOH) have been employed in an HTPH system [6], with buffering and neutralization accomplished by simply adding a stoichiometric amount of NaOH or HCl to the hydrolyzate before enzymatic hydrolysis. However, this approach resulted in a decrease in hydrogen ion concentration due to the ion-exchange

reaction between inorganic cations associated with the bound and free anions contained in wood and hydrogen ions in the applied solution [16]. This drop in acid concentration from neutralization was especially apparent at low pH and with small amounts of solution [16, 17]. Thus, neutralization capacity should be accounted for when buffering the hydrolyzate or subsequent enzymatic hydrolysis will suffer and give results that do not truly reflect differences in biomass recalcitrance. However, because titration of the hydrolyzate to adjust the pH is labor and time intensive, it is not practical for application to HTPH system. Therefore, an efficient and effective neutralizing and buffering method was needed to expand the range of applicability of HTPH to thermochemical pretreatment with dilute acid.

In this work, a novel buffering method was proven to successfully adjust the pH value of biomass slurries from dilute acid pretreatment to an appropriate range for co-hydrolysis. Then, dilute acid pretreatment followed by co-hydrolysis was applied to poplar and switchgrass in an HTPH format. Sugar release was measured for various pretreatment conditions and enzyme dosages to demonstrate that dilute acid HTPH can reproducibly screen performance over a range of conditions. Furthermore, favorable pretreatment and enzymatic hydrolysis conditions were identified to help select initial conditions for future studies. Finally, the dilute sulfuric acid HTPH system was employed to reveal differences in sugar release and recalcitrance of four Aspen samples that varied in age and composition, and the results were compared to those from hydrothermal pretreatment.

3.3 Materials and Methods

3.3.1 Biomass feedstocks

Two kinds of biomass, *Panicum virgatum* and *Populus trichocarpa*, more commonly known as switchgrass and poplar wood, were the primary substrates for this study. The switchgrass was from Pierre, South Dakota. The BioEnergy Science Center (BESC) provided the poplar which was then debarked, split, and chipped (Yard Machine 10HP, MTD Products Inc., Cleveland, OH). The resulting poplar wood chips and switchgrass were both knife milled (Model 4, Wiley Mill, Thomas Scientific, Swedesboro, NJ) through a 1 mm screen. After that, both materials were air dried for approximately one month followed by sieving to collect fractions with a particle size between 20-mesh (<0.85 mm) and 80-mesh (>0.180 mm) (RX-29, W.S. Tyler, Mentor, OH). Particles larger than 20-mesh were collected and sieved again, and the resulting 20-80 mesh fraction was mixed with the previously obtained 20-80 mesh fraction. The composition was analyzed according to NREL Laboratory Analytical Procedures [18]. As summarized in Table 3.1, the resulting switchgrass contained 32.4 % glucan and 21.2 % xylan. The poplar contained 46.5% glucan and 20.3% xylan.

Several Aspen (*P.tremuloides*) samples were also tested in this study. A cross-section of Trembling Aspen (*Populus tremuloides*) tree classified as 20-30 years in age was obtained from Benchmark International in Alberta, Canada [19]. The wood was debarked and fractionated into its individual annual rings that were labeled as 1 to 26 from pith to bark, according to the relative year in which that ring was formed. All sections were milled to pass through a 20-mesh screen (<0.85mm). The bark sample, as

well as samples 7, 11, and 14, were selected to use in this study because they offer a range of glucan, xylan, and lignin contents, as shown elsewhere [19].

3.3.2 Pretreatment in conventional tube reactors

Biomass was soaked in 0.5% (w/w) or 1% (w/w) sulfuric acid solutions at room temperature overnight to allow full penetration. To establish baseline performance at a 5% (w/w) solids concentration, the equivalent of 0.1 g of dry biomass in the soaked slurry was then added to conventional tube reactors along with enough of the appropriate acid solution to give 2 g total weight. These reactors were made from 150 mm lengths of 12.5 mm OD Hastelloy tubing with a 0.8255 mm wall thickness and stainless steel end caps (Swaglok, San Diego, CA). Each tube reactor had an internal volume of approximately 14 mL. Teflon plugs (McMaster-Carr, Santa Fe Spring, CA) were inserted in each end to avoid acid corrosion of the stainless steel caps. The tubes were heated in a 4-kW model SBL-2D fluidized sand bath (Techne, Princeton, NJ), as described elsewhere [20]. After pretreatment at 160℃ for 5, 10, 20, or 40 min, the reactions were quenched by submerging the reactors in room temperature water. The reactors were then opened, and 8 mL of deionized (DI) water was added to each reactor to dilute the hydrolyzate for subsequent enzymatic hydrolysis. All hydrolyzate was then collected in a 15 mL centrifuge tube (Corning Life Science, Fisher Scientific) and centrifuged for 10 min at 4,200 g (Allegra X-15R, Beckman Coulter, Fullerton, CA) to separate the solid and liquid. The liquid was collected for pH measurement.

3.3.3 Preparation of a buffer solution

1M citrate buffer was prepared by adding 1-4 mL of 37% (w/w) hydrochloric acid (Sigma-Aldrich, St-Louis, MO) to 40 mL 1M trisodium citrate (Sigma-Aldrich, St-Louis, MO) to produce a buffer solution for adjusting the pH of the hydrolyzate into the proper pH range following pretreatment. The buffering method was tested by adding 75 μL of the prepared 1M citrate buffer into 1350 μL sulfuric acid solutions that had pH values that mimicked the liquid resulting from dilute acid pretreatment. The final mixture corresponded to an approximately 0.05M final buffer concentration, and the pH value was determined.

3.3.4 pH measurements

All pH measurements were performed using a MI-414 Micro-combination pH electrode (Microelectrodes, Bedford, NH) coupled with a Core Module robotics platform (Freeslate, formerly Symyx Technologies, Sunnyvale, CA). The pH meter was calibrated using four standard buffer solutions with pH values of 2.0, 4.0, 7.0, and 10.0 (Fisher Scientific, Fair Lawn, NJ). A series of sulfuric acid solutions with gradient pH values from approximately 1.5 to 3.0 were prepared in 2 mL high recovery glass vials (Agilent, Santa Clara, CA, USA) loaded in a 6×8 rack on the robotics platform. Their pH was measured automatically by running a pre-coded program.

3.3.5 Dilute sulfuric acid pretreatment and enzymatic co-hydrolysis in HTPH system

Dilute sulfuric acid pretreatment and enzymatic co-hydrolysis were performed on all samples using a high throughput pretreatment and co-hydrolysis (HTPH) system described elsewhere [7]. The HTPH system is based on a 96 well plate format, but the wells are made of Hastelloy to withstand the temperatures and pressures of thermochemical pretreatment. HTPH pretreatments were performed at a 5% (w/w) solids loading with a total reaction mass of 90 mg in each well, corresponding to 4.5 mg of dry biomass. Biomass loading was accomplished with the solid and liquid dispensing robotics platform (Core Module Standard Configuration 2 equipped with Sartorius WZA65-CW balance, Freeslate, Sunnyvale, CA). Next, 85.5 μL of dilute sulfuric acid solution (either 0.5% (w/w) or 1% (w/w) concentration) was pipetted into each well with an 8 channel pipette (30-300 μL, Eppendorf North America, Hauppauge, NY). A flat pre-cut Silicone gasket (thickness 1.5875 mm, durometer hardness A40) was placed over the top of the wells to cover their openings, and the assembly was placed between bottom and top plates made of 304 stainless steel. The resulting "sandwich" was then clamped together using four 1/4 inch-20 threaded bolts (6.35 mm-20) placed in each corner of the two plates, with spring washers (flat load 1,500N) and wing nuts to allow rapid closing and opening [7]. After sitting at room temperature overnight, the plate assemblies were inserted into a custom-built steam chamber [7] for pretreatment at a temperature of 160^{o}C at times of 5, 10, 20, or 40 min. Upon reaching the target pretreatment time, the chamber was flooded with cold water to quench the reaction.

After the well plate was removed from the chamber and opened, 360 µL (8 channel pipette, 30-300 µL, Eppendorf) of DI water was added to each well to dilute the hydrolyzate and bring the total volume to 450 µL. Then, 32-35 µL of a mixture containing citrate buffer (1M, pH = 5.0), sodium azide, and enzymes (cellulase, xylanase, and β-glucosidase) was added to each well, depending on the enzyme loading. The final concentrations of citrate buffer and sodium azide were 0.05 M and 0.01 g/L, respectively. The total final reaction volume was 482 – 485 µl. The pH of the resulting hydrolyzate was adjusted to a range of 4.7 to 4.9 for enzymatic hydrolysis by addition of the prepared buffer mixture.

Cellulase (Spezyme CP®, protein concentration 116 mg/ml, activity 58 FPU/ml, Lot # 3016295230) and Multifect® xylanase (protein concentration 42 mg/ml, Lot# 4900667792), both from Genencor, a division of Danisco, now DuPont, Palo Alto, CA, were mixed at a protein ratio of 3:1. Three levels of enzyme loadings measured as mg cellulase+xylanase protein /g glucan+xylan in the original raw materials were employed: 75 mg+25 mg (low), 105 mg+35 mg (medium), and 135 mg+45 mg (high). These were supplemented with β-glucosidase (Novozyme®188, activity-665 CBU/ml) at an activity ratio of 1.5 : 1 (CBU:FPU) to enhance cellobiose hydrolysis. The activity and protein numbers assumed in this study were previously reported by Dien et al., (2008). After enzyme addition, the 96 well plate assembly was clamped shut again and held in an incubation shaker (Multitron Infors-HT, ATR Biotech, Laurel, MD) at 50^{o}C and 150 rpm for 72 h. All experiments were carried out in quadruplicate. After 72 h of enzymatic hydrolysis, the plates containing slurries in the individual wells of the well plate were

centrifuged for 10 min at 4,200 g (Allegra X-15R, Beckman Coulter, Fullerton, CA) using a 96 well plate carrier adaptor (Microplate carriers SX4750, VWR International, West Chester, PA) to separate the solids and liquid. 260 µL of the solids free supernatant liquid was then pipetted into 500 µL polyethylene HPLC vials for sugar analysis.

3.3.6 Sugar analysis

Sugar concentrations were measured by high performance liquid chromatography (1200 series, Agilent Technologies Inc., Santa Clara, CA). An Aminex HPX-87H column (BioRad, Hercules, CA) heated to 65^{o}C was used on a separation module (1200 series,) equipped with a refractive index detector (G1362A, Agilent Technologies Inc., Santa Clara, CA) and using 5 mM sulfuric acid as the mobile phase. For screening purposes, all sugars that fell under the xylose peak were included in the resulting xylose concentration, even though a minor amount of additional sugars such as mannose, fructose, and galactose may also have coeluted with the xylose.

3.4 Results and Discussion

3.4.1 Buffering method

A major challenge for thermochemical pretreatments in HTPH systems is adjusting the pH value prior to subsequent enzymatic hydrolysis, as well as eliminating or minimizing the effect of inhibitors released during pretreatment. Unlike hydrothermal pretreatment, due to the residual acid, the buffer capacity of the citrate buffer solution used in NREL Laboratory Analytical Procedures [21] is not enough to bring the hydrolyzates from dilute acid pretreatment to an appropriate range for enzymatic hydrolysis (data not shown). In previous work that demonstrated the concept of "co-

hydrolysis" on a 100 mL-scale [11], a 50 wt% NaOH solution was used to titrate slurries from pretreatment with chemicals such as dilute sulfuric acid to pH 5 prior to enzymatic hydrolysis. However, titration was tedious and would be too labor intensive and impractical for application to HTPH systems.

Herein, an alternative method was developed. First, pretreatment was performed with a 90 mg reaction weight at 5% w/w solids loading for sulfuric acid concentrations of 0.5% or 1%w/w, and the pretreated slurries were then diluted by adding 360 μL DI water to produce a 1% solid (~0.5wt% cellulose) concentration. Figure 3.1 compares the one-step neutralization and buffering method with the previous buffer method by titration. The major benefit of the new approach is two-fold. First, a higher solids loading that more closely mimics larger scale applications is used in pretreatment without increasing the actual amount of biomass required. Second, less acid was added, reducing the amount of buffering required to bring the hydrolyzate to an appropriate pH range. Third, dilution reduced the concentration of possible inhibitors to enzymatic hydrolysis. Trisodium citrate was chosen for a single step neutralization and buffering approach because of its wide buffering capacity as long as the ratio and concentration of conjugated acid-base pair is well-controlled [22]. According to the National Renewable Energy Laboratory Analytical Procedure (NREL-LAPs) [21], a suitable pH range for enzymatic hydrolysis of lignocellulosic biomass is 4.8 to 5.0, which falls in the buffering range of the conjugated pair of monosodium citrate(H_2A^-) and disodium citrate(HA^{2-}). To adjust pH in a single step, a buffer solution containing H_2A^- and HA^{2-} but with a higher pH than the final target value was added to the pretreated slurry. The excess

hydrogen ions (H^+) in the pretreated hydrolyzate adjusted the ratio of the conjugated pair and led to the desired pH for enzymatic hydrolysis. In this way, neutralizing and buffering were accomplished simultaneously.

3.4.2 Determining pH in pretreated hydrolyzates

As previously stated, a buffer solution containing monosodium citrate (H_2A^-) and disodium citrate (HA^{2-}) with a slightly higher pH than the final target pH was capable of neutralizing and buffering the slurries from dilute acid pretreatment. However, due to the complexity of the buffer system, the exact pH value required for the buffer solution must be determined empirically. Furthermore, the anions associated with inorganic cations in biomass can neutralize part of the mineral acid, further necessitating pH measurements rather than simply calculating the pH based on the original acid loading [16, 17]. To accomplish this, poplar and switchgrass were pretreated in tube reactors at various conditions, and the diluted hydrolyzate was then collected to measure the resulting pH values, as displayed in Table 3.1. For poplar pretreated at 160℃ for 5 to 40 min at a 0.5% (w/w) sulfuric acid concentration, the pH value of the diluted hydrolyzate ranged from 1.88 to 1.93, while at 1% (w/w) sulfuric acid loading, pH values varied from 1.66 to 1.69, depending on the pretreatment time. For switchgrass pretreated at the same conditions, the pH ranged from 2.00 to 2.06 and 1.66 to 1.69 for 0.5% (w/w) and 1% (w/w) acid concentrations, respectively. As demonstrated in Table 2, the pH value of the pretreated hydrolyzate was primarily determined by the original sulfuric acid loading. The type of biomass and pretreatment time had minor impacts on the final pH value. The diluted hydrolyzate from pretreatment with 0.5% (w/w) sulfuric acid tended to have a pH

value between 1.8 and 2.1, while that from pretreatment with 1% (w/w) sulfuric acid had pH values that usually fell between 1.6 and 1.7. These results provided a reference on how to prepare the proper buffer solution.

3.4.3 Testing and confirmation of the proposed buffering method

To prove that the proposed buffering method properly prepared the pretreated slurry for subsequent enzymatic hydrolysis, sulfuric acid solutions with pH values ranging from 1.5 to 3.0 were prepared to mimic the acidity of diluted hydrolyzate from pretreatment. All of the concentrations and volumes used were proportional to the actual conditions in the HTPH system but scaled up by factor of three for easy operation and testing. As such, 75 μL of 1M citrate buffer was well mixed with 1350 μL acid solution for pH measurement.

Table 3.3 shows the pH of buffer solutions A through E, sulfuric acid solutions, and the final mixture. In this table, mixtures with a pH value between 4.5 and 5.0 are highlighted in bold to indicate their suitability for enzymatic hydrolysis. The results suggest that for pretreated hydrolyzate solutions with a pH value higher than 2.15, a generic buffer (1M, pH = 4.5) had enough capacity to adjust the pH to the desired value when the final buffer concentration was 0.05M. For solutions with pH values ranging between 1.8 and 2.1, corresponding to the 0.5 % w/w sulfuric acid concentration in pretreatment, both buffer C (1M, pH = 4.85) and buffer D (1M, pH=4.55) effectively brought the final pH to a range appropriate for enzymatic hydrolysis. Although the pH value was slightly off, buffer C with acid solutions at a pH of 2.13 and buffer B (1M, pH = 5.09) with acid solutions at a pH of 1.66 were also effective. For solutions with a lower

pH range of 1.5 to 1.7, such as for hydrolyzates resulting from pretreatment with 1% w/w sulfuric acid, buffer B achieved a final pH between 4.7 and 4.9.

These results confirmed the neutralizing and buffering method developed in this paper was effective for diluted pretreatment hydrolyzates at 160℃ for 5 to 40 min with either 0.5% w/w or 1% w/w sulfuric acid concentrations on both switchgrass and poplar wood. Test results demonstrated the feasibility of the method, as well as the preferred buffer solution. Buffer C (1M, pH = 4.85) and buffer B (1M, pH = 5.09) were selected due to their ability to achieve a final pH close to 4.8 when 75 μL of buffer was added to 1350 μL of pretreated hydrolyzate over a range of pretreatment conditions . Table 3.3 can also serve as a reference when preparing buffer solutions, since 0.5% (w/w) and 1% (w/w) are two of the most commonly used sulfuric acid loadings for biomass pretreatment [23]. For lower acid loadings, such as 0.2% (w/w), a generic buffer solution (1M, pH=4.5) would be sufficient.

3.4.4 Results from dilute acid HTPH of switchgrass and poplar

After showing the neutralization and buffering concept worked effectively in adjusting and controlling pH for enzymatic hydrolysis, the performance of HTPH and larger scale conventional reactors were compared for application of dilute acid pretreatment and hydrolysis to poplar and switchgrass. In this case, both materials were pretreated at 160℃ with 0.5% w/w and 1% w/w sulfuric acid concentrations for 5, 10, 20, and 40 min, followed by co-hydrolysis at three enzyme loadings of 75 mg+25 mg (low - L), 105 mg+35 mg (medium - M), and 135 mg+45 mg (high - H) of cellulase+xylanase/g

glucan+xylan in the raw material [11] . The hydrolyzate was then diluted and buffered as described previously.

Figure 3.2a shows the glucan, xylan and total sugar yields from combined pretreatment and co-hydrolysis for switchgrass using 0.5%w/w acid for pretreatment. The highest glucan yield (99 ±2.0%) was achieved for pretreatment at 160 ℃ for 20 min with 0.5% acid, while the highest xylan yield (99 ±1.6%) and total sugar yield (98 ±1.9%) were for pretreatment at 160℃ for 10 min at enzyme loading of 135 mg+45 mg of cellulase+xylanase/g glucan+xylan in the raw material.

Figure 3.3a shows similar results for poplar wood. In this case, the highest glucan yield (84 ±1.5%) was for pretreatment at 160 ℃ for 40 min with 0.5% sulfuric acid, while the highest xylan yield (78 ±2.1 %) and total sugar yield (72 ±1.0 %) were from pretreatment at 160 ℃ for 10 min with 0.5% acid. In Figures 3.2b and 3.3b, it can be seen that increasing the acid loading to 1% w/w increased the glucan yield compared to the results obtained with 0.5% (w/w) acid. However, with 1% w/w acid loading at the same pretreatment temperature and time, xylan degradation began noticeable at 10 min and got progressively worse with increasing pretreatment time for both poplar and switchgrass.

Figures 3.2 and 3.3 also demonstrate that all three enzyme loadings resulted in very similar sugar yields for a given biomass and pretreatment condition. Furthermore, all three loadings allowed differentiation of performance between different pretreatment conditions, as well as between poplar and switchgrass.

3.4.5 Selection of pretreatment and enzymatic hydrolysis conditions for screening studies

The primary HTPH goal is to provide a rapid screening tool for initial indications of sugar release from different biomass-pretreatment-enzyme combinations in order to identify viable strategies to overcome biomass recalcitrance and improve sugar yields. For screening purposes, identifying biomass samples with high sugar yields at a sub-optimal pretreatment condition is usually favored because it not only minimizes sugar degradation and inhibitor production but also allows for differentiation between biomass samples with variable recalcitrance. Therefore, pretreatment at 160 ℃ for 5 to 10 min with 0.5% sulfuric acid was selected as a suitable screening condition. Acid loadings higher than 1% w/w are not recommended because hemicelluloses degradation can be quite high even at short pretreatment times, and very precise residence time control would be needed to maximize yields. Although only one pretreatment temperature, 160 ℃, was tested in this study, the time required to achieve similar yields at different temperatures can be estimated from the combined severity parameter [24]. For example, times of 20 to 40 min and 1.3 to 2.5 min would be estimated to give similar yields at 140 ℃ and 180 ℃, respectively, as the highest yield identified at 160^{o}C. However, the time required for pretreatment at 140 ℃ would not allow processing of large numbers of samples in a short period of time, while pretreatment at 180 ℃ must be performed with equipment capable of very rapid heating and cooling and tightly controlled residence times to avoid degradation of sugars. Considering all of these factors, pretreatment at160 ℃ for 5 to 10 min with 0.5% w/w acid was selected.

In selecting the enzyme loading for screening purposes, it is important to keep in mind that enzymatic hydrolysis in this study was performed in the same reactor as pretreatment, and dilute acid pretreatment generates inhibitors that hamper enzymatic hydrolysis [25, 26]. Due to this, a significantly higher enzyme loading was employed compared to conventional washed solids hydrolysis to offset the effects of potential inhibitors in the pretreated slurry as a result of not separating the solids and liquids following pretreatment [11, 27]. The higher loading will help ensure that enzyme activity is not the limiting factor in screening studies, regardless of sample variability and allow a clearer interpretation of sugar release data with respect to characteristics of the biomass samples [27]. As demonstrated by Figures 3.1 and 3.2, all three enzyme loadings, 75 mg+25 mg, 105 mg+35 mg, and 135mg+45mg cellulase + xylanase/g glucan+xylan in raw material gave similar trends in sugar yields, and all met the requirement of effectively converting cellulosic biomass to sugars without obscuring differences between performance with different biomass materials. Given that the three levels of enzyme loadings tested here gave similar trends in sugar yields at various pretreatment conditions, the low level enzyme loading was selected.

3.4.6 Application of dilute acid HTPH to Aspen wood rings

The primary application of HTPH systems is for screening large numbers of samples in order to select those with desired properties, such as lower recalcitrance and higher sugar yields. To evaluate the ability of dilute sulfuric acid HTPH systems to differentiate performance differences among samples, experiments were performed on four Aspen samples that differed in maturity and composition [19]. Pretreatment in the

multiwell plate was performed with a 0.5% sulfuric acid concentration at 160 ℃ for 5 min, and subsequent co-hydrolysis was carried out for 72 h at 50 ℃ with an enzyme loading of 75 mg+25 mg cellulase+xylanase/ g glucan+xylan in the raw material, as established previously. The results in Figure 3.4 show that sample 14, which was from the mature section of the tree and contained the most glucan and least lignin, gave the highest glucan yield of 82.6±3.3%. Sample 7, (juvenile wood) displayed the highest xylan yield of 97.2±5.0% of the four samples. When considering total glucan+xylan yields, samples 11 (90.0±2%) and 14 (88.3±1.9%) performed very similarly and slightly better than sample 7 (82.6±2%). The bark sample showed both the lowest glucan and xylan yields at 57.0±4.2% and 73.0±3.7%, respectively.

Figure 3.4 also compares results from dilute acid pretreatment at 160 ℃ with a 0.5%w/w sulfuric acid concentration for 5 min with previous work by our group for hydrothermal (water-only) pretreatment at 160 ℃ for 70 min [19]. The enzyme loading for both co-hydrolysis experiments was 75 mg +25 mg cellulase+xylanase / g glucan+xylan. Overall, the sugar yield trends are in good agreement. Furthermore, the HTPH results showed that dilute acid could reduce the pretreatment time from about 70 min for hydrothermal processing to about 5 min without sacrificing accuracy or obscuring differences between biomass samples. Sugar yields from the four Aspen samples demonstrated that dilute acid HTPH was capable of discerning differences in recalcitrance among samples.

3.5 Conclusions

A novel one step buffering and neutralizing method was developed and proven to simultaneously neutralize and adjust the pH value of slurries resulting from pretreatment prior to whole slurry enzymatic co-hydrolysis. By 1) concentrating the pretreatment slurry to a 5%w/w solids loading, 2) diluting it to 1%w/w solids for co-hydrolysis, and 3) adding a buffer solution with the appropriate pH, buffering and neutralization were possible within the limited volume of the HTPH reaction vials. This method allowed us to extend the HTPH concept to dilute acid pretreatment, thereby providing an additional tool to screen large numbers of biomass candidates and processing conditions to identify combinations that better overcome biomass recalcitrance and improve the economics of ethanol production from cellulosic biomass.

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Table 3.1 Glucan, xylan, and lignin contents in switchgrass, poplar wood, and aspen wood

	Switchgrass	Poplar	Aspen7*	Aspen11*	Aspen14*	Aspen bark*
Glucan	32.4	46.5	37.3	45.7	46.1	16.4
Xylan	21.2	20.3	15.3	17.4	17.8	8.8
Lignin	18.8	23.4	29.5	27.3	21.5	32.7

*Full dataset reported elsewhere (DeMartini and Wyman 2011.).

Table 3.2 pH value of diluted hydrolyzates produced by dilute sulfuric acid pretreatment of switchgrass and poplar.

Biomass	Acid loading (%w/w)	Pretreatment time (min)	pH of diluted Hydrolyzate	Buffer	pH after neutralization
Switchgrass	0.5	5	2.00	C	4.60
		10	2.00		4.60
		20	2.04		4.83
		40	2.06		4.90
	1	5	1.64	B	4.58
		10	1.66		4.60
		20	1.66		4.98
		40	1.68		5.01
Poplar	0.5	5	1.92	C	4.80
		10	1.93		4.82
		20	1.97		4.83
		40	2.09		4.90
	1	5	1.62	B	4.85
		10	1.63		4.88
		20	1.65		4.92
		40	1.66		4.92

Table 3.3 pH values of buffer, dilute acid solutions, and mixtures of the two*

	Acid	1	2	3	4	5	6
Buffer	pH	2.41	2.13	1.83	1.66	1.55	1.47
A	5.65	-	-	5.56	5.31	5.07	**4.76**
B	5.09	-	5.41	5.12	**4.88**	**4.63**	4.35
C	4.85	5.31	5.17	**4.89**	**4.65**	4.40	-
D	4.55	5.10	**4.96**	**4.72**	4.50	-	-
E	4.24	**4.66**	**4.57**	-	-	-	-

*Mixture: 75µL of 1M citrate buffer was mixed with 1350µL sulfuric acid solution

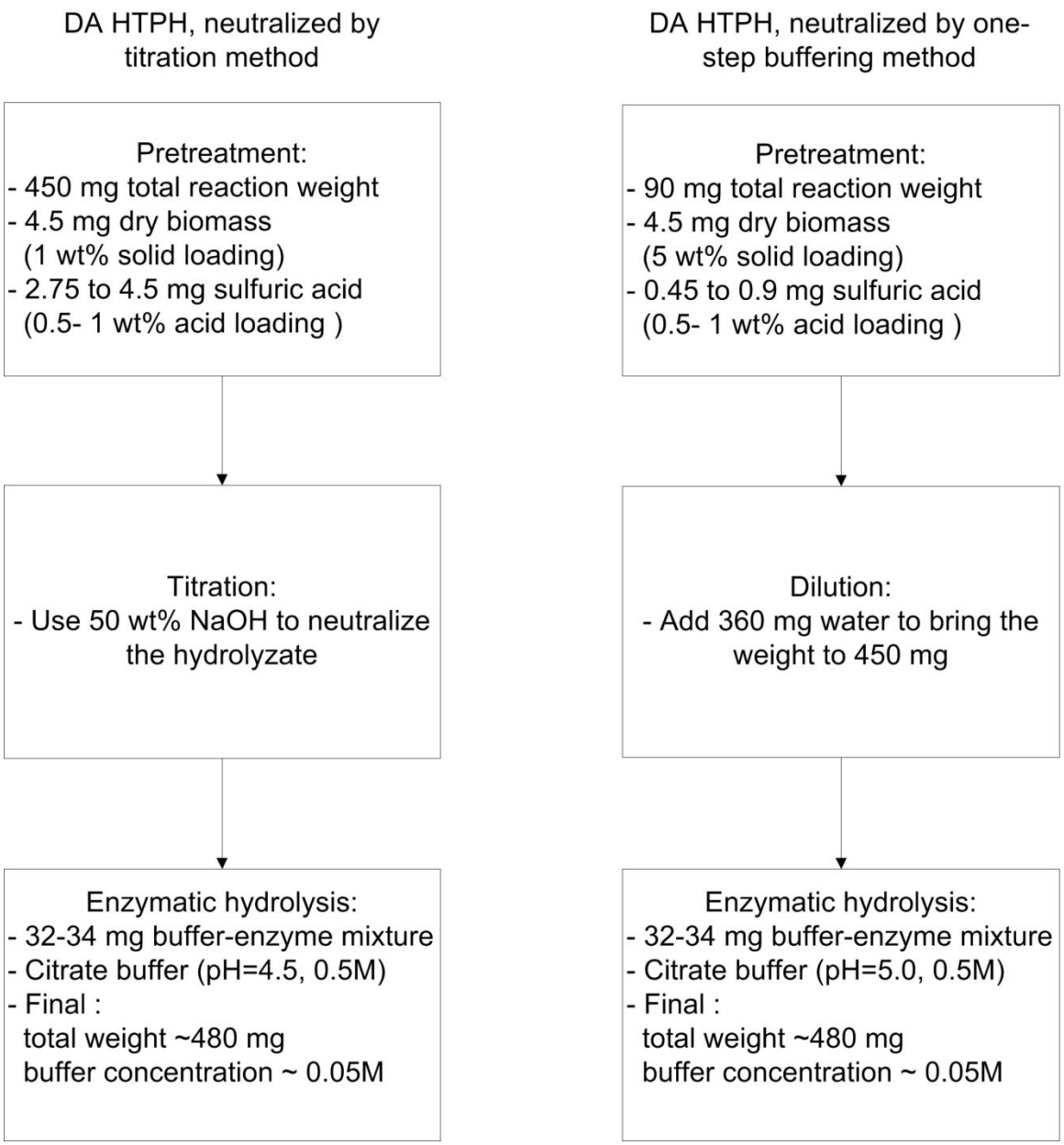

Figure 3.1 Flowchart of thermochemical pretreatment in HTPH system and the neutralization by titration or one-step buffer method.

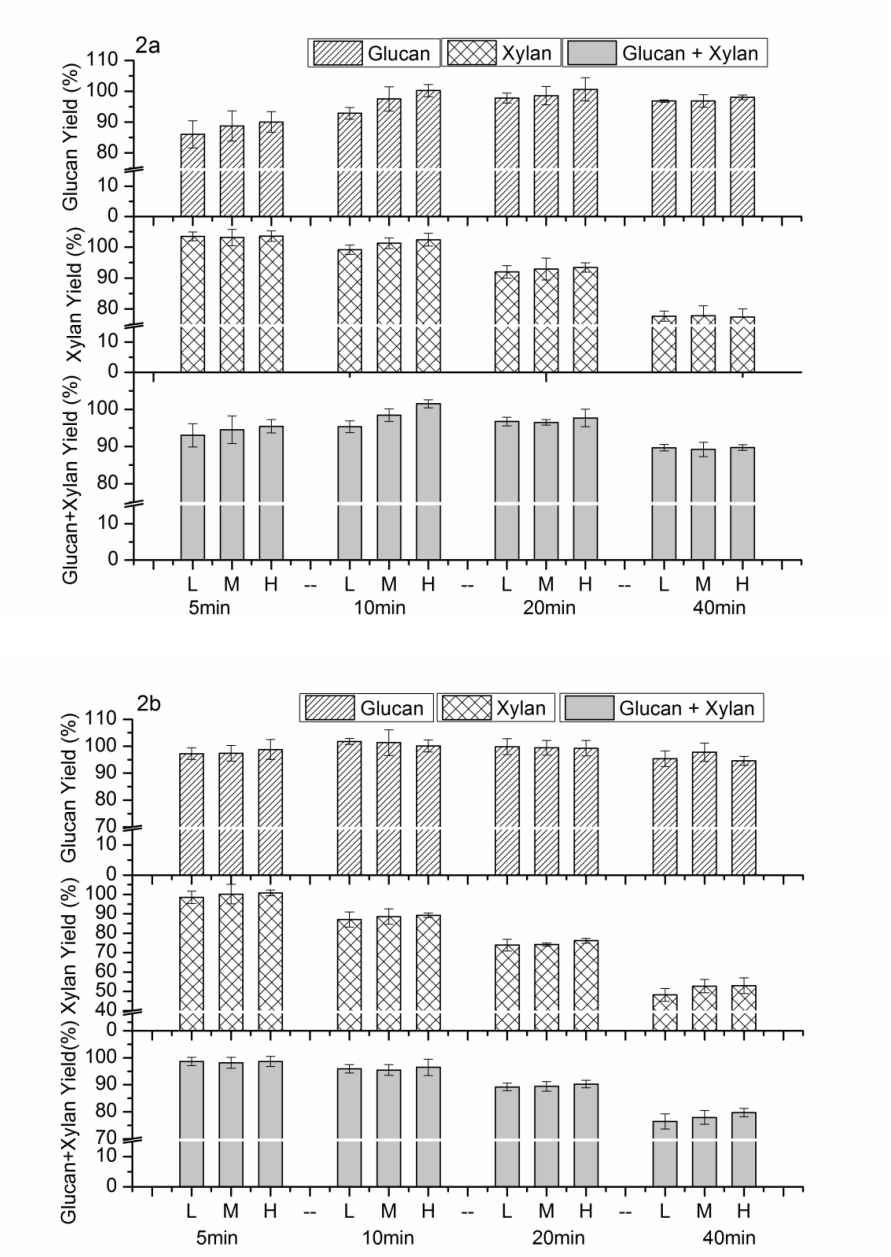

Figure 3.2 Glucan, xylan, and total sugar (glucan+xylan) yields at 5, 10, 20, and 40 min pretreatment from switchgrass at 160^{o}C with (a) 0.5% and (b) 1.0% (w/w) acid loading. L, M, and H represent the following enzyme loadings: Low-75+25 mg, Medium-105+35 mg, and High-145+35 mg of cellulase+xylanase/g glucan+xylan in raw material. The error bars represent the standard deviation of three replicates for the multi well-plate experiments

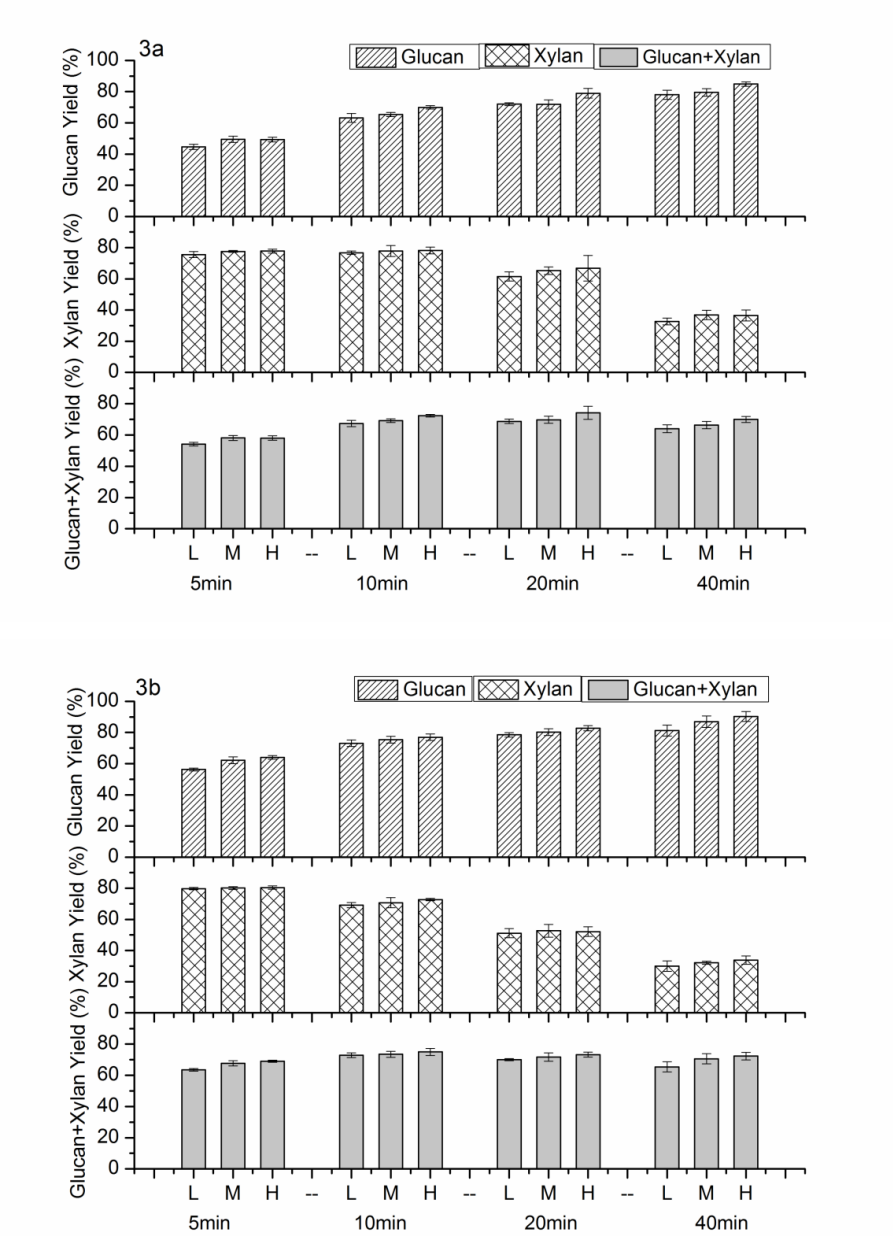

Figure 3.3 Glucan, xylan, and total sugar (glucan+xylan) yields at 5, 10, 20, and 40 min pretreatment times from poplar at 160 ^{o}C with (a) 0.5% and (b) 1% (w/w) acid concentration. L, M, and H represent enzyme loadings: Low-75+25 mg, Medium- 105+35 mg, and High-145+35 mg of cellulase+xylanase/g glucan+xylan in raw material. The error bars represent the standard deviation of three replicates for the multi well-plate experiments.

