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Factors Contributing to Recalcitrance of Poplar to Deconstruction

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

Doctor of Philosophy

in

Chemical and Environmental Engineering

by

Samarthya Bhagia

June 2016

Dissertation Committee:

Dr. Charles E. Wyman, Chairperson
Dr. Eugene A. Nothnagel
Dr. Ian Wheeldon

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The Dissertation of Samarthya Bhagia is approved:

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_____

Committee Chairperson

University of California, Riverside

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DEDICATIONS

This dissertation is dedicated to my mummy, Dr. Minoo Bhagia.

ABSTRACT OF THE DISSERTATION

Factors Contributing to Recalcitrance of Poplar to Deconstruction

by

Samarthya Bhagia

Doctor of Philosophy, Graduate Program in Chemical and Environmental Engineering
University of California, Riverside, June 2016
Dr. Charles E. Wyman, Chairperson

Cellulosic ethanol as a transportation fuel can cut greenhouse gas emissions for a sustainable earth and lower dependence on fossil fuels. However, successful penetration into an oil dominated market can only come through cost-competitiveness with gasoline, but biological conversion of cellulosic biomass to fuels is currently stymied by the need for high enzyme doses to realize commercially relevant yields for the widely pursued biological route of pretreatment followed by enzymatic hydrolysis to produce fermentable sugars. Achieving high sugar yields at low enzyme loadings can benefit from a better understanding of factors that contribute to biomass recalcitrance to deconstruction. When poplar varieties with lower lignin content due to a rare natural mutation associated with lignin biosynthesis were subjected to a high throughput pretreatment and co-hydrolysis platform, they gave higher sugar yields than standard poplar, but temperature pretreatment severity affected their rankings. In another approach to understand factors responsible for recalcitrance, application of flowthrough and batch pretreatment with dilute acid and just liquid hot water to standard poplar indicated that

flowthrough pretreatment solubilized and removed 65 to 70% of the lignin before it could react further to low solubility lignin rich fragments that otherwise deposit on biomass in batch operations and hinder enzyme action. In subsequent work, combinations of enzymes and bovine serum albumin applied to solids produced by aqueous flowthrough and batch pretreatments revealed how polysaccharides in poplar were successively deconstructed layer by layer through pretreatment and subsequent hydrolysis by cellulase and xylanase combinations and through BSA addition. The standard strong acid hydrolysis procedure for measuring carbohydrate and lignin content in biomass was shown to be robust over a range of particle sizes, reaction times, and filtration strategies. Finally, elimination of shaking resulted in enzyme loadings of only 5 mg protein/g glucan able to realize nearly complete cellulose conversion, similar yields to those achieved with shaking if surfactants were added. This surprising result suggest that surfactants protect enzymes from deactivation that only occurs in shaken flasks at low enzyme to substrate ratios.

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Chapter 1

Introduction

1.1 The Need for Sustainable Fuels

World energy consumption has tripled in the last fifty years (Tverberg, 2012). The world energy demand is expected to have an average growth of 1.1% every year, and increase by 37% from today to 2040, primarily due to emerging economies in developing countries like China and India (IEA, 2015). Human development index, a factor that counts standard of living, gross domestic product per capita, longevity, and educational attainment, is closely linked with primary per capita energy consumption (UNDP, 2008). Today, about 87% of the world energy demand is met by fossil fuels that include petroleum, natural gas, and coal, and will likely rise in the future due to increase in total energy consumption (IEA, 2015). There is no doubt that fossil fuels will eventually be depleted in about 75 to 235 years (Mohr et al., 2015; Shafiee & Topal, 2009), as the time scale for their regeneration is more than 10^5 times larger than their consumption (Tester, 2005). At the current rate of consumption, petroleum is going to be the most short-lived, as little as 35 years, followed by natural gas and then coal (Shafiee & Topal, 2009). There is uncertainty in prediction of Hubbert oil peak, which some say has already passed, or is going to occur in the near future (Abas et al., 2015; Chapman, 2014). But in any case, it is becoming harder to recover petroleum (Murray & King, 2012), and may lead to inflation in prices leading to economic crisis. As reserves diminish, non-OPEC countries will face strained relations and strategic vulnerability with OPEC countries as they control about 81% of proven reserves (Metz et al., 2007), many of which are politically unstable (Furman & Sperling, 2013). Other than this, the environmental damage by fossil fuels is a big concern today. 65% of total greenhouse gas emissions come from carbon

dioxide through combustion of fossil fuels (EPA, 2016). 25 to 30% of carbon dioxide released from surface remains in the atmosphere after equilibrating with ocean for two to twenty centuries (Archer et al., 2009), and is the primary cause of radiative forcing. Since 1880, when proper data collection began, surface temperature of earth has risen by 0.8°C and has been strongly linked to carbon dioxide emission due to anthropogenic activities that started since the industrial revolution in the 1800s. This is a very large change considering that 1 to 2°C drop in average temperature led to a Little Ice Age in the past (NASA, 2014).

Transportation sector alone consumes little more than 60% of petroleum worldwide, out of which roughly 50% comes from gasoline use by light duty vehicles and amounts to about 90 million barrels per day (1 oil barrel = 42 US gallons) (WEC, 2011). Reducing such high consumption of gasoline requires replacing it with some other onboard energy storage system from a renewable source. Potential of solar energy is anywhere between 1500 quads and 47000 quads per year. The total energy we consume today is 524 quads and will go up to 820 quads by 2040 (EIA, 2013). One way to utilize solar energy is to use photovoltaics that generate electrical energy and is stored as chemical energy in batteries that drive the electric engine. However, this process requires plenty of earth metals and energy for manufacturing of semiconductors and batteries (Crabtree & Sarrao, 2009).

The other way is to combine six molecules of carbon dioxide, six molecules of water, and solar energy together and form one molecule of glucose and six molecules of oxygen through a process that originated 3.5 billion years ago known as photosynthesis in

plants (Blankenship, 2010). Glucose then serves as an intermediate for biosynthesis of all organic compounds by plant cells. Bulk of the plant is made up of three main components by weight, cellulose, hemicellulose and lignin, and the rest from pectin, extractives, and ash. Cellulose is the largest polymer that is made entirely through polymerization of glucose, hemicellulose from polymerization of a variety of sugars, and lignin is a heterogeneous polymer designed from aromatics, and thus is popularly known as lignocellulosic biomass. Greater than 65% of the bulk of the plant is made of sugars and these can be fermented to ethanol (Lynd et al., 1999). According to recent estimates, we may be able to use roughly 1000 mha (million hectares) for growing dedicated bioenergy crops worldwide on marginal agricultural land (Cai et al., 2011). Considering that about 100 gallons or 2.38 barrels of ethanol can be produced from one dry metric ton of biomass (Wyman, 2007), and with a conservative productivity of 10 dry metric tons per hectare in one year, we can produce about 67 million barrels of ethanol per day. Energy density of ethanol is one-third of gasoline. But ethanol average octane number is between 96 and 113 which is higher than gasoline with average octane number of 86-96, which allows higher compression ratios and thus estimated 15% higher efficiency in spark ignition engine (Bailey, 1996) and a reason other than going "green" for Indianapolis 500 to fuel its race cars with 100% ethanol (Holmseth, 2008). Thus, near 52 million gasoline equivalent barrels per day out of 90 from gasoline may be replaced by cellulosic ethanol. Most of the technological needs for running cars on ethanol have already been addressed as high blends of ethanol with gasoline have been successfully used in flexible fuel vehicles by Brazil for the past 37 years that was a result of the oil embargo of 1973

(Potter, 2008). Although ethanol is already used today as a blend with gasoline in US, and is restricted by the E10 "blend wall," it comes from corn kernels through fermentation of starch in them. Corn ethanol may or may not be sustainable in the long run as the amount of energy gained over energy spent in its production is 4 to 9 mega joules per liter rather than 23 mega joules per liter for cellulosic ethanol (Farrell et al., 2006). Corn ethanol reduces carbon dioxide equivalent greenhouse gas emission by 13% versus 88% for cellulosic ethanol (Farrell et al., 2006). Even low input high diversity native grassland perennials can easily sequester net 3 tons of carbon dioxide per hectare in one year in soil and roots (Tilman et al., 2006). Assuming plantation area of 1000 mha worldwide, carbon dioxide in the atmosphere can be reduced from 404 ppm today to 350 ppm in 140 years, and to pre-industrial level of 283 ppm in 315 years. Plants might be the least expensive way to sequester carbon dioxide.

1.2 Biological Conversion of Cellulosic Biomass to Ethanol

The widely accepted biological route of cellulosic ethanol production was used in this work. Briefly, lignocellulosic biomass is processed mechanically to the appropriate size and fed to a pretreatment stage followed by enzymatic hydrolysis stage for sugar recovery, and these sugars are then fermented to make ethanol. Market penetration and establishment of cellulosic ethanol however can come only through cost-competitiveness with gasoline. Currently, due to nautral resistance of plants to deconstruction, otherwise called recalcitrance, relatively large amounts of costly enzymes are required to breakdown cellulose in pretreated biomass that reduces the profitability from cellulosic ethanol. Therefore, this biological process was studied to understand factors that

contribute to recalcitrance in biomass faced by enzymes, with the overall goal of maximizing sugar yield while keeping processing costs low.

One way to learn about these factors can be through regulating genes that control lignin or xylan biosynthesis in plants. If these mutations are able to successfully lower recalcitrance through reducing lignin content or modifying the architecture of lignocellulosic biomass, they can be used to generate next generation biofuel feedstocks (Chen & Dixon, 2007). However, one other way to achieve this target can be through finding plants that have natural mutations that may affect sugar yields and this genetic information can be used to generate better candidates (Studer et al., 2011). In another approach, behavior of lignin removal/relocation with pretreatment can be investigated. Lignin has a tendency to redeposit onto cellulose in batch pretreatment process (Li et al., 2014). However, in flowthrough pretreatment this re-deposition is reduced or prevented due to continuous removal of solubilized products. This can help in gaining valuable insight into biomass deconstruction with pretreatment for improving such processes. Enzymatic hydrolysis on such flowthrough pretreated solids with reduced lignin can be compared with batch pretreated solids for finding features that affect deconstruction. Also, mass balances for sugars are critical to account for their fate when being deconstructed from biomass through pretreatment followed by enzymatic hydrolysis. Closing mass balances requires measuring both solid and liquid phases for amount of sugars. While sugars in liquid phase can be quantified with good certainty, standard strong acid hydrolysis procedure for determining sugars content of solids may have significant variability that has never been studied in spite of its popularity. Moreover,

enzymatic hydrolysis process can be improved through use of surfactants but needs investigation at low enzyme loading to complement the many studies of surfactants already done at high enzyme loading. Thus, sugar yield from natural plants with a rare mutation suspected to affect sugar yields, was studied with regard to aqueous pretreatment processes, effect of enzymes on solids produced by these processes, procedure sugar and lignin determination in solids, and impact of surfactants in enzymatic hydrolysis.

1.3 Thesis Organization

Poplar (*Populus trichocarpa*) was used throughout this work as the source of lignocellulosic biomass. It is a hardwood species, other than grass species Swtichgrass (*Panicum virgatum*) for research within the BioEnergy Science Center (BESC). Chapter 2 discusses characteristics of lignocellulosic biomass, the process of cellulosic ethanol production and prior work related to this dissertation. Chapter 3 discusses how poplar varieties with reduced recalcitrance can affect sugar yield. A few rare poplar varieties were discovered within BESC that carried a natural mutation that affects lignin biosynthesis. Lignin content and structural characteristics can affect recalcitrance of lignocellulosic biomass to deconstruction. This is the first study of its kind to discuss ranks of a single species of biofuel feedstock with varying recalcitrance and pretreatment severity. Chapter 4 compares sugar yields and lignin removal of standard poplar from aqueous flowthrough pretreatment with batch pretreatment followed by enzymatic hydrolysis. Removal of liquefied products from solids with flowthrough pretreatment allows for understanding of features contributing to recalcitrance of biomass. Chapter 5

involves work on the use of various combinations of enzymes (cellulases and xylanases) and bovine serum albumin (BSA) on solids generated by aqueous flowthrough and batch pretreatments. This approach gave insight into factors that limit enzymatic hydrolysis yields through varying the enzyme dose, supplementing xylanase to cellulase, and blocking surface of lignin with BSA from causing unproductive loss of enzyme. Chapter 6 discusses the variations in carbohydrates and lignin contents in poplar found through the NREL (National Renewable Energy Laboratory, Golden CO) standard strong sulfuric acid hydrolysis procedure. Sugar and lignin mass balances hinge on this analytical procedure and was investigated for expected variation in the data when experimental conditions are modified drastically. Chapter 7 discusses how small quantity of surfactant can cause large improvements in saccharification of cellulose at low enzyme loading. Many other experiments were carried out to elucidate the possible mechanism of surfactant in enzymatic hydrolysis. The last Chapter 8 summarizes main findings and conclusions from this dissertation.

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Chapter 2

Pretreatment Options for Reducing Recalcitrance of Lignocellulosic Biomass

2.1 Abstract

Cellulosic ethanol holds the potential to fulfill our need of a sustainable renewable transportation fuel to reduce gasoline consumption and to mitigate the emissions of greenhouse gases such as carbon dioxide. Currently, the cost of producing cellulosic ethanol is higher than gasoline which challenges its successful penetration into oil dominated market of today. Biological route of recovering sugars from lignocellulosic biomass involves use of carbohydrate hydrolyzing enzymes but requires a pretreatment step to make biomass more amenable to enzymes and microorganisms for realizing high sugar yields. Currently, the high cost of cellulosic ethanol from this biological route is due to energy or chemical cost associated with pretreatment step, and high doses of relatively expensive enzymes. Cost reductions can come through knowledge of biomass deconstruction through application of various pretreatment technologies and their effect on subsequent enzymatic hydrolysis and fermentation steps. Thus, this chapter discusses the salient features of promising pretreatment technologies that reduce recalcitrance of lignocellulosic biomass to deconstruction by enzymes.

2.2 Introduction

Lignocellulosic biomass contains three major components, cellulose, hemicellulose, and lignin, by weight along with minor amounts of pectin, extractives, and ash. Cellulose and hemicellulose are a resource of sugars, and lignin for aromatics or solid fuel (Lynd et al., 1999). Cellulosic ethanol for use as a renewable transportation fuel can be made from fermentation of sugars locked in the form of cellulose and hemicellulose in lignocellulosic biomass (Mosier et al., 2005). However, the largest

polymer in biomass, cellulose, has a stable structure and can be solubilized only at temperatures above 220°C (Bobleter, 2004; Yan et al., 2014). Hemicellulose sugars are more susceptible to solubilization at relatively lower temperatures and/or low pH conditions (Bobleter, 1994). The drawback of solubilizing cellulose at very high temperatures is that there is significant degradation of sugars at high temperatures that lowers the overall sugar yield from biomass (Faith, 1945). Moreover, fermentability of sugar streams accompanied by sugar degradation products such as furfural and 5-hydroxylmethylfurfural is difficult as they inhibit growth of ethanol producing microorganisms (Wyman, 2007). Also, high temperature operations increase the capital and operating costs (Yang & Wyman, 2007b). On the other hand, enzymatic saccharification that uses fungal and/or bacterial enzymes can be employed to completely solubilize cellulose at a much lower temperature of ≤55°C and at a much higher selectivity. This biological route is currently the most promising route for high yield of sugars for fermentation to cellulosic ethanol (Figure 2.1). However, enzymatic hydrolysis of biomass without any prior treatment results in very low yields due to inaccessibility of bulky enzymes to penetrate biomass, and high coverage of hemicellulose and lignin on to cellulose surface that limits enzyme binding sites. Therefore, a pretreatment step is used before enzymatic hydrolysis that increases surface area and may remove or modify hemicellulose and lignin that leads to high sugar yields by enzymes (Lynd et al., 1999).

Even though this process can realize high sugar yields, its commercialization requires sugar recovery at low cost that is currently mired by larger than economical dose of enzymes (Wyman, 2007). Thus, this review will discuss various pretreatment options

and their salient features for assisting enzymes in deconstruction of lignocellulosic biomass. Pretreatments on a broad level can be categorized as mechanical, thermochemical, and biological pretreatments. However, some studies have reported using combination of mechanical with thermal and/or chemical pretreatment, and also thermochemical combined with biological pretreatment to aid in enzymatic deconstruction of biomass. Overall, the best pretreatment is the one that achieves lowest cost of cellulosic ethanol.

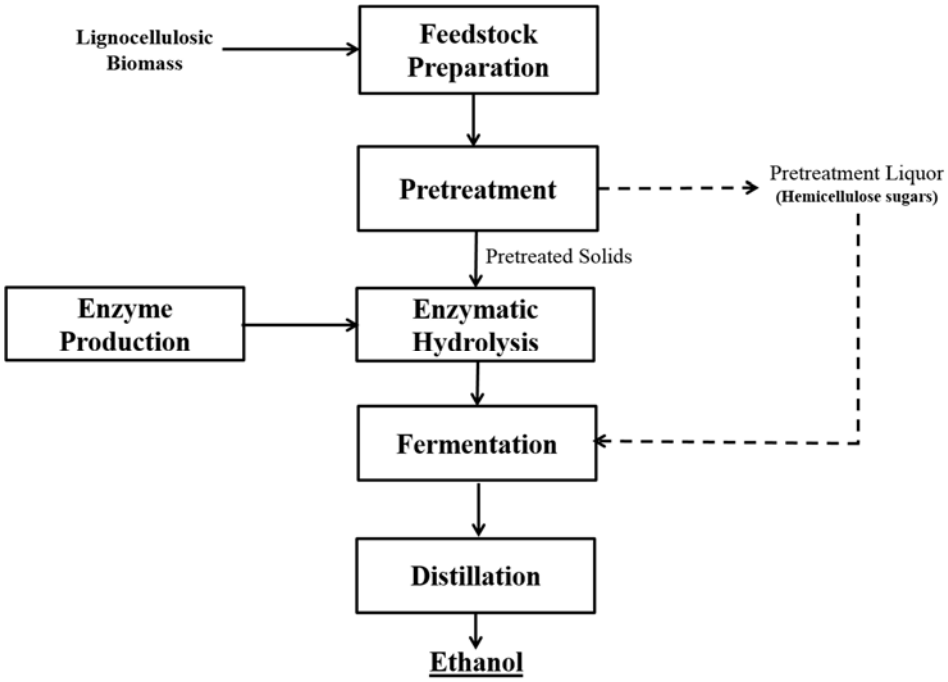

Figure 2.1. Biological route of cellulosic ethanol production.

2.3 Pretreatment Options

2.3.1 Mechanical Pretreatment

Many mechanical or physical pretreatments fractionate biomass into small sizes through milling. This increases surface area of biomass and reduces heat and mass transfer limitations. Forces acting on biomass through mechanical pretreatment can be divided into compression, impact, shear, and friction forces. Knife, hammer, pin, and centrifugal milling act through impact and shear forces. Ball milling reduces size through impact and compression forces, jet milling through impact and friction forces, and extruders through shear forces (Barakat et al., 2014). Ball milling in particular reduces crystallinity of cellulose that enhances enzymatic deconstruction significantly. Ball milling pretreatment of oil palm biomass realized 85% total sugar yield from enzymatic hydrolysis (Zakaria et al., 2014). One study tested chemical ball milling in acidic and alkaline environments and found that alkaline ball milling that delignified corn stover gave the best improvement of 110% in enzymatic hydrolysis efficiency (Lin et al., 2010).

Thermo-mechanical pretreatments involve use of extruders at higher temperatures (Lamsal et al., 2010; Yoo et al., 2011). This process requires grinding of biomass and then feeding to an extruder that causes disruption of cell wall structure due to shear which may lead to improved enzymatic saccharification. The advantage of such an extrusion process is that pretreatment can be carried out at high solids loading. Screw speed, compression ratio, and barrel temperature have an effect on digestibility of biomass (Karunanithy & Muthukumarappan, 2009; Zheng & Rehmann, 2014). One study reported thermo-mechanical extrusion for pretreatment of soybean hulls (Yoo et al., 2011).

Soybean hulls mixed with processing aids such as corn starch or ethylene glycol with a final moisture content of 20 to 25% were extruded in a twin-screw extruder at temperature between 80 and 140°C. Processing aids were needed in this extrusion process to improve flow of soybean hulls with low moisture, otherwise biomass burned or blocked the die of extrusion press. However, through increasing the moisture content to 40%, cellulose digestibility of 95% could be achieved without any processing aid. Moreover, the extruder had problems operating above 140°C temperature due to high pressures (Yoo et al., 2011).

In ultrasonic pretreatment, hydrodynamic shear forces produced through ultrasonic waves (20 kHz to 10 mHz) fractionate and increase surface area of biomass (Nitayavardhana et al., 2008; Sasmal et al., 2012). In one study, the highest reducing sugar yield was 22 g per 100 g cassava sample (Nitayavardhana et al., 2008). They also found that sugar yield was higher when enzyme was added before ultrasonication than after. Ultrasonication at a frequency of 40 kHz for 5 minutes at 60°C improved ethanol concentration of corn meal by about 11% (Nikolić et al., 2010). Another study used ultrasonication to cut down long time of lime pretreatment (Sasmal et al., 2012).

2.3.2 Leading Thermochemical Pretreatments

2.3.2.1 Liquid Hot Water (LHW) Pretreatment

Liquid hot water (LHW) pretreatment is carried out in only water as a solvent and usually between temperatures of 140 and 220°C (Bobleter, 1994; William & Schoen, 1952). High temperature leads to removal of hemicellulose but small amount of lignin

(Bobleter, 2004). Removal of hemicellulose has been correlated to enzymatic digestibility (Yang & Wyman, 2004). At high temperatures, acetic acid released from acetylated xylan in hemicellulose can lower the pH between 3 to 4 which aids in hydrolysis of hemicellulose (Yang & Wyman, 2007a). Optimum sugar yields from biomass are however, only achieved through pretreatments at temperatures above 180°C (Bin et al., 2004; Garlock et al., 2011). Also, a large portion of hemicellulose sugars recovered in LHW pretreatment liquor are in oligomeric form which requires a subsequent hydrolysis step for conversion into monomeric sugars for fermentation (Zhang et al., 2015).

2.3.2.2 Dilute Acid (DA) Pretreatment

Dilute acid pretreatment is usually carried out in 0.5 to 2 wt% sulfuric acid in water. Optimum sugar yields are realized at much lower temperatures than LHW and results in very high hemicellulose removal but small amount of lignin removal from solids (Grohmann et al., 1987). However, low pH pretreatments can degrade sugars into products such as furfural and 5-hydroxylmethylfurfural that are inhibitors to ethanol producing organisms. Moreover, acidic sugar stream needs neutralization prior to fermentation. Neutralization with lime produces gypsum that is a low value product. However, recovery of hemicellulose sugars in monomeric form and high enzymatic digestibility of dilute acid pretreated biomass are benefits of dilute acid pretreatment (Mosier et al., 2005; Yang & Wyman, 2007a).

2.3.2.3 Flowthrough Pretreatment

Flowthrough pretreatment is a percolation type pretreatment where solvents such as liquid hot water and dilute acid are passed through a reactor bed filled with biomass at high temperatures (Bobleter, 1994; Hörmeyer et al., 1988). Figure 2.2 shows the experimental setup of flowthrough pretreatment (Liu & Wyman, 2005). The advantage of such a pretreatment is that products are continuously removed from the reactor as they are formed. Unlike batch operations, this does not give time for hemicellulose to degrade. The key advantage of this pretreatment is that along with hemicellulose, lignin is also removed from the reactor and prevents re-deposition onto cellulose. Therefore, the solids produced by flowthrough pretreatment have lower lignin content than those produced by batch pretreatment performed at the same temperature, time, and aqueous conditions. The lower lignin content in flowthrough pretreated solids greatly improves cellulose digestibility by enzymes (Yang & Wyman, 2004). However, flow of liquid produces a dilute sugar stream that increases cost of downstream processes.

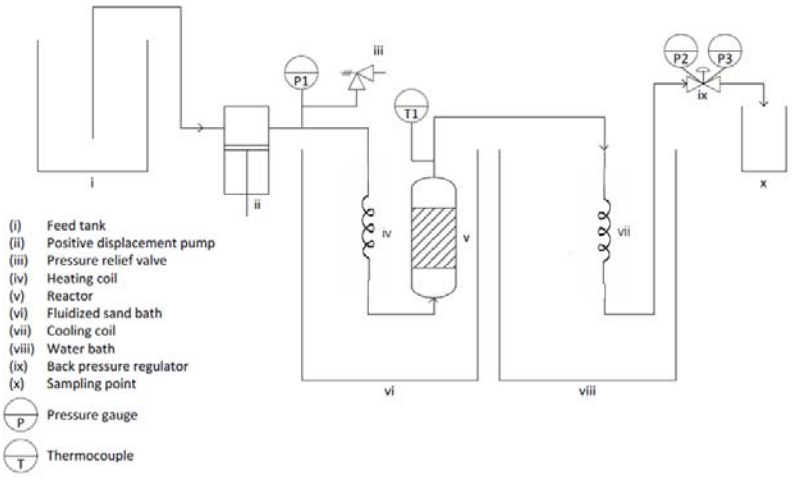

Figure 2.2. Schematic diagram of flowthrough pretreatment technology

2.3.2.4 Steam Explosion Pretreatment

Steam explosion pretreatment involves direct heating of biomass with steam and may involve use of acid or sulfur dioxide (Brownell & Saddler, 1987). Gaseous sulfur dioxide can penetrate biomass uniformly and rapidly compared to sulfuric acid (Shi et al., 2011). However, sulfur dioxide gas may pose a safety hazard (Yang & Wyman, 2007a). Greater than 95% recovery of glucose and 62 to 82% recovery of pentose sugars was reported for douglas-fir for steam pretreatment at 200°C for 5 minutes in 4% SO_2 (Kumar et al., 2010).

Steam explosion is a technology was developed in 1926 by William Mason (Mason, 1926). Steam explosion is carried out by heating the biomass with steam in the 180 to 240°C temperature range under pressure of 1 to 3.5 MPa followed by explosive decompression that may fracture cell walls or increase pore size for improved enzyme accessibility (Brownell & Saddler, 1987; Mackie et al., 1985). Steam explosion pretreatment without acid or SO_2 can lead to about 50% degradation of pentose sugars. However, biomass impregnation with SO_2 or H_2SO_4 leads to much lesser degradation (Mackie et al., 1985).

2.3.2.5 Lime Pretreatment

Lime pretreatment is carried out using slaked lime (calcium hydroxide) in oxidative or non-oxidative conditions. Oxidative conditions allow high lignin removal. Lower temperatures require longer time for pretreatment. Loading of lime is determined by adding excess lime in the initial pretreatment and excess lime calculated through titration with hydrochloric acid after pretreatment (Sierra et al., 2009). Lime consumption

increases with temperature and in oxidative conditions (Kim & Holtzapple, 2005). Three categories of this pretreatment exist. In short-term category, biomass is pretreated with lime at temperatures between 100 to 160°C and can last up to 6 hours with or without oxygen. In long term category, biomass is pretreated at 55 to 65°C up to 8 weeks in presence or absence of aeration. The third category involves boiling the aqueous slurry for 1 hour and maybe carried out in presence of oxygen or air (Falls & Holtzapple, 2011; Sierra et al., 2009). In one study, lignin removal of about 60% was achieved for switchgrass using short term lime pretreatment at 120°C for 3 hours under 100 psi oxygen and at a loading of 1 g lime per g biomass (Garlock et al., 2011). In a long term strategy, about 48% lignin removal was achieved for corn stover pretreatment at 55°C after 16 weeks in non-oxidative conditions with a lime consumption of near 0.06 g per g biomass. However, in oxidative conditions, lignin removal increased to 87.5%. This same study found that there was no inhibitory effect of pretreatment liquor on yeast fermentation to ethanol (Kim & Holtzapple, 2005).

2.3.2.6 Ammonia Fiber Expansion (AFEX)

Ammonia fiber expansion or expansion pretreatment uses liquid ammonia at a loading of 1 to 3 kg per kg biomass and pretreatment temperature of 60 to 100°C followed by explosive decompression that expands ammonia gas (Alizadeh et al., 2005). This causes swelling of biomass and partial loss of crystallinity of cellulose (Mosier et al., 2005). Unlike neutral and acidic pretreatments, AFEX does not physically remove much hemicellulose and lignin, however, it makes the biomass more digestible by enzymes by relocating lignin and hemicelluloses and creating larger pores (Balan et al.,

2009). Moreover, this pretreatment is more effective in reducing recalcitrance for grasses but is not much effective for woods (McMillan, 1994). In one study, AFEX pretreatment of switchgrass at loading of 1:1 ratio of ammonia to biomass at 100°C for 5 minutes led to 93% glucan conversion by enzymatic hydrolysis (Alizadeh et al., 2005). Residual ammonia in biomass solids can be used as a nitrogen source by the yeast (Yang & Wyman, 2007a). Due to the large amount of ammonia used for pretreatment, its recovery becomes important to keep pretreatment costs low (Tao et al., 2011; Yang & Wyman, 2007a).

2.3.2.7 Ammonia Recycle Percolation (ARP)

Ammonia recycle percolation is a flowthrough-type pretreatment where 5-15% aqueous ammonia is passed through a reactor bed filled with biomass between 80 and 180°C and recirculated. This process separates cellulose, hemicellulose, and lignin from each other (Iyer et al., 1996; Yoon et al., 1995). ARP pretreatment of hybrid poplar at 180°C for 1 h with 10% ammonia at a flow velocity of 1 ml/min led to 95% digestibility of resulting solids after 72 h of enzymatic hydrolysis (Yoon et al., 1995). In one study with corn stover, ARP pretreatment at 170°C for 90 minutes with 15% aqueous ammonia at 5 ml/min caused 85% delignification and digestibility of 92% from enzymatic hydrolysis at an enzyme loading of 10 FPU/g glucan after 72 hours (Kim & Lee, 2005a).

2.3.2.8 Soaking in Aqueous Ammonia (SAA)

Soaking in aqueous ammonia involves batch pretreatment of biomass with aqueous ammonia at temperatures less than 100°C for 6 to 48 hours (Kim & Lee). SAA

pretreatment can cause large lignin removal but small hemicellulose removal from biomass (Kim & Lee, 2005b; Kim & Lee). In one study, SAA pretreatment of rapeseed straw with 20% ammonia at 69°C for 14 hours resulted in about 50% lignin removal and 60% digestibility of cellulose in pretreated solids from enzymatic hydrolysis at an enzyme loading of 30 FPU (filter paper unit) + 30 CBU (cellobiose unit) per gram glucan (Yoon et al., 1995). In another study, SAA pretreatment of corn stover at room temperature for long times (10 to 60 days) without mixing at atmospheric pressure resulted in 55 to 74% lignin removal and 15% xylan removal and an overall ethanol yield of 77% based on glucan and xylan in substrate (Kim & Lee, 2005b).

2.3.2.9 Alkaline Hydrogen Peroxide (AHP)

Alkaline hydrogen peroxide uses hydrogen peroxide with sodium hydroxide at pH of 11.5 for pretreatment of biomass that removes lignin and hemicellulose from solids. In alkaline conditions, hydrogen peroxide decomposes to form hydroxyl and perhydroxyl radicals that remove lignin from biomass (Azzam, 1989). AHP pretreatment of sugar cane bagasse was found to be optimum at loading of around 7% (v/v) of peroxide for 1 hour at 25°C. The pretreated solids resulting from this condition gave the highest glucan yield of 69% from enzymatic hydrolysis at 3.5 FPU + 1 CBU per gram of pretreated biomass (Rabelo et al., 2011). In another study with sugar cane bagasse, AHP pretreatment with 2% peroxide at 30°C removed 50% lignin and most of the hemicellulose after 8 h (Azzam, 1989). About the same lignin and hemicellulose removal was found with AHP pretreatment of several agricultural residues such as wheat straw and corn stover at 25°C with 1% peroxide at pH 11.5 after 24 hours. Near theoretical

saccharification of cellulose in AHP pretreated solids was achieved through enzymatic hydrolysis at high enzyme loading (Gould, 1984).

2.3.2.10 Pretreatments in Organic Solvents

Batch pretreatment of biomass at 160 to 180°C in organic solvents with acid catalyst enables high hemicellulose removal along with lignin. This produces cellulose rich pretreated solids that may be completely digested by enzymes (Zhao et al., 2009). The most popular of these pretreatments is called organosolv pretreatment that frequently applies equal ratio of ethanol to water in dilute sulfuric acid. Without catalyst, hemicellulose and lignin removal are generally low. Holtzapple and Humphery (1984) It was found that butanol was as effective as ethanol in organosolv pretreatment of poplar (Holtzapple & Humphrey, 1984). Moreover, they found that ethanol with $FeCl_3$, H_2SO_4, and NaOH catalysts caused high lignin removal but cellulose saccharification was much lower with $FeCl_3$ and H_2SO_4. One peculiar nature of these organosolv pretreatments in acidic conditions is that lowering the organic solvent below 50% can cause as much as 20% removal of crystalline cellulose along with lignin and hemicellulose. Hemicellulose degradation becomes high at low organic solvent (methanol, ethanol, and butanol) to water ratios (Chum et al., 1988; Holtzapple & Humphrey, 1984). Another approach utilized concentrated phosphoric acid in organic solvent (acetone or ethanol) and water mixtures. Concentrated phosphoric acid makes crystalline cellulose amorphous and this kind of pretreatment at 50°C for 1 hour led to 88% enzymatic digestibility of cellulose in bamboo at an enzyme loading of 1 FPU per gram glucan (Sathitsuksanoh et al., 2010). Other than primary alcohols, polyhydroxyalcohols such as glycerol and ethylene glycol

have also been used (Lee et al., 2011; Sun & Chen, 2008; Thring et al., 1990). Sun and Chen (Sun & Chen, 2007) found greater than 70% lignin removal and 95% enzymatic digestibility of cellulose from pretreatment of wheat straw at loading of 15 g glycerol per g biomass carried out at 240°C for 4 hours. Other organic solvents such as ethers, ketones, dimethylsulfoxide, and phenols can also be used (Zhao et al., 2009). Recently, it has been found that tetrahydrofuran (THF) can be a good organic solvent for pretreatment of biomass. Batch pretreatment of corn stover at a temperature of 150°C for 25 minutes in 1:1 ratio by volume of THF to water in 0.5 wt% sulfuric acid, led to ethanol yield of 89.2% from simultaneous saccharification and fermentation (SSF) at 11% glucan solid loading and at an enzyme loading of 15 mg protein per g glucan after 120 hours (Nguyen et al., 2016).

