IMPACT OF ALKALINE HYDROGEN PEROXIDE PRETREATMENT ON CELL WALL PROPERTIES THAT CONTRIBUTE TO IMPROVED ENZYMATIC DIGESTIBILITY OF STRUCTURAL CARBOHYDRATES TO BE UTILIZED FOR BIOFUEL PRODUCTION

By

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ABSTRACT

IMPACT OF ALKALINE HYDROGEN PEROXIDE PRETREATMENT ON CELL WALL PROPERTIES THAT CONTRIBUTE TO IMPROVED ENZYMATIC DIGESTIBILITY OF STRUCTURAL CARBOHYDRATES TO BE UTILIZED FOR BIOFUEL PRODUCTION

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Lignocellulosic plant material is an attractive option as a source of sugars that can be converted to fuels such as ethanol due to it being an abundant and renewable resource. One of the more compelling process schemes to do this is the biochemical conversion platform, where enzymes are used to hydrolyze sugar polymer bonds and release monomeric sugars that can be used by fermenting organisms to produce the desired fuel. However, due to the recalcitrant nature of lignocelluloses, a pretreatment step is usually required before hydrolysis to improve cell wall polysaccharide accessibility to enzymes in order to facilitate enzyme catalysis and ultimately cell wall deconstruction to soluble sugar monomers. Within this pretreatment step it is necessary to increase polysaccharide accessibility by removing or redistributing lignin and hemicelluloses and increasing cell wall porosity. This work investigates pretreatment, primarily alkaline and alkaline hydrogen peroxide (AHP) pretreatment, in two ways: 1) as a unit operation integrated with enzymatic hydrolysis and fermentation for a complete conversion process and 2) as a tool for investigating cell wall properties that are important for improved deconstruction, more specifically, enzymatic digestibility. Two studies in each category are presented in this work.

In the first, corn stover and switchgrass were AHP pretreated over a range of pretreatment conditions to understand the space of changes that take place during the process; specifically, the impact of H_2O_2 loading, feedstock, pretreatment time, solids loading and scale

were determined on compositional changes of solid biomass, inhibitor release and pretreatment effectiveness measured by enzymatic digestibility.

In the next study, soluble sugars from a sweet sorghum were simultaneously extracted while the remaining lignocellulose in bagasse was alkali pretreated in a novel countercurrent diffusion extraction/pretreatment technique. The carbohydrates in the bagasse were then hydrolyzed with enzymes and the hydrolyzate was combined with the extraction juice and fermented. Near 100% soluble sugar extraction was achieved and a glucose yield of 70% was obtained on the pretreated bagasse. An ethanol concentration of 21 g/L was obtained corresponding to 85% ethanol yield indicating that this combined technique has potential.

In the last two studies, absorbed water within the solid matrix of corn stover and switchgrass, AHP and liquid hot water (LHW) pretreated, was quantified by water retention value (WRV) and settling volume and found to be linearly correlated with glucose yield after hydrolysis. Results indicate that AHP and LHW pretreatment can increase water binding to biomass surfaces and increase swelling, which is indicative of increased surface accessible not only to water molecules, but also to enzymes.

The follow up study expanded the range of AHP and LHW pretreatment conditions and included ammonia fiber expansion (AFEX) pretreatment of corn stover and switchgrass and found that linear regression of WRV with glucose yield does not fit for all pretreatment types and conditions. WRV for AFEX pretreated material does linearly correlate with glucose yield, however, not with the same slope as AHP and LHW pretreated material. A multiple linear regression model was developed to include composition features of the pretreated biomass with WRV and yielded much better prediction results across all pretreatments and conditions.

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KEY TO ABBREVIATIONS

GHG: greenhouse gas

HDI: human development index

RFS: Renewable Fuel Standard

AFEX: ammonia fiber expansion

AHP: alkaline hydrogen peroxide

BSA: bovine serum albumin

HMF: hydroxymethyl furfural

DSC: differential scanning calorimetry

WRV: water retention value

LHW: liquid hot water

HPLC: high performance liquid chromatography

NREL: National Renewable Energy Laboratory

SG: switchgrass

CS: corn stover

ANOVA: analysis of variance

GLBRC: Great Lakes Bioenergy Research Center

YNB: yeast nitrogen base

OD600: optical density at 600 nm

BET: Brunauer-Emmett-Teller theory

NMR: nuclear magnetic resonance

FTIR-ATR: Fourier transform infrared spectroscopy – attenuated total reflectance

DVS: dynamic vapor sorption

CBM: carbohydrate-binding module

MLR: multiple linear regression

AIC: Akaike information criteria

BIC: Bayesian information criteria

IR: infrared

PLS: partial least squares

PRESS: predicted residual sums of squares

Introduction

Motivation – Biofuels and Sustainable Energy

Fossil fuels provide 80% of energy needs worldwide, with primary sources including coal, natural gas and petroleum (EIA, 2011). Although nonpetroleum sources of liquid fuels are currently utilized (i.e. coal to liquid, gas to liquid, biofuels and kerogen), the overwhelming majority of liquid fuels consumed in the U.S. come from petroleum (see Figure 1) (Outlook, 2010). According to the U.S. Energy Information Administration's International Energy Outlook 2013 report, the transportation sector will account for over 60% of the projected energy demand growth worldwide in the next 30 years (EIA, 2011), and in the U.S., the transportation sector accounts for as much as 70% of the total national petroleum consumption (see Figure 1). As demand and price for liquid fuels increase, petroleum in particular, other sectors outside of transportation and industrial usage will be able to use other sources of energy (EIA, 2011). However, commerce is completely dependent on high-energy density liquid fuels and is essential for shipping via aviation, ocean shipping and land freight (Dale & Ong, 2012), not to mention the huge infrastructure investments within the transportation sector which alone invests upwards of $10 billion per year just in retail infrastructure dedicated to liquid fuels, and this does not include infrastructure required for production and distribution (Melaina et al., 2013). Therefore, transitioning to other forms of energy storage (i.e. electric, hydrogen and other non-drop-in liquid fuels) that can be used for transportation will be gradual, and liquid fuels will continue to be essential for the foreseeable future, especially fuels that are compatible with the current production, distribution and retail pipeline. Currently, the only options for renewable liquid fuels are those produced from plant material, and the most promising sustainable source of plant material is non-food cellulosic biomass (Dale & Ong, 2012). The sustainability of this biomass-

based liquid fuel system is very important, and sustainability is not simply limited to being renewable.

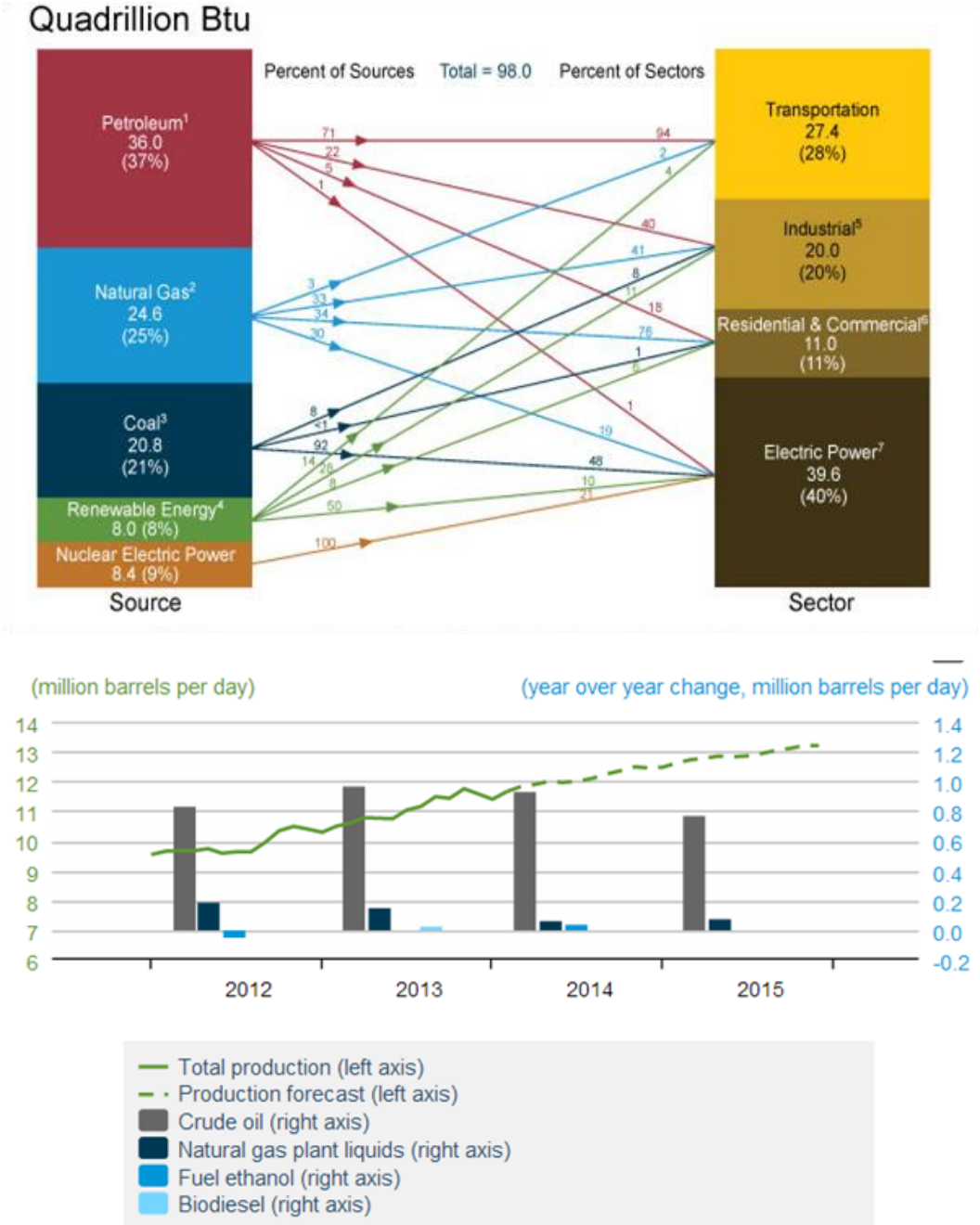

Figure 1: U.S. energy consumption by source and sector (top), and U.S. crude oil and liquid fuel production (bottom). Top figure published in U.S. Energy Information Administration Annual Energy Review 2010, bottom figure published in U.S. Energy Information Administration Short-Term Energy Outlook.

Sustainable energy by definition is the provision of energy to meet current demands without compromising the ability to meet future needs. And sustainability more generally refers to the wellbeing across the domains of environment, social equity, and economics. A truly sustainable energy system will promote wellbeing in all three of these areas. Biofuels are a renewable resource because they can be replenished on a reasonable time scale (every year) compared to fossil fuels (thousands of years) by growing biomass every year, making it a more resource responsible system compared to using petroleum (Narayan, 2001). Biofuels may also have the environmentally friendly benefit of being carbon neutral because CO_2 released during combustion of the fuel would be reincorporated into newly growing biomass material; therefore, biofuels use may actually reduce net carbon emissions compared to fossil fuels. These two environmental benefits are indicative of a sustainable system. However, whether these benefits would be realized is still debated and the subject of much research. For example, there is the potential issue of a net negative energy balance between fossil fuel consumption and bioethanol production in the current biorefining processes; meaning that more energy from fossil fuels is required to produce bioethanol than is contained in the bioethanol itself. This really means that a bioethanol plant is more of a conversion process between fossil fuels, such as natural gas, to ethanol. This would negate the claimed environmental benefit of a renewable biofuel system because of fossil fuel consumption and negate the carbon neutral system it potentially has; however, even with a net negative energy balance, the biofuel system could still reduce dependence on fossil fuels from foreign sources. Furthermore, there is the controversial issue that indirect land-use changes may have a significant impact on greenhouse gas (GHG) emissions, especially in the early stages of biomass production for biofuels (Melillo et al., 2009). This means that the initial clearing of marginal land to be used for biofuel crops could release a

large amount of sequestered carbon in both biomass and soil yielding a net increase is GHG emissions. However, accurate estimates of the amounts of released carbon are still hotly debated, and the relative importance of this released carbon is not known (Wang & Haq, 2008). Furthermore, even if there is a benefit in CO_2 emission reduction from a biofuel-based fuel system, N_2O from fertilizer may be a problem as it is likely to increase in emissions as more land is used to grow crops for fuels, at least with our current agricultural practices (Melillo, et al., 2009). In spite of these challenges, biofuels have a great potential to improve the environmental sustainability of our energy production practices.

In addition to environmental sustainability, biofuels also have the potential benefit of reducing dependence on foreign oil and producing domestic jobs for growing biofuel crops and producing the biofuel itself. However, economic drivers are not always easy to control or predict and there is concern of the potential exploitation of developing countries with cheap farmable land. For example, the African continent is particularly attractive for growing biomass to be used for biofuels because it has a lot of rain-fed land that is largely uncultivated and relatively cheap. However, many of the African countries are poor and already have a difficult time supplying enough food to their population, and therefore would need to be careful in regulating food and biofuel crop production (Greiler, 2007). If done carefully, the social benefits of a biofuel system have the potential to enhance the wellbeing of many developing countries. Bruce Dale et. al. have said that "…commerce has been recognized as a key element in trade that enable greater wealth and therefore greater human development" (Dale & Ong, 2012). Dale et. al. showed a positive correlation between power consumption per capita and the Human Development Index (HDI), which uses life expectancy, education and income to quantify the level of development in countries and is used as a policy basis for determining a countries ability

to provide for its citizen's wellbeing around the world (Dale & Ong, 2012). The clear positive correlation between power consumption and HDI is a compelling argument that providing a means for developing countries to produce their own liquid fuels for consumption and export has the potential, if done right, to improve their quality of life and promote social equity.

The economic sustainability of first generation liquid biofuels, in particular ethanol, has been shown to be tractable in Brazil and the U.S. Brazil produces 5.6 billion gallons of ethanol from sugar cane annually and the U.S. has a production capacity of corn ethanol near 14 billion gallons annually. The price of ethanol to produce is comparable to gasoline; in the U.S. in 2013, the price of ethanol ranged from $1.50 to $3.30 per gallon (Babcock, 2012). However, the price of ethanol is largely determined by fluctuations in corn (or sugar cane in Brazil) prices and natural gas cost, which is used in production. In fact, the cost per gallon to produce ethanol from corn is less than $1.00 depending on the price of natural gas (McAloon, Taylor, Yee, Ibsen, & Wooley, 2000). However, over the last decade, there has been a fivefold increase in biofuel production worldwide that has coincided with a sharp increase in food commodity prices sparking the "food vs fuel" controversy which incriminates the use of cropland to grow biofuel crops causing a rise in food prices (HLPE, 2013). The use of cellulosic plant material in place of starch or sugar based plants has been proposed as a viable alternative that could circumvent the food vs. fuel debate completely by being a cheaper source of raw material that can be grown on less attractive, marginal land (Zilberman, Dale, Fixen, & Havlin, 2013). However, deconstruction of cellulosic biomass to its carbohydrate components is more difficult than it is for high sugar and starch crops such as sugar cane and corn. Therefore, there are several conversion process challenges that require mitigation before cellulosic ethanol becomes an economically attractive option.

Challenge - Cellulosic Ethanol

The production of liquid fuels from renewable biomaterials is a popular and important topic in both ecopolitics, as discussed above, and the research world. In the U.S., legislation is in place to promote the development of a renewable fuel industry with the hopes it will provide a domestically sustainable source of liquid transportation fuels while also reducing dependence on foreign oil (Gonzalez-Garcia, Moreira, & Feijoo, 2010; Schell, Riley, & Petersen, 2008). The Environmental Protection Agency (EPA) is responsible for ensuring that transportation fuels sold in the U.S. contain a minimum amount of renewable fuels. To do this, the Renewable Fuel Standard (RFS) was developed under the Energy Policy Act of 2005 which mandates that an increase in renewable fuel blended with petroleum based transportation fuels is to increase from 9 billion gallons in 2008 to 36 billion gallons in 2022. To reach this goal, the mandate includes 16 billion gallons from cellulosic and agricultural waste-based biofuels by 2022 (Alvira, Tomas-Pejo, Ballesteros, & Negro, 2010). In 2011, only 7 million gallons of ethanol was produced from cellulosic material of the 13.5 billion gallons of biofuels produced in that year, indicative of the challenge that exists in order to reach the RFS mandated target for these cellulose based fuels (Igathinathane, Pordesimo, Womac, & Sokhansanj, 2009). The current challenge is to develop processes for converting cellulosic biomass to liquid fuels at a cost that is competitive with starch-based processes.

In the U.S., almost all of the 13.5 billion gallons of fuel produced in 2011 from biomaterial was ethanol, and almost all of that was produced from corn starch (only 0.3 billion gallons was biodiesel). The process for converting cellulosic biomass to ethanol is more challenging than the process for corn starch or other sugar rich materials due to the so called recalcitrant nature of lignocelluloses. Recalcitrance refers to the three components of

lignocelluloses: lignin, hemicellulose and cellulose combining to make an ultrastructure that is very resistant to biological degradation. Deconstructing lignocellulosic material to release the polymerized sugars cost effectively is a primary challenge to moving this technology into an industrially feasible process (S. Banerjee et al., 2010). One of the more compelling process schemes to do this is the biochemical conversion platform, where enzymes are used to hydrolyze sugar polymer bonds and release monomeric sugars that can be used by fermenting organisms to produce fuels like ethanol or butanol (Garcia, Pakkila, Ojamo, Muurinen, & Keiski, 2011), or commodity chemicals like lactic acid, succinic acid, and xylitol (Adsul, Singhvi, Gaikaiwari, & Gokhale, 2011; FitzPatrick, Champagne, Cunningham, & Whitney, 2010; Saha, 2003). However, due to the recalcitrant nature of lignocelluloses, a pretreatment step is usually required before hydrolysis to improve enzyme access to sugar polymers and thus improve sugar polymer saccharification. Therefore, to convert lignocelluloses to fuel ethanol requires 3 operations: pretreatment, enzyme hydrolysis and fermentation.

The goal of pretreatment is 3 fold: increase polysaccharide accessibility by redistributing or removing lignin and hemicelluloses, increase material porosity, and decrease cellulose crystallinity so that enzymes in the subsequent step can access the sugar polymers to perform their hydrolysis reactions. Pretreatment can be grouped into a few different categories: physical pretreatments, chemical pretreatments and biological pretreatments (Cheng & Timilsina, 2011; Hendriks & Zeeman, 2009; Yang & Wyman, 2008). Physical pretreatments include mechanical grinding or explosive techniques such as steam explosion and ammonia fiber expansion (AFEX). Chemical pretreatments include acid and alkali hydrolysis reactions that are typically performed at higher temperatures (100-190 ^{o}C) as well as oxidative pretreatments such as alkaline hydrogen peroxide (AHP). Biological pretreatments use various species of fungi to degrade lignin and

cellulose to make them more amenable to the enzymes in the next step (Adsul, et al., 2011). All of these pretreatment techniques have advantages and disadvantages compared to the others which has made it difficult for a clear front runner to emerge. Therefore, research on all of these technologies is still worthwhile and relevant.

This work will investigate the use of alkali and AHP pretreatment both practically from a process standpoint for producing biofuels, as well as fundamentally by examining how AHP affects lignocelluloses and how these effects translate into improvements in enzymatic digestibility (Figure 2). Specifically, corn stover and switchgrass, which are two popular grass feedstocks advocated to be used at large scale in cellulosic ethanol production, will be AHP pretreated at more industrially relevant conditions than have been done previously and pretreatment effectiveness will be evaluated. Additionally, the impact of AHP pretreatment on composition changes, downstream process inhibitor formation, and the water-cell wall interactions of biomass will be investigated to gain fundamental understanding of biomass property changes that are important for developing an economically feasible conversion process. Following is a literature review describing what has been done previously with AHP pretreatment and the important process conditions that impact pretreatment effectiveness and relevant work on enzyme hydrolysis, fermentation and biomass characterization especially concerning water – biomass interactions.

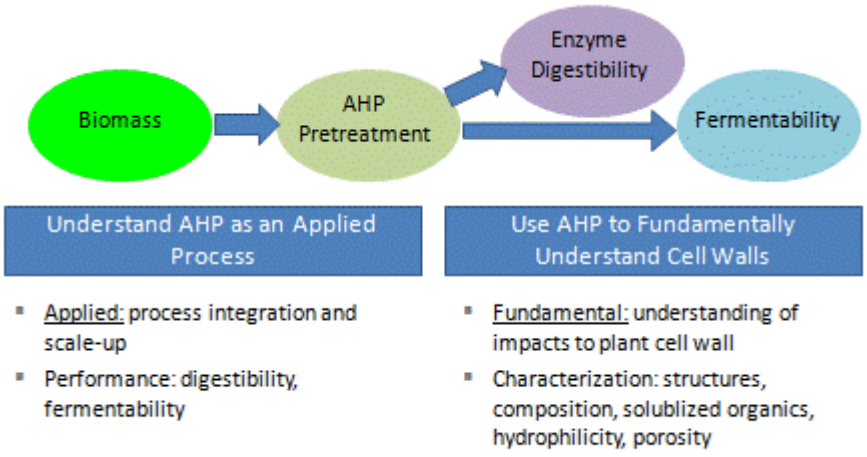

Figure 2: The scope of this research will investigate AHP pretreatment integration with enzyme hydrolysis and fermentation for different biomass feedstock and be used as a tool to understand cell wall characteristics that contribute to processing effectiveness based on digestibility and fermentability

Background Literature Review – AHP Pretreatment, Hydrolysis and Fermentation

Research on AHP being used as a pretreatment for lignocellulosic ethanol production as well as animal feed amelioration began as early as the 1980's and is based on existing processes already used in the pulp and paper industry for pulp bleaching (Gould, 1984, 1985; Gould & Freer, 1984). Typically, bleaching is done in multiple stages, where earlier stages are responsible for most of the delignification and later stages eliminate chromophores thereby brightening pulp (Reeve, 1996). H_2O_2 bleaching is performed at solids concentrations ranging from 12% to 30% with peroxide loadings from 1% to 4% typically performed at 90 ^oC (atmospheric bleaching) for up to 6 h. However, higher temperature bleaching above atmospheric pressure can significantly reduce the required bleaching duration (Bajpai, 2012). Peroxide bleaching processes will sometimes have metal removing steps prior to help stabilize the peroxide, which has been shown to then preferably remove chromophoric structures while leaving the lignin structure intact, effectively brightening pulp without delignifying (Suchy &

Argyropoulos, 2002). The key difference between pulp bleaching and AHP pretreatment is the use of a single stage in pretreatment instead of multiple as in bleaching because the primary goal is delignification, which is largely achieved at the beginning of bleaching, and not brightening. AHP pretreatment is done by mixing biomass with hydrogen peroxide and a strong base, typically NaOH, at a pH of 11.5; the concentrations of biomass and H_2O_2 can be varied. Studies have shown that during pulp bleaching, H_2O_2 will degrade to form reactive oxygen species such as hydroxyl radicals and superoxide ions which are responsible for the oxidation of phenolic compounds such as lignin. The formation of these radicals is optimal at pH 11.5, which is the pKa of H_2O_2 (Agnemo & Gellerstedt, 1979; Gellerstedt & Agnemo, 1980; Gellerstedt, Hardell, & Lindfors, 1980). The degradation products of lignin can also produce quinines which, themselves, can further increase the degradation of H_2O_2 to radicals increasing the consumption of H_2O_2 for the process (Agnemo & Gellerstedt, 1979; Gellerstedt, et al., 1980). Therefore, AHP has the advantage of being a delignifying pretreatment where lignin is separated from the sugar polymers by being solubilized or degraded. AHP has the further advantage of being performed at room temperature and atmospheric pressure reducing energy input requirements compared to other high temperature and pressure pretreatment technologies (Banerjee, Car, Scott-Craig, Hodge, & Walton, 2011). Important process variables that affect AHP pretreatment performance are H_2O_2 concentration, pH, temperature, pretreatment time and solids concentration. These pretreatment variables will have impacts on the structural and compositional components of lignocellulosic biomass that are important for the downstream performance of hydrolysis and fermentation. The following will describe the effects of pretreatment conditions on pretreatment performance based on these biomass characteristics and downstream processes.

AHP Pretreatment - pH Control

The desire to operate AHP pretreatment at pH 11.5 for reasons mentioned above presents a process challenge in that the pH is not constant during pretreatment due to two factors: acetic acid released from the biomass will lower pH, and the formation of hydroxide from H_2O_2 decomposition will raise the pH. Gould et. al have shown that for the pretreatment conditions they used on wheat straw, the pH will increase from 11.5 to 12 at high H_2O_2 concentrations in 6 hours (Gould, 1985). However, experiments done in our lab on switchgrass and corn stover have shown that the pH can decrease as much as 3 units or increase as much as 1 unit depending on the H_2O_2 concentration, or more accurately, depending on the ratio of the H_2O_2 concentration to the biomass concentration; at a high enough ratio the hydroxide formation will be higher than acetic acid release and the pH will increase, and at a low enough ratio hydroxide formation will be lower than acetic acid released and the pH will decrease. The ratio at which pH migration will change direction is likely feedstock specific and depends on acetic acid content. Not controlling pH and performing AHP pretreatment at pH values lower than 11.5 will reduce pretreatment effectiveness (Gould, 1985); performing at pH values higher than 11.5 will yield better results than the lower pH pretreatments, however, this becomes an alkali pretreatment and the beneficial effects of H_2O_2 are not observed. Therefore, to fully utilize the H_2O_2 during pretreatment it is important to maintain pH at 11.5 by the addition of alkali or acid during the pretreatment process or by using a buffer.

Temperature and Pretreatment Time

As mentioned previously, AHP pretreatment is typically performed at room temperature, or more accurately, without the addition of heat. However, the pulp and paper industry will increase the temperature during bleaching to improve paper brightness and/or to improve H_2O_2

effectiveness in lignin oxidation (Loureiro, Domingues, Fernandes, Carvalho, & Evtuguin, 2012). Therefore, studies have been done to investigate the effect of temperature on AHP pretreatment performance. Saha et. al. examined the effect of temperature and pretreatment time on pretreatment effectiveness based on sugars released after enzyme hydrolysis. The study found that at 25 ^{o}C pretreatment improvement stopped after 6 hours, but at 35 ^{o}C there was not much improvement after 3 hours of pretreatment for wheat straw at 9% solids (w/v) (Saha & Cotta, 2006). Similar results were found by Rabelo and coworkers using bagasse as the feedstock at 4% solids (w/v); for higher pretreatment temperatures, shorter pretreatment times were required to reach maximum achievable digestibility (Rabelo, Maciel, & Costa, 2008), which has also been known in the pulp bleaching process to be true (Bajpai, 2012). A thorough investigation of temperature and pretreatment time is missing in the literature; performing AHP pretreatment at higher temperatures may also affect lignin oxidation, sugar degradation and downstream inhibitor formation.

Hydrogen Peroxide and Solids Concentrations

Much of the previous research on AHP pretreatment used solids concentrations ranging from 1% to 10% (w/v) with the exception of one study using ~40% solids in which they used a modified extruder to mix the high solids slurry (Gould, 1985). H_2O_2 loadings typically ranged from 10% to 50% on a wt H_2O_2/wt biomass basis. These conditions yielded positive results for lignin removal and improved enzymatic digestibility for a range of biomass feedstocks. Gould performed AHP pretreatment at relatively dilute concentrations of H_2O_2, as low as 1% (w/v), and low solids concentrations, anywhere from 1 – 10% (w/v); this study showed lignin removal as high as 50% and enzyme hydrolysis improvements of glucose yields from 30% for untreated material to over 90% for pretreated wheat straw and kenaf (Gould, 1984). Subsequent studies

using similar pretreatment conditions resulted in similar results for wheat straw, corn cob, corn husk, alfalfa hay, soy bean stover, and others (Gould, 1984, 1985; Gould & Freer, 1984; Gould, Jasberg, Fahey, & Berger, 1989). However, these low solids conditions are not desirable for an industrial process which would lead to large volumes of wastewater that would require treatment, and dilute downstream product concentrations that would have high energy requirements for separation (Modenbach & Nokes, 2012). Therefore, higher solids concentrations during pretreatment are essential for AHP pretreatment to be industrially viable. Numerous studied have investigated the impact of performing pretreatment and hydrolysis at high-solids loadings (Hodge, Karim, Schell, & McMillan, 2008; Jorgensen, Vibe-Pedersen, Larsen, & Felby, 2007; Kristensen, Felby, & Jorgensen, 2009; Lu et al., 2010; J. Zhang et al., 2010). Table 1 shows a summary of AHP pretreatment conditions investigated in the literature, it should also be noted that most studies washed the biomass following pretreatment effectively removing any inhibitors to hydrolysis, which would not be practical in an large scale process with so much water usage.

Table 1: AHP pretreatment conditions summary of select publications

Year Pub	Feedstock	H_2O_2 Loading	Solids Loading	Washed Biomass
1983-85	Corn Cob, Husk, Stalk Foxtail, Alfalfa Hay Kenaf, Wheat Straw	30 - 50% g/g	2-6% w/v	Yes
1987	Wheat Straw	5% g/g	45% w/w	Yes
2006	Wheat Straw	50% g/g	9% w/v	No
2007	Cotton Stalk	20% g/g	10% w/v	Yes
2008	Wheat straw, Bagasse	25 - 50% g/g	4-8% w/v	Yes
2009	Corn Stover	25% g/g	4% w/v	Yes
2011	Corn Stover	12-50% g/g	2-10% w/v	Yes
		Too High	Too Low	Impractical

The first attempt at a high solids AHP pretreatment utilized an extruder, as mentioned previously, to allow for sufficient mixing at solids concentrations as high as 40% (w/v) (Gould, et al., 1989). The extruder was used to mix wheat straw, H_2O_2, and NaOH solution for about 15 sec, and then the mixed slurry was sealed into a plastic bag for the duration of the 24 h pretreatment time. Glucose yield improvements for the wheat straw were from 50% glucose yield for untreated material to over 80% glucose yield for AHP pretreated material; these results were better than those of previous studies at lower solids loadings. Under these conditions the temperature of the slurry after exiting the extruder reached upwards of 80 ^{o}C either due to friction of mixing or from exothermic H_2O_2 degradation. The authors suggested the higher temperature could explain the improvement of pretreatment efficacy compared to previous work, however, they did not explain why this should be the case. Therefore, it was not clear if the improved pretreatment performance was a result of higher solids conditions, improved mixing in the extruder, higher temperatures or a combination of all of these.

Scale-up from the bench scale of AHP pretreatment has also been attempted in the context of improving biomass digestibility in rumen animals. 70-90 kg of wheat straw was AHP pretreated in large stainless steel vats at approximately 3-5% solids and 30% H_2O_2 loading (Kerley, Fahey, Berger, Merchen, & Gould, 1987; Meeske, Meissner, & Pienaar, 1993). However, because the biomass was used as animal feed, comparing the results of pretreatment performance based on enzyme hydrolysis is not possible. In any case, the H_2O_2 conditions and solids concentrations used in these studies are too high and too dilute respectively to be industrially feasible. Banerjee et al performed AHP pretreatment on 1 kg of corn stover using 15% (w/v) solids and 12.5% H2O2 loading and were able to achieve glucose yields upwards of

75%; this demonstrated for the first time that AHP scale-up can be done reproducibly with good results and at much more industrially relevant conditions (Banerjee et al., 2012).

Gould and coworkers also showed in early studies of AHP pretreatment that much of the H_2O_2 degrades to oxygen and water, indicating unutilized potential for lignin oxidation (Gould, 1985). The incorporation of oxygen into biomass by oxidation was evaluated by measuring the O_2 evolved during pretreatment; the difference in measured O_2 evolution from the theoretical amount that should have been evolved based on stoichiometry was considered to be incorporated into the biomass. For the AHP conditions they used on kenaf at a solids concentration of 2% (w/v), they found ~20% H_2O_2 incorporation. They also found that improvement of incorporation, to ~40%, was achieved simply by performing pretreatment at a higher solids concentration and further, that the initial rate of O_2 evolution was increased at this higher solids concentration (6% w/v). These results are comparable to results observed in our lab using corn stover where we measured ~20% H_2O_2 incorporation. This indicates there is a lot of room for improvement of H_2O_2 utilization; perhaps even higher solids loadings can increase utilization, but to what extent is not certain. Another possible avenue is using catalysts to focus oxidization on desirable biomass reactions instead of with H_2O_2 degradation products (Z. Li et al., 2012).