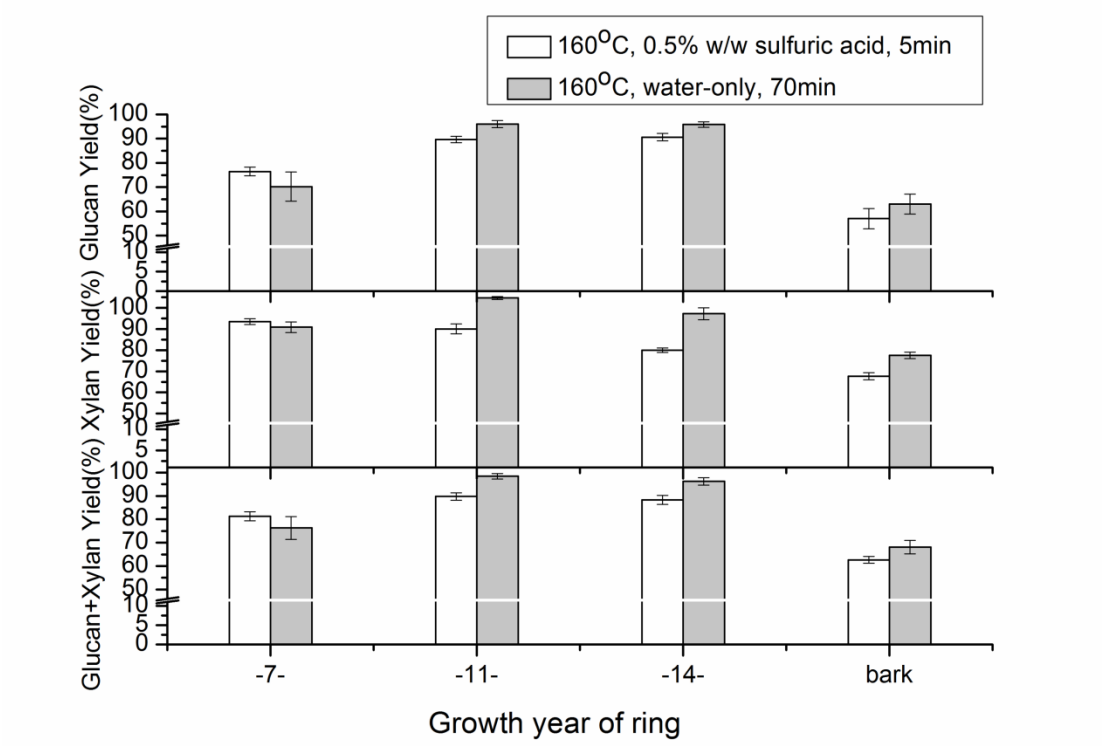

Figure 3.4 . Glucan, xylan, and total sugar (glucan+xylan) yields of aspen wood samples 7, 11, 14, and bark pretreated at 1608C with 0.5% (w/w) H2SO4 for 5min or 160 ^{o}C with water only for 70min. The enzyme loading for co-hydrolysis for both was 75+25 mg of cellulase+xylanase/g glucan+xylan in raw material. The error bars represent the standard deviation of three replicates for the multi well-plate experiments

Chapter 4. A Mild Two-stage Pretreatment Followed by Enzymatic Hydrolysis of Lignocellulosic Biomass for Producing Fermentable Sugars

This whole chapter will be submitted to the journal of "BioEnergy Research" or a similar journal under the following citation: Gao X, Kumar R, and Wyman CE. "A mild two-stage pretreatment followed by enzymatic hydrolysis of lignocellulosic biomass for producing fermentable sugars"

4.1 Abstract

Biomass recalcitrance is considered as the main hurdle to low cost conversion of lignocellulosic biomass to fuels, thus overcoming recalcitrance is essential to economically feasible and environmentally sustainable biomass conversion. Dilute acid and hydrothermal pretreatments have shown promise in removing hemicelluloses and increase glucan digestibility. However, conditions that maximize individual sugar yields do not usually occur at the same pretreatment conditions as those that maximize total sugar yields from both pretreatment and enzymatic hydrolysis. Herein, a mild two-stage pretreatment was applied, with low temperature dilute acid pretreatment in the first stage followed by hydrothermal pretreatment at higher temperatures in the second stage to achieve high glucan digestibility and total sugar yields from the combined pretreatment and enzymatic hydrolysis steps while keeping xylan degradation low. A high-throughput pretreatment and co-hydrolysis system (HTPH) was employed to screen for promising combinations of pretreatment conditions. Then identified conditions were applied in larger scale laboratory equipment to validate the results and provide more comprehensive comparisons of performance with the two-stage system to conventional one-stage pretreatment in terms of sugar yields and mass balance.

4.2 Introduction

Contraction of dwindling conventional petroleum reserves and their accelerating consumption coupled with environmental issues should encourage research and development of promising alternative energy resources [1]. Lignocellulosic biomass, including agricultural and forestry residues and herbaceous and woody crops, provides the only sustainable resource for large-scale and low-cost production of liquid fuels and organic chemicals [2, 3]. Many advances have been made in application of enzymes and microbes to convert lignocellulosic biomass into fermentable sugars that can in turn be fermented into liquid fuels and other valuable products [4, 5]. Although bioconversion of lignocellulosic biomass presents important opportunities for achieving low cost, it remains a challenge to overcome the primary obstacle to low cost fuels: the plant's natural resistance to deconstruction by enzymes or microbes [6, 7].

Pretreatment is an essential step in conversion of lignocellulosic biomass to sugars and applies chemical, thermal, or mechanical force to break down the physical barrier and/or alter the chemical structure and components of the plant cell walls, thereby making biomass more accessible to enzymes in a hydrolysis step[8, 9]. Dilute sulfuric acid pretreatment is one of the leading technologies in terms of combined cost and performance [10]. Employing 0.1 to 2.0 wt% sulfuric acid at temperatures of about 140 to 200^{o}C for residence times from 2 to 40 min effectively removes hemicellulose and recovers its component sugars at high yields while increasing cellulose digestibility for subsequent biological conversions such as enzymatic hydrolysis with cell free enzyme [11-13]. Pretreatment with just water, also known as hydrothermal pretreatment, is one

of the oldest approaches and relies on heating water to elevated temperatures (160-240 ℃) under pressure to provoke alteration of biomass structure[14, 15]. Similar to dilute acid pretreatment, hydrothermal pretreatment primarily solubilizes hemicellulose to make the cellulose more accessible to enzymes [16].

The ultimate goal is to achieve the highest possible sugar yields from the combined operations of pretreatment and subsequent enzymatic hydrolysis and not focus on yields from just one of the two unit operations. However, due to structural and chemical differences of glucan and xylan, conditions that maximize sugar yields from either pretreatment or enzymatic hydrolysis alone are usually different from conditions that maximize the yields of both these sugars from the two steps combined[17]. In fact, conditions that maximize sugar recovery from xylan are usually less severe than conditions that give high yields of glucose from cellulose/glucan [18], and yields of both sugars are compromised to maximize overall sugar yields when pretreatment is carried out in a single stage. Therefore, to address the contradiction between the relatively mild conditions appropriate to achieve high xylose yields and the more severe conditions needed to achieve high glucan digestion, two-stage pretreatment can increase overall yields of fermentable sugars from biomass. In line with this, relatively mild pretreatments have been applied to maximize recovery of xylan followed by harsher pretreatment to hydrolyze a portion of the glucan and increase the enzymatic digestibility of the glucan-enriched residues, as summarized in Table 4.1[18-26]. Application of such a two-stage strategy was beneficial to virtually full recovery of sugars from lignocellulosic materials.

Most of the two-stage pretreatment strategies developed involved use of dilute acid at high temperatures and short retention times, as summarized in Table 4.1. But these conditions can present problems, including side and degradation reactions to form products that reduce sugar yields and inhibit enzymatic and microbial action and requirements for construction of reactors from exotic materials of construction to resist corrosion at high temperature in the presence of dilute acid [27]. Only a few pretreatments applied relatively mild conditions, such as use of just water in both pretreatment stages[18, 26] and sulfuric acid combined with ethanol at temperature below 100 ^{o}C for pretreatment [23]. Unfortunately, such low-severity pretreatments suffer from low fermentable sugar yields and lower cellulose digestibility [27]. Yet, low temperatures and somewhat longer residence times can provide greater pretreatment flexibility in an alternate configuration to previous approaches [28]. For example, reducing materials-of-construction costs for pretreatment reactors would be one of the benefits of employing low severity conditions in pretreatment [28]. Therefore, to combine favorable attributes of two-stage pretreatment with low severity conditions and overcome their problems, a mild two-stage pretreatment was devised and demonstrated in this study.

In this work, a mild two-stage pretreatment was applied, with low temperature dilute acid pretreatment in the first stage followed by hydrothermal pretreatment at higher temperatures in the second stage. A novel high throughput pretreatment and co-hydrolysis (HTPH) system developed at UCR through the BioEnergy Science Center (BESC) program was applied first to screen for promising combinations of pretreatment

conditions based on the goal of achieving high glucan digestibility and total sugar yields from the combined pretreatment and enzymatic hydrolysis steps while keeping xylan degradation low. Then conditions identified with the HTPH system were applied in larger scale laboratory equipment to validate the results and provide more comprehensive comparisons of performance with the two-stage system to that possible by conventional one-stage pretreatment. Mass balances were also developed at the more promising conditions to validate the results.

4.3 Materials and Methods

4.3.1 Biomass feedstock

Switchgrass, *Panicum virgatum,* was from Pierre, South Dakota was employed for all the experiments in this study. It was knife-milled (Model 4, Wiley Mill, Thomas Scientific, Swedesboro, NJ) through a 1 mm screen. After that, all materials were air dried for approximately one month followed by sieving to collect fractions with a particle size between 20-mesh (<0.850 mm) and 80-mesh (>0.180 mm) (RX-29, W.S. Tyler, Mentor, OH). Particles larger than 20-mesh were collected and sieved again, and the resulting 20-80 mesh fraction was mixed with the previously obtained 20-80 mesh fraction. The composition of the switchgrass was 34.1 wt% glucan, 22.2 wt% xylan, and 19.6% lignin on a dry weight basis, and the moisture content in the material was about 5 wt%.

4.3.2 Enzymes

Cellulase (Spezyme® CP, BCA protein concentration 116 mg/ml, activity 58 filter paper units (FPU)/ml, Lot # 3016295230) and Multifect® xylanase (protein concentration 42 mg/ml, Lot# 4900667792), both from Genencor, a division of Danisco, now DuPont Biosciences, Palo Alto, CA, were mixed at a protein ratio of 3:1 for enzymatic hydrolysis. These were supplemented with β-glucosidase (Novozyme®188, activity-665 cellobiase unit CBU/ml) at an activity ratio of 1.5:1 (CBU:FPU), which earlier have been shown to be enough to alleviate cellulase inhibition by enhancing cellobiose hydrolysis [12, 29]. The activity and protein numbers assumed in this study were previously reported [30].

4.3.3 Screening by high throughput pretreatment and co-hydrolysis (HTPH)

A novel high throughput pretreatment and co-hydrolysis system (HTPH) developed previously at UCR [31]was employed for initial evaluation of the effect of various combinations of sulfuric acid concentrations, reaction temperatures, and reaction times summarized in Table 3.2 on pretreatment performance. The choice of conditions were based on reported work [32] and modified by several trial and error experiments. Figure 4.1 outlines the flowchart for the two-stage pretreatment approach employed when using laboratory scale batch reactor. However, two major changes were made in the operation of the HTPH system. For one, the solid–liquid separation step was accomplished by centrifugation and decanting in the HTPH system, versus filtration in batch reactor. The second was that enzymatic co- hydrolysis was applied directly to the solids from Stage 2 pretreatment without separation or washing.

HTPH pretreatments were performed at a 1 wt% solids loading with a total reaction mass of 450mg in each well, corresponding to 4.5 mg of dry biomass. Biomass addition to the HTPH wells was accomplished by a solid and liquid dispensing robotics platform (Core Module Standard Configuration 2 equipped with Sartorius WZA65-CW balance, Freeslate, Sunnyvale, CA). Next, 445 µL of 1wt% dilute sulfuric acid solution was pipetted into each well with an 8 channel pipette (30-300 µL, Eppendorf) [33]. A flat pre-cut silicone gasket (thickness 1.5875 mm, durometer hardness A40, McMaster, Los Angeles, CA) was placed over the top of the wells to cover their openings, and the assembly was placed between top and bottom plates made of 304 stainless steel. The resulting "sandwich" was then clamped together using four 1/4 inch-20 threaded bolts (6.35 mm-20) placed in each corner of the two plates, with spring washers (flat load 1,500N) and wing nuts to facilitate rapid closing and opening[31].

After sitting at room temperature overnight to assure thorough acid penetration, the plate assemblies were inserted into a custom-built steam chamber [31] for pretreatment at temperatures of 100 and 120 ℃. Upon reaching the target pretreatment time, the chamber was flooded with cold water to quench the reaction.

For the reaction performed at 60 ℃, a water bath (Polystat temperature controller, Cole-Parmer, Vernon Hills, IL) was used to heat the reactors up to that temperature and hold it there. The plate assemblies were placed in the water bath with the water level about 1mm lower than top of the vials to ensure good heat transfer and avoid leaking.

After Stage 1 pretreatment with dilute sulfuric acid at low temperature, the well plate was opened, and the plates containing slurries in the individual wells were

centrifuged for 10 min at 4,200 g (Allegra X-15R, Beckman Coulter, Fullerton, CA) using a 96 well plate carrier adaptor (Microplate carriers SX4750, VWR International, West Chester, PA) to separate the solids and liquid. 300 µL of the solids free supernatant liquid was then pipetted into 500 µL polyethylene HPLC vials for sugar analysis. To wash the solids, 300 µL of DI water was pipetted into each vial and then the amount of 300 µL was decanted from the solids following another centrifugation. This washing step was repeated 3-5 times until the pH of the liquid was neutral.

The washed solids from Stage 1 pretreatment were then subjected to Stage 2 hydrothermal pretreatment with just hot water. 300 µL of DI water was pipetted into each well, and the well plate was sealed as described previously. The total reaction volume for Stage 2 pretreatment was back to 450 mg as initial. Heating and cooling were with the steam chamber in the same manner as for Stage 1 pretreatment.

After the well plate was removed from the chamber and opened, 30 µL of a mixture of sodium citrate buffer (1M, pH = 5.0), sodium azide, and enzymes (cellulase, xylanase, and β-glucosidase) was added to each well. The final concentrations of citrate buffer and sodium azide were 0.05 M and 0.1 g/L, respectively. The total final reaction volume was 480 µL.

To overcome possible inhibition by compounds formed and released in pretreatment [29, 34], the enzyme loadings for co-hydrolysis were slightly higher than those normally applied: (75 mg cellulase+ 25mg xylanase) / (g glucan+xylan) in the original raw materials[31, 35]. The enzyme mixtures were supplemented with β-glucosidase at an activity ratio of 1.5 : 1 (CBU:FPU) to enhance cellobiose hydrolysis.

After enzymes addition, the 96 well plate assembly was clamped shut again and placed in an incubation shaker (Multitron Infors-HT, ATR Biotech, MD) for further reaction at 50 ℃ and 150 rpm for 72 h. All experiments were carried out in quadruplicates. After 72 h of enzymatic hydrolysis, the plates containing slurries in the individual wells of the well plate were centrifuged to separate the solids and liquid. 300 µL of the solids free supernatant liquid was then pipetted into 500 µL polyethylene HPLC vials (Cat. 98842, Grace Davavison Discovery Science, Dearfield, IL) for sugar analysis.

4.3.4 Pretreatment in Parr reactor

The reaction conditions summarized in Table 4.3 were selected based on performance in the HTPH screening experiments for pretreatment in a 1 L Parr reactor to confirm the HTPH trends and provide more complete analyses. The high pressure cylindrical Parr reactor employed was constructed of Hastelloy C (Parr Instruments, Moline, IL) and was equipped with a 3.5 in diameter helical ribbon impeller on a two-piece shaft driven by a variable speed DC motor to ensure a proper heat and mass transfer. A K-type thermocouple ((Extech Instruments, 421501) with a 1/8-in stainless steel probe (Omega Engineering Co., Stamford, CT) was mounted through the top of the reactor to monitor the inside temperature.

To pretreat a batch of switchgrass in the Parr reactor, aqueous slurries with 5 wt% switchgrass and 1 wt% sulfuric acid solution were allowed to soak at room temperature overnight prior to use. This presoaked slurry was loaded into the reactor, the vessel was closed tightly, and the helical impeller was set to an agitation speed of 200 rpm. The

reactor was suspended by a chain hoist mounted on a wall crane to allow adjustment of its position in a temperature controlled air fluidized sand bath (SBL-2D, TECHNE, Princeton, NJ) that was set to a temperature at least twice as high as the target reaction temperature. At the start of a run, the reactor was lowered into the sand bath for rapid heat-up of the agitated contents to the target temperature, with the heating time of approximately 2-3 min not included in the pretreatment reaction time. As the temperature inside the reactor approached the desired reaction temperature, the reactor was raised until its bottom was about 1 to 2 cm above the hot sand surface. The start of the reaction was defined at the time the temperature inside the reactor reached the target value. The temperature was maintained within ±2 ℃ of that target value by lowering or raising the reactor in the sand bath with the chain pulley system. If the inside temperature became higher than the target temperature, DI water was sprayed on the outer wall of the reactor to avoid overheating. Following pretreatment, the reactor was immediately transferred to a room temperature water bath for cooling. Once the temperature was below 50 ℃, the stirrer is turned off, and the reactor was opened [36].

The pretreatments run at 80 ℃ for 24 h employed a different strategy. In this case, the slurry was presoaked overnight in a 250 mL heavy wall pressure bottle and sealed with a silicon cap and 20 mm open center seals (Supelco, Bellefonte, PA). Three reactions were operated at the same time to obtain enough pretreated material for subsequent enzymatic hydrolysis and analysis. A gyrotory water bath shaker (Model G76Dm New Brunswick scientific Co. Inc., Edison, NJ) set at 81 ℃ with rotation speed at 200 rpm was used to heat the reactors and maintain temperature.

The solids from Stage 1 pretreatment were separated and collected by vacuum filtration through 12.5 cm diameter Whatman no.1 filter paper in a Buchner funnel. The solids were washed several times with DI water to remove chemical residues and free sugars. Solids and liquids were both stored at 4 ℃ until further analysis.

Stage 2 pretreatment was performed on the washed solids from Stage 1 following the same operations as described previously.

4.3.5 Enzymatic hydrolysis

In accordance with the National Renewable Energy Laboratory (NREL) Laboratory Analytical Procedures[37], enzymatic hydrolysis was conducted in triplicate in 50 mL Erlenmeyer flasks at a solids loading of 1 wt% glucan in 0.05M citrate buffer (pH = 4.9) with 1 mg/mL sodium azide. The slurries were incubated at 50^{o}C for 72 h in a shaker incubator (Multitron Infors-HT, ATR Biotech, MD) at 150 rpm. Enzyme loadings with cellulase to xylanase protein ratio of 3:1 were 60 mg total cellulase + xylanase protein / g glucan in the raw biomass. β-glucosidase was supplemented at an activity ratio of 1.5 : 1 (CBU:FPU). To determine the amount of sugar generated from enzymatic hydrolysis, 400 μL samples were drawn, filtered through 0.2 μm nylon filter vials (Alltech Associates Inc., Deerfield, IL), pipetted into 500 μL polyethylene HPLC vials, and then stored at 4 ℃ until analysis.

4.3.6 Compositional analysis of solid and liquid

All chemical analysis procedures applied in this work for determining solids composition and sugar concentrations in the liquid followed the well-established and

widely used laboratory analytical procedures (LAPs) of the National Renewable Energy Laboratory (NREL) [38, 39]. The major components of solids were determined by a two step acid hydrolysis. And the sugar concentration in liquid was determined by a 4 wt% acid hydrolysis, which converted all oligomers to monomers for quantification.

4.3.7 Sugar analysis

To determine sugar concentrations, liquid samples along with appropriate calibration standards were run on a Waters Alliance HPLC system (Model e-2695, Waters Corporation, Milford, MA) employing an Aminex HPX-87H column (Bio-Rad Laboratories, Life Science Research, Hercules, CA). Samples were processed at an eluent (5 mM sulfuric acid) flow rate of 0.60 ml/min using a refractive index (RI) detector (Model 2414, Waters Corporation, Milford, MA). The chromatograms were recorded and processed with Empower® 2 software (Waters Corporation, Milford, MA).

4.3.8 Sugar yields and mass balances

Yields of glucan, xylan, and the total of the two that were captured as monomers and oligomers in solution from Stage 1 and Stage 2 pretreatment and subsequent enzymatic hydrolysis were calculated as:

$$Gluan\ Yield\ (\%) = 100 \times \frac{(GH\ (g) + CB(g) * 1.053)/1.111}{Glucan\ \ in\ raw\ sample\ (g)}$$

$$Xylan\ Yield\ (\%) = 100 \times \frac{XH\ (g)/1.136}{Xylan\ \ in\ raw\ sample\ (g)}$$

$$Total\ Sugar\ Yield\ (\%) = 100 \times \frac{(GH\ (g) + CB(g) * 1.053)/1.111 + XH\ (g)/1.136}{Glucan + Xylan\ \ in\ raw\ sample\ (g)}$$

in which GH, CB, XH, represent glucose, cellobiose (if any), and xylose released from each step, and 1.053, 1.111, and 1.136 are the mass conversion factors for hydrolysis of cellobiose to glucose, cellulose to glucose, and xylan to xylose, respectively. The mass of sample used here was on a dry weight basis. The yield of solid samples left after Stage 1 and Stage 2 pretreatments was defined as:

$$Solid\ Yield\ (\%) = 100 \times \frac{Total\ dry\ solids\ after\ reaction\ (g)}{Total\ dry\ solids\ before\ reaction\ (g)}$$

And for mass balance, the sugar recovery was calculated as the following, the total sugar detected in all stream referred to all liquids from each step of pretreatment and enzymatic hydrolysis, and the sugar in solid residues after enzymatic hydrolysis:

$$Sugar\ Recovery\ (\%) = 100 \times \frac{Total\ sugar\ detected\ in\ all\ stream}{Total\ sugar\ in\ raw\ sample\ (g)}$$

4.4 Results and Discussion

For clarity in reporting these results, the terms Stage 1 and Stage 2 refer to pretreatment of switchgrass at low temperature with dilute acid (Stage 1) and at high temperature with just water (Stage 2), as illustrated in Figure 4.1. In addition, high temperature hydrothermal pretreatment was applied to switchgrass as a control. Thus, the two reactions labeled Ref 1 and Ref 2 in Table 4.3 were considered as only going through a single pretreatment. Monomers and oligomers of glucose and xylose released from each stage are reported in terms of the equivalent mass of glucan and xylan. All yields were normalized to the total potential glucan and xylan in the raw feedstock prior to pretreatment.

4.4.1 HTPH screening for favorable pretreatment conditions

Although the performance for two-stage pretreatment should be judged in terms of total sugar yields from Stage 1 and Stage 2 pretreatments plus from subsequent enzymatic hydrolysis, it would be a very tedious and inefficient to measure sugar yields from the numerous combinations of conditions by conventional laboratory scale batch reactors. Thus, a dilute acid high throughput pretreatment and co-hydrolysis system (HTPH) was first applied to screen promising combinations of acid concentrations, temperatures, and times for Stage 1 and Stage 2 pretreatments to identify those that gave the best yields. The 96 well-plate HTPH system developed by UCR through the BESC program allowed running 96 parallel reactions [40] for the two-stage pretreatment. But because the HTPH system was not originally designed for two-stage pretreatment and uses small quantities, interpretation of sugar yield data should focus more on identifying overall yield trends that point toward favorable conditions than on the actual sugar yields.

Stage 1 pretreatment in the HTPH system was performed at temperatures of 100 and 120 ℃ with 1, 2, and 5 wt% sulfuric acid. However, only 5wt% acid was employed for reaction at 60 ℃ because the reaction times to high yields were too long to be considered practical at lower acid concentrations. Then, the solids from all Stage 1 operations were washed and used for Stage 2 hydrothermal pretreatment in the HTPH system at 160 ℃ for 0, 5, 15 and 30min. Finally, 100 mg enzyme/g raw material was added to each well plate for enzymatic co-hydrolysis, as described in the Materials and Methods section.

Figures 4.2 a-d summarize the xylan and glucan yields from these combinations of two-stage pretreatments and co-hydrolysis. The yields from Stage 2 and co-hydrolysis are combined as yields from Stage 2 could not be measured for the HTPH system. As suggested by Figure 4.2a, more than 70% of the xylan could be released from Stage 1 pretreatment at the given conditions. Higher temperatures and acid concentrations generally increased xylan yields from Stage 1 pretreatment, but the more severe conditions, for example, 120°C with 5 wt% acid resulted in more degradation. When combined with appropriate Stage 2 pretreatment and co-hydrolysis, close to 100% of the xylan could be obtained in the liquid (data not shown here but more details of xylan release pattern will be discussed in later).

Figures 4.2b-d suggest that the total glucan yields from Stage 2 pretreatment coupled with co-hydrolysis were strongly influenced by the conditions applied in Stage 1 pretreatment. In general, more severe conditions in Stage 1 required less severe Stage 2 conditions to achieve good glucan digestibility. For Stage 1 pretreatment at 100 ℃ and 120 ℃, 1% and 2% sulfuric acid loadings followed by Stage 2 hydrothermal pretreatment at 160 ℃ were effective in making cellulose digestible. However, 5% sulfuric acid loadings at temperature above 100 ℃ increased sugar loses in the following stages (Figure 4.2c, d). For in Stage 1 pretreatment at 60 ℃ for 24hrs, only a 5 wt% acid concentration was applied in the HTPH system, with a resulting glucan yield of 78% (Figure 4.2b). Stage 2 hydrothermal pretreatment at 160 ℃ for 30min resulted in higher glucan yields from all solids than possible for shorter Stage 2 pretreatment times of 5 and 15 min when favorable Stage 1 conditions were applied.

4.4.2 Selection of pretreatment conditions

The primary goal of employing the HTPH system in this study was to rapidly screen for conditions that gave the highest total xylose plus glucose yields from the two - stage pretreatment strategy combined with enzymatic hydrolysis. In order to the highest possible xylan and glucan yields with less acid and lower sugar degradation, several conditions were selected and slightly altered for the larger pretreatment runs with the 1 liter Parr reactor, as summarized in Table 4.3. The use of 1 wt% sulfuric acid was selected for Stage 1 pretreatment at 80, 100, and 120 ℃ (X1, Y1, and Z1) because it effectively released xylan without noticeable sugar degradation in Stage 1 or Stage 2 pretreatments. Instead of using 60 ℃ with 5 wt% sulfuric acid as run in the HTPH system, 80 ℃ at a 1wt % acid loading was employed to apply the same acid loading at all three temperatures in Stage 1

Stage 2 hydrothermal pretreatment at 160 ℃ for 30min was applied to the solids from Stage 1. In addition, Stage 2 hydrothermal pretreatment of Stage 1 solids was also run at180 ℃ for 30min to determine if a higher temperature in Stage 2 would increase yields. All conditions run are summarized in Table 4.3.

4.4.3 Sugar yields of two-stage pretreatment combined with enzymatic hydrolysis

The glucan, xylan, and total sugar yields from Stage 1, Stage 2, and enzymatic hydrolysis at an enzyme loading of 60 mg enzymes protein/ g glucan in the raw biomass are presented in Figure 4.3 and Table 4.4. For the two-stage pretreatment performed here, which applied a low temperature dilute acid pretreatment in Stage 1 followed by hydrothermal pretreatment at higher temperatures in Stage 2,approximately 85% of the

potential xylan could be liberated and recovered from the Stage 1 plus Stage 2. When combined with enzymatic hydrolysis, close to 100% xylan yield was achieved. In studies of single stage pretreatment followed by enzymatic hydrolysis reported in the literature, the highest xylan yields usually did not coincide with the highest glucan yields, and neither one occurred at the same conditions as for the maximum total sugar yields [17]. However, application of a two-stage pretreatment strategy could realize very high glucan yields with little loss of xylan. For example, Stage 1 pretreatment at 80 ℃ with 1wt% sulfuric acid for 1440min followed by Stage 2 hydrothermal pretreatment at 180 ℃ for 30 min resulted in yields of 91.4% for glucan, 97.2% for xylan, and 93.9% for the sum of the two for the combined operations of pretreatments together with enzymatic hydrolysis (condition X2b in Figure 4.3 and Table 4.4). Thus, compared with conventional one stage hydrothermal pretreatment at the same high temperature of 180 ℃ for 30 min (Ref 2 in Figure 4.3 and Table 4.4), application of the low temperature with dilute acid in Stage 1followed by higher temperature reaction in Stage 2 achieved both high xylan and glucan yields.

Stage 1 pretreatment with dilute acid at low temperature not only improved recovery of xylan but also reduced the temperature required in high temperature Stage 2 pretreatment to achieve high glucan digestibility. As can be seen from the results with Stage 1 pretreatment over the range of 80 to 120 ℃ with 1wt% sulfuric acid followed by Stage 2 hydrothermal pretreatment at 160 ℃ for 30min combined with enzymatic hydrolysis (X2a, Y2a, and Z2a in Figure 4.3 and Table 4.4), the glucan, xylan and total sugar yields were all significantly higher than for one stage hydrothermal pretreatment at

160 ℃ for 30min followed by enzymatic hydrolysis. For the latter, the sugar yields suggested the conditions were too mild to overcome biomass recalcitrance, resulting in production of a limited amount of fermentable sugars. But applying a low temperature Stage 1 pretreatment before the 160 ℃ Stage 2 pretreatment, the glucan, xylan and total sugar yields were increased by approximately 30%, 50%, and 40% respectively. This suggested a possibility of lowering the pretreatment temperature but still achieve rational glucan digestibility. A similar enhancement in sugar yields was observed for Stage 2 pretreatment at 180 ℃ by comparing X2b, Y2b and Z2b with Ref 2 in Figure 4.3. The Stage 2 with a higher temperature was necessary for glucan digestibility, though Stage 1 removed large portion of xylan. The enzymatic hydrolysis was performed on solids from low temperature dilute acid pretreatment as Stage 1 with 1 wt% acid loading from 80 to 120 ℃ (X1, Y1, and Z1) directly with the same enzyme loadings as applied on solids from two-stage pretreatment, it turned out that glucan yields were very limited (30-55%), as shown in Table 4.4.

Stage 1 and Stage 2 pretreatment should be considered together with subsequent enzymatic hydrolysis to select conditions that result in the highest total xylan and glucan yields. Although a higher temperature Stage 1 pretreatment released more xylan from this stage, it did not necessarily lead to higher overall sugar yields. For instance, as seen in Table 4.4 and Figure 4.3, dilute acid pretreatment (1 wt% acid loading) at 100 ℃ (Y1) and120 ℃ in just Stage 1 pretreatment (Z1) resulted in a higher xylan yield than operation of Stage 1 at 80 ℃ (X1). However, when we integrate yields from Stage 2 pretreatment and from enzymatic hydrolysis with the results for Stage 1, the highest total sugar yields

were obtained from condition such as Stage 1 at 80 ℃ with 1wt% acid for 1440 min and Stage 2 at 180 ℃ with just water (X2b). Given same temperature at Stage 2 at 180 ℃, application of 100 ℃ for 200min (Y2b) and 120 ℃ for 150min (Z2b) to Stage 1 did not achieve as high sugar yields as X2b. This outcome could be due to the overall effect of the two-stage pretreatment being more harsh and generating inhibitors that negatively affected enzyme performance for hydrolysis [41, 42].

Comparing Figures 4.2 and 4.3 shows that sugar yields from the Parr reactor and HTPH system were not identical at the same operating conditions. These deviations may result from differences in solids and enzyme loadings for the two reactor systems, difficulties in solid-liquid separation between Stage 1 and Stage 2 pretreatment, and the necessity to apply the co-hydrolysis strategy for the HTPH system. It should be kept in mind that the HTPH system was not originally designed for multi-step reaction. But overall, the HTPH system gave comparable trend in sugar release as later obtained from 1L Parr reactor for same pretreatment conditions. And it helped to eliminate conditions that caused sugar losses. As a result, the HTPH system provided valuable information and reference in selecting conditions in the two-stage pretreatment.