2.3.2.11 Pretreatment with Ionic Liquids

Ionic liquids are salts whose melting points are below 100°C. Many alkylimidazolium salts carry the ability to solubilize crystalline cellulose in biomass through disrupting its strong hydrogen bonding network. However, cellulose solubilization is dependent on the ionic liquid to water ratio. (Tadesse & Luque, 2011). 1-ethyl-3-methylimidazolium acetate ([Emim][Ac]) was able to completely solubilize southern pine softwood in 46 hours and red oak hardwood in 25 hours at 110°C at a biomass loading of 5% in nearly pure ionic liquid (Sun et al., 2009). Dissolution of lignin has been found to be dependent on the nature of anions (Pu et al., 2007). Biomass dissolved in ionic liquid after pretreatment requires use of an anti-solvent to precipitate cellulose (Shill et al., 2011). Water can be added to ionic liquid to precipitate cellulose but formation of a gel phase

makes its recovery tedious. Instead equal ratios of water and acetone can be used to avoid gel formation at room temperature. Further, evaporation of acetone precipitates lignin leaving ionic liquid behind. A newer approach involves using an aqueous solution of a kosmotropic anion such as phosphate, carbonate, or sulfate as an anti-solvent. This separates the system into three phases, a solid cellulose phase, a salt rich aqueous phase, and an ionic liquid rich phase with most of the lignin (Shill et al., 2011; Sun et al., 2009). Precipitated cellulose from this process is highly digestible by enzymes (Tadesse & Luque, 2011). One major drawback of using imidazolium based ionic liquids is that even a small amount leftover in precipitated cellulose can deactivate enzymes (Wang et al., 2011). This problem has been tackled with use of cholinium amino acids ionic liquids (Hou et al., 2012).

2.4 Biological Pretreatment

2.4.1 Overview

Evolution of terrestrial plants began millions of years ago with development of aromatic compounds to protect plants from UV damage. Plants have evolved for a millions years and the Darwinian process has led to a cell wall architecture in plants which protects it from biological, thermal and chemical attack. Nevertheless, a wide variety of fungi and bacteria have established metabolic pathways to survive on living and dead plant matter. Wood decaying microorganisms have been investigated as early as 1815 due to the negative impacts on the structural strength of timber (Bowden, 1815). Certain white-rot fungi can deconstruct plant cell walls in weeks. These fungi also find application in bio-pulping, bio-bleaching, detoxification of thermochemical pretreatment

hydrolysate, and in decontaminating the environment free from xenobiotics and organic wastes. Recently, molecular clock analyses of white-rot fungi has provided a possible explanation in the sharp slowdown of coal formation at the end of Permo-carboniferous era as these fungi developed lignin decaying mechanisms during that time (Floudas et al., 2012). Only recently, their potential for pretreatment of lignocellulosic biomass has been realized due to the need of biofuels. Biological pretreatment of lignocellulosic biomass is a branch of pretreatment which uses wood decaying microorganisms or their lignolytic enzymes to facilitate easier plant cell wall deconstruction leading to enhanced sugar yields. Cell-free lignolytic enzymes have not yet been used for biological pretreatment as their titers are low in fermentation which makes them very expensive to use for pretreatment. One of the major advantages of biological pretreatment is that it is performed at ambient temperatures unlike thermochemical pretreatments which are carried out anywhere between 100°C to 220°C. Another advantage is that some white-rot fungi can degrade >90% of lignin in biomass. A third advantage is that it can be carried out as a solid state fermentation (SSF) process which has several advantages over submerged fermentation (SmF). SSF has low water usage, less capital cost, low energy expenditure, less expensive downstream processing, and lower wastewater output than SmF (Krishna, 2005).

2.4.2. Classes of Rot-fungi

Wood-rot fungi are generally divided into white-rot, brown-rot, and soft-rot fungi based on chemical and morphological changes in the wood they decay (Daniel & Nilsson, 1998). Some lesser known mold and stain fungi also exist in nature that thrive on wood and cause discoloration due to pigmentation with only minor soft-rot type decay (Wilcox, 1970). The white-rot and brown-rot fungi belong to the fungal class, basidiomycetes. Soft rot fungi belong to ascomycetes class.

2.4.2.1 White-rot fungi

Wood degraded by white-rot fungi can appear bleached due to their capacity to remove lignin (Wong, 2009). White-rot fungi carry the unique ability to incorporate lignin derived compounds into their metabolism. This can be helpful to reduce the lignin content of biomass which may lead to easier breakdown of plant cell walls. They can be divided into the ones that simultaneously remove all cell wall components or selectively degrade hemicellulose and lignin (Blanchette, 1984a). Biological pretreatment requires organisms that selectively mineralize lignin over wood polysaccharides to prevent sugar loss. Some species of white rot such as *Pleurotus eryngii*, *Hapalopilus rutilans*, *Pleurotus ostreatus* selectively mineralize lignin which makes them promising candidates for biopretreatment due to minimal sugar losses. Sugar loss has been found to be much higher for white-rot fungi such as *Phanaerochete chrysprosium*, *Phlebia radiata*, *Fomes fomentarius,* and *Trametus versicolor* (Chi et al., 2007). Daniel et al. using cryo-field emission scanning electron microscopy showed that the wild type white rot fungus

Phlebia radiata degraded all cell wall components, whereas, the cellulase lacking mutant removed only lignin and hemicellulose (Daniel et al., 2004).

2.4.2.2 Brown-rot fungi

Brown-rot fungi prefer utilizing biomass polysaccharides over lignin for growth which limits their application for biological pretreatment. Brown rot fungi prefer cellulose and hemicellulose over lignin and prolonged periods of decay, wood can appear brown and brittle due to removal of cellulose and hemicellulose. They highly modify lignin to reach wood sugars without causing appreciable loss of lignin (Goodell, 2003).

2.4.2.3 Soft-rot fungi

Savory (1954) found wood degraded by certain fungi to appear very soft and coined the term 'soft-rot fungi'. The observation was made on wet wood in that study. Ironically, salt treated transmission poles decayed by soft-rot fungi were actually found to be harder than brown or white rot fungi. They cause considerable loss of sugars by modifying lignin to breakdown hemicellulose and cellulose. Soft-rot fungi can survive in extreme conditions. It was found that only soft-rot fungi were the rot fungi decaying historical expedition huts in Antarctica and that only when ice melted for a short period in summer (Blanchette et al., 2004). *Trichoderma reesei*, the fungus from which carbohydrate active enzymes are produced in today's enzymatic hydrolysis process of cellulosic ethanol, is a soft-rot fungus.

2.4.3 Wood Degradation by Bacteria

Biological pretreatment utilizes fungi rather than bacteria. Even though bacteria can decay wood in environmental conditions where many fungi cannot survive (Blanchette, 1995); they can take several years before wood damage is evident (Clausen, 1996). Bacterial decaying wood does not show serious damage on a macroscopic scale but data shows that increase the permeability of wood to liquids. Bacteria can operate under either aerobic conditions or near anaerobic conditions such as on waterlogged wood (Blanchette & Hoffmann, 1994). Bacteria can also be found to exist with soft rot fungi in decaying wood. Actinomycetes such as *Streptomyces viridosporus*, *Streptomyces badius*, *Thermomonospora mesophila* are found in soil to solubilize lignin. These aerobic bacteria secrete heme peroxidases which can depolymerize lignin into low molecular weight compounds (Brown & Chang, 2014; Godden et al., 1992). Some other bacteria that inhabit wood include *Clostridium xylanolyticum, Bacillus polymyxa, Cellulomonas, and Pseudomonas sp.* and have powerful cellulases and pectinases. Bacteria are categorized according to their type of decay as erosion, tunneling or cavity forming. Erosion bacteria travel from the lumen into secondary cell wall whereas tunneling bacteria migrates within the cell walls (Blanchette, 1995; Singh & Butcher, 1991). Cavity forming bacteria can form irregular diamond shaped cavities (Björdal et al., 1999).

2.4.4 Characteristics and Mechanism of Wood Depolymerization by Fungi

Brown rot fungi prefer degrading gymnosperms whereas white-rot fungi are found to degrade angiosperms (Tuor et al., 1995). Also, the type of wood can influence

the nature of degradation by the same fungus (Blanchette, 1984b). Lignin and hemicellulose in gymnosperms are different than angiosperms. Gymnosperm hemicellulose in secondary walls is composed of galactoglucomannan and glucuronoarabinoxylan. Dicot angiosperms (hardwoods) have glucuronoxylan as the major hemicellulose (Scheller & Ulvskov, 2010). Lignin in hardwoods is made of syringyl (S) and guaiacyl (G) lignin units with traces of *p*-hydroxyphenyl (H), whereas, gymnosperms (softwood) contain mostly G lignin with minor quantities of S and H units (Boerjan et al., 2003). Blanchette et al. found that *Acer* and *Tilia* wood degraded by white-rot fungus *Phellinus kawakalni* had lower S to G ratio than sound wood (Blanchette et al., 1988). However, Skyba et al. found that white-rot fungus C. *subvermispora* preferentially degraded G lignin over S lignin in poplar (Skyba et al., 2013). Rigsby et al. also found preferential degradation of G lignin in Bermuda grass by *C. subvermispora* (Akin et al., 1995). The hemicellulose and lignin composition may play a role in preference of a fungus to grow on hardwood or softwood (Blanchette, 1991; Rypáček, 1977).

Rot-fungi depolymerize wood polymers via enzymatic as well as non-enzymatic mechanisms. White-rot fungi produce extracellular lignin peroxidases, manganese peroxidases, and versatile peroxidases that are generally absent in brown rot-fungi (Goodell, 2003). Leonowicz et al. divided the white-rot fungal enzymes into three groups. The first group of enzymes is involved in direct attack on wood polymers. These include cellulases, hemicellulases, and ligninases. The second group comprises of the enzymes that work in synergy with the first group enzymes but do not attack the wood polymers

directly. This group includes enzymes such as superoxide dismutase (SOD) and glyoxal oxidase (GLOX) which can generate H_2O_2 for LiP-mediated catalysis and Fenton-type reactions (Kersten, 1990; Leonowicz et al., 1999; Wei et al., 2010). The enzymes in the third group are called feedback-type enzymes which connect the metabolic chains for assimilation of compounds derived from wood. This third group includes enzymes such as glucose 1-oxidase, aryl alcohol oxidase (AAO), veratryl alcohol oxidase (VAO), pyranose 2-oxidase, cellobiose:quinone oxidoreductase and cellobiose dehydrogenase (CDH) (Leonowicz et al., 1999).

Ligninases found in white-rot fungi are lignin peroxidases, versatile peroxidases, manganese peroxidases, and laccases. They have broad specificity and are able to perform redox reactions on lignin and lignin-like compounds. Nitrogen limitation has been associated with induction of lignolytic activity. Availability of carbohydrates can sustain ligninase secretion although high concentrations of ligninase production can be achieved only with sufficiently available nitrogen (Bonnarme & Jeffries, 1990). Lignin peroxidases (LiPs) are heme glycoproteins with a molecular mass in the 40 KDa range and closely resemble horseradish peroxidase (Niku-Paavola et al., 1988).

Blanchette (1984) found wood decayed by several white-rot fungi to accumulate large amounts of manganese oxide which formed black spots in delignified white regions (Blanchette, 1984a). Manganese is now known to play a crucial role in biodegradation of lignin due to extracellular production of manganese dependent peroxidases by white-rot fungi (Perie & Gold, 1991). Manganese peroxidases (MnPs) are also heme glycoproteins with a highly specific Mn^{2+} binding site. MnP catalyzes the oxidation of Mn^{2+} chelates to

Mn^{3+} chelates which are highly reactive. These in turn act as low molecular weight compounds known to attack phenolic lignin. The molecular weight of purified MnPs from *Bjerkandera sp.*, *Phanerochaete chrysosporium*, *Physisporinus rivulosus* and *Phlebia radiata* was found to be between 45-50 kDa (Jarvinen et al., 2012). Mn^{2+} has been shown to regulate both MnP and LiP activity (Bonnarme & Jeffries, 1990).

Versatile peroxidases (VPs) are a newer class of ligninases also known as hybrid peroxidases found in white-rot fungi that contain both LiP and MnP substrate interaction sites (Ruiz-Duenas et al., 2001). Oxidation of *p*-dimethoxybenzene to benzoquinone by VP from *P. eryngii* demonstrated that this VP has a high redox potential with a mechanism similar to LiP (Camarero et al., 1999). NMR studies have shown that the overall fold and heme pocket structure of VP are very similar to those of LiP and MnP. There is a single Mn^{2+} site per VP molecule (Banci et al., 2003). VP from *P. eryngii* can oxidize Mn^{2+} with a similar efficiency as MnP. VP can also oxidize veratryl alcohol (VA) at a similar rate as LiP. However, LiP has a stronger affinity for VA than VP resulting in higher oxidation efficiency. Despite these similarities this VP was found to directly oxidize high redox potential dyes whereas LiP needed the presence of VA for the redox reaction (Perez-Boada et al., 2005).

Laccases are exceptionally versatile enzymes and are distributed in all domains of life (Claus, 2004). Rot fungi laccases are blue multi-copper phenol oxidases with broad specificity. They can oxidize a variety of aromatic compounds while converting molecular oxygen to water (Manda et al., 2007). Four laccases isolated from *Pleurotus ostreatus* had molecular weights ranging from 50 to 80 KDa. Laccases can function in

generating low molecular weight compounds that carry out non-enzymatic attack on lignin or participate in generation of iron chelators to promote a Fenton-type reaction (Wei et al., 2010). Laccases may not be able to cleave non-phenolic lignin due to their low redox potential (Eggert et al., 1997). However, a white-rot fungus *Pycnoporus cinnabarinus* which does not produce LiP has been found to secrete a high redox potential laccase that along with a metabolite 3-hydroxyanthranilic acid can depolymerize synthetic lignin (Li et al., 2001).

Diffusible agents or low molecular weight compounds (LWMC) such as veratryl alcohol and oxalate carry out non-enzymatic oxidation reactions involved in deconstruction of cell walls. Fenton-type chemistry is believed to produce hydroxyl radical through LMWCs that carries out the attack (Forney et al., 1982). Highly reactive hydroxyl radicals can nonspecifically react with wood polymers (Forney et al., 1982). Removal of a hydrogen ion from sugar residue in the cellulose or hemicellulose chain can lead to formation of carbon-centered radicals on the residue which may rapidly react with dioxygen to produce peroxide radical. If the peroxide radical does not contain α-hydoxyl group on the same carbon, it can proceed through a series of oxidoreductions leading to cleavage of the polysaccharide chain. Methoxy group in lignin can leave as methanol with hydroxyl ion attack. If it attacks the aromatic carbon ring hydroxylation can take place (Hammel et al., 2002). The white-rot fungi of the genera *Polyporu poria*, *Fome,* and *Trametes* depolymerize lignin of beech, pine, and spruce to p-hydroxybenzoate, p-coumarate, vanillate, ferulate, 4-hydroxy-3-methoxyphenylpyruvate and the aldehydes vanillin, dehydrodivanillin, syringaldehyde, and coniferaldehyde (Cain et al., 1968).

Demethoxylation and hydroxylation enzymatic reactions can form protocatechuates and catechols (Wojtas-Wasilewska et al., 1983). These compounds can be converted into lactones by fungal oxygenases. Lactones can then be substrates for synthesis of succinate and acetyl-CoA (Cain et al., 1968).

2.4.5 Future of biological pretreatment

Biological pretreatment using microorganisms that degrade plant biomass can take several weeks to achieve optimum lignin mineralization. This is too slow for an industrial pretreatment process. A possible situation may emerge due to contamination with external microorganisms in an industrial-scale biological pretreatment process due to easier access to wood sugars. Also, wood from various species may be degraded differently by the same fungus (Blanchette, 1984b). Therefore, selection of the appropriate combination of feedstock and organism is critical to achieve selective lignin mineralization. Better fungal strains using recombinant DNA technology as well as better understanding of their mechanisms to degrade lignin are required to induce faster biological pretreatment rates or industrial production of ligninases.

2.5 Conclusions

Features of mechanical, thermochemical, and biological pretreatments of lignocellulosic biomass were discussed. Mechanical pretreatments have been envisioned to be more expensive than thermochemical pretreatments, however, no thorough techno-economic analysis of this pretreatment type has been carried out to show otherwise. Biological pretreatments have the advantage that they can be performed at near ambient

temperatures but white-rot fungi eventually may cause sugar loss due through assimilation of sugars for growth after successful degradation of lignin. However, the bigger problem of utilizing rot-fungi for biological pretreatment is the lengthy time of several weeks that is not suitable for an industrial process. In future, achieving high titers of lignin degrading enzymes from white-rot fungi can be beneficial for supplementing them to cellulases and xylanases from hypersecreting mutant of *Trichoderma reesei*. Thermochemical pretreatments show better success and practicability than mechanical or biological pretreatment. Among thermochemical pretreatments, use of expensive chemicals in pretreatments that use organic solvents, sodium hydroxide, hydrogen peroxide, high amount of ammonia, and ionic liquid may overshadow their benefits unless they can be successfully recycled. Aqueous pretreatments such as liquid hot water, dilute acid, and steam pretreatment have the advantage as they are more environmental friendly than other chemical based pretreatments. In spite of removal of high quantities of hemicellulose from biomass through these pretreatments, large amount of lignin remains in the pretreated biomass, therefore, moderate to high enzyme doses are required to solubilize cellulose into glucose. In the future, improved understanding of recalcitrance of biomass will lead to development of better pretreatment technologies.

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Chapter 3

Natural Genetic Variability Reduces Recalcitrance in Poplar*

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3.1 Abstract

Lignin content and structure are known to affect recalcitrance of lignocellulosic biomass to chemical/biochemical conversion. Previously, we identified rare *Populus trichocarpa* natural variants carrying a loss-of-function mutation in the novel 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase-like transcriptional regulator of the phenylpropanoid pathway leading to significantly reduced lignin content. Because this lignin alteration may affect recalcitrance, 18 rare variants along with 4 comparators and BESC standard *Populus* were analyzed for composition of structural carbohydrates and lignin. Sugar yields from these plants were measured at 5 process conditions: one for just enzymatic hydrolysis without pretreatment and four via our combined high-throughput hot water pretreatment and co-hydrolysis (HTPH) technique. Many of these rare variants displayed much high sugar release than standard poplar. Furthermore, low lignin comparator showed a 15% increase in total sugar yield compared to the high lignin comparator at the most severe HTPH condition. Overall, relative variant rankings varied greatly with pretreatment severity, but poplar deconstruction was significantly enhanced when the pretreatment temperature was increased from 140 °C and 160 °C to 180 °C at the same pretreatment severity factor. Rare natural poplar variants had significantly lower recalcitrance than standard poplar. Lignin content in these natural variants had a strong effect on sugar yields. Because of the significant effect of processing conditions on performance rankings, selection of the best performing biofuels feedstocks should be based on sugar yields tested at conditions that represent industrial practice. From feedstock perspective, the most consistent variants, SKWE-24-2 and GW-11012, will

provide key insights into the genetic improvement of versatile lignocellulosic biofuels feedstock varieties.

3.2 Introduction

Lignocellulosic biomass provides an enormous low cost source of trapped sugars and aromatics that is the only known option for large-scale production of liquid transportation fuels that commerce values so highly. However, until reduced greenhouse gas emissions and other externalities are properly recognized and rewarded, commercial success of sustainable fuels and chemicals depends on cost competitiveness with conventional fossil based oil and gas products. In the case of utilizing lignocellulosic biomass, the challenge to competitiveness is to reduce costs of overcoming its recalcitrance to deconstruction into fermentable sugars (Lynd et al., 1999). Because plant deconstruction to sugars is essentially the major economic obstacle to producing ethanol from lignocellulosic biomass, less recalcitrant plants would have an economic advantage compared to more recalcitrant counterparts (Chen & Dixon, 2007). Accordingly, in recent years, the scientific community has intensified research into understanding and modifying factors that control recalcitrance of lignocellulosic biomass feedstocks such as poplar and switchgrass. A frequently applied approach is to lower recalcitrance by genetic modification of wild plants to generate superior varieties. An alternative is to identify naturally occurring low recalcitrance varieties by sampling across wide species ranges. Recent advances in linkage disequilibrium-based association genetics and quantitative trait loci (QTL) mapping have made it possible to identify genetic markers

for phenotypic traits that affect biomass recalcitrance (Muchero et al., 2015). These combined approaches have recently shown that some poplar varieties have a naturally occurring mutation in a 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase-like transcription factor that increases the carbon flux towards flavonoid biosynthesis and reduces lignin biosynthesis (Wellington Muchero et al., 2016). In addition, reducing lignin content through downregulation of lignin biosynthesis enzymes in alfalfa has been previously shown to lower recalcitrance, thus improving sugar yields (Chen & Dixon, 2007).

Among the many options for valorizing lignocellulosic biomass, enzymatic hydrolysis has been a leading choice for research as well as emerging lignocellulosic biomass commercial-scale processing because of its ability to realize high sugar yields from crystalline cellulose (Brown & Brown, 2013; Brown et al., 2015). However, this route typically requires biomass pretreatment prior to enzymatic hydrolysis to recover sugars at high yields essential for lowering cost of biofuel production. Because coupling these two operations by conventional laboratory methods is time and labor intensive, our previously developed high-throughput pretreatment and co-hydrolysis (HTPH) technique provides a useful tool for rapidly screening large numbers of biomass candidates for sugar yield performance (Studer et al., 2010). HTPH pretreats biomass first in a custom-made metal 96 well-plate with water at high temperatures, after which, carbohydrate active enzymes are added to the whole pretreatment slurry to hydrolyze polysaccharides left in the pretreated solids to sugars. In addition to allowing rapid screening of large numbers of biomass materials, the small amounts of biomass required (as low as 10 mg

biomass for approximately 3 mg well loadings with 3 replicates) makes it possible to study recalcitrance in the limited quantities often available for experimental plants. Because carbohydrate active enzymes (CAZys) are strongly inhibited by sugars in the pretreatment liquor as well as those released during enzymatic hydrolysis (Holtzapple et al., 1990; Kumar & Wyman, 2014; Ximenes et al., 2011; Yang et al., 2006), HTPH is generally carried out at a very high loading of CAZys (0.1 gram per gram of sugars in biomass) to be sure differences in the digestibility of substrate by enzymes is not masked by enzyme inhibition.

In their study of poplar variants, Studer et al. (Studer et al., 2011) showed that lignin content as well as syringyl to guaiacyl (S/G) ratio affected sugar yields from natural poplar variants. To build upon these findings, we measured the sugar yield performance of rare poplar varieties with natural genetic alterations in lignin biosynthesis. Eighteen rare poplar variants, 4 comparators and the BESC (BioEnergy Science Center) standard were selected for this study. The HTPH conditions employed by Studer et al. were adapted as a starting point for our work but extended by adding a higher pretreatment severity condition at a similar CAZy loading. Because lower pretreatment temperatures are desirable to reduce associated costs such as reactor wall thickness and difficulty for biomass feeding at high pressures (Karl, 2000; Rautalin et al., 1992), temperatures of 140, 160, and 180 °C were included over the same severity range to determine if the rare variants could maintain high yields at more economically desirable lower temperatures. With this experimental design, we sought to determine (1) how changes in inherent properties of rare variants, comparators, and standard poplar

impact sugar yield trends and (2) the effect of processing conditions on sugar release from a variety of poplar variants with different lignin characteristics. To our knowledge, the resulting study is the first of its kind to report sugar yield performance for a large number of native poplar variants with naturally reduced recalcitrance and determine how rankings of a single species varies with processing conditions of importance in selecting top feedstock candidates for production of biofuels and bioproducts.

3.3 Materials and Methods

3.3.1 Plant Materials

Four-year old *P. trichocarpa* variants were harvested from a field site in Clatskanie, Oregon. Stem segments were immediately dried to constant weight and milled through a 20-mesh screen by a knife mill (Model No. 3383-L20, Thomas Scientific, Swedesboro, NJ, USA). Details of field establishment and growth conditions are described elsewhere (Evans et al., 2014; Muchero et al., 2015). Table 3.1 provides a list of the poplar variants used in this study. The moisture content after milling was an average of 6.75 % ± 0.69 % for the variants as determined by a halogen moisture analyzer (HB43-S; Mettler Toledo, Columbus, OH). The moisture content was taken into account to calculate sugar yields from biomass on a dry basis. Comparator variants lacking the target mutation were grown, harvested and processed under the same conditions and were selected to represent the population range of lignin content [3]. The BESC standard poplar (BESC STD), a conventional poplar feedstock, was used as a reference point for the performance of the rare variants.

Table 3.1 List of poplar variants

Field Sites	Clatskanie, OR
1	BESC-35
2	BESC 35-II
3	BESC-283
4	BESC-352
5	BESC-876
6	BESC-877
7	GW-9920
8	BESC-5
9	BESC-8
10	BESC-100
11	BESC-167
12	BESC-292
13	GW-9782
14	GW-9947
15	GW-11054
16	CHWH-27-2
17	CHWH-27-3
18	SKWE-24-2
Comparators	
High Lignin	BESC-316
Low Lignin	GW-11012
	BESC-97
	GW-9762

3.3.2 Compositional Analysis

The compositions of unpretreated poplar variants were determined by following the standard NREL LAP "Determination of Structural Carbohydrates and Lignin in Lignocellulosic Biomass (Sluiter et al., 2008)." Three replicates were run for each variant.

3.3.3 Processing Conditions

Due to numerous mention of conditions in our results and discussion, from here on we refer to process conditions by their representative alphabetical symbols, similar to those mentioned in Table 3.2: **A** represents 'no pretreatment and enzymatic hydrolysis for 120 hours', **B** represents 'HTPH at 140°C for 264.4 min ($\log_{10}R_0 = 3.6$)', **C** represents 'HTPH at 160°C for 68.1 min ($\log_{10}R_0 = 3.6$)', **D** represents 'HTPH at 180°C for 17.6 min ($\log_{10}R_0 = 3.6$)', and **E** represents 'HTPH at 180°C for 44.1 min ($\log_{10}R_0 = 4.0$)'. As reported by others for pretreatment with just hot water, R_0 is defined as t*exp((T-100)/14.75)) in which t is the pretreatment time in minutes and T is the temperature in °C. The $\log_{10}$ of R_0 is called the pretreatment severity factor (Overend et al., 1987).

Table 3.2 Hydrothermal pretreatment conditions applied to poplar variants.

Condition Representative Symbol	Condition Name	Pretreatment Severity Factor ($log_{10}R_0$) [1]	Temperature (°C)	Time (minutes)	Co-hydrolysis Enzyme Loading	Hydrolysis Time (hours)
A	No pretreatment, only enzymatic hydrolysis	N/A	N/A	N/A	75 mg cellulase protein + 25 mg xylanase protein per gram glucan + xylan in raw biomass. Assuming 66.6% glucan + xylan in biomass.	120
B	HTPH 140 °C $logR_0 = 3.6$	3.6	140	264.4		24
C	HTPH 160 °C $logR_0 = 3.6$	3.6	160	68.1		24
D	HTPH 180 °C $logR_0 = 3.6$	3.6	180	17.6		24
E	HTPH 180 °C $logR_0 = 4.0$	4.0	180	44.1		24

[1] $R_0 = t*exp((T-100)/14.75))$, where t is the time in minutes and T is the temperature in °C. N/A- not applicable. $log_{10}R_0$ is called the pretreatment severity factor

The high-throughput pretreatment and co-hydrolysis (HTPH) procedure was performed as described by Studer et al. (Studer et al., 2010). Approximately 4.5 ± 0.15 mg of biomass was loaded into Hastelloy 96 well plate using either a Freeslate® Core Module II® high-throughput robot equipped with a Sartorius® WZA65-CW balance using powder dispensing methods (Freeslate, Sunnyvale, CA) or with a Mettler-Toledo® MX-5 microbalance (Mettler-Toledo, LLC, Columbus, OH). Each variant had four replicates, i.e., four wells were filled with the same sample type. Four similar plates were prepared for each of the conditions **B** to **E**.

Biomass solid loading was adjusted to be 1% by adding 445.5 µL of EMD® Millipore® Milli-Q® water. The plates were then sealed, and biomass was allowed to soak overnight at room temperature. The 96 well chassis plates were inserted into our custom-made high pressure chamber that was heated by saturated steam generated in a 75 kW boiler (FB-075-L Fulton Companies, Pulaski, NY) at the appropriate pressure (Studer et al., 2010). The steam chamber was pre-heated to the desired temperature for 30 minutes prior to adding the plates to reduce the pretreatment ramp-up time to 2 to 3 seconds. When the desired pretreatment time was reached, the steam inlet valve was closed, steam was released from the chamber, and water was quickly filled in the chamber to stop the reaction. The plates were then unsealed, and 30 µL of enzyme mixture was added that contained sodium citrate buffer and sodium azide broad-spectrum antibiotic (Sigma-Aldrich, St. Louis, MO, USA). The sodium citrate buffer and sodium azide concentration in the final solution were 0.05 M and 0.02% respectively. The final enzymatic hydrolysis conditions in the well plate were the same as NREL LAP "Enzymatic Saccharification of Lignocellulosic Biomass (Selig et al., 2008)." An enzyme loading of 75 mg protein of cellulase + 25 mg protein of xylanase per gram glucan + xylan in the unpretreated biomass was applied, assuming all samples had an average of 66.6% glucan + xylan content (dry basis) in unpretreated poplar. The cellulase used was Accellerase® 1500 (BCA protein content 82 mg/ml), and the xylanase was Accellerase® XY (BCA protein content 51 mg/ml), both generously provided by DuPont Industrial Biosciences, Palo Alto, CA, USA. The protein contents of these commercial preparations were determined by the standard BCA method (Smith et al., 1985a). Co-hydrolysis was carried out at 50°C

and 150 rpm for 24 hours in an incubator shaker (Multitron Infors®HT Biotech, Laurel, MD). Enzymatic hydrolysis for condition A (no pretreatment) was carried out in 25 ml Erlenmeyer flasks with a reaction volume of 10 ml, and three replicates were run at the same conditions as for HTPH but for 120 hours. The liquors after HTPH or enzymatic hydrolysis for condition **A** were analyzed for sugar concentrations using a Waters® Alliance HPLC (model e2695, Waters Co., Milford, MA) equipped with a Waters® 2414 RI detector and an Aminex® HPX-87H column (Bio-Rad Life Science, Hercules, CA) conditioned at 65°C with a 5 mM sulfuric acid mobile phase at flow rate of 0.6 ml/min.

3.3.4 Yield Calculations

Sugar yields (glucan, xylan, or glucan + xylan) mentioned throughout this article represent the amount of sugars recovered compared to the amount in unpretreated (raw) biomass (dry basis) on a mass basis, unless otherwise specified. All sugar yields were calculated based on their polymeric form throughout this article by using anhydrous factors to account for the mass of water added when polysaccharides are converted into monosaccharides. Thus, the 'an' in the sugar names (or glycans) refers to their anhydrous forms. This approach facilitates adding yields directly and closing mass balances as structural carbohydrates (cellulose and hemicellulose) are in their polymeric form in lignocellulosic biomass. The stoichiometric conversion factor from glucose to glucan is 0.9, and from xylose to xylan is 0.88.

The formulae employed are as follows;

Sugar (glycan) yield %

$$(\text{mg per 100 mg biomass}) = \frac{\begin{array}{c}\text{Concentration of sugar from HPLC } \left(\frac{\text{mg}}{\text{mL}}\right) \\ * \text{ Total volume including moisture in biomass (mL)} \\ * \text{ 0.9 or 0.88 anhydrous factor for glucose or xylose respectively}\end{array}}{\text{Biomass on a dry basis (mg)}} * 100$$

Absolute change or absolute percent = Sugar Yield% of B - Sugar Yield% of A

$$\text{Relative percent change} = \frac{\text{Sugar Yield\% of B - Sugar Yield\% of A}}{\text{Sugar Yield\% of A}} * 100$$

3.3.5 Statistical Analysis

Statistical analysis was applied to all comparators and rare variant but not the BESC standard poplar. Thus, the standard poplar data and curve should be viewed as an additional layer on the data plots. The box plots show the effect of pretreatment on the entire poplar population. The points plotted on the left of each box plot show differences between comparators, rare variants, and standard poplar. Figure 3.1 provides depth in how to interpret the box plots for sugar yields. Tukey's original method of box plots (Tukey, 1970), with whiskers extending to maximum and minimum values up to 1.5 times of interquartile range, was used. Graphs were designed on Originlab® v. 6.4 or 2015 statistics and graphing software. Table 3.3 shows the average imprecisions, i.e., the arithmetic mean of standard errors for individual plants.