Biomass Structural and Compositional Changes

After biomass has been pretreated, understanding the structural and compositional changes that take place and how they relate to improved enzyme digestibility is important. Composition refers to the constituent content including glucan, xylan, and lignin as well as minor components such as acetate, proteins and ash. How these components combine together are the structural characteristics and include degree of polymerization, lignin-carbohydrate complexes, cellulose crystallynity, lignin location, surface area and hornification (Kristensen, Thygesen,

Felby, Jorgensen, & Elder, 2008; Spinu, Dos Santos, Le Moigne, & Navard, 2011). AHP pretreatment impacts all of these factors which in turn impacts enzymatic digestibility. It is also worth noting that structural and compositional changes can occur during feedstock storage and transportation (Inman, Nagle, Jacobson, Searcy, & Ray, 2010).

Gould and coworkers examined the structural effects of AHP on cellulose using Xray diffraction and found that AHP "loosens" the three dimensional structure of lignocelluloses by removing (solubilizing) lignin and hemicellulose, but it has no effect on the crystallinity of cellulose (Martel & Gould, 1990). The removal of lignin and hemicellulose are the primary compositional components affected by AHP pretreatment, while cellulose is largely unchanged and remains insoluble. There is also a significant fraction, 10-30%, of other extractable material that is solubilized during AHP pretreatment that include proteins, acetate, ash and others. The alkali in AHP is responsible for saponification reactions that cleave acetate bonds on the hemicelluloses which then increases their solubility (Kerley, Fahey, Berger, Gould, & Baker, 1985; Pedersen & Meyer, 2010); as much as 20% of hemicellulose has been shown in the literature to solubilize for AHP pretreatment of rye straw (Fang, Sun, & Tomkinson, 2000; Girio et al., 2010), and in our lab hemicellulose removal has been observed between 20% and 40% depending on pretreatment conditions and feedstock. Lignin solubilization is due to H_2O_2 oxidation reactions evinced by the minimal amount of lignin solubilized under alkali only conditions (Janker-Obermeier, Sieber, Faulstich, & Schieder, 2012). It has been suggested that in grasses, which have high phenolic hydroxyl contents, destruction of alkyl-aryl ethers would increase lignin solubilization during AHP pretreatment (M. Y. Li et al., 2012). The removal of hemicelluloses and lignin components leads to an improvement in cellulose digestibility suggesting that lignin and hemicellulose synergistically occlude cellulose from cellulase

enzymes. This was shown by Selig and coworkers using corn stover at 4% solids (w/v) where AHP pretreatment was performed at different temperatures to remove different amounts of lignin. They found by adding different mixtures of cellulose and hemicellulose degrading enzymes that lignin is not solely responsible for blocking cellulose from enzymes and that there is some lignin-hemicellulose interaction that together inhibits the enzymes (Selig, Vinzant, Himmel, & Decker, 2009).

Enzymatic Hydrolysis

Following pretreatment, sugar polymers are hydrolyzed using enzymes to release fermentable, monomeric sugars. Because of the heterogeneous composition of lignocellulosic biomass, a system of enzymes is required which work synergistically to break bonds and expose more enzyme starting locations for hydrolysis and to reduce product inhibition. The primary enzyme activities required for lignocellulose hydrolysis are glucanase enzymes and xylanase enzymes; improved digestibility can be achieved by including accessory enzymes with activities toward minor components of lignocellulosic biomass such as glucuronidases and arabinosidases as well as pectinases. These accessory enzymes have been shown to improve sugar yields for glucose and xylose at relatively low fractions of the enzyme cocktail (~20%) (Banerjee et al., 2010). Some of the key enzyme related factors that limit efficient hydrolysis include: a) product inhibition, b) unproductive binding of cellobiohydrolases to cellulose, c) hemicellulose and lignin association with cellulose that blocks enzyme activity, d) enzyme adsorption to lignin, and e) loss of enzyme activity due to denaturation, mechanical shear, proteolytic activity or low thermal stability (Jorgensen, Kristensen, & Felby, 2007). In addition to these, there are substrate related factors that hinder enzyme hydrolysis as well including lignin composition, cellulose crystallinity, available surface area, porosity, cell wall thickness, and changes in accessibility

during hydrolysis (Alvira, et al., 2010). Furthermore, there are process-condition-related factors that impact enzymatic hydrolysis such as enzyme loading, solids concentration, and hydrolysis time. All 3 of these groups of factors can be interrelated in regards to impact on digestibility; for example, Zhu et. al. showed that for short hydrolysis times, lignin content did not matter when cellulose crystallinity was low, and for longer hydrolysis times, crystallinity did not matter when lignin content was low (Rollin, Zhu, Sathitsuksanoh, & Zhang, 2011; Zhu, O'Dwyer, Chang, Granda, & Holtzapple, 2008). Lignin inhibits enzymes in 3 different ways: 1) physically blocking enzyme access to sugar substrates, 2) unproductively and irreversibly binding enzymes, and 3) forming inhibitory compounds during pretreatment (Sewalt, Glasser, & Beauchemin, 1997). Cellulase enzymes have been shown to have stronger binding affinities for lignin than for cellulose substrates. It has also been shown that the enzymes can denature on the surface of lignin suggesting that lignin somehow destabilizes the enzymes, and that this destabilization increases with temperature (Rahikainen et al., 2011). Studies have also found that the severity of lignin-enzyme binding is different depending on the pretreatment method used. For example, acid pretreatments show more enzymatic deactivation due to lignin binding than hot water pretreatment suggesting that the physicochemical properties of lignin are different after the 2 methods and there subsequent binding capabilities are different also (Kristensen, Borjesson, Bruun, Tjerneld, & Jorgensen, 2007; Rahikainen, et al., 2011). The lignin binding effect has been shown to be reduced by the addition of surfactants or bovine serum albumin (BSA) that act as lignin blockers preventing enzymes from binding; alternatively, higher enzyme loadings would have the same effect by sacrificing a fraction of enzymes for lignin binding (Eriksson, Borjesson, & Tjerneld, 2002; Kristensen, et al., 2007; Yang & Wyman, 2006; Y. Q. Zhang, Xu, Zhang, & Li, 2011).

In order for the biochemical conversion of lignocelluloses to ethanol to be economically viable, both pretreatment and enzymatic hydrolysis steps will need to be performed at high solids concentrations to reduce reactor volumes and wastewater streams. This will also allow for ultimately higher sugar concentrations and thus higher ethanol concentrations which would reduce the energy requirements for downstream separation. However, high solids hydrolysis presents several challenges including higher sugar concentrations that lead to product inhibition, higher inhibitor concentrations, mass transfer limitations, and difficulty mixing. The solids effect is a catch-all term referring to a decrease in enzymatic digestibility at higher solids concentrations (Kristensen, et al., 2009).

Fermentation Inhibitors

Much of the work examining the effectiveness of AHP pretreatment uses enzyme hydrolysis sugar yield as the rubric for comparison. However, fermentability of hydrolyzate is another important factor to consider for determining pretreatment effectiveness and for comparing different pretreatment technologies. However, this approach is problematic since different fermenting species and strains may be more suitable for different pretreatment liquors and subsequent hydrolyzates. Using fermentability as a measure of pretreatment effectiveness complicates the comparisons that are made. However, quantifying known fermentation inhibitors after pretreatment and hydrolysis is still important. Certain compounds are known to be inhibitive to fermenting organisms and have been shown to be present after AHP pretreatment. Qureshi and coworkers have shown that the sodium concentrations reached during AHP pretreatment are inhibitory to the acetone-butanol-ethanol fermenting organisms *Clostridium acetobutylicum* and *Clostridium beijerinckii* (Qureshi, Saha, Hector, & Cotta, 2008). In addition, weak acids, furan derivatives and phenolic compounds are known to inhibit

fermentation (Luo, Brink, & Blanch, 2002; Palmqvist & Hahn-Hagerdal, 2000a; Persson et al., 2002). Weak acids (such as acetic, formic and levulinic acids) in undissosiated form are liposoluble and can transport across the cell membrane where they can dissociate depending on pKa and intracellular pH. This internal acidification in addition to other acid interactions with cellular processes can inhibit fermentation (Pampulha & Loureirodias, 1990). Hydroxymethyl furfural (5-HMF) and furfural inhibits glycolytic enzymes and contributes to the accumulation of acetaldehyde which has been suggested as the reason for growth inhibition in the presence of furfural (Palmqvist & Hahn-Hagerdal, 2000b). The mechanisms of phenolic inhibition to fermenting organisms is not well understood but may be due, in part, to partitioning in cell membranes damaging membrane selectivity (Heipieper, Weber, Sikkema, Keweloh, & Debont, 1994; Palmqvist & Hahn-Hagerdal, 2000b). The inhibitor compounds and concentrations vary with biomass feedstock, pretreatment technology and pretreatment conditions. Detoxification of hydrolyzates prior to fermentation by phenolic compound removal using laccase treatment and anion exchange techniques has been shown to remove as much as 80% of phenolics (Palmqvist & Hahn-Hagerdal, 2000b).

Cell Wall and Water Interactions

The interaction of water with biomass material during pretreatment and hydrolysis can be an important factor for processing, especially for enzymatic hydrolysis. Figure 3 shows the difference in water sorption between AHP pretreated corn stover and switcgrass; both materials were pretreated at the same solids concentration (15% w/v), however, there is essentially no free water in the corn stover sample, while the switchgrass sample is much less absorbent and has more free water. Knowing that corn stover has a higher enzymatic digestibility than switchgrass led to the hypothesis that this biomass-water interaction may be a useful indicator for how

susceptible a material is to hydrolyzing enzymes. Figure 4 illustrates the idea that certain cell walls properties such as lignin content and cellulose crystallinity contribute to cell wall hydrophilicity which in turn can impact water penetration and thus enzyme penetration into the cell wall ultimately impacting enzymatic digestibility.

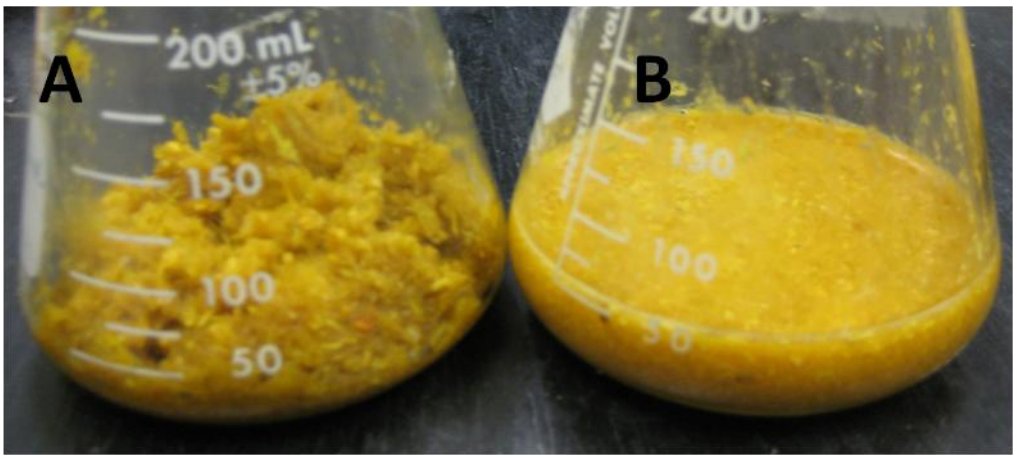

Figure 3: AHP pretreated A) corn stover and B) switchgrass water sorption comparison

For woody biomass, it is known that water will sorb to specific sites, hydroxyl and carboxyl groups, in a layered or clustered formation (Berthold, Olsson, & Salmen, 1998; Froix & Nelson, 1975; Olsson & Salmen, 2004). For some materials, 1-1.3 water molecules can sorb to a single hydroxyl group (Olsson & Salmen, 2004). Water will interact first with readily available hydroxyl groups, and then as the material swells, more hydroxyl groups become exposed allowing for further water adsorption to these groups (Berthold, et al., 1998; Froix & Nelson, 1975). Water bound to cellulose primarily occurs in amorphous regions, and it has been shown that bound water decreases with increasing crystallinity (Hatakeyama, Nakamura, & Hatakeyama, 2000; Nakamura, Hatakeyama, & Hatakeyama, 1981) and the amorphous regions are removed easily at the beginning of hydrolysis (Vyas, Pradhan, Pavaskar, & Lachke, 2004). The diffusion of enzymes and hydrolysis products through amorphous regions is higher than

crystalline regions, which explains, in part, why hydrolysis rates are slower at later stages of hydrolysis when most of the remaining cellulose is more crystalline. However, cellulose and lignin do not contribute as much to water binding as hemicelluloses and pectins, likely due to higher amounts of hydroxyl, carboxyl and other charged groups in hemicellulose components of biomass (Lund, Sjostrom, & Brelid, 2012; Weber, Kohlhepp, Idouraine, & Ochoa, 1993).

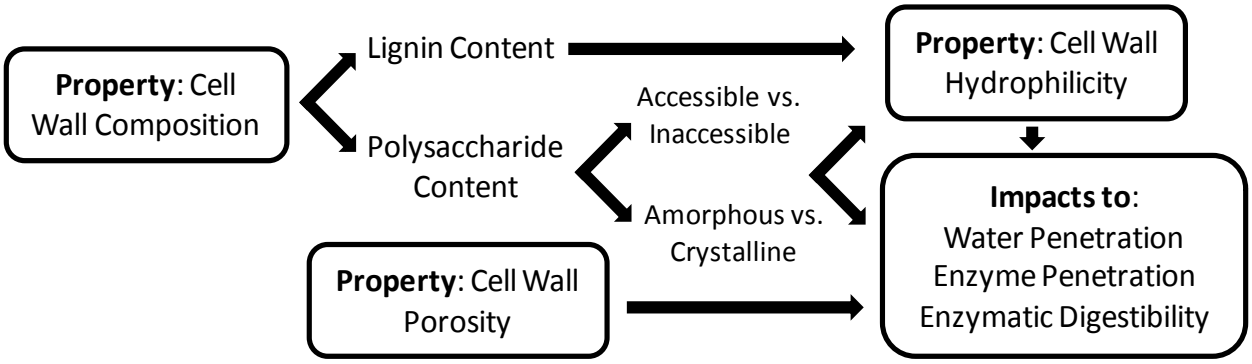

Figure 4: Diagram of various cell wall properties that contribute to cell wall hydrophilicity of biomass and ultimately enzymatic digestibility

Different types of feedstock, feedstock composition and even plant cell type can lead to different water sorption behavior. For example, it has been shown that guaiacyl lignin can restrict fiber swelling more so than syringyl lignin, therefore, differences in the amounts of these two lignins would certainly lead to different water sorbing behavior (Ramos, Breuil, & Saddler, 1992). In addition to differences between plant species, there are differences in water sorption within plants themselves. Igathinathane et al, examining water sorption behavior in different locations in the plant, found that the stalk pith region of corn stover did not hold as much water as other areas such as stalk leaf perhaps due to entrained air preventing water penetration. Furthermore, different regions of the stover would equilibrate with water differently depending on properties (Igathinathane, Womac, Sokhansanj, & Pordesimo, 2005). Furthermore, certain

regions of corn stover have been shown to have different energy states such that water binding sites will fill up preferentially (Igathinathane, Womac, Sokhansanj, & Pordesimo, 2006, 2007).

Absorbed water in fibers is classified into three groups: water of constitution, imbibed water and free water. Water of constitution is strongly held by the fiber surface by hydrogen bonds and forms a monolayer on the surface. Imbibed water is the additional water held by fiber when the relative water vapor is increased to 100%. And free water is that which is held after reaching fiber saturation (Akinli-Kogak, 2001). Water of constitution and imbibed water are also termed non-freezing and freezing water respectively based on their behavior during differential scanning calorimetry (DSC) at below freezing temperatures; non-freezing water will not freeze at temperatures well below 0 ^{o}C, and freezing water will have different freezing temperatures below 0 ^{o}C depending on the size of the pore in which it is trapped (Kaewnopparat, Sansernluk, & Faroongsarng, 2008). Because of this, freezing water quantification using DSC allows for the quantification of pore size distribution. Yu et al showed that delignification of wood will tend to increase pore volume to a certain point, quantified by freezing water with DSC. They also showed a correlation between increasing freezing water, and thus accessible pore volume, and carbohydrate digestibility; however, the pattern varied depending on material and delignification technique (Yu, Jameel, Chang, & Park, 2011). Similar results have been shown correlating pore volume with digestibility where pore volume was determined using a solute exclusion technique; they found that larger total accessible pore volume allowed for faster initial hydrolysis rates (Grethlein, 1985).

Several methods have been developed to quantify biomass and water interactions which are used as important indicators of fiber properties. The water retention value (WRV) (Maloney, Laine, & Paulapuro, 1999), settling volume (Alince & Robertson, 1974) and water activity

(Fardim, Liebert, & Heinze, 2013) are examples of such metrics that have standardized methods for quantifying the amount of pore water versus free water, fiber stiffness, and water sorption respectively. The WRV is a centrifugation method where a pad of wet biomass is drained using centrifugation at a specified speed and duration; the value is determined by the amount of water remaining in the biomass after centrifugation (Scallan & Carles, 1972). The settling volume provides a metric for distinguishing differences between inter-fiber friction, and chemical treatments that increase settling volume also tend to increase dewatering rates (Hubbe & Heitmann, 2007). The settling volume quantifies the relative height of biomass to total slurry height when it is allowed to settle in a solution of water for a specified solids concentration and duration. Water activity is another method for quantifying the interaction of water with biomass and is typically used in the food industry as a way to predict bacterial growth on food by quantifying the amount of water absorbed in food that is available for bacterial utilization leading to growth and food spoilage (Berg & Bruin, 1978). This metric is based on the energy state of water bound or trapped in a solid material based on the partial pressure of water molecules contained in the porous solid material.

For the context of this work, biomass hydrophilicity will include water association with biomass due to spatial confinement within the biomass matrix (macro-scale), within nano-scale pores, and physicochemical association with the solid fiber surfaces. Several cell wall properties can contribute to changes in hydrophilicity. For example, delignification and hemicellulose removal have been shown to increase pore volume and leave behind empty space for water molecules to occupy, which allows for increased water penetration and thus water swelling (Akinli-Kogak, 2001; Grethlein, 1985). Water molecules also adsorb to biomass surfaces through polar groups, specifically hydroxyl and carboxyl functional groups (Olsson & Salmen,

2004). Cell wall composition, specifically lignin and hemicellulose content, can both contribute to cell wall hydrophilicity, and pretreatment impacts composition and therefore swelling behavior (Grabber, Hatfield, & Ralph, 2003). Polysaccharide accessibility and crystallinity can impact cell wall hydrophilicity due to water access to cell wall surfaces and cellulose swelling; these properties are also known to be impactful to enzymatic digestibility (Chundawat et al., 2011). Also, cell wall porosity will be indicative of water and enzyme penetration and therefore, perhaps, enzymatic digestibility. The hypothesis is therefore, that quantifiable water and biomass interactions may incorporate impacts of composition, porosity and hydrophilic properties of the cell wall and be a more useful indicator of enzymatic digestibility than any one group of these properties on their own.

Much of the work examining water-cell wall properties has focused on woody biomass which is more commonly used in the pulp and paper industry; water sorption behavior of grasses is largely missing in the literature and would be a valuable addition to the breadth of knowledge for biofuel production from this type of feedstock. Specifically, how water sorption behavior impacts process operations of pretreatment and hydrolysis and, as this work will show, how water sorption can be used as an indicator of pretreatment effectiveness and a predictor of enzymatic digestibility.

Contents of this Dissertation

Chapter 1 will show results of AHP pretreatment on two different feedstock, corn stover and switchgrass, investigating the impact of H_2O_2 loading, solids loading, pretreatment time and scale on pretreatment effectiveness determined by sugar yields following enzymatic hydrolysis, and on changes to biomass composition and inhibitor release and solublization. Understanding

the reaction space of these pretreatment conditions will allow for further optimization of AHP pretreatment as a process.

Chapter 2 will discuss the potential for a novel combined sugar extraction and alkali pretreatment process of sweet sorghum that could be used for biofuel production. Soluble, readily fermentable sugars of sweet sorghum are easily extracted with hot water, however, structural polysaccharides are unutilized and left intact after this process. Performing an alkali extraction to remove soluble sugars and pretreat bagasse, which can then be enzymatically hydrolyzed, would provide an additional sugar stream for fermentation. This work will present results for this combined process concerning sugar extraction efficiency and pretreatment efficacy based on hydrolysis yield and show fermentability of the combined extraction juice and hydrolyzate.

In chapter 3, the impact of AHP pretreatment and liquid hot water (LHW) pretreatment on the hydrophilic properties of biomass will be investigated, specifically WRV, settling volume, and water activity and how these properties relate to certain composition or structural properties such as carboxylic acid content and surface charges as well as to enzymatic hydrolysis and enzyme binding. It will be shown that for the conditions of AHP and LHW pretreatment used here, cell wall swelling increases with increasing pretreatment severity, and that swelling quantified by WRV and settling volume linearly correlate well with enzyme binding and glucose yield. Therefore, water swelling of biomass may be a useful indicator of pretreatment effectiveness and enzymatic digestibility.

In chapter 4, a continuation of the work presented in chapter 3 will be discussed where an even larger range of AHP and LHW pretreatments as well as results for AFEX pretreatment are

performed on corn stover and switchgrass, to determine if the strong linear correlation between WRV and glucose yield is universal across these materials. It will be shown that the linear correlation does not hold for the more extreme pretreatment conditions and for the AFEX pretreated material when regressed with the AHP and LHW pretreated material. However, by performing multiple linear regression (MLR) that includes composition information, for example glucan content, as well as WRV, a much better linear predictive model can be achieved for the conditions used here.

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Chapter 1 – Impact of AHP pretreatment on corn stover and switchgrass composition, inhibitor release and enzymatic digestibility

Introduction

Cellulosic biomass is a promising source of renewable material that can be used to produce fuels such as ethanol or butanol (Garcia, Pakkila, Ojamo, Muurinen, & Keiski, 2011), or commodity chemicals such as lactic acid, succinic acid, and xylitol (Adsul, Singhvi, Gaikaiwari, & Gokhale, 2011; FitzPatrick, Champagne, Cunningham, & Whitney, 2010; Saha, 2003). Deconstructing lignocellulosic material to release polymerized sugars cost effectively is a primary challenge to moving cellulosic fuel production technology into an industrially feasible process (S. Banerjee et al., 2010). One of the more compelling process schemes to do this is the biochemical conversion platform, where enzymes are used to hydrolyze sugar polymer bonds and release monomeric sugars that can be used by fermenting organisms to produce the desired products. However, due to the recalcitrant nature of lignocelluloses, a pretreatment step is usually required before hydrolysis to improve cell wall polysaccharide accessibility to enzymes in order to facilitate enzyme catalysis and ultimately cell wall deconstruction to soluble sugar monomers. Within this pretreatment step it is necessary to increase polysaccharide accessibility by removing or redistributing lignin, increasing cell wall porosity and decreasing cellulose crystallinity. There are many pretreatment technologies to do this and they can be grouped into a few different categories including physical, chemical and biological pretreatments (Cheng & Timilsina, 2011; Hendriks & Zeeman, 2009; Yang & Wyman, 2008). Physical pretreatments include mechanical grinding and particle size reduction or explosive techniques such as steam explosion and ammonia fiber expansion (AFEX). Chemical pretreatments include acid and alkali hydrolysis reactions that are typically performed at higher temperatures (100-190 ^{o}C) as

well as oxidative pretreatments such as alkaline hydrogen peroxide (AHP) which oxidize lignin, and to some extent carbohydrates. Biological pretreatments use various species of fungi to degrade the lignin and cellulose to make it more amenable to the enzymes in the next step (Adsul, et al., 2011). All of these pretreatment techniques have advantages and disadvantages depending on the criteria of evaluation (Dale & Ong, 2012), and research in many of these pretreatment technologies is still worthwhile and relevant.

Research on AHP being used as a pretreatment for lignocellulosic ethanol production is based on existing processes already used in the pulp and paper industry for pulp bleaching (Gould, 1984, 1985; Gould & Freer, 1984), and has been investigated for both application in fuel production and animal feed amelioration (Gould, 1984; Kerley, Fahey, Berger, Merchen, & Gould, 1987). Pulp bleaching is a multiple stage delignification and brightening process, and in H_2O_2 bleaching, earlier stages are responsible for most of the delignification, while later stages eliminate chromophores which results in pulp brightening (Reeve, 1996). H_2O_2 bleaching is performed at solids concentrations ranging from 12% to 30% with peroxide loadings from 1% to 4% at 90 ^oC (atmospheric bleaching) for up to 6 h, however, higher temperature bleaching performed above atmospheric pressure can significantly reduce the required bleaching duration (Bajpai, 2012). Peroxide bleaching processes will sometimes have prior metal removing steps to help stabilize the peroxide, which has been shown to then preferably remove chromophoric structures while leaving the lignin structure intact, effectively brightening pulp without delignifying (Suchy & Argyropoulos, 2002). Therefore, key difference between pulp bleaching and AHP pretreatment, besides the operation difference that bleaching uses multiple stages and pretreatment is typically one, is that the primary goal of bleaching is pulp brightening, which

occurs in later stages, and the goal of pretreatment is delignification, which occurs at early stages where brightening is minimal.

Therefore, AHP pretreatment is done by mixing biomass with hydrogen peroxide and a strong base, typically NaOH, at a pH of 11.5 where the oxidant acts as a delignifying agent rather than a brightening agent. Studies have shown that during pulp bleaching, H_2O_2 will degrade to form hydroxyl radicals and superoxide ions which are the reactive agents responsible for the oxidation of phenolic compounds such as lignin; the formation of these radicals is optimal at pH 11.5, which is the pKa of H_2O_2 (Agnemo & Gellerstedt, 1979; Gellerstedt & Agnemo, 1980; Gellerstedt, Hardell, & Lindfors, 1980). Maintaining the pH at 11.5 during the process is important because pH will increase or decrease depending on the H_2O_2 concentration used and the biomass being pretreated (Gould, 1985); this is likely due to the acetic acid content of the biomass which is released during pretreatment, the oxidation of cell wall polymers to organic acids, and the generation of hydroxyl anions from H_2O_2 degradation. Not controlling pH and performing AHP pretreatment at pH values lower than 11.5 will reduce pretreatment effectiveness (Gould, 1985). Performing pretreatment at pH values higher than 11.5 can yield better results than those performed at lower pHs. However, this becomes an alkali pretreatment and the beneficial effects of H_2O_2 are not observed (Li, Chen, Hegg, & Hodge, 2013). Therefore, to fully utilize H_2O_2 during pretreatment it is important to maintain pH at 11.5 by the addition of alkali, or acid depending on the direction of pH migration, during the pretreatment process. The degradation products of lignin can also produce quinones which, themselves, can further increase the degradation of H_2O_2 to radicals increasing the consumption of H_2O_2 for the process (Agnemo & Gellerstedt, 1979; Gellerstedt, et al., 1980). Thus, AHP has the advantage of being a delignifying pretreatment where lignin is separated from the sugar polymers by being

solubilized or degraded. In pulp bleaching the temperature is increased above ambient temperature to improve H_2O_2 effectiveness in lignin oxidation (Loureiro, Domingues, Fernandes, Carvalho, & Evtuguin, 2012). However, AHP as a pretreatment has typically been performed at room temperature and atmospheric pressure reducing energy input requirements (Banerjee, Car, Scott-Craig, Hodge, & Walton, 2011). Important process variables that affect AHP pretreatment performance are H_2O_2 concentration, pH, temperature, pretreatment time and solids concentration. These pretreatment conditions will have impacts on the structural and compositional components of lignocellulosic biomass that are important for the downstream performance of hydrolysis and fermentation.

Grasses such as corn stover, switchgrass, miscanthus, wheat straw, and sugar cane bagasse are examples of attractive sources of lignocelluose for biofuel production because they are either agricultural waste products or designated energy crops that would minimize competition with or disruption of food crop markets. Grassses are particularly susceptible to alkali pretreatments because their cell walls contain ferulic acid ether-linked to lignin as well as higher free phenolic contents of lignin that make them highly soluble in alkali (Lapierre, Jouin, & Monties, 1989). Lignin content has been shown to be negatively correlated to glucan digestibility for grasses with a range of lignin properties, indicative of the contribution lignin has toward recalcitrance (M. Y. Li et al., 2012). However, the relative contribution of different cell wall components to recalcitrance is not universally the same. For example, DiMartini et. al. showed that xylan in switchgrass contributed more to recalcitrance than xylan in popular, while lignin in poplar contributed more than in switchgrass (DeMartini et al., 2013). Lignin, xylan, and ferulate removal in grasses (M. Y. Li, et al., 2012) are important outcomes of AHP pretreatment and are linked to the digestibility of the cell walls. We hypothesize that xylan and

lignin removal are correlated for grasses following AHP pretreatment, and that removal of both components is synergistic. For this work we will investigate the compositional changes that take place during AHP pretreatment of corn stover and switchgrass under different hydrogen peroxide conditions and relate these changes to sugar yield. Specifically, lignin and xylan removal will be correlated to each other and to ferulic acid removal. In addition, AHP pretreatment will be scaled-up from the bench scale, 8 g of biomass, to 1kg of corn stover and performed at higher solids concentrations (>15% w/w) to evaluate potential pretreatment improvement at these more industrially relevant conditions.

Methods

Bench-Scale Alkaline Hydrogen Peroxide Pretreatment

Pretreatment of corn stover and switchgrass was performed using four different loadings of H_2O_2on biomass: 0%, 6%, 12.5% and 25% (g H_2O_2/g biomass). The pH was maintained at 11.5 by the periodic addition of aliquots of 5 M sodium hydroxide. All four pretreatment conditions were performed in duplicate using 8g of biomass (dry basis) at 15% solids (w/v) which is equivalent to 12.6% to 13% (w/w) depending on the H_2O_2 condition. Samples were prepared in 250 mL Erlenmeyer flasks and placed in an incubator at 30^0C with shaking at 180 rpm. The flasks were covered with parafilm to prevent evaporation and to allow for expansion as the pressure in the flasks increased with O_2 evolution. Pretreatment was stopped at 24 h by diluting the sample to approximately 10% solids (w/w) with water and adjusting the pH to 4.8 using concentrated sulfuric acid in preparation for enzymatic hydrolysis.

Scaled-up Alkaline Hydrogen Peroxide Pretreatment

Pretreatment of corn stover was scaled-up from the bench scale using an industrial bowl mixer to pretreat 1 kg of biomass. Pretreatment was performed using 12.5% H_2O_2 loading (g H_2O_2/g biomass) at pH 11.5 and solids concentrations of 15%, 25%, 35%, 45% and 55% (w/v) which is equivalent to 12.7%, 19.2%, 24.7%, 29.3%, and 33.2% (w/w) respectively. The appropriate amount of water, 30% H_2O_2 (v/v) solution and 5 M sodium hydroxide solution were added to the bowl mixer followed by the 1 kg of corn stover and the slurry was mixed well for several minutes. For the first 30 min the slurry would periodically be mixed as needed to prevent the contents from overflowing the mixing bowl as the oxygen evolution caused the slurry to rise. The pH was periodically adjusted back to 11.5 using appropriate amounts of 5 M sodium hydroxide at 3, 6, 9, 12, and 24 h with thorough mixing; samples were also collected at these times in duplicate and prepared for enzymatic hydrolysis by diluting with water to approximately 10% solids (w/w) and adjusting the pH to 4.8 using concentrated sulfuric acid.

Enzymatic Hydrolysis

Hydrolysis of all corn stover and switchgrass samples was performed by diluting the AHP delignification slurries with water to 10% (w/w) solids and adjusting the pH to approximately 4.8 using concentrated sulfuric acid as mentioned above. Then an aliquot of 1 M citric acid buffer was added to give a concentration of 50 mM citric acid buffer in the sample flasks. Tetracycline and cyclohexamine were added to make a concentration of 10 mg/L each to prevent microbial contamination. Next, an enzyme mixture of Accellerase 1000, Multifect Xylanase and Pectinase was added in a protein mass ratio of 4.4:1.7:1, respectively, at an enzyme loading of 30 mg enzyme/g glucan; this optimized enzyme ratio was determined by Banerjee et al. (G. Banerjee et al., 2010) and the protein contents of the enzymes were based on

the Bradford assay. Samples were then mixed well and placed in a shaking incubator at 50°C with 180 rpm shaking for seven days. Sugar concentrations in the hydrolysate were determined by HPLC using the method described in the NREL / TP 510-42618 protocol and converted to glucose yields based on the solids content in the reaction vessel and glucan content in the undelignified biomass.