4.4.4 Mass balance for two-stage pretreatment

A mass balance for two-stage pretreatment with Stage 1 at 80 ℃, 1wt% sulfuric acid for 1440 min, Stage 2 at 180 ℃ for 30 min with water only, and followed by enzymatic hydrolysis of 60 mg protein/g glucan in raw (X2b) was presented in Figure 4.4. On the basis of 100 g switchgrass containing 56.34g glucan and xylan, two-stage pretreatment combined with enzymatic hydrolysis released 52.8 g of fermentable sugars

into liquid expressed as equivalent glucan and xylan in the original biomass. 2.14g sugars remained in the solid residues. Overall, 97.7% of glucan, 97.3% of xylan were recovered in the mass balance. Total acid and water consumption were 20 g and 3470 g, respectively, at the condition given. Water used to wash the solids was not included. Mass balance of two-stage pretreatments performed in other conditions can be obtained from the sugar yields in Table 4.4 and solids yield as well compositions of residue in Table 4.5.

4.4.5 Considerations in implementation of the two-stage pretreatment strategy

Dilute sulfuric acid pretreatment is considered a promising option because of its high xylan recovery and ability to increase glucan digestibility [8]. However, handling its high corrosiveness at the high temperatures needed to be effective in single stage pretreatment requires exotic and expensive materials-of-construction for reactors. In addition, xylan is degraded at conditions that maximize recovery of total sugars in a single stage [17]. To overcome these limitations, a two-stage pretreatment strategy was applied, in which Stage 1 was performed at low temperatures (below 100 ℃) with dilute sulfuric acid, followed by hydrothermal pretreatment at higher temperatures (160-180 ℃) in Stage 2. A major advantage of this approach compared to single stage sulfuric acid pretreatment is to enhance glucan digestibility while also increasing xylan yields. Another benefit is to avoid use of dilute acid at high temperatures while achieving nearly complete xylan removal. Even though a high temperature second stage is needed to realize high digestibility of the pretreated solids, materials-of-construction that can accommodate hydrothermal pretreatment would be much less expensive [43]. In addition,

lower temperatures and associated pressures (160 to 180 ℃ = 617 to 1000 kpa) can be applied for Stage 2 pretreatment than would be needed for single stage hydrothermal pretreatment to realize the highest possible xylan yields (>200 ℃ =1554 kpa)[44], reducing the thickness and cost of reactors. It should also be kept in mind that it is far more challenging to pump solids against pressures needed at 200 ℃ vs. those associated with 160 to 180 ℃ operation. And about 12 to 23% less heat is needed for the latter than the former temperature. Even though dilute acid is required for the first stage pretreatment, the low temperatures and pressures close to or even below atmospheric can cut the materials-of-construction costs, with use of fiberglass or other quite inexpensive materials possible. Because the maximum in the yield vs. time curve is broader at lower temperatures, reactor control should be less challenging in applying low temperature Stage 1 conditions commercially. Finally, low temperature pretreatment with dilute acid avoids mixing acid effectively at high temperatures where degradation reactions become very important. It also overcomes corrosion/erosion concerns with high pressure solids feeders if acid is mixed with biomass prior to high temperature pretreatment. Although the reactions reported here were performed at low solids to accommodate limitations in small scale pretreatment reactors, experience of the senior author of this paper shows that similar performance can be expected at higher solids concentrations by using appropriate Pandia or other configurations [45-48]. However, a careful economic evaluation must be made to be sure improvements in yields, reactor materials-of-construction, solids feeders, vessel wall thickness, and other aspects can justify additional costs for a wash step between the two pretreatment stages and a larger Stage 1 low pressure vessel.

4.5 Conclusions

Performance data was developed for a two-stage pretreatment strategy that applied dilute acid pretreatment at low temperatures first to release mostly hemicellulose sugars followed by subjecting the solids to a high temperature hydrothermal pretreatment to increase glucan digestibility. A major advantage of this strategy was to obtain high yields of sugars from glucan and xylan from the overall two-stage pretreatment coupled with enzymatic hydrolysis and low sugar degradation during pretreatment. A HTPH system was employed to screen a wide range of Stage 1 pretreatment conditions to identify the most promising possibilities. More detailed data was then developed in a 1 liter Parr reactor to validate the findings and provide more detailed insights. As a result, Stage 1 pretreatment at 80 ℃ with 1wt% sulfuric acid for 1440 min followed by Stage 2 hydrothermal pretreatment at 180 ℃ for 30min proved to be a favorable combination of pretreatment conditions. When coupled with enzymatic hydrolysis at 60mg of protein / g glucan, 91.4% of the glucan, 97.2% of the xylan, and 93.9% of the total of the two was recovered from switchgrass as sugars in solution.

4.6 Acknowledgements

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Table 4.1 Summary of prior studies of two-stage pretreatment of biomass with dilute acid and/or hot water.

	Biomass	Stage1			Stage2		
		Temp (℃)	Time (min)	Sulfuric Acid loading (wt%)	Temp (℃)	Time (min)	Chemical loading (wt%)
Nguyen et al., 2000 [19]	70% white fir, 30% ponderosa pine	180,210	1.5-4	2.5	180,210	1.5-4	Sulfuric Acid, 2.5, 2.66
Kim , 2005 [20]	Hemlock sawdust	190	2.5	1.1	205,210	1.3-2.25	Sulfuric Acid, 2.6, 3.5
Kim et al., 2005 [21]	Mixed hemlock hog fuel/pin chips (85:15 dry wt)	190	2.5	1.1	210	1.9	Sulfuric Acid, 2.5
Boesch et al., 2010 [22]	Spruce (*Pica abies*)	190-210	3-7	1	210-220	2-10	1
Söderström et al., 2003 [25]	Spruce	180	10	0.5	180-220	2-10	1, 2
Söderström et al., 2002 [24]	Spruce	180-220	2.5-10	SO2, 3 wt%	190-220	2.5-10	SO_2, 3
Papatheofanous et al., 1995 [23]	Wheat straw	99.5±1	10-60	0.5-2.5N	81 ±2	90	2N H2SO4 in aqueous ethanol (62.5-87.5 EtOH v/v)
Yu et al., 2010 [26]	Eucalyptus	180-200	20	water	180-240	0-60	water
Lee et al., 2010 [18]	Coastal Bermuda grass	150, 160	60,30	water	170	60	water

Table 4.2 Summary of two-stage pretreatment conditions screened in the UCR high throughput pretreatment and co-hydrolysis (HTPH) system.

	Temp (℃)	Time (min)	Solid loading (wt %)	Sulfuric acid loading (wt %)
Stage1	60	1440	1	5
	100	200	1	1, 2, 5
	120	150	1	1, 2, 5
Stage2	160	0, 5 , 15, 30	<1	0

Table 4.3 Pretreatment conditions applied in 1 L Parr reactor

		Label	Temp (℃)	Time (min)	Solid loading (wt %)	Sulfuric acid concentration (wt %)
Two-stage pretreatment	Stage1	X1	80	1440	5	1
		Y1	100	200	5	1
		Z1	120	150	5	1
	Stage2	X2a,X2b	160, 180	30	5	-
		Y2a,Y2b	160,180	30	5	-
		Z2a, Z2b	160, 180	30	5	-
One stage pretreatment	-	Ref 1	160	30	5	-
		Ref 2	180	30	5	-

Table 4.4 Summary of sugar yields (% of maximum possible) 1 from each stage and from enzymatic hydrolysis at 60 mg protein/g glucan in raw biomass for two-stage pretreatment in the 1 L Parr reactor plus one stage hydrothermal pretreatment for the two controls.

		X1			**Y1**			**Z1**		**Ref1**	**Ref2**
	G	6.0			7.8			7.8		-	-
Stage1	X	55.6			79.0			78.9		-	-
	G+X	25.6			35.9			35.9		-	-
			X2a	**X2b**		**Y2a**	**Y2b**		**Z2a**	**Z2b**		
	G	-	1.8	2.7	-	1.5	3.1	-	1.5	3.4	5.4	6.6
Stage2	X	-	15.3	31.7	-	10.0	12.3	-	2.9	6.9	23.1	66.6
	G+X	-	7.1	14.2	-	4.8	6.7	-	2.1	4.8	12.4	30.3
	G	-	7.8	8.7	-	9.3	10.9	-	9.3	11.2	-	-
Stage1+2	X	-	70.9	87.3	-	89.0	91.3	-	81.8	85.8	-	-
	G+X	-	32.7	39.8	-	40.7	42.6	-	38.0	40.7	-	-
Enzymatic Hydrolysis	G	30.5	52.2	82.7	33.9	54.6	72.9	55.8	57.2	66.8	28.3	77.3
	X	12.3	27	9.9	7.8	11.6	6.4	6.5	12.6	5.8	15.9	15.8
	G+X	23.3	42.2	54.1	23.6	39.5	46.7	36.4	39.6	40.4	23.4	50.9
Total [2]	G	36.5	60.3	91.4	41.7	63.9	83.8	63.6	66.5	78.0	33.7	83.9
	X	67.9	98.9	97.2	86.8	100.6	97.7	85.4	94.3	91.6	39.0	82.4
	G+X	48.9	74.9	93.9	59.9	80.2	89.3	72.3	77.6	81.1	35.8	81.2

[1]Glucan (G), xylan(X) and glucan + xylan(G+X) yields were calculated as described in *Material and Methods* section

[2]Total: sugar yields from pretreatment (Stage 1, Stage 2) combined with enzymatic hydrolysis

Table 4.5 Summary of solid yields and compositions of raw switchgrass and solids following pretreatment of switchgrass in the Parr reactor at conditions defined in Table 4.3.

	Solid Yield (%)			Components, wt% dry basis		
	Stage1	Stage2	Total	Glucan	Xylan	K-Lignin
Raw	-	-	100	34.1	22.2	19.6
X1	73.6	-	73.6	44.0	14.2	24.1
X2a	73.6	83.4	61.4	54.0	10.3	25.6
X2b	73.6	70.0	51.5	58.9	4.6	26.5
Y1	72.3	-	72.3	45.7	12.5	25.5
Y2a	72.3	81.9	59.2	49.8	8.5	26.0
Y2b	72.3	70.6	51.0	57.0	3.0	27.1
Z1	59.2	-	59.2	52.9	6.3	29.4
Z2a	59.2	95.1	56.3	56.1	5.4	28.0
Z2b	59.2	87.3	51.7	56.1	2.9	30.0
Ref 1	81.5	-	81.5	39.4	21.4	20.0
Ref 2	58.5	-	58.5	50.3	10.1	24.0

Solid yield = 100% × Total dry solids after pretreatment (g)/Total dry solids before pretreatment (g)
Total solid yield = Stage1 solid yield (%) × Stage2 solid yield (%)

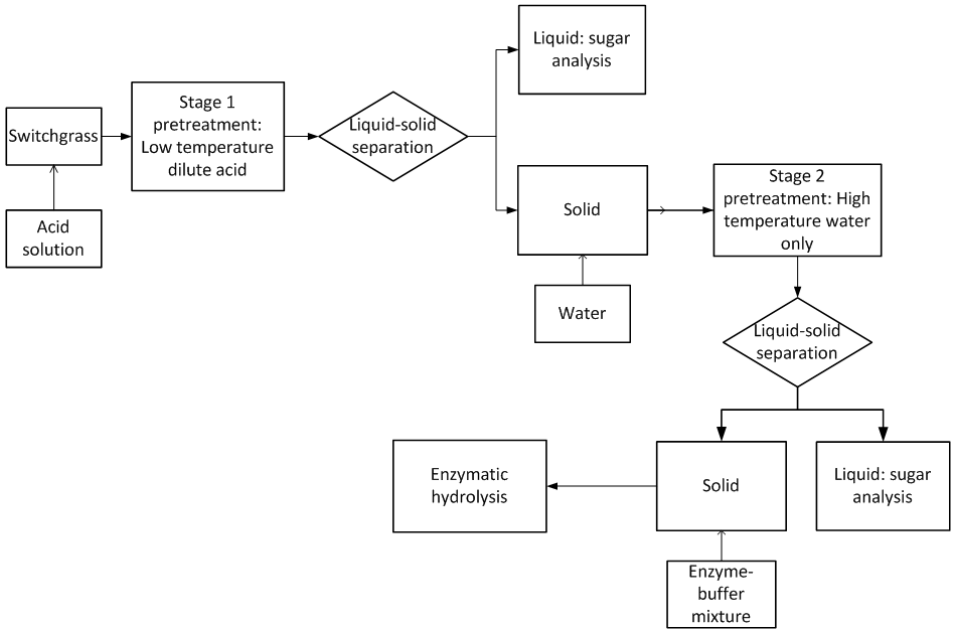
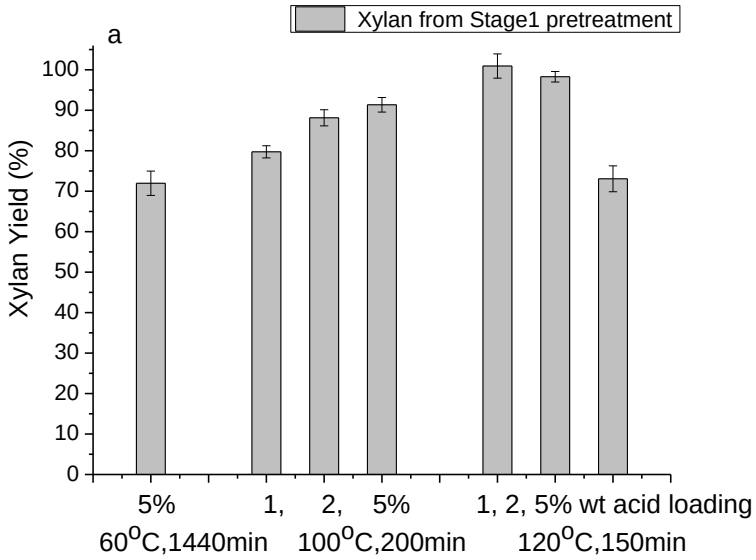
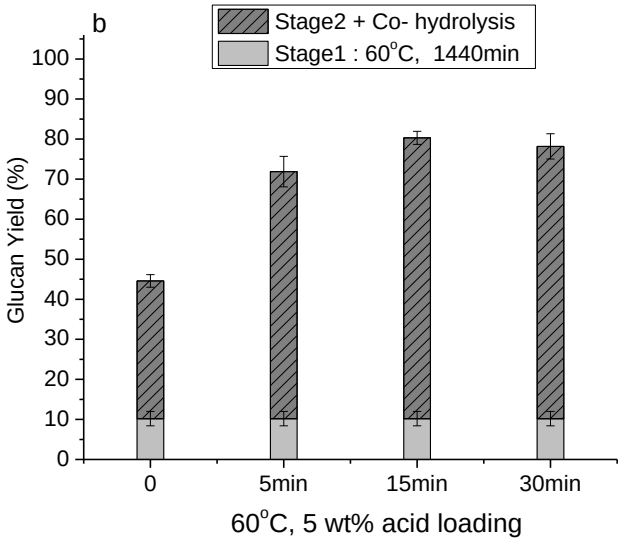


Figure 4.1 Flowchart of two-stage pretreatment and enzymatic hydrolysis approach applied in this study when using Parr reactor. For HTPH system, the liquid-solid separation between Stage 1 and Stage 2 pretreatment was replaced by centrifuge and decanting to remove liquid. And co-hydrolysis was performed after Stage 2 pretreatment thus no liquid- solid separation was performed in between.


a
Xylan from Stage1 pretreatment
Xylan Yield (%)
0
10
20
30
40
50
60
70
80
90
100
5%
1,
2,
5%
1, 2, 5% wt acid loading
60°C,1440min
100°C,200min
120°C,150min


b
Stage2 + Co- hydrolysis
Stage1 : 60°C, 1440min
Glucan Yield (%)
0
10
20
30
40
50
60
70
80
90
100
0
5min
15min
30min
60°C, 5 wt% acid loading

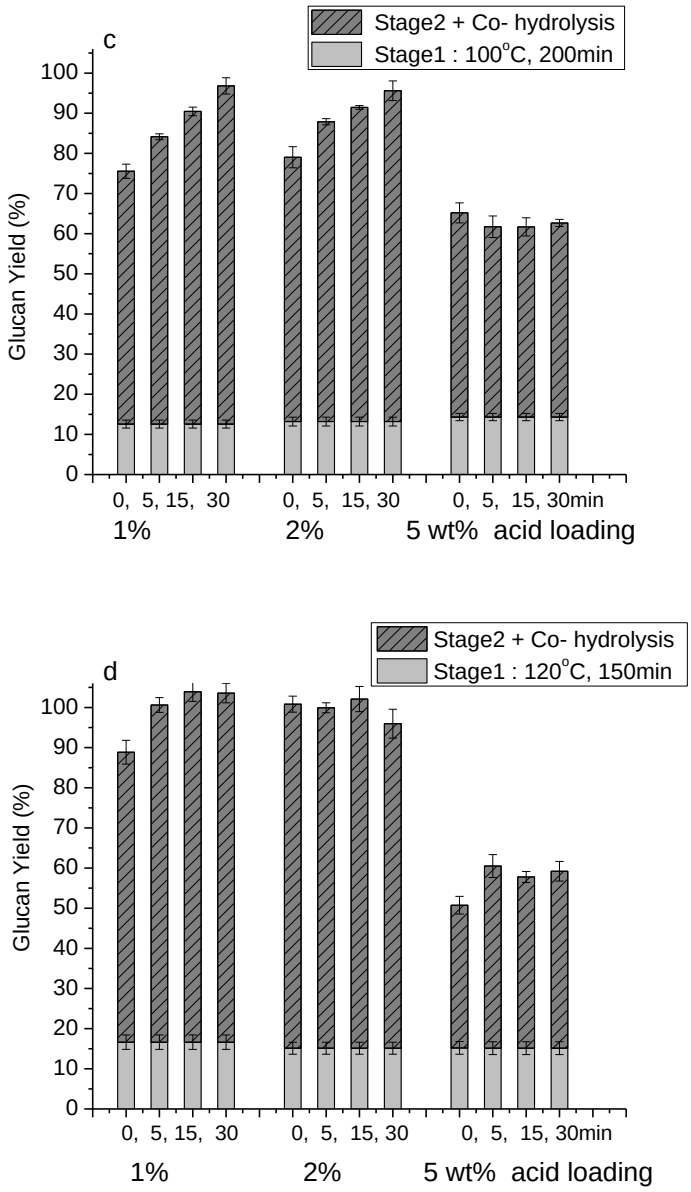


Figure 4.2 Sugar yields from HTPH system. a: Xylan yields from Stage 1 pretreatment at 60, 100, 120 ℃ with 1, 2, 5 wt% sulfuric acid performed in the HTPH system for 1440, 200 and 150 min. b, c, and d: Glucan yields from Stage 1 pretreatment at 60, 100, 120 ℃ , respectively, with 1, 2, 5 wt% sulfuric acid and Stage 2 pretreatment at 160 ℃ followed by enzymatic co-hydrolysis at 100 mg enzymes protein/g glucan in raw biomass. Error bars represent standard deviations of quadruplicates

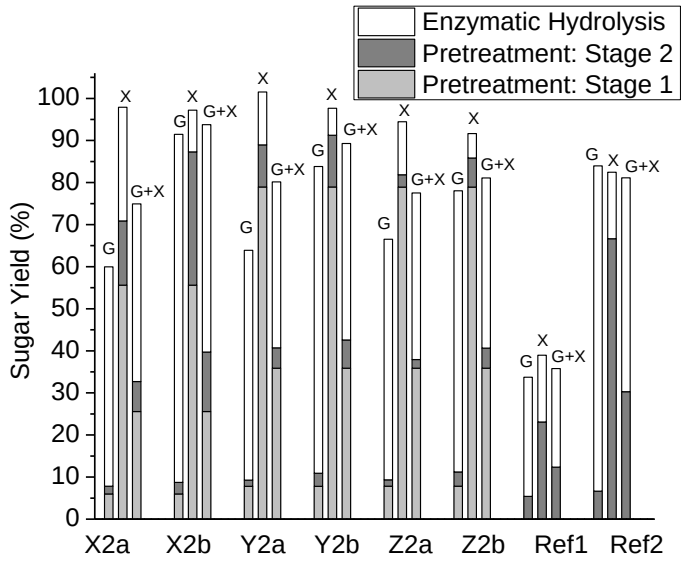


Figure 4.3 Glucan (G), xylan (X), and glucan+xylan (G+X) yields from two-stage (Stage 1 at 80, 100, 120 ℃ with 1 wt% sulfuric acid and Stage 2 at 160 and 180 ℃ for 30 min) vs. one-stage pretreatment at 160 and 180 ℃ for 30 min, followed by enzymatic hydrolysis with 60 mg enzyme protein/g glucan in the raw biomass

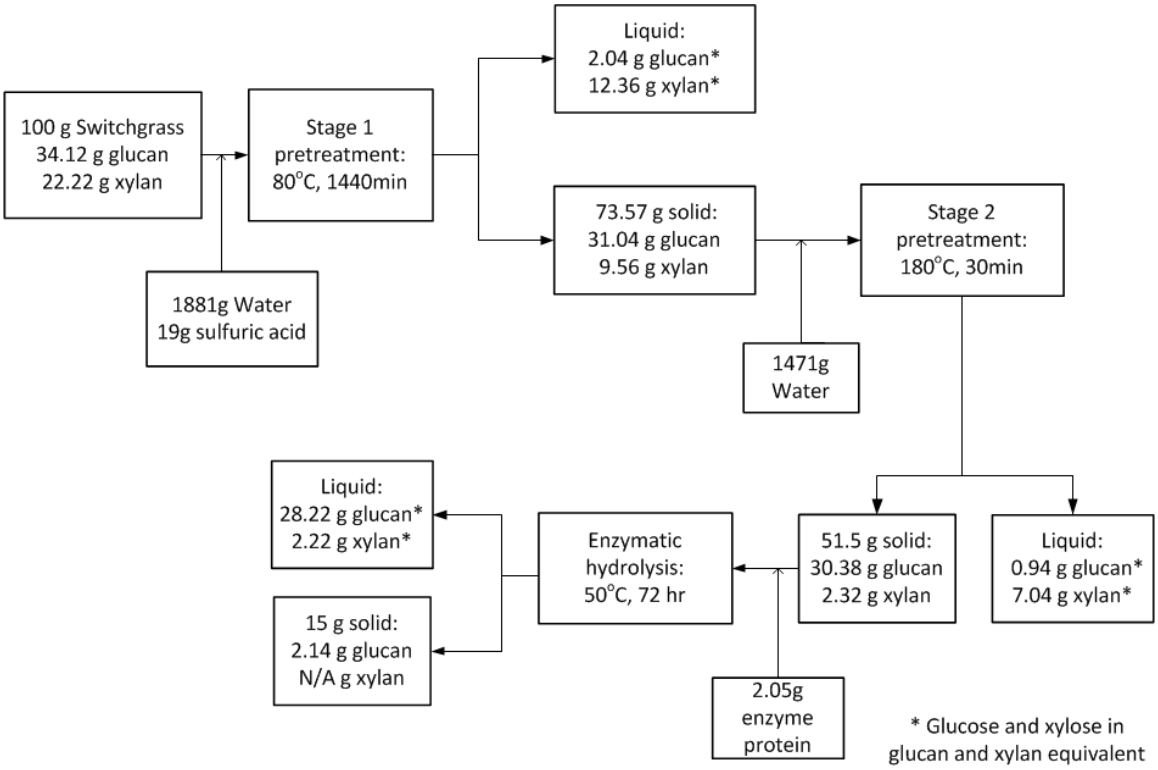


Figure 4.4 Mass balance of two-stage pretreatment with Stage 1 at 80 ℃, 1wt% sulfuric acid for 1440 min, Stage 2 at 180 ℃ for 30 min with water only, and followed by enzymatic hydrolysis of 60 mg protein/g glucan in raw. In liquid portion, the glucose and xylose were represented by glucan and xylan equivalent

Chapter 5. Understanding the Impact of Low vs. High Temperature Pretreatments on Enzymatic Digestibility of Switchgrass

This entire chapter will be submitted to "Bioresource Technology" or a similar journal with the following citation: Gao X, Kumar R, Pattathil S, Ma T, Pu Y, Ragauskas A, Hahn M, Ding S, and Wyman CE. "Understanding the Impact of Low vs. High Temperature Pretreatments on Enzymatic Digestibility of Biomass."

5.1 Introduction

Lignocellulosic biomass provides the only sustainable resource for large-scale and low-cost production of liquid transportation fuels and holds the potential to significantly reduce the world's dependence of petroleum [1-3]. The major barrier hindering cost effective conversion of lignocellulosic biomass to renewable fuels and chemicals is the plant's natural resistance to deconstruction by enzymes, microbes, or chemicals, which has been labeled as biomass recalcitrance [4]. The complexity of plant cell wall structures is responsible for biomass recalcitrance and limits efficient enzymatic hydrolysis, with such factors as the rigid structure of cellulose, the amorphous and complicated structure of both hemicellulose and lignin, and covalent and non-covalent interactions between polysaccharides and non-carbohydrate polymers` such as the lignin-carbohydrate complexes (LCCs) that shed on the cellulose microfibril all thought to play roles [5, 6].

Pretreatment is an essential step in reducing biomass recalcitrance and preparing the biomass for downstream biological operations. Among several options, thermochemical pretreatments are typically preferred for commercial utilization because of their ability to achieve high sugar yields with relatively low energy demands and capital costs [7-9]. Over the past few decades, various thermochemical pretreatments have proved capable of reducing biomass recalcitrance and increasing biomass accessibility to enzymes in a cost effective way [8, 10-12], with dilute sulfuric acid pretreatment being one of the most promising choices [9, 12].

Typically, dilute acid pretreatments employ about 0.1 to 2.0 wt% sulfuric acid at temperatures of about 140 to 200 ℃ for residence times from 2 to 40 min. At these conditions, a large part of the hemicellulose fraction of the biomass and a small fraction of lignin are solubilized, with a significant increase in enzymatic digestibility of the cellulose rich solids left behind [7, 13, 14]. Similar structural alternations of biomass are also observed for hydrothermal pretreatments that employ pressurized hot water at about 160-240 ℃ [15, 16]. In many ways, the mechanism of hydrothermal pretreatment appears very similar to that with dilute acid pretreatment, as both take advantage of proton-catalyzed cleavage of glycosidic bonds. For hydrothermal pretreatment, water dissociation increases with temperature, leading to an increase in the concentration of catalytic hydronium ions that, coupled with greater energy at high temperatures, catalyze hydrolysis of hemicellulose and some cellulose at favorable pretreatment conditions [17]. As a result, higher temperatures are typically needed for hydrothermal pretreatment to give similar high sugar yields as dilute acid [12]. A two-stage hydrolysis has also been employed in which dilute acid was first applied at relatively low temperatures followed by application of stronger acid at higher temperatures as a means to selectively remove hemicellulose and maximize yields of both the xylose and glucose[18].

Understanding the influence of pretreatment on modification of physiochemical properties of biomass and how they reduce recalcitrance and increase enzymatic digestibility could be very valuable in suggesting new routes to improve biomass conversion [6, 19, 20]. Although the improved digestibility of pretreated biomass has often been related to hemicellulose removal and/or lignin alteration for dilute acid and

hydrothermal pretreatments [13, 14], other concurrent physiochemical changes are also thought to contribute to reducing recalcitrance, including increased surface area and pore volume and melting and relocation of lignin[21, 22]. Many previous studies presented correlations between some compositional and structural features and biomass digestibility[23-27], but the complexity of plant cell wall structures and interdependence of structural and compositional changes resulting from pretreatment make it not yet possible to conclude which physicochemical alternations played the most important roles in reducing biomass recalcitrance, due to the. Moreover, conclusions often depend on the choice of substrate, pretreatment methods, and harshness of conditions employed.

In this study, switchgrass were processed in a two-stage pretreatment that employed low temperature dilute acid pretreatment first to remove and recover most of the hemicellulose sugars followed by hydrothermal pretreatment of washed solids from the first stage at higher temperatures to improve cellulose digestion. The mild conditions applied removed different levels of xylan and lignin, resulting in significant variability in the digestibility of the glucan (mostly cellulose) left in the solids. The composition and structural features including residual hemicellulose, substrate accessibility, and cellulose and lignin distribution on the surface were characterized for the solids from both pretreatment stages to find changes that could account for changes in enzymatic effectiveness. Correlations between enzymatic digestibility and compositional and structural changes of the substrate were sought to shed light on features that had the greatest impact on reduction of biomass recalcitrance in pretreatment. We also sought to understand how those features that had the greatest impact on recalcitrance were

controlled by temperature, time, and acid loading in thermochemical and hydrothermal pretreatments. The identification of key factors that influence biomass recalcitrance and how to control them can be invaluable in rational design of pretreatments that are effective in preparing biomass for biological conversion.

5.2 Materials and Methods

5.2.1 Switchgrass

The switchgrass, *Panicum virgatum,* used in this study was from Pierre, South Dakota. Upon arriving at our site, it was knife milled (Model 4, Wiley Mill, Thomas Scientific, Swedesboro, NJ) to pass through a 1 mm screen. After that, all materials were air dried for approximately one month followed by sieving to collect fractions with a particle size between 20-mesh (<0.850 mm) and 80-mesh (>0.180 mm) (RX-29, W.S. Tyler, Mentor, OH). Particles larger than 20-mesh were collected and sieved again, and the resulting 20-80 mesh fraction was mixed with the previously obtained 20-80 mesh fraction. The moisture content in the switchgrass was about 5 wt%.

5.2.2 Enzymes

Cellulase (Spezyme® CP, BCA protein concentration 116 mg/ml, activity 58 filter paper units (FPU)/ml, Lot # 3016295230) and Multifect® xylanase (protein concentration 42 mg/ml, Lot# 4900667792), both from Genencor, a division of Danisco, now DuPont Biosciences, Palo Alto, CA, were mixed at a protein ratio of 3:1 for enzymatic hydrolysis. These were supplemented with β-glucosidase (Novozyme®188, activity-665 cellobiase unit CBU/ml) at an activity ratio of 1.5:1 (CBU:FPU), which earlier have been shown to

be enough to alleviate cellulase inhibition by enhancing cellobiose hydrolysis [13, 28]. The activity and protein numbers assumed in this study were previously reported [29].

5.2.3 Pretreatment and enzymatic digestion of switchgrass

The pretreatment conditions applied along with label to designate each are summarized as Table5.1. All pretreatments were performed in a high pressure 1L cylindrical Parr reactor made of Hastelloy C (Parr Instruments, Moline, IL), with operations as described in previous reports from our group [30, 31].

To minimize the effects of end product inhibition on digestion yields that would otherwise blur following changes in substrate by pretreatment, enzymatic hydrolysis was conducted in triplicate at a solids loading corresponding to 1 wt% glucan, with other ingredients being 0.05M citrate buffer (pH = 4.9) and 1 mg/mL sodium azide in 50 mL Erlenmeyer flasks[32]. The slurries were incubated at 50 ℃ for 72 h in a shaker incubator (Multitron Infors-HT, ATR Biotech, MD) at 150 rpm. Enzyme loadings were 45 mg cellulase + 15 mg xylanase protein / g glucan in the raw biomass with β-glucosidase supplemented at an activity ratio of 1.5 : 1 (CBU:FPU) to enhance cellobiose hydrolysis and reduce its adverse effect on yields. To determine the amount of sugar generated during enzymatic hydrolysis, 400 μL samples were drawn, filtered through 0.2 μm nylon filter vials (Alltech Associates Inc., Deerfield, IL), pipetted into 500 μL polyethylene HPLC vials, and then stored at 4℃ until analysis.

5.2.4 Composition analysis

All chemical analysis procedures for determining solid compositions and sugar concentrations in liquid streams were according to the well-established and widely used laboratory analytical procedures developed by the National Renewable Energy Laboratory (NREL's LAPs) [33, 34]

5.2.5 Simons' staining

A modified Simons' stain assay was applied to raw and pretreated switchgrass according to reported procedures [35]. Orange (Pontamine Fast Orange 6RN) and blue (Pontamine Fast Sky Blue 6BX) dyes were obtained from Pylam Products (Garden City, NY). First, 1% w/v orange dye solution was prepared as the filtrate from passing the purchased dye through a 100 K ultrafiltration membrane (Millipore, Bedford, MA) employing an Amicon ultrafiltration apparatus (8200, Millipore, Bedford, MA) under 28 psi nitrogen gas pressure until 20% of the original solution was left. The 1% w/v blue dye solution was prepared by filtering the purchased solution through a 50 K membrane in Millpore filtration tubes (Millipore, Bedford, MA) to remove impurities. Then, 1.0 mL each of the orange and blue dye solutions retained in the filter was dried in a 50 ℃ vacuum oven for 5 days, and the solid residue was weighed to determine the concentration of the filtered solution. The corresponding result was then used to dilute the filtered orange dye solution to the concentration required (10 mg/mL) for Simons' stain test.

For the Simon's stain test, 100 mg of biomass samples was weighed into a 20mL serum glass bottle (Wheaton, Millville, NJ), followed by adding 1.0 mL of phosphate

buffered saline solution (pH=6, 0.3 M PO_4^{3-},1.4 M NaCl). Then, 10 mg/mL each of orange dye and blue dye solutions were added in increasing volumes (0, 0.25, 0.50, 0.75, 1.0, 1.5 mL) to each tube containing biomass samples and buffer solution to maintain 1:1 mass ratios of orange and blue dyes but at increasing concentrations. Following that, deionized (DI) water was added to each bottle to make the final volume 10.0 mL. Then the bottles were sealed with silicon caps and 20mm open center seals (Supelco, Bellefonte, PA). The bottles were incubated in a 70 ^{o}C gyrotory water bath shaker (Model G76Dm New Brunswick scientific Co. Inc., Edison, NJ) shaking at 200 rpm for 6 h. At the completion of this time, the supernatant was removed and centrifuged at 10,000 rpm for 8 min (Model 5424, Eppendorf North America, Hauppauge, NY). UV absorbance of the supernatant was measured with a SpectraMax M2e UV-Vis spectrophotometer (Molecular Devies, Sunnywale, CA) at 455 nm (wavelength1) and 624 nm (wavelength 2). The concentration of the orange and blue dyes in the supernatant was calculated using the following two equations (based on Lambert-Beer law for a binary mixture) [35]:

$$A1 = \varepsilon_{O1} L\, C_o + \varepsilon_{B1} L\, C_B$$

$$A2 = \varepsilon_{O2} L\, C_o + \varepsilon_{B2} L\, C_B$$

in which A1 and A2 are solution absorptions at 455 and 624nm; ε_{O1}, ε_{O2}, ε_{B1}, and ε_{B2} are extinction coefficients of orange and blue dye at 455 and 624nm, respectively, as determined by preparing standard calibration curves at each wavelength; L is the light path length of 1.0 cm; and C_O and C_B are the concentrations of orange and blue dye in the supernatant. The amount of dye adsorbed by biomass was calculated by deducting the

mass of dye in the supernatant from the mass of the dye initially added, with the total adsorption reported as mg of dye per gram of biomass.

The adsorption data was non-linearly fit to the following Langmuir equation:

$$[C_{ads}] = \frac{C_{max}.[C_f]}{1/K_{ads} + [C_f]}$$

in which $[C_{ads}]$ is the amount of adsorbed dye in mg dye/g substrate, $[C_f]$ is the free dye concentration in mg/mL, C_{max} is the maximum adsorption capacity in mg/g substrate, and K_{ads} is the adsorption constant equal to $[CS]/[C_f][S]$, where [CS] is the dye adsorbed substrate concentration in mg/mL, [S] is the dye-free substrate concentration in m g/mL [35].