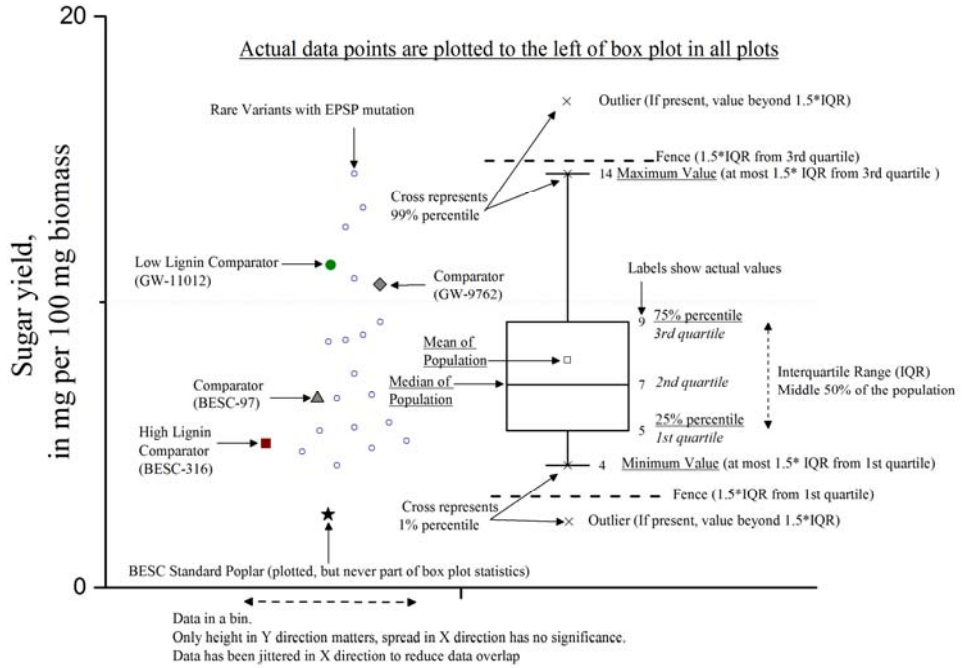

Figure 3.1 Understanding box plots used in this study

Table 3.3 Average imprecision % in measurement.

Condition		Glucan	Xylan	Glucan + Xylan	Lignin
	Composition	0.19	0.06	0.20	0.33
A	No pretreatment enzymatic hydrolysis	0.13	0.05	0.14	N/A
B	HTPH 140°C $logR_0$ = 3.6	0.59	0.35	0.69	N/A
C	HTPH 160°C $logR_0$ = 3.6	0.72	0.37	0.81	N/A
D	HTPH 180°C $logR_0$ = 3.6	0.97	0.42	1.05	N/A
E	HTPH 180°C $logR_0$ = 4.0	0.49	0.23	0.54	N/A

N/A- not applicable

3.4 Results and Discussion

Figure 3.2 shows the distribution of the mass of structural components glucan, xylan, glucan + xylan, and Klason lignin (acid insoluble lignin) for each of the poplar variants harvested from Clatskanie before processing. The glucan composition ranged from 41% to 47% with median and mean values of 43% and 44%, respectively. The xylan contents in unpretreated variants were between 15% and 20% with median and mean being 16% and 17%, respectively. Taken together, the total glucan + xylan composition ranged from 58% to 65%, with median and mean values of 59.5% and 60%, respectively. The Klason lignin contents of the rare variants ranged between 18% and 21% and were lower than for the BESC standard poplar (BESC STD). Both the mean and median lignin contents for the rare variants were the same: 19.8%. On the other hand, the high lignin comparator, BESC-316 had 23.3% and was almost 5% higher than the low lignin comparator, GW-11012 (18.4%) in raw biomass. The two other comparators (BESC-97 and GW-9762) had lignin contents toward the lower end of approximately 20%. BESC STD had the highest lignin content (24.5%) among all the plants tested. It is interesting to note that the glucan and xylan amounts varied independently; that is to say, plants with the highest glucan content did not have the highest amount of xylan (data not shown). Figure 3.3 presents a bar plot of the differences from the mean of lignin content for all plants. Because lower lignin content allows for a higher amount of carbohydrates in these poplar variants (Novaes et al., 2010), plants with lower lignin content would be expected to reduce fuel production costs due to a higher potential fuel yields and lower transportation costs per amount of product. Another consideration is that although *S.*

cerevisiae and other yeast can realize high ethanol titers from glucose, native yeast lack the ability to metabolize pentose, and even though strains have been developed to co-utilize xylose and glucose (Balan, 2014), a high cellulose content in raw material can still be beneficial for facilitating conversion to ethanol or other products (Laluce et al., 2012; Wei et al., 2015).

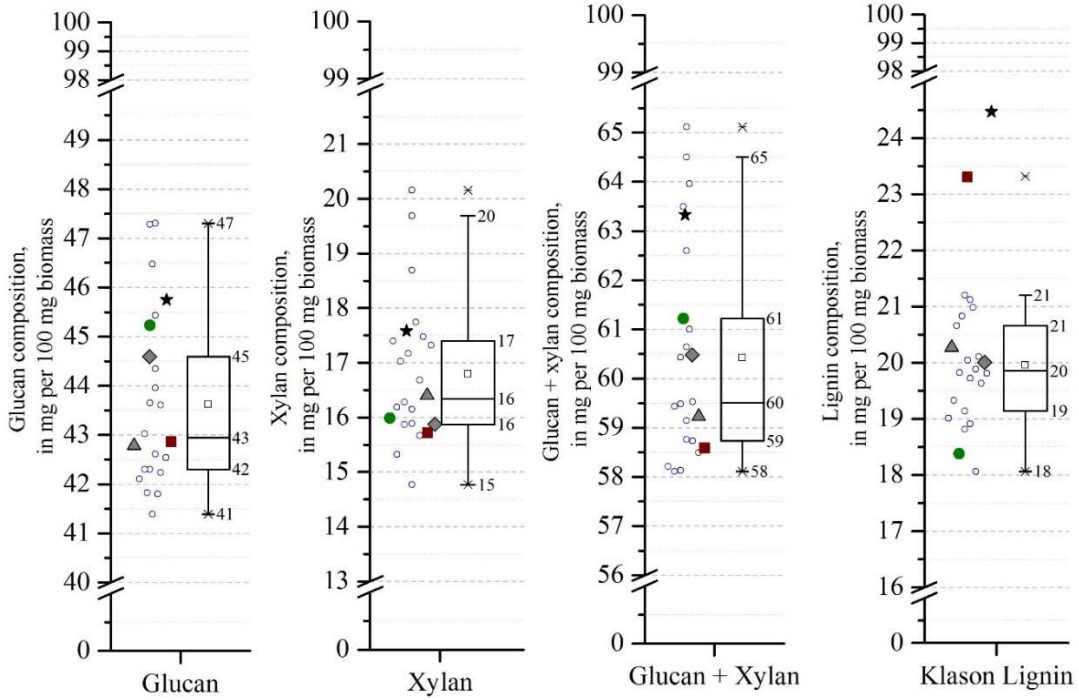

Figure 3.2 Structural composition of poplar variants.
From left to right, these graphs show glucan, xylan, glucan + xylan, and Klason lignin compositions, respectively, of the raw poplar variants. Open circles represent rare variants with the EPSP synthase-like transcription factor mutation, solid circles represent the low lignin comparator (GW-11012), solid squares represent the high lignin comparator (BESC-316), and the triangle and polygon represent BESC-97 and GW-9762 comparators, respectively. BESC standard poplar is shown with a solid star. The square inside the box plot represents the mean values of the population. All samples except BESC standard were part of the descriptive box plot statistics.

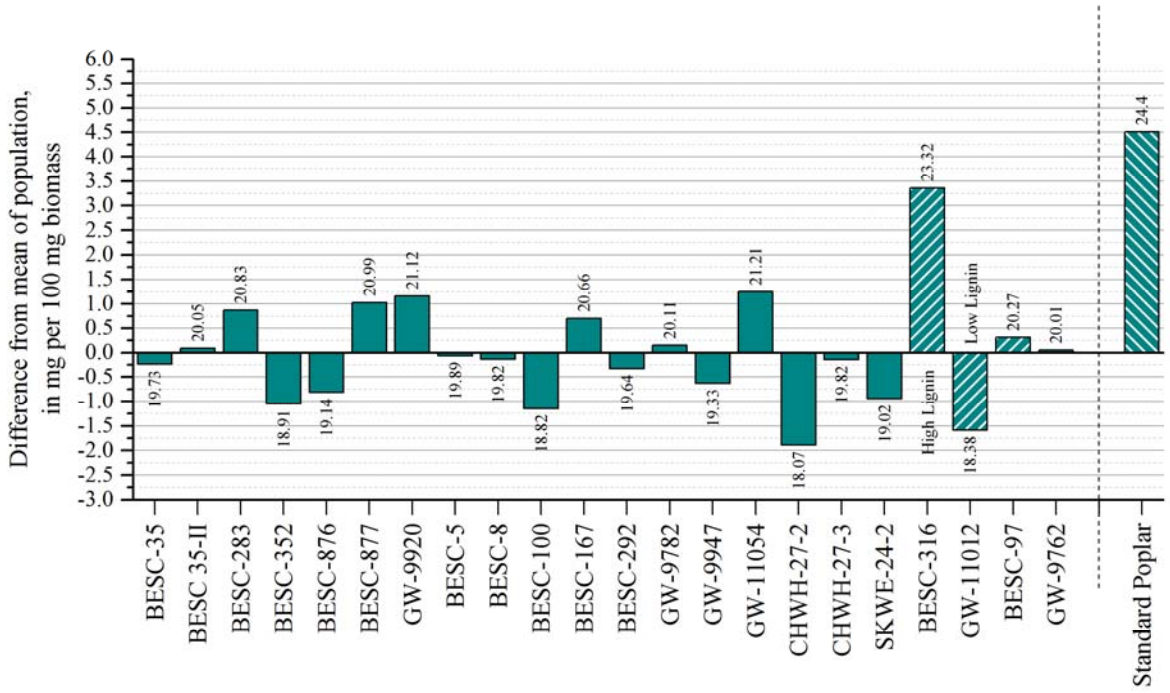

Figure 3.3 Klason lignin composition of Clastkanie plants and BESC standard poplar.
Columns are represented as percent relative difference from mean of the population. Units are in mg per 100 mg biomass (dry basis). Column data labels show actual Klason lignin values. Population mean is 19.96%. Average imprecision in measurement was 0.35%. Columns with pattern are comparators and do not have the natural mutation. BESC standard poplar, shown for comparison, was not part of the statistic, and was not grown in Clatskanie, OR

Figure 3.4 shows the xylan yields for conditions **A** to **E**. For the entire population, xylan yields from enzymatic hydrolysis without pretreatment (condition **A**) were poor, with maximum yield of 2% being achieved with 100 mg total protein loaded per gram glucan + xylan in unpretreated poplar. At any of the HTPH conditions **B** through **E**, there was about 4 to 6% difference between minimum and maximum xylan yield among samples (not including BESC standard). HTPH conditions **B** (140°C), **C** (160°C), and **D** (180°C) are at the same pretreatment severity factor ($\log R_0 = 3.6$). Among these conditions, xylan yields increased by 1 absolute percent when temperature was raised from 140°C to 160°C, and by 2 absolute percent from 160°C to 180°C. Near theoretical xylan yields could be achieved when the severity factor was increased from 3.6 to 4.0 (condition **D** to **E**). It is interesting to note that although increasing severity enhanced xylan yields for all samples, the ability of hot water pretreatment to solubilize a large portion of the xylan in all conditions resulted in small differences in xylan yield among samples.

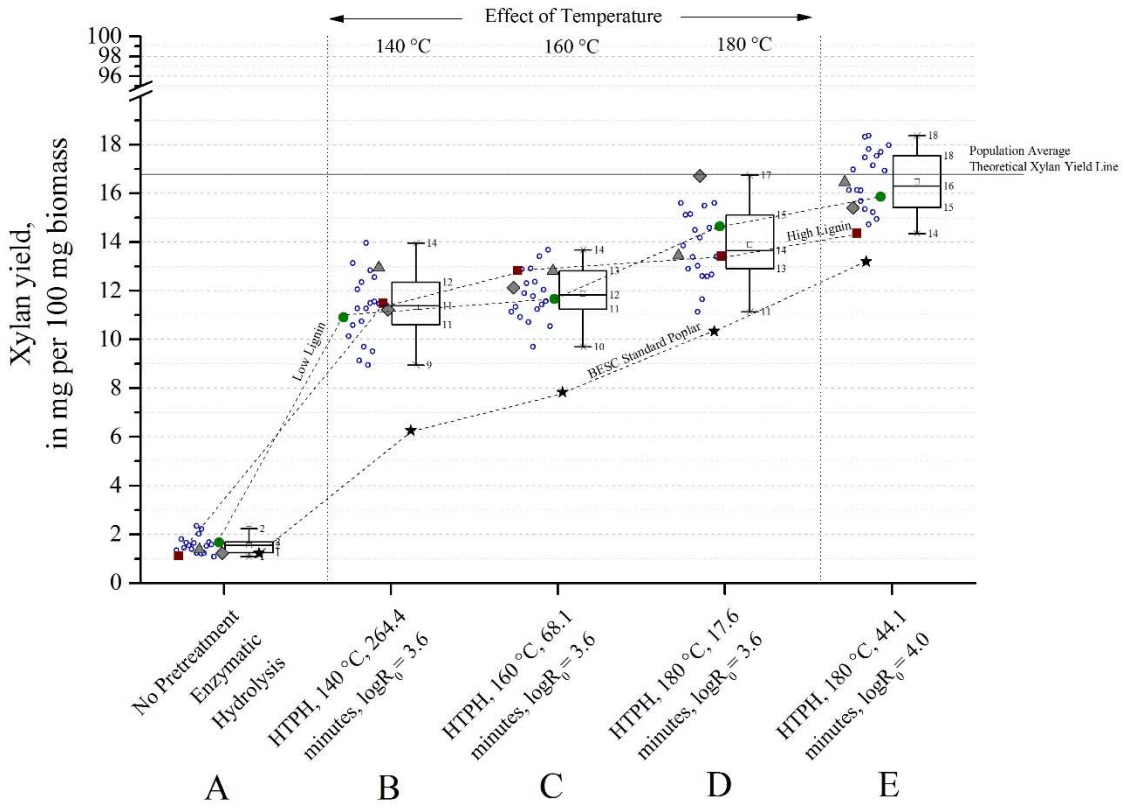

Figure 3.4 Xylan yields of poplar variants.
Effect of pretreatment on xylan yields from poplar variants. Five process conditions are shown. **A:** sugar release from enzymatic hydrolysis for 120 hours without prior pretreatment; **B:** HTPH at 140°C for 264.4 min ($logR_0 = 3.6$); **C:** HTPH at 160°C for 68.1 min ($logR_0 = 3.6$); **D:** HTPH at 180°C for 17.6 min ($logR_0 = 3.6$); and **E:** HTPH at 180°C for 44.1 min ($logR_0 = 4.0$). Co-hydrolysis for all HTPH conditions was performed for 24 hours at an enzyme loading of 75 mg cellulase protein + 25 mg xylanase protein per gram glucan + xylan in raw biomass. Open circles represent rare variants with the EPSP synthase-like transcription factor mutation, solid circles represent the low lignin comparator (GW-11012), solid squares represent the high lignin comparator (BESC-316), and the triangle and polygon represent BESC-97 and GW-9762 comparators, respectively. BESC standard is shown with a black star. The square inside the box plot represents the mean of the population. All samples except BESC standard were part of the descriptive box plot statistics.

Figures 3.5 and 3.6 show that glucan and glucan + xylan yields for 120 hours of enzymatic hydrolysis without prior pretreatment (condition **A**) ranged between 4 and 14%, and 6 and 16%, respectively. The highest 14% glucan yield and 16% glucan + xylan yield achieved from the entire population were from one of the rare variants (GW-9920). For comparison of enzymatic hydrolysis yields without pretreatment, the BESC standard poplar had a maximum glucan + xylan yield of 6%, the high lignin comparator BESC-316 had a similar yield of 6%, and the low lignin comparator GW-11012 had a yield of 13%. Thus, these data suggest that some degree of pretreatment is essential for poplar conversion to be economically viable, as the best total sugar yield achieved with enzymatic saccharification alone was only 16% for GW-9920. We can see that solubilization of structural sugars is largely incomplete without pretreatment, even with such a large dose of fungal enzymes. On the other hand, near theoretical yields could be achieved for some of the samples by proper selection of pretreatment conditions (Figure 3.6, condition **E**). Thus, the ability of aqueous pretreatments such as hot water and dilute acid to remove most of the xylan, alter lignin, and increase cellulose accessibility substantially improves sugar release by fungal enzymes (Chundawat et al., 2011a; Kumar & Wyman, 2013; Mosier et al., 2005).

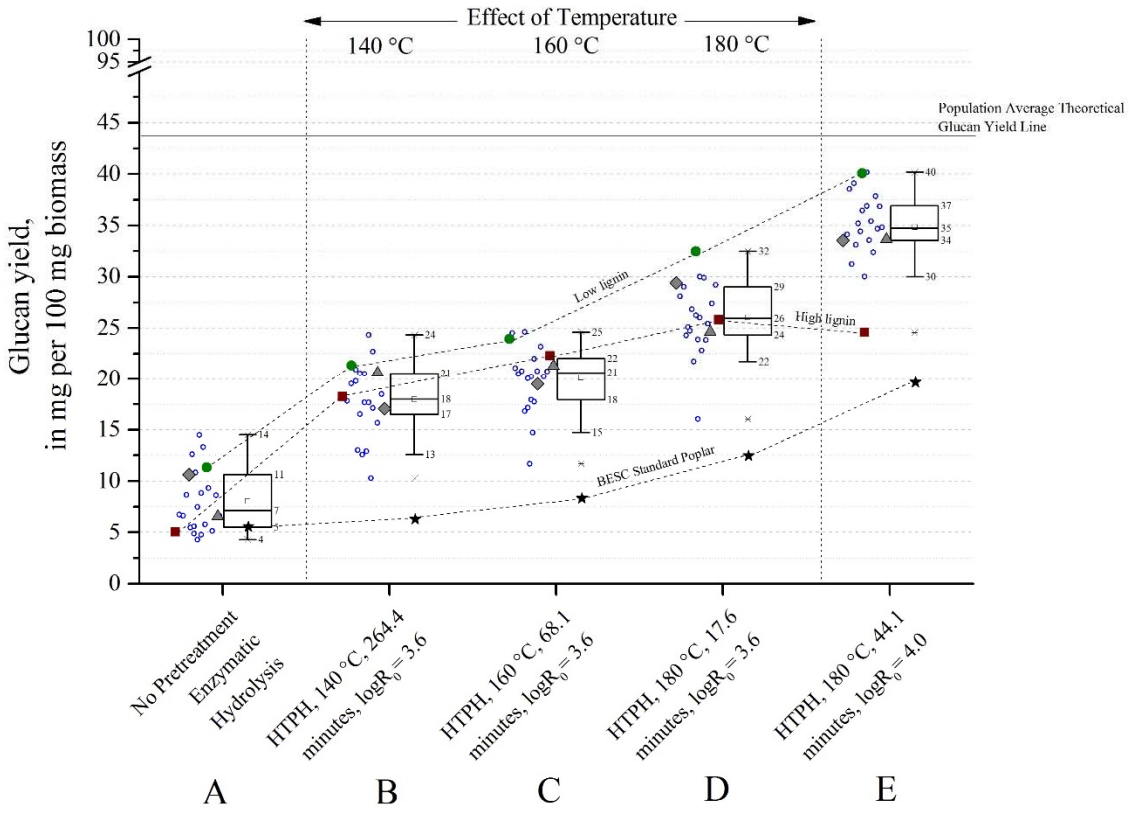

Figure 3.5 Glucan yields of poplar variants.
Effect of pretreatment on glucan yields from poplar variants. Five process conditions are shown. **A:** sugar release from enzymatic hydrolysis for 120 hours without prior pretreatment; **B:** HTPH at 140°C for 264.4 min ($logR_0 = 3.6$); **C:** HTPH at 160°C for 68.1 min ($logR_0 = 3.6$); **D:** HTPH at 180°C for 17.6 min ($logR_0 = 3.6$); and **E:** HTPH at 180°C for 44.1 min ($logR_0 = 4.0$). Co-hydrolysis for all HTPH conditions was performed for 24 hours at an enzyme loading of 75 mg cellulase protein + 25 mg xylanase protein per gram glucan + xylan in raw biomass. Open circles represent rare variants with the EPSP synthase-like transcription factor mutation, solid circles represent the low lignin comparator (GW-11012), solid squares represent the high lignin comparator (BESC-316), and the triangle and polygon represent BESC-97 and GW-9762 comparators, respectively. BESC standard is shown with a black star. The square inside the box plot represents the mean of the population. All plants except BESC standard were part of the descriptive box plot statistics.

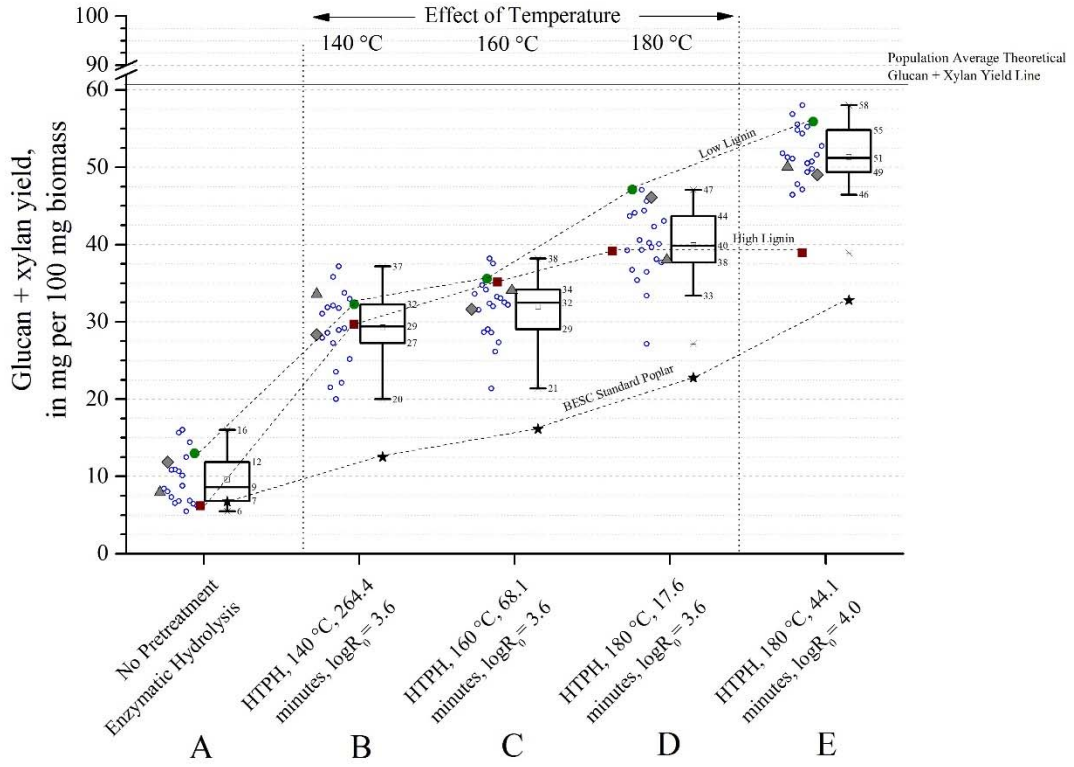

Figure 3.6 Glucan + xylan yields of poplar variants.
Effect of pretreatment on glucan + xylan yields from poplar variants. Five process conditions are shown. A: sugar release from enzymatic hydrolysis for 120 hours without prior pretreatment; B: HTPH at 140°C for 264.4 min (logR0 = 3.6); C: HTPH at 160°C for 68.1 min (logR0 = 3.6); D: HTPH at 180°C for 17.6 min (logR0 = 3.6); and E: HTPH at 180°C for 44.1 min (logR0 = 4.0). Co-hydrolysis for all HTPH conditions was performed for 24 hours at an enzyme loading of 75 mg cellulase protein + 25 mg xylanase protein per gram glucan + xylan in raw biomass. Open circles represent rare variants with the EPSP synthase-like transcription factor mutation, solid circles represent the low lignin comparator (GW-11012), solid squares represent the high lignin comparator (BESC-316), and the triangle and polygon represent BESC-97 and GW-9762 comparators, respectively. BESC standard is shown with a black star. The square inside the box plot represents the mean of the population. All plants except BESC standard were part of the descriptive box plot statistics.

At any of the conditions **A-E,** there was a 10 to 11% and 10 to 17% difference between minimum and maximum of glucan yields (Figure 3.5) and glucan + xylan yields (Figure 3.6) respectively among samples (not including BESC standard). These figures

show that yields of glucan, or glucan + xylan changed little between conditions **B** (HTPH at 140°C) and **C** (HTPH at 160°C) that correspond to the same pretreatment severity factor ($logR_0 = 3.6$). In particular, the mean and median of glucan yields both increased by 3 absolute percent, and glucan + xylan yields by 3 absolute percent when the temperature was raised from 140 °C to 160 °C at the same severity factor. However, the situation changed when the HTPH temperature was increased to 180ºC (condition **D**) at the same pretreatment severity factor. In this case, raising the temperature from 160°C to 180°C increased the mean and median of glucan yields by 5 absolute percent, and glucan + xylan yields by 8 absolute percent. Figure 3.6 clearly shows that glucan + xylan yields for poplar varieties can easily be increased by 10 absolute percent or more by increasing the temperature to 180ºC (condition **D**) from 140ºC (**B)** or 160°C (**C**).

These results point to the need to better appreciate the meaning of the pretreatment severity factor that combines time and temperature into a single variable that can facilitate trade-offs between pretreatment time and temperature (Overend et al., 1987). A key to this approach is that xylan release from cellulosic biomass can be modeled as a first order reaction with a rate constant that has an Arrhenius temperature dependence. The value of 14.75 corresponds to the reaction rate doubling with a 10°C temperature rise [12]. However, the significant increase in sugar yields at 180°C (condition **D**) compared to 140°C (condition **B**) and 160°C (condition **C**), shows that pretreatment severity factor cannot always correctly project how the reaction will respond to varying temperature. This outcome is not surprising in that it would be expected that the bonds between different constituents of the hemicellulose complex would have

different activation energies. As a result, the rate constants for breaking these different bonds would not be necessarily expected to all follow the same 10°C rule, with the outcome that yields were higher for pretreatment at 180°C than at the lower temperatures even though the severity factor was the same.

Another important consideration was to understand how yields from the variants responded to an increase in severity factor. In this case, when the severity factor was increased from 3.6 to 4.0 (condition **D** to **E**), overall glucan yields increased by 10% (Figure 3.5). As previously mentioned, xylan yields jumped up by virtually 100% (Figure 3.4). As a result, the trends in glucan yield and glucan + xylan yield look somewhat similar at the high severity factor. Because almost all the glucan was conserved in the solids produced by hot water pretreatment at the conditions tested (data not shown) and poplar contains far more glucan than xylan, glucan + xylan yields were largely determined by glucan yields from enzymatic hydrolysis. In addition, the fact that xylan yields were virtually 100% of theoretical for many of the variants at condition **E**, up to 99% of theoretical glucan + xylan yields could be achieved for one of the rare variants (BESC-35), followed by 94% from SKWE 24-2.

Figures 3.4 to 3.6 as well as Tables 3.4 and 3.5 show that many of the variants with reduced lignin content can be high sugar yielding candidates compared to BESC standard poplar. Going in order from conditions **A** to **E,** first increasing temperature at the same severity and followed lastly by a higher severity at the highest temperature shows that glucan yields in rare variants increased from 3 to 16 absolute percent compared to standard poplar. Xylan yields for the same variants increased from 1% to 4%, and glucan

+ xylan rose from 4% to 20%. It can be seen from Table 3.5 that the highest glucan + xylan yield from rare variants can be as high as 26 absolute percent and 77 relative percent compared to standard poplar. The relative increase in sugar yields for the rare variants increased in moving to condition **B** (140 °C) followed by conditions **C** (160 °C) and **D** (180 °C) for the same severity and **E** for a higher severity at (180 °C). Both Tables 4 and 5 show that within the four HTPH conditions, the relative increase dropped in going from condition **B** to **E**. It is important to note that rare variants showed a greater relative increase in sugar yields at lower temperatures than standard poplar.

Table 3.4 Relative and absolute percent changes in mean of sugar yields among rare variants compared to the high lignin comparator, low lignin comparator, and BESC standard poplar.

		High Lignin Comparator			Low Lignin Comparator			Standard Poplar		
Condition	Condition Details	Glucan	Xylan	Glucan + Xylan	Glucan	Xylan	Glucan + Xylan	Glucan	Xylan	Glucan + Xylan
A	No pretreatment enzymatic hydrolysis	59 (3)*	44 (1)	56 (4)	-30 (-4)	-6 (-1)	-27 (-4)	44 (3)	30 (1)	42 (3)
B	HTPH 140°C $logR_0 = 3.6$	-3 (-1)	-2 (-1)	-3 (-1)	-18 (-4)	4 (1)	-11 (-4)	181 (12)	80 (5)	131 (17)
C	HTPH 160°C $logR_0 = 3.6$	12 (-3)	-9 (-2)	-11 (-4)	-18 (-5)	1 (1)	-12 (-5)	137 (12)	51 (4)	95 (16)
D	HTPH 180°C $logR_0 = 3.6$	-2 (-1)	3 (1)	1 (1)	-22 (-7)	-7 (-1)	-17 (-8)	106 (14)	33 (4)	73 (17)
E	HTPH 180°C $logR_0 = 4.0$	44 (11)	17 (3)	34 (14)	-13 (-5)	6 (1)	-8 (-4)	80 (16)	27 (4)	59 (20)

*Values in parentheses represent absolute change.

Table 3.5 Relative and absolute percent changes in best sugar yields among rare variants compared to the high lignin comparator, low lignin comparator, and BESC standard poplar.

		High Lignin Comparator			Low Lignin Comparator			Standard Poplar		
Condition	Condition Details	Glucan	Xylan	Glucan + Xylan	Glucan	Xylan	Glucan + Xylan	Glucan	Xylan	Glucan + Xylan
A	No pretreatment enzymatic hydrolysis	188 (10)*	111 (2)	161 (10)	29 (4)	39 (1)	24 (4)	161 (9)	92 (2)	137 (10)
B	HTPH 140 °C $logR_0 = 3.6$	34 (7)	22 (3)	26 (8)	14 (3)	29 (4)	16 (5)	287 (19)	123 (8)	196 (25)
C	HTPH 160 °C $logR_0 = 3.6$	11 (3)	7 (1)	9 (4)	3 (1)	18 (3)	8 (3)	196 (17)	75 (6)	137 (23)
D	HTPH 180 °C $logR_0 = 3.6$	17 (5)	17 (3)	17 (7)	-8 (-3)	7 (1)	-4 (-2)	141 (18)	51 (6)	100 (23)
E	HTPH 180 °C $logR_0 = 4.0$	64 (16)	29 (5)	50 (20)	1 (1)	16 (3)	4 (3)	105 (21)	40 (6)	77 (26)

*Values in parentheses represent absolute change.

As shown in Tables 3.4 and 3.5, the mean and maximum glucan + xylan yields from rare variants increased by a relative 34% and 50%, respectively, compared to the high lignin comparator (BESC-316) at condition **E**. For the high lignin comparator BESC-316, low glucan and glucan + xylan yields were only clearly evident at condition **A**, where yields were less than the 25% percentile, and condition **E** where it was an outlier. For conditions **B**, **C,** and **D** that all were at a $\log R_0$ of 3.6, its yields were not the lowest in the population. At condition **B**, the yield of high lignin comparator was close to median of the population. At condition **C,** its yield rose above the 75 percentile and dropped down to close to the median (50% percentile) at condition **D**. Some of the rare variants had significantly lower sugar yields than the high lignin comparator at these conditions. The box plots in Figures 3.5 and 3.6 show that glucan and glucan + xylan yields for the low lignin comparator (GW-11012) were above the 75 percentile of the population for all conditions **A** to **E**. The absolute and relative change in the mean of glucan + xylan yields from rare variants were negative compared to the low lignin comparator (GW-11012) (Table 3.4), as GW-11012 had one of the highest glucan + xylan yields (Figure 3.6). At conditions **D** and **E,** it ranked highest in glucan yield among all samples. However, some of the rare variants gave relative 4 to 24% higher glucan + xylan yields than the low lignin comparator (Table 3.5). The same three conditions in Figure 3.5 show that the differences in glucan yield between low and high lignin comparator were only 2 to 3 absolute percent at the lower temperatures **B** (140°C) and **C** (160°C) but increased to 7 absolute percent at **D** (180°C). At the higher severity factor condition **E**, this difference increased to 15 absolute percent. Going back to the plot for distribution of

Klason lignin content in Figure 3.2, we see that lignin contents for rare variants and three comparators (GW-11012, BESC-97, and GW-9762) are within a range of 3 absolute percent. These samples with only a 3% difference in lignin content were found to have a 10 absolute percent variation in glucan + xylan yield. One of the rare variants (CHWH-27-2, Table 3.2) had lignin content lower than but close to the low lignin comparator (GW-11012). Thus, although these rare variants share a common mutation in the EPSP-like transcription factor, variation in sugar yields can be attributable to other genetic differences such that factors other than lignin content alone have significant effects on deconstruction.

Standard poplar had an almost 5 absolute percent higher lignin content (24.5%) than the mean of lignin content of the rare variants (19.8%) but was similar to that for the high lignin comparator BESC-316 (23.3%). In fact, BESC standard poplar showed such a low sugar yield that statistical analysis found it as an outlier at all conditions compared to any of the variants, including the comparators. One would expect that if only lignin content affected sugar yield, then BESC-316 and BESC STD would exhibit similar yields, but this was clearly not the case as the total xylan plus glucan yields for BESC-316 were 6 to 19 absolute percent higher than for BESC STD for the full range of HTPH conditions **B** to **E**.

Since the BESC STD was a much older plant at harvest, the observations suggest that environmental factors, harvest age, growth site, harvest season, climate, and soil conditions can affect phenotypic plasticity and cause significant difference cell wall characteristics and subsequent sugar yields (McKown et al., 2014; Savolainen et al.,

2007). Although standard poplar is not a true comparator to the variants, the differences in yields still point to an important result; that there can be extreme differences in sugar yields within the same species, possibly due to differences in age or phenotypic plasticity. However, we still observed significant differences in samples grown, harvested and processed under the same conditions suggesting that genetic variability confers major differences in cell wall recalcitrance and provides opportunities for improving lignocellulosic biofuels feedstocks.