Scaled-up pretreated corn stover hydrolysis samples were prepared by removing 150 g, 100 g, 75 g, 70 g and 60 g of pretreatment slurry from the mixing bowl for the 15%, 25%, 35%, 45% and 55% (w/v) solids conditions respectively; these samples were then diluted to 10% (w/w) solids and the pH was adjusted to 4.8 using concentrated sulfuric acid as mentioned above. Then 75 g of the diluted slurry was transferred to shake flasks in duplicate where citrate buffer, tetracycline, cyclohexamide and the enzymes were added as described above. The flasks were then placed in an incubator at 50^{o} C with 180 rpm shaking for 48 hours with samples taken periodically for HPLC analysis.

Composition Analysis

Composition of solid biomass before and after pretreatment, as well as after enzymatic hydrolysis, was determined using a modified version of the NREL / TP 510-42618 two stage acid protocol for quantifying structural carbohydrates and lignin in biomass (B. H. A. Sluiter, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, and D. Crocker, 2010) with minor modifications as described by Li et al. (M. Li et al., 2012). Briefly, quantities of biomass, 72% sulfuric acid and water were reduced to 1/3 the amounts specified in the protocol to allow for the use of smaller volume pressure tubes for autoclaving. Thus 0.1 g of washed biomass was added to a pressure tube along with 1 mL of 72% acid and placed in a 30^{0}C water bath with periodic stirring for 1 h. Then 28 mL of water was added and the tubes were autoclaved at 120^{0}C for 1 hour. Contents of

the tube were filtered through Whatman 54 filter paper and the solids were dried in an oven at 105^0C and weighed to determine Klason lignin content. The liquid fractions were prepared for HPLC analysis and sugar concentrations were determined using an Aminex HPX-87H column from BioRad according to the NREL procedure.

The composition of the hydrolyzate following pretreatment and enzymatic hydrolysis was determined by using the NREL/TP-510-42623 protocol for the determination of sugars, byproducts, and degradation products in liquid fraction process samples (B. H. A. Sluiter, R. Ruiz, C. Scarlata, J. Sluiter, and D. Templeton, 2006). Briefly, the hydrolyzate was centrifuged at 4500 g for 15 min and the liquid decanted. Then 3 mL of liquid sample was added to a pressure tube along with 0.105 mL of 72% sulfuric acid; then the tubes were autoclaved at 120^0C for 1 hour. The liquid contents of the tube were prepared for HPLC analysis and sugar concentrations were determined using an Aminex HPX-87H column from BioRad according to the NREL procedure. The difference in sugar contents between the liquid composition samples and the enzymatic hydrolysis samples is the amount of solublized oligomeric carbohydrates in the hydrolyzate.

HPLC Analysis

Glucose and xylose concentrations were determined by HPLC using an Aminex HPX-87H column (#125-0140, Bio-Rad, Hercules, CA) operating at 65 ^{o}C, using a 0.05 M sulfuric acid mobile phase, at a flow rate of 0.6 mL/min, and detection by refractive index. This method can separate most sugars, ethanol, xylitol, and acetate. Manose and galactose comprised less than 2% of the total biomass and is counted as xylose because xylose, galactose, and manose co-elute on the HPX-87H column.

The concentrations of ferulic acid and p-coumaric acid in the pretreatment liquor after AHP pretreatment were determined by HPLC using a Discovery C18 column (126489-04, Supelco, Bellefonte, PA) operating at 40^{o}C using a step-gradient of 1% (v/v) acetic acid and methanol at a flow rate of 0.25 ml/min. The mobile phase started with 100% of the 1% (v/v) acetic acid in water and 0% methanol for 2min then step-wise increased the methanol concentration by 2.5% every minute up to 40% and held for 2 min. Then the column was flushed by increasing to 50% methanol for 3 min before being returned to 100% acetic acid. The elution times were 12.4 min and 14.2 min for p-coumaric acid and ferulic acid respectively.

Results and Discussion

Hydrogen Peroxide Effects

AHP pretreatment of corn stover and switchgrass was performed using four different H_2O_2 loadings, 0%, 6%, 12.5% and 25% (g H_2O_2/g biomass) to determine the effect of H_2O_2 on xylan and lignin removal/solubilization, enzymatic digestibility, and hydroxycinnamic acid release. Table 2 shows the compositions following delignification of glucan, xylan, and lignin for both types of biomass and their respective glucose yields. AHP pretreatment increased glucan content by as much as 15% for corn stover and 18% for switchgrass. Lignin content was reduced by as much as 15% for corn stover and 13% for switchgrass, which corresponds to 65% and 77% lignin removal respectively. Figure 5A shows the glucose and xylose yields after 7 days of hydrolysis for both corn stover and switchgrass as a function of H_2O_2 loading. Glucose yield increased from 26% to 85% for corn stover over the range of conditions used and 15% to 52% for switchgrass and there is a clear positive correlation between sugar yield and H_2O_2 loading. Sugar yields were higher for corn stover than switchgrass for both glucan and xylan, however, the improvement from the 0% H_2O_2 loading condition to the 25% H_2O_2 loading

condition is similar between the two materials. Therefore, under the conditions of this study, 25% H_2O_2 loading improved glucan digestibility by approximately 35% over alkali alone for both corn stover and switchgrass. However, corn stover improved almost 60% from untreated material compared to 40% improvement for switchgrass. Figure 5B shows the same sugar yields as a function of lignin removal, and again a near linear correlation is observed for both types of biomass for this range of conditions. However, Li et. al. found a sigmoidal relationship between lignin content and glucose yield for a range of different grass species (M. Y. Li, et al., 2012). This particular switchgrass is not particular well suited for AHP pretreatment evidenced by the relatively low glucose yield, 52%, at the most severe pretreatment condition. However, other types of switchgrass may perform better. For example, the Cave-In-Rock cultivar, which is used here, has been shown to exhibit lower in vitro ruminant digestibilities relative to improved upland cultivars such as Shawnee (Sanderson & Burns, 2010) or to lowland cultivars (Burns, Godshalk, & Timothy, 2008).

Table 2: Glucan, xylan and lignin contents of untreated and AHP pretreated corn stover and switchgrass along with glucose yields

	Corn Stover	AHP Delignified (g H_2O_2 / g biomass)			
	Untreated	0 g/g	0.06 g/g	.125 g/g	25 g/g
Glucan	33.2%± 0.7%	37.5%± 0.1%	37.0%± 1.0%	44.0%± 0.4%	48.5%± 0.4%
Xylan	21.5%± 0.1%	22.7%± 0.3%	23.5%± 0.4%	23.7%± 0.4%	25.2%± 0.4%
Klason Lignin	20.5%± 0.6%	19.0%± 0.4%	16.8%± 1.3%	12.9%± 1.6%	11.8%± 1.6%
Glucose Yield	26.4%± 0.7%	48.2%± 0.1%	51.0%± 0.2%	64.6%± 0.2%	84.7%± 0.9%
	Switchgrass	AHP Delignified (g H_2O_2 / g biomass)			
	Untreated	0 g/g	0.06 g/g	.125 g/g	25 g/g
Glucan	27.4%± 0.3%	33.7%± 0.1%	36.6%± 0.4%	37.5%± 0.5%	45.0%± 0.5%
Xylan	20.5%± 0.1%	25.2%± 0.1%	27.0%± 0.1%	28.0%± 0.3%	24.7%± 0.2%
Klason Lignin	21.9%± 0.3%	19.3%± 0.4%	18.2%± 1.2%	14.3%± 0.1%	8.9%± 0.4%
Glucose Yield	15.2%± 0.5%	20.1%± 0.1%	29.9%± 1.3%	47.8%± 0.2%	52.2%± 1.2%

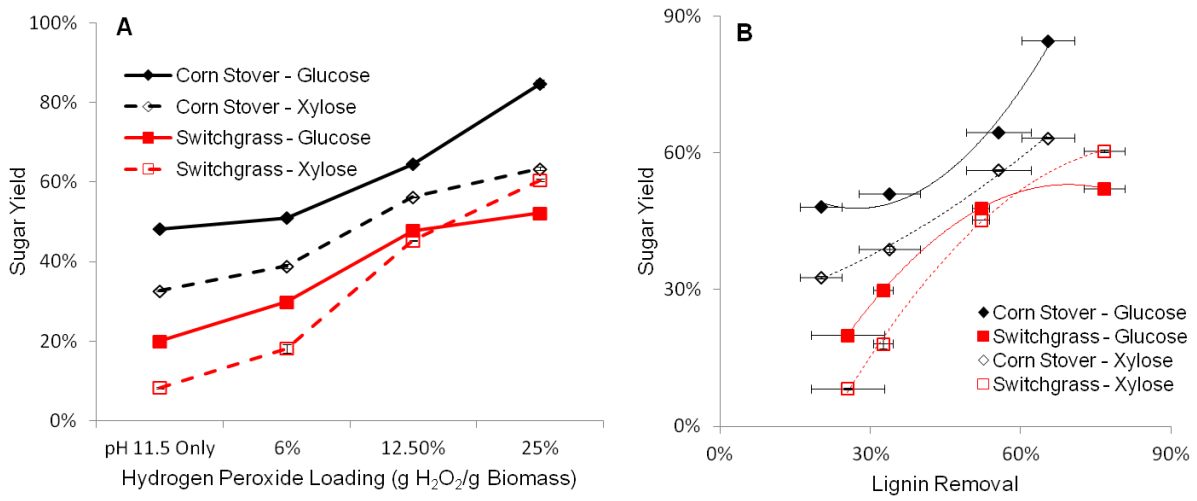

Figure 5: Glucose and xylose hydrolysis yields as a function of A) hydrogen peroxide loading and B) lignin removal

Ferulic acid and p-coumaric acid are known to be ester linked to carbohydrates and ester or ether linked to lignin and they are important for cell wall reticulation. (Iiyama, Lam, & Stone, 1990). Figure 6 shows the release of ferulic and p-coumaric acids as a function of H_2O_2 loading during AHP pretreatment for both corn stover and switchgrass. Again, near linear increases in acid release were observed as a function of hydrogen peroxide loading. Ferulic acid release expressed as a percentage of the original biomass ranged from 0% to 0.3% for corn stover and 0% to 0.2% for switchgrass, and p-coumaric acid ranged from 0.15% to 1% for corn stover and 0% to 0.7% for switchgrass. Larger amounts of ferulic acid were released with higher H_2O_2 concentrations and this may be due to cleavage of bonds more resistant to alkali alone or it may be that as more surface area is exposed larger quantities of easy-to-release ferulates are exposed (Hartley & Ford, 1989). It may also be that H_2O_2 oxidation of lignin allows for the release of ferulic acid that normally would not be fully releasable due to a mechanism of formation where ferulate radicals cross-couple with lignin radicals (Hatfield, Ralph, & Grabber, 1999).

Correlating ferulic acid release with enzymatic digestibility is difficult because of other impacts on cell wall properties that improve hydrolysis such as lignin and xylan solubilization and lignin hydrophobicity (Grabber, Hatfield, & Ralph, 2003; Grabber, Ralph, & Hatfield, 1998b). Howerever, the improvement of digestibility with increased release of phenolics has been known for some time (Grabber, Ralph, Lapierre, & Barriere, 2004; Hartley & Ford, 1989; Hatfield, et al., 1999), and ferulate cross-links between lignin are thought to inhibit enzymatic hydrolysis rate but not necessarily the extent of saccharification (Grabber, Ralph, & Hatfield, 1998a). Ferulic acid that is ether-linked to lignin is also ester-linked to cell wall carbohydrates (Iiyama & Lam, 2001), therefore, the more difficult-to-remove ferulic acid is an indication of more cross-linking which corresponds to increased recalcitrance (Jung, Samac, & Sarath, 2012). Uncondensed lignin containing only beta-O-4 bonds absorbs more flat on the cellulose surface, thus covering more surface area and occluding cellulose access to enzymes (Besombes & Mazeau, 2005). Further, Zhang et al. have suggested that p-coumaric acid esterification occurs primarily in the uncondensed lignin associated with syringyl lignin and corresponds to a decrease in cell wall digestibility (Zhang et al., 2011). Therefore, these results show that AHP pretreatment can remove more ferulates and p-coumarates than alkali alone, and this may be a useful method for quantifying these different types of cross-links.

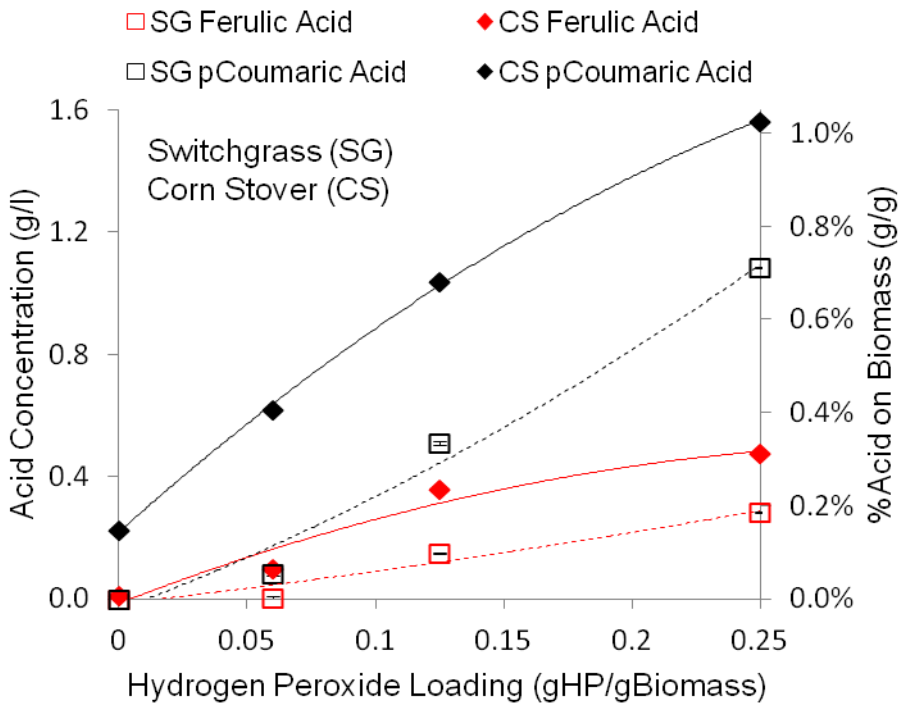

Figure 6: Ferulic and p-coumaric acid release (solubilization) as a function of hydrogen peroxide loading during AHP delignification

Figure 7A shows the relationship between lignin and xylan removal for corn stover and switchgrass for all the AHP pretreatment conditions used. Interestingly, there is a linear correlation, R^2=0.915, between xylan and lignin removal when combining data from both types of biomass instead of having different slopes of linearity between the different biomasses, which was common for other correlations between properties investigated. Figure 7B shows the correlation between lignin removal and ferulic acid release from both corn stover and switchgrass, and there is a linear correlation between these variables for both materials, however, the slopes are different. These linear relationshipshave been observed for other AHP pretreated grasses and is thought to be evidence that they are removed concurrently during pretreatment (M. Y. Li, et al., 2012). This is reasonable since ferulic acid and xylan are linked to lignin, it would

be expected that their removal would be concurrent. It is interesting that xylan and ferulic acid removal trends have different slopes for corn stover and switchgrass which would indicate that there is a difference in the amount of cross-linking between these two components, corn stover having more xylan-lignin cross-linking than switchgrass.

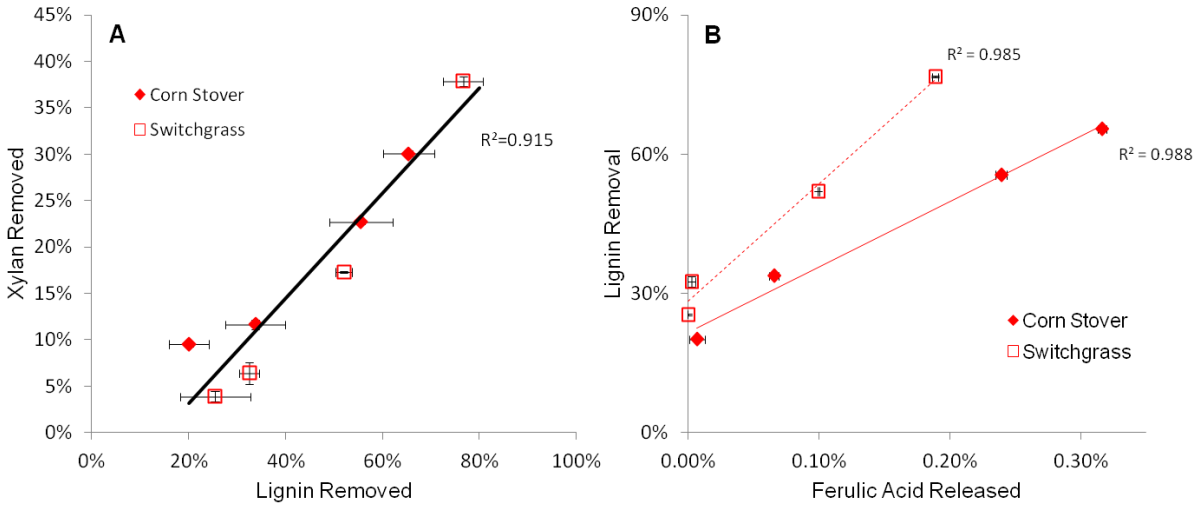

Figure 7: Correlation between A) xylan and lignin removal for corn stover and switchgrass and between B) lignin and ferulic acid removal

Figure 8 shows the fractions of monomeric xylose and solubilized oligomeric xylan released following AHP pretreatment and enzymatic hydrolysis of corn stover and switchgrass as a function of H_2O_2 loading. Oligomeric xylan comprised from 15% to 35% of the solubilized xylan for corn stover and 2% to 23% for switchgrass depending on the H_2O_2 loading. The presence of xylo-oligomers in the hydrolyzate is known to be strongly inhibitory to cellulose hydrolysis (Brienzo, Carvalho, & Milagres, 2010; Qing & Wyman, 2011). Unhydrolyzed xylan following AHP pretreatment and enzymatic hydrolysis may be due to substitutions such as ferulic acids, arabinans, and glucuronic acids on the xylan backbone that limit enzyme hydrolysis

beyond chains of a certain length by physically occluding the enzyme. Arabinose substitution on xylan has been shown to negatively impact digestibility (F. Li et al., 2012).

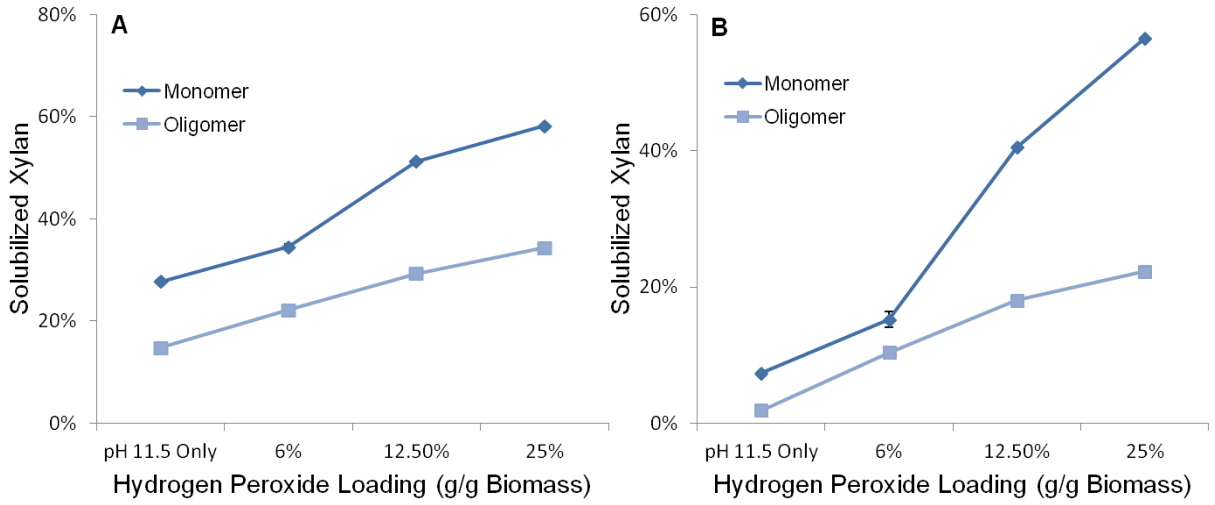

Figure 8: Fraction of solubilized xylan following AHP pretreatment and enzymatic hydrolysis that is monomeric and oligomeric as a function of H_2O_2 loading for A) corn stover and B) switchgrass

Figure 9 shows the kinetics of hydrogen peroxide consumption during AHP delignification for two different hydrogen peroxide loading conditions, 0.15 and 0.25 g/g. There is an initial drop in detectable H_2O_2 at time 0, likely due to the deprotonation of some fraction of the hydrogen peroxide at the relatively high pH. Much of the H_2O_2 is consumed, 40% and 60%, for the 15% and 25% g/g conditions respectively, within the first 3 hours, and the remaining being consumed within 24 hours. Gould et. al. also showed that ~20% of the oxygen in H_2O_2 is incorporated into kenaf for the AHP conditions they used at a solids concentration of 2% (w/v), and that improvement of incorporation, to ~40%, could be achieved simply by performing pretreatment at a higher solids concentration, 6% w/v, and further, that the initial rate of O_2 evolution was increased at this higher concentration (Gould, 1985). Observations in our lab have shown up to 40% oxygen incorporation in corn stover (data not shown).

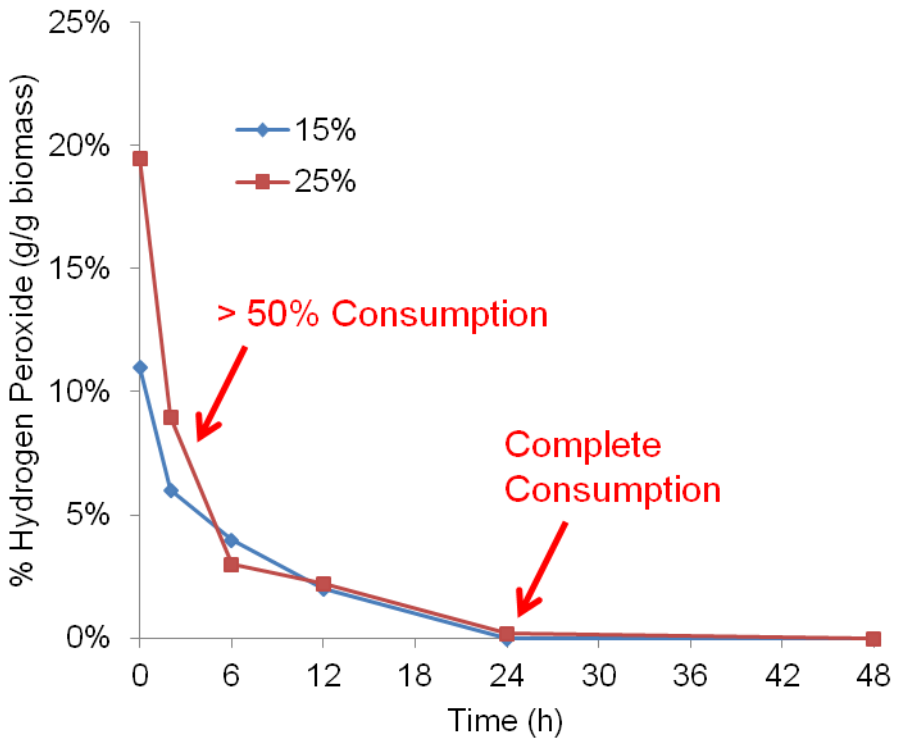

Figure 9: Hydrogen peroxide utilization with time for 0.12 and 0.25 g/g hydrogen peroxide loading

Figure 10 shows the sodium hydroxide consumption during AHP pretreatment of corn stover (A) and switchgrass (B) with and without hydrogen peroxide at 2% and 8% solids concentrations. For both materials saturation is approached where consumption no longer increases as alkali loading is increased. This is indicative of the limit in acid release from biomass. Also, when the solids loading is increased, the saturation level of consumed alkali also increases indicating that higher solids loading perhaps improves the effectiveness of pretreatment, although the reason for this is not clear. It could simply be that at higher solids concentrations with a constant alkali loading the liquid phase alkali concentration is higher and therefore the pretreatment is more effective at removing acid groups from the biomass.

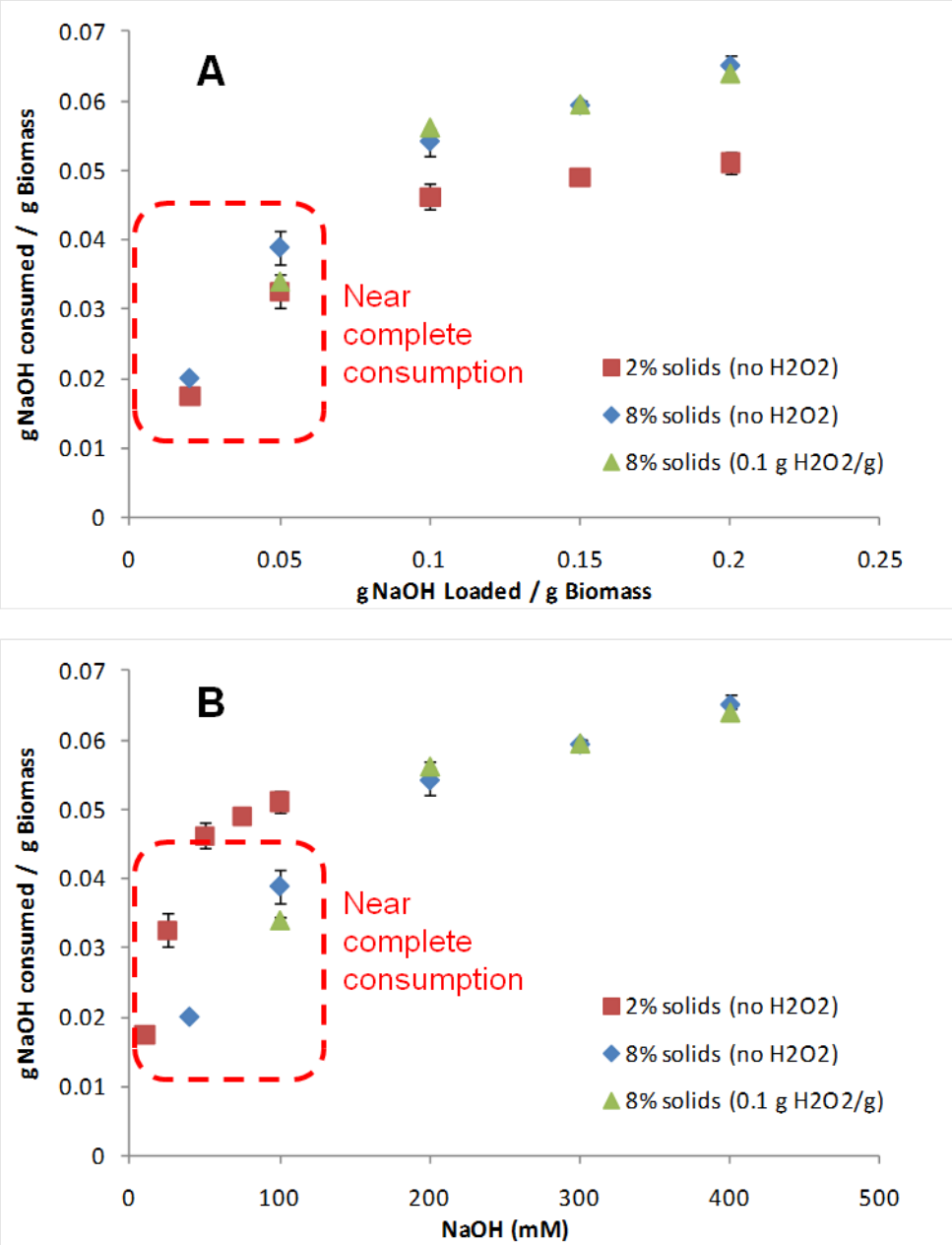

Figure 10: Sodium hydroxide consumption during AHP delignification for corn stover (A) and switchgrass (B) for 2% and 8% solids concentration

High Solids Scale-up

Much of the AHP pretreatment work that has been done in the literature was performed at the bench-scale on the order of several g of biomass pretreated at a time. Banerjee et. al. were among the first to report results of AHP pretreatment at a larger scale, 1 kg of corn stover, and

pretreatment was integrated with enzymatic hydrolysis and fermentation (Banerjee et al., 2012). A glucose yield of 75% was achieved in their work for an H_2O_2 loading of 0.125 g/g biomass, and a solids concentration starting at 13% (w/w), and further, no washing was performed after pretreatment indicating that the impact of inhibitors formed during pretreatment was relatively minimal. In continuation of that work, and to determine the effects of solids concentration at the1 kg scale on AHP pretreatment effectiveness, corn stover was pretreated as described above using an industrial bowl mixer at solids concentrations >15% w/v and enzymatically hydrolyzed using the same conditions for 48 h and then compared based on sugar yield. Figure 11 shows the 48 h yields of glucose (A) and xylose (B) for the different pretreatment times as a function of solids concentration. For 3 h of pretreatment, glucose yield increased with increasing solids concentration up to 45% then it decreased at 55%, and this pattern is less pronounced for 9 h and for 24 h of pretreatment. ANOVA, and Tukey's test analysis of the data indicate that there are statistical differences between yield values with some acceptions for the longer pretreatment times, where some values cannot be considered statistically different based on 95% confidence (additional statistical analysis of the data set is shown in the appendix of this chapter.) However, it can be said that higher solids concnetraitons reduces pretreatment times required to reach maximal levels of digestibility. It should be noted that the temperature of the pretreatment increased with solids concentration from 25 ^{o}C for the 15% condition to 85 ^{o}C for the 55% condition, likely due to the exothermic degradation of H_2O_2. This is likely a consequence of higher liquid phase concentration of H_2O_2 at higher solids concentrations and the increase in heat dissipation times at the larger scale. Saha et. al. examined the effect of temperature and pretreatment time on pretreatment effectiveness based on sugars released after enzyme hydrolysis and found that at 25 ^{o}C pretreatment improvement stopped after 6 h, but at 35 ^{o}C there

was not much improvement after 3 h of pretreatment for wheat straw at 9% solids (w/v) (Saha & Cotta, 2006). The same trend is observed for xylose yield as glucose shown in Figure 11B, however, the maximum achieved yield is at 25% solids and not 45% as the case for glucose yield. This may be due to an increased susceptibility of xylan to oxidative degradation in the liquid phase compared to glucan due to xylan solubilization during pretreatment and the presence of higher liquid concentrations of H_2O_2 at higher solids concentrations. The susceptibility of solubilized glucan may also be the reason for decreased yields for the 55% solids condition. Porro et. al. have shown that at a high enough NaOH concentration, around 8%, Na-cellulose starts to become soluble (Porro, Bedue, Chanzy, & Heux, 2007). Therefore, it is possible that any solubilized cellulose may be oxidatively degraded especially at the higher solids concentration conditions. However, the oxidative degradation of sugar was not directly determined for these conditions and is simply a hypothesis. It should also be noted that by running hydrolysis for longer times, conversion values may be improved by as much as 5-10%; the 2 day hydrolysis times were chosen for simplicity as a basis for comparison.

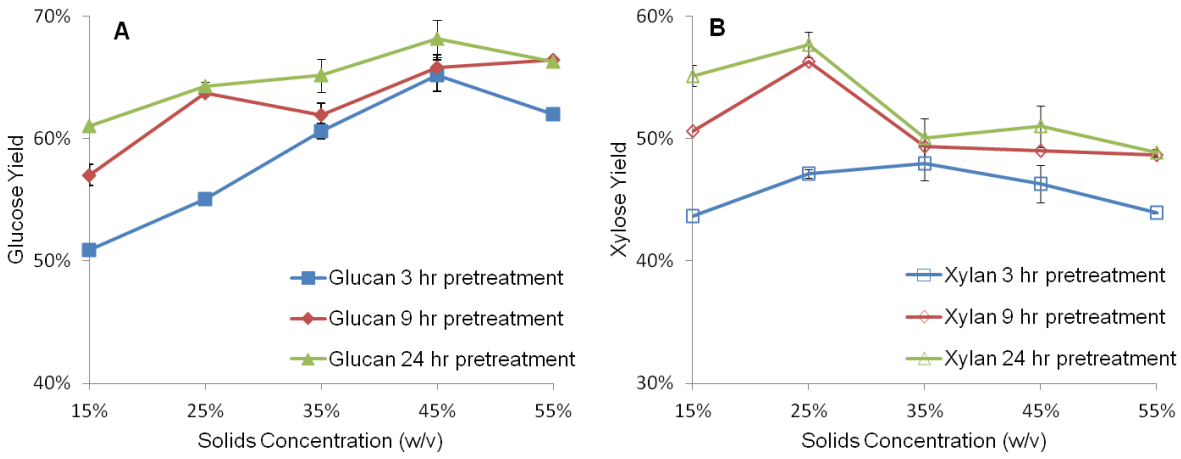

Figure 11: A) glucose yield and B) xylose yield of AHP pretreated corn stover as a function of solids loading and pretreatment time for the large scale pretreatment

Quantification of ferulic and p-coumaric acids in the high solids pretreatment samples was not performed during the scale-up experiments describe above. Therefore, small-scale high solids pretreatments of corn stover were performed at a low and high temperature mimicking the least and most severe temperatures experienced during the scale-up experiments. Figure 12 shows the concentrations of p-coumaric and ferulic acids as a function of pretreatment solids concentration for two different pretreatment temperatures, 25 ^{o}C and 85 ^{o}C. As the solids loading increases the liquid-phase concentrations of both acids saturates (A). However, this does not represent the maximum amount of these compounds in the biomass material as indicated in Figure 12B; as the solids concentration is increased, the amount of acids released per mass of biomass decreases indicating that these compounds are either degraded further or the solubility limit has been reached and they are simply precipitating out of solution. Which of these is responsible has not been determined. However, the higher pretreatment temperature increases both acid concentrations suggesting the increase is due to increased solubility at the higher temperature.