5.2.6 Glycome profiling

Glycome Profiling is an ELISA-based method that applies plant glycan-directed monoclonal antibodies (mAbs) to identify cell wall carbohydrate components isolated from sequential extractions with increasingly harsh chemical reagents [36-38]. About 250 mg (dry weight) extractive-free sample for each of the raw and pretreated switchgrass samples were obtained by sequentially washed with absolute ethanol and acetone. The washed residues were dried in a vacuum oven overnight and subjected to extraction steps in 10 mg/mL suspensions on the starting dry biomass weight basis. First, 50 mM ammonium oxalate (pH=5.0) was used to suspend and incubate the bioass overnight with constant mixing at room temperature. After incubation, the mixture was centrifuged at 3400 g for 15 min. The resulting supernatant was decanted and saved as the oxalate fraction. Following the same protocol, the pellet was then subjected to

additional extractions using in turn 50 mM sodium carbonate (pH 10) containing 0.5% w/v sodium borohydride, and 1 M KOH, 4 M KOH, each containing 1% w/v sodium borohydride. The pellet remaining after the 4 M KOH extraction was then treated with sodium chlorite (100 mM) in order to breakdown lignin polymers into smaller components, as described previously[38]. In the end, the pellet left after the sodium chlorite treatment was subjected to a final extraction with 4 M KOH containing 1% w/v sodium borohydride to isolate material that had previously been secured within the walls by lignin (4 M KOH PC). No further analyze was performed on the resulting residual. The extracts from 1 M KOH, 4 M KOH, and 4 M KOH PC were neutralized with glacial acetic acid. All extracts were dialyzed against four changes of DI water (with an approximate sample to water ratio of 1:60) for 48 h at room temperature and then lyophilized. After estimating the total sugar contents of the cell wall extracts by the phenol-sulfuric acid method, the extracts were dissolved in DI water to a concentration of 0.2 mg/mL. Next, 50 μL each of all diluted extracts to the same sugar concentration of 20 μg/mL were loaded onto ELISA plates and allowed to evaporate overnight at 37 ^{o}C until dry. The ELISAs were performed as described using an array of 155 monoclonal antibodies specific to epitopes from most major groups of plant cell wall polysaccharides [38]. Negative controls consisting of water blanks without antigen were included in all assays and their absorbance subtracted from all samples. None of the monoclonal antibodies used here showed background in the ELISA assays. ELISA data are presented as heat maps in which antibodies are grouped based on a hierarchical clustering analysis of their binding specificities against a diverse set of plant glycans. Monoclonal antibodies

were obtained from the Complex Carbohydrate Research Center collection (available through CarboSource Services; http://www.carbosource.net).

5.2.7 Surface characterization by Tof-SIMS

The relative surface abundance of raw and pretreated switchgrass were obtained by Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS). Ground samples were pressed into thin pellets by a hydraulic press. Spectra were obtained by a ION-TOF TOF-SIMS V instrument (ION-TOF, Münster, Germany) equipped with a Bi liquid metal ion gun (LMIG) as a primary ion source and a O_2 sputter ion source[39]. The Bi_3^+ primary ion beam was rastered over the sample (500×500 μm^2) for positive spectra and charge compensation was subsequently applied using pulsing low-energy (25 eV) electrons onto the sample between successive primary ion pulses.

Three data points were acquired from each sample to reduce site specificity. Because secondary ion yields vary with the chemical environment of different samples, the relative intensity of the species was normalized as the sum of its primary ion fragments [40].

5.2.8 Bright field imaging

The raw and pretreated switchgrass samples were suspended in DI water at room temperature. The images were obtained by a bright field imaging microscopy with 20x lens.

5.3 Results

5.3.1 Compositions of solids following pretreatment of switchgrass

Table5.2 summarizes compositions of raw and pretreated switchgrass and xylan and lignin removal during each pretreatment. As shown, the pretreatments applied mainly removed xylan, correspondingly increasing glucan and lignin contents in the resulting solid residues compared to the raw material. Since the conditions were not harsh, xylan removal was incomplete but still greater than 70% from most of the solids. The exceptions were pretreatment at 80 ℃ with 1wt% acid for 1440 minutes (X1) that gave a slightly lower 57% xylan removal and hydrothermal pretreatment at 160 ℃ for 30 minutes (HT 1) that only removed 37.1% of the xylan. Lignin removal was limited from the solids produced by dilute acid or hydrothermal pretreatment, ranging from approximately 10 to 30%, depending on conditions. These changes are typical of dilute acid and hydrothermal pretreatments in many previous studies [23, 41].

Hemicellulose and lignin removal did not follow a consistent trend with pretreatment conditions, as shown in Figure 5.1. High xylan removal can be achieved by both low and high temperature pretreatment, as long as dilute acid and long enough reaction time and high was provided to low temperature pretreatment. In contrast, lignin removal for pretreatment with hot water or dilute acid was more dominated by temperature since lignin removal of more than 10% could only be achieved at high temperatures (160 or 180 ℃ in this case). For example, xylan removal from 160 ℃ hydrothermal pretreatment for 30 minutes (HT1) was much lower than for other conditions, but lignin removal was higher than for low temperature (80-120 ℃) dilute

acid pretreatment (X1,Y1, Z1). When the solids produced by low temperature dilute acid pretreatment (X1, 80 ^{o}C, 1 wt% acid, 1440 min) were subjected to hydrothermal pretreatment at 160 ^{o}C (X2a) or 180 ^{o}C (X2b) for 30min, additional lignin was removed.

5.3.2 Enzymatic digestibility of pretreated switchgrass

The enzymatic digestibilities, defined as the 72 h glucan yield from enzymatic hydrolysis at an enzyme loading of 45 mg cellulase +15 mg xylanase / g glucan in the original biomass with beta-glucosidase supplementation, are shown Figure5.2. For pretreatments at 80, 100, and 120 ^{o}C with 1wt % sulfuric acid (X1,Y1, Z1), glucan digestibilities were 33.5%, 36.8%, and 63.8%, respectively, all higher than for untreated material (14.4%) but to a different degree. When the solids from low temperature dilute acid pretreatment at these three conditions were subsequently subjected to hydrothermal pretreatment at a higher temperature of 160 ^{o}C for 30min, the glucan digestibility improved to varying degrees, as shown by X2a, Y2a and Z2a in Figure 5.2. A comparison of X1(80 ℃, 1 wt% acid, 1440min) with X2a (X1 subjected to 160 ℃, 30min) and Y1 (100 ℃, 1 wt%, 200min) with Y2a (Y1 subjected to 160 ℃, 30min) shows that the glucan digestibility increased by close to 30%. However, hydrothermal pretreatment of Z1 (120 ℃, 1 wt%, 150min) at 160 ℃ for 30min did not enhance the digestibility very much. Application of a higher temperature of 180 ℃ for hydrothermal pretreatment of the solids resulting from low temperature dilute acid pretreatment increased digestibility more. For example, as shown for X2b in Figure 5.2, subjecting the solids from dilute acid pretreatment with 1 wt% acid at 80 ℃ to 180 ℃ hydrothermal pretreatment increased the glucan digestibility to 92.9%, 59.4% higher than without the

second stage (X1) and 33.6% higher than from second stage hydrothermal pretreatment at 160 ℃ (X2a). Hydrothermal pretreatment of switchgrass at 160 ℃ for 30min (HT1) by itself resulted in a relatively low glucan digestibility of only 30%. While temperature in hydrothermal pretreatment was180 ℃ for 30 minutes (HT2), it gave a higher glucan yield of 85.1%.

5.3.3 Digestibility vs. xylan and lignin removal

Figure5.3 shows enzymatic digestibility of solids from the different pretreatments plotted against xylan (Figure 5.3a) and lignin (Figure 5.3b) removal. The overall trend shows that glucan yields increase with both xylan and lignin removal. However, it is also apparent that glucan digestibility varies significantly for similar amounts of xylan removal, as shown for pretreatment at low temperature with just dilute acid, low temperature with dilute acid followed by high temperature at 160 ℃ with water, and high temperature hydrothermal pretreatment at 180 ^{o}C (points Y1, X2a, and HT2, respectively). On the other hand, digestibility displays a much more nearly monotonic trend with lignin removal for all points except from hydrothermal pretreatment at160 ℃ (HT1).

5.3.4 Glycome profiling

To better understand structural differences of residual hemicellulose of solids produced at different pretreatment conditions, samples with different digestibilities at almost identical xylan removals (Y1, X2a, and HT2 in Figure 5.3) were characterized by glycome profiling. The sample from low temperature (80 ^{o}C) dilute acid pretreatment (X1) was also characterized because of its similar digestibility to that for Y1 despite

having 20 % less xylan removal and 5% more lignin removal. Raw switchgrass was also characterized for comparison.

The glycome profiling technique employed glycan-directed monoclonal antibodies (mAbs) to monitor the structure and extractability of non-cellulosic wall polysaccharides to help reveal structural differences in hemicellulose residues produced by application of different pretreatment conditions to switchgrass. As shown in Figure5.4, pretreatment at all conditions resulted in several discernible changes compared to untreated switchgrass. First, pectin and arabinogalactan epitopes recognized by the AG, RG-I/AG and pectin backbone antibody groups were reduced significantly. Second, the loss of almost all lignin-bound arabinogalactan and xylan epitopes in the chlorite extract suggested that pretreatment disrupted these lignin-carbohydrate associations in the cell wall. These observations are consistent with results from previous hydrothermal pretreatment of poplar wood [37]. There were no substantial differences in the structure of the residual hemicellulose for all four pretreated samples, and only the strength of epitope binding to antibodies had the decreasing trend of HT2<X1, Y1< X2a.

5.3.5 Simons' staining

Simons' staining is semi-quantitative method based on the competitive adsorption of two dyes (orange and blue) to estimate the available surface area of lignocellulosic substrates [42, 43]. An orange dye with larger size and stronger affinity penetrates larger pores and displaces blue dye with smaller size but weaker affinity for cellulose hydroxyl groups [44]. The ratio of maximum adsorption capacities of orange and blue dyes (O/B ratio) provides an estimate of the surface accessibility of porous substrates.

In this study, Simons' staining was applied to the same samples characterized by Glycome profiling, that is, samples with almost identical xylan removal but large differences in digestibility (Y1, X2a, and HT2) and the sample with similar digestibility to that for Y1 but different levels of xylan and lignin removal (X1). Figure 5.5 shows dye adsorption of raw and pretreated switchgrass and a plot of enzymatic digestibility against the O/B ratio. Figure 5.5a shows that orange dye adsorption increased, blue dye dropped slightly, and total dye adsorption increased for the pretreated samples compared to results with untreated switchgrass. The increase in O/B ratio from 0.8 for raw switchgrass to 1.4-1.8 for the pretreated samples suggests that more orange than blue dye adsorbed to pretreated biomass. Figure 5.5b shows that substrates with higher digestibility also displayed higher O/B ratios at the same extent of xylan removal.

5.3.6 Tof-SIMS

Because of its unique ability to analyze solid surfaces, time of flight secondary ion mass spectrometry (Tof-SIMS) was employed to obtain information about relative cellulose contents on the surface [45]. Figure 5.6 shows the relative cellulose content on the surface and its relationship to enzymatic digestibility of glucan in raw and pretreated biomass. The increased ion counts of cellulose suggest that more cellulose was exposed to the surface by pretreatment. In addition, the change in surface morphology and chemistry may permit higher yields of secondary ion fragments from the biomass surface [45].

5.3.7 Imaging

Light field microscopy imaging provided a visual comparison of the change in morphology of raw and the pretreated samples. As shown Figure 5.7, the surface appearance of all samples was similar, but the darkness appeared different. The untreated raw material appeared clean and transparent, but pretreated samples became darker in the following trend: raw switchgrass < X1, Y1 < X2a < HT2. Samples subjected to higher temperatures in two-stage (X2a,) or one stage hydrothermal (HT2) pretreatment showed darker colors than samples from low temperature 1 wt% acid pretreatment at 80 ℃ and 100 ℃ (X1, Y1). The darkness was likely caused by migration and relocation of lignin from the cell wall [46].

5.4 Discussion

Dilute acid and hydrothermal pretreatments both employ hydronium ions catalyzed reactions to cleave linkages between polysaccharides and lignin-carbohydrate complexes, fully or partially releasing hemicellulose and lignin [16, 47]. The main parameters used to describe such pretreatments are temperature, H^+ concentration, and residence time. Kinetic models have been built around these three parameters, such as use of severity factor or combined severity factor to describe the effect of pretreatment conditions on release of major components and enzymatic digestibility of the remaining solids [48-51]. However, it was also found that sugar yields from enzymatic hydrolysis of pretreated solids cannot be reliably predicted by the calculated severity factor [52]. The complex structure of the biomatrix in plant cell walls responsible for biomass recalcitrance makes modeling and prediction via one-dimensional severity unreliable.

Temperature and acid appear to act differently in pretreatment with the result that their effects are not totally interchangeable.

Multiple structural and compositional substrate features have been proposed to affect biomass recalcitrance and enzymatic digestibility [6], and contrary conclusions were sometimes drawn from experiments using different biomass types and pretreatment methods and conditions, further reflecting the complexity of biomass pretreatment. In some research, hemicellulose removal by dilute acid and hydrothermal pretreatment was believed to increase accessibility and benefit enzymatic hydrolysis [48, 53]. But it was also noticed that enzymatic digestibility did not continue to increase after about 80% of xylan was removed [53]. In addition, no clear relationship was seen between digestibility and xylan removal when different pretreatment methods were compared [41]. In this work, application of dilute acid at low temperature produced distinctly different glucan digestibility compared to results with high temperature hydrothermal or dilute acid pretreatments at comparable xylan removals, as shown in Figure 5.3a. Although dilute acid pretreatment at 100 ℃ with 1 wt% acid (Y1), hydrothermal pretreatment at 180 ℃ (HT2), and two-stage pretreatment with 1% acid at 80 ℃ followed by a hydrothermal pretreatment at 160 ℃ (X2a) all removed about 75% of the xylan, the enzymatic digestibility differed by almost 50%, suggesting factors other than hemicellulose removal are responsible for enhancement in digestibility. These observations indicate that xylan removal alone does not dominate digestion but that other substrate properties should be considered. For example, it has been suggested that xylan removal may be a marker of lignin disruption during pretreatment [13]. Many believe that lignin contributes to

biomass recalcitrance by shielding cellulose from enzymes and by non-productive binding of enzymes to reduce enzyme effectiveness [6, 26]. Previous studies have shown that enzymatic digestibility of cellulose could be related to lignin removal [14, 26], consistent with the trends in Figure 5.3b.

Though both hemicellulose and lignin removal could enhance digestibility, their effects did not appear to be coincident for dilute acid and hydrothermal pretreatments, as shown in Figure 5.1 and discussed above. The different responses in xylan and lignin removal to acid and temperature were attributed to differences in structural properties and associated reaction mechanisms. The principle mechanism of hemicellulose hydrolysis is the H^+ catalyzed cleavage of glycosidic bonds to form sugar oligomers and monomers that are highly soluble in water and easily removed [54]. The mechanism of lignin removal was thought to involve a phase transition around its glass transition temperature [55, 56]. In addition, the idea that lignin could condense or re-polymerize to form solid deposits during cooling was reinforced by observation of lignin droplets on the surface of pretreated solids [57]. Although a very wide temperature range has been reported to be applicable, the hydrophobic nature of lignin made it more difficult to remove in dilute acid or hydrothermal pretreatments unless a sufficiently high temperature was employed [13, 57]. From this perspective, both hemicellulose and lignin removal can more effectively eliminate lignin carbohydrate linkages to increase the cellulose exposure and digestibility [23]. However, because efficient removal of each component requires different conditions, removal of lignin and xylan do not occur simultaneously, and

tailoring conditions such as by the two-stage approach employed here to remove each sequentially can be more effective.

Surface accessibility has been recognized as a more important indicator of glucan digestibility than simply removing xylan and/or lignin [24, 53]. In line with this, we observed a positive correlation between substrate accessibility as characterized by the O/B ratio measured by the Simons' staining method and glucan digestibility (Figure 5.5b). The Simons' staining results are thought to serve as a useful indicator of the accessibility of the cellulosic substrate to enzymes [43], and samples subjected to higher temperature hydrothermal pretreatment (HT 2) either by itself or as the second stage of a two-stage pretreatment (X2a) strategy had higher accessibility as measured by the O/B ratio than solids from only low temperature pretreatment (X1 and Y1). The surface distribution of cellulose obtained from Tof-SIMS was consistent with this observation.

The results in this study show that xylan removal by low temperature dilute acid pretreatment can initially increase the digestibility of pretreated switchgrass as compared to the raw material. However, application of higher temperature further increased accessibility and glucan digestibility as more lignin and LCCs were removed. It should also be kept in mind that because such substrate features are affected by pretreatment methods and conditions, removal or alternation of one factor during pretreatment is usually accompanied by changes in other substrate features. Therefore, describing the contribution by each factor separately may not give an objective view.

5.5 Conclusions

In this study, low temperature dilute acid pretreatment, high temperature hydrothermal pretreatment, and the combination of the first followed by the second were applied to switchgrass to understand whether removal of xylan alone was sufficient to achieve high digestibility of the glucan left in the pretreated solids. The results show that xylan can be removed at relatively mild conditions with addition of dilute acid at low temperature or hot water pretreatment without acid. But removing only xylan was insufficient to fully unlock the biomatrix that hinders enzyme accessibility. On the other hand, the enzymatic digestibility represented by 72 h glucan yields correlated with lignin removal and was even more directly related to surface accessibility. Thus, although hemicellulose removal by either dilute acid or hydrothermal pretreatments does not require high temperatures, the temperature must be high enough to increase of substrate accessibility and achieve high enzymatic digestibility.

5.6 Acknowledgements

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Table 5.1 Pretreatment conditions applied in Parr reactor

		Label	Temp (℃)	Time (min)	Solid loading (wt %)	Sulfuric acid loading(wt %)	R or CS
Two-stage pretreatment	Stage1	X1	80	1440	5	1	1.88
		Y1	100	200	5	1	1.61
		Z1	120	150	5	1	2.07
	Stage2	X2a,X2b	160, 180	30	5	-	3.24,3.83
		Y2a	160	30	5	-	3.24
		Z2a	160	30	5	-	3.24
One stage pretreatment	-	HT1	160	30	5	-	3.24
		HT2	180	30	5	-	3.83

Severity: R=log{t* exp[(T-100)/14.75]}
Combined Severity: CS=R- pH

Table 5.2 Summary of solid yield and composition of raw and pretreated switchgrass

	Solid yield (%)			Wt% dry basis			Removal (%)	
	Stage1	Stage2	Total	Glucan	Xylan	Lignin	Xylan	Lignin
Raw	-	-	100	34.1	22.2	19.6	-	-
X1	73.6	-	73.6	44.0	14.2	24.1	57.0	9.5
X2a	73.6	83.4	61.4	54.0	10.3	25.6	72.9	19.8
X2b	73.6	70.0	51.5	58.9	4.6	26.5	89.5	30.4
Y1	72.3	-	72.3	45.7	12.5	25.5	73.6	5.9
Y2a	72.3	81.9	59.2	49.8	8.5	26.0	82.6	21.5
Z1	59.2	-	59.2	52.9	6.3	29.4	84.2	11.2
Z2a	59.2	95.1	56.3	56.1	5.4	28.0	87.0	19.6
HT 1	81.5	-	81.5	39.4	21.4	20.0	37.1	16.8
HT 2	58.5	-	58.5	50.3	10.1	24.0	73.2	28.4

Solid yield = 100% × Total solid after pretreatment (g)/Total solid before pretreatment (g)

Total solid yield = Stage1 solid yield (%) × Stage2 solid yield (%)

Xylan removal (%) = 1- (%Xylan in pretreated solids / % Xylan in raw biomass) × Solid yield

Lignin removal (%)= 1- (%Lignin in pretreated solids / % Lignin in raw biomass)× Solid yield

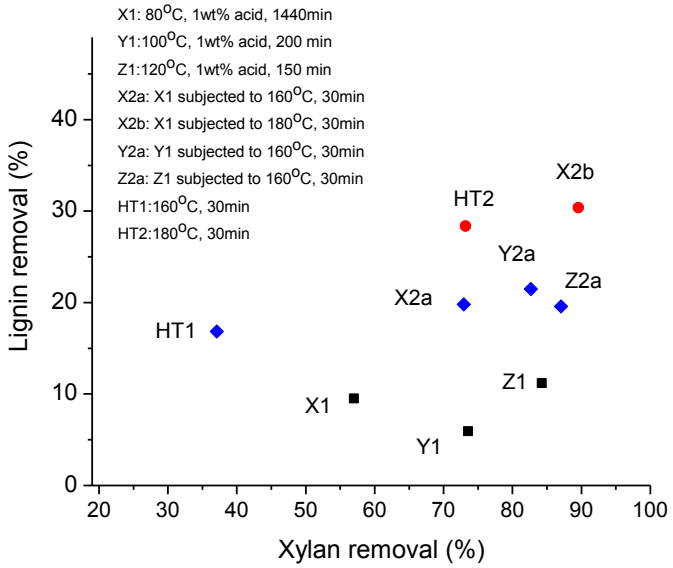

Figure 5.1 Relationships between xylan removal and lignin removal from switchgrass for low temperature dilute acid, hot water, and two-stage pretreatments.

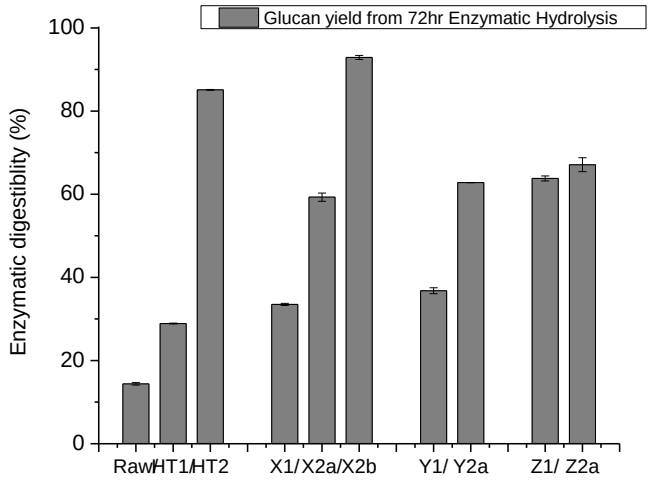

Figure 5.2 Enzymatic digestibility of raw and pretreated samples from low temperature, dilute acid pretreatment (X1-80 ℃,1 wt% acid, 1440min; Y1-100 ℃, 1 wt% acid, 200min; Z1-120 ℃, 1 wt% acid,150min), two-stage pretreatment (X2a-X1 subjected to 160 ℃ for 30 min; Y2a-Y1 subjected to 160 ℃ for 30 min, Z2a-Z1 subjected to 160 ℃ for 30 min, X2b-X1 subjected to 180 ℃ for 30 min), and hydrothermal pretreatment (HT1-160 ℃ for 30 min and HT2-180 ℃ for 30min). Enzymatic hydrolysis was performed at an enzyme loading of 45mg cellulase+15mg xylanase enzyme protein/g glucan with β- glucosidase supplement in the raw biomass at 50 ℃ for 72 h. The error bars are standard deviations calculated from triplicate runs.

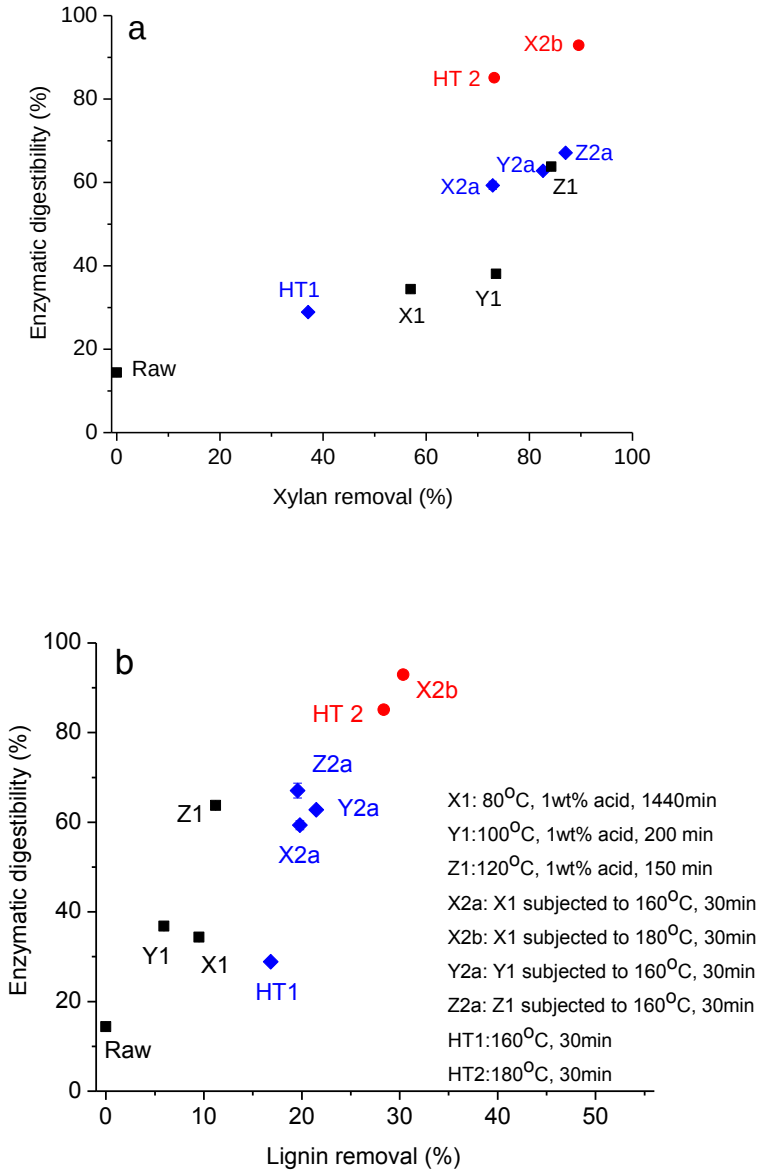

Figure 5.3 Enzymatic digestibility of solids from pretreatment of switchgrass plotted against **a**. xylan removal and **b**. lignin removal for pretreatment conditions listed in Table 5. 1. Enzymatic hydrolysis was performed at an enzyme loading of 45 mg cellulase+15 mg xylanase enzyme protein/g glucan with β- glucosidase supplement in the raw biomass at 50 ℃ for 72 h

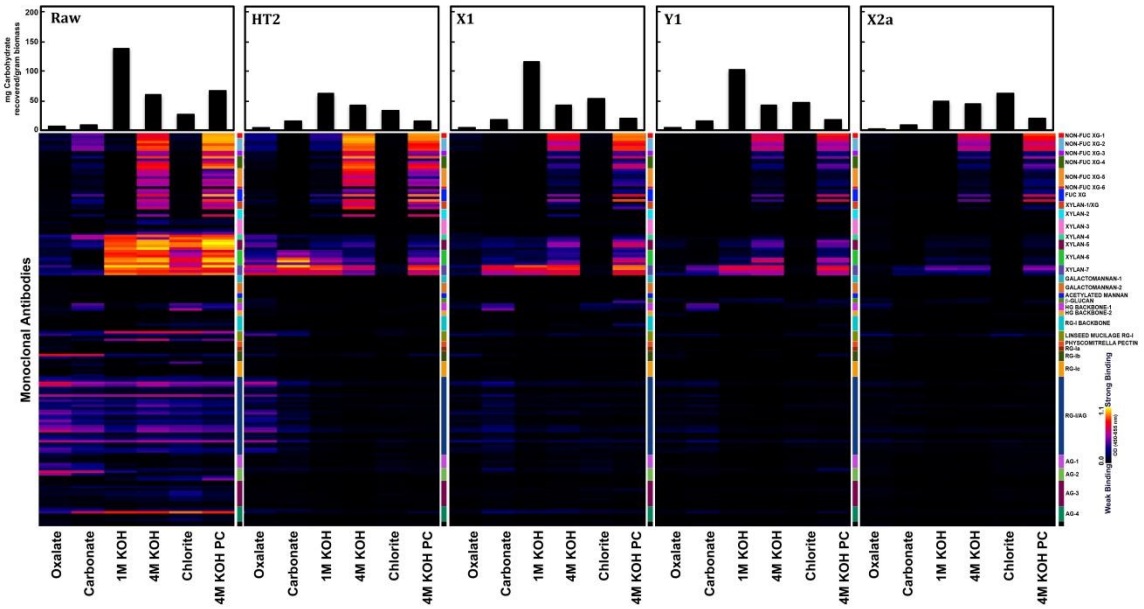

Figure 5.4 Glycome profiling of raw switchgrass and solids resulting from low temperature dilute acid, hydrothermal, and two-stage pretreatments at the conditions listed in Table5. 1. Extracted materials released from each biomass sample by sequential extraction with various reagents (as labeled at the bottom of each map) were loaded onto the ELISA plates and screened against an array of plant glycan-directed monoclonal antibodies. The legend panel on the right displays the nature of the polysaccharides predominantly recognized by these mAbs. Antibody binding is represented as colored heat maps, with black signifying no binding and light yellow representing the strongest binding. The bar graphs at the top indicate the amount of material recovered at each extraction step per gram of alcohol insoluble residue (AIR).

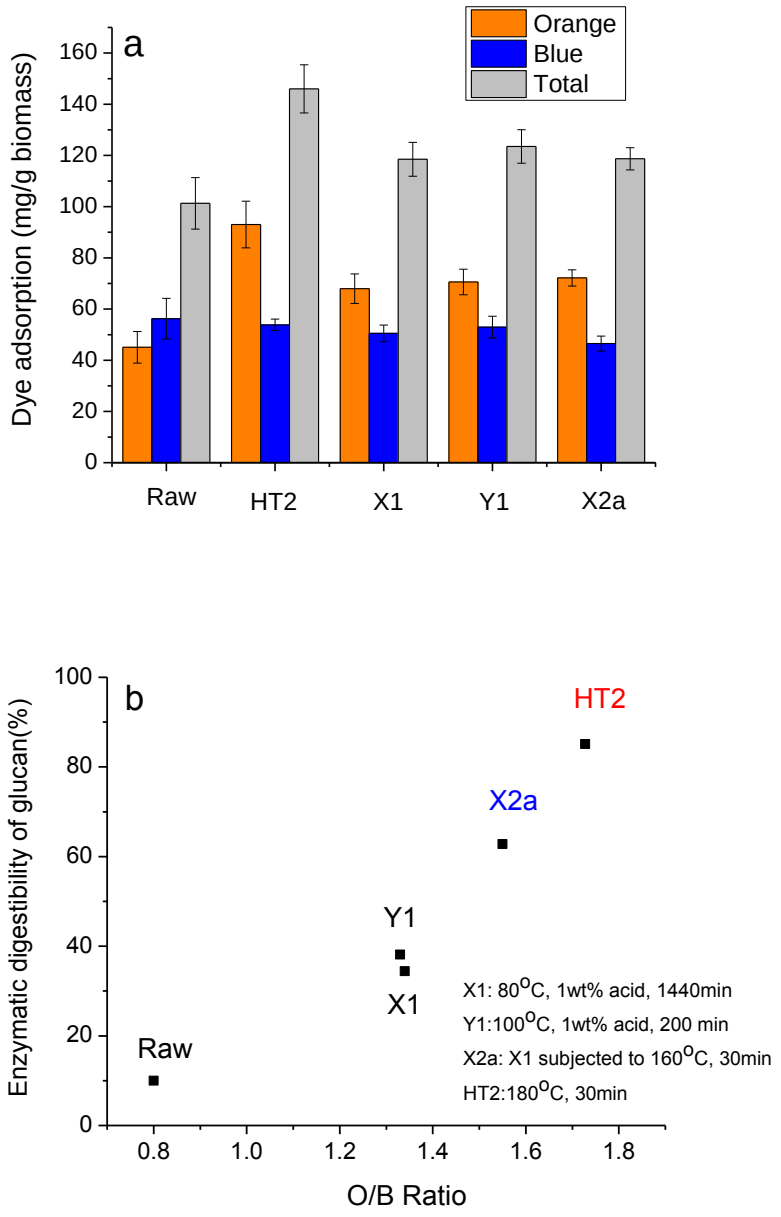

Figure 5.5 . **a**. Simons'stain results to estimate biomass pore surface area by the amount of absorbed dye, mg dye/g of sample. **b**. Relative enzyme accessibility represented by the ratio of absorbed large dye to small dye, [mg orange dye/g sample] / [mg blue dye/g sample] in the Simons' stain method plotted against glucan yield from 72 h enzymatic hydrolysis at an enzyme loading of 45mg cellulase+15mg xylanase enzyme protein/g glucan with β- glucosidase supplement.

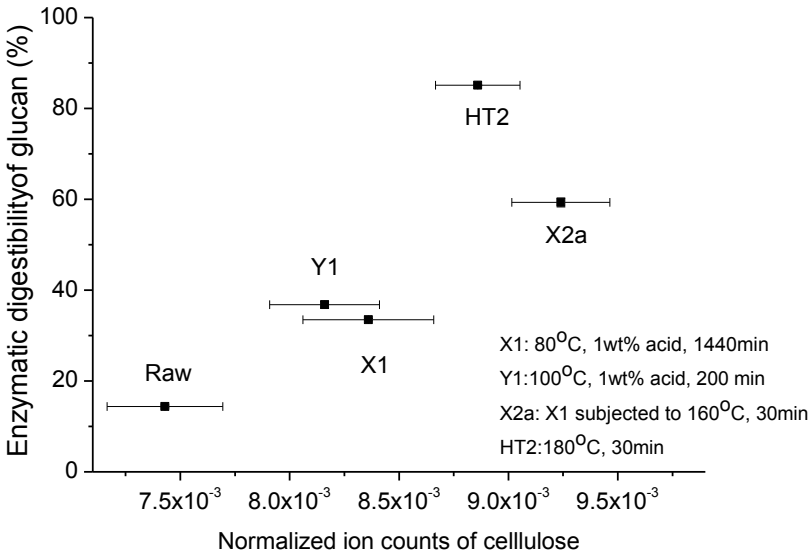

Figure 5.6 Relationship between relative ion intensity of cellulose from raw and pretreated materials and enzymatic digestibility measured as glucan yields from 72 h enzymatic hydrolysis at an enzyme loading of 45mg cellulase+15mg xylanase enzyme protein/g glucan with β-glucosidase supplement.

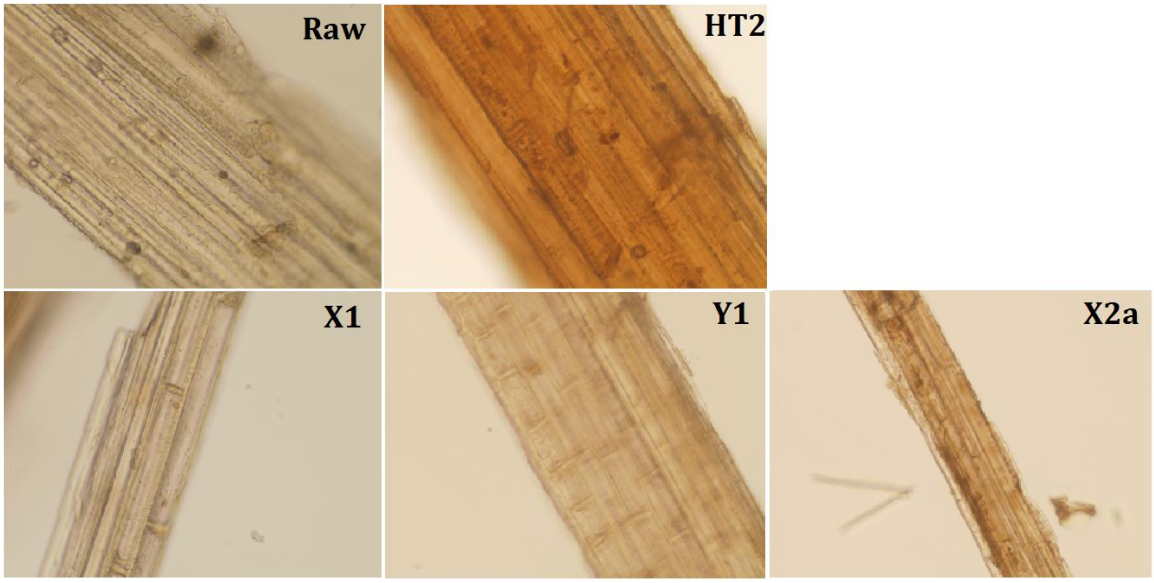

Figure 5.7 Bright field images of raw and pretreated switchgrass. The pretreatment conditions are listed in Table5. 1.