As noted earlier, xylan yields did not change noticeably with plant variety in that a high percentage of xylan was always recovered. Thus, as shown in Figure 3.4, trends in xylan yields with change from low to high lignin comparators was unclear for lower temperature pretreatments at a severity parameter of 3.6 (conditions **B** and **C**). In fact, the high lignin comparator BESC-316 showed about a 1 absolute percent higher xylan yield than the low lignin comparator GW-11012 for these two conditions. However, for both low severity (condition **D**) and high severity (condition **E**) pretreatments at 180°C, the low lignin comparator had about 1 absolute percent higher xylan yields than from the high lignin comparator. Xylan yields as a percentage of the theoretical maximum were 85% and 91% from the high and low lignin comparators, respectively, at condition **D** and increased to 91% and 99%, in that order, at condition **E**. Overall, glucan + xylan yields were much greater from the low lignin comparator than from the high lignin comparator due to larger differences in glucan yields rather than xylan yields. At the most severe HTPH (condition **E**), the theoretical glucan + xylan yields ranged from 73% to 99% for the rare variants, but the range dropped to 47% to 76% at the lower severity factor

condition **D** even though the temperature was the same. GW-9762 and BESC-97, the two comparators that do not have the EPSP-like natural mutation, had lignin contents and sugar yields similar to the rare variants.

Figure 3.7 summarizes the overall ranking of yields of sugars from xylan plus glucan together for all the samples included in this study. We can clearly see that glucan, xylan, and glucan + xylan yield ranks changed drastically with process conditions. For example, GW-9920 ranked 1st in glucan + xylan yield for enzymatic hydrolysis without prior pretreatment (condition **A**) but dropped to 19th out of 22 total for high severity pretreatment at 180°C (condition **E**). On the other hand, the glucan + xylan yield rank of BESC-35 rose from 11 to 1 when moving from condition **A** to condition **E.** Thus, we can see that within the range of pretreatment conditions applied, severity factor and temperature both impacted sugar yield ranks. Low lignin comparator GW-11012 was one of the more consistent performers in that glucan + xylan ranks stayed between 1 and 6 out of 22 for all five process conditions. Rare variant SKWE 24-2 was also a consistent top performer as it displayed a high rank among the pretreatment conditions but average without pretreatment.

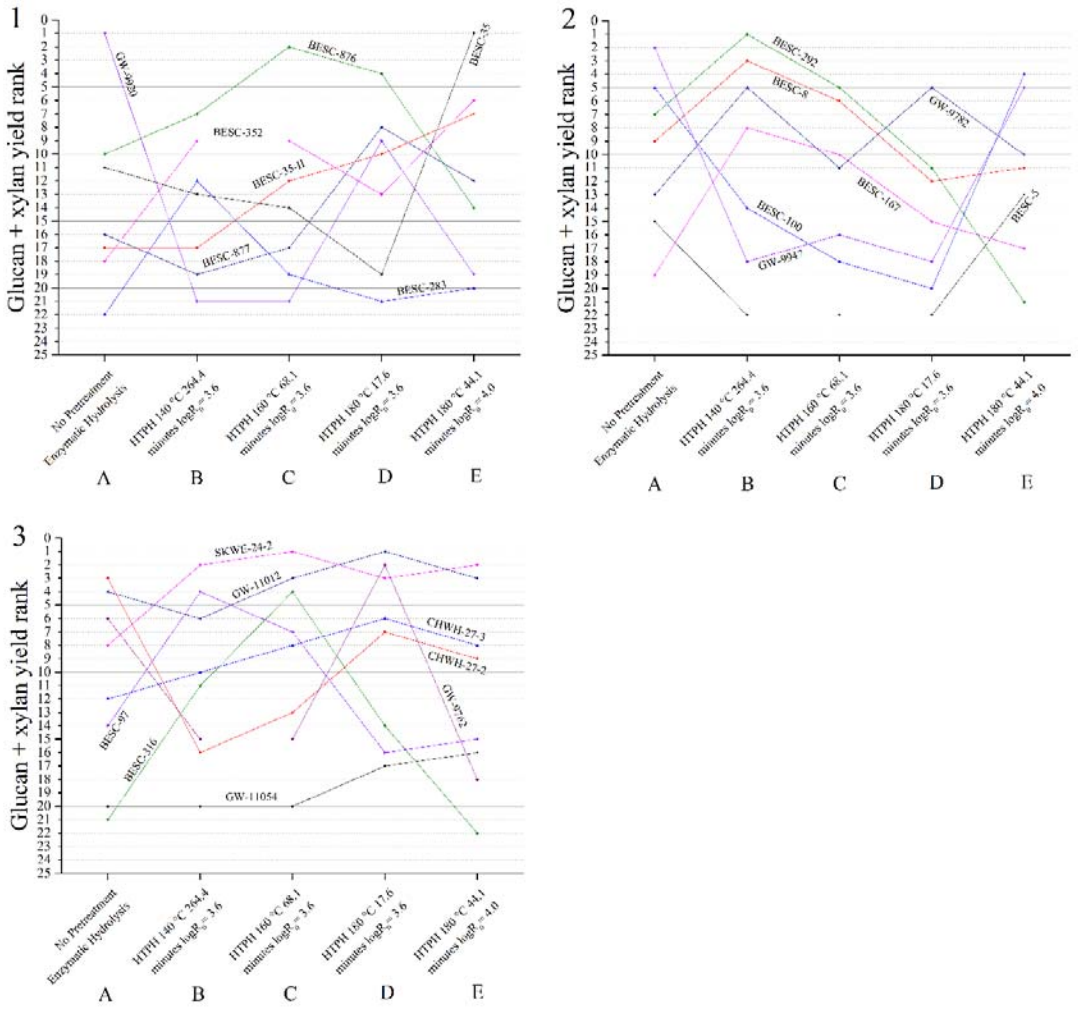

Figure 3.7 Sugar yield ranking of poplar variants.
Graphs 1, 2, and 3 represent the total glucan + xylan yield ranks for 22 variants from at different processing conditions. The highest yield is rank as 1, and the lowest yield is ranked as 22. Conditions from left to right are (1) enzymatic hydrolysis for 120 hours without prior pretreatment, (2) HTPH at 140°C for 264.4 min (logR0 = 3.6), (3) HTPH at 160°C for 68.1 min (logR0 = 3.6), (4) HTPH at 180°C for 17.6 min (logR0 = 3.6), and (5) HTPH at 180°C for 44.1 min (logR0 = 4.0). Enzymatic hydrolysis for all HTPH experiments was applied for 24 hours. The enzyme loading for all conditions was 75 mg cellulase protein + 25 mg xylanase protein per gram glucan + xylan in the raw biomass.

Figure 3.7 also shows that although CHWH-27-2 ranked 3rd from the best yields when enzymatically hydrolyzed without prior pretreatment (condition **A**), its performance fell to 9th when subjected to the highest severity pretreatment prior to enzymatic hydrolysis (condition **E**). On the other hand, the low lignin comparator GW-11012 rose from the 4th rank without pretreatment (condition **A**) to 3rd rank at for the most severe pretreatment (condition **E**). This shift in performance strongly suggests that factors other than lignin content have a major impact on sugar yields. It is important to remember that the lignin wall in lignocellulosic biomass can be viewed in terms of three major characteristics: lignin amount, lignin strength, and lignin location. For the first, high lignin contents have been shown to negatively affect sugar yields (Studer et al., 2011). Strength, the second characteristic, depends on several underlying factors that affect nature of bonding. For one, the ratio of syringyl to guaiacyl units (S/G) affects the strength of lignin bonds in that higher S/G ratios increase carbon to oxygen (β-O-4) linkages that are more susceptible to breakdown than carbon to such carbon bonds as β-β, β-5, β-1, and 5-5 (Boerjan et al., 2003; Sannigrahi et al., 2010). Interactions with carbohydrates in lignin carbohydrate complexes (LCC) also affect lignin strength (Pu et al., 2011). The third category, location, refers to lignin distribution in native plant cell walls. Complex bond cleavage and re-association reactions occur in lignin when biomass is subjected to such high temperature processes as aqueous pretreatment. These reactions alter the lignin distribution and are thought to move lignin from the interior of cell walls to the outer surface where it can still affect enzymatic deconstruction of cellulose (Li et al., 2013; Selig et al., 2007). The shift in one or more of these lignin attributes might be

responsible for the high lignin comparator showing poor relative performance without pretreatment (condition **A**) and for high severity HTPH at 180 °C (condition **E**) while still performing relatively well when pretreated at moderate severity conditions (**B**, **C,** and **D**). This result also suggests that high lignin content may not universally result in very low yields within plant populations.

These findings indicate that the optimum processing conditions can vary considerably even for a single species of plants. That is to say, plants that perform well without pretreatment may not be the best performing plant with pretreatment because yields are too low to be economically attractive without pretreatment for all the poplar varieties tested. Making choices based on results from enzymatic hydrolysis without pretreatment is likely to eliminate feedstocks that have the greatest yield potential. Therefore, screening of plants for top candidates for cellulosic ethanol or biomass-derived products should be based on small scale tests that mimic processing conditions to be applied in a commercial scale biofuel plant.

3.5 Conclusions

There was an overall 10 to 17 absolute percent difference between minimum and maximum total sugar yield for poplar variants. Some rare natural variants and reduced lignin poplar plants displayed significantly reduced recalcitrance in that they displayed up to a 26 absolute percent and 77 relative percent higher total sugar yield than possible from standard poplar. Thus, these plants promise to be superior feedstocks for biological processing into sugars that can be fermented into ethanol or other products. However,

yields from all poplar varieties were highest when liquid hot water pretreatment was applied at a temperature of 180°C than at 140 or 160ºC, even though the severity was held constant. The low lignin comparator achieved a 15 absolute percent increase in total sugar yields compared to the high lignin comparator at the most severe hot water pretreatment condition at 180°C. However, this negative impact of high lignin content was not as evident at the lower severity HTPH condition. Furthermore, while lignin content strongly affected glucan yields, it had limited effect if any on xylan yields. Overall, the large variance in poplar variant ranks with processing conditions shown here indicates that the choice of poplar feedstocks should be based on tests that mimic expected operating conditions and not based on performance without pretreatment or fully optimizing conditions.

3.6 Abbreviations

HTPH: High-throughput hot water pretreatment and co-hydrolysis **EPSP:** 5-enolpyruvylshikimate-3-phosphate **BESC:** Bioenergy Science Center **BESC STD:** BESC standard poplar

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Chapter 4

Flowthrough Pretreatment with Very Dilute acid Provides Insights into High Lignin Contribution to Biomass Recalcitrance*

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4.1 Abstract

Flowthrough pretreatment is capable of removing much higher quantities of hemicellulose and lignin from lignocellulosic biomass than batch pretreatment performed at otherwise similar conditions. Comparison of these two pretreatment configurations for sugar yields and lignin removal can provide insights into lignocellulosic biomass deconstruction. Therefore, we applied liquid hot water (LHW) and extremely dilute acid (EDA, 0.05%) flowthrough and batch pretreatments of poplar at two temperatures and the same pretreatment severity for the solids. Composition of solids, sugar mass distribution with pretreatment, sugar yields, and lignin removal from pretreatment and enzymatic hydrolysis were measured. Flowthrough aqueous pretreatment of poplar showed between 63 to 69% lignin removal at both 140°C and 180°C, while batch pretreatments showed about 20 to 33% lignin removal at similar conditions. Dilute acid did not significantly enhance lignin removal from solids with flowthrough pretreatment at either of the pretreatment temperatures. However, extremely dilute acid batch pretreatment did realize greater than 70% xylan yields largely in the form of monomeric xylose. Close to 100% total sugar yields were measured from LHW and EDA flowthrough pretreatments and one batch EDA pretreatment at 180°C. The high lignin removal by flowthrough pretreatment enhanced cellulose digestibility compared to batch pretreatment, consistent with lignin being a key contributor to biomass recalcitrance. Furthermore, solids from 180°C flowthrough pretreatment were much more digestible than solids pretreated at 140°C despite similar lignin and extensive hemicellulose removal for both pretreatments. Results with flowthrough pretreatment show that about 65 to 70% of the lignin is

solubilized and removed before it can react further to form low solubility lignin rich fragments that deposit on the biomass surface in batch operations and hinder enzyme action. The leftover 30 to 35% lignin in poplar was a key player in biomass recalcitrance to enzymatic deconstruction and it might be more difficult to dislodge from biomass with lower temperature of pretreatment. These results also point to the possibility that hemicellulose removal is more important as an indicator of lignin disruption than in playing a direct role in reducing biomass recalcitrance.

4.2 Introduction

Photosynthesis is nature's way of capturing and storing solar energy in the form of plant biomass. Sun's energy pumps the reaction uphill to combine carbon dioxide and water that have no heating value to form glucose and other plant components (Tester, 2005). Plant biomass is an enormous renewable energy resource that can be utilized as a raw material for conversion of the sugars and aromatics that comprise much of biomass into commodities, with fermentation of sugars to ethanol receiving considerable attention for fulfilling future fuel needs. The structural portion of plants contains three major components, cellulose, hemicellulose, and lignin and is referred to as lignocellulosic biomass. Cellulose and hemicellulose can be converted into sugars by carbohydrate active enzymes while lignin can be broken down into aromatics. Enzyme driven deconstruction typically requires prior pretreatment to achieve commercially viable product yields, which, *inter alia*, may remove or alter the hemicellulose and lignin in biomass to increase the surface area of polysaccharides accessible to enzymes (Lynd et al., 1999). Although cellulose may be altered in aqueous pretreatments, most of the

original structure remains in the pretreated solids. Very high temperatures or high chemical concentrations are required to thermochemically breakdown cellulose into glucose, but increased sugar losses via parasitic degradation pathways result in low glucose yields (Torget; Robert W.). However, as noted in an opinion article outlining important needs for advancing cellulosic ethanol "Just as the three most important contributions in real estate are location, location, location, the three most important factors for commodity products are yield, yield, yield"(Wyman, 2007). Fortunately, cellulase enzymes are capable of breaking down most of the cellulose in pretreated biomass with little subsequent degradation, thereby achieving the high yields essential to commercial competitiveness. Furthermore, enzymatic hydrolysis is the only known technology that has the potential to convert crystalline cellulose, the largest polymer in lignocellulosic biomass, into glucose at near theoretical yields. The most significant obstacle to commercializing such biological based processes is the cost of enzymes due to the large doses needed to obtain high sugar yields from pretreated biomass (Grohmann et al., 1990; Wyman, 2001; Wyman, 2007). Settling for lower yields to reduce enzyme loadings shifts the cost burden to the feedstock and pretreatment. Thus, it is vital to understand and advance pretreatment and enzymatic hydrolysis technologies for biomass deconstruction and their integration to devise new approaches to reduce the amount of enzyme required to achieve high sugar yields.

Numerous mechanical and chemical pretreatment options have been studied and developed over the years. Among the chemical types, pretreatments can be broadly divided into aqueous pretreatments and solvent based pretreatments, with some

comparative studies providing more details (Mosier et al., 2005; Wyman et al., 2005a; Wyman et al., 2005b). Aqueous pretreatments such as those with liquid hot water (LHW) and dilute acid have the advantage that they do not use organic solvents and are usually carried out in batch reactors in laboratory studies. Organic solvents, in spite of some pretreatment benefits, can be expensive and raise issues about fire and pressure containment hazards. Moreover, carryover of residual organic solvent to downstream operations may inhibit microbes and/or enzymes (Agbor et al., 2011). Aqueous batch pretreatments can remove a large portion of hemicellulose, modify but not remove much of the lignin, increase biomass accessible surface area, and increase cellulose digestibility by enzymes (Grohmann et al., 1986).

Most laboratory pretreatments are performed in batch operations and commercial continuous pretreatments generally transport the liquid and solids together. However, flowthrough pretreatment can be valuable for deciphering deconstruction mechanisms occurring during aqueous pretreatments, as continuous removal of soluble fractions from biomass provides valuable insights into the sequence in which components are removed and the effect on the features of the remaining solids that can be compared to results for batch pretreatments at otherwise similar conditions. Pioneered by the late Dr. Ortwin Bobleter of the University of Innsbruck, Austria (Bobleter, 1994; Bobleter et al., 1980; Bonn et al., 1983; Hörmeyer et al., 1988), the distinguishing feature of flowthrough pretreatment is that both lignin and hemicellulose are removed from biomass during pretreatment, in contrast to batch pretreatments that remove relatively little lignin (Bobleter & Concin, 1979; Liu & Wyman, 2003; Mok & Antal, 1992). This difference is

believed to result from lignin that is covalently attached to hemicellulose oligomers initially moving into solution with the highly soluble oligomers for both batch and flowthrough pretreatments. It has been further hypothesized that the lignin-hemicellulose oligomers bonds break when held at reaction conditions for batch pretreatments, with the low solubility lignin fragments then condensing onto the solids biomass. On the other hand, flowthrough pretreatment removes these lignin-oligomer fragments before they have time to breakdown to lower solubility products (Donaldson et al., 1988). As a result of enhanced lignin removal by flowthrough pretreatment, digestion of the glucan left in flowthrough solids by carbohydrate active enzymes can achieve near theoretical glucan yields (Yang & Wyman, 2004). Furthermore, flowthrough pretreatment has shown that flow rate and solids concentration enhance xylan solubilization beyond that predicted by models that only account for acid concentration, temperature, and time in accordance with Saeman's and other first order kinetic models (Bin et al., 2004; Kobayashi & Sakai, 1956; Liu & Wyman, 2003; Liu & Wyman, 2004; Saeman, 1945).

Even though flowthrough pretreatment produces solids that are more digestible by enzymes than solids from batch pretreatment, the large amount of water needed dilutes the sugar stream and leads to high costs for downstream sugar concentration or fermentation product recovery. In addition, the high heat capacity of water results in excessive energy consumption or high capital costs for effective heat exchange. Flowthrough operations are also more complex than batch. In spite of these challenges, there has been interest in scaling up flowthrough pretreatment from bench scale to pilot scale. Recently, Kilpelainen et al. (Kilpeläinen et al., 2014) successfully applied hot

water flowthrough pretreatment to spruce and birch at a 300 liter scale and discussed the impact of channeling in the biomass bed, an important topic in continuous pretreatment system design. In addition, partial flowthrough systems are applied commercially for wood chip pretreatment. Interest in flowthrough systems is also growing for extracting compounds such as flavonoids, terpenes, phytosterols, tannins and protein from biomass. Extraction of the small amounts of active medicinal ingredients from herbal plants can be particularly desirable, with extractions in room temperature water or solvents important to avoid degrading medicinal qualities (Wang & Weller, 2006). Flowthrough pretreatment may enhance extraction yields and throughput by improving mass transfer rates, while the limited time in solution could allow higher temperature operation that reduce manufacturing costs. The high value of such intermediates and nutraceuticals could potentially compensate for the challenges in downstream processing of dilute solutions.

Most flowthrough pretreatments were carried out with just hot water at high temperatures (LHW). Although such operations provide interesting insights, the deconstruction patterns observed may not be applicable to lower temperatures that would be more desirable for commercial pretreatments. For example, lower temperature pretreatments would lower energy costs, and resulting lower pressures would reduce wear and tear on solid biomass feeding systems and vessel wall thickness and associated capital costs (Rautalin et al., 1992; Wyman, 2013). This study, therefore, focused on investigating deconstruction of plant biomass for flowthrough pretreatment at lower temperatures and severities than applied in the past to understand factors that could

account for less effective pretreatments at more commercially relevant conditions when applied to poplar, a fast growing hardwood and model research plant focused on by the Bioenergy Science Center (BESC), Oak Ridge, TN. This comparative study is unique in its examination of sugar yields and lignin removal for application of two pretreatment configurations, aqueous pretreatment with very dilute acid in addition to just hot water, and a temperature 40°C lower than applied in previous studies, resulting in pretreated solids with distinctive features.

4.3 Materials and methods

4.3.1 Materials

Bioenergy Science Center standard poplar (BESC STD) provided by Oak Ridge National Laboratory (ORNL), Oak Ridge, TN, USA was knife milled through a 1 mm screen (Model 4 Wiley Mill Thomas Scientific, Swedesboro, NJ, USA) by the National Renewable Energy Laboratory (NREL), Golden, CO, USA. The moisture content of BESC STD was 7.3% as determined by a halogen moisture analyzer (HB43-S; Mettler Toledo, Columbus, OH).

4.3.2 Pretreatments

The reaction conditions for batch and flowthrough pretreatments are summarized in Table 4.1. Batch pretreatments were carried out at a 2% poplar solids loading in a 1L Parr® reactor (Parr Instruments, Moline, IL, USA) equipped with dual pitch-blade turbine type impellers turning at 200 rpm to mix a total mass of 700 g. Extremely dilute acid (EDA) pretreatments were carried out with 0.05 weight% of sulfuric acid based on liquid

mass, i.e., 686 g, with biomass moisture content included in the liquid mass. Milled biomass was used as-is (no Soxhlet extraction) and soaked overnight to ensure good moisture penetration into the poplar before pretreatment. The Parr reactor was rapidly heated in a fluidized sand bath (Model# SBL-2D, 4 kW, Techne Corp., Princeton, NJ) set at 360°C with the temperature maintained at target values as measured by a K-type thermocouple (Omega Engineering Co., Stamford, CT) inserted in the reactor by adjusting the level of the reactor vessel in contact with sand. For both batch and flowthrough pretreatments, time zero for pretreatment was when the temperature was within 2°C of the required pretreatment temperature, and the deviation in pretreatment temperature was less than 1°C. When the chosen pretreatment time was reached, the vessel was quickly removed from the sand bath and quenched in a large water tank at 10°C (Liu & Wyman, 2004; Lloyd & Wyman, 2005).

Because the flowthrough pretreatment system used in this study was described previously (Yang & Wyman, 2004), only key points will be outlined here. The 1/2" ID by 6" long reactors were loaded with 1 gram of BESC STD on a dry basis, and an extremely dilute acid (0.05%) solution (EDA) was continuously fed to the flowthrough reactor. Although batch dilute acid pretreatments are typically performed in 0.5 to 1.0 wt% percent sulfuric acid, it was necessary to limit the acid concentration to 0.05 wt% because higher acid concentrations would rapidly corrode our SS 316 stainless steel flowthrough reactor system at the temperatures run. This limitation proved fortunate as side-by-side comparisons of results from batch and flowthrough pretreatment with liquid hot water (LHW) and extremely dilute acid (EDA) at moderate pretreatment severity provided

unanticipated insights into biomass deconstruction. A 20 mL/min flow rate applied to the flowthrough reactor with an internal volume of 19.2 ml resulted in a linear velocity of 15.8 cm/min and a liquid residence time of 0.93 minutes. The temperatures listed in Table 4.1 for flowthrough reactions were maintained by setting the fluidized sand bath temperature 9°C higher than the target value. A back pressure gauge controlled the reactor pressures at 40.6 psig and 160 psig for 140°C and 180°C, respectively.

Following pretreatment, the liquid was removed from the solids by filtration with Whatman No. 1 filter paper and thoroughly washed with deionized water until neutral pH wash water resulted to be sure no acid, soluble sugars, or other solubilized products were left in the pretreated solids.

4.3.3 Biomass Composition and Pretreatment Liquor Analysis

For compositional analysis, the wet solids were dried at 37°C for several days until the moisture content dropped to about 4 to 7%. This moisture content was accounted for in the dry weight calculation. Biomass composition and determination of oligomeric sugars were determined according to standard NREL procedures "Determination of Structural Carbohydrates and Lignin in Biomass" (Sluiter et al., 2008) and "Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples" (Sluiter et al., 2006), respectively. All sugar analyses were carried out on a Waters® e2695 Separations Module equipped with a Waters® 2414 RI detector and a Biorad® Aminex® HPX-87H column conditioned with a 5 mM sulfuric acid mobile phase at 65°C.

4.3.4 Enzymatic Hydrolysis

Untreated and washed pretreated solids were enzymatically hydrolyzed for 120 hours with 100 mg of Accellerase 1500® cellulase protein per gram glucan in the unpretreated biomass. Enzymatic hydrolysis of never-dried solids was performed according to standard NREL procedure, "Enzymatic Saccharification of Lignocellulosic Biomass" (Selig et al., 2008). Accellerase 1500® enzyme that had an 82 mg/ml protein content as determined by the standard BCA method (Smith et al., 1985a) was generously provided by DuPont Industrial Biosciences, Palo Alto, CA, USA. UV-vis spectrophotometry of acid soluble lignin was carried out by a Spectramax® M2e Plate Reader (Molecular Devices, Sunnyvale, CA, USA) equipped with SoftMax® Pro data acquisition software in a Costar® UV 96 well pate with absorbance of water blank taken into account for correction of sample absorbance. Three replicates of each sample were kept in a 96 well plate for measurements. A 25 L/(gram cm) absorption coefficient and 240 nm wavelength were employed in line with the standard NREL procedure (Sluiter et al., 2008) to calculate acid soluble poplar lignin concentrations by the Beer Lambert Bouguer Law. Three replicates were kept for each sample for all standard biomass procedures. Graphs and statistical analyses were carried out using Originlab® v. 2015 statistics and graphing software.

Table 4.1 Process Conditions

Identification number	Aqueous condition	Type	Severity factor	Temperature (°C)	Time (min)
1	Liquid hot water	Flowthrough	$\log_{10}R_0 = 3.4$	140	192
2		Flowthrough		180	12
3		Batch		140	192
4		Batch		180	12
5	Extremely dilute acid (0.05%)	Flowthrough	logCS = 1.4	140	192
6		Flowthrough		180	12
7		Batch		140	192
8		Batch		180	12

4.3.5 Calculations

The mass of each sugar was converted to the mass of its corresponding anhydrous form by multiplying glucose values by 0.9 and xylose measurements by 0.88 to account for the mass of water added to each during hydrolysis. Enzymatic hydrolysis loading was based on mg of protein per gram glucan in the unpretreated biomass to allow fair comparison of the effect of overall enzyme loadings on performance for each pretreatment. Mass units were in grams, volumes in liters, and concentrations in grams per liter. Stage 1 and Stage 2 refer to the pretreatment and enzymatic hydrolysis stages, respectively.

1. Mass of polymeric sugar in solids before pretreatment = Dry weight of biomass fed to pretreatment reactor * fraction of polymeric sugars in unpretreated biomass

2. Mass of total liquid before pretreatment = Mass of water added to the reactor + (fraction moisture * wet weight of biomass added to the reactor) + mass of acid (if added)

3. Solid yield% =

$$\frac{\text{Wet weight of biomass solids recovered from pretreatment reactor} * (1 - \text{moisture content fraction}) * 100}{\text{Dry weight of unpretreated biomass fed to reactor}}$$

4. Mass of liquid after pretreatment = Mass of total liquid before pretreatment + [(1-solid yield fraction)*dry weight of biomass fed to pretreatment reactor]

5. Volume of liquid after pretreatment =

$$\frac{\text{Mass of liquid after pretreatment (g)}}{\text{Measured density of liquid after pretreatment } (\frac{\text{g}}{\text{L}})}$$

6. Stage 1 sugar yield =

$$\frac{\text{Concentration of monomeric sugar from HPLC} * \text{Volume of liquid after pretreatment} * \text{Anhydrous correction factor} * 100}{\text{Mass of polymeric sugar in solid before pretreatment}}$$

7. Lignin or xylan removal =

$$\frac{[\text{dry weight of biomass fed to pretreatment} * \text{lignin or xylan fraction of unpretreated biomass}] - [\text{dry weight of pretreated solids from the reactor} * \text{lignin of xylan fraction of pretreated solids}]}{[\text{dry weight of biomass fed to pretreatment} * \text{lignin or xylan fraction of unpretreated biomass}]}$$

8. Enzyme loading =

$$\frac{\text{mg of protein per gram glucan in enzymatic hydrolysis flask}}{\text{glucan yield fraction after pretreatment}}$$

9. Enzymatic saccharification efficiency% =

$$\frac{\text{Concentration of monomeric sugar from HPLC} * \text{anhydrous correction factor} * \text{total reaction volume of enzymatic hydrolysis flask}}{\text{mass of glucan or xylan in enzymatic hydrolysis flask}}$$

10. Stage 2 sugar yield =

$$\frac{\text{Enzymatic saccharification efficiency\%} * \text{mass of glucan or xylan fed to stage 2}}{\text{Mass of polymeric sugar in solid before pretreatment}}$$

11. Total Stage 1+2 glucan or xylan yield = Stage 1 yield% + Stage 2 yield%
12. Total Stage 1+2 (glucan + xylan) yield = Stage 1 glucan + xylan yield% + Stage 2 glucan + xylan yield%

4.4 Results and Discussion

4.4.1 Solids Compositions and Yields

Figure 4.1 shows glucan, xylan and total lignin (acid insoluble lignin plus acid soluble lignin) contents in unpretreated and pretreated solids, with each chart accompanied by the percent of the original mass recovered as solids after pretreatment (solids yield%). Most of the glucan is contained in cellulose, the core component of lignocellulosic biomass, and a small amount as xyloglucan in the primary cell wall of hardwoods like poplar (Ebringerova et al., 2005). Most of the hemicellulose in poplar is composed of xylose. As shown, raw BESC standard poplar (BESC STD) contained 45.75% glucan, 17.5% xylan, 24.4% acid insoluble lignin (AIL) (also known as Klason lignin or K-lignin), and 3.6% acid soluble lignin (ASL). It can be seen on going from left to right in Figure 4.1 that solid yields dropped with increasing temperature for both LHW and EDA batch pretreatments. In addition, solid yields dropped by 5% for higher temperature batch LHW pretreatment and 9% for higher temperature batch EDA pretreatment. Furthermore, flowthrough pretreatments displayed different trends in solid yields than batch pretreatments in that those from batch pretreatments dropped with

increasing temperature and with addition of acid while solid yields from LHW flowthrough pretreatments at both 140°C and 180°C remained at 53% and only changed from 51% to 49% when the temperature was increased from 140°C to 180°C for EDA flowthrough pretreatments. In all situations, about half of the hardwood biomass was solubilized at these moderate severities.

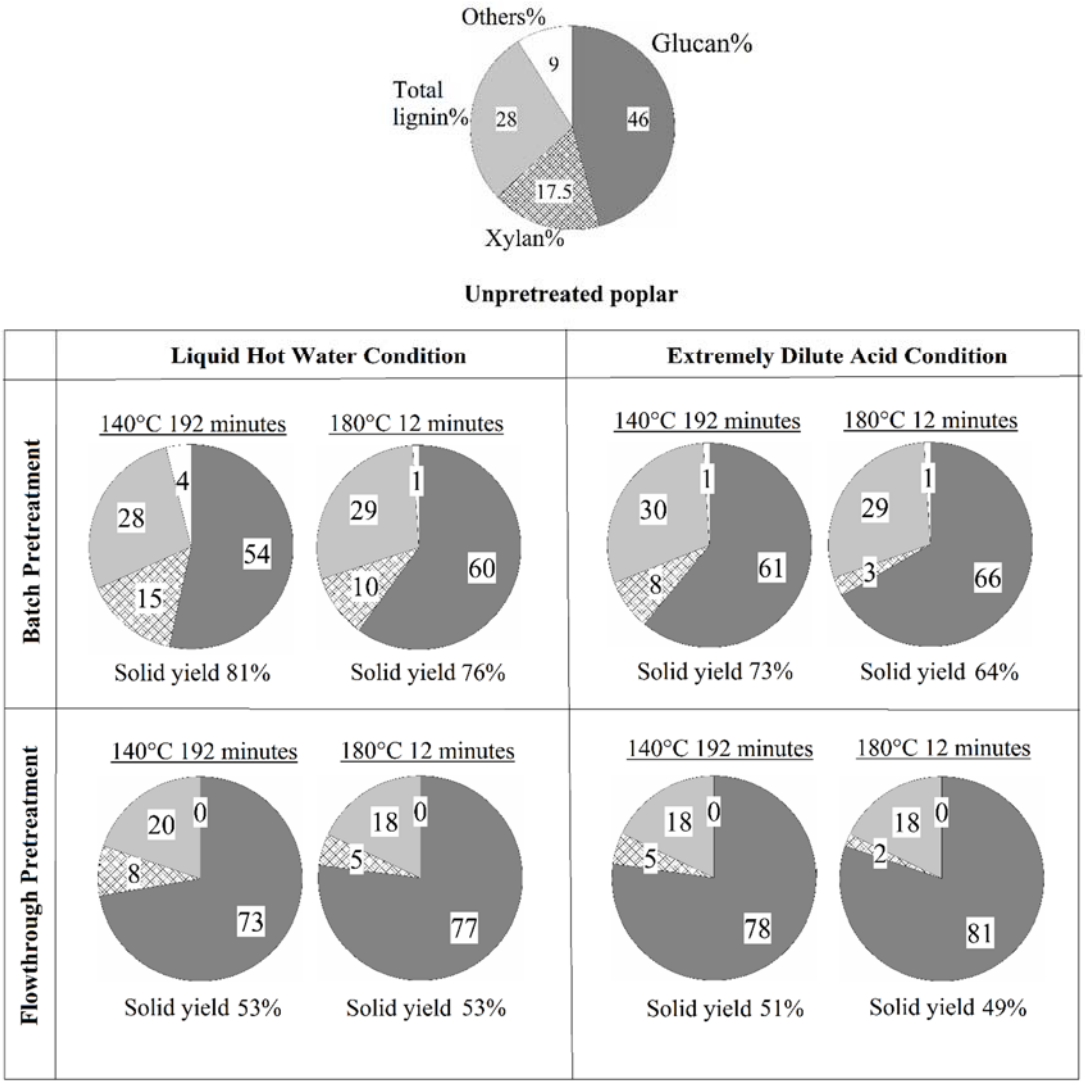

Figure 4.1 Composition and solid yields.
Composition of poplar prior to pretreatment and yields and compositions of solids produced by liquid hot water (LHW) and extremely dilute acid (EDA) batch and flowthrough pretreatments.