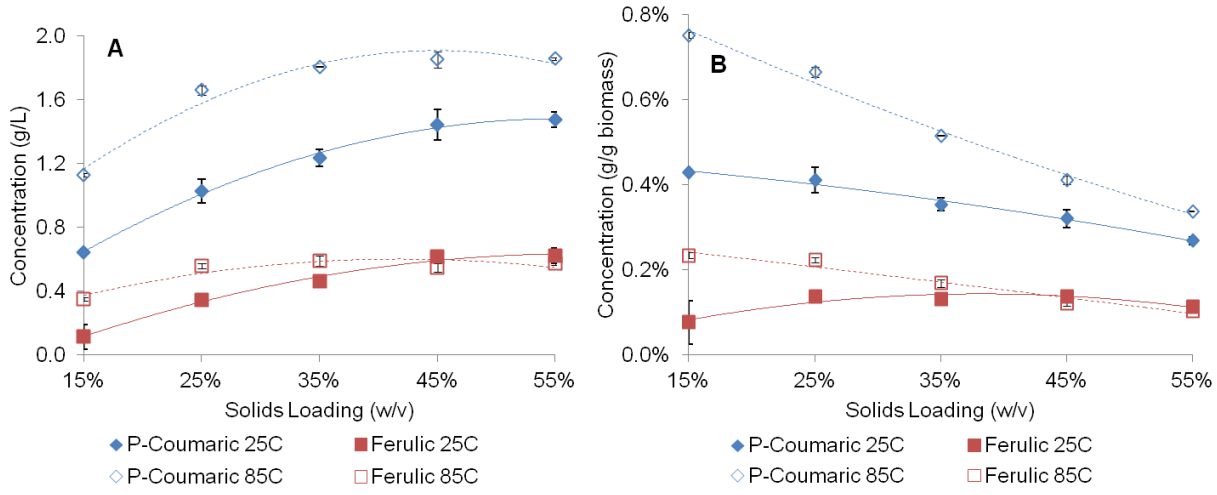

Figure 12: Cinnamic acid released expressed as A) g/L liquid phase concentration and B) g/g corn stover concentration as a function of solids concentration and temperature during small scale AHP pretreatment of corn stover

Conclusions

AHP pretreatment of corn stover and switchgrass improved enzymatic glucose and xylose yield ~30% over mild alkali alone for the conditions used in this work. Corn stover glucose yields reached as high as 85% while switchgrass was only 52%. These results indicate that the switchgrass used, Cave-in-Rock, is not well suited for AHP pretreatment, since typically sugar yields of 80% or higher are desired. In addition to H_2O_2 loading significantly impacting enzymatic digestibility, there was also noted impacts on lignin and xylan removal, and ferulic and p-coumaric acid release. Lignin removal ranged from 20-65% and 25-77% for corn stover and switchgrass respectively over the range of conditions used. And xylan removal ranged from 9-30% and 4-37% for corn stover and switchgrass respectively. Interestingly, lignin and xylan removal were compellingly linearly correlated suggesting they are removed concurrently, which has been suggested elsewhere (M. Y. Li, et al., 2012). Ferulic and p-coumaric acids were released in increasing amounts with increasing H_2O_2 loadings suggesting that alkali alone is not

sufficient to remove all of these compounds from biomass. Oligomeric xylan accounted for a notable amount of solubilized xylan during AHP pretreatment, as high as 35% for corn stover and 23% for switchgrass, which is important because xylo-oligomers are known to be inhibitive to cellulases. AHP pretreatment of corn stover was performed at the 1 kg scale at solids concentrations higher than have been reported before, up to 55% w/v, with positive results. Glucose yields near 70% could be achieved in just two days of hydrolysis, and higher yields could be achieved for longer hydrolysis times. It was found that the temperature of slurries increased significantly with solids concentration and that sugar degradation, particularly xylan, may be a consequence of performing pretreatment at these high solids conditions. Ferulic and p-coumaric acid concentrations increase as well with increasing solids concentrations, however, some of the acid may precipitate out of solution as the solubility limit is reached for these compounds. These results demonstrate that AHP pretreatment may be well suited for some grasses, but perhaps not all, and that performing pretreatment at higher solids concentrations is possible with good results compared to lower solids concentrations at small scale. However, H_2O_2 is expensive and for this process to become more feasible, peroxide addition will need to be reduced, which could be achieved if it can be used at higher efficiency.

Acknowledgments

I would like to thank Alex Smith for performing the work on NaOH and H_2O_2 consumption presented in this chapter.

APPENDIX

Appendix: Statistical Analysis – Scale-up AHP pretreatment of corn stover

Statistical analysis includes the response variables glucose and xylose yield following enzymatic hydrolysis and the factors solids concentration, pretreatment time and hydrolysis time with 5, 5, and 6 levels respectively. Table 3 shows the factors and level values. ANOVA analysis on all the data was performed to determine differences in values of the response values. Table 4 below shows the ANOVA tables for glucose and xylose yield. The p values are all 0.000 indicating that the null hypothesis (all mean values are the same) should be rejected, which indicates that mean values are not the same within each factor. This is expected since each factor has been shown to impact the response variables. Therefore, an interaction plot was generated for each response variable, and Figures 13 and 14 shows these interaction plots for glucose and xylose yield for all three factors and their respective levels.

The interaction between pretreatment time and hydrolysis time for glucose yield in Figure 13 (bottom right graph) shows that increasing pretreatment time increases the glucose yield at all six different hydrolysis times. The same result is observed for xylose yield in Figure 14 where the increase is more evident. The interaction between solids concentration and hydrolysis time (upper right graph) shows that increasing solids loading increases yield for some of the solids concentrations. In particular the higher solids loadings at later hydrolysis times produced the highest glucose yields, however, 25% and 35% solids concentration produced the highest xylose yields across all hydrolysis times. This indicates that increasing solids concentration improves glucose yield but decreases xylose yield likely due to oxidative degradation. The solids concentration and pretreatment time interaction (leftmost graph) indicates again that solids concentration improves glucose yield but too high a concentration will decrease xylose yield.

Table 3: list of factors and levels for design of experiments statistical analysis

Factors	Levels
Solids Concentration (% w/v)	15%, 25%, 35%, 45%, 55%
Pretreatment Time (h)	3, 6, 9, 12, 24
Hydrolysis Time (h)	2, 4, 6, 8, 24, 48

Table 4: ANOVA tables for glucose (top) and xylose (bottom) yield including all three factors

Anova Table for Glucose Yield

Source	DF	Seq SS	Adj SS	Adj MS	F	P
%Solids	4	0.10277	0.10277	0.02567	34.25	0.000
Pretreat	4	0.06789	0.06789	0.01697	22.63	0.000
Hydrol	5	4.34762	4.34762	0.86952	1159.14	0.000
Error	286	0.21454	0.21454	0.00075		
Total	299	4.73282				

Anova Table for Xylose Yield

Source	DF	Seq SS	Adj SS	Adj MS	F	P
%Solids	4	0.35698	0.35698	0.08925	190.39	0.000
Pretreat	4	0.16829	0.16829	0.04209	89.76	0.000
Hydrol	5	0.9798	0.9798	0.19596	418.04	0.000
Error	286	0.13406	0.13406	0.00049		
Total	299	1.63914				

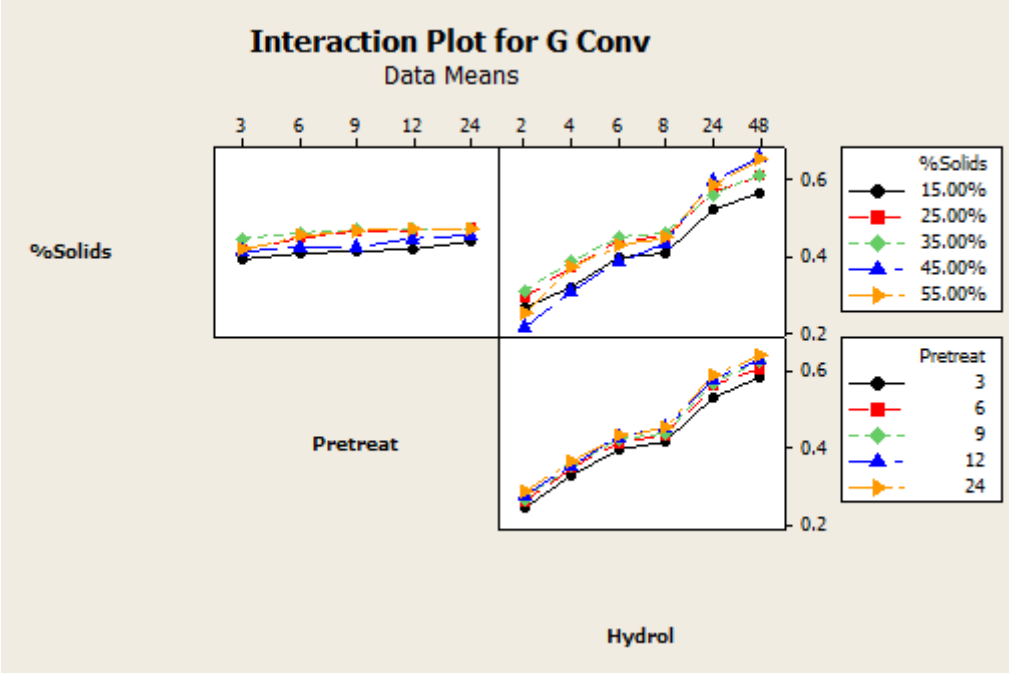

Figure 13: Interaction plot for glucose yield

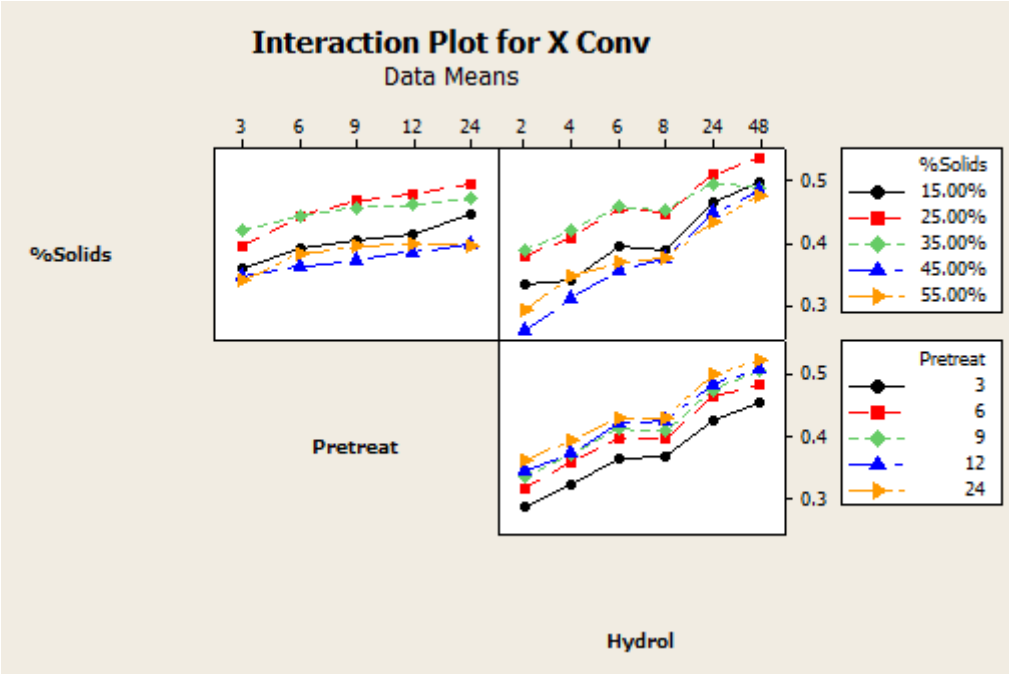

Figure 14: Interaction plot for xylose yield

The Tukey Method for pairwise comparisons within each factor at each factor level was used to determine which conditions yielded statistically significant differences in glucose and xylose yield values. Table 5 shows the summary results of the Tukey Test assigning alphabetic labels to levels that have statistically different means. These results indicate that only 25% and 35% solids have similar means for glucose and xylose yields, and 45% and 55% solids for xylose yields are statistically similar. Glucose and xylose yields are statistically different for the short pretreatment times of 3 and 6 h and then there is some overlap for the later times of 9, 12 and 24 h. Hydrolysis time, as expected, yielded statistically different means for all times investigated. However, these results include all three factors and levels, and may give different results if only one hydrolysis time was used. To test this, a pairwise t-test was performed on only the 48 h hydrolysis time for glucose yield. Table 6 shows the resulting groupings, and it is apparent that overlapping groupings increases with pretreatment time, which indicates that there are less statistical differences between glucose yield values at different solids loading as pretreatment time increases. This is indicative of the fact that pretreatment has reached maximal effectiveness at longer pretreatment times.

Table 5: Summary of groupings using Tukey's Test for all three factors and their levels based on 95% confidence interval

%Solids	Glucose Grouping	Xylose Grouping	Pretreat	Glucose Grouping	Xylose Grouping	Hydrol	Glucose Grouping	Xylose Grouping
0.15	C	B	3	D	D	2	F	E
0.25	A	A	6	C	C	4	E	D
0.35	A	A	9	BC	B	6	D	C
0.45	B	C	12	AB	B	8	C	C
0.55	A	C	24	A	A	24	B	B
						48	A	A

Table 6: Summary of groupings for glucose yield using a pairwise t-test for 48 h hydrolysis time and select pretreatment times: 3, 9, 24 hours

Solids	3 hours	9 hours	24 hours
15	A	A	A
25	B	B	A B
35	C	B C	A B C
45	D	B D	B D
55	C	D	C D

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Chapter 2 – Bench scale combined diffuser type extraction and alkali pretreatment of sweet sorghum sugars and bagasse

Introduction

With the projected increase in demand for liquid fuels, predominantly used for transportation, the production of ethanol has increased significantly worldwide in an effort to meet demands while decreasing reliance on petroleum (Babcock, 2012). Most ethanol produced utilizes a sugar platform wherein carbohydrates are extracted from sugar or starch rich crops, such as sugar cane and corn, which are then fermented to make ethanol. Another potentially valuable feedstock, particularly in North America where sugar cane does not grow very well, is sorghum, specifically sweet sorghums which have huge breeding potential and, like sugar cane, have high sugar contents that are readily fermentable to ethanol (G. Eggleston, Cole, & Andrzejewski, 2013). Furthermore, sweet sorghums require a third less water and less fertilizer than corn making them even more attractive as a bioenergy feedstock (G. Eggleston, et al., 2013). Life-cycle analysis has shown that sorghum-based ethanol can have a significant reduction on green house gass (GHG) emissions and reduced fossil energy consumption, as much as 72% and 84% respectively compared to petroleum (Cai, Dunn, Wang, Han, & Wang, 2013). The downside is that sweet sorghums can lose their sucrose content quickly, often within hours of being cut (G. Eggleston, et al., 2013) which would require that stalks be processed on site and sugars be stabilized or perhaps fermented at a local location before being transported to a centralized biorefinery for further processing. In addition to the non-structural carbohydrates such as glucose, sucrose, fructose and starch which comprise around 40% or more of the total carbohydrate content of sweet sorghums and are relatively easy to extract, as much as 50-60% of the dry plant material is structural carbohydrates such as cellulose and hemicelluloses (Rooney,

Blumenthal, Bean, & Mullet, 2007). However, there is a lack of processing technologies that are geared toward the utilization of both the non-structural and structural carbohydrates that would make using this crop commercially attractive (G. Eggleston, et al., 2013).

Sweet sorghum can be processed similar to sugar cane (Webster, Hoare, Sutherland, & Keating, 2004; Woods, 2000). In fact, a study on sweet sorghum harvesting, milling and extraction using a sugar cane mill was done in Australia and it was found that sweet sorghum can be harvested to the same bulk density as cane and that cane mills can achieve Brix extraction upward of 75%, which is a typical maximum purity for sorghum (G. Eggleston, et al., 2013), compared to 88% for sugar cane (Webster, et al., 2004). Therefore, existing technologies for sugar cane can be used, albeit modified, for processing of sweet sorghum to obtain relatively high Brix extractions, however; bagasse carbohydrates are left unused or are burned and used for plant process energy. Cane milling and sugar extraction is typically done in one of a few different ways: tandem roller-mills, screw press extraction, or diffuser extraction. Tandem roller mills squeeze juice from the stalk, however, at low effeciencies (~85%) (Gnansounou, Dauriat, & Wyman, 2005). Screw press extraction can squeeze juice from chipped or ground stalks but efficiency is also low, ~65%, and the feed rate is too slow for large-scale use (Weitzel, Cundiff, & Vaughan, 1989). Diffuser extraction of shredded stalks is the most common technique and has efficiencies as high as 98% for cane, although impurity content is higher too (Rein, 1995). Disadvantages of diffusion are higher levels of sand in the baggase, and longer equipment start up and shut down procedures due to larger cane holding capacity within the equipment. However, the capital cost ratios of 1:1.5 or higher have been estimated for diffusion to milling plant style sugar extraction (Rein, 1995). Diffuser extraction technology could also be used to facilitate utilization of carbohydrate polymers such as starch or cellulose. It may be possible to

add amylases during the diffusion extraction to facilitate starch hydrolysis to fermentable sugars (G. Eggleston, et al., 2013), or alkali to simultaneously perform extraction and pretreatment. This would yield extraction juice with higher impurity content, but if the goal is to make a fermentable sugar stream for biofuels, that may not be an issue.

Cane sugar streams typically require some stabilization, clarification and evaporation to syrup if the juice is to be stored or transported to a separate facility for fermentation than the milling/extraction site (Eggleston, et al., 2013). The stability of sweet sorghum juices and syrups has not been investigated as extensively as those from cane, and there is a lot of opportunity for research in this area especially for utilization to make biofuels where the requirements for stability may not be as stringent. Furthermore, if lignocellulosic sugars are to be used in addition to the extractable ones, there are other issues that would need to be addressed. Challenges in performing fermentation of lignocelluosic hydrolyzates include hydrolyzate toxicity, the desire to co-ferment glucose and xylose, the desire to perform pretreatment and hydrolysis at high solids concentrations to yield high ethanol titers, and the need the achieve high overall yield cost effectively. Hydrolyzate toxicity to fermentation depends on the pretreatment technology employed and even the feedstock used where inhibitors include organic acids, phenolics, furans and inorganics (Luo, Brink, & Blanch, 2002; Palmqvist & Hahn-Hagerdal, 2000). For example, high temperature acid pretreatments can degrade xylose to furfural and formic acid, while mannose, galactose and glucose are known to degrade to hydroxymethylfurfural and levulinic acid (Palmqvist & Hahn-Hagerdal, 2000). Other potential inhibitors include the hydroxycinnamic acids ferulic and p-coumaric acid. Ferulic acid is ester-linked between xylan and lignin and p-coumaric acid is acetylated to the side chains of lignin (Harris & Stone, 2008). Alkali and acid pretreatments are known to cleave these bonds releasing the acids (shown in

chapter 1). Combining extracted juice or syrup from sweet sorghum with lignocellulosic hydrolyzate has the advantage of diluting inhibitor concentrations in the hydrolyzate with the juice, and as long as sugar concentrations are comparable in the two streams, high titers of ethanol would still be possible.

In this chapter, a baseline of liquid hot water (LHW) and alkali pretreatment impacts on sorghum bagasse will be established based on composition changes and enzymatic digestibility improvement. Then a combined sugar extraction and alkali pretreatment technique will be performed on sweet sorghum integrated with enzyme hydrolysis and fermentation as a proof of concept for a potential process scheme utilizing both the extractable and structural carbohydrates in sorghum for biofuel production.

Methods and Materials

Biomass

A sweet sorghum bioenergy hybrid (TX08001) was obtained from John Gill at Texas A&M, bred to have high biomass yield and not necessarily high extractable sugar content. Sorghum was milled to pass a 5 mm screen and dried to a moisture content of ~8%. Water extractives and sugar content as well as structural carbohydrate and lignin content of the sorghum and bagasse were determined using the NREL analytical protocols NREL/TP-510-42619 and NREL/TP-510-42618 respectively, the later performed with modification as described previously (Li et al., 2012). Bagasse was prepared by washing 15 g of sorghum at a time with 600 mL of 80 ^{o}C water using a Buchner funnel with a 200 mesh porous base. The bagasse was then allowed to air dry before further use in composition analysis and batch pretreatment and hydrolysis experiments.

LHW and Alkali Pretreatment

Air dried bagasse was pretreated using two conditions of LHW and alkali pretreatments. LHW pretreatment was performed at 15% solids (w/v) by adding 6 g of bagasse and 40 mL of water into an Ace Glass pressure tube (8648-162) and sealed with the thred-cap. Tubes were then placed in an autoclave for 1 h at 120 ^{o}C for the first condition, and a 5 L M/K Systems digester (M/K Systems, Inc., Peabody, MA) was used to heat tubes to 160 ^{o}C for 1 h for the second condition. The pretreated bagasse was then diluted to 10% solids (w/v) and placed in flasks in preparation for hydrolysis. Alkali pretreatment was performed in shake flasks at 15% solids (w/v) and NaOH loadings of 0.1 and 0.06 g NaOH/g bagasse. The flasks were incubated at 80 ^{o}C for 1 h in a water bath, and then diluted to 10% (w/w) and the pH was adjusted to 5.5 using concentrated sulfuric acid in preparation for enzymatic hydrolysis.

Sugar Extraction and Alkali Pretreatment

Sugar extraction was performed using a series of five Buchner funnels and filter flasks; the Buchner funnels had a 3.8 cm diameter, 200 mesh stainless steel porous base. 5 g of fresh sorghum was introduced at one end of the series of funnels, designated stage #5, and moved sequentially to the right during the process, and 25 mL of fresh water was introduced at the other end, designated stage #1 and moved sequentially to the left during the process (see Figure 17 for schematic of process, and a picture of the apparatus and process is shown in the appendix to this chapter as Figure 21). This work will be investigating scheme 2 illustrated in Figure 17. Therefore, at stage#2 0.51 mL of 5 M NaOH solution is added to the extraction water from stage #1 and the slurry of biomass, water and NaOH was incubated at 80 ^{o}C for 60 min in a water bath before continuing to the next stage. Extraction juice after filtering at stage #5 was filter sterilized using a 0.22 µm stericup and stored at 4 ^{o}C until fermentation was performed. The extracted

sugar at each stage was determined using the HPLC protocol described in the NREL/TP-510-42618 method. The bagasse exiting stage #1 was immediately prepared for enzymatic hydrolysis.

Enzymatic Hydrolysis

For the batch LHW and alkali pretreated bagasse samples, pretreated material was diluted to 10% (w/w) solids and the pH adjusted to 5.5 as needed. Then hydrolysis samples were prepared as follow: citrate buffer, tetracycline and cyclohexamide were added to 50 mM and 10 mg/L respectively, and CTEC2 and HTEC2 were added in a protein ratio of 2:1 for an enzyme loading of 15 mg enzyme/g glucan. Samples were then incubated at 50 ^{o}C for 7 days. For the combined sugar extraction and pretreatment process, the bagasse leaving stage #1 was diluted to ~18% solids (w/w) and the pH adjusted to 5.5 using concentrated sulfuric acid. Citrate buffer, antibiotics and enzymes were then added and samples were incubated as described above; hydrolysate was then filter sterilized using a 0.22 µm stericup and stored at 4^{o}C until fermentation was performed. Sugar concentrations in the hydrolysates were determined by HPLC using the method described in the NREL / TP 510-42618 protocol and converted to glucose and xylose yields based on the glucan and xylan contents of the untreated bagasse.

Fermentation

Fermentation of a combination of the extracted juice and hydrolysate from the scheme 2 extraction and alkali pretreatment process was performed using *S. cerevisiae* GLBRC Y73 yeast strain. Juice and hydrolysate were mixed in a 60:40 v/v ratio (which is same ratio of liquid exiting both stages of the process) and yeast nitrogen base (YNB) and urea were added to the mixture to yield concentrations of 1.67 and 2.27 g/L respectively to serve as nutrients. The yeast seed culture was prepared by inoculating 50 mL of YNB medium (10 g/L yeast extract, 20 g/L

peptone and glucose) with the glycerol stock of Y73, and then incubating for 24 h at 30 ^{o}C with 150 rpm shaking. After 24 h, 10 mL of culture was transferred aseptically to 60 mL of the juice/hydrolysate mixture in a shake flask, in duplicate. The flasks were covered with fermentation locks, sparged with nitrogen, and incubated at 30 ^{o}C with 150 rpm shaking for 7 days. Samples were collected every 24 hours to determine OD600 and sugar and ethanol concentrations using HPLC as described above.

Results and Discussion

Figure 15 shows the sugar composition of the sweet sorghum TX08001, including the water extractable and the structural carbohydrates. This sorghum is a bioenergy hybrid designed to have high biomass yield and not extractable sugar, therefore, only 44% extractives on a dry weight basis is reasonable. The goal if this work is to utilize as much of the structural glucan and xylan as possible for fermentation to ethanol along with the extractable sucrose, glucose and fructose, which together accounts for 57% of the dry material. To determine a baseline of how digestible the bagasse is to enzymes, LHW and alkali pretreatment were performed on the bagasse after washing out the extractives as described above. Table 7 shows the impact of the two LHW and alkali pretreatment conditions on solids removal during pretreatment and the composition of glucan, xylan and lignin after pretreatment. Glucan contents are similar for the alkali and the higher temperature LHW pretreatment conditions. Also, lignin content is noticeably higher for LHW than alkali, which is expected since alkali pretreatment is known to solubilize some lignin, while LHW does not, but does redistribute it on the biomass surface. Figure 16 shows the glucan and xylan yields of enzymatic hydrolysis of the four pretreatments and untreated bagasse. Digestibility improvement from the untreated condition is largest for the more severe alkali condition for glucan (87%) and the more severe LHW condition for xylan

(40%). Regardless of pretreatment type or condition, glucan digestibilities were improved to near 70% or higher over the 50% yield of untreated bagasse, and this is without attempting to optimize the pretreatment conditions or the enzyme mixture for hydrolysis. Therefore, this material may still have much potential for improvement.

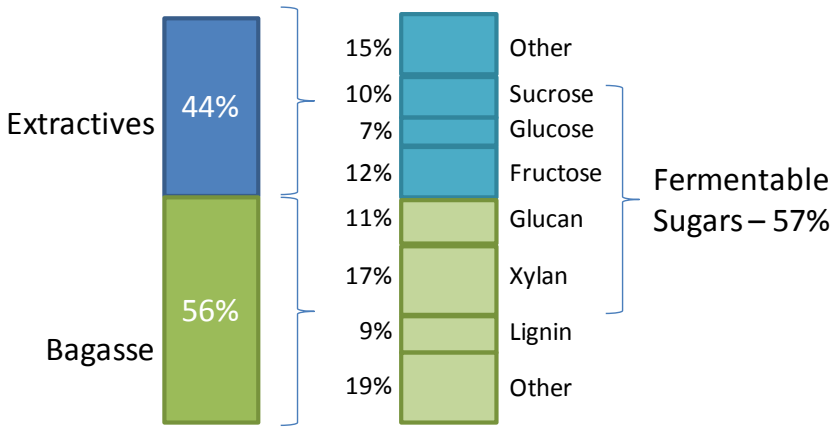

Figure 15: Composition of carbohydrates, both soluble and structural, in the sweet sorghum

Table 7: Impact of LHW and alkali pretreatments on solubilized material during pretreatment (removed) and glucan, xylan and lignin content of residual solids after pretreatment

Pretreat Condition	Removed	Glucan	Xylan	Lignin
Alkali 0.1 g/g NaOH	29.5%	61.3%	42.1%	15.3%
Alkali 0.06 g/g NaOH	22.5%	63.9%	36.5%	15.6%
LHW 160 C for 1 h	36.7%	64.5%	37.1%	26.3%
LHW 120 C for 1 h	24.0%	55.5%	41.7%	23.0%

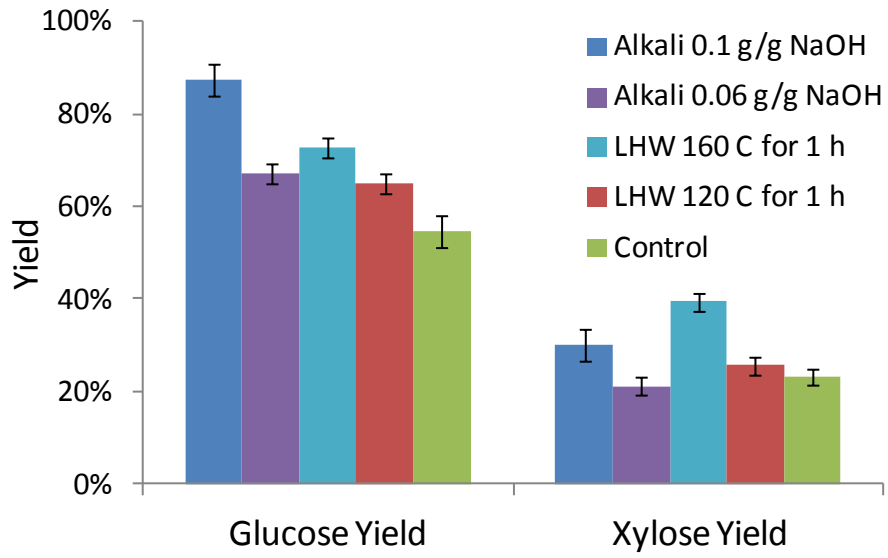

Figure 16: Glucose and xylose yields for LHW pretreated, alkali pretreated and untreated bagasse

Figure 17 illustrates a few different process integration schemes for generating sugar streams of different compositions depending on the source. Scheme 1 is similar to the batch process performed above where sugar extraction is done, typically a diffuser type process, followed by separate pretreatment on the bagasse, which depending on the pretreatment technology, could generate a stream of solubilized xylan or lignin for example with alkali pretreatment. The pretreated solids could then be hydrolyzed to make a stream of glucose and xylose from structural carbohydrates leaving a solid residue relatively high in lignin content and unhydrolyzed carbohydrates that could be burned for energy. In scheme 2, a combined sugar extraction with alkali pretreatment would be done by adding alkali to the wash water. Combined alkali extraction and pretreatment may promote a more stable juice that is not susceptible to infection from *Leuconostoc mesenteroides* which is the major contributor to microbial deterioration of sugar crops such as sugar cane and sugar beets (Eggleston, Monge, & Ogier, 2003). The juice stream would not be as clean, meaning it would contain Na+, lignin and maybe some xylan, which would not be desirable in conventional sugar extraction where the sugar is

used to make high purity products like crystallized sugar or syrup, but for ethanol fermentation that would not be an issue, and lower purity juices may be sufficient. Other types of pretreatment could be used in this scheme including enzyme addition, hemicellulases or other accessory enzymes, or amylases to hydrolyze starch could be added during washing to make the bagasse more amenable to the cellulolytic hydrolysis process following. In scheme 3, pretreatment is combined with extraction again, but the pretreatment liquid is separated before reaching the less extracted sorghum upstream thus providing a separate sugar stream that leaves the juice free of impurities the pretreatment would generate.