Chapter 6. The Effects of Recycling the Pretreatment Liquid from Low Temperature Pretreatments on Sugars Degradation in Liquid Hydrolyzate and Resulting Solids Digestion

6.1 Introduction

In chapter 4, a mild two-stage pretreatment strategy was proposed, and some preliminary results were obtained to prove its viability. The two-stage strategy applied low temperature dilute acid pretreatment as Stage 1 to remove most of the hemicellulose with high yields and followed with a high temperature hydrothermal pretreatment to substantially increase cellulose digestibility. Thus, most of the hemicellulose was removed at a low enough temperature that degradation was minimal in the first stage while also minimizing the amount of hemicellulose left in the solids that could be degraded at the higher temperature Stage 2 conditions. The solids from the second stage pretreatment were then ready for enzymatic hydrolysis with high sugar yields.

Although this pretreatment strategy combined with enzymatic hydrolysis obtained high yields of sugars from both glucan and xylan, consideration should be given to how it could be employed commercially. One important aspect is to minimize water consumption to keep pretreatment energy costs as low as possible. In addition, less water consumption translates into higher final product concentrations, thereby reducing the size of fermentation and other process vessels as well as reducing energy requirements for product recovery by distillation or other operations. Most conceptual process designs for production ethanol from lignocellulosic biomass recycle key streams to reduce fresh water consumption, decrease waste water production, and save energy for distillation [1-4]. Many of these previous studies obtained recirculation liquid from a stillage unit for evaporation and condensation of liquid [2]. The broth from fermentation was also recirculated to enzymatic hydrolysis to increase the ethanol concentration [3]. The

effects of the increased concentration of volatile and non-volatile substances in the recycled stream on hydrolysis and fermentation were studied as well [2, 5, 6].

Pretreatment chemical catalysts were also recycled for thermochemical pretreatment, especially for those technologies using expensive chemicals. For ionic liquid pretreatment, in particular, concentrated water-structuring salt solution (such as K_3PO_4 or K_2HPO_4) was used as an anti-solvent to drive phase separation of ionic liquid from the solid and salt phases. The residual water in the ionic liquid rich phase was then evaporated before ionic liquid recycle [7]. Ammonia recycle has also been favored in ammonia related pretreatment methods [8, 9]. However, very little attention has been devoted to recycle and reuse of the liquid produced in dilute acid pretreatment.

In this study, the possibility of recycling the liquid from Stage 1 pretreatment was investigated to provide supporting information for Chapter 4. Experiments were performed to determine the degree of sugar loss due to recycle, and substrate digestibility for recycled liquid was compared to that without recycle. A simple mass balance was applied to determine how much recycle of the liquid following low temperature Stage 1 pretreatment could increase sugar concentrations and lower water and acid consumption per gram of biomass in the two-stage pretreatment strategy.

6.2 Materials and Methods

6.2.1 Biomass feedstock

The switchgrass, *Panicum virgatum,* used in this study was from Pierre, South Dakota and provided by the BioEnergy Science Center (BESC). It was knife milled (Model 4, Wiley Mill, Thomas Scientific, Swedesboro, NJ) to pass through a 1 mm

screen. After that, all materials were air dried for approximately one month followed by sieving to collect fractions with a particle size between 20-mesh (<0.85 mm) and 80-mesh (>0.180 mm) (RX-29, W.S. Tyler, Mentor, OH). Particles larger than 20-mesh were collected and sieved again, and the resulting 20-80 mesh fraction was mixed with the previously obtained 20-80 mesh fraction. The moisture content in the switchgrass material was about 5 wt%.

6.2.2 Pretreatment of switchgrass fresh acid or mixture recycled liquid with fresh acid

Pretreatment of switchgrass was performed with fresh acid or mixture of recycled liquid with fresh acid, as summarized in Table 6.1 . Recycled liquid from pretreatment at 80℃ with 1wt% acid for 1440min and from reaction at 120℃ with 1wt% acid for 150min were mixed with fresh 1 wt% sulfuric acid at volume ratios of 0:100, 50:50, 75:25 and 100:0. Heating and cooling as well as liquid-solids separation and collection were performed as described above. Aqueous slurries at 5 wt% switchgrass and 1 wt% sulfuric acid solution or a mixture of recycled liquid with fresh 1wt% acid added to make up the reaction liquid were allowed to soak at room temperature overnight prior to use.

Reactions at 120 ℃ were performed in Hastelloy (C276) tube reactors assembled from 150mm lengths of 12.5 mm OD Hastelloy tubing with a 0.8255 mm wall thickness and stainless steel end caps (Swaglok, San Diego, CA). Each tube reactor had an internal volume of approximately 14mL. Teflon plugs (McMaster-Carr, Santa Fe Spring, CA) were inserted in each end to avoid acid corrosion of the stainless steel caps. The tubes were immersed in a 22.8 cm id × 35 cm deep 4-kW model SBL-2D fluidized sand bath

(Techne, Princeton, NJ) set at 1 ℃ higher than the target temperature. To monitor the temperature over the course of the reaction, an additional tube reactor with a thermocouple probe inserted 2 in. deep in the center was used in parallel with the other reactors. The tubes were held for a specified amount of time, removed from the sand bath, and immediately immersed into a room temperature water bath to quench the reaction. The reaction time was determined as the elapsed time from when the temperature in the tube with the thermocouple reached 2 ℃ below the target temperature until the moment of quenching. After cooling, the tube was opened, and the liquid and solids were separated by filtration. The solid was washed three times using approximately 100mL room temperature deionized (DI) water. Both liquid and solids were collected and stored in 4 ℃ for until further analysis.

The reactions at 80℃ employed a different method. The slurry was presoaked overnight in a 250 mL heavy wall pressure bottle and sealed by a silicon cap and 20 mm open center seals (Supelco, Bellefonte, PA). A gyrotory water bath shaker (Model G76Dm New Brunswick scientific Co. Inc., Edison, NJ) was used for heating and maintaining the temperature. The water bath was set at 81 ℃ with rotation speed at 200 rpm to ensure the heat and mass transfer.

After pretreatment, the liquid and solid were separated by vacuum filtration through 12.5 cm diameter Whatman No.1 filter paper in a Buchner funnel. The solids were washed with DI water several times to remove chemical residues and free sugars. Both solid and liquid were stored at 4 ℃ until further analysis and utilization.

6.2.3 Sugar degradation test and pretreatment by recycled liquid

A sugar degradation test was performed to understand if sugars were lost due to their recycle with the liquid. 10 mL of liquid from pretreatment, which containing sugars and acid, was held at the pretreatment temperature for the same amount of time as used in pretreatment. Thus, the liquid from 120 ℃, 150 min pretreatment was heated back up to 120 ℃ and held there for 150min. In total 12 tubes were run as a group, with two tubes removed at 30, 60, 80, 120, and 150 min of reaction. Similarly, liquid from 80 ℃, 1440 min pretreatment was reheated to 80 ^{o}C and held there for 1440 min. Once again, 12 bottles were used for this experiment, with two removed at each of the following times: 60, 150, 300, 600, 1320, and 1440 min.

6.2.4 Enzymes

Cellulase (Spezyme® CP, BCA protein concentration 116 mg/ml, activity 58 filter paper units (FPU)/ml, Lot # 3016295230) and Multifect® xylanase (protein concentration 42 mg/ml, Lot# 4900667792), both from Genencor, a division of Danisco, now DuPont Biosciences, Palo Alto, CA, were mixed at a protein ratio of 3:1 for enzymatic hydrolysis. These were supplemented with β-glucosidase (Novozyme®188, activity-665 cellobiase unit CBU/ml) at an activity ratio of 1.5:1 (CBU:FPU), which earlier have been shown to be enough to alleviate cellulase inhibition by enhancing cellobiose hydrolysis [10, 11]. The activity and protein numbers assumed in this study were previously reported [12].

6.2.5 Enzymatic hydrolysis

In accordance with the National Renewable Energy Laboratory (NREL) Laboratory Analytical Procedure[13], enzymatic hydrolysis was conducted in triplicate at a solids loading corresponding to 1 wt% glucan in 0.05M citrate buffer (pH = 4.9) containing 1 mg/mL sodium azide in 50 mL Erlenmeyer flasks. The slurries were incubated at 50^{o}C in a shaker incubator (Multitron Infors-HT, ATR Biotech, MD) at 150 rpm for 168 h. The enzyme loading was in 45mg cellulase + 15mg xylanase/ g glucan in the raw biomass, with β-glucosidase added at an activity ratio of 1.5 : 1 (CBU:FPU) to enhance cellobiose hydrolysis and reduce end-product inhibition.

Samples were taken at 1,6, 24, 72, and 168 h. To determine the amount of sugar generated from enzymatic hydrolysis, 400 μL samples were drawn, filtered through 0.2 μm nylon filter vials (Grace Davision Discovery Science, Deerfield, IL), pipetted into 500 μL polyethylene HPLC vials (Cat. 98842, Grace Davavison Discovery Science, Dearfield, IL , and stored at 4 ^{o}C until analysis.

6.2.6 Compositional analysis of solid and liquid

All chemical analysis procedures applied in this work for determining solid composition and concentrations of sugar monomer and oligomers in liquid were according to the well-established and widely used Laboratory Analytical Procedures (LAPs) of the NREL [14, 15].

6.2.7 Sugar analyses

For determination of sugar concentrations, samples along with appropriate calibration standards were run on a Waters Alliance HPLC system (Model e-2695, Waters Corporation, Milford, MA) employing an Aminex HPX-87H column (Bio-Rad Laboratories, Life Science Research, Hercules, CA). Samples were processed at an eluent flow rate of 0.60 ml/min using a refractive index (RI) detector (Model 2414, Waters Corporation, Milford, MA). The chromatograms were recorded and processed with Empower® 2 software (Waters Corporation, Milford, MA).

6.2.8 Calculations

The glucan and xylan yields from pretreatment and glucan yield from enzymatic hydrolysis were calculated as following:

$$Gluan\ Yield\ (\%) = 100 \times \frac{(GH\ (g) + CB(g) * 1.053)/1.111}{GP\ (g)}$$

$$Xylan\ Yield\ (\%) = 100 \times \frac{XH\ (g)/1.136}{XP\ (g)}$$

$$Solid\ Yield\ (\%) = 100 \times \frac{Solid\ after\ pretreatment\ (g)}{Solid\ before\ pretreatment\ (g)}$$

When the pretreatment was performed with recycled liquid, the amounts of glucose, xylose, and cellobiose used for yields calculation were as the following:

$$GH,XH,CB\ (g) = GH,XH,CB_{\ after\ reaction}\ (g) - GH,XH,CB_{\ before\ reaction}\ (g)$$

Usually, the amount of cellobiose was negligible at the reaction conditions run.

The amounts of gluco- and xylooligomers in the liquid were calculated as:

$$G_{3+} = GH'(g) - GH(g) - 1.053 \times CB(g)$$

$$X_{2+} = XH'(g) - XH(g)$$

Where GH, CB, and XH represent glucose, cellobiose, and xylose in the liquid, respectively; GH' and XH' are the glucose and xylose after posthydrolysis, respectively; GP and XP stand for glucan and xylan available in the raw biomass (for yields from pretreatment)or pretreated biomass(for yields from enzymatic hydrolysis), respectively; and the factors 1.111, 1.136, and 1.053 account for the mass gained during hydrolysis of glucan to glucose, xylan to xylose, and cellobiose to glucose, respectively.

6.3 Results and Discussion

6.3.1 Sugar degradation test

Because hydrolysis of hemicellulose to oligomers and monomers is typically followed by breakdown of these dissolved sugars [16], recycle of liquid from the Stage 1 pretreatment would not be very attractive if sugar degradation was very great. Therefore, tests were run to assess the amount of sugar lost when liquid from pretreatment was reused. The liquids from the two pretreatment conditions listed in Table 6.1, 80 ℃ for 1440 min with 1 wt % acid (X4) and 120 ℃ for 150min with 1 wt% acid (Z4), were heated up to the same pretreatment temperature and time again to mimic the recycle process. Figure 6.1 and 6.2 show the concentrations of monomer and oligomers of xylose and glucose in each liquid from the mimicked recycle. These results show that no or little sugar degradation occurred when the recycled liquid from the previous batch containing sugars was undergone the different length of pretreatment times. For the liquid sample prepared at 80 ℃ for 1440 min (X4) shown in Figure 6.1, both total xylose

and glucose remained constant for a 1440 min reaction at 80 ℃. It is also important to notice that the oligomers were gradually converted into monomers in the process, increasing the concentration of xylose monomer from inital.7 mg/mL to 7.2 mg/mL after 1440 min. The amount of glucose monomer only increased from initial 0.39 to 0.61mg/mL with much less released in solution during pretreatment, and about half of the glucose in the liquid remained as oligomers. As shown in Figure 6.2 for reaction of the pretreatment liquor at 120 ^{o}C for 150 min (Z4), no sugar degradation was observed, and the concentrations of both monomers and oligomers did not change notably. The results suggested that at the low temperatures applied in Stage 1 pretreatment, the extended residence time for recycle did not lead to significant sugar loss but did increase breakdown of oligomers to monomers.

6.3.2 Effect of pretreatment using fresh acid vs. recycled liquid on solids digestibility

Stage 1 pretreatment was performed using mixtures of fresh acid solution and recycle liquid over the range of volume ratios shown in Table 6.1. Figures 6.3 and 6.4 compare the sugar yields from pretreatment at 80 ℃ for 1440 min and 120 ℃ for 150 min, respectively, for these different mixtures and show that mixtures of recycled liquid and fresh acid (X2-X4, Z2-Z4), at volume ratios of 50:50, 75:25 and 100:0 were as effective as fresh acid (X1, Z1) in terms of sugar yield released from pretreatment. In particular, xylan yields from pretreatment using recycled liquid were comparable to those from pretreatment with fresh acid. Although glucan yields from pretreatments with recycled liquid were not quite as high as those with fresh acid, the values were still very close.

The enzymatic digestibilities of solids resulting from pretreatment of all the mixtures of recycled liquid and fresh acid solution are also compared in Figure 6.5 and Figure 6.6 to provide a measure of any changes in the effectiveness of pretreatment. The solids were washed with DI water prior to enzymatic hydrolysis, and no differences in inhibition were observed during enzymatic digestion of the washed solids, indicating that recycled liquid did not generate any additional insoluble inhibitors. Thus, at the same temperature and time, solids resulting from pretreatment of switchgrass with recycled liquid (X2-X4, Z2-Z4) behaved the same in enzymatic hydrolysis as solids produced by pretreatment with fresh acid (X1, Z1). As a result, sugar yields from both glucan and xylan from enzymatic hydrolysis were unaffected. It should be kept in mind that although the 168 h digestibilities of the solids from the low temperature Stage 1pretreatment were low (glucan yields <70%), the two-stage strategy employs Stage 2 pretreatment at higher temperature to increase the yields substantially(glucan yields >95%).

6.3.3 Glucan and xylan contents of solids from pretreatment

The solids yields and glucan and xylan contents of solids from pretreatment using different proportions of recycled liquid and fresh acid are shown in Table 6.2. The solids from pretreatments at 80 ℃ for 1440min all have similar amounts of glucan and xylan in the solids from pretreatment regardless of whether fresh acid or recycled liquid were used (X1-X4). The same trend was also observed for solids produced by 120 ℃ pretreatment (Z1- Z4).

6.3.4 Possible process configuration for hydrolyzate recycle

The results from sugar degradation for recycle and pretreatment performance supported the possibility of employing recycle of hydrolyzate liquid from one pretreatment to subsequent pretreatments to reduce acid use and increase sugar concentrations. Figure 6.7 shows an initial mass balance based on recycle of liquid hydrolyzate from Stage 1 pretreatment in the two-stage strategy. All numbers used here are projections based on experiment results since an actual recycle process has not been tested.

Figure 6.7 shows an approach in which the liquid hydrolyzate recovered following Stage 1 pretreatment is used for subsequent Stage 1 pretreatment of additional biomass. The result is that all hydrolyzate liquid (④) from the prior pretreatment was recycled and with some supplementary acid solution(⑥) to subsequent pretreatment with10 wt% solids loading. The sugar contents and water, acid amount in each stream in the flowchart were summarized in Table 6.3. The overall water and sulfuric acid use can be reduced from 8.9g to 5g /g dry biomass and 0.09g to 0.05 g/g dry biomass, respectively. In addition, reusing the liquid from the first Stage 1 pretreatment for the next produces a more concentrated sugar stream. Provided the sugar degradation is low, as shown by the results in Figure 6.1, 6.2 and the mixture of recycle liquid and fresh acid give virtually the same sugar yields as using fresh acid (Figure 6.3, 6.4), the sugar concentration in liquid 2 can be expected to approximately double from what it was leaving the first Stage 1 pretreatment. Although Figure 6.7 illustrates the approach applied to just one recycle, this could be repeated for additional pretreatments if

appropriate. In all cases, solids left after any of the Stage 1 pretreatment operations are sent to Stage 2 for high temperature pretreatment to enhance the digestibility of the solids.

6.4 Summary

This study measured the effect of recycling the liquid from pretreatment at mild conditions on sugar yields. These results showed that sugar degradation was insignificant when the liquid from one Stage 1 pretreatment was used in a second pretreatment at the same low temperature. In addition, pretreatments with recycled liquid hydrolyzate that contained acid and sugars gave comparable sugar yields from both pretreatment and enzymatic hydrolysis and similar glucan and xylan contents in solid as pretreatment with fresh acid. Third, a material balance on a possible configuration to recycle liquid showed that consumption of fresh water and acid can be reduced and more concentrated sugar stream achieved by replacing the fresh water with recycle liquid from pretreatment. Overall, these results showed no negative effects on either sugar yields from pretreatment or solids digestibility when using recycled liquid in pretreatment. However, further research is needed to better understand recycle of liquid hydrolyzate from Stage 1 pretreatment at low temperatures. For example, it would be beneficial to optimize the recycle ratio in concert with downstream operations.

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Table 6.1 Summary of reaction conditions and percentages of recycled liquid and fresh acid employed for each pretreatment

Number	Solution (v:v)		Pretreatment Conditions
	Recycled liquid	1 wt% sulfuric acid	
X1	0	100%	5 wt% solid loading, 80 ℃, 1440 min
X2	50%	50%	
X3	75%	25%	
X4	100%	0	
Z1	0	100%	5 wt% solid loading 120 ℃, 150 min
Z2	50%	50%	
Z3	75%	25%	
Z4	100%	0	

Table 6.2 Solid recoveries following pretreatment with different amounts of recycled acid and glucan and xylan contents of unpretreated switchgrass and washed solids resulting from pretreatment with different mixtures of fresh acid and recycled hydrolyzate from Stage 1

Substrate	Conditions Temp /recycled liq: fresh acid (v:v)	Solid yield (%)	Component, % dry basis	
			Glucan (%)	Xylan (%)
Raw switchgrass	-	N/A	34.1 ± 0.1	22.2 ±0.1
X1	80 ℃/fresh acid	73.6	45.4 ±1.0	13.9 ±0.2
X2	80 ℃/50:50	74.1	43.9 ±0.03	14.0 ±1.2
X3	80 ℃/75:25	74.9	43.1 ±0.2	14.0 ±0.1
X4	80 ℃/recycled liquid	75.2	41.9 ±0.1	13.8 ±0.1
Z1	120 ℃, fresh acid	59.2	52.9 ±0.0	6.3 ± 2.7
Z2	120 ℃/50:50	60.0	50.0 ±1.5	7.7 ±0.2
Z3	120 ℃/75:25	61.5	48.4 ±0.6	6.7 ±0.1
Z4	120 ℃, recycled liquid	60.9	50.1 ±1.3	6.6 ±2.7

Table 6.3 A representative mass balance for Stage 1 pretreatment at 80 ℃ with 1wt% sulfuric acid for 1440 min at a 10 wt% solid loading with hydrolyzate liquid recycle

Stream (g)	1	2	3	4	5	6	7	8	9
Solid	100	-	73.6	-	100	-	74.9	-	148.5
Glucan	34.1	-	33.4	0.7	34.1	-	32.3	2.5	65.7
Xylan	22.2	-	10.2	12	22.2	-	10.4	23.8	20.6
Water	-	891	109.3	787.7	-	109.3	111	786	220.3
Acid	-	9	1.1	7.9	-	1.1	1.12	7.9	2.2

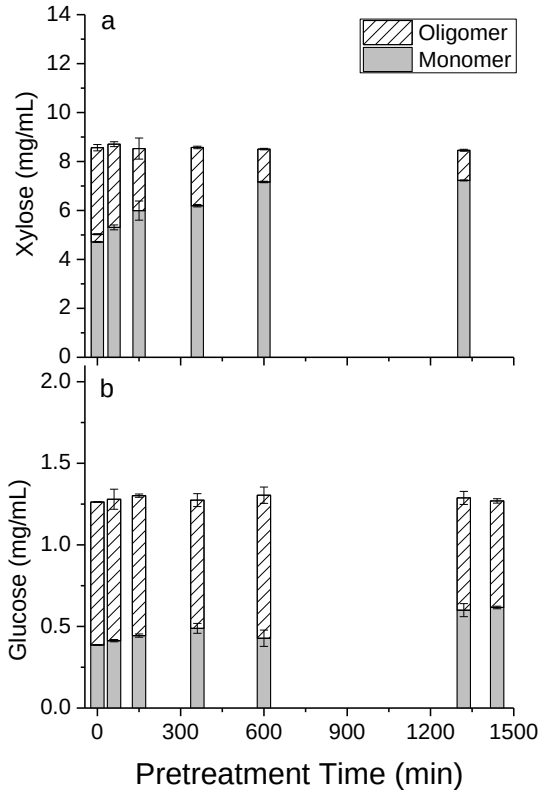

Figure 6.1 Effect of pretreatment (80 ℃, 1wt% acid solution) time on (**a**) xylose and (**b**) glucose concentrations in recycled liquid X4. The solids free liquid was from pretreatment performed at 5wt% switchgrass solids loading in 1wt% acid solution at 80^{o}C for 1440 min. The oligomers concentration is measured in terms of monomer equivalents.

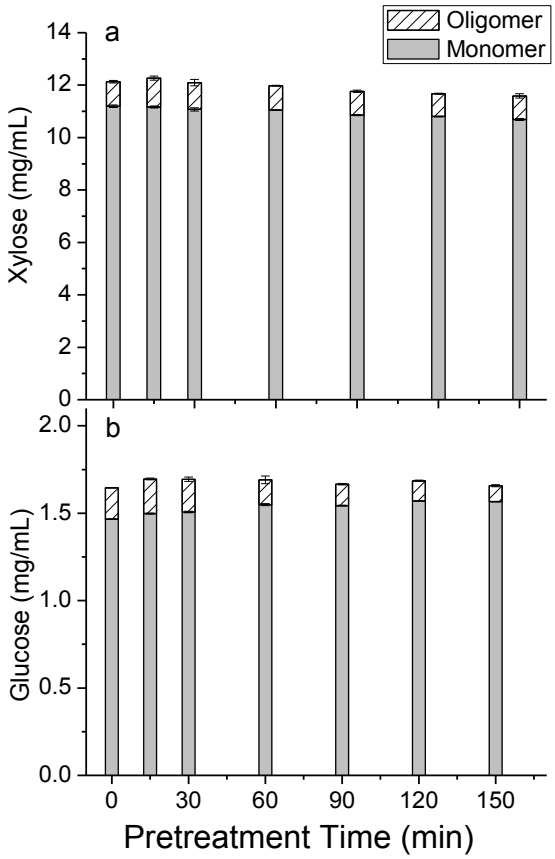

Figure 6.2 Effect of pretreatment (120 ℃, 1wt% acid solution) time on (**a**) xylose and (**b**) glucose concentrations in recycled liquid Z4. The solids free liquid was from pretreatment performed at 5wt% switchgrass solids loading in 1wt% acid solution at 120^{o}C for 150 min. The oligomers concentration is measured in terms of monomer equivalents.

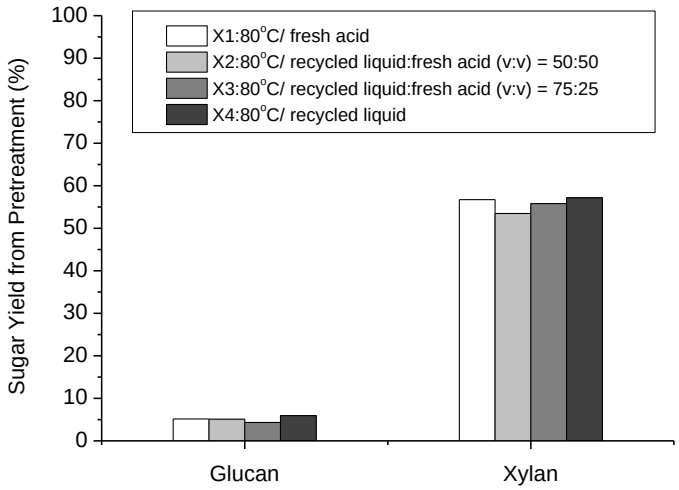

Figure 6.3 Glucan and xylan yields from switchgrass pretreatment at 5wt% solids loading in fresh 1wt % sulfuric acid (X1) or with a mixed solution of recycled liquid with acid (X2,X3,X4) at 80 ℃ for 1440 min.

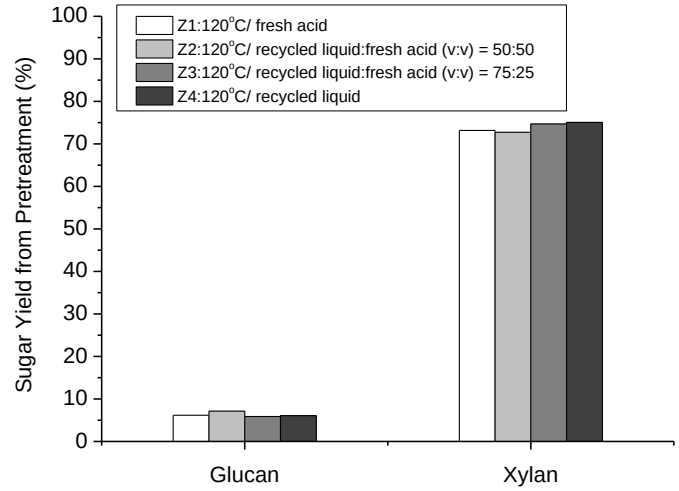

Figure 6.4 Glucan and xylan yields from switchgrass pretreatment at 5wt% solids loading in fresh 1wt % sulfuric acid (Z1) or with a mixed solution of recycled liquid with acid (Z2,Z3,Z4) at 120 ℃ for 150 min.

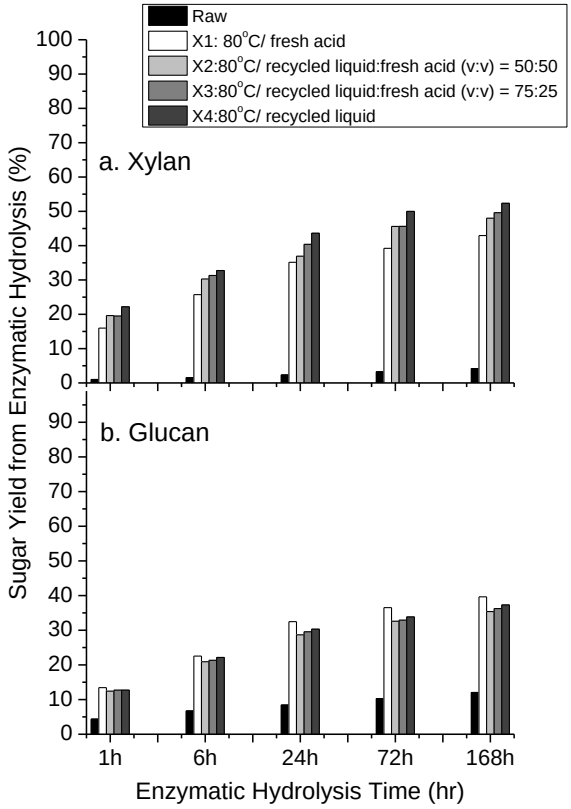

Figure 6.5 Glucan and xylan yields from enzymatic hydrolysis of raw switchgrass and the solids resulting from pretreatment of switchgrass in fresh 1wt% acid solution, recycled liquid, or mixture of these two (X1-X4) at 80 ℃ for 1440 min for enzyme loadings of 60 mg protein/g glucan in the unpretreated biomass

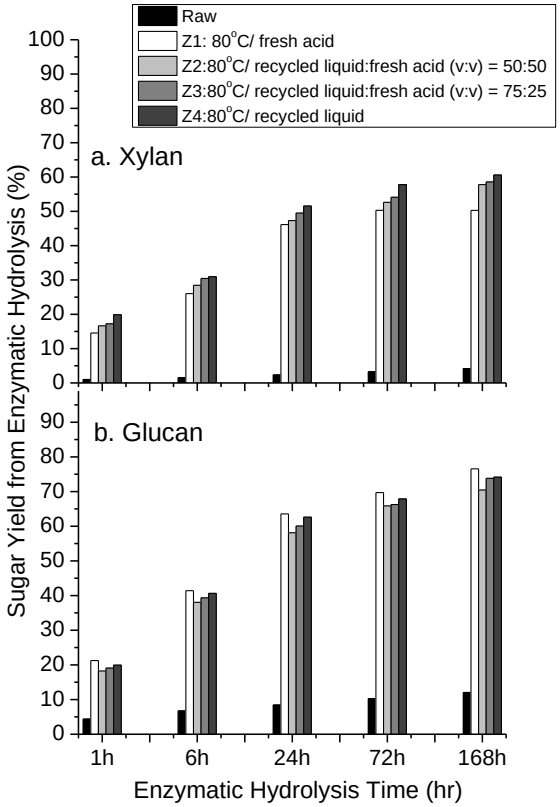

Figure 6.6 Glucan and xylan yields from enzymatic hydrolysis of raw switchgrass and the solids resulting from pretreatment of switchgrass in fresh 1wt% acid solution, recycled liquid, or mixture of these two (Z1-Z4) at 120 ℃ for 150 min for enzyme loadings of 60 mg protein/g glucan in the unpretreated biomass.

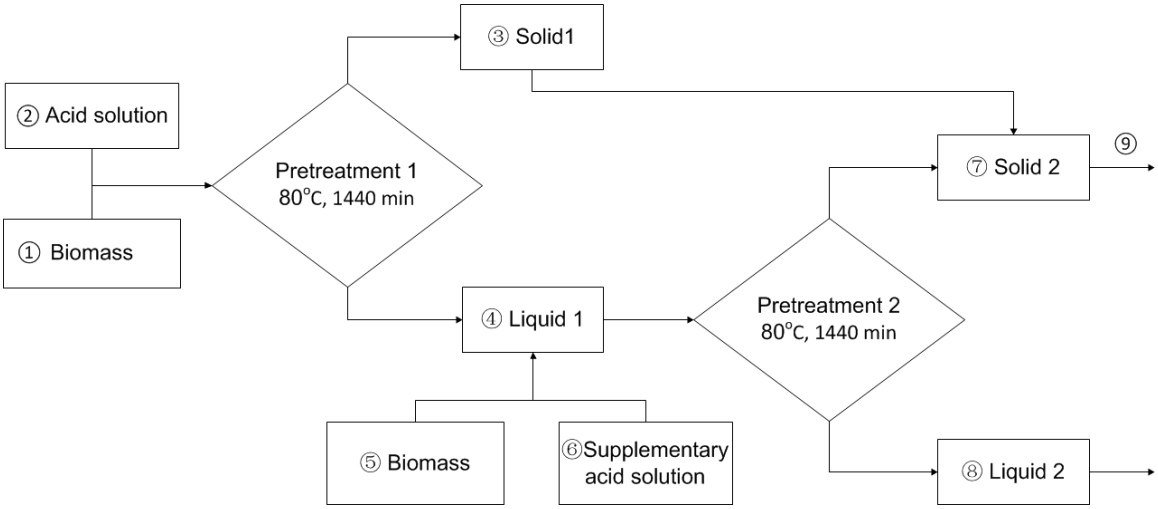

Figure 6.7 A representative flowchart for Stage 1 pretreatment at 80 ℃ with 1wt% sulfuric acid for 1440 min at a 10 wt% solid loading with hydrolyzate liquid recycle. The material balance for each stream is shown in Table 6.3.

Chapter 7. Fast Hemicellulose Quantification via a Simple One-Step Acid Hydrolysis

This whole chapter will be submitted to “Biotechnology and Bioengineering” or a similar journal under the following citation: Gao X, Kumar R, and Wyman CE. “Fast Hemicellulose Quantification via a Simple One-Step Acid Hydrolysis”

7.1 Abstract

As the second most common polysaccharides in nature, hemicellulose has received much attention in recent years for its importance in biomass conversion in terms of producing high yields of fermentable sugars and value-added products, as well as its role in reducing biomass recalcitrance. Therefore, a time and labor efficient method that specifically analyzes hemicellulose content would be valuable to facilitate the screening of biomass feedstocks. In this study, a one-step acid hydrolysis method was developed, which applied 4 wt% sulfuric acid at 121 ℃ for 1 h to rapid quantify of XGM content in various types of lignocellulosic biomass. This method gave statistically identical results in XGM contents compared to results from conventional two-step acid hydrolysis while significantly shortening analysis time.

7.2 Introduction

Lignocellulosic biomass, including agricultural and forestry residues and herbaceous and woody crops [1], provides the only sustainable resource for large-scale and low-cost production of liquid fuels and organic chemicals that are currently produced from dwindling and nonrenewable fossil resources[1-4]. However, the plant's recalcitrance to deconstruction by enzymes or microbes is the primary obstacle to low cost biological production of renewable fuels from lignocellulosic biomass [5, 6]. Therefore, versatile approaches are applied to make the conversion from biomass to fuels or chemical more commercially viable. On one hand, optimization or improvement of key operations, including developing effective pretreatment technologies and improving the enzymes and microbes applied, can play an important role in reducing recalcitrance [7,

8]. On the other hand, attention to selection of biomass species with reduced recalcitrance and genetic modification of biomass for less recalcitrance also needs to be addressed [9, 10]. In pursuing these objectives, accurate and rapid determination of composition, particularly sugar content, is essential for identifying plant-pretreatment-biocatalyst combinations with performance advantages [11].