Because increasing flow rate and not just temperature was previously shown to reduce solid yields (Liu & Wyman, 2003; Liu & Wyman, 2004), it is important to note that the flow rate of 20 ml/min applied here to a 0.5" by 6" reactor resulted in a linear velocity of 15.8 cm/min and liquid residence time of 0.93 minutes. By comparison, when

Mok and Antal (Mok & Antal, 1992) applied a flow rate of 1 ml/min at a lower linear velocity of around 6 cm/min and somewhat greater liquid residence time of 1.26 minutes in a 0.46 cm by 7.6 cm reactor, biomass solubilization values were between 40 and 60% with LHW flowthrough pretreatment at 230°C after 2 minutes for 10 biomass species including a solid yield of around 50% for poplar. LHW flowthrough pretreatment of corn stover at 200°C for 12 to 20 minutes by Liu and Wyman (Liu & Wyman, 2004) resulted in a solid yield between approximately 50 to 55% at a linear velocity of 10.7 cm/min and residence time of 1.68 minutes. On the other hand, when Bobleter and co-workers (Bonn et al., 1983) applied flow rates between 11 to 14 ml/min to a larger 560 ml reactor vessel containing aspen hardwood at 180°C, the resulting residence time of between 40 and 50 minutes produced a 57% solids yield after 30 minutes. Another paper reported solid yields between 48 and 58% from pretreatment of poplar at temperatures of 180 and 200°C for residence times of 2 and 0.67 minutes through application of 3 and 9 ml flow rates in a 6 mL flowthrough reactor (Bobleter, 2004; Bobleter et al., 1981). After 20 minutes in the Liu and Wyman study (Liu & Wyman, 2004), 45% of the corn stover was solubilized at 200°C. Thus, these results from several independent studies show that flowthrough pretreatment from 140°C to 230°C at LHW severity factors between 3.4 and 4.1 solubilizes roughly half of the biomass. Furthermore, this result does change significantly at much lower severities due to limited hemicellulose and lignin solubilization or at very high severities that trigger cellulose degradation. Our preliminary work in applying a flow rate of 20 mL/min at 140°C for just 12 minutes, the latter two conditions corresponding to a low severity factor $\log_{10}R_0$ of 2.2, gave a 90% solid yield

in LHW versus 78% in EDA higher temperatures (data not shown). Cellulose solubilization is known to increase drastically at temperatures above 240°C and can reach 100% at a severity factor 5.5 (Yan et al., 2014).

Aqueous pretreatments can solubilize a large fraction of the xylan and other compounds that comprise hemicellulose. As shown by the top row of Figure 4.1, batch LHW and EDA pretreatments both reduced xylan content in the solids by 5% more at 180°C than at 140°C. As shown by the bottom row of Figure 4.1, raising the temperature for flowthrough pretreatment from 140°C to 180°C reduced the solids xylan content by 3%. However, the xylan content in pretreated solids from EDA was 3 to 7% lower than solids from LHW for both batch and flowthrough pretreatments. Furthermore, Figure 4.1 shows that the xylan content was 1 to 7 absolute percent lower for flowthrough pretreatment than batch pretreatment at otherwise identical temperatures and times. These results suggest that mass transfer plays an important role in hemicellulose solubilization that will be discussed further in xylan yields section.

The high removal of xylan and lignin in these pretreatments translated into enhanced glucan content in the pretreated solids, as shown in Figure 4.1. Furthermore, the glucan content for solids from batch pretreatment with both LHW and EDA increased by 5 to 6% when the temperature was increased from 140°C to 180°C. For flowthrough pretreatment, the higher temperature increased glucan content by 4%. Figure 4.1 also shows that the flowthrough solids were 15 to 19 absolute percent richer in glucan than batch pretreated solids due to the low xylan and lignin content. On the other hand, the percent lignin in solids from batch pretreatment either remained at the 28% value of

unpretreated poplar or increased to 30%. Overall, the distinctive feature in comparing the compositions of solids from flowthrough and batch pretreatments of poplar in Figure 4.1 is the much lower lignin content of 18 to 20% for the former vs. 28 to 30% for the latter.

4.4.2 Xylan Removal from the Solids and Yields in Solution

Consistent with the trends in Figure 4.1, Figures 4.2 and 4.3 show that mass of xylan solubilized increased with temperature and use of acid in both reactor configurations. This outcome is expected in that Saeman's first order hemicellulose hydrolysis model predicts that the xylan solubilization rate increases with temperature and hydronium ion concentration (Kobayashi & Sakai, 1956; Saeman, 1945). Overall, xylan removal ranged from 32% to 94%, with flowthrough pretreatment removing more than batch at otherwise equivalent conditions. Figure 4.3 shows LHW and EDA could remove about 5 to 14 grams and 12 to 15 grams xylan, respectively, out of 17.5 grams in the unpretreated raw poplar. It is important to note that the mass of sugars in the liquors was not quantifiable for 140°C flowthrough pretreatments as the liquid was too dilute ($\sim 10^{-5}$ g/L) for the extended time required for pretreatment. The performance for flowthrough pretreatment at 140°C therefore had to be judged based on xylan removal from the solids. An additional file (Additional File 1) shows material balances for all process conditions corresponding to the data in Figure 4.3.

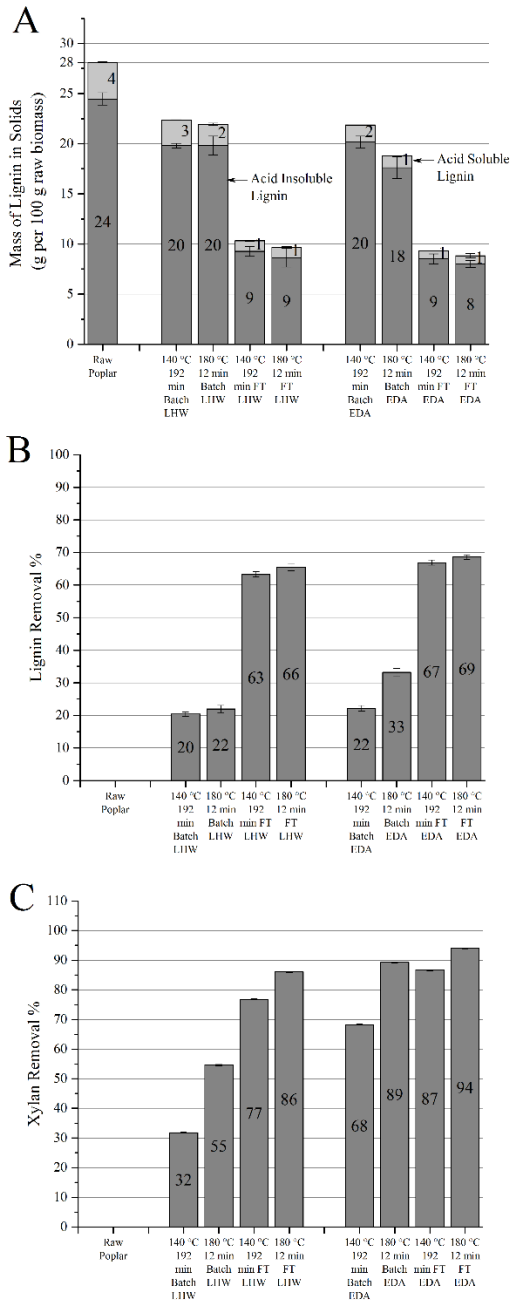

Figure 4.2 Mass of lignin, and lignin and xylan removal.
Mass of lignin left in solids (A) and lignin (B) and xylan (C) removal from solids for batch and flowthrough (FT) pretreatments with just hot water (LHW) and extremely dilute acid (EDA).

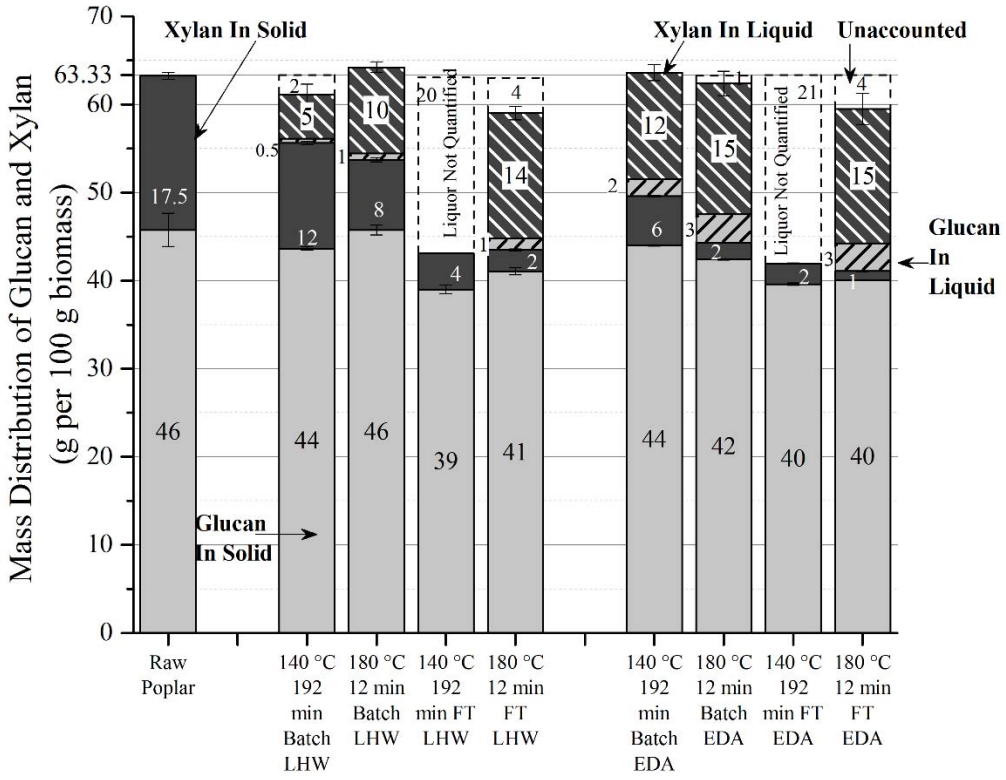

Figure 4.3 Mass distribution of glucan and xylan
Mass balance on glucan and xylan left in solids and solubilized in the liquid based on 100 grams of initial poplar fed to pretreatment at selected temperatures and times for pretreatment with just hot water (LHW) and 0.05% sulfuric acid (EDA). Sugars could not be accurately measured for flowthrough pretreatment at 140°C due to high volumes of water required for the long reaction times required.

In Figure 4.2, the range of xylan removal was greater at LHW conditions (32 to 86%) than at EDA conditions (68 to 94%). However, xylan removal increased by about 34 to 36% in going from LHW to EDA batch pretreatments for both temperatures run. For flowthrough pretreatments, the increase from LHW to EDA was only about 8 to 10%. In either case, the increase in xylan removal from LHW to EDA was the same for both

temperatures. Figure 4.2 also shows that LHW and EDA flowthrough pretreatments at 140°C removed about 77% and 87%, respectively, of total xylan in raw biomass, while increasing the temperature to 180°C increased xylan removal to 87 and 94% for LHW and EDA, respectively. Thus, flowthrough pretreatments removed large quantities of xylan even at 140°C while batch pretreatment at 140°C removed only 55% of the xylan for LHW and 68% xylan for EDA.

Figure 4.4 shows that LHW flowthrough pretreatment at 180°C achieved a 81% xylan yield while Figure 4.5 indicates that LHW batch operation at otherwise identical conditions only realized a xylan yield of 56%. However, EDA operation at 180°C shrunk the difference in yields considerably in that batch operation increased substantially to 84% while in light of its already high yields from LHW operation, EDA could only enhance yields slightly relative to LHW operation to 87%. The smaller difference in xylan yield from flowthrough vs. batch EDA pretreatments at 180°C is likely due to reaching a highly recalcitrant hemicellulose core that cannot be readily deconstructed. In either event, as noted in the past, first order homogenous kinetic models cannot explain why hemicellulose solubilization was so much greater for flowthrough than batch pretreatment at 180°C (Jacobsen & Wyman, 2002). Such models also cannot account for xylan removals from flowthrough pretreatment being so much closer for a 40°C temperature difference than from batch pretreatment at the same severity factor. These results strongly suggest that mass transfer plays an additional role for xylan removal beyond what reaction kinetics alone can account for. It has been shown that a 10 ml/min flow rate to a flowthrough reactor increased mass transfer coefficients by 90% compared

to those for stirred batch pretreatment of corn stover at 180°C (Bin et al., 2004). Brennan and Wyman (Brennan & Wyman, 2004) developed two mass transfer models to explain hemicellulose hydrolysis, with one attributing the difference to shrinking the liquid film thickness. The other biphasic leaching model suggested that by continually preventing equilibrium between xylan in the biomass solids and its concentration in the pretreatment liquor, xylan removal is solubilized until exhaustion. Another branched pore leaching model, developed from leaching of water soluble organic carbon from soil (Cao et al., 1999), was able to describe hemicellulose hydrolysis based on mass transfer alone.

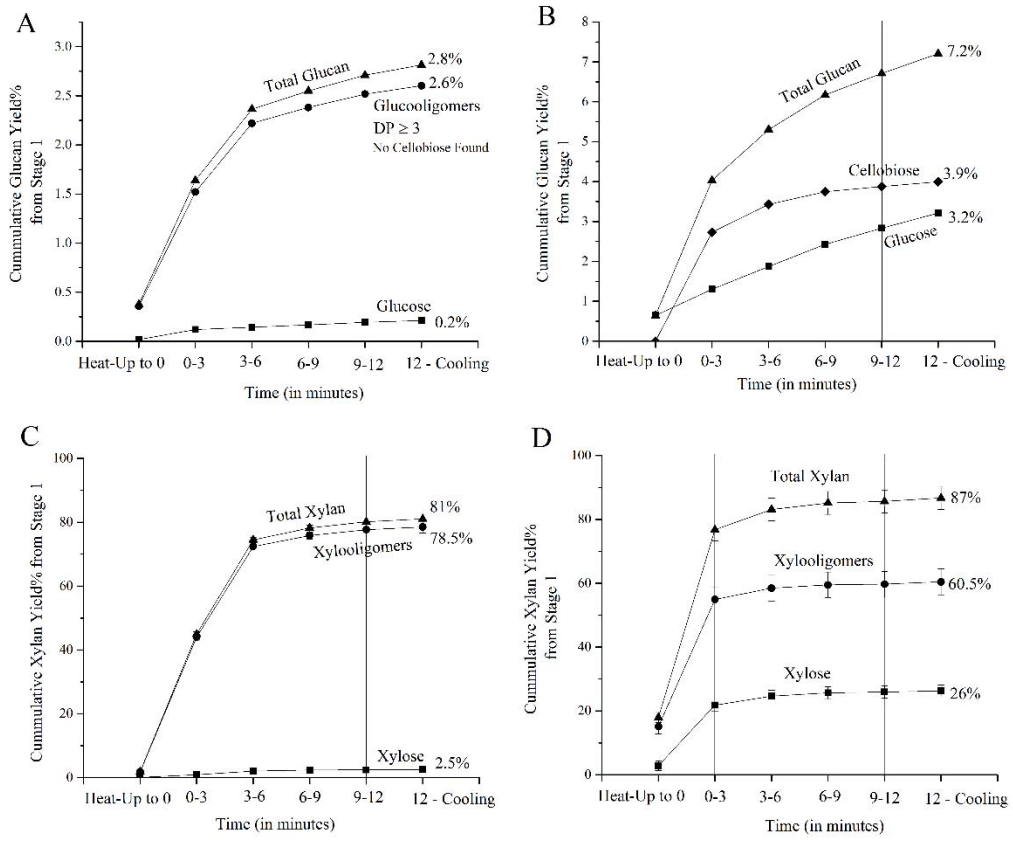

Figure 4.4 Glucan and xylan yields for flowthrough pretreatment at 180°C. Yields of glucan recovered in solution as monomers, cellobiose, and higher oligomers versus time for LHW (A) and EDA (B) pretreatments at 180°C. Yields of xylan recovered in solution as monomers and xylooligomers versus time for LHW (C) and EDA (D) pretreatments at 180°C.

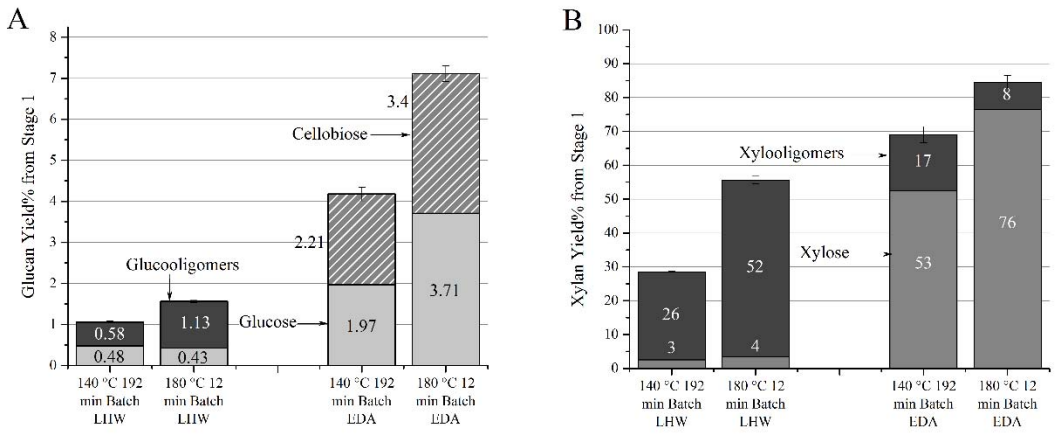

Figure 4.5 Glucan and xylan yields for batch pretreatment
Yields of glucose, cellobiose, and higher glucooligomers from batch LHW and EDA pretreatments at 140 and 180ºC (A) and of xylose and higher xylooligomers (B).

The 86% xylan removal at 180°C for 12 minutes of LHW flowthrough pretreatment was higher than the 62% value reported for operation at 180°C for 8 minutes and 80% at 200°C for 16 minutes by Archambault-Leger, Shao, and Lynd (Archambault-Leger et al., 2012b) for poplar. However, this difference could be due to the fact that they applied a flow rate of 30 ml/min in their 2.1 cm internal diameter flowthrough reactor to produce a linear velocity of about 8.66 cm/min and a residence time of about 1.85 minutes compared with our velocity of 15.8 cm/min and residence time of 0.96 minutes. Mok and Antal (Mok & Antal, 1992) reported 100% hemicellulose removal from solids and about 90% hemicellulose sugar recovery for LHW flowthrough pretreatment of 10 biomass species at 230°C for 2 minutes. They pointed out that their results were not strongly dependent on temperature or time within the range of 200 to 230°C for 0 to 15 minutes at a flow rate of 1 ml/min. Liu and Wyman's study (Liu & Wyman, 2004) of

LHW flowthrough pretreatment of corn stover at 200°C showed that increasing the linear velocity from 2.8 to 10.7 cm/min increased the rate of biomass solubilization and xylose recovery, although both parameters eventually reached the same values after 16 minutes.

Figure 4.5 shows that only 3 to 4% of the solubilized xylan was monomeric xylose with all the rest being oligomers for LHW batch pretreatments at both temperatures. However, addition of a small amount of acid increased the percent monomeric xylose to 53% at 140°C and 76% at 180°C. Production of monomers is advantageous in that they are more universally suitable for yeast uptake to produce ethanol while oligomers are not (Wyman, 1996). The small amount of acid also enhanced overall xylan solubilization in addition to converting more of the xylooligomers to xylose.

Figure 4.4C and 4.4D show liquor profiles versus time of LHW and EDA, respectively, flowthrough pretreatment at 180°C. Xylooligomers were predominant for 180°C LHW flowthrough pretreatment with limited monomeric xylose (2.5%). Although we were unable to characterize the extremely dilute liquor for 140°C LHW flowthrough pretreatment, most of the xylan is expected to be oligomeric for those conditions as well. On the other hand, 180°C EDA flowthrough pretreatment produced about 30% of the xylan in solution as monomers (26% out of total 87% xylan). Previous characterization of the degree of polymerization (DP) of xylooligomers produced by LHW flowthrough pretreatment of corn stover at 200°C for 10 minutes showed that high linear velocities such as 19.7 cm/min (25 ml/min) resulted in DPs greater than 30 for most of the

xylooligomers while longer residence times and lower flow rates or higher temperatures reduced the xylooligomer DP (Yang & Wyman, 2008).

The fast and slow reacting hemicellulose model introduced by Kobayashi and Sakai (Kobayashi & Sakai, 1956) for dilute acid conditions and improved by others to include sugar oligomer intermediates could help understand these patterns in xylose and xylooligomer yields from LHW and EDA flowthrough pretreatments. Past fits of this model to data attributed about 65% of the reacted xylan to be fast reacting for many biomass types (Wyman et al., 2005c), and Shen and Wyman (Shen & Wyman, 2011) showed that modeling the fast reaction to oligomers as a reversible first order reaction improved the data fit. For the latter, the fast reacting xylan fraction can either form xylooligomers reversibly or react directly to xylose, while the slow reacting xylan forms xylooligomers that breakdown to xylose that eventually decompose into furfural. The model showed that the slowest relative step for 180°C LHW pretreatment was xylooligomer conversion to xylose, while the slowest relative step for 160°C dilute acid pretreatment was xylose conversion to furfural. In addition, the rate constant for dilute acid breakdown of xylooligomers to xylose was about 100 times greater than for LHW. They also showed that the rate constant for direct xylan conversion to xylose was about 5 times greater for dilute acid than LHW. Thus, these and other insights gained by this model predict patterns similar to those observed in this study of high amounts of oligomers for LHW, more monomers for EDA and enhanced yields from EDA pretreatment. Furthermore, the release of 20% of the total initial xylan as xylose and 55% as xylooligomers in the first 3 minutes from EDA flowthrough pretreatment at 180°C

shown in Figure 4.4 is consistent with conclusions by a number of studies that about 65% to 70% of the xylan is more easily hydrolyzed while the rest would take a longer time to appear in solution.

4.4.3 Glucan Removal from the Solids and Yields in Solution

The material balances presented in Figure 4.3 reveal that very little glucan was solubilized by any of the batch pretreatments, resulting in the dissolved glucan yields in Figure 4.5 for LHW being only about 1% and 1.5% at 140°C and 180°C, respectively, while those for EDA increased only slightly to about 4% and 7% for the same temperature order. The liquor from LHW batch pretreatment contained both glucose and glucooligomers other than cellobiose, but only glucose and cellobiose was measurable in the liquid from batch EDA operations. For 180°C flowthrough pretreatment, the LHW yields presented in Figure 4.4 were 0.2% for glucose and 2.6% for glucooligomers with no cellobiose, while only glucose and cellobiose were observable in the EDA flowthrough pretreatment liquor. Total glucan yields were both about 7% for EDA batch and EDA flowthrough pretreatment at 180°C, with the yields continually increasing with time. A possible source for the 2 to 4% monomeric xylose yields and less than 2% glucan yields from LHW pretreatments could be the loose structures of xyloglucans and amorphous cellulose in the primary cell walls (Blaschek et al.). Perhaps small amounts of glucose were also derived from cellulose in the secondary cell walls. The patterns of glucose release have similarities to predictions by fast reacting xylan models in that (1) almost all of glucan and xylan from LHW pretreatment is in oligomeric form, (2) glucose and xylose are released right from the very start for 180°C EDA flowthrough

pretreatment, and (3) glucan yields were enhanced by about 5% for both batch and flowthrough EDA pretreatments.

4.4.4 Lignin Removal

As noted above, a distinctive characteristic of flowthrough pretreatment is lignin removal, as evidenced by Figure 4.2 showing that flowthrough pretreatments significantly enhanced lignin removal to 63 to 69% compared to only 20 to 33% for batch pretreatments. For batch pretreatment at 140°C, little difference was realized in lignin removal between LHW (20%) and EDA (22%), but increasing the temperature to 180°C enhanced lignin removal to 33% for batch EDA operation while having little effect on lignin removal for LHW (22%). On the other hand, 180°C LHW and EDA flowthrough pretreatments achieved lignin removals of 66 and 69% respectively. Surprisingly, lower temperatures did not reduce lignin removals much in the flowthrough mode compared to 180°C. At 140°C, lignin removals from flowthrough pretreatments were 63% in LHW and 67% in EDA. Thus, out of 28 grams of total lignin in unpretreated poplar, about 14 to 15 grams was solubilized with flowthrough pretreatment at either temperature, as shown in Figure 4.2A. By comparison, Liu and Wyman (Liu & Wyman, 2004) found 65% lignin removal from corn stover at velocities of 2.8, 5.2, and 10.7 cm/min at 200°C, and Archambault-Leger, Shao, and Lynd measured about the same lignin removal from poplar at 220°C with LHW flowthrough pretreatment at a linear velocity of 8.66 cm/min and a 1.84 minute residence time (Archambault-Leger et al., 2012a). These tradeoffs among temperature and flow rate could be explained by the influence of a number of factors on the glass transition temperature (T_g) of lignin including drastic reductions due

to hydrogen bonding with water (Goring, 1963; Hatakeyama & Hatakeyama, 2010; Ko et al., 2015a; Ramiah & Goring, 1965; Salmén, 1984), the type of lignin (Olsson & Salmén, 1992), and the lignin extraction method. Thus, our results show that coupling a higher linear velocity of 15.8 cm/min with lower temperature operation was as effective in lignin removal as lower velocities combined with higher temperatures in these prior studies. Enhanced lignin removal at 140°C by flowthrough pretreatment suggests that the glass transition temperature for the poplar used here could be as low as 140°C. Furthermore, the high velocities applied for flowthrough pretreatment likely prevented pseudo lignin deposition onto solids reported for batch pretreatments at these moderate severities (Kumar et al., 2012; Sannigrahi et al., 2011) and resulted in a substantial drop in lignin contents for the solids left after flowthrough pretreatment.

These results indicate that about 65 to 70% of the lignin being less recalcitrant to deconstruction and subject to being removed if re-deposition is prevented. It is interesting to note that the more easily removed lignin fraction has a similar value to the fast reacting xylan fraction calculated for so many different biomass types. However, the coincidence may end there as most of the kinetically slower hemicellulose fraction can be solubilized at longer pretreatment times while the remaining lignin fraction has not been shown to be so amenable to deconstruction. Removal of a large fraction of the lignin by flowthrough pretreatment might be made possible by its bonding to far more soluble xylan. On the other hand, about 30 to 35% of the lignin may not be tightly associated with hemicellulose and therefore not swept from the system with hot water even when catalyzed by very dilute acid concentrations. This remaining fraction could be the most

recalcitrant to deconstruction at aqueous conditions. This hypothesis is reinforced by about 65% of the lignin linkages in hardwood being β-O-4 bonds that are more easily broken than others (Nimz, 1974) to form fragments subject to release by flowthrough pretreatment. It is worth noting that some of the results summarized above are for corn stover in which such linkages makeup about 60% of the total (Min et al., 2014; Wang et al., 2015). Thus, the relatively easy breaking of some lignin-lignin bonds coupled with the high solubility of lignin-hemicellulose fragments and the ability of flowthrough pretreatment to remove these fragments before breaking of lignin-hemicellulose bonds allows less soluble lignin to condense back on the solids results in high removal of lignin by flowthrough pretreatment. On the other hand, if the lignin eventually breaks away from the hemicellulose and condenses onto the solids for batch systems, the extent of hemicellulose solubilization would be indicative of the degree of cycling of lignin from the solids into solution and back onto the solids in batch pretreatments, thereby disrupting lignin shielding of cellulose from enzyme action. In addition, although virtually 100% of hemicellulose can be removed by flowthrough and batch pretreatments with or without adding acid, these results point to about 30 to 35% of the lignin being highly recalcitrant to removal in aqueous environments.

4.4.5 Enzymatic Hydrolysis

Figure 4.6 shows yields of glucan, xylan, and glucan plus xylan recovered in solution from both LHW and EDA pretreatments (Stage 1) combined with subsequent enzymatic hydrolysis (Stage 2) of the pretreated solids. A high enzyme loading of 100 mg protein per gram glucan in unpretreated biomass was applied to the pretreated solids

as our focus was on understanding substrate features that contribute to recalcitrance while not being distracted by enzyme limitations due to such factors as enzyme inhibition or loss of activity. Without any pretreatment, even at this high enzyme loading, Stage 1+2 glucan + xylan yields (total sugar yield) was 10% at best. On the other hand, among the four batch pretreatment conditions applied, EDA at 180°C stood out in achieving near theoretical glucan yield. (Liu et al., 2011). One reason for this superior performance could be that the 33% lignin removal for this condition was about 11 to 13% higher than that for the other three batch pretreatments. Raising the temperature from 140°C to 180°C had a greater effect on increasing total glucan yields for EDA batch pretreatments (52% vs. 100%) than for LHW batch pretreatments (27 vs. 52%). Among flowthrough pretreatments, nearly 100% sugar deconstruction could be achieved at 180°C for both LHW and EDA conditions. However, in spite of high lignin removal, the digestibility of solids produced by 140°C LHW and EDA flowthrough pretreatments were 35% and 54% compared to 68% and 65% at 180°C respectively. Thus, lignin removal by flowthrough pretreatment or lignin relocation by batch pretreatment are not sufficient to realize high digestion by enzymes. The results from this study point to the likelihood that the more recalcitrant 30 to 35% of the lignin that could not be removed by flowthrough pretreatment or relocated by batch operation is likely a primary contributor to biomass recalcitrance which require higher temperatures to affect such lignin properties as surface spread on cellulose, strength of association with cellulose, and hydrophobicity. However, other factors could play a role such as the overall cellulose surface area that is available for adsorption by the carbohydrate binding module (CBM) of fungal cellulases,

specifically the hydrophobic face of cellulose crystals, might be affected by differences in pretreatment pH and temperature.

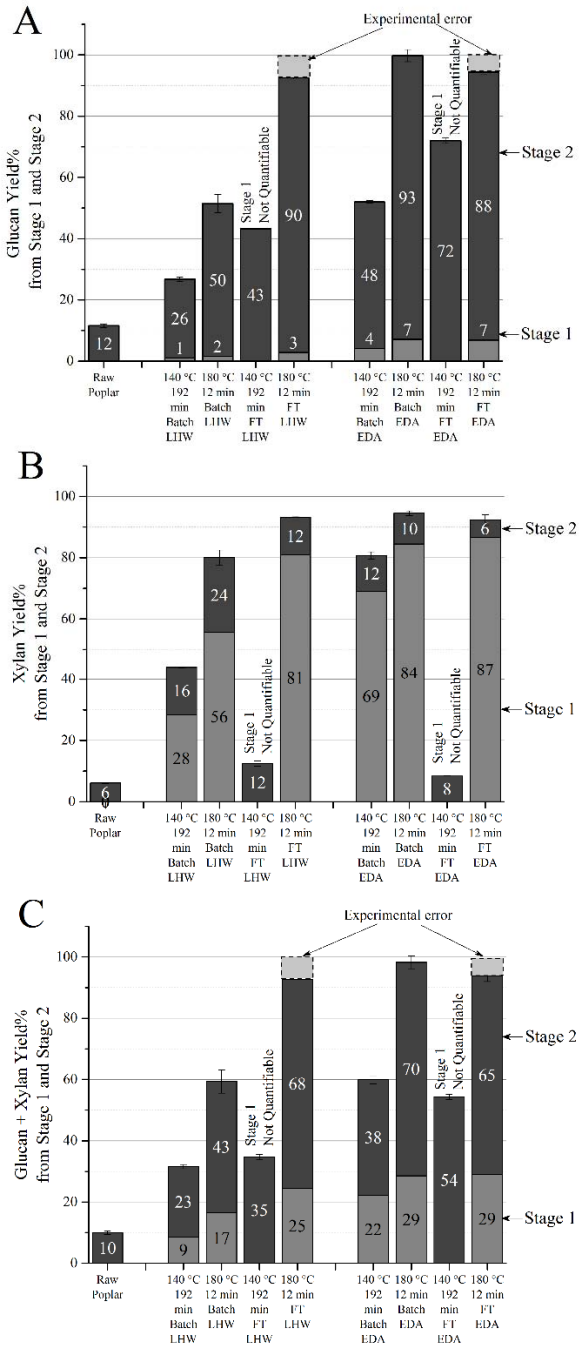

Figure 4.6 Stage 1 and Stage 2 sugar yields
Glucan (A), xylan (B), and glucan + xylan (C) yields from Stage 1 and Stage 2 combined. Stage 2 enzymatic hydrolysis was performed with 100 mg cellulase per gram glucan initially in the raw poplar using flasks shaken at 150 rpm for 120 hours at 50°C.

4.5 Conclusions

The results from this study suggests that about 30 to 35% of the lignin in poplar that could not be removed by flowthrough pretreatment with or without adding acid is an important contributor to the recalcitrance of biomass to enzymatic digestion. These results also point to the possibility that hemicellulose removal is more important as an indicator of lignin disruption than in playing a direct role in reducing biomass recalcitrance. Moreover, solids from 180°C flowthrough pretreatment were much more digestible than solids pretreated at 140°C despite similar lignin and extensive hemicellulose removal for both, suggesting that higher temperatures play a vital role in improving cellulose digestibility through modifying lignin and/or other barriers. Adding a small amount of acid disproportionately enhanced hemicellulose solubilization for both batch and flowthrough pretreatments. Use of extremely dilute acid in batch pretreatment led to high recovery of xylan in the form of monomeric xylose.

4.6 Abbreviations

LHW: Liquid hot water **EDA:** Extremely dilute acid **BESC:** Bioenergy Science Center **BESC STD:** BESC standard poplar

4.7 Acknowledgements

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4.9 Additional Information

4.9.1 Material balances

This additional information shows detailed material balances for Stage 1 (pretreatment) and Stage 2 (enzymatic hydrolysis) for all process conditions.