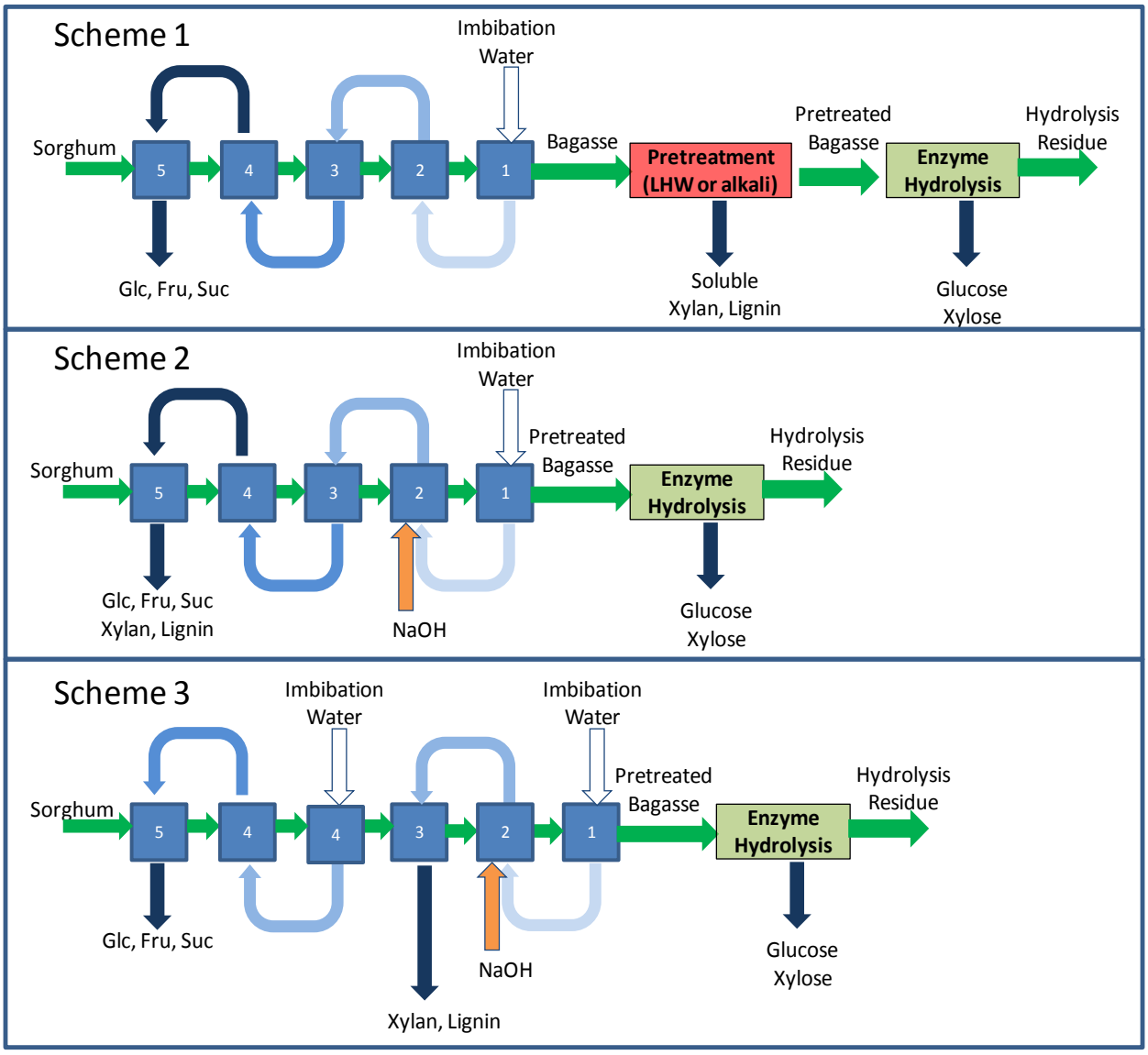

Figure 17: potential process schemes for integrating sugar extraction and bagasse pretreatment and hydrolysis of sorghum to generate various sugar streams that can be utilized for fuel production, or other sugar-based products

As a proof of concept, scheme 2 using 0.06 g NaOH/g bagasse alkali pretreatment during sugar extraction followed by enzymatic hydrolysis and fermentation of the combined juice and hydrolysate was performed as described above. Figure 18 shows a non-exhaustive mass balance flow diagram of the whole process indicating sugar concentrations in the juice and hydrolysate and the amounts of water and solid residue exiting each operation. The juice and hydrolysate

were combined at a liquid ratio of 60:40, which does dilute the sucrose and fructose concentrations in the juice and the glucose and xylose concentrations in the hydrolysate where they are higher. Figure 19 shows the sugar profile for the extraction stages and the pH value at each stage. The process does reach near 100% washing efficiency as the sugar concentration at stage 5 is close to 0. The sugar concentrations at stage #5 are maximal for the solids concentration used for washing. However, the particle size of sorghum during extraction is important and does impact the yield of sugar extraction (Jia, Chawhuaymak, Riley, Zimmt, & Ogden, 2013).Water extraction which recycles the water, has been shown to remove sugars more efficiently than the press method (Jia, et al., 2013). The leaves and pith can absorb juice lowering the extraction yield (G. Eggleston, et al., 2013; Whitfield, Chinn, & Veal, 2012). It is also interesting to note that the pH drops from 12.5 at the start of the pretreatment at stage #2 to 5.2 at stage #5. This is fortuitous because the desired pH for fermentation is 5.5, which means no pH adjustment of the juice is required after extraction. Figure 20A shows the fermentation results. All of the sucrose, glucose and fructose are used in the first 16 hours, indicating that inhibition of the juice/hydrolysate is minimal, and ~80% of the xylose was utilized after 7 days. An ethanol concentration of 21 g/L was obtained which gave a yield of 85% which is comparable to other results using this same Y73 strain (Liu et al., 2014). Achieving higher sugar concentrations during extraction was not possible using the apparatus setup developed here due to limitations in dewatering. Therefore, to determine how fermentable higher concentratrions of the mixed sugar streams may be, rotary evaporation was used to concentrate the sugar mixture, and then fermentation was performed as before. The results are shown in Figure 20B. An approximate 6-fold increase in sugar concentration was achieved by rotary evaporation to a total sugar concentration of 332 g/L. Yeast growth was notably slower than the more dilute condition where

3 days was required before OD 600 absorbance reached saturation. Sugar consumption was also significantly slowed where approximately 15-20 g/L of sucrose, fructose and glucose were still remaining after 5 days of fermentation, compared to complete consumption of these sugars in less than 18 hours for the more dilute condition. However, an ethanol concentration of 80 g/L was achieved representing an ethanol yield of 46%. Acetic and lactic acid are known to be inhibitive to fermentation, and acetic acid more so than lactic acid (Graves, Narendranath, Dawson, & Power, 2006), however, acetic acid was not detected in the concentration sugar mixture, likely due to it evaporating during concentrating with rotary evaporation.

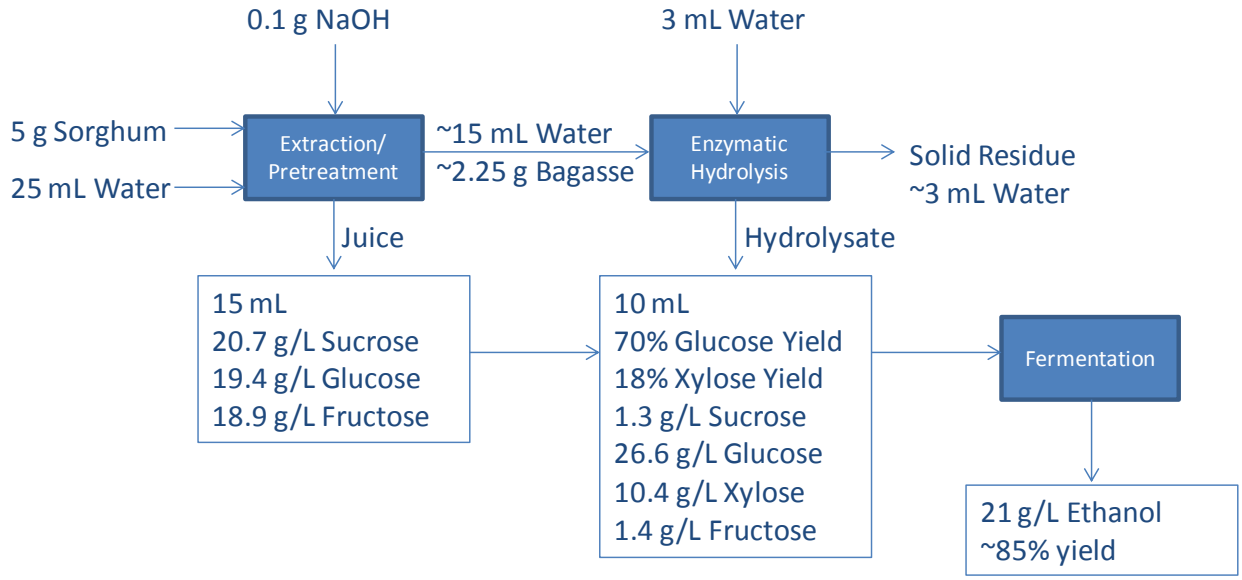

Figure 18: selected mass balance and sugar concentrations in juice and hydrolyzate for the combined sugar extraction/pretreatment process integrated with enzyme hydrolysis and fermentation

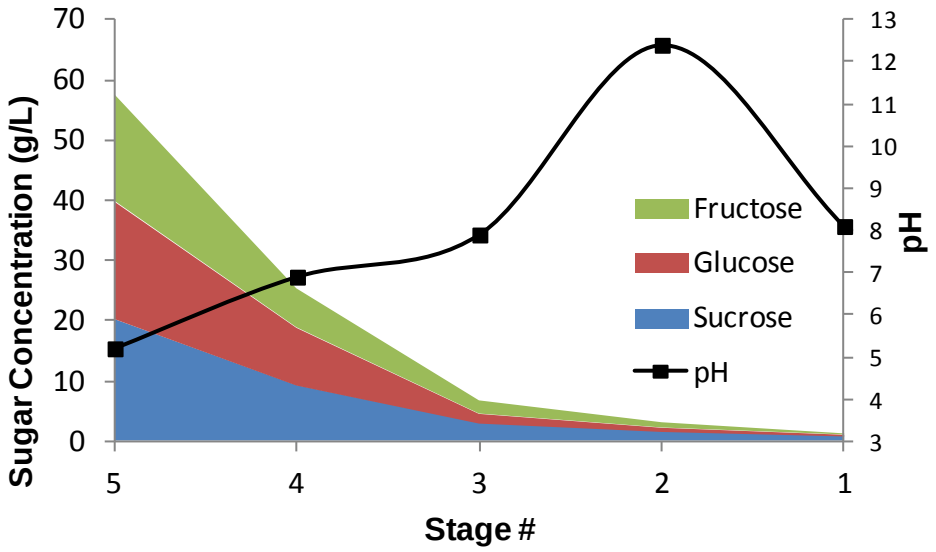

Figure 19: sugar profile during extraction for each stage and pH change during the process

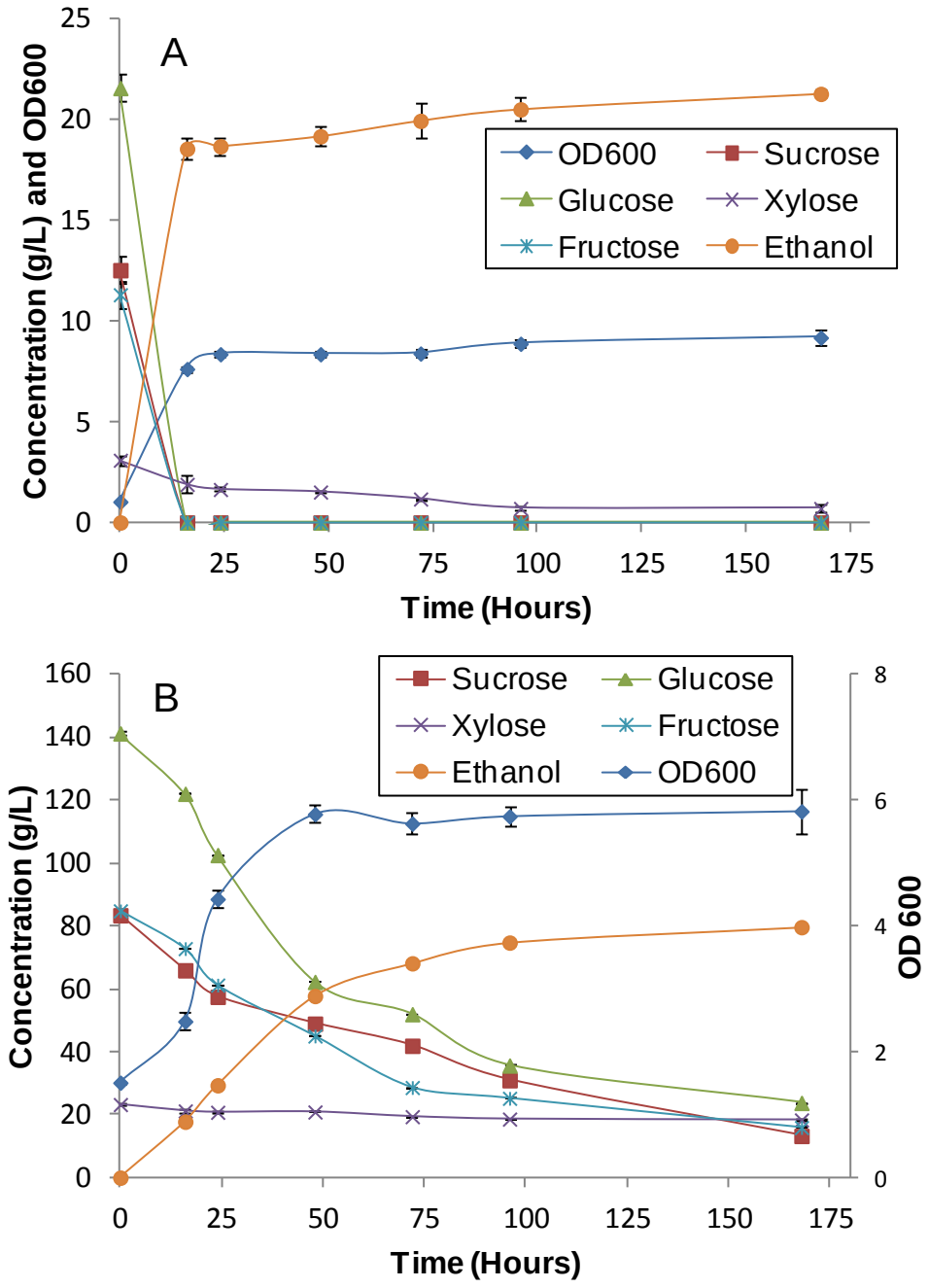

Figure 20: sugar utilization during fermentation of juice/hydrolysate combination using *S. cerevisiae* GLBRC Y73 strain on A) combined juice and hydrolyzate and B) 6-fold increase in concentration by rotary evaporation

Conclusions

The digestibility of a sweet sorghum bagasse was determined for a select set of conditions using LHW and alkali pretreatment. Glucose yield improvements ranged from 15% to 35% depending on the pretreatment and condition compared to untreated bagasse. A maximum yield of 87% was achieved for alkali pretreatment using an NaOH loading of 0.1 g NaOH/g bagasse. Xylose yields, however, were considerably lower, ranging from 20% to 40% for the conditions used. Yield improvement from those observed here could be achieved with pretreatment condition and enzyme optimization specific to this bagasse material. A combined sugar extraction and alkali pretreatment integrated with enzyme hydrolysis and fermentation of the sweet sorghum was also investigated to demonstrate that mixed sugar streams that would normally be considered impure for sugar product utilization could be used for bioethanol production. The combined extraction/pretreatment process produce juice with near 100% extraction efficiency that required no pH adjusted prior to fermentation, and the subsequent hydrolysis of the bagasse reach sugar yields comparable to the batch processes, 70% glucose yield and 18% xylose yield. Combining the juice stream with the hydrolyzate stream provided a fermentable mixed sugar solution that achieved an ethanol concentration of 21 g/L at an ethanol yield of 85%, and 80 g/L at a yield of 46% for a concentrated mixed sugar solution using rotary evaporation. This demonstrates the potential of combining sugar extraction with pretreatment of bagasse where the end goal is a fermentable sugar stream and not necessarily a pure one.

APPENDIX

Appendix: Description of combined extraction/pretreatment setup

Figure 21 below shows the apparatus setup for performing the sugar extraction of sorghum and alkali pretreatment of the resulting bagasse. Fresh sorghum is introduced at stage #5 where it is washed with the liquid from stage #4 before the solids remaining are moved to the right to stage #4. The juice that is passed thru the fresh bagasse is then collected in the beaker to the left. Fresh water is introduced at stage #1 washing the bagasse from stage #2. The bagasse after being washed at stage #1 is then collected in the beaker to the right. At stage #2, the liquid from stage #1 is mixed with 0.51 mL of 5 M NaOH and slurried with the begasse coming from stage #3 and incubated for 1 h at 80 ^{o}C before being filtered thru the funnel at stage #2. Therefore, sorghum/bagasse moves processively from left to right and water right to left until enough juice and bagasse have accumulated to perform hydrolysis and fermentation.

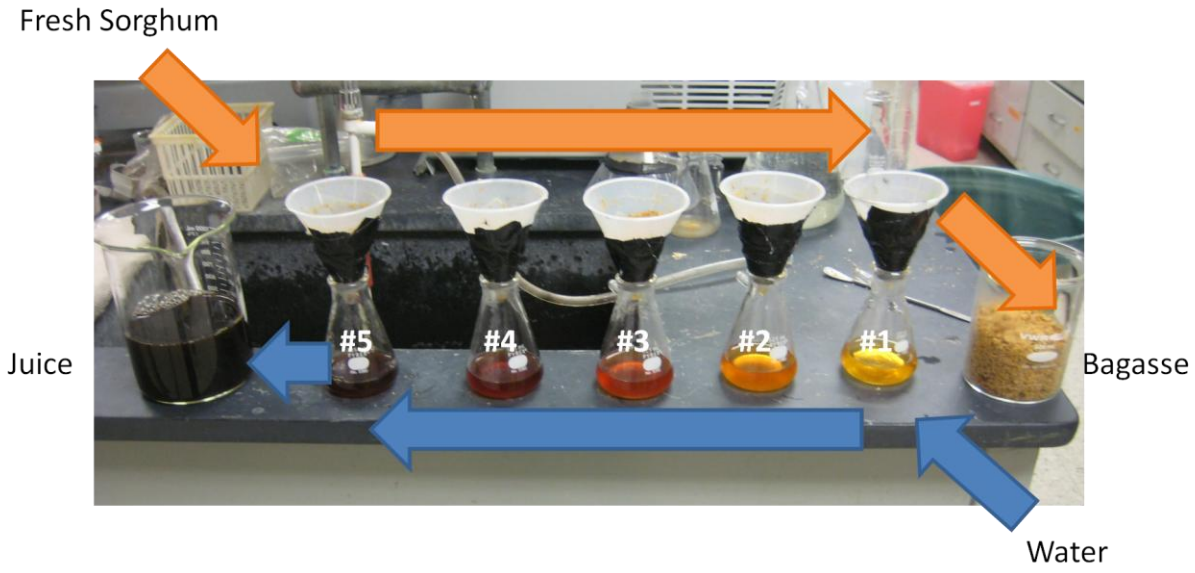

Figure 21: Extraction setup showing the stage # and the direction of water/juice and sorghum/bagasse flow

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Chapter 3 – Impacts of delignification and hot water pretreatment on the water induced cell wall swelling behavior of grasses and its relation to cellulolytic enzyme hydrolysis and binding

This work has been published as original research in February 2014, in *Cellulose*, Volume 21, Issue 1, and Pages 221-235.

Introduction

The majority of terrestrial carbon in the biosphere is thought to be sequestered within plant cell walls (Gilbert 2010). However, this vast resource of reduced carbon is used primarily by humans for its existing structural value, as a fuel for combustion, or as a ruminant feed rather than for the value contained in its existing chemical constituents. This is due to the recalcitrance of the cell wall to deconstruction by chemical and biological treatments which is set by features that are both structural and chemical and cut across the length scales at the molecular, macromolecular, and cellular levels (Ding, Liu et al. 2012). One route for the production of biofuels is via the biological conversion of plant cell wall polysaccharides through a pretreatment, enzymatic hydrolysis of the cell wall polysaccharides, and fermentation of these monomers to a biofuel such as ethanol (Alvira, Tomas-Pejo et al. 2010). The enzymatic hydrolysis of cell wall polysaccharides requires that an active cellulolytic enzyme be able to penetrate into the cell wall, bind to its substrate, and perform catalysis. Fundamentally, this can be considered as a combination of the related properties of both porosity and target glycan accessibility.

The quantification of porosity in combination with knowledge of other cell wall properties should yield important information about the enzymatic digestibility and glycan accessibility. However, compared to other properties such as bulk composition, cell wall

porosity and indirect measures of glycan accessibility have received significantly less attention primarily due to the challenges relating to their accurate quantification. An important concept when considering the porosity of plant cell walls is that, as a matrix of polymers crosslinked primarily by non-covalent forces, these porous structures act as swellable hydrogels (O'Neil and York 2003), particular in unlignified or low-lignin tissues. As such, the solvent properties influence the swelling of the cell wall and hence the porosity and glycan accessibility to enzymes. A variety of techniques are available for quantifying porosity and surface area in porous materials (Papadopoulos, Hill et al. 2003), however techniques for plant cell wall porosimetry have more restrictions due to the requirement for a hydrated material to maintain pore integrity (Pönni, Vuorinen et al. 2012). As such, common techniques such as BET or mercury intrusion are unsuitable, while methods that can be performed in the hydrated state include differential scanning calorimetry of "bound" water freezing point depression (Yu, Jameel et al. 2011), solute exclusion (Thompson, Chen et al. 1992; Ishizawa, Davis et al. 2007), and ^{1}H NMR by either cryoporosimetry (Ishizawa, Davis et al. 2007), diffusion (Topgaard and Söderman 2001), or relaxation (Andreasson, Forsström et al. 2005). Complementary to these, a number of methods provide metrics for the strength of water association with plant cell walls including water retention value (WRV) (Scallan and Carles 1972; Maloney, Laine et al. 1999), settling volume (Alince and Robertson 1974; Hubbe and Heitmann 2007), "freeness" or the rate of water drainability (Helmerius, von Walter et al. 2010), and water activity (Fardim, Liebert et al. 2013; Selig, Thygesen et al. 2013).

The characterization of porosity in delignified woody plant cell walls generated by chemical pulping, cell wall swelling, and the diffusion of charged cationic or amphiphilic polyelectrolytes into pores for paper "sizing" has been covered in the literature in the context of

chemical pulping and papermaking for the role these phenomena play in influencing the drainability of delignified pulps, fiber-fiber adhesion, wettability or hydrophobicity of paper, and the colloidal stability of process liquors among many others (Scallan 1983; Scallan and Tigerström 1992; Hubbe and Heitmann 2007). In the context of cellulosic biofuels, pretreated, delignified, and or mechanically refined woody biomass fibers have been characterized with respect to wet porosity (Grethlein 1985; Thompson, Chen et al. 1992; Koo, Treasure et al. 2011; Yu, Jameel et al. 2011; Wang, He et al. 2012) and water retention (Luo and Zhu 2011; Luo, Zhu et al. 2011; Wang, He et al. 2012; Hoeger, Nair et al. 2013; Jones, Venditti et al. 2013), and these properties have generally been shown to be strongly correlated to the enzymatic yield of glucose. Relative to the cell walls of woody biomass, which comprise the most industrially significant fiber source, less literature is available on the porosity and swellability of the cell walls of graminaceous monocots (grasses) that include some of the most promising feedstocks for bioenergy processes (e.g. corn stover, switchgrass, miscanthus, etc.) with several publications characterizing changes in wet-state porosity as a function of pretreatment condition for corn stover (Ishizawa, Davis et al. 2007) and sugar cane bagasse (Junior, Milagres et al. 2013) while one study investigated the WRV of dilute acid-pretreated and enzymatically hydrolyzed corn stover with inconclusive results (Roche, Dibble et al. 2009).

Relative to the cell walls of woody plants, the grasses have significantly different compositions and organizations at the cellular and macromolecular levels (Ding, Liu et al. 2012). For example, parenchymatous tissue comprises a significant fraction of the pith of grasses such as corn stover and sugar cane bagasse that is not present in wood (Lois-Correa 2012). These tissues are known to be low in lignin, thin-walled, and substantially more digestible by rumen microbiota (Akin, Rigsby et al. 1993; Wilson, Mertens et al. 1993) as well as cellulolytic

enzymes following a pretreatment (Zeng, Ximenes et al. 2012) or a chemical delignification (Ding, Liu et al. 2012) than the other cell types in grasses. These same tissues are typically removed in a "de-pithing" step when non-wood fibers such as sugar cane bagasse are used as a feedstock for chemical pulping due to their poor strength properties and extreme hygroscopicity (Zanutti 1997) which results in poor drainability of pulped fibers during papermaking. It has been shown that sugar cane bagasse is able to sorb as much as 20 times its weight in water, while depithed bagasse holds only five times (Lois-Correa 2012). Other work has shown that corn stover pith has higher equilibrium moisture contents (Igathinathane, Womac et al. 2005; Igathinathane, Womac et al. 2007) than either the leaves or rinds over the entire range of relative humidities. Early work with alkaline hydrogen peroxide (AHP) delignification of wheat straw demonstrated that nearly completely delignifed wheat straw was capable of retaining three times more water than untreated wheat straw when subjected to filtration (Gould 1985).

From this it is clear that plant cell wall water swellability, lignin content, and enzymatic digestibility should be strongly correlated properties, yet these relationships have not been systematically explored, particularly for potentially important bioenergy feedstocks such as the grasses. For this work, we investigate these relationships by employing a range of pretreated grasses that include combinations of alkaline hydrogen peroxide (AHP) delignification and liquid hot water (LHW) pretreatment that result in significant alterations of cell wall properties and subsequent enzymatic hydrolysis yields. These sets of untreated and pretreated plant cell wall materials are characterized with respect to their WRV and the settling volume and correlated to enzymatic glucose yields and enzyme binding. Additionally, the impact of AHP delignification on water activity during dynamic vapor sorption will be shown for corn stover as

well as significant differences in carboxylic acid contents for select materials, while FTIR is employed to yield information about compositional changes.

Materials and Methods

Biomass

The biomass feedstocks used in this work included switchgrass (*Panicum virgatum*, cv. Cave-in-Rock) and corn stover (*Zea mays* L Pioneer hybrid 36H56) as reported in our previous work (Li, Foster et al. 2012). The biomass was milled to pass a 5 mm screen (Circ-U-Flow model 18-7-300, Schutte-Buffalo Hammermill, LLC) and air-dried to a moisture content of ~5% before any treatments were performed. The composition of structural carbohydrates and lignin of all material was determined by the NREL / TP 510-42618 protocol with minor modifications as described by Li et al. (Li, Foster et al. 2012).

LHW Pretreatment

LHW pretreatment of corn stover and switchgrass was performed in a 5 L M/K Systems digester (M/K Systems, Inc., Peabody, MA). A total of 500 g of biomass (dry basis) was loaded into three cylinders (7 cm diameter x 35 cm height) fabricated from 200 mesh corrosion-resistant 304 stainless steel cloth (McMaster-Carr Inc., Cleveland, OH), which were then placed into the digester with 4 L of water. The digester was then programmed to heat up to 160°C at a heat rate of 0.8°C /min, holding for 5 min, followed by cool-down for one hour at a rate of ~1°C /min. The pretreated biomass was washed by soaking in clean water using a large bucket while the biomass was still inside the stainless steel containers. The solids were then air-dried and composition analysis was performed prior to AHP delignification and enzymatic hydrolysis.

AHP Delignification

Delignification of corn stover and switchgrass, both untreated and LHW pretreated, was performed using four different conditions of hydrogen peroxide to biomass loadings, 0%, 6%, 12.5% and 25% (g H_2O_2 /g biomass). All four conditions were performed in duplicate using 8 g of biomass (dry basis) at 15% (w/v) which is equivalent to 12.6% to 13% (w/w) depending on the H_2O_2 loading condition. Samples were prepared in 250 mL Erlenmeyer flasks and placed in an incubator at 30°C with shaking at 180 rpm. The flasks were sealed with parafilm to prevent evaporation but to allow for some expansion as the pressure in the flasks increased with O_2 evolution. The pH was adjusted back to 11.5 during pretreatment at 3, 6, and 9 hours with aliquots of 5 M NaOH as the pH would decrease during the process for the conditions used. Delignification was stopped after 24 hours by diluting the sample to 10% (w/w) solids and adjusting the pH to approximately 4.8 using concentrated sulfuric acid in preparation for enzymatic hydrolysis.

Enzymatic Hydrolysis

Hydrolysis of all corn stover and switchgrass samples was performed by diluting the AHP delignification slurries with 25 mL of water to 10% (w/w) solids and adjusting the pH to approximately 4.8 using concentrated sulfuric acid as mentioned above. Then an aliquot of 1 M citric acid buffer was added to give a concentration of 50 mM citric acid buffer in the sample flasks. The antibiotics tetracycline and cyclohexamine were added to make a concentration of 10 mg/L each to prevent microbial contamination. Next, an enzyme mixture of Accelerase 1000, Multifect Xylanase and Pectinase (Genencor, Inc., Palo Alto, CA) was added in a protein mass ratio of 4.4:1.7:1, respectively, at an enzyme loading of 30 mg enzyme/g glucan; this optimized enzyme ratio was determined by Banerjee et al. (Banerjee, Car et al. 2010) and the protein

contents of the enzymes were based on the Bradford assay. Samples were then mixed well and placed in a shaking incubator at 50°C with 180 rpm shaking for 7 days. Sugar concentrations in the hydrolysate were determined by HPLC using the method described in the NREL / TP 510-42618 protocol and converted to glucose yields based on the solids content in the reaction vessel and glucan content in the undelignified biomass. Glucose yield is percent of glucose released per glucose in the undelignified biomass.

Water Retention Value

Water retention value (WRV) was determined according to a modified version of TAPPI UM 256. For this the biomass samples were filter-washed with a fabricated Buchner funnel containing a 200 mesh stainless steel screen at the bottom as the porous base. The solids remaining after delignification were washed with approximately 700 mL of deionized water and vacuum-filtered to a moisture content of approximately 80%. Next, approximately 2.5 g of this wet biomass was inserted into a spin-column (Handee Spin Column Cs4, Thermo Scientific) modified to have a 200 mesh stainless steel screen as the membrane directly under the biomass. The spin columns were then centrifuged at 900 x g for 15 min (the TAPPI method uses 30 min.) The drained biomass was then weighed in an aluminum tray and placed in an oven at 105°C for 3 hours, and then weighed again. The WRV is the ratio of the mass of water remaining in the biomass after centrifuging divided by the mass of dry biomass. Samples were measured in triplicate and errors bars represent standard deviations.

Settling Volume

The AHP delignified slurries were filter-washed on the fabricated Buchner funnel as described above. Approximately 0.5 g of the wet solids remaining were transferred to a 20 mL scintillation vial and approximately 7 mL deionized water was added to achieve a solids

concentration of 6.5% w/w accounting for the moisture content of the wet solids. The vials were vortexed for 30 seconds and allowed to settle for 1 hour. The height of the settled solids and the height of solids and liquid slurry were measured, and the settling volume was determined as the ratio of the height of solids to the height of total slurry as reported in the literature (Riedlberger and Weuster-Botz 2012). Samples were measured in duplicate and the error bars represent the two values measured.

Enzyme Binding

AHP delignified corn stover slurries were filter-washed on a fabricated Buchner funnel as described above. Wet solids remaining were then air-dried for several days. Next, 0.5 g of dried biomass were placed in a 15 mL centrifuge tube for a total of 5 tubes per sample material. A combined volume of 10 mL of water, Cellic CTec2 (Novozymes, Bagsværd, DK) and 1 M citrate buffer at pH 5.5 was added to each tube for a solids concentration of 5% (w/v). The pH of 5.5 was selected because of the findings of Lan et. al. which showed that for lignocellulosic substrates, the optimal pH range for hydrolysis is between 5.5 and 6.2 (Lan et. al. 2013). The enzyme loading range in the 5 tubes was 0, 5, 10, 15, and 20 mg CTec2/g biomass. The samples were then placed in a 4°C cold room for 4 hours in a rotary mixer. Following incubation, protein in the supernatant was assayed by the Bradford assay (Fischer Scientific) using BSA as a standard, and corrected for background protein from the biomass by subtracting the absorbance of the sample with no enzyme added. Unbound protein in the liquid was calculated for each tube, and the bound enzyme fraction was determined as the difference of this unbound concentration from the initial enzyme concentration. The fraction of bound enzyme for each sample material was determined by regressing total enzyme concentration with the bound

enzyme concentration, where the slope of the regression line is the fraction of bound enzyme for each material.