Lignocellulosic biomass is a complex material that is composed of three major components: cellulose, hemicellulose, and lignin. Cellulose is a linear polysaccharide of β-1,4-glucose units, which are linked by intra- and inter-molecular hydrogen bonds to form a crystalline structure [12]. Lignin is a cross-linked and three dimensional phenolic polymer [13]. Hemicellulose is the second most common polysaccharides in nature and makes up about 20-35 wt% of lignocellulosic biomass [14]. Unlike cellulose, hemicellulose is not chemically homogeneous, and the chemical nature depends on the source. In general, the dominant component of hemicellulose from hardwoods and agricultural plants is xylan, while mannan is prevalent in softwoods. The xylan backbone is a major ingredient that is composed of 1,4-linked β-xylose units[15]. Xylan branching varies from species to species, typically with arabinose sugar acids as well as acetyl groups [16]. Hemicellulose is amorphous and hydrophilic and therefore more easily removed from cell walls than the cellulose polysaccharide.

Utilization of hemicellulose has received much attention in recent years for its importance in efficient and low-cost conversion of lignocellulosic biomass to fuel ethanol and other value-added products, with both biological and chemical strategies applied for hemicellulose conversion [15]. Xylose and xylooligomers are often the major products

from pretreatment and enzymatic hydrolysis of hemicellulose. Application of chemicals, such as sulfuric acid, at mild conditions is also capable of generating xylose monomer and oligomers from hemicellulose. Although traditional microorganisms, such as *S. cerevisiae and Z. mobilis* ferment glucose to ethanol rapidly and efficiently, they cannot ferment other pentose, such as xylose. However, several recombinant strains have been developed to successfully ferment pentoses to ethanol under both laboratory and industrial conditions [17]. Value-added products, or reactive intermediates (RIs) such as xylitol, furfural, and levulinic acid for the production of chemicals and polymers can also be generated from hemicellulose polysaccharides via appropriate catalytic approaches [18, 19].

Some studies suggested that the content as well as composition of hemicellulose also affects cell wall bioconversion [20]. The cross linkages between hemicellulose and cellulose microfibrils as well as lignin-carbohydrate linkages (LCCs) are believed to sterically hinder enzyme attack [21, 22]. Transgenic *Arabidopsis* with less methyl groups on glucoronoxylan side chains released more xylose than the wild type control at lower severity conditions [23]. Reduced glucuronoxylan content in genetically modified poplar was also reported to result in increased cellulose digestibility [24].

Due to the importance of hemicellulose in biomass conversion in terms of producing high yields of fermentable sugars and value-added products, as well as its role in reducing biomass recalcitrance, a time and labor efficient method that specifically analyzes hemicellulose content would be valuable. A well-established and widely-used procedure from the National Renewable Energy Laboratory (NREL) employs two-step

acid hydrolysis to breakdown structural carbohydrates into components that can be quantified by chromatography and gravimetric methods to accurately determine total sugar content in cellulosic biomass [25]. This method uses 72 wt% sulfuric acid in a first step followed by 4 wt% sulfuric acid in a second step to hydrolyze both cellulose and hemicellulose to sugars that can then be quantified via HPLC or other methods. This procedure takes approximately 3 h to measure the sugar content in one sample.

In this work, a one-step acid hydrolysis method was developed and was found to be effective for fast quantification of the hemicellulose sugars in biomass. The method applied 4wt% sulfuric acid to hydrolyze biomass samples at 121 ℃ for 1 h. Then the sugars released were measured to determine the hemicellulose sugar content. The method was applied to compounds enriched in hemicellulose and to agricultural, herbaceous, and woody biomasses, and the results were compared with those from a conventional two-step acid hydrolysis.

7.3 Materials and Methods

7.3.1 Materials

Five hemicellulose compounds, four biomass standards, three typical biomass materials, pure cellulose with different crystallinity, and starch were used in this study. Beechwood xylan (Lot BCBS8393V) was purchased from Sigma Chemicals（St. Louis, MO). Glucomannan was a dietary fiber derived from the root of the konjac plant (Konjac foods, Sunnyvale, CA). Carob galactomannan (Lot 10501b), wheat arabinoxylan (Lot

20301b), and xyloglucan (Lot 100402) were all purchased from Megazyme International (Wicklow, Ireland).

Sugarcane bagasse (NIST 8491), Eastern cottonwood (NIST 8492), Monterey pine (NIST 8493), and Wheat straw (NIST 8494) standard biomass materials of known composition as established by the National Institute of Standards and Technology (NIST) were obtained from NIST. The particle size range of the NIST standards was 20 to 74 mesh (0.19mm – 0.85mm). All biomass materials were well mixed and dried overnight in a 105 ℃ oven before analysis.

The three typical biomass materials tested were corn stover, switchgrass, and poplar wood. Corn stover was obtained from Michigan State University Farms (East Lansing, MI, USA). This stover was harvested in September 2008 from corn hybrid NK 49-E3 (Syngenta, Basel, Switzerland), a typical CS hybrid grown in the Great Lakes Region. The switchgrass, *Panicum virgatum*, and poplar wood, *Populus trichocarpa*, were provided by the BioEnergy Science Center (BESC). The poplar was debarked, split, and chipped (Yard Machine 10HP, MTD Products Inc., Cleveland, OH). The corn stover, switchgrass, and poplar wood chips were further knife milled (Model 4, Wiley Mill, Thomas Scientific, Swedesboro, NJ) through a 1 mm screen. After that, all materials were air dried for approximately one month followed by sieving to collect fractions with a particle size between 20-mesh (<0.85 mm) and 80-mesh (>0.180 mm) (RX-29, W.S. Tyler, Mentor, OH). Particles larger than 20-mesh were collected and sieved again, and the resulting 20-80 mesh fraction was mixed with the previously obtained 20-80 mesh fraction.

Microcrystalline and amorphous cellulose as well as starch were also tested by conventional two-step acid hydrolysis and the new one-step approach. Pure cellulose (Avicel®PH101, Cat No. 11365, Lot 1094627) was purchased from FMC Corporation, Philadelphia, PA. Regenerated amorphous cellulose (RAC) was prepared from Avicel PH 101 according to a method reported by Zhang and coworkers [26]. Starch powder (Batch #076K0181) from potato was from Sigma Chemicals (St. Louis, MO). 72 wt% sulfuric acid (Lot.19093268) used for acid hydrolysis was from Ricca Chemicals (Arlington, Texas Sigma).

7.3.2 Composition analysis

The major components of all the materials were determined by the well-established laboratory analytical procedures (LAPs) "Determination of structural carbohydrates and lignin in biomass" refined by NREL researchers [25]. The method is based on two-step acid hydrolysis, in which 0.3±0.01 g biomass is first hydrolyzed by 3 mL 72 wt% sulfuric acid at 30 ℃ for 1 h, followed by its dilution to 4 wt% acid for additional hydrolysis at 121 ℃ for 1 h. A set of sugar recovery standards (SRS), summarized in Table7.1, were prepared to correct for losses due to sugar degradation during the second acid hydrolysis. After hydrolysis, about 700 μL of liquid was drawn, centrifuged to separate solids from the liquid in a microcentrifuge (Model 5424, Eppendorf North America, Hauppauge, NY) at 14,600 rpm for 5 min, pipetted into 500μL polyethylene HPLC vials (Grace, Deerfield, IL), and then stored at 4℃ until analysis for sugar content.

7.3.3 Hemicellulose analysis by one-step acid hydrolysis

The hemicellulose content of all materials was also determined by the fast one-step acid hydrolysis method developed here and compared with values determined by conventional NREL method. The fast hemicellulose quantification used 0.3±0.01 g biomass (dry weight) that was hydrolyzed in 87 mL of 4 wt% sulfuric acid hydrolysis at 121 ℃ for 1 h. Then the liquid was drawn, centrifuged, and stored at 4 ℃ until analysis for sugar content.

7.3.4 Component removal by one-step acid hydrolysis

After one-step acid hydrolysis of corn stover, switchgrass, and poplar wood, the solid residues were collected by filtration, dried in a 105 ℃ oven overnight, and weighed to calculate the solid yield. Then, the composition of the solid residues was determined by NREL LAPs [25] for component removal calculation.

7.3.5 Sugar analysis

For analysis, samples along with appropriate calibration standards were run on a Waters Alliance HPLC system (Model e-2695, Waters Corporation, Milford, MA) employing an Aminex HPX-87H column (Bio-Rad Laboratories, Life Science Research, Hercules, CA). Samples were processed at an eluent of 5mM sulfuric acid with flow rate of 0.60 ml/min using a refractive index (RI) detector (Model 2414, Waters Corporation, Milford, MA). The chromatograms were recorded and processed with Empower® 2 software (Waters Corporation, Milford, MA).

7.3.6 Calculation of sugar content, solid yield, and component removal

The glucan, xylan, and arabinan contents were calculated as:

$$Gluan\ Content\ (\%) = 100 \times \frac{(GH\ (g) + CB(g) * 1.053)/1.111}{Sample\ (g)}$$

$$Xylan\ Content\ (\%) = 100 \times \frac{XH\ (g)/1.136}{Sample\ (g)}$$

$$Arabinan\ Content(\%) = 100 \times \frac{AH\ (g)/1.136}{Sample\ (g)}$$

Galactose and mannose have similar retention times and responses as xylose in the Aminex HPX-87H column. However, when the three sugars did not coelute, the content of each was determined as:

$$Galactan\ Content\ (\%) = 100 \times \frac{GaH\ (g)/1.111}{Sample\ (g)}$$

$$Mannan\ Content\ (\%) = 100 \times \frac{MH\ (g)/1.111}{Sample\ (g)}$$

Otherwise, the content was reported as XGM (xylan+galactan+mannan), which was calculated by the following equation:

$$XGM\ Content\ (\%) = 100 \times \frac{XH\ (g)/1.136}{Sample\ (g)}$$

in which GH, CB, XH, AH, GaH and MH represent glucose, cellobiose (if any), xylose , arabinose, galactose, and mannose released during acid hydrolysis, and 1.053, 1.111, and 1.136 are the mass conversion factors for cellobiose to glucose, glucose (mannose or galactose) to cellulose (mannan or galactan), and xylose (arabinose) to xylan (arabinan), respectively. The mass of sample used here was on a dry weight basis.

The solid yield of samples after one-step acid hydrolysis was defined as:

$$Solid\ Yield\ (\%) = 100 \times \frac{Total\ dry\ solid\ after\ acid\ hydrolysis\ (g)}{Total\ dry\ solid\ before\ acid\ hydrolysis\ (g)}$$

The components (glucan, xylan, and lignin) removed in one-step acid hydrolysis was calculated as:

$$Glucan\ removal\ (\%) = 1 - \frac{\%\ Glucan\ in\ solid\ after\ one\ step\ acid\ hdyrolysis}{\%\ Glucan\ in\ raw\ biomass} \times Solid\ yield$$

$$Xylan\ removal\ (\%) = 1 - \frac{\%\ Xylan\ in\ solid\ after\ one\ step\ acid\ hydrolysis}{\%\ Xylan\ in\ raw\ biomass} \times Solid\ yield$$

$$Lignin\ removal\ (\%) = 1 - \frac{\%\ Lignin\ in\ solid\ after\ one\ step\ acid\ hdyrolysis}{\%Lignin\ in\ raw\ biomass} \times Solid\ yield$$

7.3.7 Statistical analysis

The xylan content (XGM) in corn stover, switchgrass, poplar wood, and the four NIST standards was determined by the conventional two-step acid hydrolysis (NREL LAP) and the new one-step acid hydrolysis using quadruplicate measurements. To test whether the xylan contents from the two methods were statistically the same, an equivalence test was performed. First, a two-tailed F-test was performed to check if the variances of two sample populations were the same:

$$F_{calculate} = \frac{S^2_{the\ larger\ value}}{S^2_{the\ smaller\ value}}$$

where S is the sample standard deviation, n is the number of samples, and the degree of freedom is n-1 for both the numerator and the denominator. $F_{calculate}$ was compared with the critical value at the 10% significance level ($\alpha = 0.1$). If the $F_{calculate} \leq F_{critical}$, then the variances of the two sample populations were accepted as equal. If the $F_{calculate} > F_{critical}$, then the variances of the two sample populations were considered to be different.

In addition, the two-tailed t-test was used to test if the mean values of the two sample populations were the same. As for the F-test, if the variances of the two populations were determined to be equal, then t was calculated as:

$$t_{calculate} = \frac{\bar{X}_1 - \bar{X}_2}{\left(\frac{s_p^2}{n_1} + \frac{s_p^2}{n_2}\right)^{\frac{1}{2}}}$$

in which $s_p = \frac{(n_1-1)s_1^2+(n_2-1)s_2^2}{n_1+n_2-2}$, the degree of freedom is df=n1+n2-2. When the variances of the two populations were not equal, then t was calculated as:

$$t_{calculate} = \frac{\bar{X}_1 - \bar{X}_2}{\left(\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}\right)^{\frac{1}{2}}}$$

with the degree of freedom calculated by the following equation to the closest integer larger then the calculated value:

$$df = \frac{\left(\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}\right)^2}{\frac{(s_1^2/n_1)^2}{n_1} + \frac{(s_2^2/n_2)^2}{n_2}}$$

In this equation, $\bar{X}_1, \bar{X}_2$ are average values of the samples, S_1^2 and S_2^2 are variances of the samples, and n1 and n2 are the number of tests in a sample.

The value of $t_{calculate}$ was compared to the critical value at the10% ($\alpha = 0.1$) significance level. If $t_{calculate} \leq t_{critical}$, then the one-step and two-step acid hydrolysis methods were considered to give statistically the same xylan content. Otherwise, the xylan contents determined by the one- and two-step acid hydrolysis were considered to not be statistically equal.

7.4 Results and Discussion

7.4.1 Selection of conditions for one-step method

The one-step acid hydrolysis applied acid loadings of 2, 4, and 6 wt% at 121 ℃ for 1 h on beechwood xylan, corn stover, switchgrass, and poplar wood and the resulting xylan plus galactan plus mannan(XGM) were compared with the values obtained from conventional two-step hydrolysis. For both acid loading of 2 and 6 wt% with one-step hydrolysis, the XGM value were much lower than that determined by the two-step acid hydrolysis (not shown). Moreover, the sugar recovery standards run with 6wt% showed very severe degradation of xylose (approximately 30%), which suggested that 6 wt% acid loading was not a proper choice [27]. Only 4 wt% acid loading at 121 ℃ for 1h gave comparable XGM results as the two-step acid hydrolysis. As a result, this condition was chosen for the following tests.

7.4.2 Composition of hemicellulose compounds

The one-step acid hydrolysis was first applied to the five compounds rich in hemicellulose, and the results were compared to compositional data from the conventional two-step acid hydrolysis (NREL-LAP). As shown in Figure7.1, one-step hydrolysis at 121 ℃ with 4 wt% sulfuric acid for 1h gave virtually identical sugar contents as conventional two-step acid hydrolysis for all five hemicellulose compounds, in that the sugar contents measured by one-step acid hydrolysis were in agreement with the conventional method and the standard deviations were also comparable. Overall, there was no discernible trend that suggested over- or under-estimation of the sugar compositions by the one-step acid hydrolysis method. Regardless of the type of

hemicellulose-rich compound, the one-step method was capable of complete deconstruction of the hemicellulose to monomeric sugars for quantitative analysis.

7.4.3 Xylan and arabinan content of NIST standards and lignocellulosic biomass samples

Next, the xylan and arabinan contents of the four NIST standards were measured by one-step acid hydrolysis, and the results were compared with the reference value provided by NIST [28-31] as well as the results from application of the conventional NREL methods in our laboratory. As shown in Figure7.2, both the xylan plus galactan plus mannan (XGM) and arabinan contents determined by one-step acid hydrolysis were comparable to results from the conventional method and the reference value.

The XGM contents measured by one-step and conventional two-step acid hydrolysis were further tested for statistical equivalence at a 10% significance level (α=0.1) by applying the widely used two-tailed t-test. However, the F-test at the 10% significance level (α=0.1) was applied first to test the equivalence of variances from the two methods as shown in Table7.2. The critical value of F at α=0.1 with df =3 for both sample populations is 9.28 according to an F-table. Based on the results from F-test, the variances of XGM content from one-and two-step acid hydrolysis were not equal for sugarcane bagasse, cottonwood, wheat straw, and poplar wood, but equal for Monterey pine, corn stover, and switchgrass. Then the t-test was employed because the sample size was small (< 30), with the distribution of the population assumed to be normal or approximately normal. There are two equations to calculate the t value as explained in the Methods section, with one generally used when the variances of the two populations

are assumed equal and the other when the variances are not equal. In the following, the t-test at significance level of 10% (α=0.1) was applied to calculate the t value $|t_{calculate}|$ which was then compared to the critical value from a t-distribution table. As shown in Table7.3, $|t_{calculate}|$ values of all samples were smaller than $t_{critical}$, suggesting that the XGM contents of all the NIST standards measured by the rapid one-step acid hydrolysis and the conventional two-step acid hydrolysis were statistically equivalent.

Corn stover, switchgrass, and poplar wood are feedstocks that hold great potential to support large-scale fuel production. The XGM and arabinan contents for these three species were evaluated by both the one-step acid hydrolysis and the conventional method, with the results shown in Figure 7.3. The XGM and arabinan contents from one-step acid hydrolysis were in good agreement with those determined by conventional method. In addition, Tables 7.2 and 7.3 show that the F-test and t-test, respectively, proved that these two analysis methods produced statistically equivalent XGM contents.

Overall, the one-step acid hydrolysis method gave statistically identical XGM and arabinan contents as the conventional two step approach for different biomass species, including agricultural residues (corn stover, sugarcane bagasse, and wheat straw), grasses (switchgrass), softwood (Monterey pine), and hardwood (poplar wood and eastern cottonwood). Thus, the one-step acid hydrolysis provides a viable method for rapid evaluation of the hemicellulose content in many different types of biomass. Compared with the conventional method for structural sugar measurement, the one-step acid hydrolysis reduces the analysis time by approximately half because only a 1h hydrolysis at 121 ℃ is needed, instead of 1 h at 30 ℃ followed by 1 h at 121 ℃. In addition, the one-

step method employs only a 4 wt% sulfuric acid solution and not concentrated sulfuric acid, reducing the hazard of dealing with concentrated sulfuric acid. Moreover, the one-step acid hydrolysis method can also be integrated with the high throughput small-scale compositional analysis system, which employs only 3.0 mg samples in 1.5 mL glass vials for measurement [11]. Thus, the one step approach can be an appealing method in terms of labor and time efficiency, especially when dealing with large numbers of samples, for example, genetic mutants with modified hemicellulose structure and/or content.

7.4.4 One-step acid hydrolysis on crystalline, amorphous cellulose and starch

Figures 7.2 and 7.3 show that other than XGM and arabinan, minor amounts (<5%) of glucan were also released in one-step acid hydrolysis for some biomasses. The most possible source of the glucan release was from β-glucan and hemicellulose side chains. In addition, starch in biomass would also be a possible source of glucose release in one-step acid hydrolysis. Starch is a storage component in most biomass, and its content can vary with part of the plant and harvest season and time of day [32]. The content of starch is typically low in mature wood or field dried herbaceous crops but varies from 2-8 wt% in green material of switchgrass, as reported by Decker et al[32]. Crystalline cellulose, however, is very unlikely to be a large source of glucan release from 4 wt% acid hydrolysis at 121 ℃ for 1 h due to its high recalcitrance at these conditions [12, 33].

To gain an understanding of how cellulose and starch behave in one-step acid hydrolysis, glucose release from crystalline cellulose (Avicel) and regenerated amorphous cellulose (RAC) as well as starch from potato was measured in terms of

glucan equivalents, as shown in Table7.4. At the given conditions, starch was completely hydrolyzed into glucose. In contrast, as expected, negligible amounts of glucan were released from crystalline cellulose (Avicel). The solids recovery data in Table7.4 also suggest that one-step acid hydrolysis hydrolyzed starch completely but not crystalline cellulose.

RAC was also subjected to both one-step and conventional two-step acid hydrolysis. The glucan content in RAC was first determined by conventional two-step acid hydrolysis as 75.2 ±0.6%. One-step acid hydrolysis partially released glucose from RAC, and not all the RAC was recovered after one-step hydrolysis. However, it should be kept in mind that the structure of RAC regenerated from phosphoric acid swollen cellulose disrupted hydrogen bonds so they were different from the amorphous region in natural cellulose in biomass [26]. Considering the tightly packed structure of crystalline of crystalline cellulose, we anticipate that the glucan released from one-step acid hydrolysis was not from the cellulose in biomass.

7.4.5 Components removal by one-step acid hydrolysis

The composition of the starting raw material and the material recovered from one-step acid hydrolysis as well as the solid yield are summarized in Table7.5. These results show that 48.0%, 54.9%, and 68.4% of the corn stover, switchgrass, and poplar wood were recovered from hydrolysis with 4 wt% acid at 121 ℃ for 1 h. Although it is known that acid treatment of biomass mainly removes hemicellulose from biomass, one-step acid hydrolysis also partially removed glucan and lignin, as shown in Table7.5. And as discussed before, the glucan may have come from the hemicellulose, β-glucan, and/or

starch in biomass. The method almost completely removed xylan, with only negligible amounts left in biomass: 0.9% for corn stover, 0.9% for switchgrass, and 1.0% for poplar wood. Therefore, one-step acid hydrolysis is a promising method for removal of hemicellulose from the solids so its content can be measured in terms of dissolved sugars.

7.5 Conclusions

A one-step acid hydrolysis method was developed for rapid quantification of XGM content in various types of lignocellulosic biomass. By hydrolysis with 4wt% sulfuric acid at 121 ℃ for 1 h, the hemicellulose was almost totally released from various types of biomass as sugar monomer that could then be quantified by HPLC. This method gave statistically identical results in XGM contents compared to results from conventional two-step acid hydrolysis while significantly shortening analysis time. Thus, the one-step acid hydrolysis method provides a rapid and simple approach for xylan (and other) hemicelluloses quantification.

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Table 7.1 Sugar recovery standards and their concentrations range used in the methods applied.

Name	Vendor	Lot Number	Concentration (mg/mL)
D (+)-Glucose	Sigma – Aldrich, St Louis, MO	089K00601	1 - 4
D (+)-Xylose	Acros Organic, New Jersey,	A0308408	1 - 4
L (+)-Arabinose	Alfa Aesar, Ward Hill, MA	10162224	1 - 4
D (+)-Mannose	Acros Organic, New Jersey,	A0308014	1 - 4
D (+)-Galactose	Acros Organic, New Jersey,	A0244833	1 - 4

Table 7.2 Application of F-test at 10% significance level to determine the variance of XGM content from one- and two-step acid hydrolysis methods were statistically the same.

	Conventional two-step acid hydrolysis			One-step acid hydrolysis			$F_{calculate}$	$F_{critical}$	$S_1^2 = S_2^2$
	$\bar{X}_1$	S_1	n_1	$\bar{X}_2$	S_2	n_2			
Corn stover	23.1	0.84	4	22.4	0.32	4	6.79	9.28	yes
Switchgrass	22.0	1.04		21.4	0.57		3.35		yes
Poplar wood	20.7	2.51		21.4	0.60		17.54		no
Sugarcane bagasse	21.1	0.11		20.9	0.43		14.82		no
Eastern cottonwood	17.4	0.17		18.0	0.98		34.69		no
Monterey pine	18.6	0.70		17.3	1.21		2.97		yes
Wheat straw	20.4	0.06		19.6	1.44		641.1		no

$\bar{X}$: average of four independent measurements
S: standard deviation of four independent measurements
n: number of samples
$F_{critical}$ is at 10% significance level (α=0.1)

Table 7.3 The results of t-test performed to compare the equivalence of XGM contents from one- and two-step acid hydrolysis methods.

Sample	df	$\lvert t_{calculate} \rvert$	$t_{critical,0.1}$	Equivalence
Corn stover	6	1.449	1.943	yes
Switchgrass	6	1.009	1.943	yes
Poplar wood	5 (4.46)	0.544	2.015	yes
Sugarcane bagasse	5 (4.54)	0.833	2.015	yes
Eastern cottonwood	5 (4.23)	1.262	2.015	yes
Monterey pine	6	1.922	1.943	yes
Wheat straw	5 (4.01)	1.133	2.015	yes

df- degree of freedom, the value of df was calculated as introduced in Methods section. The numbers between brackets are the calculated value of df when variance of two sample sets were unequal and the closest integer larger than the calculated value was used as df

$t_{critical}$ at 10% significance level

Table 7.4 Summary of glucan release and solid yields from one- and two-step acid hydrolysis of Avicel, RAC, and starch.

		Avicel	RAC	Starch
Conventional two-step acid hydrolysis	Solid yield	0	0	N/A
	Glucan (%)	98.4 ± 0.4	75.2 ± 0.6	N/A
One-step acid hydrolysis	Solid yield	97.0 ± 1.0	56.7 ± 1.7	0
	Glucan (%)	2.7 ± 0.2	14.6 ± 2.2	97.0 ± 1.4

N/A- not analyzed

Table 7.5 Summary of the raw biomass and residual solids compositions, and components removal and solid yields of various feedstocks after one-step acid hydrolysis.

	Composition	Corn Stover	Switchgrass	Poplar
Raw	Solid (g)	100	100	100
	Glucan (%)	34.3 ±0.3	33.1 ±1.0	46.9 ±1.4
	XGM (%)	23.1 ±0.8	22.0 ±1.0	20.7 ±2.5
	K-Lignin (%)	17.2 ±1.0	18.8 ±1.2	23.4 ±1.0
Residual solids after one-step acid hydrolysis	Solid (g)	48.0 ±0.4	54.9 ±1.2	68.4 ±0.1
	Glucan (%)	63.2 ±0.2	59.0 ± 0.8	65.7 ±0.3
	XGM (%)	0.9 ±0.07	0.9 ±0.4	1.0 ±0.05
	K-Lignin (%)	29.0 ±1.3	29.0 ±1.2	29.0 ±1.2
Removal by one-step hydrolysis	Solid (g)	52	45.1	31.6
	Glucan (%)	10.7 ±0.9	2.9 ±1.4	4.15 ±0.6
	XGM (%)	98.0 ±0.1	97.8 ±1.1	96.7±0.2
	K-Lignin (%)	20.1 ±2.2	18.1 ±2.45	15.9 ±2.3

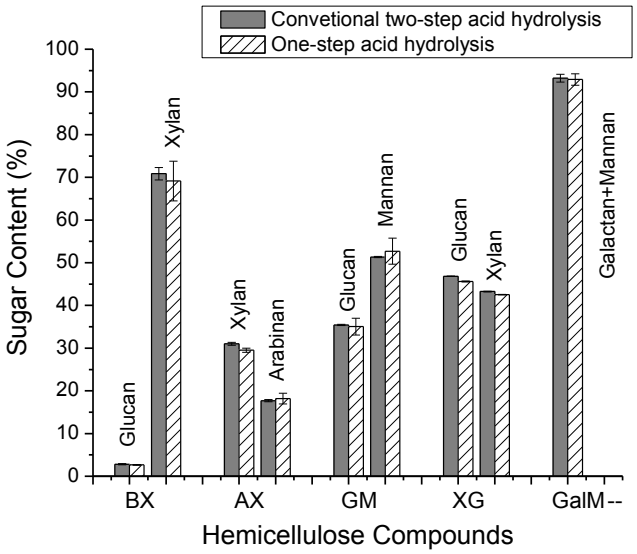

Figure 7.1 Sugar contents of Beechwood xylan (BX), glucomannan (GM), galactomannan(GalM), arabinoxylan(AX), and xyloglucan (XG) as determined by one-step and conventional two-step acid hydrolysis methods. The compositions are displayed as mass percent, and the error bars represent standard deviation from 4 independent measurements

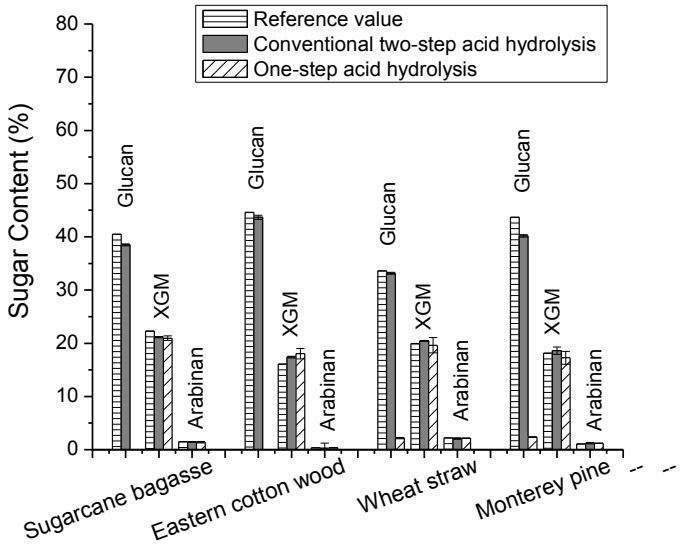

Figure 7.2 Glucan, xylan plus galactan and mannan (XGM), and arabinan contents of the NIST standards determined by the one-step and conventional two-step acid hydrolysis methods, and their reference values. The compositions are displayed as mass percent, and the error bars represent standard deviation from 4 independent measurements

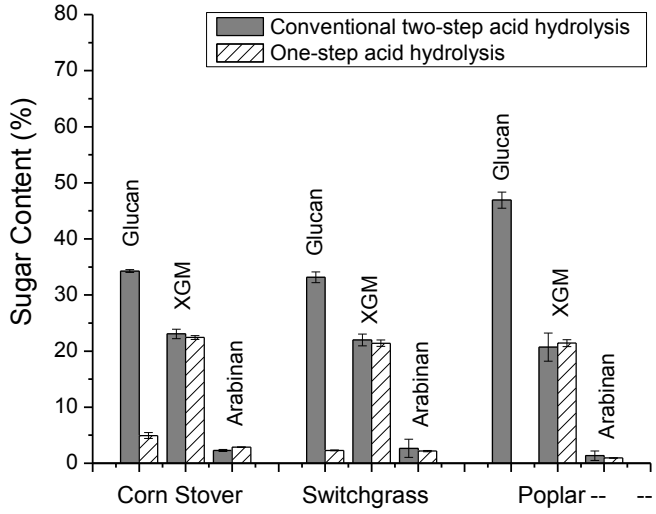

Figure 7.3 Glucan, xylan plus galactan and mannan (XGM), and arabinan contents in corn stover, switchgrass, and poplar wood determined by the one-step and conventional two-step acid hydrolysis methods. The compositions are displayed as mass percent, and the error bars represent standard deviation from 4 independent measurements.

Chapter 8 Comparison of Enzymatic Reactivity of Corn Stover Solids Prepared by Dilute Acid, AFEX™, and Ionic Liquid Pretreatments

This whole chapter will be submitted to "Biotechnology for Biofuel" or a similar journal under the following citation: Gao X., Kumar R., Singh S., Simmons B., Balan V., Dale B., and Wyman CE. "Comparison of Enzymatic Reactivity of Corn Stover Solids Prepared by Dilute Acid, AFEX™, and Ionic Liquid Pretreatments**"**

8.1 Abstract

Pretreatment is essential to realize high product yields from biological conversion of naturally recalcitrant cellulosic biomass, with thermochemical pretreatments often favored for cost and performance. In this study, dilute sulfuric acid (DA), ammonia fiber expansion (AFEX^{TM}), and ionic liquid (IL) thermochemical pretreatments that have distinct differences in their effects on biomass were applied to corn stover to understand how the resulting changes in substrate features influenced sugar yields.

Corn stover was pretreated by DA, AFEX^{TM}, IL, and enzymatic digestion was performed on the pretreated solids at low to high protein loadings with ratios of cellulase, xylanase, and pectinase enzymes optimized for each pretreatment. Avicel cellulose, regenerated amorphous cellulose (RAC), and beechwood xylan were also subjected to enzymatic hydrolysis. Initial hydrolysis rates and sugar release over 120 h of hydrolysis, cellulase accessibility to cellulose, and analysis of the amount of oligomers released over enzymatic hydrolysis were measured. Ionic liquid pretreated corn stover displayed the highest initial reactivity at all enzyme loadings and the highest final digestibility for a low enzyme loading of 3 mg protein/g glucan in the raw material. However, increasing the enzyme loading to 12 mg/g glucan resulted in dilute acid and AFEX^{TM} pretreated corn stover attaining higher cellulose digestions. Hydrolyzate from AFEX^{TM} pretreated corn stover had the highest proportion of xylooligomers, while ionic liquid produced the most glucooligomers. However, the amounts of both oligomers dropped with increasing enzyme loadings and hydrolysis times. Ionic liquid pretreated corn stover had the highest accessibility to enzymes as measured by the maximum cellulase adsorption capacity.

Substrate reactivity and digestibility were affected by substrate features and interactions between substrate and enzymes for all three pretreatment technologies. Initial hydrolysis rates were highest for greater lignin removal, a greater portion of amorphous cellulose, and high enzyme accessibility. The final glucan yield, however, was also affected by xylooligomers released from xylan during hydrolysis. Overall, no single factor could account for enzymatic digestion performance for the three pretreated materials.

Keywords: corn stover, accessibility, cellulase, oligomers, pretreatment, hydrolysis

8.2 Background

Lignocellulosic biomass, including agricultural and forestry residues and herbaceous and woody crops [1], provides the only sustainable resource for large-scale and low-cost production of liquid fuels and organic chemicals that are currently produced from dwindling and nonrenewable fossil resources [1, 2]. To realize the high yields and low costs vital to commercial success [3], cellulosic biomass must be pretreated prior to enzymatic hydrolysis, and the choice of pretreatment not only affects enzymatic digestion performance but impacts upstream and downstream processing steps as well [1, 4]. To overcome the natural recalcitrance of cellulosic biomass, several biological, chemical, thermochemical, physical pretreatment methods have been applied. Nevertheless, thermochemical pretreatments are often preferred due to their low energy demand and capital costs and better performance. Among thermochemical pretreatments, several employing inexpensive chemicals and reagents show promise. An ideal pretreatment, however, should reduce biomass recalcitrance and increase biomass accessibility to enzymes in a cost effective way [5]. Among several options, only hemicellulose or lignin removal and/or alternation by dilute acids or base promise reasonable costs so far [3, 6, 7]. In particular, dilute sulfuric acid (DA) and ammonia fiber expansion (AFEX^{TM}) pretreatments are currently among the most promising from a combined cost and performance perspective [1].

Dilute sulfuric acid pretreatment effectively removes and recovers hemicellulose and partially disrupts and relocates lignin, while increasing cellulose digestibility [8-10]. The AFEX^{TM} process pretreats biomass with anhydrous liquid ammonia at high pressure

and moderate to high temperatures. Following pretreatment for a given time, the pressure is rapidly released resulting in biomass structure disruption and partial cellulose decrystallization, presumably resulting in enhanced cellulose digestibility [11-13]. Lately, the use of certain ionic liquids, such as those based on imidazolium cations, to dissolve cellulose has received attention as promising pretreatment solvents [14]. In this case, biomass is exposed to the ionic liquid 1-ethyl-3-methylimidazolium acetate, at certain temperatures and pressures and then an anti-solvent is added to the solution to precipitate biomass. It is believed that this ionic liquid disrupts the native cellulose crystalline structure and chemical linkages, thus reducing biomass recalcitrance [15-17].