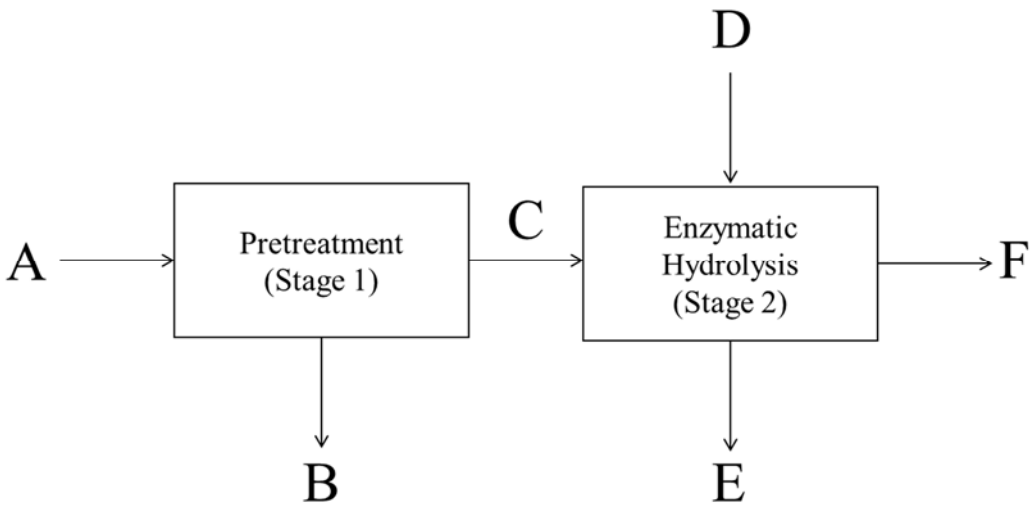

Figure 4.7. Material balance diagram

100 kg biomass basis
Enzyme after reaction assumed to remain in biomass residue

Table 4.2 Materials in Stream A (in kilograms)

Condition	Mass of Solid	Glucan in Raw	Xylan in Raw	Lignin in Raw	Others in Raw	Acid to Stage 1	Water To Stage 1
No pretreatment	100	46	18	28	9	0	0
140 °C 192 min Batch LHW	100	46	18	28	9	0	4900
180 °C 12 min Batch LHW	100	46	18	28	9	0	4900
140 °C 192 min FT LHW	100	46	18	28	9	0	396000
180 °C 12 min FT LHW	100	46	18	28	9	0	36000
140 °C 192 min Batch EDA	100	46	18	28	9	2	4898
180 °C 12 min Batch EDA	100	46	18	28	9	2	4898
140 °C 192 min FT EDA	100	46	18	28	9	198	395802
180 °C 12 min FT EDA	100	46	18	28	9	18	35982

Table 4.3 Materials in stream B (in kilograms)

Condition	Total Mass of Liquid	Glucose in Pretreatment Liquor	Cellobiose in Pretreatment Liquor	Glucooligomers in Pretreatment Liquor	Xylose in Pretreatment Liquor	Xylooligomers in Pretreatment Liquor	Solublized Lignin in Pretreatment Liquor	Others in Pretreatment Liquor
No pretreatment	0	0	0	0	0	0	0	0
140 °C 192 min Batch LHW	4919	0	0	0	0	5	6	6
180 °C 12 min Batch LHW	4924	0	0	1	1	9	6	8
140 °C 192 min FT LHW	396047	N/A	N/A	N/A	N/A	N/A	18	9
180 °C 12 min FT LHW	36047	0	0	1	0	14	18	9
140 °C 192 min Batch EDA	4927	1	1	0	9	3	6	8
180 °C 12 min Batch EDA	4936	2	2	0	13	1	9	8
140 °C 192 min FT EDA	396049	N/A	N/A	N/A	N/A	N/A	19	9
180 °C 12 min FT EDA	36050	1	2	0	5	11	19	9

Table 4.4 Materials in stream C (in kilograms)

Condition	Mass of Solid	Glucan in Pretreated Solid	Xylan in Pretreated Solid	Lignin in Pretreated Solid	Others in Pretreated Solid
No pretreatment	100	46	18	28	9
140 °C 192 min Batch LHW	81	44	12	22	3
180 °C 12 min Batch LHW	76	46	8	22	1
140 °C 192 min FT LHW	53	39	4	10	0
180 °C 12 min FT LHW	53	41	2	10	0
140 °C 192 min Batch EDA	73	45	6	22	1
180 °C 12 min Batch EDA	64	42	2	19	1
140 °C 192 min FT EDA	51	40	2	9	0
180 °C 12 min FT EDA	50	40	1	9	0

Table 4.5 Materials in stream D (in kilograms)

Condition	Enzyme to Stage 2	Water to Stage 2	Citrate Buffer to Stage 2	Antibiotic to Stage 2	Total Reaction Mass of EH
No pretreatment	5	4435	59	1	4600
140 °C 192 min Batch LHW	5	4257	56	1	4400
180 °C 12 min Batch LHW	5	4459	59	1	4600
140 °C 192 min FT LHW	5	3792	50	1	3900
180 °C 12 min FT LHW	4	3989	53	1	4100
140 °C 192 min Batch EDA	4	4363	58	1	4500
180 °C 12 min Batch EDA	4	4076	55	1	4200
140 °C 192 min FT EDA	4	3893	51	1	4000
180 °C 12 min FT EDA	4	3894	52	1	4000

Table 4.6 Materials in stream E (in kilograms)

Condition	Total Mass of EH Liquor	Glucose in EH liquor	Xylose in EH Liquor	Buffer in EH Liquor	Antibiotic in EH Liquor
No pretreatment	4502	5	1	59	1
140 °C 192 min Batch LHW	4329	12	3	56	1
180 °C 12 min Batch LHW	4546	23	4	59	1
140 °C 192 min FT LHW	3865	20	2	50	1
180 °C 12 min FT LHW	4086	41	2	53	1
140 °C 192 min Batch EDA	4446	22	2	58	1
180 °C 12 min Batch EDA	4176	42	2	55	1
140 °C 192 min FT EDA	3979	33	1	51	1
180 °C 12 min FT EDA	3988	40	1	52	1

Table 4.7 Materials in stream F (in kilograms)

Condition	Total Mass of Residue	Glucan in Residue	Xylan in Residue	Lignin in Residue	Others in Residue	Enzyme In Residue
No pretreatment	98	41	17	28	8	5
140 °C 192 min Batch LHW	71	32	9	22	3	5
180 °C 12 min Batch LHW	54	23	4	22	0	5
140 °C 192 min FT LHW	35	19	2	10	0	5
180 °C 12 min FT LHW	14	0	0	10	0	4
140 °C 192 min Batch EDA	54	23	4	22	1	4
180 °C 12 min Batch EDA	24	0	0	19	1	4
140 °C 192 min FT EDA	21	7	1	9	0	4
180 °C 12 min FT EDA	12	0	0	9	0	4

Chapter 5

Effects of Dilute Acid and Flowthrough Pretreatments and BSA Supplementation on Enzymatic Deconstruction of Poplar by Cellulase and Xylanase*

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5.1 Abstract

To help understand factors controlling the recalcitrance of lignocellulosic biomass to deconstruction to sugars, poplar hardwood was pretreated with liquid hot water (LHW) and extremely dilute acid (EDA) at 140 and 180°C in batch and flowthrough reactors. The resulting solids were then subjected to enzymatic hydrolysis by eight combinations that included, 5, 15, and 100 mg cellulase, co-addition and sequential addition of 30 mg xylanase and 15 mg cellulase, co-addition of 15 mg cellulase and 30 mg BSA, and pre-addition of 100 mg BSA followed by 15 mg cellulase or co-addition of 15 mg cellulase and 30 mg xylanase based on one gram glucan in unpretreated biomass. For LHW pretreatment followed by enzymatic hydrolysis with just cellulase at a loading of 100 mg per gram glucan, the highest overall sugar yields were 35 and 58% from batch operation at 140 and 180°C, respectively, and 66 and 100% from LHW flowthrough operation for these temperatures. On the other hand, with the same cellulase loading, EDA increased maximum overall sugar yields to 58 and 100% for batch and 88 and 100% for flowthrough pretreatments at 140 and 180°C, respectively. However, co-addition of 30 mg xylanase and 15 mg cellulase resulted in 0 to 11% higher sugar yield than sequential addition 30 mg xylanase for 4 days followed by 15 mg cellulase in all solids. 100 mg BSA supplementation to 15 mg cellulase increased overall yields from LHW and EDA pretreated solids by up to 7% and 19%, respectively, and was 4 to 10% more effective for flowthrough than for batch solids. BSA supplementation had the greatest impact on yields from solids prepared by batch pretreatments at 180°C with EDA. However, BSA affected glucan but not xylan yields. Consistent with conclusions by others, flowthrough

pretreatment produced less recalcitrant solids than did batch operation, but using very dilute acid reduced recalcitrance even more. In any event, operation at 180°C was needed to achieve complete solubilization of sugars. Co-addition of xylanase to cellulase proved to be a better strategy than adding xylanase followed by cellulase. BSA enhanced yields more from solids produced by flowthrough pretreatment than by batch, and adding extremely dilute acid enhanced yields from enzymatic hydrolysis even more. Moreover, BSA supplementation only affected yields from glucan and not from xylan.

5.2 Introduction

Production of sustainable liquid fuels from plant biomass is vital for reducing greenhouse gas emissions and attaining energy sustainability and independence (Gupta, 2008). Cellulose, hemicellulose, and lignin are the three main structural components that make up the bulk of this resource. Cellulose is the longest polysaccharide, contains only glucose, and represents about 35 to 50 weight% of the structural fraction. Hemicellulose, about 10 to 30 weight%, is also formed from sugars but its constitution varies among hardwoods, softwoods, and grasses. Lignin, about 10 to 30 weight%, is polymerized from nine carbon aromatic monomers and has a very heterogeneous composition and architecture (Lynd et al., 1999). The sugars in cellulose and hemicellulose offer an enormous potential resource for fermentation into ethanol and other products, and the aromatics in lignin could be valuable for conversion into chemicals or fuels. However, deconstruction of biomass into sugars and aromatics from lignocellulosic biomass requires overcoming its recalcitrance to thermal or biological processing that currently results in high overall conversion costs. Hemicellulose and lignin in particular present

important obstacles to biological processing as they cover cellulose in unprocessed biomass and impede access of bulky enzymes to cellulose. As a result, some type of pretreatment is generally needed to disrupt the hemicellulose-lignin shield and achieve economically viable sugar yields.

Aqueous batch pretreatments such as liquid hot water and dilute acid solubilize a large portion of hemicellulose, only a small amount of lignin, and limited quantities of cellulose. Through this process, pretreated solids have much increased cellulose surface area and pore accessibility due to swelling, hemicellulose solubilization and shrinking of lignin through coalescence. Although pretreatment lowers recalcitrance and aids in enzymatic hydrolysis of cellulose into glucose that may reach near theoretical yields (Wyman et al., 2005c), pretreatment and enzymatic hydrolysis are the most expensive steps for recovery of fermentable sugars for biological processing and require improvements to reduce pretreatment and/or enzyme costs (Klein-Marcuschamer et al., 2012) while still achieving commercially viable sugar yields from cellulose and hemicellulose combined.

Enzymes can adsorb onto lignin which lowers the amount of active enzyme available for hydrolysis. One strategy to prevent this loss can be through blocking of the surface of lignin by bovine serum albumin (BSA) or Tween® 20 (non-ionic surfactant) (Brethauer et al., 2011; Eriksson et al., 2002; Yang & Wyman, 2006). In one-study, pre-addition of 1% solution of BSA in enzymatic hydrolysis of dilute acid pretreated corn stover (1% H_2SO_4, 140°C, 40 min) increased yield from 82 to 92% at an enzyme loading of 15 FPU cellulase per gram cellulose after 72 hours (Yang & Wyman, 2006). In another

study, pre-addition of 300 mg BSA per g glucan in unpretreated biomass to enzymatic hydrolysis of dilute acid pretreated poplar (0.02% H_2SO_4, 190°C) raised glucose yields from about 55 to 63% at a dose of 20 mg enzyme per gram glucan in unpretreated biomass after 72 hours (Kumar & Wyman, 2009). In yet another study, blocking lignin through pre-addition of 50 mg BSA per gram of mixed hardwood chips pretreated in liquid hot water at 220°C for 15 minutes increased enzymatic hydrolysis glucose yield from around 18% to 72% at an enzyme loading of 8 mg protein per gram glucan (Ko et al., 2015a; Ko et al., 2015b). However, these studies that employed BSA were carried out on solids pretreated in severe conditions. Unproductive loss to lignin in less severe pretreatments and lower temperatures has not been studied. Moreover, solids pretreated in aqueous conditions at high pretreatment severities have low xylan content. It is not known how residual xylan in moderately pretreated solids affects cellulose deconstruction by cellulase. Moreover, such studies have never been carried out flowthrough pretreated biomass.

Thus, moderately pretreated poplar hardwood solids were produced through liquid hot water and extremely dilute acid pretreatments (0.05% acid) in batch and flowthrough reactors at 140°C for 192 minutes and 180°C for 12 minutes. Our previous study focused on sugar and lignin mass balances of poplar from these pretreatments, and enzymatic hydrolysis at high enzyme loading of 100 mg cellulase per gram glucan in unpretreated biomass (Bhagia et al., 2016b). A distinct characteristic of flowthrough pretreated solids was that they were lower in lignin content than batch pretreated solids as flowthrough pretreatment reduces or prevents lignin re-deposition (Yang & Wyman, 2004). First,

three cellulase loadings of 5, 15 and 100 mg cellulase (per gram glucan in unpretreated biomass) were employed to learn how cellulase loading affects deconstruction in these solids. Since many solids had significant amount of xylan in pretreated solids, 30 mg xylanase was supplemented through co-addition or sequential addition to 15 mg cellulase for their effect on xylan yields as well as glucan yields. Next, 30 mg of xylanase co-added to 15 mg cellulase was replaced with 30 mg BSA to distinguish between its catalytic effects and unproductive loss to lignin. Finally, pre-addition of high loading of 100 mg BSA to poplar solids was done to block lignin followed by enzymatic hydrolysis with just 15 mg cellulase or co-addition of 15 mg cellulase and 30 mg xylanase. In all, these experiments explore deconstruction of polysaccharides in poplar layer by layer through moderate pretreatment followed by enzymatic hydrolysis in many enzyme formulations and protein supplementation strategies.

5.3 Materials and Methods

5.3.1 Materials

Bioenergy Science Center standard poplar (BESC STD) that had been provided to University of California at Riverside (UCR) by Oak Ridge National Laboratory (ORNL) (Oak Ridge, TN, USA) was used in this study after knife milling through a 1 mm screen (Model 4 Wiley Mill of Thomas Scientific Company at Swedesboro NJ). DuPont Industrial Biosciences (Palo Alto, CA) graciously supplied Accellerase® 1500 cellulase (Batch# 4901298419 Genencor®, DuPont Industrial Biosciences) and Multifect® Xylanase (Lot# 301-04021-015, Genencor®, DuPont Industrial Biosciences). Protein contents of these commercial preparations as determined by the standard BCA method

(Smith et al., 1985a) were 82 mg/ml for Accellerase® 1500 and 42 mg/ml for Multifect® Xylanase. Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (St. Louis MO).

5.3.2 Pretreatments

Flowthrough and batch pretreatments were applied with just hot water (LHW) and 0.05 wt% sulfuric acid (EDA) at 140 and 180°C in the combinations summarized in Table 5.1 with the times adjusted to keep the severity parameter value at 3.4 in LHW and combined severity of 1.4 in EDA to gain a perspective of the effect of temperature on recalcitrance. Details of the pretreatment methods have been previously described (Bhagia et al., 2016b).

Table 5.1: Pretreatment configurations and conditions.

#	Aqueous condition	Type	Severity factor[1]	Temperature (°C)	Time (min)
1	Liquid hot water	Flowthrough	$\log_{10}R_0$ = 3.4	140	192
2		Flowthrough		180	12
3		Batch		140	192
4		Batch		180	12
5	Extremely dilute acid (0.05%)	Flowthrough	logCS = 1.4	140	192
6		Flowthrough		180	12
7		Batch		140	192
8		Batch		180	12

[1] R_0 = t*exp((T-100)/14.75)), where t is the time in minutes and T is the temperature in °C. N/A- not applicable. $\log_{10}R_0$ is called the pretreatment severity factor. logCS = $\log_{10}R_0$ – pH is called the combined severity factor.

5.3.3 Solids Composition

Prior to compositional analysis, biomass solids were dried at 37°C for several days until the moisture content dropped to about 4 to 7%, with this moisture content taken into account for calculation of dry weight. Composition of biomass was then measured according to standard NREL procedure "Determination of Structural Carbohydrates and Lignin in Biomass (Sluiter et al., 2008)." A UV-vis spectrophotometry with a Spectramax® M2e Plate Reader (Molecular Devices, Sunnyvale, CA, USA) equipped with SoftMax® Pro data acquisition software in a Costar® UV 96 well pate with absorbance of water blank taken into account for correction of absorbance in sample was employed to measure acid soluble lignin. Three

replicates for each sample in 96 well plate were kept for measurements. An absorption coefficient of 25 L/gram.cm for a 240 nm wavelength was applied per the NREL standard procedure (Sluiter et al., 2008) to calculate acid soluble lignin concentration of poplar using the Beer Lambert Bouguer law.

As reported in our previous sugar and lignin mass balances for pretreatment (Stage 1) coupled with hydrolysis at high enzyme loadings (Stage 2) (Bhagia et al., 2016b), lignin can condense onto solids after solubilization during batch pretreatment, while continuous flow of liquid during flowthrough pretreatment removes a large portion of solubilized lignin from the reactor before it can condense. Thus, flowthrough solids have a much lower lignin content, as shown by the balance among glucan, xylan and total lignin for solids in Table 5.2.

Table 5.2 Composition of unpretreated poplar and solids resulting from each of the pretreatments outlined in Table 5.1.

	Glucan%	Xylan%	Total Lignin%
Unpretreated Poplar	46	17.5	28
140 °C 192 min Batch LHW	54	15	28
180 °C 12 min Batch LHW	60	10	29
140 °C 192 min FT LHW	73	8	20
180 °C 12 min FT LHW	77	5	18
140 °C 192 min Batch EDA	61	8	31
180 °C 12 min Batch EDA	66	3	29
140 °C 192 min FT EDA	78	5	18
180 °C 12 min FT EDA	81	2	18

5.3.4 Enzymatic Hydrolysis

Enzymatic hydrolysis was performed in duplicates according to the NREL standard procedure "Enzymatic saccharification of lignocellulosic biomass" (Selig et al., 2008) at a 1% glucan loading, 50°C, 150 rpm, and a total reaction volume of 10 ml. All enzymatic hydrolysis runs were carried out for 120 hours after cellulase addition. To follow enzymatic hydrolysis progress, 0.5 ml aliquots were withdrawn 4 hours after

addition of enzymes and then after every 24 hours. All protein loadings mentioned throughout this study were based on milligrams of protein per gram glucan in the poplar prior to pretreatment.

Three loadings of commercial cellulase shown in Table 5.3 were applied to the pretreated solids. However, because pretreatment at moderate severity left more xylan in solids than pretreatment at optimum higher severity conditions, the next set of enzyme loadings listed in Table 5.3 added commercial xylanase to cellulase to learn how much of the residual xylan could be solubilized and how cellulose solubilization is impacted by xylanases. Both enzymes were added at the same time in one set of experiments and sequentially in another to determine if the addition strategy affected sugar yields. Because enzyme unproductively adsorbs on lignin in pretreated biomass and amphiphilic substances such as BSA and Tween reduce such enzyme loss by blocking lignin (Eriksson et al., 2002; Yang & Wyman, 2006), bovine serum albumin (BSA) was added along with cellulases and xylanases in another set of experiments to understand if it could enhance enzyme effectiveness, particularly at lower enzyme loadings than typically applied. In the experiments with high BSA doses, we were interested in learning how cellulose deconstruction is impacted if unproductive binding of enzymes to lignin is minimized. The final condition listed in Table 5.3 replaced xylanase with the same amount of BSA to separate the catalytic effects of xylanase from loss to lignin.

Table 5.3 Loadings and timings of cellulase, xylanase, and BSA additions to pretreated poplar solids.	
Condition	**All protein loadings were based on mg per gram glucan in unpretreated biomass**
1	5 mg cellulase
2	15 mg cellulase
3	100 mg cellulase
4	100 mg BSA addition for 12 hours followed by 15 mg cellulase
5	Co-addition of 15 mg cellulase and 30 mg xylanase
6	30 mg xylanase for 4 days followed by 15 mg cellulase
7	Co-addition of 15 mg cellulase and 30 mg BSA
8	100 mg BSA addition for 12 hours followed by co-addition of 15 mg cellulase + 30 mg xylanase

5.3.5 Sugars Determination

Waters® e2695 Separations Module with detection on Waters® 2414 RI detector provided the platform for all sugar analyses. This HPLC was equipped with a Biorad® Aminex® HPX-87H column conditioned at 65°C and used 5 mM sulfuric acid for the mobile phase.

5.3.6 Calculations

The mass of each sugar was converted to the mass of the corresponding anhydrous form by multiplying glucose values by 0.9 and xylose measurements by 0.88 to account for the mass of water added during hydrolysis. Enzymatic hydrolysis loadings were based on mg of protein per gram glucan in the unpretreated biomass to keep overall enzyme loadings consistent for each pretreatment. Mass units were in grams, volumes in liters, and concentrations in grams per liter. Stage 1 and Stage 2 refer to the pretreatment and enzymatic hydrolysis stages, respectively. Yields were calculated by the following expressions:

1. Stage 1 sugar (glucan + xylan) yield =

$$\frac{\begin{array}{c}(\text{absolute glucan } + \text{ xylan in unpretreated biomass}) - \\ (\text{absolute glucan } + \text{ xylan in pretreated biomass solids})\end{array}}{\text{absolute glucan } + \text{ xylan in unpretreated biomass}} * 100$$

2. Enzyme loading =

$$\frac{\text{mg of protein per gram glucan in enzymatic hydrolysis flask}}{\text{glucan yield fraction after pretreatment}}$$

3. Enzymatic saccharification efficiency% =

$$\frac{\begin{array}{c}\text{Concentration of monomeric sugar from HPLC} * \text{anhydrous correction factor} * \\ \text{total reaction volume of enzymatic hydrolysis flask}\end{array}}{\text{mass of glucan or xylan in enzymatic hydrolysis flask}}$$

4. Stage 2 sugar yield =

$$\frac{\text{Enzymatic saccharification efficiency\%} * \text{mass of glucan or xylan fed to stage 2}}{\text{Mass of polymeric sugar in solid before pretreatment}}$$

5. Overall glucan or xylan yield = Stage (1+2) glucan or xylan yield = Stage 1 yield% + Stage 2 yield%
6. Overall sugar yield = Stage (1+2) (glucan + xylan) yield = (Stage 1 glucan + xylan yield%) + (Stage 2 glucan + xylan yield%)

Throughout this paper, sugar yield is defined as the total glucan + xylan yield, and Stage 1 + Stage 2 yield mean the overall yield from pretreatment combined with enzymatic hydrolysis. Pretreatment sugar yields were calculated by dividing the difference between the initial content of each sugar in the raw biomass and the amount left in the pretreated solids by the amount of that sugar in the raw poplar solids, with all amounts based on compositional analysis of the solids. This approach was necessary because the very low sugar concentrations in the liquors from 180°C and 140°C flowthrough pretreatments could not be measured accurately. However, because sugar degradation is low at these moderate pretreatment conditions, these sugar yields allow for fair comparisons of results from different pretreatment conditions. Stage 2 enzymatic hydrolysis yields were determined based on the mass of sugars released into the liquid by enzymatic hydrolysis as these could be measured through HPLC accurately. Mass balances that validate these approaches have been determined previously (Wyman et al., 2005a; Wyman et al., 2009).

5.3.7 Sugar Yields from Enzymatic Hydrolysis without Pretreatment

It is important to note that the poplar used in these experiments was very recalcitrant to enzymatic deconstruction without pretreatment. In particular, sugar yields were 4, 7, and 12% from addition of 5, 15, and 100 mg cellulase protein/g glucan,

respectively. In addition, xylanase and BSA supplementation to 15 mg cellulase did not realize sugar yields higher than 8% from unpretreated poplar.

5.4 Results and Discussion

Glucose and xylose yields from LHW and EDA pretreatments and subsequent enzymatic hydrolysis of the pretreated solids with the enzyme loadings outlined in Table 5.3 are pictured as "onion" diagrams in Figure 5.1 through 5.6. Onion diagrams were employed to visually illustrate how different layers of polysaccharides were "peeled off" first by pretreatment and then by enzymes, with the order of release providing a perspective on the transition from less to more recalcitrant components.

5.4.1 Sugar yields from Just Pretreatment

Figure 5.1 shows that Stage 1 sugar yields were only slightly higher from LHW pretreatment at 180°C than at 140°C for both batch and flowthrough reactor configurations. However, flowthrough pretreatment achieved about twice as high Stage 1 sugar yields with LHW (30 to 31%) as LHW batch operation (12 to 15%).

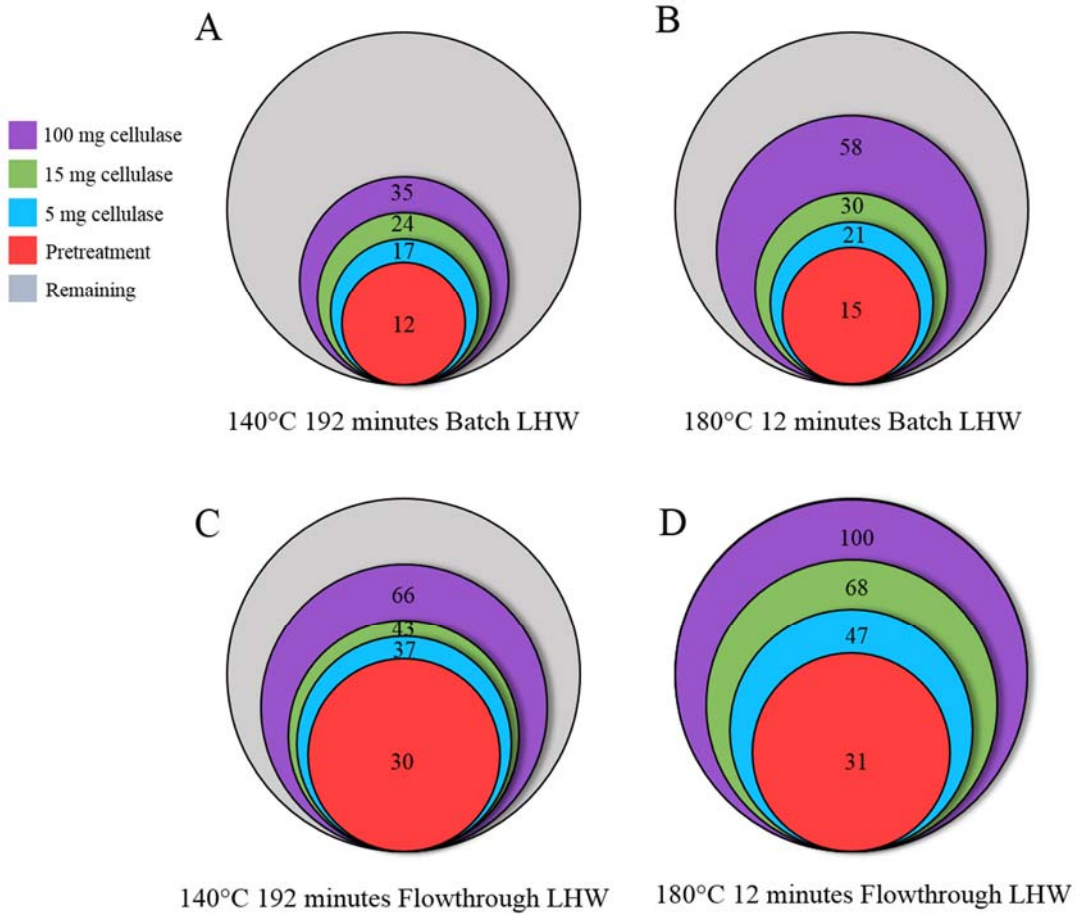

Figure 5.1 Effect of cellulase loadings of 5, 15, and 100 mg protein/g glucan in raw poplar on yields of total glucan plus xylan sugars from solids resulting from liquid hot water (LHW) pretreatments. A and B are for batch pretreatments at 140°C and 180°C, respectively, while C and D are for flowthrough pretreatments at 140°C and 180°C, respectively. Sugar yields from just pretreatment are in red, and the additional sugar yields resulting from subsequent addition of 5, 15 and 100 mg cellulase protein/g glucan in poplar are shown in blue, green, and purple, respectively. Data labels represent overall sugar yield.

Differences in pretreatment sugar yields between batch (20 to 30%) and flowthrough pretreatment (34 to 35%) were lower for EDA pretreatments in Figure 5.2 than for LHW conditions in Figure 5.1. Furthermore, because EDA operation increased xylan yields from 68% for LHW pretreatment to 94%, kinetic and mass transfer xylan removal rates were more similar than for LHW conditions. In addition, overall glucose plus xylose Stage 1 yields increased from 20% at 140°C to 30% at 180°C for EDA batch pretreatment. The highest sugar yield from just pretreatment was for EDA flowthrough operation at 140 (34%) and 180°C (35%). For both LHW and EDA batch pretreatments, most of the sugar was from xylan as Stage 1 glucan yields were low (2 to 7%). Nonetheless, flowthrough pretreatment produced higher glucan yields than batch operations (10 to 13%), as shown in the "pretreatment" columns in Tables 5.4 through 5.6. Because xylan yields were much higher from flowthrough than batch pretreatment, the xylan contents in flowthrough pretreated solids reported in Table 5.2 were much lower.

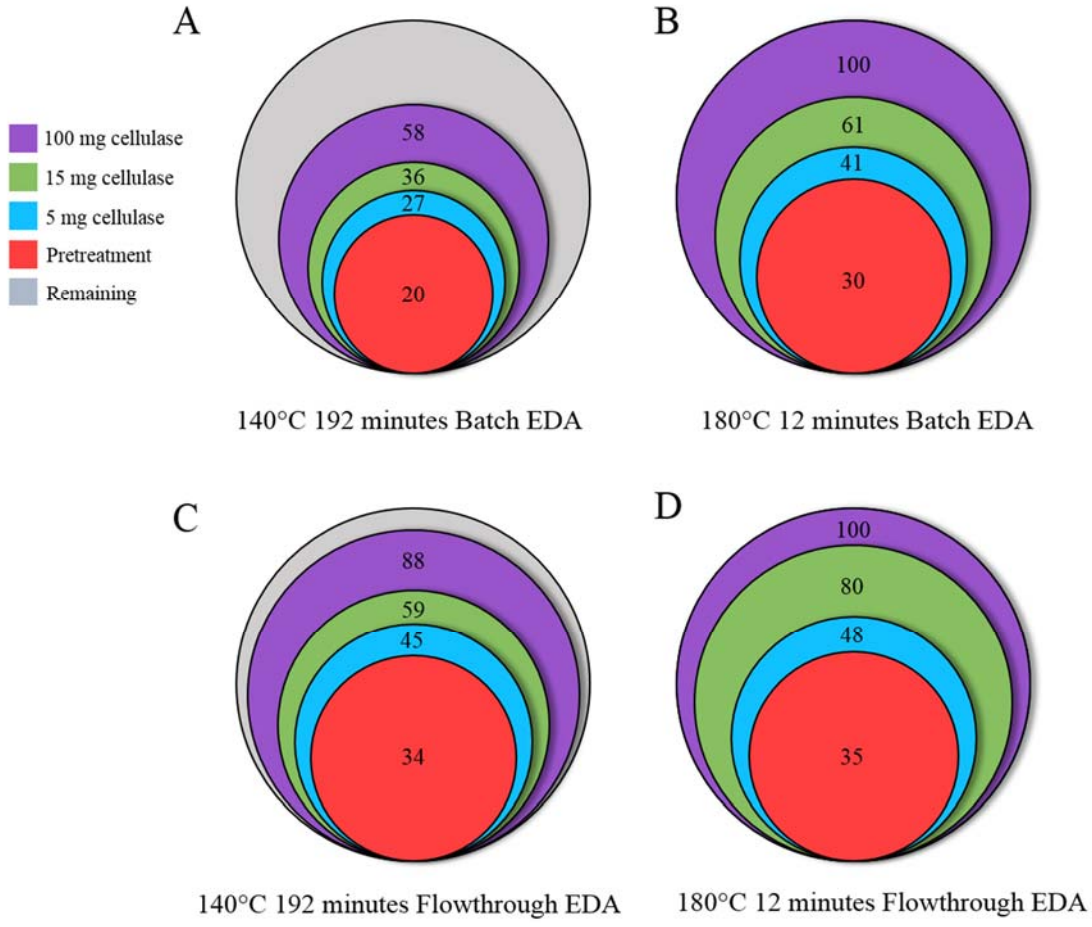

Figure 5.2 Effect of cellulase loadings of 5, 15, and 100 mg protein/g glucan in raw poplar on yields of total glucan plus xylan sugars from solids resulting from extremely dilute acid (EDA) pretreatments. A and B are for batch pretreatments at 140ºC and 180°C, respectively, while C and D are for flowthrough pretreatments at 140 and 180°C, respectively. Sugar yields from just pretreatment are in red, and the additional sugar yields resulting from subsequent addition of 5, 15 and 100 mg cellulase protein/g glucan in poplar are shown in blue, green, and purple, respectively. Data labels represent overall sugar yield.

Table 5.4 Overall xylan yields resulting from application of the pretreatment conditions listed in Table 5.1 followed by enzymatic hydrolysis with the enzyme loadings and mixtures listed in Table 5.3.

	Pretreatment Yield	Overall Yield								
		5 mg cellulase *(5C)*	**15 mg cellulase *(15C)***	**100 mg cellulase *(100C)***	**Co-addition of 15 mg cellulase and 30 mg BSA *(15C+30BSA)***	**100 mg of BSA for 24 hours followed by 15 mg cellulase *(100 BSA + 15C)***	**Co-addition of 15 mg cellulase and 30 mg xylanase *(15C+30X)***	**30 mg xylanase *(30X)***	**30 mg xylanase for 4 days followed by 15 mg cellulase *(30X + 15 C)***	**100 mg BSA for 24 hours followed by co-addition of 15 mg cellulase and 30 mg xylanase *(100BSA +15C+30X)***
Unpretreated Poplar	N/A	3	3	6	3	3	3	3	3	3
140 °C 192 min Batch LHW	32	36	41	48	39	41	46	41	44	45
180 °C 12 min Batch LHW	55	61	68	79	65	68	76	68	71	75
140 °C 192 min FT LHW	77	80	81	89	82	82	87	81	86	86
180 °C 12 min FT LHW	86	87	91	98	90	91	91	88	93	91
140 °C 192 min Batch EDA	68	70	73	80	72	74	73	71	73	73
180 °C 12 min Batch EDA	89	89	92	99	91	94	92	90	91	93
140 °C 192 min FT EDA	87	87	90	95	91	91	92	88	92	92
180 °C 12 min FT EDA	94	94	96	100	94	98	95	94	94	96

Table 5.5 Overall glucan yields resulting from application of the pretreatment conditions listed in Table 5.1 followed by enzymatic hydrolysis with the enzyme loadings and mixtures listed in Table 5.3.