Dynamic Vapor Sorption

Dynamic vapor sorption of untreated and air-dried, pretreated corn stover was performed at 25℃ using an AquaLab Vapor Sorption Analyzer (Decagon Devices, Pullman, WA).

Potentiometric Titration

Corn stover and switchgrass solids after AHP delignification were washed and air-dried before being milled with a Wiley mill to pass a 40 mesh screen. The method used is similar to Biliuta et. al. (Biliuta, Fras et al. 2011) where 200 mg milled solid samples were added to a solution consisting of 5 mL of 0.1 M KCl and HCl and 25 mL of water and the pH adjusted to 2.5. The pH was then titrated using a Brinkmann 716 DMS Titrino from 2.5 to 11 using 1 M KOH and the carboxylic acid content was determined as the mmol equivalent of KOH added per g of biomass.

FTIR-ATR

FTIR of solid biomass before and after pretreatment and delignification was performed using a Perkin Elmer Spectrum One FT-IR spectrometer and the Perkin Elmer Universal ATR Sampling Accessory. Air-dried biomass samples were first milled using a Wiley mill to pass a 40 mesh screen. Samples were placed on the FTIR diamond and covered with aluminum foil and the pressure arm was adjusted to 70 bar. Spectra were collected in the transmittance mode between 650 and 4000 cm-1 at a resolution setting of 4 cm-1 using 16 scans per sample. Individual spectra were normalized by mean-centering and scaling with respect to the spectra standard deviation (Robert, Marquis et al. 2005) to better compare spectra between conditions.

Results and Discussion

Biomass Pretreatment and Delignification

Combinations of alkaline hydrogen peroxide (AHP) delignification and liquid hot water (LHW) pretreatment of corn stover and switchgrass were performed in order to generate materials with a range of compositions, susceptibilities to enzymatic hydrolysis, as well as a diverse set of other cell wall properties such as water sorption that may allow for the correlations between these properties to be quantified. The compositions of the 20 materials generated by this combination of pretreatments and biomass feedstocks are presented in Table 8 along with the glucose conversions shown in the appendix to this chapter. These data show that AHP delignification preserves the majority of the cellulose and xylan while solubilizing lignin which has been well-established in the literature (Gould 1985). The LHW pretreatment is shown to solubilize xylan while preserving cellulose and lignin, which is also well-known from the literature whereby the improvement in digestibility can be attributed to xylan removal by autocatalyzed acid hydrolysis and lignin relocalization due to its increased mobility at elevated temperature (Selig, Viamajala et al. 2007). This relocalized lignin is known to occlude access to polysaccharides and has been shown to be overcome by additional, subsequent delignification (Selig, Vinzant et al. 2009).

Differences in macroscopic appearance of the slurries of 15% (w/v) corn stover and switchgrass subjected to AHP delignification (12.5% w/w H_2O_2 loading) are presented in Figure 22. These two materials show a striking difference in their apparent hygroscopicity with the AHP-delignified corn stover, which is substantially more digestible by fungal cellulases, showing essentially no free water while the AHP-delignified switchgrass solids are able to settle out of the slurry. A number of cell wall properties, both structural and compositional, can be

hypothesized to contribute to these differences in water swelling behavior. Water is known to be associated through a number of chemico-physical phenomena with plant cell wall biopolymers and the exterior and interior surfaces of cell walls as "free" and "bound" water. Free water comprises bulk water in large pores such as the lumen, and may strongly resist removal by, for example, capillary forces. Bound water represents more thermodynamically constrained water involved in non-covalent chemical interactions with cell wall biopolymers. This bound water can consist of primary bound water or "non-freezing" water that is tightly associated with cellulose surfaces, even within cellulose crystalline regions (Matthews, Skopec et al. 2006). Secondary bound water exhibits freezing point depression, but is still capable of undergoing a solid-liquid phase change. The relative abundance of these pools of water is set by the overall surface area and porosity of the cell wall matrix and its local chemical environment.

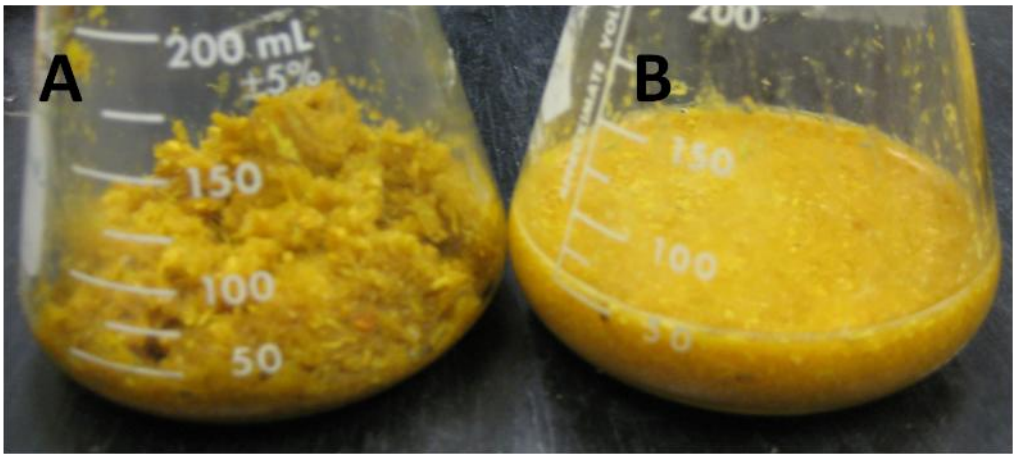

Figure 22: Observable macroscopic differences in the water swelling behavior of AHP-delignified (A) corn stover and (B) switchgrass at 15% (w/v) solids content.

Foremost among the compositional differences are lignin content and accessible amorphous polysaccharide content. Polysaccharide-associated bound water is thought to be most abundant in association with amorphous polymers (e.g. hemicelluloses or amorphous regions of

cellulose), and it is known to decrease proportionally with cellulose crystallinity (Nakamura, Hatakeyama et al. 1981; Hatakeyama, Nakamura et al. 2000). For lignin, both its total content and location strongly impact cell wall enzymatic digestibility (Grabber, Hatfield et al. 2003; Ding, Liu et al. 2012). Prior to lignification, the cell wall matrix behaves as a porous, swellable hydrogel, becoming hydrophobic and water-excluding after lignification (O'Neil and York 2003). It is well-established that delignification and hemicellulose removal can increase cell wall matrix porosity, allowing for increased water penetration and water swelling (Grethlein 1985; Akinli-Kogak 2001), and that these water-swellable, nano-scale pores are thought to exist as the voids between delaminated microfibril sheets (Fahlén and Salmén 2004). As cell wall matrix "hydrophilicity", swellability, and porosity are clearly important properties relating to polysaccharide accessibility to cellulolytic enzymes, these water properties are the subject of further investigation in this work.

Water Retention Value and Settling Volume

Quantifiable metrics of cell wall-water association, such as water retention value (WRV), incorporate many other properties such as cell wall composition and porosity, and may be a useful, simplified indicator of enzymatic digestibility. The variation in the WRV as a function of centrifugation speed was explored for untreated corn stover and switchgrass and AHP-delignified corn stover and switchgrass using the 12.5% H_2O_2 loading condition. These results are presented in Figure 23 and show a number of results of interest. The first observation is that AHP delignification more than doubles the amount of water that the biomass can hold. This substantial increase in water swelling has been identified for AHP-delignified wheat straw (Gould 1985). Figure 23 also shows that even though WRV decreases with increasing speed, the decrease is less for untreated material than for AHP-delignified material. Untreated corn stover

and switchgrass decrease from approximatly 1.5 to 1.2 while AHP delignified material decreases from 3.5 to 2.5. Similar results have been shown for cotton, where higher centrifuge speeds removed more water (Aggebrandt and Samuelson 1964). However, shorter centrifuge times will also reduce the amount of water removal. In fact, the SCAN-C 62:00 method for WRV uses a spin speed of 3000 x g for 15 min compared to the TAPPI UM 256 method of 900 x g for 30 min.

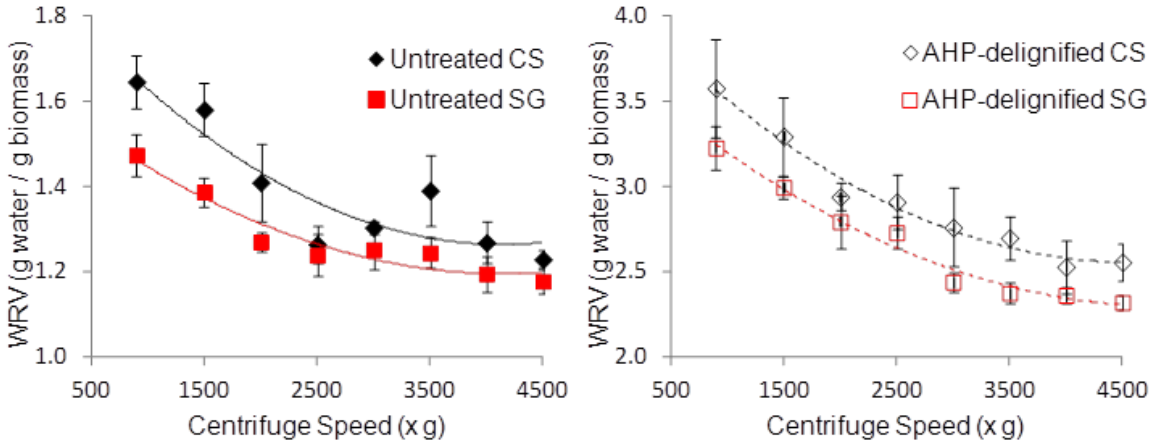

Figure 23: Water retention value (WRV) for corn stover (CS) and switchgrass (SG) before and after AHP delignification as a function of centrifugation speed for 15 min centrifugation time

A second notable observation from Figure 23 is that water swelling values tend toward an asymptote as the centrifugation speed is increased for both untreated and AHP delignified corn stover and switchgrass. This is likely due to easy-to-remove free water being largely removed before some threshold centrifugation speed and the only water remaining is bound water that is more strongly associated with the biomass. A "fiber saturation point" has been proposed to represent the moisture content at which there is no free water remaining within the cell lumina, and all remaining water is contained in the cell wall, and previous work has proposed that the WRV is a good estimate of the fiber saturation point (Scallan and Carles 1972). However,

certain properties of the bulk biomass material can impact water drainage suggesting that the WRV may not be such a good estimate of the fiber saturation point. For example, a higher packing density would be expected for material with a wider particle size distribution, and thus these materials would be able to trap more water in the biomass matrix. Also, the amount of small particles, or fines, can impact water drainage by blocking water flow pathways and increasing the water holding capacity; this is termed the choke-point hypothesis (Hubbe and Heitmann 2007).

A third observation from Figure 23 is that for all centrifugation speeds, the untreated and pretreated corn stover consistently retains more water than the corresponding untreated and pretreated switchgrass. These differences are statistically significant for all but three conditions using a t-test with an α of 0.05 (data not shown). These findings confirm the observations presented in Figure 22. For the remainder of this work, the lowest centrifugation speed tested, 900 x g, was employed to have more sensitivity in the measurements allowing larger differences between samples to be observed. Additionally, this is the standard centrifugation conditions for the established WRV protocols (TAPPI), albeit with a centrifugation time of 30 minutes rather than 15 minutes used in this work.

WRV and settling volume were next determined for corn stover and switchgrass samples that were subjected to a range of pretreatment conditions including combinations of liquid hot water (LHW) pretreatment and AHP delignification. These diverse pretreated samples showed enzymatic glucose yields after 7 days of hydrolysis ranging from 18% to 85%. The correlations between these two metrics and enzymatic glucose yield are shown in Figure 24. These correlations between the glucose yields settling volume and WRV both have a compelling linear correlation, R^2 of 0.895 and 0.900 respectively, regardless of biomass feedstock, pretreatment

type, or pretreatment condition. This is noteworthy because correlating enzymatic digestibility to carbohydrate or even lignin content does not give a clear trend for these samples (data not shown) and is therefore inconclusive as to the relative contributions of these factors toward impacting sugar yield.

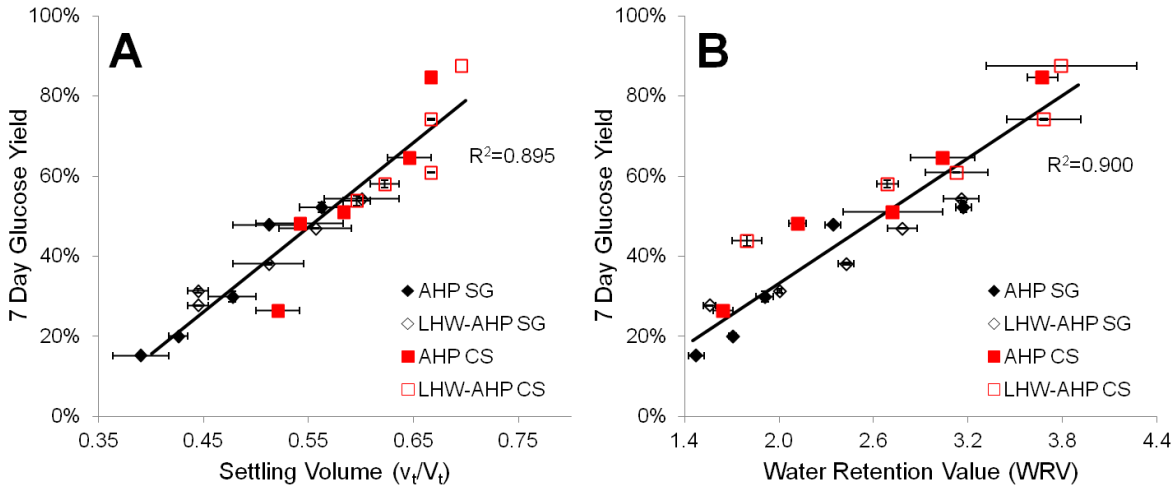

Figure 24: Corn stover (CS) and switchgrass (SG) correlation between the enzymatic glucose yield and (A) settling volume and (B) WRV

Differences in composition may be one (of many) of the factors that contributes to the differences in water swelling. Lignin has been shown to have the lowest water affinity followed by cellulose and then hemicelluloses in lignocellulosic material (Berry and Roderick 2005). However, Weber et al. have shown that cellulose does not contribute as much to WRV as hemicelluloses and lignin, and they did not observe a correlation between hemicellulose content and WRV (Weber, Kohlhepp et al. 1993); this may be because other properties of the hemicelluloses are also important factors that contribute to water retention (Lund, Sjostrom et al. 2012). As factors such as porosity and surface affinity are neglected by composition-only information, the increased settling volume and WRV may provide indirect evidence for increased porosity and polysaccharide accessibility to enzymes.

Settling volume has been studied extensively in the context of compaction and settling of activated sludge (Jenkins, Richard et al. 2004; Jones and Schuler 2012). For fibrous biomass, particle settling volume is an alternative quantification of the biomass bulk density which has been reported for bioenergy feedstocks such as wheat straw and switchgrass (Lam, Sokhansanj et al. 2008), with particle size, individual particle density, and particle-particle interaction being important in controlling the bulk density. The settling volume provides a metric for distinguishing differences between inter-fiber friction, and chemical treatments that increase settling volume also tend to increase dewatering rates (Hubbe and Heitmann 2007).

Enzyme Adsorption

Enzyme adsorption isotherms were determined for corn stover subjected to increasing AHP delignification (Figure 25A), with enzyme adsorption determined at 4°C to minimize catalytic activity. The obvious trend from this data is that the substrate has a higher binding affinity for cellulases with increasing delignification. As these are not complete isotherms and only represent the portion of the curve at low enzyme loadings before the adsorption saturates, they show an approximate linear increase in enzyme adsorption with increasing loadings to which linear trendlines are fitted. The slope of this portion of the isotherm is taken as the fraction of the protein that is bound to the biomass (mass bound protein / mass total protein). These values are plotted against the WRV and enzymatic glucose yields in Figure 25B which shows that there is a strong correlation between the bound enzyme fraction and WRV, R^2 = 0.995, and the enzymatic glucose yield, R^2 = 0.914. This result for increasing enzyme adsorption with increasing enzymatic hydrolyzability has been shown many times in the literature for many types of biomass and treatments for complex cellulase cocktails. As examples, strong correlations between protein adsorption and enzymatic glucose yield were found for

mechanically fibrillated softwood Kraft pulps (Hoeger, Nair et al. 2013), AHP-delignified rice straw (Wei and Cheng 1985), as well as many other diverse alkaline and acid pretreatments of corn stover (Kumar and Wyman 2009).

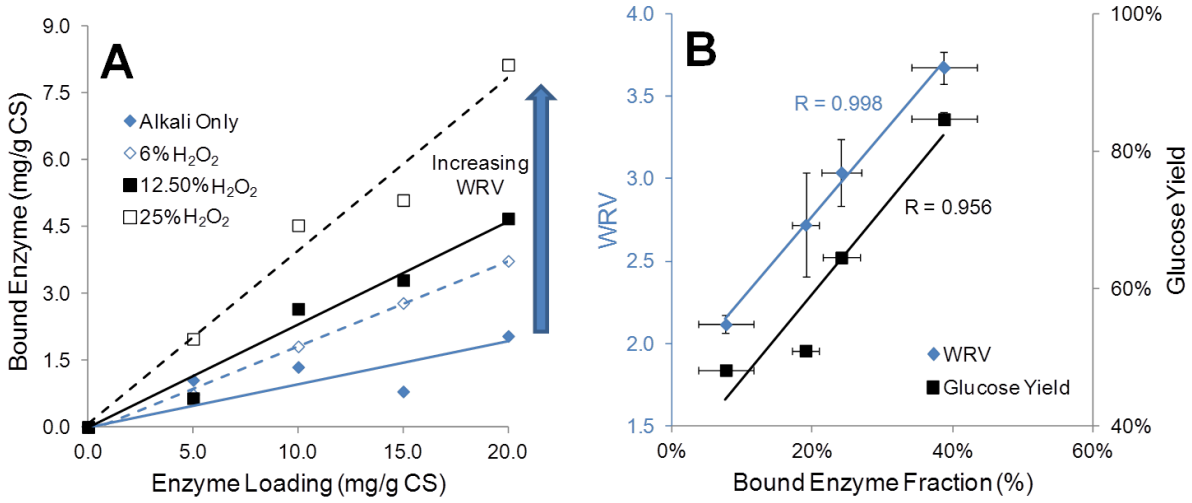

Figure 25: Impact of increased WRV on (A) enzyme binding and (B) the correlation between bound enzyme fraction and WRV and enzymatic glucose yield.

It should be considered that this is a multicomponent enzyme cocktail with many proteins with strongly differing substrate affinities and even some catalytic components such as β-glucosidase that do not contain cellulose binding modules (CBMs) and would not be expected to show strong adsorption to insoluble cell wall polymers. Additionally, protein adsorption is not necessarily correlated to the sugar yields as non-specific adsorption of enzymes to lignin is known to be important, in particular to high-lignin materials such as acid-pretreated softwoods (Tu, Pan et al. 2009).

Dynamic Vapor Sorption

Dynamic vapor sorption (DVS) was employed as a method to investigate the impact of AHP delignification on the water affinity of surfaces within the porous cell walls, how this might

relate to structural and chemical changes in the biomass, and how this could be interpreted with respect to differences in the enzymatic digestibility of these materials. A substantial literature exists for vapor sorption and its dynamics in native and modified woody biomass as reviewed by Engelund et al. (Engelund, Thygesen et al. 2013). However, the literature for grasses is less well-developed. For example, sorption isotherms (for adsorption only) have been reported for corn stover components (Igathinathane, Womac et al. 2005; Igathinathane, Womac et al. 2007), big bluestem (Karunanithy, Muthukumarappan et al. 2013), wheat straw, and reed canary grass (Nilsson, Svennerstedt et al. 2005). Adsorption-desorption hysteresis and the effect of cell wall modifications such as pretreatment on these sorption dynamics for grasses have not been reported in the literature. In this work, DVS isotherms at 25˚C were determined for untreated corn stover and AHP-delignified corn stover (12.5% H_2O_2 loading) with the results presented in Figure 26. These show changes in the moisture content of the material as a function of the relative humidity (alternatively the water activity, a_w, of the sorbed water) for water adsorption (lower curves) and desorption (upper curves).

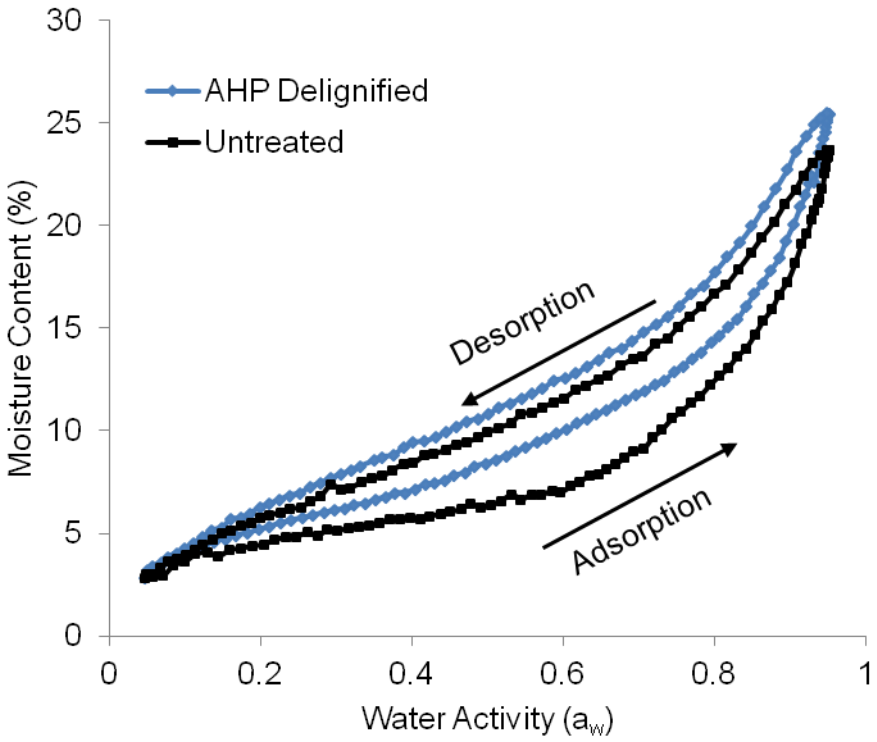

Figure 26: Water activity isotherms for untreated and AHP delignified corn stover

The data in Figure 26 show at least three notable results that deserve comment. The first result is that the AHP-delignified corn stover has a lower water activity than the untreated material for any given moisture content. In other words, the water vapor adsorption-desorption isotherms are shifted to the left following delignification. This indicates that following mild delignification, more water is present at any select moisture content in a more constrained environment. This can be interpreted as indicating that more water can be adsorbed within the cell wall pores (or on fiber surfaces) and that delignification may increase the accessible sites for water to sorb by increasing intra-cell wall porosity. The oxidative delignification may additionally introduce more hydrophilic functional groups such as carboxyl groups as a consequence of oxidation reactions, or increasing the accessibility hydroxyl groups such as in polysaccharides and in particular with non-crystalline hemicelluloses (Olsson and Salmen 2004).

DVS of different cellulose allomorphs has shown that native allomorphs of cellulose (cellulose I) offer significantly less sorption potential than other allomorphs (Selig, Thygesen et al. 2013). However, alkali concentrations used for AHP delignication in this work are not at a high enough concentration to induce the formation of the cellulose II allomorph. The second notable result from Figure 26 is that the total mass of sorbed water at 100% relative humidity (a_w of 1.0) is increased, with the equilibrium moisture content increasing from 23.7% to 25.5% following AHP delignification, again indicating a higher affinity for water. The third notable finding is that the AHP delignified corn stover also has smaller hysteresis than the untreated material. Water sorption/desorption hysteresis in swellable, porous materials has been attributed to irreversible water phase change, irreversible absorbent swelling, and capillary condensation-evaporation processes in pores where vapor condenses in the pores and is then trapped (Boki and Ohno 1991). Small hysteresis may be the result of weakly associated fiber chains and weakly bound water for material with high swelling and sorption properties, or for smaller swelling and sorption properties the opposite is true, indicating strongly associated chains and bound water. This would make sense for AHP delignified corn stover which is more enzymatically digestible than untreated corn stover, and weaker polymer and water association would allow for easier enzyme access and subsequent hydrolysis of sugar polymers for the AHP material. However, hysteresis differences may also be explained by differences in mesopore volume (Boki and Ohno 1991) which too could explain the differences between untreated and AHP delignified corn stover, where lignin and hemicelluloses have been removed with the AHP treatment, potentially increasing pore volume and allowing, again, for more enzyme access.

Cell Wall Carboxylate Content

Oxidizing pretreatments and pulp bleaching processes are well-known to introduce carboxylate groups to cell wall biopolymers. It is presumed that these functional groups may influence the chemical and structural environment within the cell wall matrix, for example, by increasing electrostatic repulsion between polymers, increasing the affinity for water within the cell wall, and resulting in an overall more porous cell wall when swollen with water. The carboxylic acid contents of AHP-delignified corn stover and switchgrass were determined by potentiometric titration with the results presented in Figure 27. This shows that estimated acid content is higher for corn stover (0.58-0.77 mmol COOH/g original biomass) relative to switchgrass (0.38-0.64 mmol COOH/g original biomass) for all conditions. Another result is that the carboxylic acid content passes through a maximum at an H_2O_2 loading 12.5% (w/w on biomass) after which it decreases.

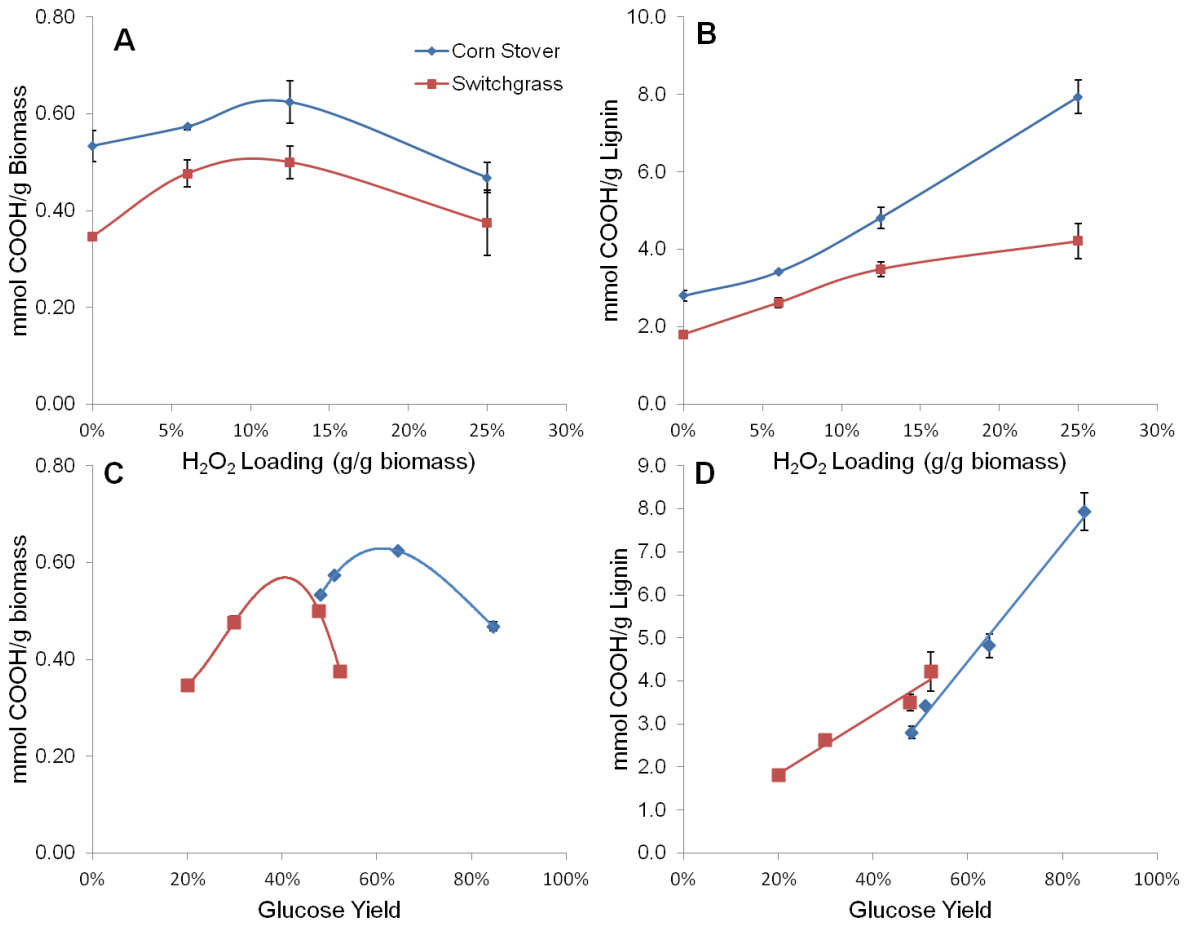

Figure 27: The carboxylic acid content determined by potentiometric titration as a function of AHP pretreatment condition on (A) a per mass biomass and (B) a per mass residual lignin basis and as a function of enzymatic glucose yield on (C) a per mass biomass and (D) a per mass residual lignin basis

Carboxylic acid groups are known to be sites for water adsorption; therefore, these data corroborate the water swelling capacities above with the exception of the 25% condition. This may be due to the fact that lignin content has been reduced so much that cellulose chains are free to swell much less inhibited than the other conditions regardless of the amount of water adsorption sites. A similar result was found by Scallan et al. where an increase in acid groups to a maximum was observed following selective delignification by Kraft pulping, then it decreased again as progressively more lignin was removed (Scallan and Tigerstrom 1992). It was

hypothesized that water will imbibe the cell wall due to osmotic pressure causing the cell wall to swell; swelling stops when osmotic pressure equals the elastic tension of the cell wall. Increased delignification by Kraft pulping has been shown to decrease elastic modulus which equates to increased swelling, and at some point a threshold was reached where lignin removal would no longer decrease the elastic modulus because of an increase in hydrogen bonding between cellulose fibers which counteracted the effect of lignin removal (Scallan and Tigerstrom 1992). Figure 27B shows the same results plotted per g lignin with the assumption that the majority of the COOH groups are introduced onto the lignin. For this, the trend is a continuous increase in COOH/g lignin with increasing H_2O_2 loading indicating that the introduction of COOH groups is indicative of lignin removal. Plotting this same data against enzymatic hydrolysis yields of glucose shows similar trends (Figure 27C, D). Specifically, the glucose hydrolysis yields show maxima (Figure 27C) relative to a carboxylate content on a per original biomass basis while a linear trend is apparent for glucose hydrolysis yields when plotted against carboxylate content on a per mass lignin basis.

FTIR-ATR

In order to understand the impact of AHP delignification and LHW pretreatment on other chemical features of corn stover and switchgrass that may contribute to water swelling, the samples were analyzed using FTIR-ATR. These results, plotted in Figure 28A, show a select region of the FTIR spectra subtracted from the spectra of untreated material for AHP delignified corn stover over the range of H_2O_2 loadings used. Peaks below 0 represent decreases in transmittance intensity for that peak from untreated material, while peaks above 0 represent increases in intensity. Peak assignments to specific functional groups and properties in biomass are difficult as even in the literature there seem to be differences depending on experiments and

materials. There are notable decreases in transmittance for peaks 1727, 1386, and 1235 which have been attributed to acetyl esters in xylan (Sun, Sun et al. 2004; Wrigstedt, Kylli et al. 2010) as well as ferulate and *p*-coumarate esters (Pan, Bolton et al. 1998), and therefore are indicative of saponification of these esters, which is expected considering the alkaline pH used during delignification. The decrease in peak 1517 indicates a decrease in lignin which is in agreement with the composition results after delignification (see Table 8) (Liu, Xu et al. 2006). Ferulate and *p*-coumarate may contribute to this peak (Nakagame, Chandra et al. 2011), which would agree with results in our lab showing an increase in ferulic and *p*-coumaric acid removal with increasing AHP severity (manuscript in preparation). The peak at 1640 represents water bending vibration (Lojewska, Miskowiec et al. 2005), showing a decrease with increasing AHP severity; this makes sense as the air-dried moisture content also decreases with increasing AHP severity due to more severe hornification because of more lignin and hemicelluloses removal. The peak at 1160 is antisymmetric bridge stretching of C-O-C groups typically for the xylan backbone (Cao and Tan 2004; Wrigstedt, Kylli et al. 2010) and the peak at 990 is for β-glycosidic bonds associated with cellulose (Robert, Marquis et al. 2005) indicating a decrease in xylan content and an increase in cellulose content which is corroborated by composition data (Table 8). The peak at 1710 may be due to carboxyl or aldehyde functionality (Lojewska, Miskowiec et al. 2005) and has been attributed to carboxylic acid groups in lignin, which is in agreement with the results in Figure 27. Figure 28B shows the same results for LHW pretreated followed by AHP delignified corn stover. There is noticeably less cellulose concentrating evinced by the smaller peak height at 990, which agrees with composition data.