Various biomass physiochemical changes resulting from the action of different leading pretreatments have been reported to enhance cellulose digestion, such as surface area, pore volume, hemicellulose removal, lignin removal and/or relocation, crystallinity reduction, and reduced cellulose degree of polymerization [18-24]. Of the many possible factors, DA pretreatment is mainly credited with enhancing digestion by hemicellulose removal and lignin relocation [10]. Creation of pore structure, and disruption of lignin-carbohydrates linkages are believed to result in enhanced digestion for AFEX^{TM} pretreatment [25, 26]. Increased surface roughness, loss of cellulose crystallinity, and expansion or even a transformation of cellulose lattice have been reported to account for the effects of ionic liquid pretreatment on biomass [15].

Enzymatic hydrolysis is one of the key steps in converting carbohydrates in lignocellulosic biomass into fermentable sugars and allows nearly theoretical yields of sugars [1, 2, 27-29]. Endoglucanases, exoglucanases and β-glucosidase as well as

supplementary enzymes such as xylanase are generally required to complete such heterogeneous reactions effectively and efficiently [30-34]. Although it is possible to realize near theoretical yields, the high doses of enzymes are expensive and still a major obstacle to commercial use [28, 29].

Hence, it is vital to understand how pretreatments affect subsequent enzymatic hydrolysis and the key features controlling enzymatic hydrolysis of solids as well as their impacts on effectiveness of enzymatic hydrolysis, especially at low enzyme loadings. For this objective, researchers from the University of California Riverside supported by the BioEnergy Science Center (BESC), Michigan State University supported by the Great Lakes Bioenergy Research Center (GLBRC), and the Joint BioEnergy Institute (JBEI) collaborated to better understand how biomass pretreatments with much different deconstruction patterns impact biological conversion to sugars and changes in chemical and structural features of biomass that could account for these changes. Comparative information was obtained based on use of a single source of feedstock and common commercial cellulase and other accessory enzymes.

In this study, we report on how the three pretreatments impact several aspects of pretreated biomass: composition change following pretreatment, sugar release patterns in enzymatic hydrolysis at different enzyme loadings, substrate accessibility to enzymes, and oligomers released over enzymatic hydrolysis. The results were then integrated to understand how these pretreatments change substrate features and their role in achieving high yield.

8.3 Methods and Materials

8.3.1 Pretreated corn stover and model compounds

Corn stover was obtained from Michigan State University Farms (East Lansing, MI, USA). The corn hybrid used was NK 49-E3 (Syngenta, Basel, Switzerland) which is a typical CS hybrid grown in the Great Lakes Region and harvested in September 2008. Solids resulting from pretreatment of the same sources of corn stover were prepared and shared within the BRC collaboration partners as follows: Dilute acid pretreatment was performed by UCR, AFEX™ by Michigan State University, and ionic liquid by JBEI. Upon receipt, the AFEX™ and ionic liquid pretreated corn stover solids were immediately refrigerated at 4°C until further analysis. Table 8.1 summarizes conditions for all three pretreatments.

Pure cellulose (Avicel®PH101, Cat No. 11365, Lot 1094627) was purchased from FMC Corporation, Philadelphia, PA. Regenerated amorphous cellulose(RAC) was prepared from Avicel PH 101 according to a method reported by Zhang and coworkers [45]. Beechwood xylan (Lot BCBS8393V) was purchased from Sigma Chemicals, St. Louis, MO. Moisture contents and compositional analysis of the corn stover solids and model compounds were performed according to NREL Laboratory Analytical Procedures [50].

8.3.2 Enzymes

Cellic® CTec2 (Batch No. VCNI0001) and Cellic® HTec2 (Batch No. VHN0001) enzymes were generously provided by Novozymes North America, Inc. (Franklinton, NC, USA), and Multifect® Pectinase (Batch No. 4861295753) was from DuPont™

Genencor® Science (Palo Alto, CA, USA). Table 8.2 shows the enzyme protein concentrations determined by the Kjeldahl method [51], with the nitrogen factor (NF) determined by equation 1:

$$NF = \%\ protein\ /\ \%\ nitrogen \quad (1)$$

in which the % protein was calculated as:

$$\%\ protein = protein\ content\ (mg/mL)\ /\ solid\ concentration\ (mg/mL) \quad (2)$$

The nitrogen content was determined by following the method described elsewhere [52]. The solid content of the enzyme solution was determined following the NREL Laboratory Analytical Procedure [53].

8.3.3 Enzymatic hydrolysis

In accordance with the NREL Laboratory Analytical Procedure[54], enzymatic hydrolysis was conducted in triplicate at a solids loading corresponding to 1% (w/w) glucan in 0.05M citrate buffer (pH = 4.9) containing 10 mg/mL sodium azide in 50 mL Erlenmeyer flasks. The slurries were incubated at 50^{o}C for 120 h in a shaker incubator (Multitron Infors-HT, ATR Biotech, MD) at 150 rpm. Enzyme loadings were 3, 6, 12, and 30 mg of total protein/g glucan in the raw biomass. The optimized enzyme combinations of Celic® CTec2, Celic® HTec2, and Multifect® Pectinase to achieve maximum sugar release for solids from DA, AFEX™, and IL pretreatments were determined by GLBRC using their novel high throughput micro-plate method [37] and are shown in Table 8.3.

Hydrolysis samples were collected at 1, 2, 4, 8, 24, 48, 72, and 120 h. To determine the amount of sugar generated from enzymatic hydrolysis, 400 μL samples

were drawn, filtered through 0.2 μm nylon filter vials (Alltech Associates Inc., Deerfield, IL), pipetted into 500 μL polyethylene HPLC vials, and then stored at 4℃ until analysis. Glucan to glucose and xylan to xylose hydrolysis yields were calculated as defined in the equations 3 and 4 below, respectively.

$$\% \text{ Glucan yield} = 100 \times ((GH\ (g) + CB\ (g) \times 1.053))/1.111)\ /GP\ (g) \quad (3)$$

$$\% \text{ Xylan yield} = 100 \times (XH\ (g)/1.136)\ /\ XP\ (g) \quad (4)$$

in which GH, CB, and XH represent glucose, cellobiose, and xylose released from enzymatic hydrolysis; GP and XP stand for glucan and xylan available in the pretreated biomass, and the factors 1.111, 1.136, and 1.053 account for the mass gained during hydrolysis of glucan to glucose, xylan to xylose, and cellobiose to glucose, respectively.

8.3.4 Basis for enzyme protein loading per g glucan in raw biomass

Consistent with the approach used by our team in prior research, enzyme loadings for all enzymatic digestion experiments in this study were based on glucan content in the original raw material [1]. This loading method allows a fair comparison of different pretreatment techniques in that providing the same amount of enzyme based on glucan content in raw biomass, a pretreatment which removes more glucan in the pretreatment step would benefit in terms of higher enzyme loading per unit glucan left in the solids to the enzymatic hydrolysis step. This comparison is particularly important for enzymatic hydrolysis at commercially viable low enzyme loadings. Herein, because of the different composition resulted from each pretreatment, the enzyme loading per gram glucan in the pretreated biomass solids varied as shown in Table 8.3. It suggests that solids from DA

and IL had higher enzyme loading per g glucan in the pretreated solids, when compared to solid from AFEX™ which did not have any lose composition of biomass.

8.3.5 Estimation of oligomers amount

To determine the total amount of glucose and xylose including oligomers generated by enzymatic hydrolysis, liquid samples following enzymatic hydrolysis for 4, 24, and 72 h were subjected to post hydrolysis according to the NREL Laboratory Analytical Procedure [55]. The slurries after enzymatic hydrolysis were centrifuged to separate solids from the liquid. Then, the liquid was incubated for 1 h with 4% sulfuric acid at 121℃ in an autoclave (Model HA300MII, Hirayama Manufacturing Corporation) along with sugar recovery standards. It is important to note that post-hydrolysis was carried out in 1.5 mL high recovery glass HPLC vials (Agilent, Santa Clara, CA) and scaled down to 1mL reaction volume [56] than conventionally performed in 125 ml pressure bottles with 5 to 20 ml liquid. Following post-hydrolysis, about 400 μL samples were withdrawn, pipetted into 500 μL polyethylene HPLC vials, and kept at 4℃ or frozen at -20℃ until further analysis.

The percentage (%) of glucooligomers with a degree of polymerization > cellobiose,

G_{3+}, was calculated as:

$$G_{3+} = 100 \times (GH'(g) - GH(g) - 1.053 \times CB(g)) / GH'(g) \quad (5)$$

X_{2+}, the percent yield of xylooligomers containing 2 or more xylose units, was calculated as:

$$X_{2+} = 100 \times (XH'(g) - XH(g)) / (XP(g) * 1.136) \quad (6)$$

Meanwhile, the percentage (%) of xylose oligomers containing 2 or more xylose units in total xylose release was calculated as:

$$\% X_{2+} = 100 \times (XH'(g) - XH(g)) / XH'(g) \quad (7)$$

The terms GH' and XH' represent glucose and xylose after post hydrolysis adjusted for losses during that method [55].

8.3.6 Sugar analysis

For analysis, samples along with appropriate calibration standards were run on a Waters Alliance HPLC system (Model e-2695, Waters Corporation, Milford, MA) employing an Aminex HPX-87H column (Bio-Rad Laboratories, Life Science Research, Hercules, CA). Samples were processed at an eluent flow rate of 0.60 ml/min using a refractive index (RI) detector (Model 2414, Waters Corporation, Milford, MA). The chromatograms were recorded and processed with Empower® 2 software (Waters Corporation, Milford, MA).

8.3.7 Enzyme adsorption

Adsorption experiments were performed at 4℃ in 0.05 M citrate buffer (pH-4.8±0.2) in 15 ml test tubes (Cat No. 430055, Fisher Scientific) with a biomass loading appropriate to achieve 1% w/w glucan with enzyme loadings of 0 to 2000 mg protein /g glucan (0 to 20 mg protein/ml). The tubes containing biomass slurry and enzyme proteins were mounted on a variable speed rugged rotator (Glass-Col, LLC, Terre Haute, IN) and equilibrated for 6 h at 40 rpm. Following equilibration, the tubes were centrifuged (Model Allegra X-15R, Beckman Coulter, Fullerton, CA) at 3500 rpm for 15min for

solid-liquid separation, the liquid was decanted, and then the tubes were dried overnight at 105℃. The adsorbed protein amount was directly determined by a nitrogen factor method described elsewhere [52]. The nitrogen content of the dried and homogenized biomass solids was measured using a Flash EATM 112 N/Protein plus CHNS/O Analyzer (CE Elantech, Lakewood, NJ) with atropine as a standard (Cat No.33835210, CE Elantech, Lakewood, NJ, US). Adsorption data was non-linearly fitted to a Langmuir model according to equation 8 [31, 57],

$$[CE] = \frac{\sigma[S_t][E_f]}{K_d + [E_f]} \qquad (8)$$

in which [CE] is the amount of adsorbed enzyme in mg/mL, $[E_f]$ is the free enzyme concentration in mg/mL, σ is the maximum adsorption capacity in mg/mg substrate, $[S_t]$ is the substrate concentration mg/mL, and K_d is the equilibrium constant equal to [C][E]/[CE].

8.4 Results and Discussion

8.4.1 Compositional analysis of DA, AFEX™, and IL pretreated corn stover

Table 8.1 summarizes the pretreatment conditions applied, compositions of raw and pretreated corn stover solids, and the amount of xylan and lignin removal during each pretreatment. It can be seen that DA removed about 87% of the xylan, resulting in solids with a very low xylan content of 6.5% and a high glucan content of 59.1%. AFEX™ pretreatment of corn stover did not solubilize much of the biomass during pretreatment, resulting in negligible compositional change. However, IL pretreatment removed > 90% of the lignin and > 20% of the xylan originally present in corn stover, which resulted in

solids with a low lignin content of only 2.7% and enhanced glucan and xylan contents of 46.9% and 29.8%, respectively. These changes in composition agree with results from many previous studies [24, 35, 36].

8.4.2 Enzymatic hydrolysis of pretreated corn stover solids and model compounds

8.4.2.1 Glucan yields

Enzymatic digestion was performed with enzyme formulations determined to maximize sugar yields by GLBRC using their high throughput micro-plate system[37]. As shown in Figure 8.1, a general observation was that hydrolysis of DA and AFEX^{TM} pretreated corn stover was rapid in the first 8 h and then started to slow down, giving the maximum yield after 72 h. The corn stover from IL had a faster initial hydrolysis rate compared to DA and AFEX^{TM} corn stover and reached the maximum yield in only 8 h.

Figures 8.2 and 8.3 compare the initial reactivity and final digestibility of corn stover from different pretreatments at four levels of enzyme loadings. The initial reactivity was determined from glucan yields in the first hour of enzymatic hydrolysis. Figure 8.2 shows that the reactivity of all substrates increased with enzyme loading and was almost linearly related to it. IL corn stover solids had the highest reactivity at all enzyme loadings followed by DA and then AFEX^{TM}. Increasing the enzyme loading increased the glucan yield from IL pretreated solids by 40% in the first hour of hydrolysis. The reactivity of DA corn stover at the highest enzyme loading of 30 mg/g glucan was 32% higher than at the lowest enzyme loading of 3 mg. AFEX^{TM} corn stover reactivity was slightly lower compared to DA at the low enzyme loading but was substantially lower

(10%) at the high enzyme loading. Thus, the reactivity of AFEX™ corn stover did not benefit from higher enzyme loading as much as other two substrates.

The digestibility, defined as the final maximum glucan yield from enzymatic hydrolysis, was also compared among substrates from different pretreatments and model compounds at four enzyme loadings, as shown in Figure 8.3. Overall, higher enzyme loading led to higher glucan yields, but the benefit varied with pretreatment. For example, as shown in the Figure 8.3, when the enzyme loading was increased from 3 to 30 mg/g original glucan, the digestibility for DA and AFEX™ corn stover increased substantially by 24.3% and 20.3% , respectively, while corn stover from IL pretreatment had only a 7% enhancement in glucan yield with increased enzyme loading. As seen in Figure 8.3, at 30 mg protein/ g glucan in raw stover with the optimal enzyme formulation, glucan yields from 72 h enzymatic hydrolysis of corn stover spanned a range of 74.5% to 93.8%, with DA pretreated corn stover showing the highest digestibility of 93.8% followed by IL (91.6 %) and then AFEX™ (82.1 %). Among model compounds, Avicel and RAC realized a 40% and 9% increase in digestibility with enzyme loading, respectively.

8.4.2.2 Xylan yields

Figure 8.4 reports xylan yields from enzymatic hydrolysis of DA, AFEX™, and IL corn stover at enzyme loadings of 3 and 30 mg/ g glucan in the raw biomass. Enzymatic hydrolysis was performed on a model compound, beechwood xylan, with loading of 3-30mg HTec2/g xylan as well. The total 72 h yield from beechwood xylan was 87% to 92% depending on the enzyme loading. However, large amounts of xylooligomers accumulated during hydrolysis, which may partially be due to absence of

β-xylosidase activity [36]. It should be kept in mind that when comparing xylan yields from enzymatic hydrolysis of solids from different pretreatments for the 0.01 g glucan/mL basis applied here, the composition variance of substrates from each pretreatment led to quite different xylan loadings. As shown in Figure 8.4, the total xylan yield for DA, AFEX^{TM}, and IL corn stover were 83%, 68%, and 77%, respectively, at an enzyme loading of 30 mg/g glucan in raw corn stover, with 9%, 14%, and 12%, respectively, being oligomers.

8.4.2.3 Percentage of oligomers released during hydrolysis

The liquid samples from 4, 24, and 72 h of enzymatic hydrolysis of corn stover from DA, AFEX^{TM}, and IL pretreatments were analyzed for longer chain length glucooligomers (> cellobiose) and xylooligomers by hydrolysis of the liquid samples from pretreatment with 4 %w/w dilute acid at 121^{o}C for 1 h . As shown in Figure 8.5, both sufficient hydrolysis time and increased enzyme loadings reduced the fraction of gluco- and xylo-oligomers. With individually optimized enzyme formulations of CTec2, HTec2, and Multifect Pectinase for each pretreatment type, the digestion of glucan to monomers was complete. At the early stage of hydrolysis and a low enzyme loading, such as 3 mg, the highest glucooligomers percentage (%) was less than 13% for AFEX^{TM} followed by 12% for IL, and 6% for DA. And as the enzyme loading was increased to 30 mg/g glucan, only trace amounts of glucooligomers, if any, could be detected at longer hydrolysis times of 72 or 120 h.

Figure 8.5 also suggests that hydrolysis of xylan was relatively incomplete. For hydrolysis of AFEX^{TM} and IL pretreated corn stover solids, approximately 20% or even

higher oligomers of the total xylose persisted even after 72h hydrolysis at an enzyme loading of 30 mg/g glucan in the raw material. Hydrolyzate from AFEX^{TM} contained the highest amount of xylooliomgers followed by IL and DA. It was also found that during enzymatic hydrolysis of pretreated corn stover, digestion of DA corn stover released the lowest percentage (%) of both cello-and xylooligomers.

8.4.3 Effect of xylan and lignin removal on enzymatic digestion

Enzymatic digestion was performed on corn stover pretreated by DA, AFEX^{TM}, and IL at low to high protein loadings with optimized ratios of CTec2, HTec2, and Multifect pectinase. The initial hydrolysis rate and final digestibility were affected by both substrate properties and enzyme loadings. Lignin and/or xylan removal as well as cellulose structure alternation influenced yields from enzymatic hydrolysis. Figure 8.6 shows how the reactivity and digestibility in terms of 1 h and 72 h glucan yields from enzymatic hydrolysis changed with lignin and xylan removal. Here we see that the initial yield at all enzyme loadings and the final digestibility at low enzyme loading correlated well with lignin removal. It has been reported that lignin is one of the key substrate features impacting enzymatic digestion of cellulosic biomass [2]. Lignin is believed to not only hinder cellulose accessibility as a result of lignin-carbohydrates linkages but also impact cellulase effectiveness by unproductive binding [24, 38, 39]. Figure 8.6 also shows that the reactivity data did not follow a clear trend with xylan removal, and digestibility was only correlated at high enzyme loading. Xylan removal has been reported to enhance glucan digestibility by improving cellulose accessibility[40] and/or reducing cellulase inhibition by xylooligomers, produced due to partial hydrolysis of

xylan [41-43]. It has been shown that xylobiose and xylooligomers with higher DP strongly inhibit enzymatic hydrolysis of pure cellulose, pure xylan, and pretreated corn stover [41], and xylooligomers were more inhibitory to cellulase than xylose or xylan for an equivalent amount of xylose as well as equal molar amounts of glucose or cellobiose[42].

Figure 8.7 records the relationship between the 72h glucan yield vs. xylooligomers concentration in the liquid from hydrolysis of each substrate, with the concentration of xylooligomers calculated on an equivalent xylose mass basis. A drop in glucan yield was observed as higher concentrations of xylooligomers accumulated. Therefore, xylan removal played an important role in the final glucan yield, especially when enzyme loading was high. But at the initial stage or low enzyme loading, other factors such as lignin removal and cellulose structure appeared to have a higher influence on digestibility.

8.4.4 Effect of physical structure on reactivity of substrate

Other than xylan and lignin removal, cellulose crystallinity is also believed to be one of the major factors limiting cellulose enzymatic hydrolysis. The reactivity data for IL corn stover showed higher initial hydrolysis rates than other substrates, probably due to reduction in crystalline cellulose content and/or altered cellulose structure that has been shown for corn stover earlier [35, 44]. This result is consistent with an earlier hypothesis that lower crystallinity had more significant influence on initial hydrolysis rate than on ultimate sugar yield [38]. Two model compounds, microcrystalline Avicel cellulose and RAC, were subjected to enzymatic hydrolysis as well. Avicel cellulose

showed the lowest hydrolysis rates (Figure 8.2) and final sugar release (Figure 8.3). RAC was a highly homogeneous substrate with disrupted hydrogen bonds [45] and consequently had much higher reactivity and final glucan yields (close to ~100%). The rapid hydrolysis of amorphous substrate could be explained as a homogeneous reaction that enabled cleavage of all β-glucosidic bonds randomly, resulting in a rapid reduction of DP [45]. Based on the hydrolysis model reported by Zhang and Lynd [32], three processes occur simultaneously when enzymes act on insoluble cellulosic substrates: (1) chemical and physical changes in the solid residue, (2) primary hydrolysis in which the solid phase is hydrolyzed into soluble cellodextrins, and (3) secondary hydrolysis in which the soluble oligomers are further hydrolyzed into monomers. Given that the rate of primary hydrolysis is much slower than the rate of secondary hydrolysis, a substrate with a fast primary hydrolysis rate should result in a faster overall rate. The experimental observation of a faster hydrolysis rate with IL pretreated corn stover suggested a similarity of IL corn stover with RAC. However, the properties of cellulose in corn stover from DA and AFEX^{TM} pretreatment might be more heterogeneous. This result could explain why IL corn stover showed a much faster initial hydrolysis rate when compared to others.

8.4.5 Adsorption of CTec2 and HTec2 on pretreated corn stover

Cellulase adsorption onto the substrate is the primary step in enzymatic degradation of cellulose [33, 46]. The adsorption parameters calculated for the Langmuir model, i.e., the maximum adsorption capacity σ and equilibrium constant K_d, are summarized in Table 8.5. We see that AFEX^{TM} corn stover had the lowest maximum

enzyme adsorption capacity for both CTec2 and HTec2, while IL corn stover had the highest values for both. These results show that enzyme adsorption onto solids and their effectiveness are affected by both substrate and enzyme features and as well as pretreatment type, consistent with information reviewed elsewhere[39, 47] Cellulose accessibility to enzyme has long been recognized as an essential factor in enzymatic hydrolysis of cellulosic biomass [48], and the hydrolysis rate or yield is often claimed to be related to enzyme adsorption [40, 47, 49]. In line with this, the reactivity of the three pretreated substrates followed the same trend as their maximum enzyme adsorption capacity for both CTec2 and HTec2.

8.5 Conclusions

Substrate reactivity and digestibility were affected by pretreatment technologies, resulting substrate features, and interactions between substrate and enzymes. No single factor absolutely dominated enzymatic digestion performance for the three pretreated materials. The high initial hydrolysis rate of IL corn stover correlated with a high degree of lignin removal, the large amorphous fraction, and high accessibility to enzymes. The final glucan yield from enzymatic hydrolysis, however, was also affected by the concentration of xylooligomers released from xylan during hydrolysis. The ionic liquid pretreated corn stover had highest glucan yield at low enzyme loadings while dilute acid pretreated corn stover, which contains the least amount of xylan, exhibited the highest digestibility at high enzyme loadings.

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Table 8.1 Pretreatment conditions, corresponding solids compositions, and component removals of corn stover pretreated by DA, AFEX™, and IL

	Pretreatment			
	None	DA	AFEX™	IL
Pretreatment conditions				
Chemicals	NA	dilute sulfuric acid	anhydrous ammonia	1- ethyl- 3- m ethyl- imidazolium acetate
Loadings	NA	0.5% wt	1:1 (Biomass: NH_3)	1:9 (Biomass: IL)
Temperature (℃)	NA	160	140	140
Time (min)	NA	20	15	180
Component (%)				
Glucan	33.4	59.1	33.5	46.9
Xylan	24.9	6.5	24.8	29.8
Arabinan	3.7	3.6	3.3	0.3
Lignin (AIL)	17.2	32.2	12.2	2.7
Component Removal (%)*				
Solid		51	0	36
Xylan	-	87.0	0.4	23.4
Lignin	-	8.2	2.8	89.9

**Solid removal (%)= 100% × (1- Total solid after pretreatment (g)/Total solid before pretreatment (g))*

Xylan removal (%) = 1- (%Xylan in pretreated solids / % Xylan in raw biomass) × Solid yield

Lignin removal (%)= 1- (%Lignin in pretreated solids / % Lignin in raw biomass)× Solid yield

Table 8.2 Description of enzymes, their protein concentration and nitrogen factor

Enzyme	Description	Protein concentration (mg/mL)	Nitrogen factor
Cellic® CTec2	Blend of cellulase, high level of β-glucosidase, and hemicellulases	138	6.09
Cellic® HTec2	Blend of hemicellulases and cellulase background	157	6.58
Multifect®Pectinase	Pectinase, cellulase, and hemicellulases	72	-

Table 8.3 Glucan recovery after pretreatment and enzyme loadings based on glucan content in the raw material

Pretreatment	Glucan yield (%)	Enzyme loading (mg protein/g glucan in raw)	Enzyme loading (mg/g glucan in pretreated)
Dilute acid	87	30	34
AFEX™	100		30
Ionic liquid	90		33

Glucan yield= Glucan in pretreated biomass(g)/ Glucan in the starting material (g)
Enzyme loading per g glucan in pretreated biomass = Enzyme loading per g glucan in raw/ glucan yield

Table 8.4. Optimized enzymes formulation on protein mass basis for DA, AFEX, and IL pretreated corn stover

Pretreatment	Cellic® CTec2	Cellic® HTec2	Multifect®Pectinase
Dilute acid	67%	33%	0
AFEX™	67%	16.5%	16.5%
Ionic liquid	39%	33%	28%

Table 8.5 Maximum CTec2 and HTec2 adsorption capacities, equilibrium constants, and correlation coefficients for solids resulting from pretreatments of corn stover by DA, AFEX™ and IL pretreatments

	Pretreatment		
	DA	**AFEX™**	**IL**
CTec2			
Maximum adsorption capacity, σ (mg/g substrate)	139	111	190
Equilibrium constants, K_d (mg/mL)	1.88	0.19	1.43
R^2	0.92	0.96	0.92
Htec2			
Maximum adsorption capacity, σ (mg/g substrate)	142	127	239
Equilibrium constants, K_d (mg/mL)	0.67	0.27	2.3
R^2	0.97	0.95	0.98

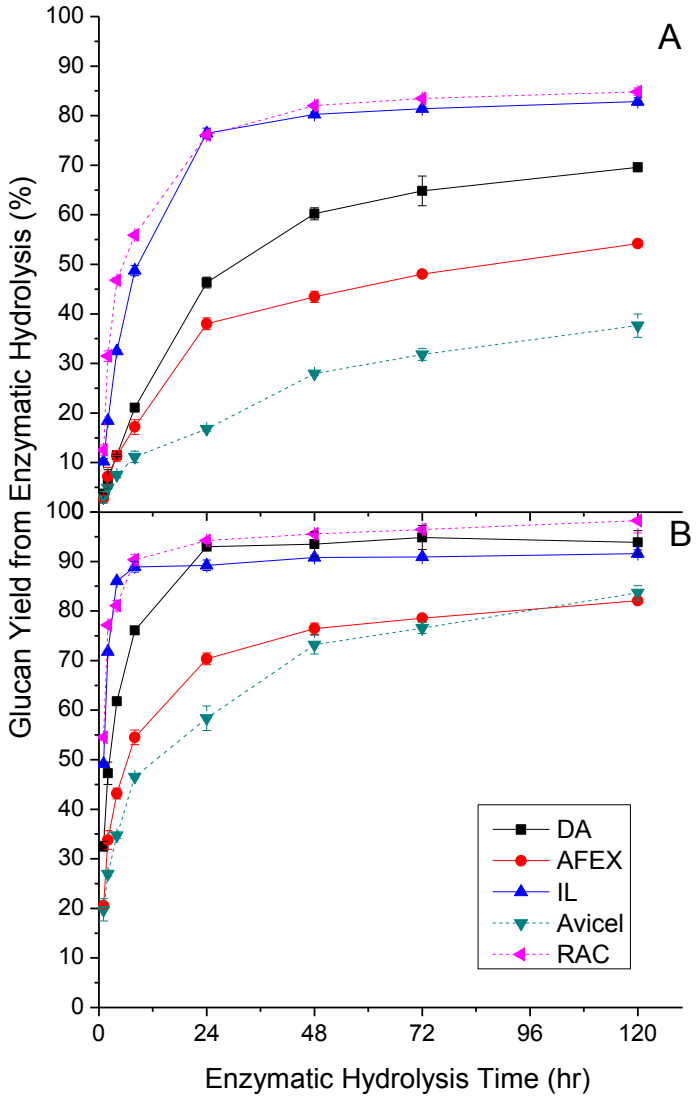


Figure 8.1 Glucan yields as glucose and glucose oligomers in solution following enzymatic hydrolysis of dilute acid (DA), ammonia fiber expansion (AFEX^{TM}), and ionic liquid (IL) pretreated corn stover, Avicel cellulose, and RAC for enzyme loadings of 3 mg (**A**) and 30 mg (**B**) enzyme protein/g glucan in the raw corn stover.

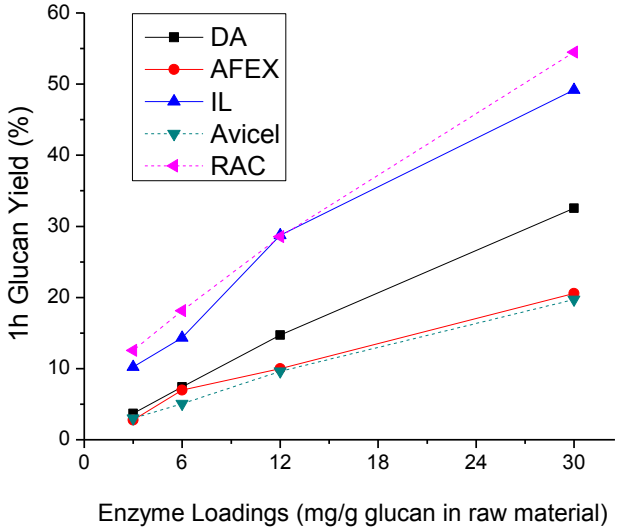


Figure 8.2 Effect of enzyme loadings on the initial (1 h) glucan yields from enzymatic hydrolysis of dilute acid (DA), ammonia fiber expansion (AFEX™), and ionic liquid (IL) pretreated corn stover; Avicel; and RAC.

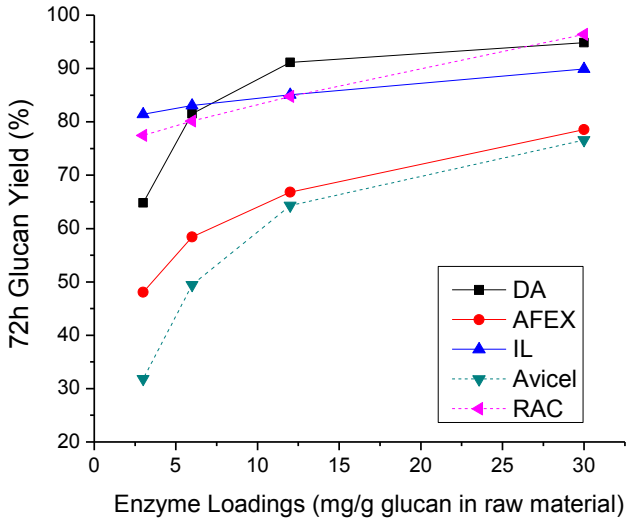


Figure 8.3 72 h glucan yields from enzymatic hydrolysis vs. enzyme loadings for dilute acid (DA), ammonia fiber expansion (AFEX™), and ionic liquid (IL) pretreated corn stover; Avicel; and RAC.

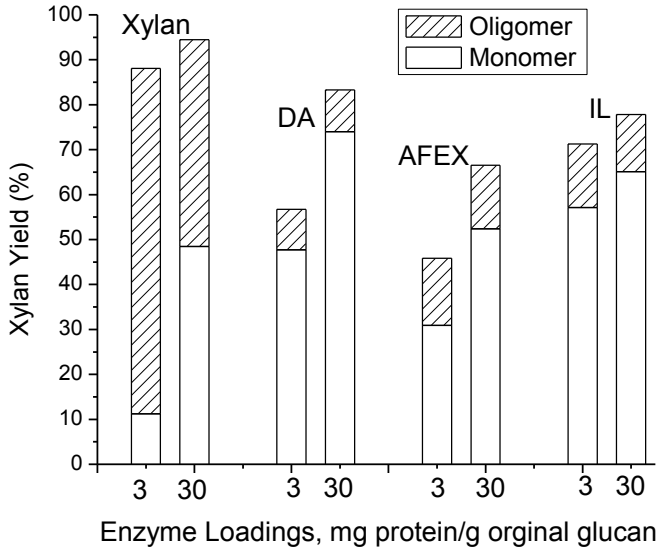
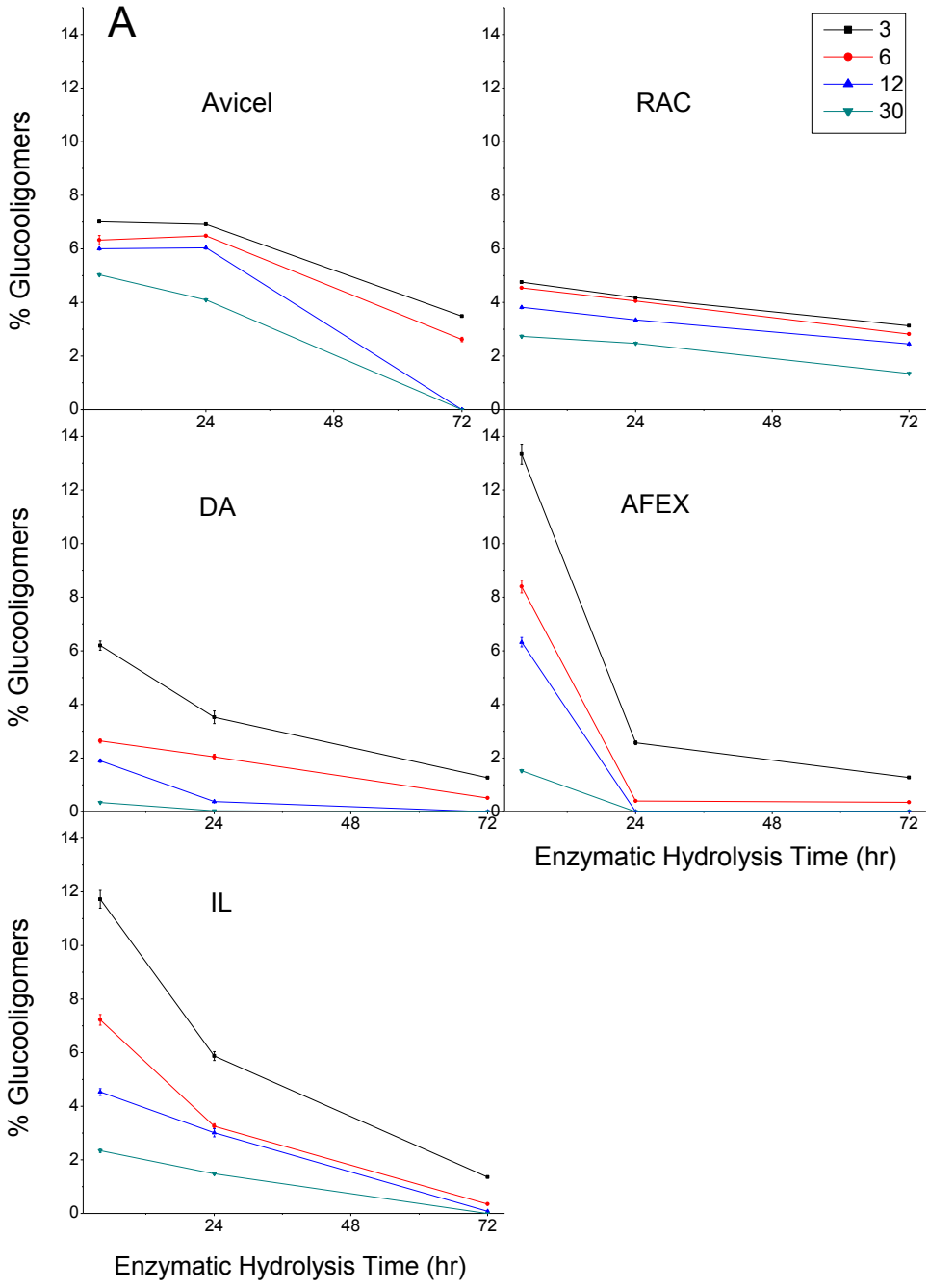


Figure 8.4 Xylan yields from 72 h enzymatic hydrolysis of beechwood xylan, and dilute acid (DA), ammonia fiber expansion (AFEX^{TM}), and ionic liquid (IL) pretreated corn stover. Note: pure xylan was only hydrolyzed with HTec2.