	Pretreatment Yield	**Overall Yield**								
		5 mg cellulase *(5C)*	**15 mg cellulase *(15C)***	**100 mg cellulase *(100C)***	**Co-addition of 15 mg cellulase and 30 mg BSA *(15C+30BSA)***	**100 mg of BSA for 24 hours followed by 15 mg cellulase *(100 BSA + 15C)***	**Co-addition of 15 mg cellulase and 30 mg xylanase *(15C+30X)***	**30 mg xylanase *(30X)***	**30 mg xylanase for 4 days followed by 15 mg cellulase *(30X + 15 C)***	**100 mg BSA for 24 hours followed by co-addition of 15 mg cellulase and 30 mg xylanase *(100BSA +15C+30X)***
Unpretreated Poplar	N/A	4	7	12	8	8	8	2	8	8
140 °C 192 min Batch LHW	5	10	17	30	15	18	23	6	20	23
180 °C 12 min Batch LHW	0	6	15	50	13	17	26	2	20	27
140 °C 192 min FT LHW	11	21	28	57	32	33	44	13	40	42
180 °C 12 min FT LHW	10	32	60	100	60	69	78	12	77	80
140 °C 192 min Batch EDA	2	10	21	50	22	26	28	3	23	33
180 °C 12 min Batch EDA	7	23	49	100	53	68	59	9	50	77
140 °C 192 min FT EDA	13	29	47	85	59	66	67	16	66	73
180 °C 12 min FT EDA	12	30	74	100	89	100	100	14	84	100

Table 5.6 Overall glucan+xylan yields resulting from application of the pretreatment conditions listed in Table 5.1 followed by enzymatic hydrolysis with the enzyme loadings and mixtures listed in Table 5.3.

	Pretreatment Yield	**Overall Yield**								
		5 mg cellulase *(5C)*	**15 mg cellulase *(15C)***	**100 mg cellulase *(100C)***	**Co-addition of 15 mg cellulase and 30 mg BSA *(15C+30BSA)***	**100 mg of BSA for 24 hours followed by 15 mg cellulase *(100 BSA + 15C)***	**Co-addition of 15 mg cellulase and 30 mg xylanase *(15C+30X)***	**30 mg xylanase *(30X)***	**30 mg xylanase for 4 days followed by 15 mg cellulase *(30X + 15 C)***	**100 mg BSA for 24 hours followed by co-addition of 15 mg cellulase and 30 mg xylanase *(100BSA +15C+30X)***
Unpretreated Poplar	N/A	4	6	10	6	6	7	2	7	7
140 °C 192 min Batch LHW	12	17	24	35	22	24	30	16	27	29
180 °C 12 min Batch LHW	15	21	30	58	28	31	40	20	35	40
140 °C 192 min FT LHW	30	37	43	66	46	47	56	32	53	54
180 °C 12 min FT LHW	31	47	68	100	68	75	81	33	81	83
140 °C 192 min Batch EDA	20	27	36	58	36	40	41	22	37	44
180 °C 12 min Batch EDA	30	41	61	100	63	75	68	32	62	81
140 °C 192 min FT EDA	34	45	59	88	68	73	74	36	73	78
180 °C 12 min FT EDA	35	48	80	100	91	99	98	36	87	99

5.4.2 Effect of cellulase loading

Figure 5.1 shows overall sugar yields from enzymatic hydrolysis of LHW pretreated solids at cellulase loadings of 5, 15, and 100 mg protein/g glucan in raw poplar. As noted in Figures 5.1A and B, overall (Stage 1+ Stage 2) sugar yields were higher from batch LHW pretreatment at 180°C than at 140°C. LHW operation at 180°C only improved overall sugar yields slightly from values achieved at 140°C for enzyme loadings of 5 and 15 mg protein/g glucan. For 140°C LHW pretreatment, 5 mg of cellulase protein increased sugar yields from 12% following pretreatment to 17% after enzymatic hydrolysis, while adding 15 mg of cellulase increased sugar yields further to 24%. At 180°C, this same sequence increased yields from 15% to 21% and on to 30%. Although 100 mg of cellulase protein had a much larger effect, overall yields leveled off at 35% for solids from batch LHW pretreatment at 140°C and at 58% for operation at 180°C. On the other hand, solids generated by flowthrough pretreatment showed considerably higher overall sugar yields realized by batch pretreatment at the same temperatures. Furthermore, sugar yields were much higher for solids from 180°C flowthrough operation than those produced at 140°C even at the lower enzyme loadings of 5 and 15 mg cellulase protein. In particular, for 140°C flowthrough pretreatment, overall sugar yields increased from 30% for just pretreatment to 37% with 5 mg cellulase and further to 43% with 15 mg cellulase. For 180°C flowthrough operation, overall sugar yields increased from 31% for just pretreatment to 47% with 5 mg cellulase and further to 68% with 15 mg cellulase. LHW flowthrough pretreatment at 180°C for 12 minutes generated the least recalcitrant solids produced by LHW, In addition, solids generated at

this pretreatment condition were the only ones that could achieve 100% sugars solubilization among all LHW solids when subjected to the high enzyme dose of 100 mg cellulase protein. Thus, higher temperature LHW pretreatment in both pretreatment configurations produced solids that had lower recalcitrance at the same pretreatment severity factor. It is important to note that glucan has the dominant effect on overall sugar yields due to the higher glucan content in poplar (composition of pretreated solids shown in Table 5.1). Overall xylan yields reported in Table 5.4 reached a maximum of 48% and 79% (based on xylan in raw poplar) with 100 mg cellulase from solids produced by 140°C and 180°C LHW batch pretreatment, respectively. Adding 100 mg of cellulase protein boosted the overall xylan yield to 89% from 140°C LHW batch pretreatment and 98% from 180°C LHW operation. The lower sugar yields from enzymatic hydrolysis of the solids produced by batch LHW pretreatment compared to flowthrough LHW operation are likely due to higher xylan and lignin contents in the former.

Figure 5.2 also reports the effect of cellulase loadings on overall sugar yields from solids produced by EDA pretreatments. As for LHW pretreatment, yields from pretreatment alone as well as overall yields were significantly higher for solids subjected to 180°C than 140°C. In fact, solids from 140°C batch EDA pretreatment were quite recalcitrant, with the maximum overall yield being only 58% despite an enzyme loading of 100 mg cellulase protein. Unlike LHW for which only the 180°C flowthrough pretreatment achieved a 100% overall sugar yield, EDA pretreatments at 180°C achieved 100% overall sugar yields for both batch and flowthrough configurations. Furthermore, even for a low loading of 5 mg cellulase protein, the difference in overall sugar yields

between 140°C (45%) and 180°C (48%) flowthrough pretreatments was minor. However, the differences increased substantially when the enzyme loading was dropped to 15 mg protein: the overall sugar yield from 180°C flowthrough pretreatment increased to 80% while that from 140°C flowthrough operation only rose to 59%.

These results provide some insights into features contributing to recalcitrance in hardwoods like poplar pretreated at aqueous conditions. Because the three solids that realized complete deconstruction to sugars were all pretreated at 180ºC, temperature plays a crucial role in reducing recalcitrance. Furthermore, despite high lignin removal by 140°C flowthrough pretreatments, high yields could not be achieved after 5 days of enzymatic hydrolysis even for the high enzyme loading of 100 mg cellulase protein. On the other hand, near theoretical overall sugar yields were possible from extremely dilute acid pretreated solids with lignin contents as high as 29%, provided the highest enzyme loading was applied. Sugar yields from flowthrough pretreatment were always superior to those from batch operation for the same pretreatment temperature, time, and aqueous condition, likely due to greater xylan and lignin removal. It was unexpected that adding so little acid to water would reduce recalcitrance of pretreated solids to subsequent enzymatic hydrolysis to the extent observed over the wide range of enzyme loadings applied.

5.4.3 Effect of Adding Xylanase

Two strategies were employed for supplementing cellulase with xylanase. First, 15 mg of cellulase protein together with 30 mg of xylanase protein per gram glucan in unpretreated biomass were added at the same time. In the second strategy, 30 mg of xylanase protein was added 4 days before addition of 15 mg of cellulase protein. Figure 5.3 shows that either of these strategies increased overall sugar yields from LHW pretreatment. However, only solids produced by180°C LHW flowthrough pretreatment realized the same overall yields (81%) for either xylanase supplementation strategy. It is interesting to note that these solids had the lowest xylan content (5%) of all LHW solids. For the other three LHW pretreatment conditions, overall yields were higher 5 days after co-addition of cellulase and xylanase than when xylanase was allowed to act alone for 4 days prior to adding cellulase followed by another 5 days of reaction. These trends for supplementing cellulase with xylanase were similar to those seen in Figure 5.1 for varying cellulase doses, that is to say, xylanase supplementation resulted in larger improvements in overall yields from solids that pretreatment had rendered less recalcitrant.

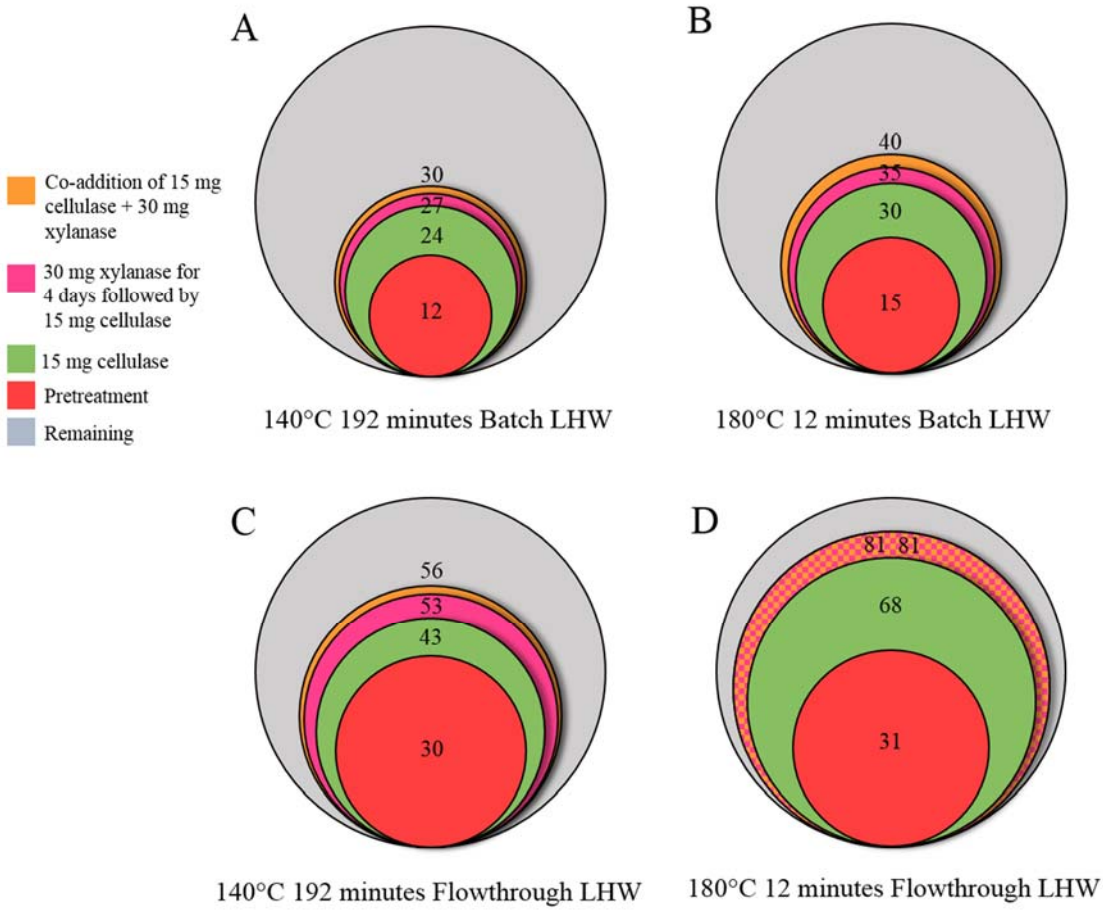

Figure 5.3 Effect of adding xylanase to cellulase on yields of total glucan plus xylan sugars from solids resulting from liquid hot water (LHW) pretreatments. A and B are for batch pretreatments at 140ºC and 180°C, respectively, while C and D are for flowthrough pretreatments at 140 and 180°C, respectively. Sugar yields from just pretreatment are in red, and overall yields with 15 mg cellulase, sequential addition of 30 mg xylanase followed by 15 mg cellulase, and co-addition of 15 mg cellulase and 30 mg xylanase are in green, pink, and orange colors, respectively. Enzymes were loaded as mg of protein per gram glucan in unpretreated biomass. Data labels represent overall sugar yields.

Crude cellulase preparations have some xylanase activity, and crude xylanase preparations have some cellulase activity (Chundawat et al., 2011b). Moreover, endoglucanase (EG 1) is highly active on xylan and not just on cellulose. For example, Gao et al. (Gao et al., 2010) reported that EG1 had a specific activity of 5.08 U on xylan compared to 8.24 U for endoxylanase. As noted in Table 5.4, 4 days of hydrolysis with xylanase alone resulted in some improvement in xylan saccharification but negligible improvement in glucan saccharification. Interestingly, overall xylan yields with 30 mg of xylanase alone were the same as with 15 mg of cellulase alone. Since crude xylanase preparation (Multifect® Xylanase) without cellulase (Accellerase® 1500) did not show significant glucan hydrolyzing ability (Table 5.5), xylan yield with just 30 mg xylanase can mean that this portion of xylan was accessible to xylanase and was not obstructed by cellulose.

Figure 5.4 shows results from supplementing cellulase with xylanase for enzymatic hydrolysis of solids pretreated at EDA conditions. For batch pretreatments, adding xylanase increased overall yields from 36 to 41% for solids produced at 140°C and from 61 to 68% for solids produced at 180°C. For flowthrough pretreatment, yields increased from 59 to 74% for 140°C and from 80 to 98% for 180°C operation. Thus, xylanase supplementation improved overall sugar yields from solids produced by flowthrough pretreatment more than from solids resulting from batch operations. Similar to results with solids produced by LHW pretreatments, higher overall yields were achieved through co-addition of cellulase and xylanase than xylanase followed by cellulase. Furthermore, increases in glucan yields accounted for most of the overall yield

improvements by xylanase supplementation to cellulase due to the high removal of xylan by EDA pretreatment leaving little xylan in the solids, as shown in Table 5.2. In fact, 180°C batch and 140 and 180°C flowthrough pretreatments removed close to 90% of the xylan with the result that of the 17.5 mg of xylan in 100 mg of unpretreated poplar, only about 1.75 mg xylan was left in these three solids. On the other hand, because 140°C EDA batch pretreatment solubilized only 68% of xylan, about 5.6 mg of xylan would be left in 100 mg of pretreated solids. However, combining 15 mg of cellulase protein with 30 mg of xylanase protein did not improve yields from any of the EDA pretreated solids compared results with 15 mg of cellulase protein alone.

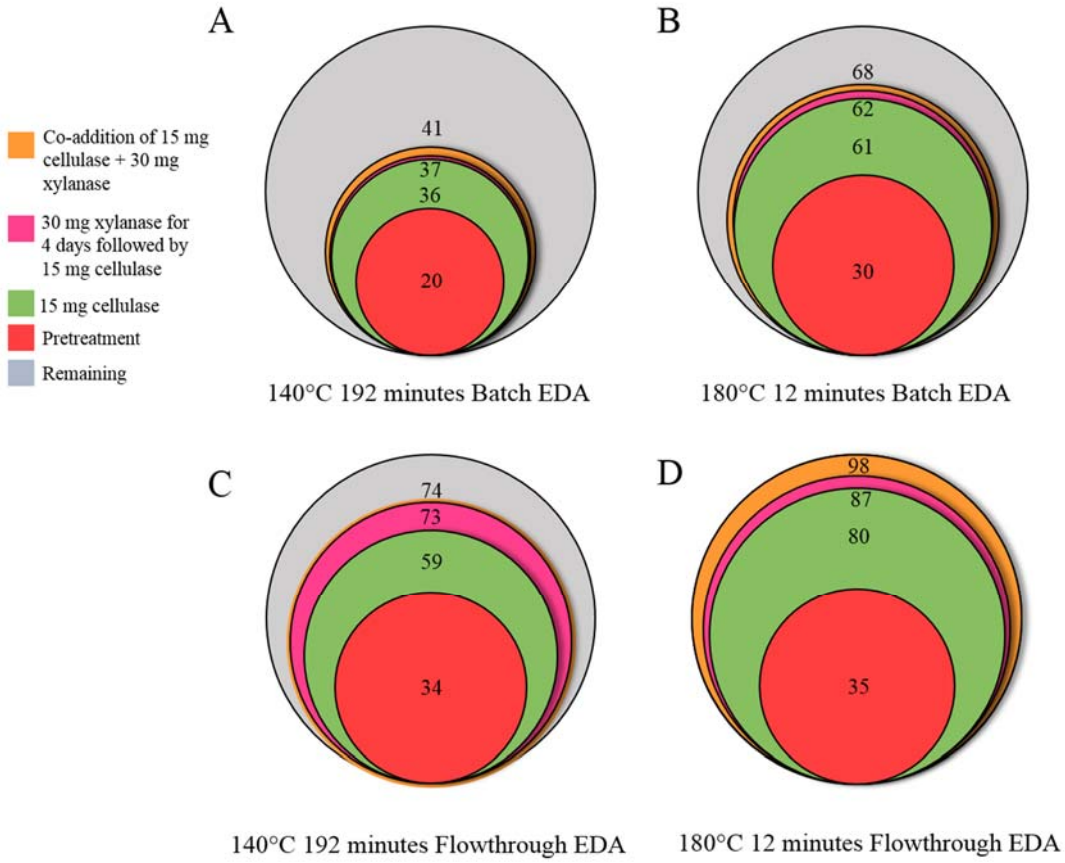

Figure 5.4 Effect of adding xylanase to cellulase on yields of total glucan plus xylan sugars from solids resulting from extremely dilute acid (EDA) pretreatments. A and B are for batch pretreatments at 140ºC and 180°C, respectively, while C and D are for flowthrough pretreatments at 140 and 180°C, respectively. Sugar yields from just pretreatment are in red, and overall yields with 15 mg cellulase, sequential addition of 30 mg xylanase followed by 15 mg cellulase, and co-addition of 15 mg cellulase and 30 mg xylanase are in green, pink, and orange colors, respectively. Enzymes were loaded as mg of protein per gram glucan in unpretreated biomass. Data labels represent overall sugar yields.

The similar or in most cases better sugar yields from co-addition of cellulase and xylanase compared to addition of xylanase prior to adding cellulase 4 days later could result from loss of activity of xylanase over the extended hydrolysis for sequential

addition. The differences could also be due to differences in adsorption for co-addition versus sequential addition of xylanase to cellulase. Because it has been reported that better or similar yields can be achieved from enzymatic hydrolysis through replacement of cellulase by xylanase (Hu et al., 2011), an extra experiment with 45 mg cellulase alone was applied to see if these enzymes were interchangeable for the solids used in this study and gave similar yields as co-addition of 15 mg of cellulase protein with 30 mg of xylanase.

5.4.4 Effect of BSA

It is known that a portion of carbohydrate active enzymes (CAZys) may be lost through adsorption to hydrophobic lignin sites (Eriksson et al., 2002; Yang & Wyman, 2006), while binding of Tween 20 (polysorbate type non-ionic surfactant) and bovine serum albumin (BSA) to lignin can block CAZys adsorption and improve cellulose saccharification. Similarly, the improvement in sugar yields by adding xylanase could result from some of the cellulase or xylanase or both blocking lignin and making the remaining enzymes more effective. Thus, to differentiate catalytic from lignin blocking effects of xylanases from enzyme loss to lignin, 30 mg BSA was used in place of the 30 mg of xylanase. Although xylanases and BSA likely have somewhat different affinities for lignin, similar performance by both would suggest that reducing enzyme losses to unproductive binding might explain why adding xylanase enhanced yields. Tables 5.4 through 6 show that glucan, xylan, and glucan + xylan overall yields dropped slightly when 30 mg of BSA was added at the same time as 15 mg cellulase to LHW batch pretreated solids compared to yields from adding 15 mg cellulase alone. Per Table 5.6,

overall sugar yields dropped from 24 to 22% when BSA as added with cellulase to solids prepared by 140°C LHW batch pretreatment and from 30 to 28% for solids resulting from 180°C LHW batch pretreatment. On the other hand, co-addition of BSA and cellulase increased sugar yields slightly from 43 to 46% for solids from 140°C LHW flowthrough pretreatment but did not improve yields from solids prepared by 180°C LHW flowthrough operation. Thus, the improvement in sugar yields by xylanase addition can be attributed to the catalytic effect of xylanase on LHW solids. Similar to solids from LHW batch pretreatments, BSA did not enhance sugar yields from solids prepared by EDA batch pretreatment at either temperature. However, adding 30 mg of BSA increased overall sugar yields from 59 to 68% with 15 mg cellulase from solids produced by 140°C EDA flowthrough pretreatment and from 80 to 91% with 15 mg cellulase from solids resulting from 180°C EDA flowthrough operation. Because addition of BSA did not improve the xylan yields reported in Table 5.4, the increase in sugar yields was just due to improved glucan release. In all, the catalytic effect of xylanases was dominant over unproductive losses to lignin in all solids other than EDA flowthrough solids.

Going further, a high loading of BSA was employed similar to other studies (Brethauer et al., 2011; Kumar & Wyman, 2009; Yang & Wyman, 2006) to block the surface of lignin from unproductive loss of enzyme. 100 mg of BSA was added to LHW pretreated solids 24 hours prior to addition of 15 mg of cellulase or to co-addition of 15 mg cellulase and 30 mg xylanase. The results plotted in Figure 5.5 reveal that adding BSA before enzymes had a negligible effect on overall sugar yields from solids pretreated by LHW batch reactors at both temperatures. However, pre-addition of BSA

did result in the following large improvements in sugar yields from solids produced by LHW flowthrough pretreatment: from 43 to 47% for 140°C and from 68 to 75% for 180°C. Adding BSA 24 hours prior to co-addition of 15 mg cellulase and 30 mg xylanase did not improve sugar yields compared to that without BSA, as evidenced by Table 5.6 and Figure 5.3. Thus, the yield improvement by BSA addition was not additive, and sugar yields higher than that shown without BSA would have occurred if BSA reduced loss of cellulase and xylanase through lignin blocking.

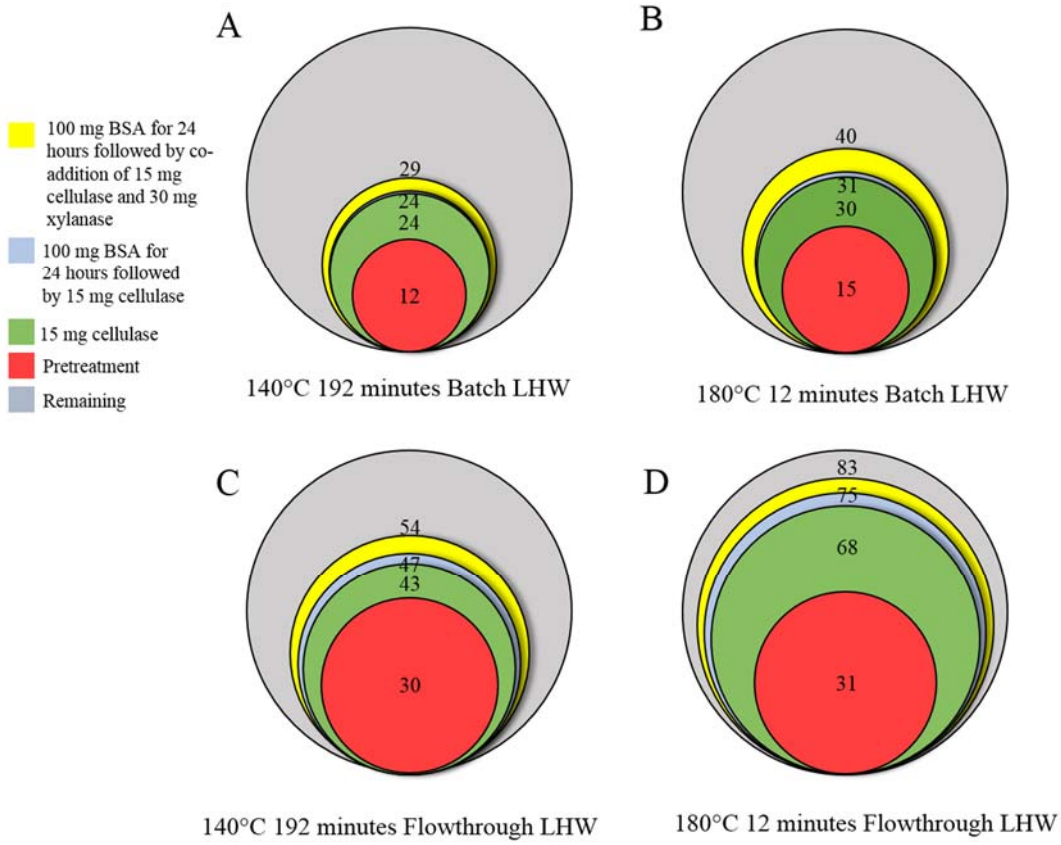

Figure 5.5 Effect of adding BSA to cellulase on yields of total glucan plus xylan sugars from solids resulting from liquid hot water (LHW) pretreatments. A and B are for batch pretreatments at 140ºC and 180°C, respectively, while C and D are for flowthrough pretreatments at 140 and 180°C, respectively. Sugar yields from just pretreatment are in red, and overall yields from adding 15 mg cellulase, 100 mg BSA for 24 hours followed by 15 mg cellulase, and 100 mg BSA for 24 hours followed by co-addition of 15 mg cellulase and 30 mg xylanase are shown in green, purple, and yellow colors, respectively. Enzymes were loaded as mg of protein per gram glucan in unpretreated biomass. Data labels represent overall sugar yields.

Data are also included in Figure 5.6 on sugar yields from EDA pretreatment followed by addition of 100 mg of BSA per gram glucan 24 hours prior to addition of 15 mg cellulase and co-addition of 15 mg cellulase and 30 mg xylanase to the EDA solids. Although sugar yields from solids prepared by 140°C EDA batch pretreatment increased

slightly from 36 to 40%, pre-addition of BSA increased the sugar yields in Figure 5.6 from 61 to 75% for solids prepared by 180°C EDA batch pretreatment. For batch EDA operation, BSA only outperformed xylanase for poplar solids pretreated at 180°C. Adding 100 mg BSA followed by 15 mg cellulase produced an overall sugar yield of 75% while the yield was 68% for co-addition of 15 mg cellulase with 30 mg xylanase without BSA in extremely dilute acid solids produced by batch pretreatment at 180°C. In 140°C EDA flowthrough solids, overall sugar yield increased from 59% with 15 mg cellulase to 73% with 100 mg BSA addition prior to addition of 15 mg cellulase. The same increase was found with addition of 30 mg xylanase to 15 mg cellulase. However, yields increased even further to 78% when 100 mg BSA was added prior to co-addition of 15 mg cellulase and 30 mg xylanase in 140°C EDA flowthrough solids. For 180°C EDA flowthrough solids, overall sugar yields reached 99% by adding 100 mg BSA prior to 15 mg cellulase. Figure 5.4 had indicated earlier that similar yields were achieved by the 15 mg cellulase and 30 mg xylanase combination without BSA.

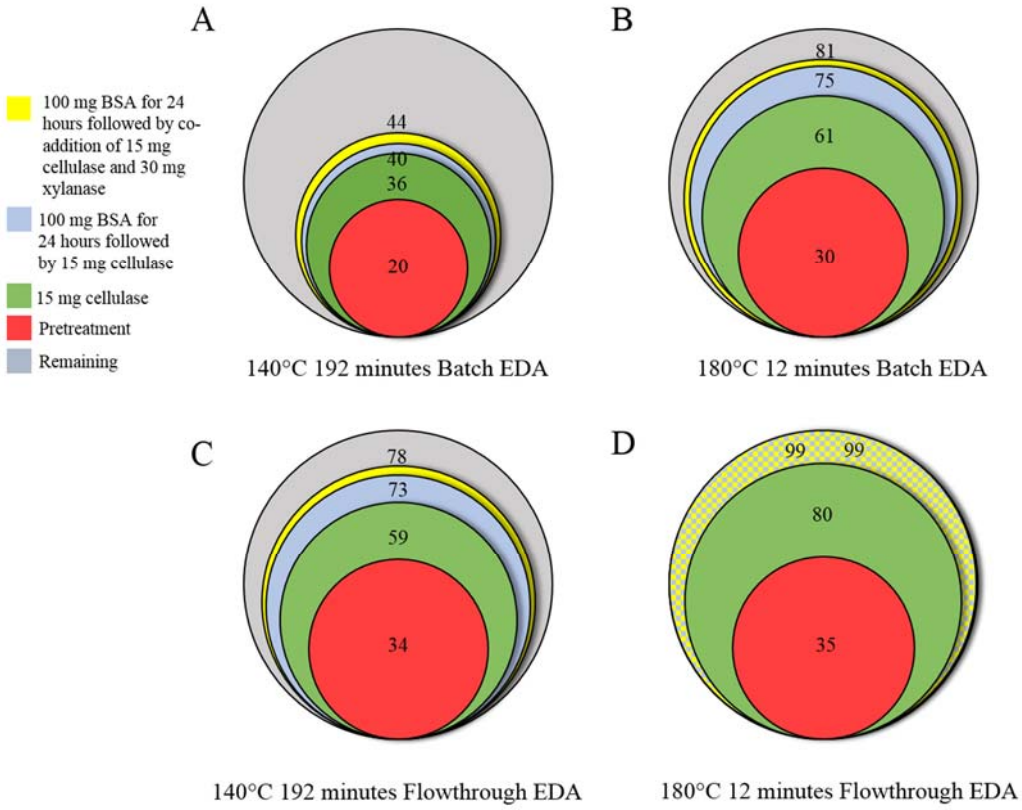

Figure 5.6 Effect of adding BSA to cellulase on yields of total glucan plus xylan sugars from solids resulting from extremely dilute acid (EDA) pretreatments. A and B are for batch pretreatments at 140ºC and 180°C, respectively, while C and D are for flowthrough pretreatments at 140 and 180°C, respectively. Sugar yields from just pretreatment are in red, and overall yields from adding 15 mg cellulase, 100 mg BSA for 24 hours followed by 15 mg cellulase, and 100 mg BSA for 24 hours followed by co-addition of 15 mg cellulase and 30 mg xylanase are shown in green, purple, and yellow colors, respectively. Enzymes were loaded as mg of protein per gram glucan in unpretreated biomass. Data labels represent overall sugar yields.

Overall for LHW solids, adding xylanase to cellulase improved overall sugar yield more than adding BSA to cellulase, while for EDA solids, adding BSA to cellulase had a similar or better effect as adding xylanase to cellulase. Thus, BSA seems to have a larger role than just reducing unproductive binding of enzyme to lignin. This

interpretation is supported by the observation that adding 100 mg of BSA to cellulase had a sizable impact on sugar yields from low lignin content solids produced by LHW flowthrough pretreatment. Co-addition of 30 mg BSA with 15 mg cellulase only improved sugar yields significantly for solids produced by EDA flowthrough pretreatment. With the exception of solids produced by 180°C EDA batch pretreatment, adding 100 mg BSA 24 hours prior to adding 15 mg cellulase and 30 mg xylanase did not improve yields more than that realized by just using 15 mg cellulase plus 30 mg xylanase. Furthermore, adding 30 or 100 mg of BSA to 15 mg cellulase only enhanced sugar yields from glucan but not xylan.

5.5 Conclusions

At moderate pretreatment severity, complete deconstruction of sugars by enzymes was possible only through pretreatment at higher temperatures followed by application of high enzyme loadings. Flowthrough pretreatment realized higher sugar yields from pretreatment as well as enzymatic hydrolysis compared to batch pretreatment at otherwise identical conditions. Extremely dilute acid pretreatment reduced recalcitrance to enzymes significantly compared to liquid hot water operation but benefited little from xylanase supplementation. Co-addition of cellulase and xylanase produced higher yields than addition of xylanase followed by cellulase. The catalytic effect of xylanase had a greater role in improving sugar yields than in blocking lignin in all solids except in extremely dilute acid flowthrough solids. In general, BSA improved sugar yields more from solids produced by flowthrough pretreatment than by batch and more from solids produced by extremely dilute acid than those produced by liquid hot water despite the

lower lignin content in flowthrough and dilute acid solids. Furthermore, BSA addition only improved sugar yields more than co-addition of cellulase and xylanase when applied to solids produced by extremely dilute acid batch pretreatment. Overall, BSA appears to have a larger role in enhancing sugar yields than just reducing unproductive binding but had no effect on xylan yields from enzymatic hydrolysis.

5.6 Abbreviations

LHW: Liquid hot water **EDA:** Extremely dilute acid **BSA:** Bovine serum albumin **BESC:** Bioenergy Science Center **BESC STD:** BESC standard poplar **CAZy:** Carbohydrate active enzymes

5.8 Acknowledgements

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Chapter 6

Robustness of Two-step Acid Hydrolysis Procedure for Composition Analysis of Poplar*

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6.1 Abstract

The NREL standard procedure for lignocellulosic biomass composition has two steps: primary hydrolysis in 72% wt sulfuric acid at 30°C for 1 h followed by secondary hydrolysis of the slurry in 4 wt% acid at 121°C for 1 h. Although pointed out in the NREL procedure, the impact of particle size on composition has never been shown. In addition, the effects of primary hydrolysis time and separation of solids prior to secondary hydrolysis on composition have never been shown. Using poplar, we show that particle sizes less than 0.250 mm significantly lowered the glucan content and increased the Klason lignin but did not affect xylan, acetate, or acid soluble lignin contents. Composition was unaffected for primary hydrolysis time between 30 to 90 minutes. Moreover, separating solids prior to secondary hydrolysis had negligible effect on composition suggesting that lignin and polysaccharides are completely separated in the primary hydrolysis stage.