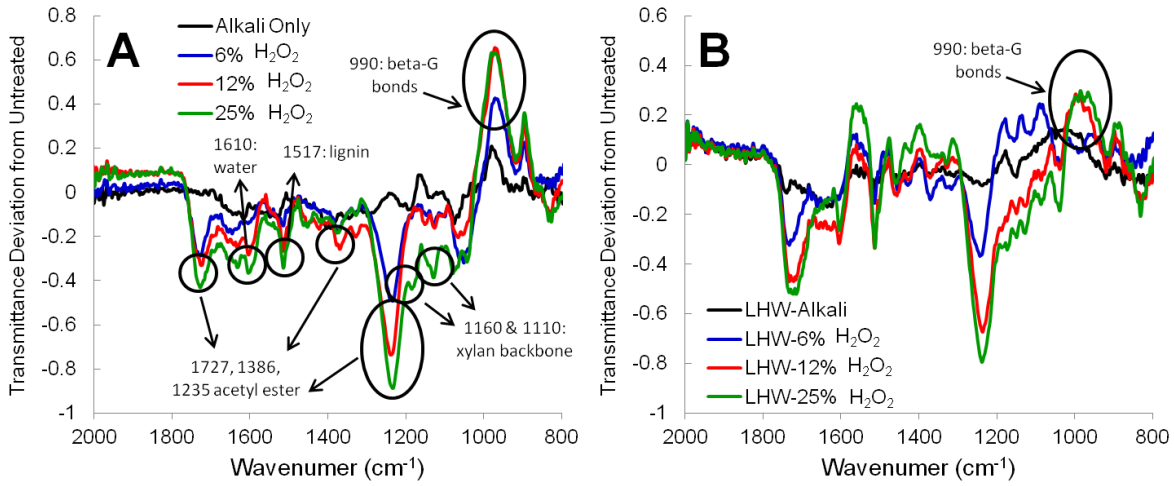

Figure 28: FTIR spectra of AHP delignified corn stover compared to (A) untreated material and to (B) LHW treated material. Results show transmittance deviation for each delignified material from the untreated material or LHW-only spectra which were used as the baselines

Conclusions

Cell wall water swelling and particle settling volume are measurements that are impacted by and can provide indirect assessments of biomass properties such as porosity and composition which have independently been shown to correlate well with enzyme accessibility and/or enzyme binding and, therefore, enzymatic digestibility. This work has shown that for combinations of LHW pretreatment and AHP delignification, two different biomass materials, corn stover and switchgrass having a range of compositions and water sorption behavior, WRV and settling volume are strong predictors of enzyme binding and enzymatic conversion. This is reasonable as water molecular association with biomass would be constrained by the same biomass properties as limit enzymatic deconstruction plant cell walls: polysaccharide accessibility, porosity, and surface water affinity. This work also showed that mild AHP delignification increased WRV up to 3-fold by removing lignin and increasing the water swellability of cell walls. It was also shown that AHP delignification increased the carboxylic acid content of corn stover and

switchgrass under some oxidative delignification conditions, which may also contribute to the increased cell wall affinity for water. Dynamic vapor sorption isotherms demonstrated that AHP-delignified corn stover exhibited an increased capacity for water sorption from the vapor phase relative to untreated corn stover, with more water present at any moisture content present in a more constrained environment, indicating more water-accessible pore volume. FTIR results corroborated what was known from composition results, that AHP delignification removed lignin and xylan and consequently concentrated the composition of cellulose. Future work will investigate a greater range of pretreatment technologies and pretreatment conditions to determine if this predictive power is universal and could be a valuable tool for evaluating pretreatment effectiveness. If this were to be the case, WRV, would be a very useful screening tool for identifying biomass feedstocks that would be more susceptible to chemical and enzymatic deconstruction.

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APPENDIX

Appendix: Supplimental data and a description of apparatus for measuring WRV

Table 8: Glucan, xylan, lignin compositions and glucose yields of corn stover and switchgrass before and after AHP delignification and LHW pretreatment

	Corn Stover					Switchgrass				
	Untreated	AHP Delignified (g H_2O_2 / g biomass)				Untreated	AHP Delignified (g H_2O_2 / g biomass)			
		0 g/g	0.06 g/g	0.125 g/g	0.25 g/g		0 g/g	0.06 g/g	0.125 g/g	0.25 g/g
Glucan	33.2% ± 0.7%	37.5% ± 0.1%	37.0% ± 1.0%	44.0% ± 0.4%	48.5% ± 0.4%	27.4% ± 0.3%	33.7% ± 0.1%	36.6% ± 0.4%	37.5% ± 0.5%	45.0% ± 0.5%
Xylan	21.5% ± 0.1%	22.7% ± 0.3%	23.5% ± 0.4%	23.7% ± 0.4%	25.2% ± 0.4%	20.5% ± 0.1%	25.2% ± 0.1%	27.0% ± 0.1%	28.0% ± 0.3%	24.7% ± 0.2%
Klason Lignin	20.5% ± 0.6%	19.0% ± 0.4%	16.8% ± 1.3%	12.9% ± 1.6%	5.9% ± 1.8%	21.9% ± 0.3%	19.3% ± 0.4%	18.2% ± 1.2%	14.3% ± 0.1%	8.9% ± 0.4%
Glucose Yield	26.4% ± 0.7%	48.2% ± 0.1%	51.0% ± 0.2%	64.6% ± 0.2%	84.7% ± 0.9%	15.2% ± 0.5%	20.1% ± 0.1%	29.9% ± 1.3%	47.8% ± 0.2%	52.2% ± 1.2%
	Liquid Hot Water Pretreated									
Glucan	40.9% ± 1.4%	37.7% ± 0.6%	46.2% ± 2.5%	51.7% ± 0.1%	63.1% ± 0.8%	39.5% ± 0.1%	31.8% ± 0.0%	43.1% ± 0.5%	46.3% ± 0.6%	53.6% ± 0.1%
Xylan	20.2% ± 0.3%	18.5% ± 0.8%	20.3% ± 0.6%	20.3% ± 1.3%	17.7% ± 0.2%	22.4% ± 0.1%	18.3% ± 0.0%	23.3% ± 0.3%	20.9% ± 0.5%	18.9% ± 0.1%
Klason Lignin	25.5% ± 2.3%	25.3% ± 1.3%	21.0% ± 1.5%	20.2% ± 0.6%	8.3% ± 0.3%	23.3% ± 0.8%	19.6% ± 0.2%	20.6% ± 0.2%	16.6% ± 0.2%	11.5% ± 0.4%
Glucose Yield	53.9% ± 1.3%	58.0% ± 0.9%	60.9% ± 0.1%	74.2% ± 0.2%	87.5% ± 0.1%	27.7% ± 0.2%	31.4% ± 0.4%	38.1% ± 0.3%	47.0% ± 0.2%	54.3% ± 0.4%

The Buchner funnel and spin column used for WRV quantification described above are shown in Figure 29 below. The diameters of the 200 mesh porous base and spin column membrane are 9 cm and 1.25 cm respectively. These dimensions are important and may be responsible for any differences in results obtained when using other equipment. Different equipment for biomass pad formation prior to centrifugation have been shown to yield different values of WRV in the SCAN-C 62:00 method.

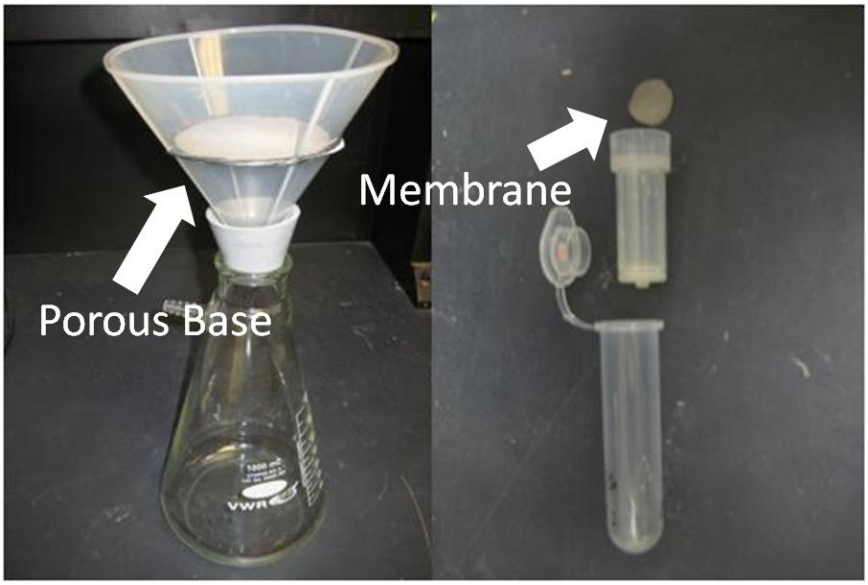

Figure 29: Fabricated Buchner funnel with 200 mesh porous base (left) and modified spin column with 200 mesh membrane (right)

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Chapter 4 – Water Retention Value of Pretreated Grasses Multiple Linear Regression Models

Introduction

The use of lignocellulosic biomass as a raw material for producing biofuels is an attractive option due to it being in abundance and a renewable resource. A promising conversion route to make fuel from lignocellulose is the biochemical pathway which utilizes polysaccharide hydrolyzing enzymes and fermenting microbes to produce fuels from cell wall carbohydrates. The challenge is that lignocellulose is difficult to degrade biologically due to its recalcitrant nature, which is a consequence of the ultrastructure created by its constituent's cellulose, hemicelluloses and lignin. Therefore, a pretreatment step is required before adding enzymes to disrupt the ultrastructure providing the enzymes access to the structural carbohydrates, promoting cell wall deconstruction. There are many pretreatment technologies being researched with a variety of chemistries and mechanisms for effective cell wall disruption that include a range of property changes of the biomass itself (Ong, Chundawat, Hodge, Keskar, & Dale, 2014). Porosity, pore volume and pore size distribution are important physical properties that can change during pretreatment which allow for increased enzyme accessibility.

Typical enzymes used for cell wall polysaccharide depolymerization include glycoside hydrolases (Lynd, Weimer, van Zyl, & Pretorius, 2002), monooxygenases (Harris et al., 2010; Langston et al., 2011; Quinlan et al., 2011), and esterases. Non-complexed fungal and bacterial glycoside hydrolases typically contain a catalytic region and a binding domain consisting of a carbohydrate binding module (CBM) from any number of evolutionarily conserved families. CBMs are necessary to bring the protein glycoside hydrolases into proximity of the substrate and orienting it for catalysis. CBM binding is thought to disrupt the local H-bonding network in the

region of the cellulose surrounding the binding site (Boraston, Bolam, Gilbert, & Davies, 2004; Nigmatullin et al., 2004), while the introduction of hydrolytic chain breaks causes further disruption (Bu et al., 2009). Enzyme access to binding sites is important and has been known to be impacted by lignin and hemicelluloses content due to physical occlusion as well as to be impacted by amorphous cellulose content measured by crystallinity (Chundawat et al., 2011). Both of these properties are measured independently and it is difficult to use composition data and crystallinity together to predict improved enzyme binding and thus enzymatic digestibility. Furthermore, these types of measurements completely omit the contribution of porosity and substrate surface affinity that would also contribute to enzyme accessibility and binding. Several techniques exist for performing pore analysis that can be used on biomass including mercury intrusion (Rigby, Fletcher, & Riley, 2002), gas permeability (Garey, Leekley, Hultman, & Nagel, 1973), scanning electron microscopy (Chinga, Helle, & Forseth, 2002), and atomic force microscopy (Mohammad, Hilal, & Seman, 2005). However, these methods typically require dry samples, and for lignocellulosic fibers, drying can cause irreversible collapse of pores, or hornification, that would not be observed if samples remained wet as they do during the conversion process. Therefore, pore analysis would be better done with undried samples, and there are methods for doing this as well, including differential scanning calorimetry (DSC) (Nakamura, Hatakeyama, & Hatakeyama, 1981; Park, Venditti, Jameel, & Pawlak, 2006), NMR cryoporometry (Gane et al., 2004), and water retention value (Weise, Maloney, & Paulapuro, 1996). These methods actually utilize water-fiber interactions and relate them to pore properties. For example, DSC defines three types of water in wet fibers: free water, freezing bound water and non-freezing bound water (Liu & De Yao, 2001; Nakamura, et al., 1981). Non-freezing bound water is that which does not crystallize at temperatures below the freezing point of water,

and it is thought to be the result of more strong hydrogen bonding with polymer surfaces as well as spatial confinement within "nanocavities" which prevent crystallization of the water (Berlin, Kliman, & Pallansc.Mj, 1970; Liu & De Yao, 2001). In fact, water molecules will first bind to polymers at water binding sites preferentially until all sites are occupied, comprising the non-freezing bound water content, before filling sorption sites for freezing bound water (Ping, Nguyen, Chen, Zhou, & Ding, 2001). Freezing bound water is that which is contained in pores and has the property of depressed melting temperatures compared to free water. These depressed melting temperatures have been correlated to different pore sizes which can be quantified based on the amount of freezing bound water they contain (Brun, Lallemand, Quinson, & Eyraud, 1977; Park, et al., 2006). The application of these wet-methods for characterizing biomass, in particular pulp, can be found extensively in the literature.

The interaction between water and pulp fibers has been an important topic in the pulp and paper industry for a long time for a variety of process related and paper quality related reasons (Akinli-Kogak, 2001; Weise, et al., 1996). For example, a higher packing density would be expected for material with a wider particle size distribution, and thus these materials would be able to trap more water in the biomass matrix. Also, the amount of fine particles can impact water drainage by blocking water flow pathways and increasing the water holding capacity; this is termed the choke-point hypothesis (Hubbe & Heitmann, 2007). In addition to these bulk properties, micro and nano-scale properties are also known to impact water interaction with biomass. Delignification and hemicelluloses removal have been shown to increase pore volume and leave behind empty space for water molecules to occupy, which allows for increased water penetration and thus increased WRV (Akinli-Kogak, 2001; Grethlein, 1985). Water molecules also absorb to biomass surfaces by hydrostatic interactions with hydroxyl and carboxyl

functional groups (Olsson & Salmen, 2004). Water properties are also used as indicators of paper quality; for example, increased fiber swelling is known to improve paper strength (Talwar, 1957). Methods have been developed in the pulp and paper industry to quantify biomass and water interactions as important indicators of fiber properties. The WRV, settling volume and freeness of pulp are examples of such methods. The WRV is a centrifugation method where a pad of wet biomass is drained using a centrifuge at a specified speed and duration; the value is determined by the amount of water remaining in the amount of biomass after centrifugation. Settling volume is a good way to view differences between inter-fiber friction and treatments that increase settling volume also tend to increase dewatering rates (Hubbe & Heitmann, 2007). The settling volume quantifies the relative height of biomass to total slurry height when it is allowed to settle in a solution of water at a specified solids concentration and duration. Water activity is another method for quantifying the interaction of water with biomass and is typically used in the food industry as a way to predict bacterial growth on food. Water activity is a measure of the energy state of water bound or trapped in a solid material based on partial pressure of sorbed water molecules. Our previous work, discussed in chapter 3, has demonstrated the changes in WRV, settling volume and water activity that take place after AHP delignification and how they can be related to enzyme digestibility (Williams & Hodge, 2014).

The quantifiable water properties of biomass discussed thus far have been used extensively for woody biomass in the pulp and paper industry, but have not been used extensively in the context of lignocellulosic biomass, particularly grasses, for biofuels. However, some examples include the work of Luo et al which has shown that WRV is a good indicator of cellulose accessibility after wet-pressed and heat-dried hornification has occurred, and that enzyme absorption to cellulose substrate correlates well with WRV for woody biomass

(Luo & Zhu, 2011; Luo, Zhu, Gleisner, & Zhan, 2011). Roche et al used the amount of entrained liquid (a variation of WRV) to determine if pore volume changed during enzymatic hydrolysis for corn stover (Roche, Dibble, Knutsen, Stickel, & Liberatore, 2009). Weber et al has shown that cellulose does not contribute as much to WRV as hemicelluloses and lignin, however, no correlation between hemicellulose content and WRV was found (Weber, Kohlhepp, Idouraine, & Ochoa, 1993); this may be because other specific properties of the hemicelluloses may be more important factors that contribute to WRV, for example hydroxyl and carboxyl contents, or charged groups which are known to increase fiber swelling (Lund, Sjostrom, & Brelid, 2012). And cell wall porosity will be indicative of water and enzyme penetration and therefore enzymatic digestibility. The hypothesis is therefore, that quantifiable water and biomass interactions may incorporate composition, porosity and hydrophilic properties of the cell wall and be a more useful indicator of enzymatic digestibility than any one group of these properties on their own. The work in chapter 3 has shown a positive, linear correlation between WRV and glucose yield for corn stover and switchgrass subjected to a range of AHP and LHW pretreatment conditions. This work will expand on our previous findings to include more extreme AHP and LHW pretreatment conditions, and also include AFEX pretreatments, to investigate how well WRV can be predictive of glucose yield over a wider range of biomass properties including composition and WRVs. A multiple linear regression (MLR) analysis will be performed to determine if the predictive capabilities of WRV can be improved by including specific biomass composition information where WRV may not be sufficient alone.

Methods and Materials

Biomass

The untreated biomass feedstocks used in this work include switchgrass (*Panicum virgatum*, cv. Cave-in-Rock*)* and corn stover (*Zea mays* L Pioneer hybrid 36H56). The biomass was milled with a Wiley Mini-Mill (Thomas Scientific) to pass a 5 mm screen and air-dried to a moisture content of ~5% before any treatments were performed. AFEX pretreated switchgrass and corn stover were obtained from Rebecca Garlock at Michigan State University. The composition of structural carbohydrates and lignin of all material was determined by the NREL / TP 510-42618 protocol with minor modifications as described by Li et al. (Li et al., 2012).

LHW Pretreatment

LHW pretreatment of corn stover and switchgrass was performed in a 5 L M/K Systems digester (M/K Systems, Inc., Peabody, MA) at three different conditions, 160°C for 5 min and 60 min, and 190°C for 5 min. The pretreatment method is the same as described in a previous paper (Williams & Hodge, 2014). Briefly, a total of 500 g of biomass (dry basis) was loaded into three cylinders (7 cm diameter x 35 cm height) fabricated from 200 mesh corrosion-resistant 304 stainless steel cloth from McMaster-Carr, which were then placed into the digester with 4 L of water. The digester was then programmed to heat up to the desired temperature, 160°C or 190°C, at a heat rate of 0.8°C /min, holding for 5 min or 60 min, followed by a cool-down for one hour at a rate of ~1°C /min. The pretreated biomass was washed by soaking in clean water for 24 hours and the solids were then air-dried.

AHP Pretreatment

Delignification of corn stover and switchgrass, both untreated, LHW pretreated, and AFEX pretreated (switchgrass only) was performed using two different conditions of hydrogen peroxide to biomass loadings, 2%, and 10% (g H_2O_2 /g biomass). Both conditions were performed in duplicate using 8 g of biomass (dry basis) at 15% (w/v). Samples were prepared in 250 mL Erlenmeyer flasks and placed in an incubator at 30°C with shaking at 180 rpm. The flasks were sealed with parafilm to prevent evaporation but also to allow for some expansion as the pressure in the flasks increased with O_2 evolution. The pH was adjusted back to 11.5 during pretreatment at 3, 6, and 9 hours with aliquots of 5M NaOH as the pH would decrease during the process for the conditions used. Delignification was stopped after 24 hours by diluting the sample to 10% (w/w) solids and adjusting the pH to approximately 4.8 using concentrated sulfuric acid in preparation for enzymatic hydrolysis.

Enzymatic Hydrolysis

Hydrolysis of all corn stover and switchgrass samples was performed by diluting the AHP pretreated slurries with 25 mL of water to 10% (w/w) solids and adjusting the pH to 4.8 using concentrated sulfuric acid as mentioned above. Then an aliquot of 1 M citric acid buffer was added to give a concentration of 50 mM citric acid buffer in the sample flasks. Antimicrobials tetracycline and cyclohexamine were added to make a concentration of 10 mg/L each to help prevent contamination of hydrolyzate. Next, an enzyme mixture of Cellic CTec2 and HTec2 was added in a protein mass ratio of 2:1, respectively, at an enzyme loading of 30 mg enzyme/g glucan and the protein contents of the enzymes were based on the Bradford assay. Samples were then mixed well and placed in a shaking incubator at 50°C with 180 rpm shaking for seven days. Sugar concentrations in the hydrolysate were determined by HPLC using the

method described in the NREL / TP 510-42618 protocol and converted to glucose yields based on the solids content in the reaction vessel and glucan content in the undelignified biomass.

Water Retention Value

Water retention value (WRV) was determined according to a modified version of TAPPI UM 256 as described in a previous work (Williams & Hodge, 2014). Briefly, the biomass samples were filter-washed using a Buchner funnel containing a 200 mesh stainless steel screen at the bottom as the porous base. The solids remaining after pretreatment and delignification were washed with approximately 700 mL of deionized water and vacuum-filtered to a moisture content of approximately 80%. Then, ~2.5 g of wet biomass was inserted into a spin-column (Handee Spin Column Cs4 from Thermo Scientific) modified to have a 200 mesh stainless steel screen as the membrane directly under the biomass. The spin columns were then centrifuged at 900 x g for 15 min. The drained biomass was then weighed in an aluminum tray and placed in an oven at 105°C for 3 hours, and then weighed again. The WRV is the ratio of the mass of water remaining in the biomass after centrifuging divided by the mass of dry biomass. Samples were measured in triplicate and errors bars represent standard deviations.

Differential Scanning Calorimetry

Solid residue after AHP pretreatment was washed with 500 mL of water using a Buchner funnel with a 200 mesh porous base and drained under vacuum to a moisture content of ~80%. Approximately 15 mg of wet biomass samples was placed into DSC aluminum pans (TA Instruments, Part #900786.901 bottom and Part #900779.901 top) and then run on DSC. The measurement of freezing bound water was performed the same to the method by Park et. al (Park, et al., 2006). Briefly, sample pans were subjected to a gradient and isothermal melting regime which started by cooling the pan to -30 ^{o}C and holding for 5 min, and then scanning at 1

^{o}C/min to -20 ^{o}C and holding again for 5 min. This procedure was continued in the same way for the higher temperatures of -15, -10, -6, -4, -2, -1.5, and -1.1 ^{o}C which relate to the melting temperature depressions that correspond to pore diameters 2.6, 4.0, 6.6, 9.9, 19.8, 26.4, and 36.0 nm respectively (Brun, et al., 1977; Park, et al., 2006). The size of the pore is related to the depressed melting temperature by the equation:

$$T_M - T_0 = -4\sigma_k \omega_k T_o / d\Delta H_s$$

Where T_0 is the 0 ^{o}C, σ_k is the surface tension between water and ice, ω_k is the specific volume of ice, ΔH_s is the specific heat of fusion (1000 kg/m^3), and d is the pore diameter (Burghoff & Pusch, 1979). The amount of water in each pore size was determined by integrating the area of each peak in the thermogram and dividing that area by the specific heat of fusion for water (334 J/g) which gives the mass of water per mass of slurry in each pore size. Figure 30 shows an example of the DSC thermograms, where the negative heat flow indicates heat being absorbed by the sample, and each peak is an endotherm.

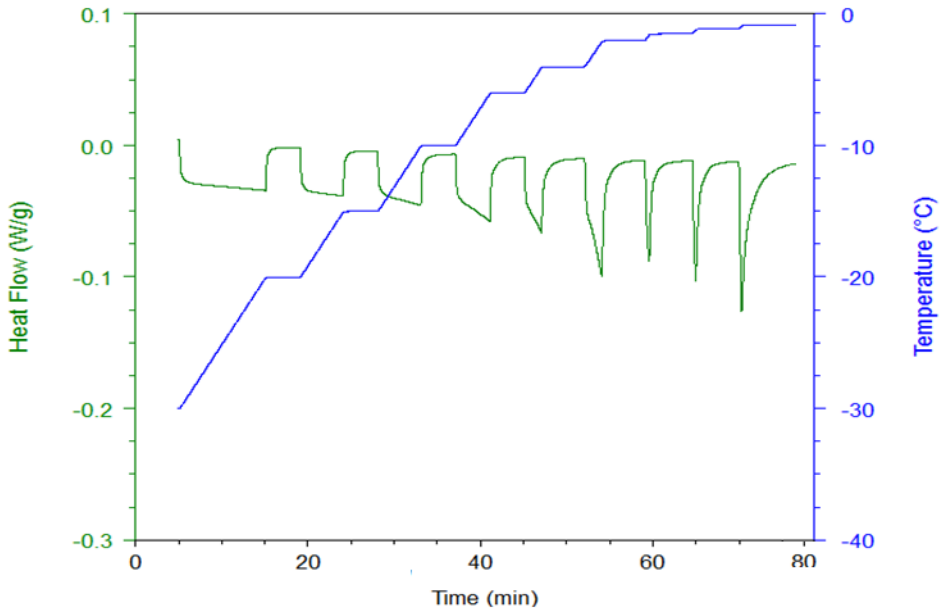

Figure 30: Example of a thermogram obtained from DSC at the different melting temperatures. The temperature regime with the heat-up and isothermal holds is shown in blue and the heat flow shown in green

Results and Discussion

AHP delignification has been shown to impact cell wall composition by removing xylan and lignin (chapter 1) and to increase cell wall/water affinity by increasing carboxylate content and likely porosity (chapter 3). The use of DSC to quantify pore size changes following AHP delignification confirms that porosity does in fact increase. Figure 31 shows the cumulative bound water (freezing water) versus the pore diameter for corn stover (A) and switchgrass (B) for the four different conditions of AHP delignification. There is a clear increase in bound water in the larger pore sizes quantified for the more severe AHP delignification conditions, indicative of increased porosity. However, the increased porosity is relatively small between the more severe conditions of AHP delignification, for example the 12% and 25% conditions for corn stover and the 6%, 12% and 25% conditions for switchgrass are very similar. In fact the average

pore diameter for the alkali, 6%, 12% and 25% AHP conditions are 5.9, 9.9, 12.7 and 14.6 for corn stover and 3.7, 12.2, 12.7, and 14.7 for switchgrass, respectively. However, the digestibilities of these different materials are notably different indicating that porosity itself is not solely responsible for differences in digestibility. Higher depression temperatures and thus pore diameters were investigated, however, endotherm peaks became erratic after temperatures of -0.5 ^{o}C. This is noteworthy because it has been suggested that there could be a significant amount of pores larger than even 400 nm (Park, et al., 2006). Furthermore, freezing bound water content has also been shown to increase with decreasing crystallinity (Nakamura, et al., 1981), which may contribute to the difficulty quantifying changes at the higher temperatures. However, this limitation may also be due to the sensitivity of the equipment or the characteristics of the pores larger than 100 nm themselves. The non-freezing bound water content was not determined for this work, but it has been shown to be constant even when the freezing bound water content is increased by making material more absorbent (Ping, et al., 2001).

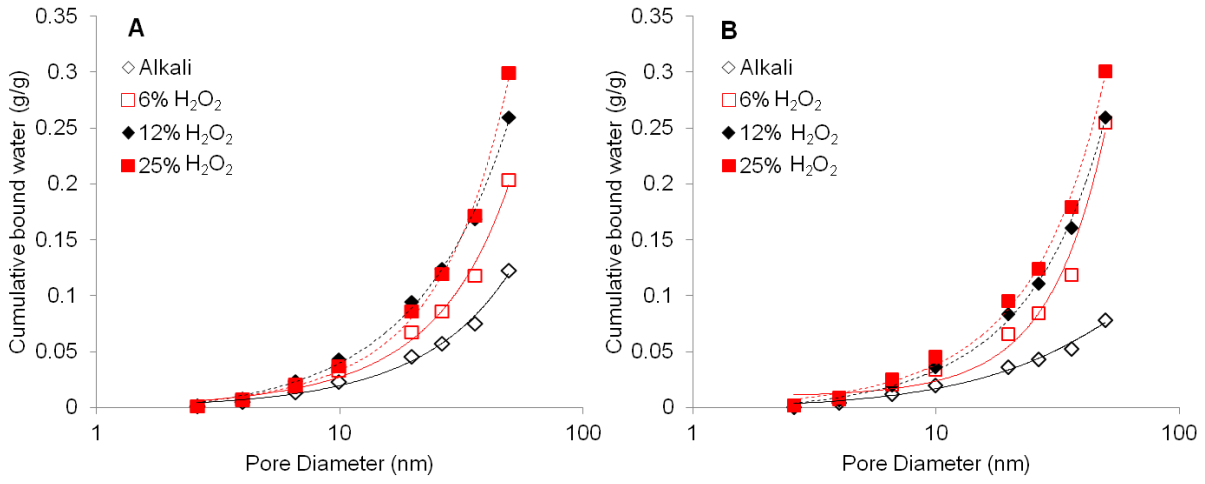

Figure 31: Cumulative freezing bound water content as a function of pore diameter for AHP delignified corn stover (A) and switchgrass (B) obtained from DSC melting temperature depression endotherms

Similar to the results shown in chapter 3, Figure 32 shows the correlation between glucose yield and WRV for AHP delignified corn stover and switchgrass (A) and LHW and AFEX pretreated switchgrass combined with AHP delignification (B); data are shown on two separate graphs to highlight the deviations from linearity for some pretreatment combinations. Figure 32A shows there is a clear linear correlation, R^2=0.951, and all data are within a 90% confidence interval (dashed lines) when both corn stover and switchgrass data points are regressed together. This is in agreement with results previously published by the author (Williams & Hodge, 2014). Figure 32B shows the linear model with confidence interval from Figure 32A again, and the results of combined LHW and AFEX pretreatment with AHP delignification. The AHP only, low intensity LHW (160 ^{o}C, 5 min) and one of the mid intensity LHW (160 ^{o}C, 1 h) results fall within the 90% confidence interval. However, the high intensity LHW (190 ^{o}C, 5 min) and the AFEX samples deviate significantly from the simple linear model. The region of biomass properties that contribute to increased WRV and digestibility improvement no longer overlap for these materials, at least in the same way as the other samples. A potential reason for this deviation may be explained by the composition changes that take place during the different pretreatment types. Figure 33 shows the relationship between xylan and glucan contents for switchgrass when performing AHP delignification, LHW pretreatment, and AFEX pretreatment with increasing intensity (for AHP this means increasing H_2O_2 concentration, and for LHW and AFEX, it means increasing temperature). AHP delignification causes an increase in xylan and glucan contents with increasing intensity due to oxidation and solubilization of lignin. Xylan, being an amorphous polysaccharide, contributes significantly to water sorption (Weber, et al., 1993), and therefore, higher xylan containing material would be expected to have higher WRV. Also, with higher glucan content, and therefore glucan

accessibility, digestibility increases as well. LHW pretreatment primarily removes or redistributes xylan, and redistributes lignin on cell wall surfaces (Holopainen-Mantila et al., 2013). Therefore, the WRV would be expected to decrease with xylan loss, but as lignin is redistributed and becomes less occlusive to enzyme access to cellulose, and because of an increase in glucan content and accessibility, digestibility will still increase. The same trend is observed following AFEX pretreatment as well; there is a decrease in xylan content, as xylan is more soluble once material is added to water for hydrolysis, and there is an increase in glucan content. This may explain why there are differences in WRV and hydrolysis yield correlations between these different pretreatment types. However, this does not necessarily mean that for LHW and AFEX pretreatments there is not a linear correlation between glucose yield and WRV.