A
Avicel
RAC
DA
AFEX
IL
3
6
12
30
% Glucooligomers
Enzymatic Hydrolysis Time (hr)
0
2
4
6
8
10
12
14
24
48
72

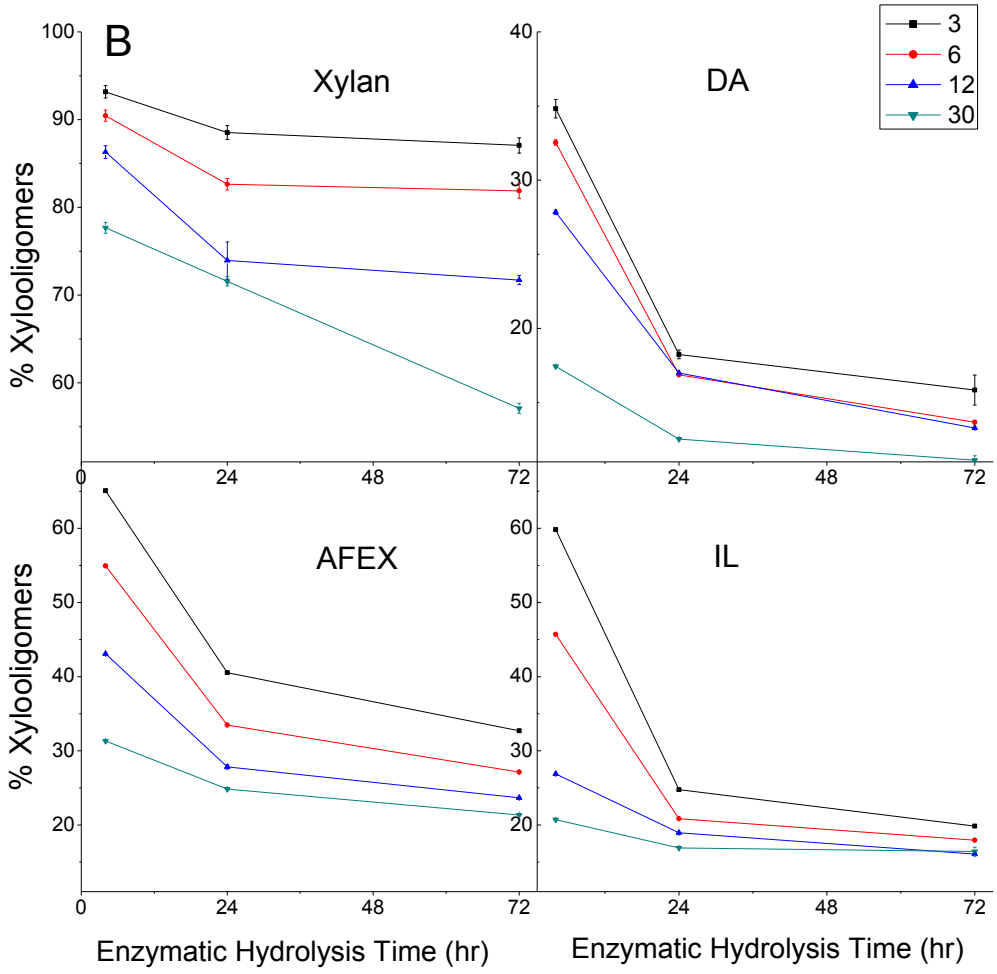


Figure 8.5 Gluco- (**A**) and xylooligomers (**B**) as a percent of the total glucose and xylose in solution following 72 h of hydrolysis at enzyme loadings of 3 and 30 mg/g glucan in the raw biomass for Avicel; RAC; Beechwood xylan; and dilute acid (DA), ammonia fiber expansion (AFEX™), and ionic liquid (IL) pretreated corn stover

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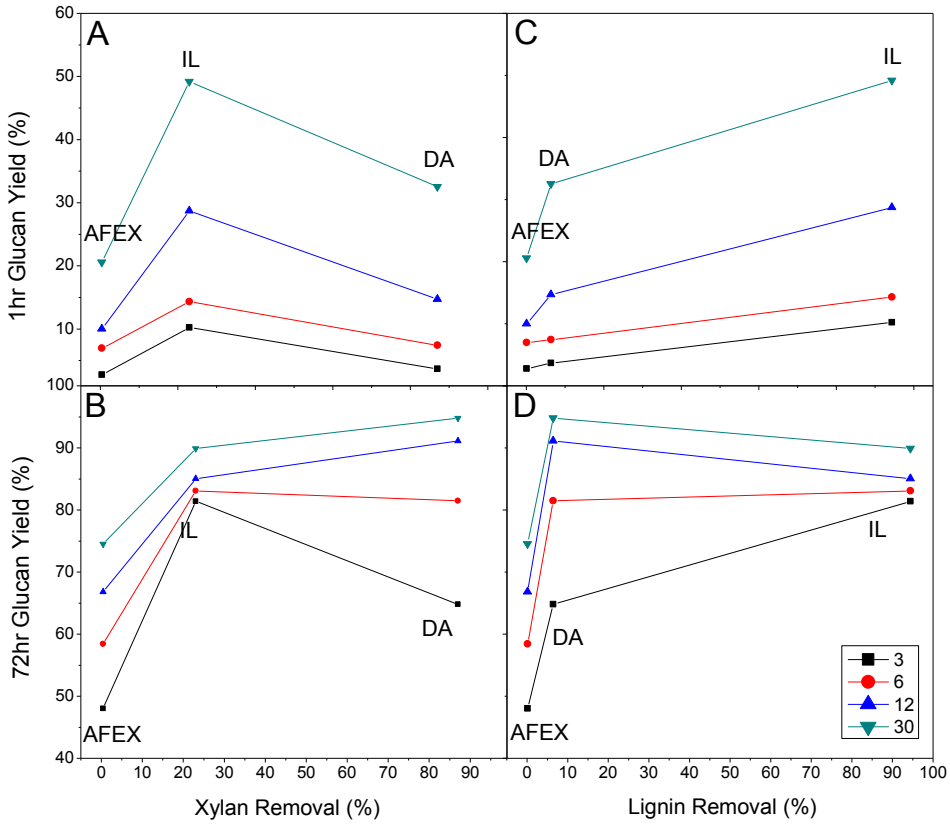

Figure 8.6 Relationships between 1 h and 72 h glucan yields from enzymatic hydrolysis of dilute acid (DA), ammonia fiber expansion (AFEX™), and ionic liquid (IL) pretreated corn stover solids vs. xylan removal (**A,B**) and lignin removal (**C,D**) at total enzyme loadings of 3, 6, 12 and 30 mg/g glucan in original corn stover.

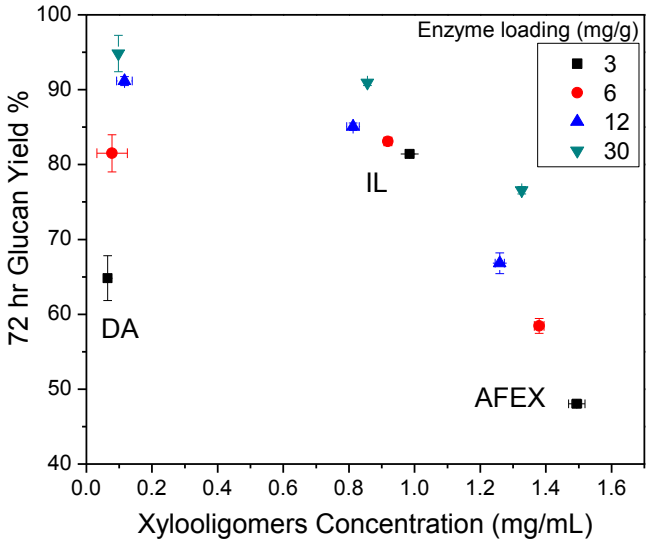

Figure 8.7 Relationship between 72h glucan yield at enzyme loading of 3, 6, 12, 30 mg/g original glucan and xylooligomers concentration for dilute acid (DA), ammonia fiber expansion (AFEX^{TM}), and ionic liquid (IL) pretreated corn stover.

Chapter 9 Laboratory Dilute Acid and Hydrothermal Treatment of Cellulosic Biomass for Its Conversion to Reactive Intermediates

This whole chapter will be submitted to "Journal of Visualized Experiments" under the following citation: Gao X, Kumar R, and Wyman CE. "Laboratory dilute acid and hydrothermal treatment of cellulosic biomass for its conversion to reactive intermediates". The format of this chapter was followed the journal's guideline.

Keywords: dilute sulfuric acid, hydrothermal, pretreatment, cellulosic biomass, conversion, reactor

9.1 Short Abstract:

Cellulosic biomass can be converted into reactive intermediates (RIs) including sugars and aldehydes for further biological or catalytic reaction into fuels and chemicals. Laboratory reactors can provide valuable tools for defining combinations of acid concentrations, temperatures, and times that maximize yields of targeted RIs appropriate for different uses.

9.2 Long Abstract:

Exposure of cellulosic biomass to temperatures of about 120 to 210 ℃ with water only or dilute sulfuric acid have been widely applied as biomass pretreatment for removing most of the hemicellulose and producing cellulose-rich solids from which high glucose yields are possible with cellulase enzyme. With similar apparatus but harsher conditions, sugar dehydration products such as furfural, 5-HMF, or levulinic acid that are valuable reactive intermediates for production of chemicals and hydrocarbon fuels can be obtained from biomass.

The exact combination of temperatures, times, and acid concentrations to maximize yields of the reactive intermediates vary with the type of biomass employed and must be determined experimentally. Pretreatment reactors exhibit considerable diversity. Small-diameter tube reactors make it possible to employ rapid heat-up and good temperature control and reduce temperature gradients; thus, they are often used to evaluate biomass recalcitrance and obtain accurate closure of material balances. Mixed

Parr reactors can be employed to prepare larger amounts of biomass than possible in small-diameter tubes. In this paper, the methods to prepare and operate biomass treatment using the two most used batch systems, tube and Parr reactors, will be introduced.

9.3 Introduction

Lignocellulosic biomass has long been recognized as a unique resource for sustainably meeting society's energy needs but must be converted into liquid or gaseous fuels if it is to integrate well with our infrastructure [1]. Various technologies are being developed for biomass conversion into fuels and chemicals, including biochemical and catalytic routes [2] .

Application of dilute sulfuric acid at temperatures of about 140 to 180^{o}C for about 5 to 20 minutes can release sugars from the hemicellulose fraction that comprises about 15 to 25% of biomass for fermentation to ethanol and other products[3]. Furthermore, the resulting solids are enriched in cellulose that made up about 35 to 50% of the original biomass and are now accessible to enzymes for breakdown into easily fermentable glucose with high yields [3].

Although dilute sulfuric acid gives somewhat higher yields, heating many forms of biomass to about 180 to 210^{o}C in just water can also be effective in recovering hemicellulose sugars while preparing the remaining solids for effective subsequent enzymatic hydrolysis [4]. Pretreatment with just water in this manner, often referred to as hydrothermal or autocatalytic pretreatment, produces a much higher fraction of

oligomers than from dilute acid pretreatment but avoids the complications associated with sulfuric acid use and neutralization.

Dilute acid treatment of sugars from hemicellulose in hardwoods, agricultural residues, and grasses that are typically rich in xylose at temperatures of about 200^{o}C can convert them into furfural for catalytic reaction into hydrocarbons that are compatible with our existing fuel infrastructure [5]. Similarly, at such temperatures, glucose released from cellulose can be reacted to 5-hydroxymethyl furfural (5-HMF) followed by levulinic and formic acid, with 5-HMF and levulinic acid being potentially valuable intermediates for making hydrocarbons as well[6, 7]. These reactions can be conducted sequentially or simultaneously with sugar release.

9.4 Protocol Text:

9.4.1 Preparation of samples

Prior to pretreatment, biomass samples are soaked for a minimum of 4 hr in a 0.5wt% acid solution at room temperature to hydrate the biomass and ascertain a uniform penetration of acid catalyst into the biomass pores. The solid loadings employed generally are 5 to 10wt% for reactions in tube reactors and 5 to 20wt% in Parr reactor.

9.4.2 Pretreatment

The size of laboratory reactors to perform pretreatment can range from 500 μL to 4 L to meet various purposes, depending on the amount of biomass processed and the purpose. Heating is provided by 4-kW SBL-2D fluidized sand baths or steam for biomass pretreatment

9.4.3 Tube reactors

Small tube reactors are usually used for screening reaction conditions that maximize the yields of desired RIs. These reactors are made of Hastelloy C276 tubing (12.5 mm O.D. and 150 mm L) with a 0.8255 mm wall thickness and stainless steel end caps. Each tube reactor has an internal volume of approximately 14 mL. For dilute acid reactions, Teflon plugs are inserted into each end to avoid acid corrosion of the stainless steel caps. A pair of tube reactors is usually treated identically, as summarized in the steps below, and then following the reactions the contents of each tube are used to determine reaction performance and/or residual solids cellulose enzymatic digestibility.

1. Prior to pretreatment, the tube reactors are assembled by inserting a Teflon plug in one end followed by securing with a screw cap.
2. The appropriate amount of milled biomass (~ 0.5 to 1g) on dry basis is loaded into tubes, and water or acid solution is added to make the required solid loading.
3. Then the tubes are sealed at the other end with a second Teflon plug and cap. The biomass and water or acid solution are mixed well and kept at room temperature for a minimum of 4 hr for the acid catalyst or water to soak into the biomass.
4. To monitor the temperature over the course of the reaction, an additional tube reactor with a thermocouple probe inserted 2 in. deep in the center is used in parallel with the other reactors.
5. A two-bath heat-up procedure is used for rapid heating and to minimize the effects of thermal transients. The tube reactors are placed into a steel basket that can hold up to 10-15 reactors. The basket is attached to an iron rod longer than

the sand bath's diameter to prevent the basket from sinking into the bath and lowered into the fluidized sand bath. The reactors are preheated to 2 ℃ lower than the target reaction temperature in one of the air fluidized sand baths with temperature set at minimum 20 ℃ above the target reaction temperature. The setting of temperature for sand bath to achieve the fastest heat up without over shooting the target temperatures is determined by trial and error.

6. Then the reactors are immediately transferred into a second sand bath set at the 1 to 2 ℃ higher than the target reaction temperature. The reaction time starts after transferring the basket into the second sand bath.
7. After pretreatment, the reactions are quenched by submerging the reactors in a room temperature water bath to allow cool down within 2 min.

9.4.4 Parr reactor

To prepare larger quantities of pretreated biomass, a high pressure 1L cylindrical Parr reactor made of Hastelloy C can be employed. Its lid is equipped with an 88.9 mm diameter helical impeller on a two-piece shaft driven by a variable speed DC motor to ensure a proper heat and mass transfer, and a K-type thermocouple is mounted in the reactor to monitor the inside temperature. The reactor is suspended by a chain hoist mounted on a wall crane for position adjustment. The following steps summarize biomass pretreatment with 1L Parr reactor:

1. The temperature controlled air fluidized sand bath is set to a temperature twice or higher than the target reaction temperature.

2. Water or dilute sulfuric acid presoaked biomass (< 20 wt% solid loading dry basis) is loaded into the reactor.
3. The reactor is then closed tightly, and the helical impeller is set to the target agitation speed of 200 rpm.
4. The reactor suspended by a chain- pulley system is lowered into the sand bath for rapid heat-up of the agitated contents to the target temperature. The heating time is approximately 2-5 min and is not included in the pretreatment reaction time.
5. As the temperature inside the reactor approaches the reaction temperature, the reactor is lifted up so the reactor bottom is about 1 and 2 cm above the sand's surface. The reaction time starts once the temperature inside the reactor reaches the target value.
6. The temperature is maintained within ±2 ℃ of the reaction temperature by lowering/lifting the reactor into/from the sand bath with a chain pulley system. If the inside temperature becomes higher than the target temperature, DI water can be sprayed on the outer wall of the reactor to avoid overheating.
7. Following pretreatment, the reactor is immediately transferred to a room temperature water bath for cooling.
8. Once the temperature is below 50^{o}C, the stirrer is turned off, and the reactor is opened.

9.4.5 Separation of pretreated solid and liquid

1. To separate the liquid from the solids, the slurry is immediately vacuum filtered with glass fiber filter paper in a fume hood.

2. The liquid is collected and stored for further analysis.
3. The solids are washed extensively with room temperature deionized water in an amount equal to about 10 times of the initial biomass weight to remove chemicals and free sugars.

9.4.6 Analysis and following operation

The liquid and solids are analyzed for their compositions following National Renewable Energy Laboratory 's Analytical Procedures [8, 9]. Different downstream operations can be applied to meet different goals [10].

9.5 Representative Results:

Figure 9.1 shows glucose and xylose concentrations over pretreatment time for dilute acid (160^{o}C with 0.5 wt % acid loading) and hydrothermal (180^{o}C) pretreatments at 5 wt% solids loadings of corn stover. It can be seen that large amounts of xylose were released by dilute acid and hydrothermal pretreatments, while most of the glucose remained in the solids. For dilute acid pretreatment at the listed conditions, xylose concentrations dropped slightly for longer pretreatment times due to degradation. On the other hand, much less degradation was observed for hydrothermal pretreatment at 180^{o}C and reaction times up to 40 min.

Figure 9.2 compares glucan digestibility of raw and pretreated corn stover at an enzyme loading of 30 mg protein of Celic® CTec2/g glucan in raw material. The glucan digestibility is defined as the glucan yield from 72 hr hydrolysis and is calculated as:

% Glucan yield = 100 × ((GH (g) + CB (g) × 1.053))/1.111) /GP (g)

in which GH and CB stand for glucose and cellobiose released from enzymatic hydrolysis. GP stands for glucan available in the pretreated biomass solids.
It can be seen that the enzymatic digestibility of raw corn stover is much lower than for pretreated corn stover. Both dilute acid and hydrothermal pretreatments can significantly increase the digestibility by more than 70%.

Figure 9.3 shows that dilute acid and hydrothermal pretreatment altered the composition of biomass. Because both pretreatments mainly removed xylan, the resulting solids had very low xylan and high glucan contents. The lignin content increased slightly, mainly due to xylan removal.

9.6 Discussion

Pretreatment of biomass plays an important role in its conversion to fuels and chemicals [11]. Dilute acid and hydrothermal pretreatments are often used on a wide variety of biomass types for their subsequent biological and chemical conversions.
The batch tube and mixed reactors described in this manuscript are commonly applied for defining reaction conditions favorable to production of reactive intermediates. However, laboratory scale apparati for dilute acid or hydrothermal pretreatment are actually quite diverse, and the reactors are not restricted to these types [12]. For instance, a novel high throughput pretreatment and co-hydrolysis (HTPH) system was recently developed in our lab and applied for screening larger sets of samples for sugar release patterns over a wide range of operational parameters with less labor and time required compared to conventional systems[13]. In another instance, a flowthrough reactor system is employed to pass hot water through a fixed bed of biomass to realize virtually theoretical yields of

hemicellulose and high levels of lignin removal [14]. Flowthrough reactors can also be employed to understand deconstruction mechanisms for lignocellulosic biomass[15]. Pretreatment of larger amounts of biomass can be carried out using direct steam injection and rapid pressure release. Such "steam gun" devices allow higher solids concentrations than possible with either tube or mixed reactors.

Although less common, other types of acid catalysts can be used for pretreatment such as nitric, phosphoric, and hydrochloric. The Parr reactor has also been used for sulfur dioxide or carbon dioxide pretreatments with minor modifications in the system's valve for gas penetration [16].

To integrate with downstream operations to desired products, pretreatment conditions, in terms of time, temperature, pressure, and catalyst loadings, should be selected carefully. As interest in biomass hydrolysis has evolved from direct sugar generation from cellulose to biomass preparation for subsequent operations, pretreatment objectives have shifted from obtaining glucose directly from cellulose to recovery of hemicellulose-based sugars and modification of cellulose for subsequent enzymatic hydrolysis[11, 17]. It is also worth noting that recent interest in producing chemicals and "drop-in" hydrocarbon fuels focuses on identifying conditions to maximize production of dehydration products as reactive intermediates from biomass [6].

9.7 Acknowledgments

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Table 9.1 Specific reagents and equipment

Name of the reagent/equipment	Company	Catalog number	Comments
72% Sulfuric acid	Sigma-Aldrich	Fluka 00647	
Celic® CTec 2	Novozymes	Batch No. VCNI0001	
Glass fiber filter paper	Fisher Scientific	09-804-110A	
C-276 tube reactor	Swagelok	Hastelloy® alloy	6 inch * ½ in tubes
Stainless steel cap	Swagelok	SS-810-C	
Teflon plugs	McMaster-Carr	Teflon® PTFE	5/8 inch diameter
Parr reactor	Parr Instruments	236HC	
Speed DC motor drive	Parr Instruments	A1750HC	
4-kW fluidized sand bath	TECHNE	model SBL-2D	
Temperature controller	TECHNE	model TC-8D	

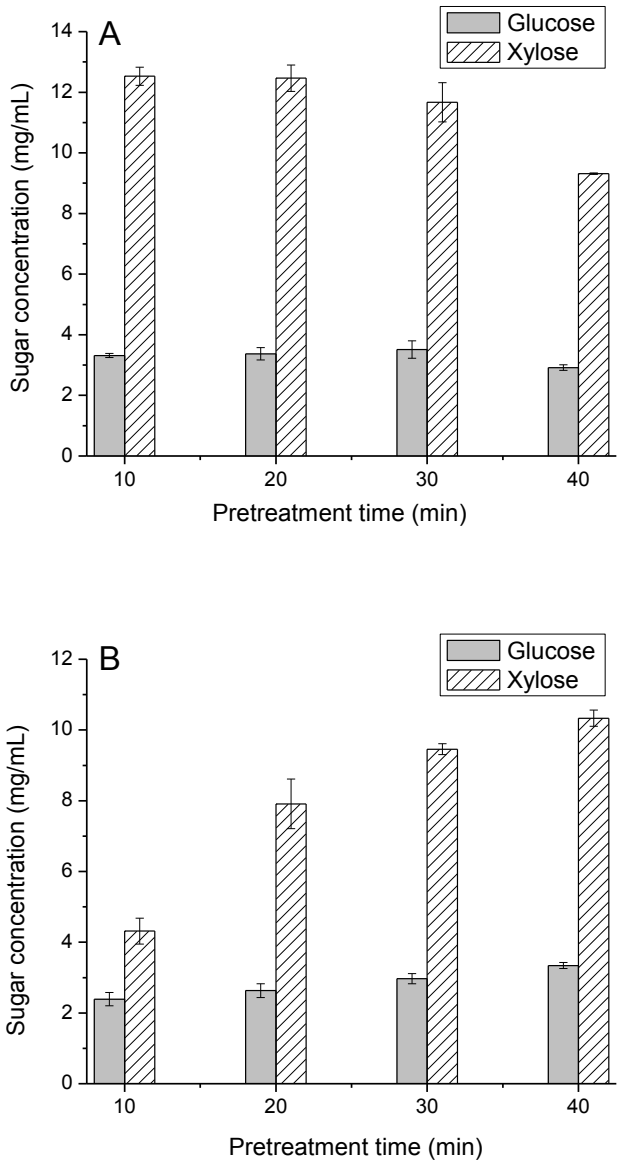

Figure 9.1 Glucose and xylose concentration vs. pretreatment time for pretreatment of 5wt% corn stover in tube reactors by (**A**) dilute acid pretreatment at 160oC with 0.5wt% acid loading and (**B**) hydrothermal pretreatment at 180 ^{o}C.

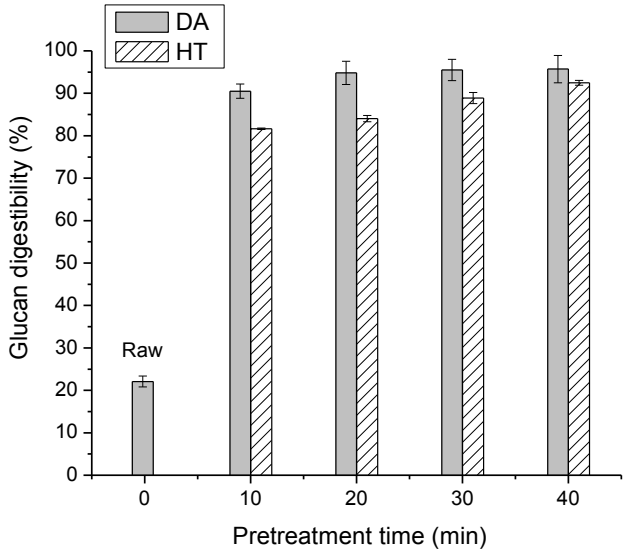

Figure 9.2 Enzymatic digestibility of glucan in raw and dilute acid (DA, 160oC with 0.5 wt % acid loading, 5wt% solids loading) and hydrothermal (HT, 180 ℃, 5wt% solids loading) pretreated corn stover for an enzyme loading of 30 mg protein/g glucan in the raw material

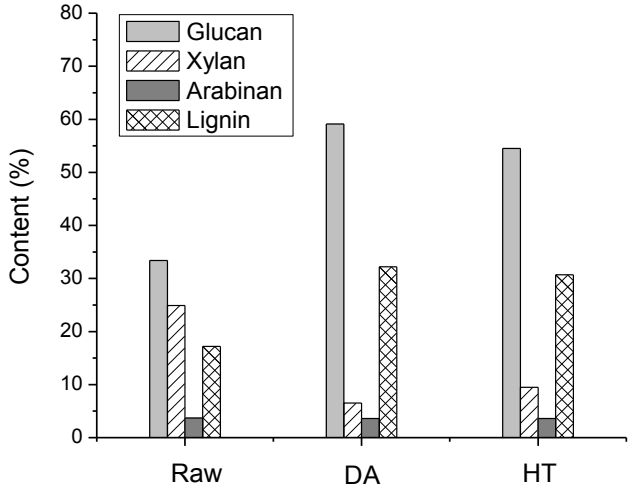

Figure 9.3 Composition of raw, dilute acid (DA, 160 ℃, 0.5%wt sulfuric acid, 20 min), and hydrothermal (HT, 180 ℃, 5 wt % solid loading, 40 min) pretreated corn stover solids.

Chapter 10. Conclusions and Future Works

10.1 Summary of Key Developments and Findings

A better understanding of relationships among biomass recalcitrance, pretreatment, and enzymatic hydrolysis would provide meaningful information and guidance for overcoming biomass recalcitrance in an economic manner. The main motivation of this thesis was to understand the influence of several leading pretreatment technologies on lignocellulosic biomass features as well as how such substrate features affect subsequent enzymatic hydrolysis. Information from such studies can help to customize pretreatment conditions and process configurations as well as the enzyme formulation and loadings to achieve high fermentable sugar yields from lignocellulosic biomass at low costs.

In light of this, application of the high throughput pretreatment and co-hydrolysis (HTPH) system, previously developed at UCR through support of the BioEnergy Science Center (BESC) by the DOE Office of Science, was extended to dilute acid pretreatment by incorporating the novel one step buffering and neutralizing method described in *Chapter 3*. Because dilute acid pretreatment offers many important advantages in rendering biomass highly susceptible to subsequent enzymatic hydrolysis, development of the dilute acid HTPH system provides an additional tool to screen large numbers of biomass candidates and processing conditions to identify combinations that overcome biomass recalcitrance better.

Assisted by the dilute acid HTPH system, a mild two-stage pretreatment, with low temperature dilute acid pretreatment in the first stage followed by hydrothermal pretreatment at higher temperatures in the second stage, was developed and its

performance was evaluated in *Chapter 4*. The HTPH system was first applied to screen for promising combinations of pretreatment conditions based on the goal of achieving high glucan digestibility and total sugar yields from the combined pretreatment and enzymatic hydrolysis steps while keeping xylan degradation low. Then conditions identified with the HTPH system were applied in larger scale laboratory equipment to validate the results and provide more comprehensive comparisons of performance with the two-stage system to that possible by conventional one-stage dilute acid and hydrothermal pretreatments. Through proper selection of conditions, high yields of both glucan and xylan from the overall two-stage pretreatment coupled with enzymatic hydrolysis and low sugar degradation during pretreatment were achieved.

Coupled with developing two-stage pretreatment, *Chapter 6* explored the possibility of recycling liquid from Stage 1 of the two-stage pretreatment to increase sugar concentrations to provide and strengthen the case for a two-stage pretreatment strategy. The results show that for recycle of liquid from one Stage 1 pretreatment for use in a second pretreatment at the same low temperature, (1) more concentrated sugar streams could be achieved; (2) sugar degradation could be insignificant; (3) comparable sugar yields from both pretreatment and enzymatic hydrolysis could be obtained; (4) similar glucan and xylan contents could be realized in pretreated solids as pretreatment with fresh acid; and (5) consumption of fresh water and acid could be reduced. Overall, these results preliminarily demonstrate the feasibility of recycling liquid in the Stage 1 pretreatment of the two-stage strategy.

Inspired by HTPH screening results from *Chapter 4*, a one-step acid hydrolysis method was developed in *Chapter 7* for rapid quantification of hemicellulose in various types of lignocellulosic biomass in terms of total xylan, galactan, and mannan (XGM) contents. By hydrolysis with 4 wt% sulfuric acid at 121 ℃ for 1h, hemicellulose was almost totally released from various types of biomass as sugar monomer that could then be quantified by HPLC. This method gave statistically identical results in XGM contents compared to results from conventional two-step acid hydrolysis while significantly shortening analysis time.

In addition to methods development, *Chapter 5* facilitated understanding how compositional and structural features of switchgrass were altered by low temperature dilute acid pretreatment, hydrothermal pretreatment, and two-stage pretreatment and their effect on biomass recalcitrance and enzymatic digestibility. Characterization of xylan and lignin removal, substrate accessibility, residual hemicellulose structure, and cellulose surface distribution of substrates from different pretreatments suggested that although large amounts of hemicellulose could be removed from biomass by low temperature proton-catalyzed pretreatments (dilute acid and hydrothermal), high temperatures were needed to realize the substantial increase in substrate accessibility that is more critical for high enzymatic digestibility.

While chapter 5 focused on compositional and structural properties of biomass altered by similar pretreatment mechanism but different severities, *Chapter 8* shifted emphasis to comparing features of pretreated substrates that affect enzymatic digestion for solids produced by widely different pretreatment technologies: dilute acid (DA),

ammonia fiber expansion (AFEX) and ionic liquid (IL). Xylan and lignin removal, oligomers released during enzymatic hydrolysis, substrate accessibility to enzymes, and their relationship with enzymatic reactivity and digestibility were integrated to understand how these different pretreatments changed substrate features and their role in achieving high yields from enzymatic hydrolysis. It was found that IL corn stover exhibited the highest reactivity (1^{st} h glucan yield) and digestibility (72 h glucan yield) at low enzyme loadings, results that correlated with high degree of lignin removal, large amorphous fractions, and high accessibility to enzymes. With higher enzyme loadings, DA corn stover showed better digestibility, which may benefit from its lower xylan content and resulting lower inhibition by xylooligomers released from xylan during hydrolysis. No single factor absolutely dominated enzymatic digestion performance for the three pretreatment methods.

10.2 Closing Remarks and Suggestion for Future Work

The thesis was focused on improving the understanding of the influence of pretreatment on substrate features and their relative importance in controlling biomass recalcitrance to aid in identifying strategies to effectively deconstruct biomass. Important learnings from this thesis and topics deserving further investigation are summarized in the following four points:

First, the enzymatic digestibility of biomass is not related simply to one or several compositional and structural features but also closely affected by the choice of pretreatment strategy. The observations from *Chapter 5* and *Chapter 8* suggest that mild dilute acid, hydrothermal, and two-stage pretreatments that share similar proton-catalyzed

mechanisms, can all achieve high levels of xylan removal from biomass, opening up the biomatrix of plant cell wall for enzymes and reducing possible inhibition from xylooligomer released during enzymatic hydrolysis. Consequently, other factors may have more important impacts on enzymatic digestibility through increasing substrate accessibility, such as greater lignin removal at higher temperatures. On the other hand, when we compared features of substrates from different pretreatments, corn stover pretreated with ionic liquids had high accessibility to enzymes, high lignin removal, a high amorphous fraction, high hydrolysis rates, and relatively high glucan yields at low enzyme loadings. However, the large amount of xylooligomers released in pretreatment and in enzymatic hydrolysis at higher enzyme loadings limited digestibility and resulted in lower yields than from dilute acid pretreatment of corn stover.

A useful perspective can be gained by considering these results in terms of the "Barrel effect". This concept is based on the analogy to "a barrel with missing pieces, for which the amount of water it can hold is not determined by the longest piece of wood on the barrel, but the shortest barrel piece of wood." Similarly, the digestibility of pretreated biomass is not determined by the substrate's most favorable features for enzymatic hydrolysis but by obstacles that have not been overcome. Thus, biomass recalcitrance seems closely related to the choice of pretreatment technologies because different pretreatments overcome biomass recalcitrance in different ways, with the result that biomass recalcitrance often depends on the choice of substrate, pretreatment methods, and the harshness of pretreatment conditions.

Second, in addition to relating enzymatic digestibility to the features of pretreated biomass, the properties of biomass gradually change during enzymatic hydrolysis, with the result that the factors controlling digestibility performance can also shift. It would be valuable to track key physiochemical properties of substrate during enzymatic hydrolysis. A previous study reported changes in the rate of enzymatic hydrolysis of Avicel with conversion via a "restart" strategy [1]. This strategy could be used to characterize substrates from different pretreatments with various enzyme loadings during enzymatic hydrolysis, thereby providing valuable information about the effects of substrate features on biological conversion of biomass.

Third, pretreatment strategies and enzyme formulations need to be customized to meet different purposes for different biomass species. As shown in *Chapter 4*, a two-stage pretreatment strategy could increase overall glucan and xylan sugar yields from coupled pretreatment and enzymatic hydrolysis. However, further research is needed to better understand how to take advantage of the low degradation of sugars for low temperature pretreatments to enhance sugar concentrations and reduce acid use. For example, it would be beneficial to optimize the recycle ratio in concert with downstream operations. And a techno-economic analysis based on NREL's model would provide a valuable perspective on its viability [2, 3].

Last but not least, applications of characterization techniques on raw, pretreated and enzymatically hydrolyzed biomass provide valuable information to understand biomass recalcitrance on multiple scales. Simple and rapid techniques for measuring substrate accessibility to enzymes, such as enzyme adsorption (*Chapter 8,* [4-6]) and

Simons' staining (*Chapter5*, [7-9]), would be particularly valuable to employ in future studies. Meanwhile, newly developed analytical and characterization methods should be integrated with high throughput methods to evaluate compositional, structural, and physiochemical properties of biomass more effectively.

10.3 Acknowledgements

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