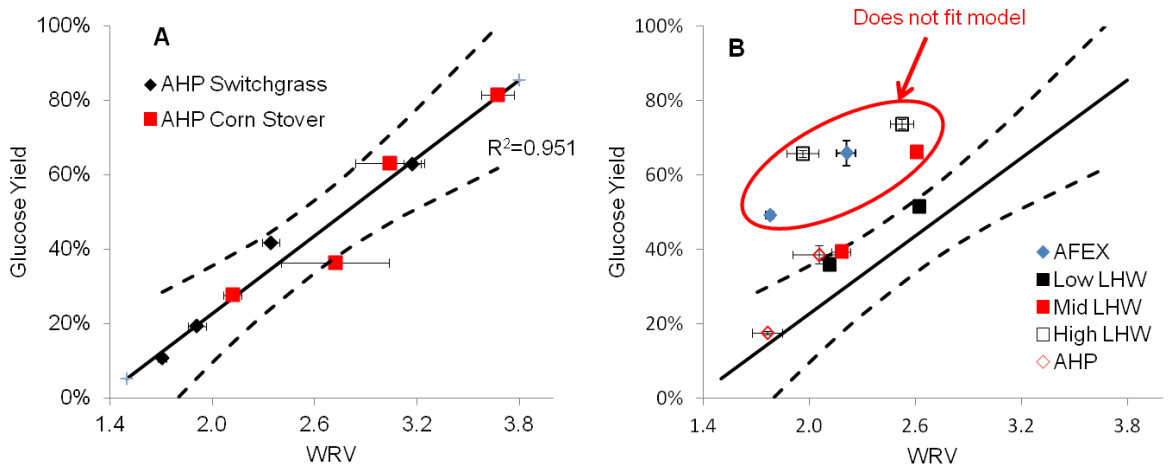

Figure 32: Correlation of glucose yield and WRV for (A) AHP delignified corn stover and switchgrass with linear regression model and 90% confidence interval shown in dashed lines and (B) the linear model from graph A and data from combined LHW and AFEX pretreatments with AHP delignification

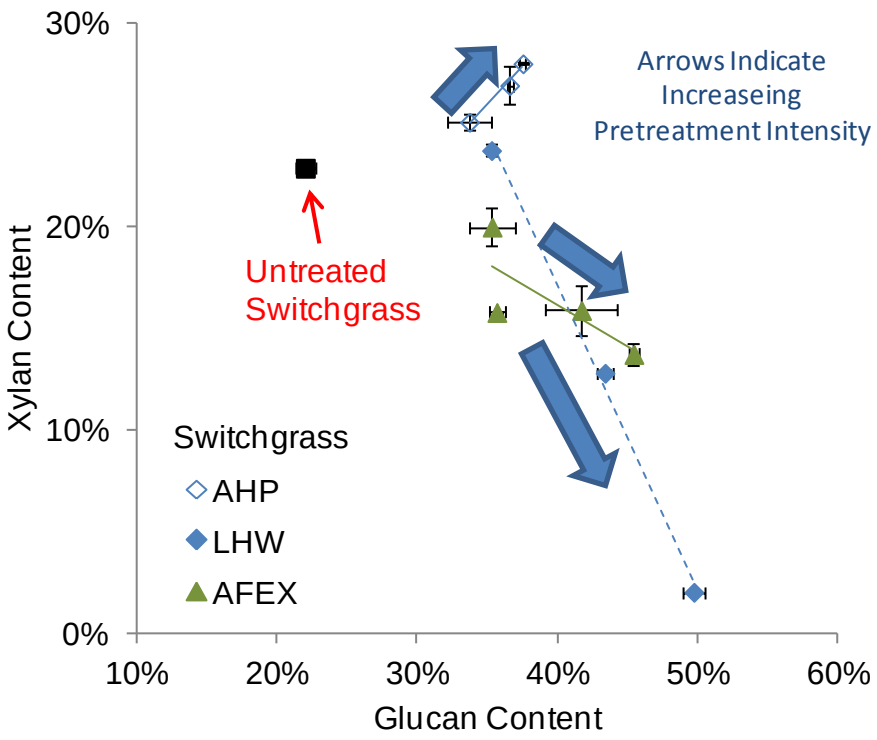

Figure 33: Relationship between xylan and glucan content changes for AHP delignification, AFEX, and LHW pretreatment of switchrass (arrows indicate increasing pretreatment type intensity, which is temperature for AFEX and LHW, and H_2O_2 loading for AHP)

Although the linear correlation did not hold true for all the different pretreatment types and conditions regressed together, WRV and glucose yield may still be linearly correlated on an individual pretreatment technology basis. Figure 34 shows the correlations between glucose yield and WRV for the AHP delignified corn stover and switchgrass shown in Figure 32A and also AFEX pretreated corn stover and switchgrass pretreated under two different conditions of ammonia loading (1.5 and 2 g/g) and four different temperatures (60, 90, 120, and 150 ^{o}C) for each ammonia condition. It is interesting to note that glucose yield for both AFEX pretreatment conditions are clearly linearly correlated with WRV but have different slopes, and different slopes from that of the AHP delignified material. This indicates that the respective pretreatments

do impact digestibility and WRV in a way that relates the two variables, but that the relationship is not universally correlated across the different pretreated biomass properties.

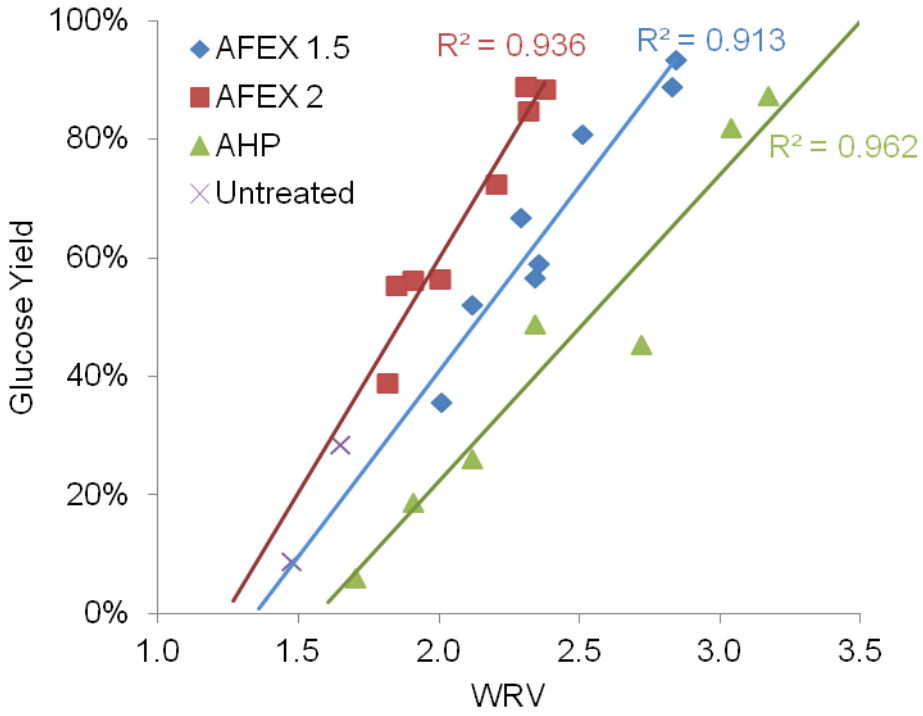

Figure 34: Correlation between glucose yield and WRV for AHP delignified corn stover and switchgrass (presented in Figure 32A) and AFEX pretreated corn stover and switchgrass at two different ammonia loadings , 1.5 and 2 g/g biomass, and a range of temperatures, 60, 90, 120, and 150 ^{o}C

The simple linear model relating glucose yield and WRV was not sufficient to explain all the data across multiple feedstocks, pretreatment technologies and conditions. It may be worthwhile to expand the linear model to include other independent predictor variables by performing multiple linear regressions (MLR). Figure 35 shows a heat map of the correlation matrix for the response variable glucose yield, and the predictor variables WRV, glucan content, xylan content, and lignin content. Glucan and xylan are both noticeably correlated to glucose yield in addition to WRV, albeit not as strongly. While lignin content is not correlated to

glucose yield, but is to xylan content. Therefore, MLR models will be investigated using combinations of the variable presented in Figure 35 to find a model capable of sufficiently fitting all the data presented so far.

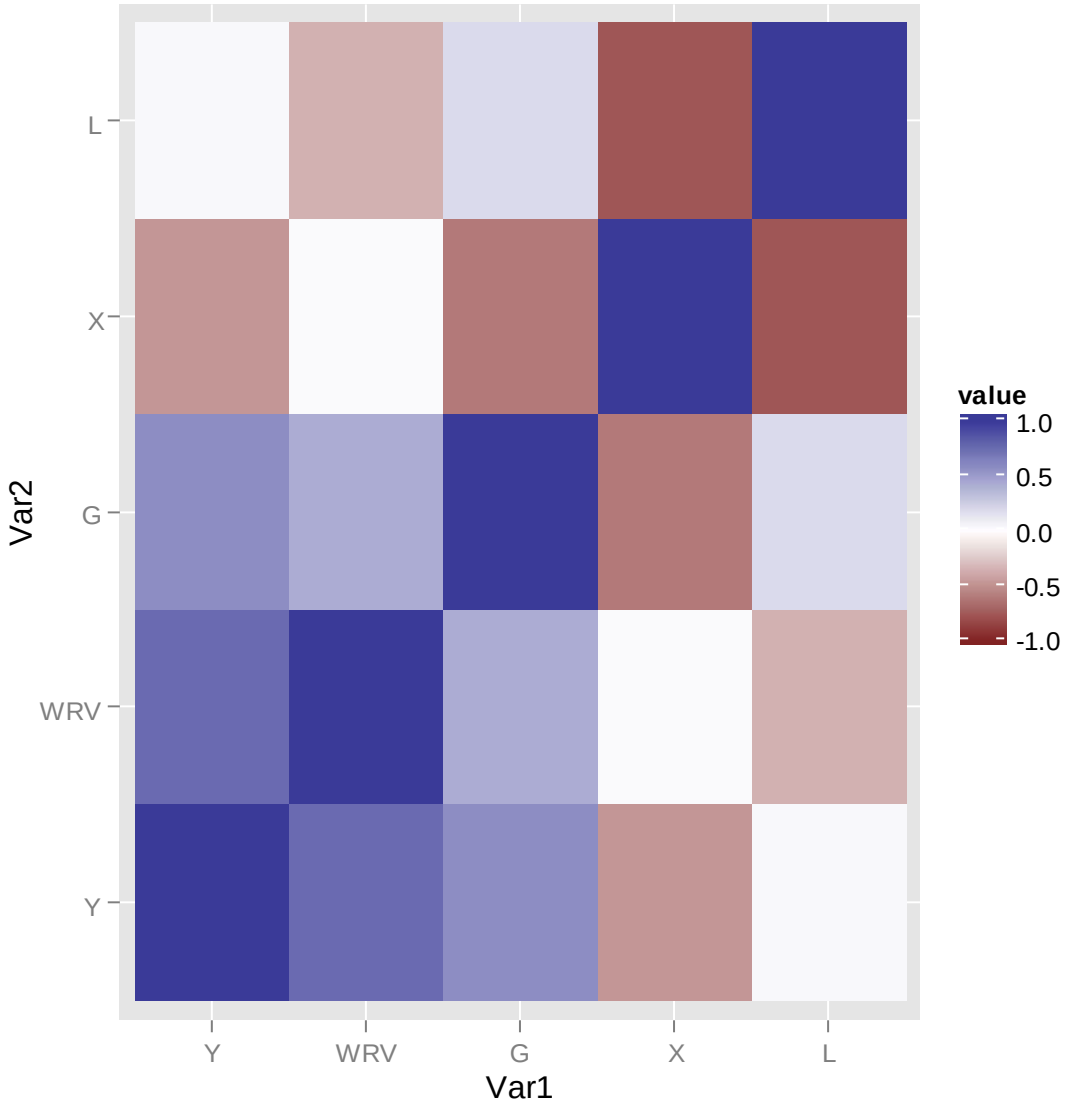

Figure 35: Heat map of correlation matrix for the response variable glucose yield (Y), and four predictor variables, WRV, glucan (G), xylan (X) and lignin (L) content

Model selection using the statistical selection criteria stepwise AIC, R^2, adjR^2, and Mallow's Cp are shown in the appendix of this chapter, and the best model is presented here.

Chapter 3 presented a simple linear model using WRV as the sole predictor variable, and that will be shown here as Model 1. The best fitting model using all the data presented thus far based on the selection criteria and model simplicity (least number of variables) is present here as Model 2, which includes WRV and xylan content after pretreatment, and has an R^2=0.900. Figure 36 shows predicted versus actual glucose yield for these two models. Other models that used more than two predictors where also analyzed as shown in the appendix to this chapter. Using WRV, glucan and xylan content or xylan and lignin content did give better models based on selection criteria. However, upon statistical analysis of these models, the glucan and the xylan contents were found to not be statistically important based on t and F-tests. The R^2 and adjusted R^2 values for all the models investigated here were on the order of 0.8 to 0.9. The fit of these models are comparable with other models reported in the literature, for example a near IR based model showed good results in which the range of R^2 values for various response models was between 0.69-0.87 for LHW, dilute acid and alkali pretreated miscanthus samples. However, the range of composition values and hydrolysis yields was not as broad as those used here (Huang et al., 2012). Another linear model based on FTIR spectra using partial least squares (PLS) regression models obtained fits from 0.84-0.99 R^2 for alkali pretreated switchgrass, big bluestem grass, several prairie grasses and corn stover (Sills & Gossett, 2012). An MLR model using lignin, acetyl, and carbohydrate content and cellulose crystallinity to predict glucan and xylan conversion for a range of enzyme loading conditions during hydrolysis and at different hydrolysis times for hybrid poplar subjected to various delignification, acetylation and decrystallization techniques to give very good predictions with R^2 of 0.95 or higher (Zhu, O'Dwyer, Chang, Granda, & Holtzapple, 2010). More complex models using artificial neural network approximations of non-linear functions for composition based variables

have also been shown to predict enzymatic hydrolysis yields very well (O'Dwyer, Zhu, Granda, Chang, & Holtzapple, 2008). Non-linear composition based models using lignin and glucan content have also been investigated with good results but for a limited range of composition values (Kim & Holtzapple, 2006).

Therefore, the MLR model presented here which uses WRV and composition information has similar utility to those presented in the literature, and may have better predictive power over a wider range of biomass composition properties due to the utilization of WRV. Furthermore, the model performed well for three different pretreatment technologies, and combinations of these pretreatments, over a range of pretreatment conditions. Much of the models found in the literature were limited to using one pretreatment type over a more narrow range of conditions.

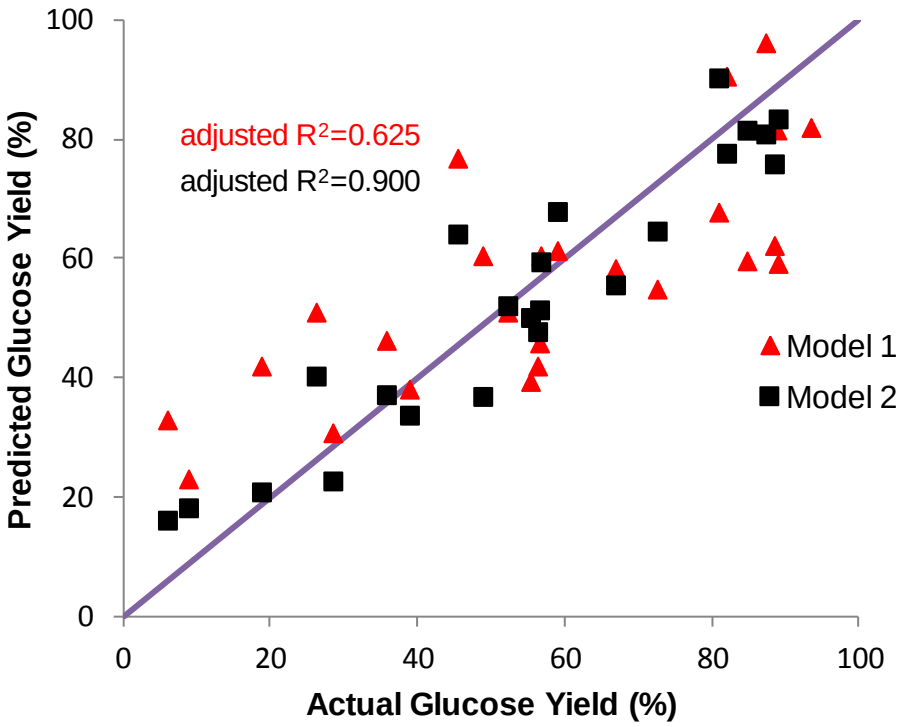

Figure 36: Predicted versus actual glucose yield for two linear regression models: model 1 includes WRV only, while model 2 includes WRV and residual xylan content after pretreatment

Conclusions

AHP delignification of corn stover and switchgrass did increase the average pore size of biomass fibers evidenced by DSC pore size distribution analysis. This corroborates with increased WRV and increased glucose yield which would be expected with increased porosity. WRV did linearly correlate well for AHP delignified and low to mild LHW pretreated corn stover and switchgrass samples as observed previously (Williams & Hodge, 2014). However, the linear correlation was not observed to more severe LHW pretreatment conditions and AFEX pretreated switchgrass. This is likely due to the relative changes in composition between lignin, xylan and cellulose that contribute to water sorption and enzyme accessibility differently. A single variable linear model, using WRV as the predictor, was not sufficient to predict glucose yield within a statistically acceptable range, R^2=0.65, for all the pretreatment types and conditions used in this work. It was found however, that AFEX pretreatment does yield material that has a compelling linear correlation between glucose yield and WRV, albeit with a different slope as that of AHP pretreated material. Therefore, by including other variables, in this case xylan content, in addition to WRV in an MLR model, the predictive power of this new multiple linear model is improved significantly, R^2=0.90.

APPENDIX

Appendix: Model Selection Using Multiple Linear Regression (MLR)

The determination of a model(s) with the best fit was done using statistical selection criteria including adjusted R^2,Mallow's Cp, and Akaike information criteria (AIC) both forward and backward step-wise. Following is a description of the analysis process for choosing the best model, which is described as Model 2 in the chapter above. Table 9 below lists all of the variables used in this analysis with the coded symbol used in most of the software outputs. Model selection begins with the full model including all four predictor variables for glucose conversion.

Table 9: Variables with coded symbols and variable descriptions

Variable	Symbol	Description
G Conv	Y	The percent of cellulose converted to glucose
WRV	WRV	Water retention value
Glucan	G	The percent glucan in residual solids
Xylan	X	The percent xylan in residual solids
Lignin	L	The percent lignin is residual solids

Analysis of Full Model

Figure 37 below shows the scatter plot matrix, residuals of the full model and the correlation matrix for the full model

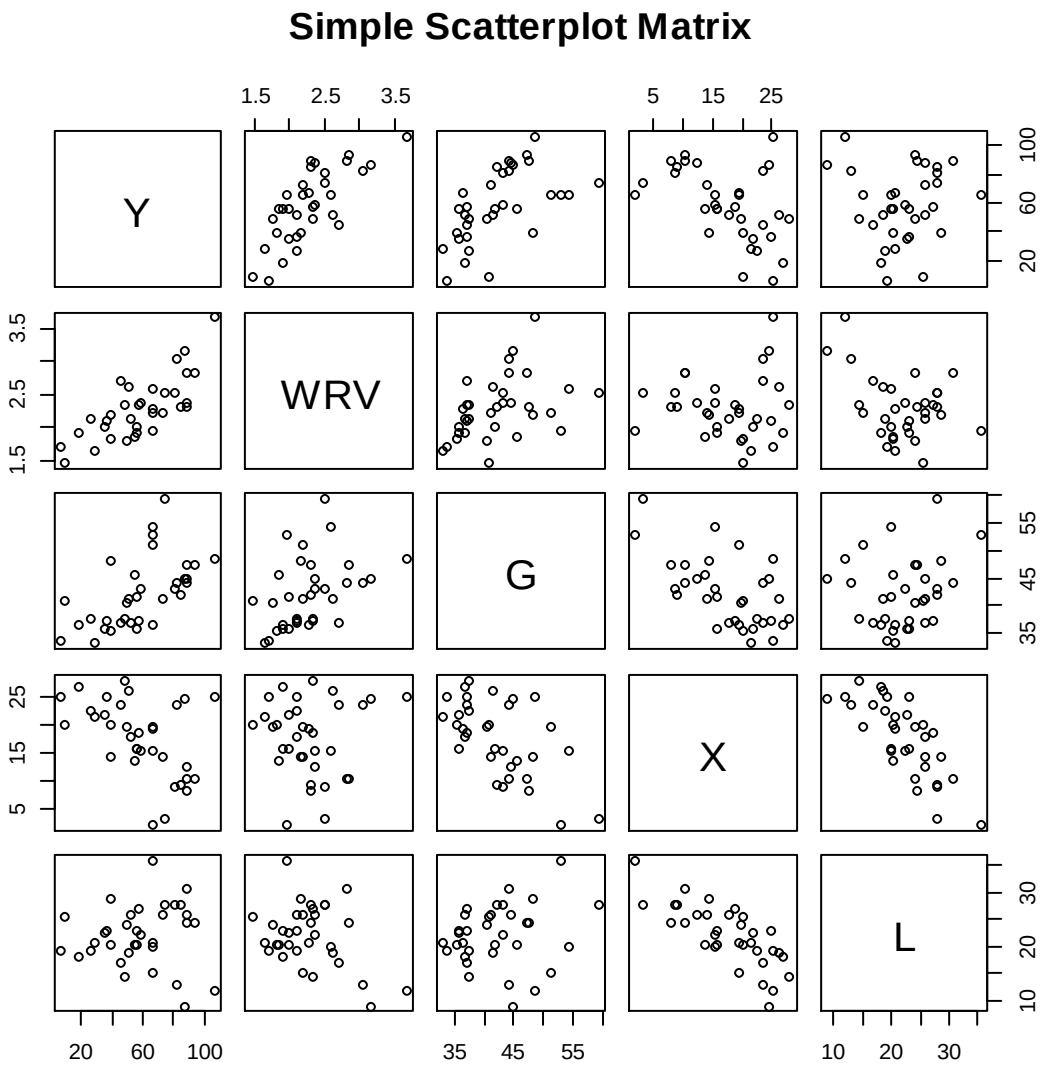

Figure 37: Scatter plot matrix for the respone variable (Y, glucose yield) and all four predictor variables

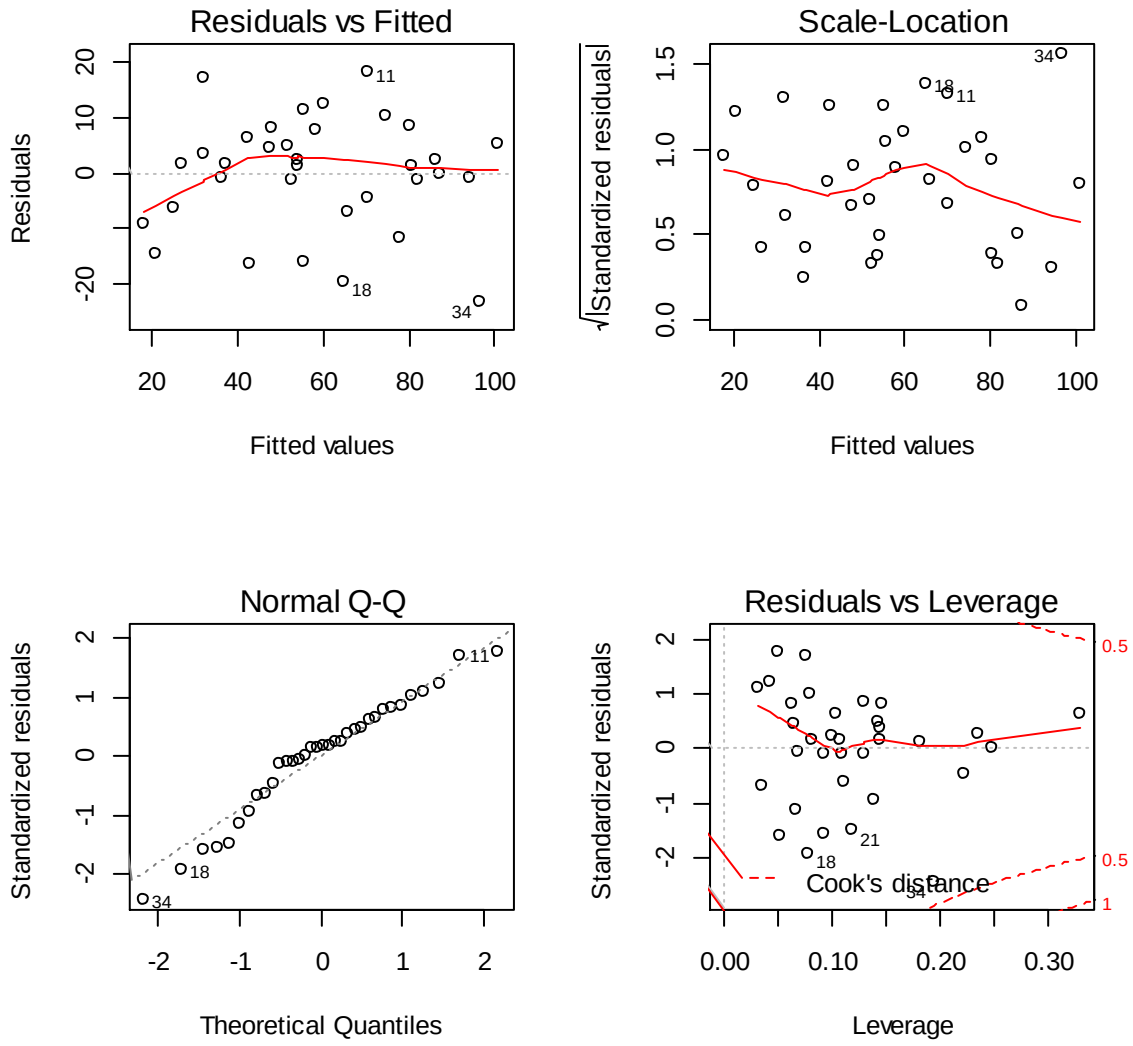

Figure 38: Residual vs Fitted plots, Normal plot and Leverage plot of all data

From the scatter plot matrix and multivariate correlations, we can see that the response is linearly associated with all of the predictors. So the first-order regression model is a good fit based on all predictor variables. Fitting the full model gives:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \varepsilon$$

or

$$\hat{Y} = 93.53 - 39.82\text{WRV} - 0.83\text{G} - 3.29\text{X} - 1.52\text{L}$$

The plot of residuals in Figure 38 doesn't show apparent evidence against normality, although there is noticeable tailing in the Normal Q-Q plot. The scatter plot matrix does show multicolinearity of the predictors, for instance, there are strong linearity between xylan and lignin.

Sub-model selection

The next step is to find some subset of predictors which is adequate to fit the response variable. Three optimal models were chosen for statistical investigation shown below, models A, B, C, and D. Model A is the full model including all four predictor variables. This model is best based on the four selection criteria investigated, AIC, Mallow's Cp, R^2, and adjR^2 all shown in Table 10. However, simpler models that only include less than all the predictors were considered as well. WRV was used in all models. Model B additionally uses glucan and xylan content, with the idea that xylan contributes more significantly to water affinity than the other components, and glucan content is an indicator of enzyme accessibility as described in the chapter above. Model C uses xylan content and lignin content in addition to WRV. And Model D uses only WRV and xylan content for reasons that will described below.

Model A: $Y = \beta_0 + \beta_1\text{WRV} + \beta_2\text{G} + \beta_3\text{X} + \beta_4\text{L} + \varepsilon$

Model B: $Y = \beta_0 + \beta_1\text{WRV} + \beta_2\text{G} + \beta_3\text{X} + \varepsilon$

Model C: $Y = \beta_0 + \beta_1\text{WRV} + \beta_3\text{X} + \beta_4\text{L} + \varepsilon$

Model D: $Y = \beta_0 + \beta_1\text{WRV} + \beta_3\text{X} + \varepsilon$

Comparison of all four models using AIC, Mallow's Cp, adjusted R^2, and R^2, is shown in Table 10 below. Model A, the full model, has the best values for all criteria shown. However, Models B, C, and D are not much behind the full model. It is reasonable then to desire to select any of these models which are simpiler in that they have fewer predictor variables, and the simplest is Model D which only contains the composition component X. To test whether G and L can be dropped from Models C and D, we determine if the coefficients β_2 and $\beta_4 = 0$. The p-values of the t test and F test are 0.36 and 0.07 for models B and C respectively (Table 11) which are both >0.05, so the hypothesis that β_2 and $\beta_4 = 0$ cannot be rejected. So it is reasonable to drop the predictor variables G and L from Models B and C, indicating that Model D is the best choice based on the selection criteria shown and for simplicity purposes.

Table 10: Selection criteria values for the four models being investigated

Model	AIC	Cp	R^2	adjR^2
Full	162.4	5	0.8533	0.8331
WRV, G, X	166.9	9.1	0.8223	0.8046
WRV, X, L	164.2	6.4	0.8362	0.8199
WRV, X	165.9	8.1	0.8175	0.8055

Table 11: Summary of t and F test for the inclusion of G and L in best model selection

t-test for Model B

```
Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  12.6678    20.8367   0.608    0.548
WRV          43.6728     4.9335   8.852 7.22e-10 ***
G            -0.4258     0.4594  -0.927    0.361
X            -2.0680     0.3862  -5.355 8.55e-06 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

F-test for Model C

```
Analysis of Variance Table

Response: Y
          Df  Sum Sq Mean Sq  F value    Pr(>F)
WRV        1 11718.0 11718.0 104.7124 2.669e-11 ***
X          1  5035.8  5035.8  45.0003 1.963e-07 ***
L          1   389.3   389.3   3.4785   0.07198 .
Residuals 30  3357.2   111.9
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Therefore, the final estimated linear regression function for Model D is

$$\hat{Y} = -3.87 + 41.17\text{WRV} - 1.82\text{X}$$

This model, Model D, is presented as Model 2 in the text of the chapter above.

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Chapter 5 - Summary of Work, Conclusions, and Future Directions

The contents of this dissertation have included practical explorations of potential process schemes and process conditions for the utilization of lignocellulosic carbohydrates for biofuel production by AHP pretreatment of grass feedstocks and alkali pretreatment integration with sorghum sugar extraction. Furthermore, this work also includes fundamental investigations of the impact of pretreatment on water/biomass interaction properties and their utility for understanding cell wall deconstruction with enzymes. In chapter 1, it was shown that AHP pretreatment delignifies and solubilizes a large fraction of lignin and xylan, and that they are removed concurrently, while leaving cellulose in tact. The effectiveness of the pretreatment is optimal at pH 11.5 and that effectiveness can be improved simply by performing pretreatment at higher solids loading, and concequently at higher temperatures. Inhibitor formation was also determined to be minimal for the feedstocks investigate here. However, to achieve relatively high digestibility improvements requires H_2O_2 at quantities that make the process cost unattractive. Future work will require strategies that reduce H_2O_2 loadings while still achieving high pretreatment effectiveness. Work in this area has been performed by colleagues using a catalytic approach where metal based catalysts improve H_2O_2 utilization, by reducing wasteful degradation reactions of the reactive oxidants, with promising successon woody biomass feedstocks (Li, Chen, Hegg, & Hodge, 2013). Another strategy is to use AHP as a delignifying post-treatment in conjunction with other pretreatment techniques such as alkali pretreatment (Liu et al., 2014). It is likely that whatever approach is taken to utilize AHP pretreatment will be feedstock specific, targeting biomass that contains a lot of alkali labile constituents and/or oxidatively susceptible lignin.

In chapter 2 it was shown that soluble sugar-rich biomass feedstock such as sweet sorghum, can incorporate existing technologies for soluble sugar extraction with lignocelluloses pretreatment to generate dirty sugar streams that are very fermentable. A combined alkali pretreatment and diffuser type soluble sugar extraction followed by separate bagasse hydrolysis utilized ~70% of the total sugars in a sweet sorghum, including both soluble and structural carbohydrates, for fermentation to ethanol at an 85% yield. Furtur work could investigate this type of approach on other sorghum types, for example high starch containing sorghums could perform extraction with amylase washes, or more alkali labile sorghums could use the technique presented here.

Chapters 3 and 4 demonstrated that biomass properties that contribute to water/biomass interactions such as swelling can be quantified and correlated to hydrolysis yield. WRV represents a useful metric for doing this, and a simple linear regression model can be developed to correlate with glucose yield for a sizable range of biomass properties. This range of properties, as well as pretreatment techniques and conditions, can be expanded and a MLR model which includes WRV and compostion information, in this case xylan content, can be used to predict glucose yield with positive results. This type of predictive model is simpler than many of the models presented in the literature and may be a useful screening tool for identifying biomass feedstocks that would be particularly well suited for effective cell wall deconstruction.

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