6.2 Introduction

The two step acid hydrolysis procedure is the benchmark for measuring the amounts of sugars in the form of cellulose and hemicellulose, and lignin in lignocellulosic biomass feedstocks and accounting for their fate as biomass progresses through pretreatment, enzymatic hydrolysis, fermentation, and other steps of biological processing of biomass. Furthermore, because sugar mass balances hinge on accurate compositional analysis, understanding uncertainties inherent in this procedure and factors that affect measured values can be valuable in interpreting the resulting compositional

data. This procedure has two major steps: 1) strong sulfuric acid hydrolysis at near room temperature followed by 2) a secondary hydrolysis at higher temperature after diluting the primary hydrolysis slurry. The ability of strong sulfuric acid to dissolve crystalline cellulose has been known for over a century. For example, in 1900, Alexander Classen of Aachen, Germany patented a process for sugar recovery in which he added three parts of 55 to 60° Baumé sulfuric acid (69.65% to 77.67 wt% H_2SO_4) to one part sawdust by mass, pressed the resulting slurry for half an hour with a hydraulic press, diluted the mixture with four more parts water, and boiled the resulting slurry for another half an hour to release glucose (Classen, 1900). Today, sugar and lignin compositions are determined based on a procedure developed by Professor Johan Peter Klason of Stockholm, Sweden in 1908 in which he added 72% sulfuric acid to biomass isolate what is now known as Klason lignin (Klason, 1908). The National Renewable Energy Laboratory (NREL) in Golden, Colorado, has documented the resulting standard procedure as "Determination of Structural Carbohydrates and Lignin in Biomass" (Sluiter et al., 2008). The first primary hydrolysis step specifies mixing 0.3 grams of biomass with 3 ml of 72 weight% sulfuric acid (4.9 grams by weight) at 30°C for 60 minutes, followed by dilution with water to reduce the acid concentration to 4%. Next, a secondary hydrolysis step holds the diluted 4% acid slurry at 121°C for another 60 minutes, at the end of which, the slurry is filtered through a ceramic crucible. The filtered liquor is neutralized for HPLC analysis for sugars and acetic acid from which the weight fractions of cellulose and hemicellulose in biomass can be calculated. The generally small amount of lignin soluble in the acid solution can be quantified by UV-Vis spectrophotometry and

is termed acid soluble lignin (ASL). Gravimetric analysis of the solid residue left in the crucible is applied to determine the acid insoluble residue (AIR) and ash content, with the acid insoluble lignin (AIL) calculated as the difference between the mass of the acid insoluble residue and ash.

Although this procedure has been a workhorse for biomass analysis for decades, it is important to understand the method's consistency in measuring the makeup of structural components in lignocellulosic biomass in different sized particles from a single biomass source. It is also important to know how robust this technique is to variations in the primary hydrolysis time from the recommended time of 60 minutes. Another concern was whether lignin carbohydrate complexes still present after the primary hydrolysis would not be accounted for in the final HPLC measurements, thereby misleading the final results.

6.3 Materials and Methods

The National Renewable Energy Laboratory (NREL), Golden, Colorado, graciously provided BESC standard poplar (BESC STD) chips for this study. These chips were knife milled through a 1 mm screen (Model 4 Wiley Mill Thomas Scientific, Swedesboro, NJ, USA) at University of California at Riverside. The moisture content of BESC STD was 7% determined by a halogen moisture analyzer (HB43-S; Mettler Toledo, Columbus, OH). No extraction was applied to the poplar prior to analysis because extractives levels in poplar are low and the purpose of this study was to identify how deviations in analysis conditions impacted compositional results for the same material.

Biomass samples were sieved through ASTM E 11 standard sieves (USA Standard Testing Sieve) of mesh sizes 20, 40, 60, 80, and a bottom collection pan that were stacked in that order and shaken by a Ro-Tap® sieve shaker (W.S. Tyler, Model# RX-29) for 30 minutes. The depth of the sample placed evenly on the number 20 sieve before shaking was less than 7 cm. The sieves prior to loading and after shaking were weighed to calculate weight fraction of biomass collected on each sieve after shaking.

Figure 6.1 outlines the standard procedure as well as the experimental strategy employed in this study. To determine the effect of varying particle sizes, the compositions of the biomass collected on each sieve after shaking (+20, -20/+40, -40/+60 and -60/+80), along with the fines (-80 fraction) collected in the bottom pan were individually analyzed. Here symbol "+" represents the fraction retained by a sieve and "-" represents the fraction that passed through a sieve. For example, -20/+40 represents the sample that passed through mesh 20 sieve but retained on mesh 40 sieve. The other conditions were the same as standard NREL procedure. In brief, about 0.3 g biomass was weighed on Fisherbrand® weighing paper (Cat. No. 09-898-12B) on analytical balance (Mettler-Toledo AB54-S, Columbus OH) and transferred into glass conical graduates (Kimble® Kimax® No. 60345, Fisher Scientific, Thermo Fisher Scientific Inc., Waltham MA). Moisture content was the same in all sieved fractions. The conical graduates were kept in water bath (Cole-Parmer StableTemp Water Bath 20L Item# EW-14575-16, Vernon Hills IL) at 30°C and stirring rods were put in them and allowed to equilibrate for 10 minutes. Then, 3 ml of 72% sulfuric acid (lot # R8191600-4A, Ricca Chemical Company through Fisher Scientific, Thermo Fisher Scientific Inc., Waltham MA) was

accurately pipetted into the conical graduates. Time was started when sulfuric acid was added to the last conical graduate. Mixing with stirring rods was done every 5 minutes. At the end of 60 minutes, the conical graduates were quickly removed from the water bath and the contents diluted with 14 ml water to stop the strong acid primary hydrolysis. This slurry was transferred to 125 ml serum bottles (Wheaton® Cat# 06-406K), and another 70 ml of water was added to the conical graduates and transferred to serum bottles to bring the total to 84 ml. The serum bottles were then sealed and autoclaved (Model HA-300MII, Hirayama Manufacturing Corp., Tokyo, Japan) at 121°C for 60 minutes. After the autoclave cooled down to 40°C, bottles were removed and filtered through crucibles of known weight. Samples of the filtered liquor were collected for HPLC analysis after which more DI water was added to each bottle to recover solids left in the bottles. The crucibles were dried at 105°C for 24 hours and then allowed to cool down in a desiccator prior to being weighed. Next, these crucibles were then placed in a muffle furnace (Thermo Fisher Scientific Inc., Isotemp® Programmable Muffle Furnace, Model 750) and ashed using the ramp-up program mentioned in the NREL procedure (Sluiter et al., 2008). The filtered liquor without neutralization was analyzed for sugars and acetic acid on Waters® e2695 Separations Module with detection on Waters® 2414 RI detector (Waters Corp., Milford MA). Bio-Rad® Aminex® HPX-87H column conditioned at 65°C was used for all separations using 5 mM sulfuric acid mobile phase at a flow rate of 0.6 ml/min. Calibration of cellobiose, glucose, xylose and acetic acid was done according to the concentrations prescribed in the standard NREL procedure. The fractions of glucose and xylose left after secondary hydrolysis of sugar recovery

standards were 0.95 and 0.9, respectively. Neutralization of secondary hydrolysis liquor was unnecessary in that the Aminex HPX-87H column uses a 5 mM sulfuric acid mobile phase and poplar contains negligible amount of galactose that could otherwise interfere with the xylose retention time.

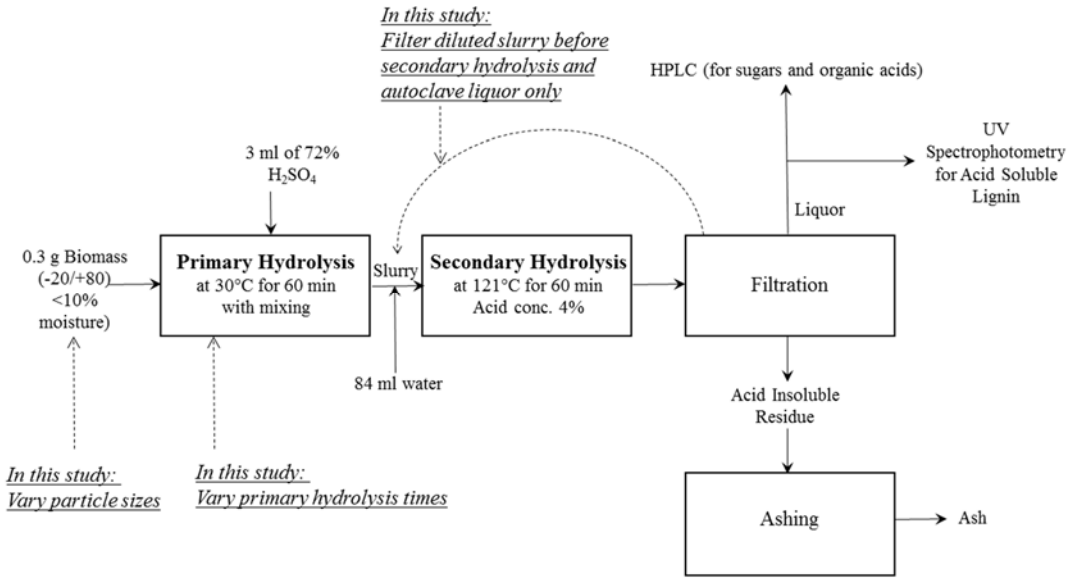

Figure 6.1 Experimental Design

For experiments to study effect of primary hydrolysis time, the same batch of biomass that had been knife-milled through 1 mm screen was used. However, each of the particle size ranges (+20, -20/+40, -40/+60 and -60/+80, and -80) were mixed in equal parts, i.e., the contribution of each of the sizes to our "mixed" poplar was one-fifth of the total weight. Primary hydrolysis of mixed poplar was performed for 30, 45, 60, 75 and 90 minutes at 30°C in exactly the same way as for the varying particle size experiments.

The third leg of this study used the same mixed poplar for varying primary hydrolysis times. Primary hydrolysis was carried out at three different times: 30, 60, and 90 minutes. After primary hydrolysis, the liquor was diluted quickly similar to the other experiments. After dilution to 4% acid concentration, approximately 10 ml of liquor was filtered through crucible. A 0.5 ml aliquot was withdrawn from each of the filtered samples and kept for HPLC analysis. The rest of the 10 ml filtered liquor was autoclaved at 121°C for 60 minutes. The liquors after autoclaving were centrifuged (Eppendorf® Microcentrifuge Model 5424, Eppendorf North America, Hauppauge, NY) at 15000 rpm for 5 minutes, followed by HPLC analysis similar to the other experiments.

Acid soluble lignin content was determined in the liquors after secondary hydrolysis in 96 well plate (Corning® UV-Transparent Microplate 3635) in triplicates for each sample with 0.3 ml in each well through Spectramax® M2e Plate Reader (Molecular Devices, Sunnyvale, CA, USA) equipped with SoftMax® Pro data acquisition software. Concentration of acid soluble lignin was calculated by the Beer-Lambert-Bougeur's Law based on absorption coefficient of 25 L/g.cm at 240 nm.

Three replicates were kept for each condition and all calculations were done following the standard NREL procedure (Sluiter et al., 2008).

6.4 Results and Discussion

6.4.1 Effect of Particle Size

The mass distribution of particle sizes after milling through 1mm screen were as follows: 4.16% between 1 and 0.850 mm (+20), 53.09% between 0.850 and 0.425 mm (-20/+40), 29.99% between 0.425 and 0.250 mm (-40/+60), 4.81% between 0.250 and

0.180 mm (-60/+80), and 7.96% less than 0.180 mm (-80). Figures 6.2A and 6.2B show the composition of each of these fractions as determined by the standard compositional analysis procedure, while Table 6.1 reports the values along with standard deviations for the data presented in these graphs. Figure 6.2A shows only about a 1% deviation in measured glucan content (as weight percent of biomass on a dry basis) from an average value of 45.2% for particle sizes ranging from 1 mm to 0.250 mm (1mm to +60 However, the average glucan content dropped significantly to 39% for a particle size less than 0.250 (-60 and less). On the other hand, acid insoluble lignin (AIL) measurements rose continuously from 21.83 % to 25.90% with decreasing particle size. Because the ash content in this poplar was too low to be measured accurately, the contents of acid soluble lignin and acid soluble residue were pretty much the same.

The high acid insoluble lignin content in the -60/+80 and -80 sizes can be due to formation of humic substances through glucose degradation as glucan% was lower in these two sizes (Hu et al., 2012). As shown in Table 6.1, the overall average AIL was 24% with 1.34% standard deviation. The data in Table 6.1 and Figure 6.2 indicates no meaningful trend in xylan content with variation in particle size. Although a xylan content of 14% was measured for the +20 fraction, xylan levels increased to about 15% in the -20/+60 range, dropped back to 14% in -60/+80 fraction, and increased to 15% in the -80 fines. Thus, the difference between the lowest and largest values for xylan content was only 1.5% while the overall average was 14.5% with 0.8% standard deviation (Table 6.1). The deviations in xylan content seem to be due to experimental error rather than particle size and was also lower than the deviations measured in glucan content. Figure

6.2B shows that neither the acid soluble lignin (ASL) nor acetate content were affected by varying the particle size, with an overall average of 3.87% with only a 0.1% deviation for ASL and 3.4% with a 0.35% standard deviation for acetate. In summary, this data indicates if a substantial portion of biomass has particle sizes below 0.250 mm, measurements of glucan and acid insoluble lignin may not truly representative of the overall biomass composition of poplar. Furthermore, it could prove valuable to check the particle size distribution if sugar mass balances cannot be closed or lignin removal seems lower than expected. Also, the strong sulfuric acid concentration could be reduced to determine if this change affects measurements of glucan concentrations.

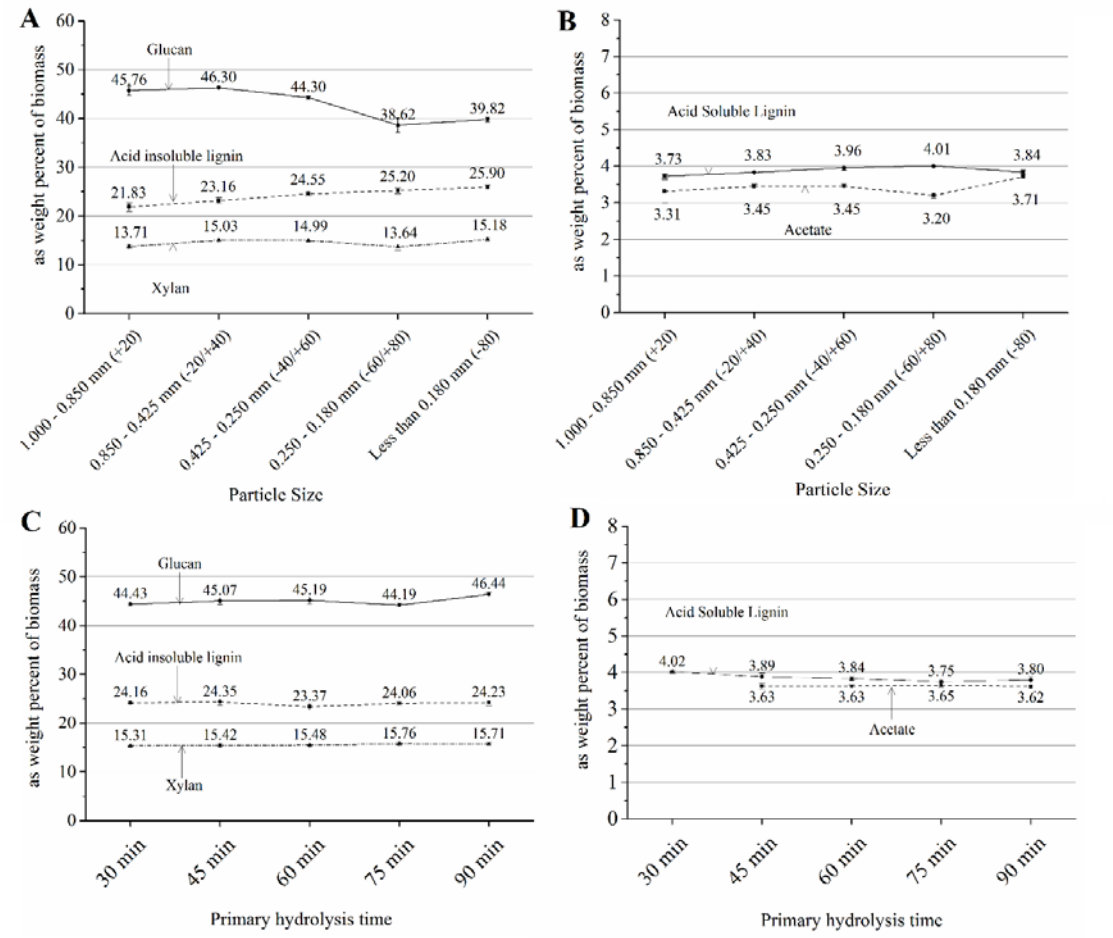

Figure 6.2. Effect of particle size and primary hydrolysis time on composition
Graph A shows glucan, xylan and AIL contents whereas graph B shows acetate and ASL contents with variation in particle size. Graph C shows glucan, xylan and AIL contents whereas graph D shows acetate and ASL contents with variation in primary hydrolysis time.

Table 6.1 The composition of different particle size fractions produced by milling and screening poplar.

Particle Size Range	Glucan%		Xylan%		Acetate%		ASL%		AIL%	
	Value	St. dev.	Value	Std. dev.	Value	Std. dev.	Value	Std. dev.	Value	Std. dev.
1.000 - 0.850 mm (+20)	45.48	0.96	13.71	0.29	3.31	0.32	3.73	0.05	21.83	0.87
0.850 - 0.425 mm (-20/+40)	46.03	0.18	15.03	0.09	3.45	0.06	3.83	0.04	23.16	0.67
0.425 - 0.250 mm (-40/+60)	44.01	0.34	14.99	0.07	3.45	0.06	3.96	0.07	24.55	0.26
0.250 - 0.180 mm (-60/+80)	38.34	1.48	13.64	0.68	3.20	0.08	4.01	0.05	25.20	0.56
Less than 0.180 mm (-80)	39.54	0.46	15.18	0.26	3.71	0.04	3.84	0.05	25.90	0.45
Overall average	42.68	1.86	14.51	0.79	3.42	0.35	3.87	0.12	24.13	1.34

6.4.2 Effect of Primary Hydrolysis Time

Since 83% of the milled biomass had particle sizes in the range of 0.850 to 0.250 mm, the effect of particle sizes outside this range might not be apparent over the primary hydrolysis time. For example, particles sizes less than 0.250 mm (-60) might appear to have lower sugar contents due to greater degradation than larger sizes when subjected to long primary hydrolysis times. Therefore, the composition was measured of a mixture of equal portions by mass of each of the size ranges (1 mm to +20, -20/+40, -40/+60, -60/+80, and less than -80) for primary hydrolysis times ranging from 30 to 90 minutes. Secondary hydrolysis after primary hydrolysis was done according to the conventional NREL method. Surprisingly, Figures 6.2C and 6.2D and Table 6.2 show no significant differences in the amounts of glucan, xylan, AIL, ASL, and acetate measured. This result shows that this procedure is robust with respect to primary hydrolysis over times that were well below and well above the recommended 60 minutes. Table 6.2 shows that overall compositional averages were similar for all components measured over the range of primary hydrolysis times applied to those for the varying particle size experiments other than for the glucan content measured for particle sizes less than 0.250 mm.

Table 6.2 The composition of poplar as measured after application of primary hydrolysis times from 30 to 90 minutes followed by secondary hydrolysis according to standard protocols. The standard primary hydrolysis time is 60 minutes.

Primary Hydrolysis Time	Glucan%		Xylan%		Acetate%		ASL%		AIL%	
	Value	Std. dev.	Value	Std. dev.	Value	Std. dev.	Value	Std. dev.	Value	Std. dev.
30 min	44.15	0.39	15.31	0.08	N.D.[a]	N.D.	4.02	0.03	24.16	0.19
45 min	44.79	0.73	15.42	0.32	3.63	0.08	3.89	0.09	24.35	0.71
60 min	44.91	0.77	15.48	0.15	3.63	0.04	3.84	0.05	23.37	0.74
75 min	43.91	0.47	15.76	0.23	3.65	0.05	3.75	0.03	24.06	0.33
90 min	46.16	0.46	15.71	0.28	3.62	0.05	3.80	0.09	24.23	0.65
Overall average	44.78	1.31	15.54	0.52	3.63	0.12	3.86	0.14	24.03	1.27

[a]N.D. Not Detemined

6.4.3 Filtration of Slurry before Secondary Hydrolysis

In the third leg of this study, a 4% primary hydrolysis slurry produced at times of 30, 60, and 90 minutes was filtered before subjecting it to secondary hydrolysis. However, unlike the brittle solids produced by secondary hydrolysis that are easy to separate in accordance with the conventional procedure, filtering the 4% slurry from primary hydrolysis was so slow due to formation of lignin sludge on the crucible that only 10 ml of 4% primary hydrolysis slurry was filtered. Thus, we could see that secondary hydrolysis significantly changed the physical nature of lignin such that the AIL content could not be measured. Before subjecting the filtered liquor to secondary hydrolysis, samples taken in aliquots were analyzed by HPLC and UV-Vis spectrophotometry to determine how the fraction of monomeric versus oligomeric sugars after dilution of primary hydrolysis liquor was affected by different primary hydrolysis times. . The results in Figures 6.3A (glucan and xylan) and 3B (acetate and ASL), as well as Table 6.3, show that the amount of monomeric glucan was negligible at the end of 30 minutes, rose to 3% after 60 minutes, and increased further to 5% by 90 minutes. However, 6% of the 16% total xylan content was monomeric at the primary hydrolysis time of 30 minutes but increased to about 9% in 60 and 90 minutes. Thus, monomeric xylan plateaued after 60 minutes, while monomeric glucan kept increasing over the entire time period considered. At the primary hydrolysis time of 60 minutes specified in the standard procedure, about 9% of xylan and only 3% of glucan was monomeric with the rest oligomers. Because the acetate content stayed at the same value of about 3.5% without secondary hydrolysis, as shown in Figure 6.3B, the first 30 minutes of primary

hydrolysis is sufficient to de-acetylate all of the hemicellulose. The figure also shows that acid soluble lignin (ASL) was slightly lower (0.2%) when analyzed after primary hydrolysis than after secondary hydrolysis, likely due to formation of sugar dehydration products in secondary hydrolysis that absorb the same wavelength range of ASL compounds.

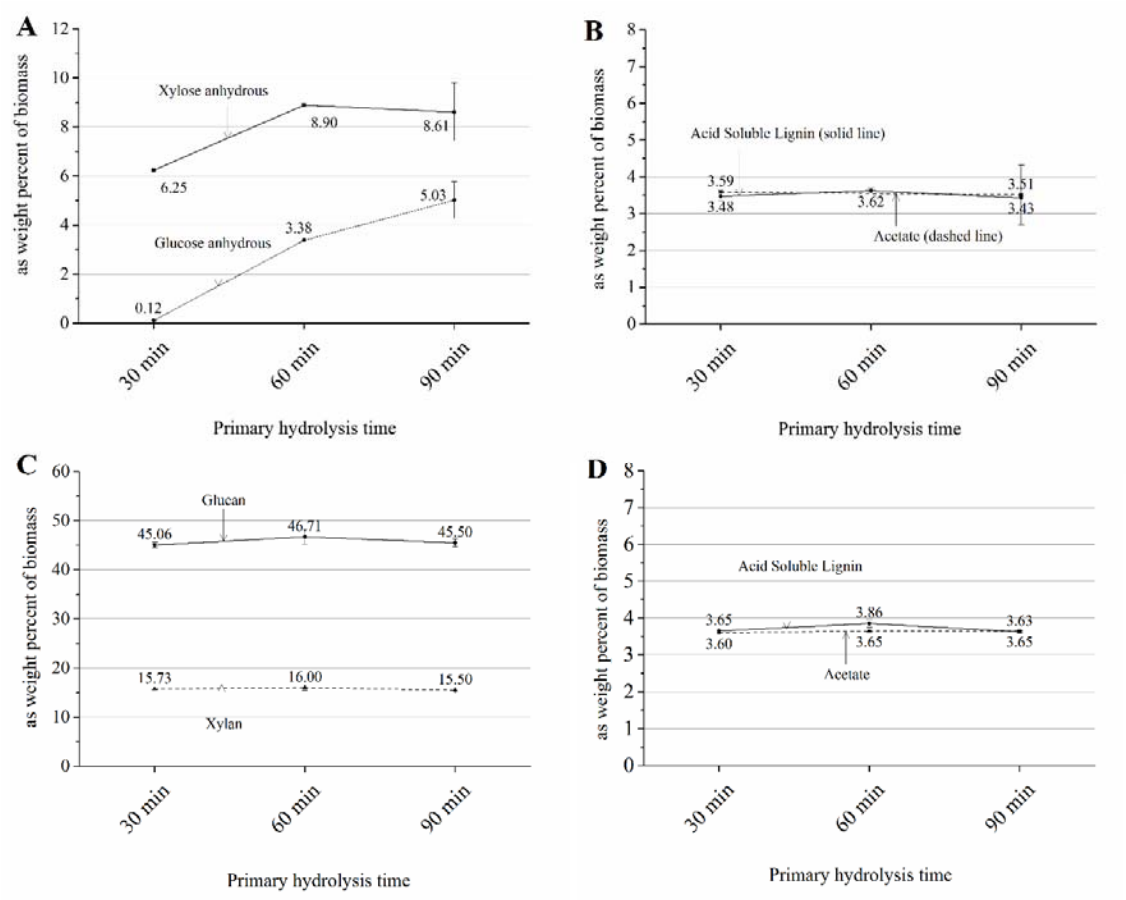

Figure 6.3 Effect of filtration of diluted primary hydrolysis slurry before secondary hydrolysis on composition
Graph A shows glucan and xylan contents in monomeric form, and Graph B shows acetate and ASL contents in diluted primary hydrolysis filtered liquor without secondary hydrolysis for three primary hydrolysis times. Graph C shows glucan and xylan, whereas Graph D shows acetate and ASL for the same filtered liquors but after secondary hydrolysis for three primary hydrolysis times.

Table 6.3 Composition of filtered and diluted primary hydrolysis liquors before and after application of the standard secondary hydrolysis procedure.

		Glucan%		Xylan%		Acetate%		ASL%	
	Primary hydrolysis time	**Value**	**Std. dev.**	**Value**	**Std. dev.**	**Value**	**Std. dev.**	**Value**	**Std. dev.**
Before secondary hydrolysis	30 min	0.12	0.01	6.25	0.03	3.59	0.03	3.48	0.14
	60 min	3.38	0.08	8.90	0.06	-	-	3.62	0.07
	90 min	5.03	0.74	8.61	1.18	3.51	0.81	3.43	0.05
After secondary hydrolysis	30 min	44.78	0.57	15.73	0.11	3.60	0.01	3.65	0.03
	60 min	46.44	1.50	16.00	0.62	3.65	0.02	3.86	0.11
	90 min	45.22	0.80	15.50	0.33	3.65	0.01	3.63	0.04

Figures 6.3C and 6.3D show results from subjecting the liquor that was filtered from the solids after primary hydrolysis of the 4% slurry to secondary hydrolysis, while Table 6.3 presents the values and standard deviations for these results. No significant difference was found in glucan, xylan, acid soluble lignin, and acetate contents whether filtration was done before or after secondary hydrolysis. The overall averages of glucan, xylan, ASL, and acetate contents listed in Table 6.3 are similar to the measurements for the different particle sizes and 3 primary hydrolysis times. Thus, the primary concentrated sulfuric acid hydrolysis step appears to completely break cellulose and hemicellulose away from the lignin in poplar even for times as low as within 30 minutes.

6.5 Conclusions

Compositional analysis of poplar particle sizes smaller than 0.250 mm showed significantly lower glucan content and higher acid insoluble lignin content than larger particle sized samples while xylan, acetate, and acid soluble lignin measurements were unaffected. The procedure was robust with respect to primary hydrolysis time in that varying the hydrolysis time from 30 to 90 minutes had little effect on composition values of mixed poplar. Compositional results for mixed poplar were similar when only diluted primary hydrolysis filtered liquor was subjected to secondary hydrolysis instead of diluted primary hydrolysis slurry. Furthermore, primary hydrolysis completely separated polysaccharides from lignin, while secondary hydrolysis depolymerized sugar oligomers to monomers.

6.6 Acknowledgements

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Chapter 7

Effect of Surfactant and Mixing on Cellulose Saccharification at Low Enzyme Loading*

*This whole chapter will be submitted under the following citation:
Bhagia, S., Kumar, R., Wyman, C.E., 2016. Effect of Surfactant and Mixing on Cellulose Saccharification at Low Enzyme Loading.

7.1 Abstract

Surfactants can improve enzymatic deconstruction of lignocellulosic biomass through reducing unproductive loss of enzyme to lignin adsorption. On pure cellulose substrates, the effects of surfactant are only apparent at low enzyme to substrate ratios. In this study, the impact and role of surfactants on enzymatic hydrolysis of cellulosic and pretreated lignocellulosic substrates was investigated at low enzyme and surfactant loadings. At 1% glucan loading of Avicel, adding 5 mg of surfactant (BSA or Tween 20) to 5 mg enzyme protein, both on the basis of one gram glucan in the solids, increased glucan yields from 60 to 92% of the theoretical maximum after 17 days of reaction at 50°C with shaking at 150 rpm. Interestingly, we found that if hydrolysis flasks were not shaken, 5 mg enzyme could reach a 95% yield after 17 days without adding surfactant, although surfactant produced faster rates with shaking. At a 15% glucan loading of Avicel, while adding 5 mg of surfactant to 5 mg of enzyme increased glucan yields from 33 to 66% for shaking at 150 rpm and 50°C, the same enzyme loading could realize a 62% yield without shaking or without surfactant. On the other hand, the surfactants had no effect on performance if the flasks were not shaken. Carrying out hydrolysis in flasks made from siliconized glass or polycarbonate, or with addition of glass powder in borosilicate glass flasks did not affect yields. The effectiveness of surfactants dropped slightly with increasing hydrolysis temperature, and surfactant was less beneficial to glucose release from cotton than from Avicel. At a low enzyme loading of 5 mg protein/g cellulose, eliminating shaking realized high glucose yields from pure cellulose without adding surfactant while surfactants only improved yields when flasks were shaken. The

beneficial effects were not due to enzyme adsorption on vessel surfaces or shear stress. Thus, the surfactant seemed to reduce enzyme activity loses at the air-liquid interface or deactivation of adsorbed enzyme on the substrate surface rather than stimulate activity.

7.2 Introduction

Biological conversion of lignocellulosic biomass to fuels or chemicals generally applies pretreatment and enzymatic hydrolysis for sugar released from lignocellulosic biomass (Lynd et al., 1999). Relatively low temperature operation, ability to achieve near theoretical sugar yields, environmental friendliness, and biocompatibility with subsequent fermentation process are some of the benefits of enzymatic hydrolysis. However, the high loadings of expensive enzymes required to achieve high sugar yield limits the profitability from commodities synthesized from biomass sugars (Wyman, 2007). At low enough enzyme loadings to keep costs reasonable, sugar yields from hydrolysis tend to be too low to be commercially attractive. Poor yields can result from biomass recalcitrance or loss of enzyme activity or likely both. For example, lignin in biomass is known to adsorb enzyme unproductively, thereby reducing reaction rates and yields (Eriksson et al., 2002; Yang & Wyman, 2006). Sugar oligomers released during biomass deconstruction are known to be strong inhibitors at low concentrations (Qing et al., 2010). Other factors including adsorption behavior (Castanon & Wilke, 1981; Converse et al., 1988; Ooshima et al., 1985), shear stress (Maa & Hsu, 1997; Reese & Ryu, 1980), and denaturation at the vessel surface (Duncan et al., 1995; Wang, 1999) and air-liquid interface (Docoslis et al., 2000; Kim et al., 1982) and can also negatively affect cellulose deconstruction.

Several works have shown that use of amphiphilic substances such as bovine serum albumin (BSA) and surfactants (often Tween) may improve sugar yields from enzymatic hydrolysis. Such improvement have been attributed to reducing non productive binding of enzymes by blocking the lignin surface, protection of enzyme by a protein-surfactant complex, cellulase stimulation, reduction in enzyme viability due to shear stress and exposure to the air-liquid interface, and reducing deactivation by facilitating desorption from cellulose surface.

The ability of surfactants to enhance glucose yields from flowthrough pretreated materials with lower lignin content than those from batch operation (Bhagia et al., 2016a) suggest that surfactants may play important roles in lignocellulosic biomass hydrolysis beyond just preventing unproductive loss of enzyme to lignin. Furthermore, most studies of enzymatic hydrolysis with surfactants focused on high enzyme loadings that are not viable commercially. Thus, this study was directed at determining how BSA protein and Tween 20 surfactant affect glucose release from cellulose at a low enzyme loading of 5 mg protein/g cellulose and to clarify how shaking impacted results.

7.3 Materials and Methods

7.3.1 Materials

Avicel (Avicel PH-101, Fluka, cat# 11365-1KG, lot# BCBN7864V), bovine serum albumin (BSA) (cat# A7906-500G, batch# 078K0730), cotton linters (cat# C6663-250G, lot# 090M0144V), and defatted soybean flour (cat# S9633, lot# SLBL7333V with an approximate protein content of 52%, 85+% dispersible and 1% fat) were purchased from Sigma-Aldrich Corporation, St. Louis, MO. Tween® 20 was purchased from Acros

Organics (code# 233362500, lot# A0226412), 125 ml Erlenmeyer flasks made of borosilicate glass for 50 ml reaction volume from Corning® Pyrex® (cat# 4985-125), 25 ml Erlenmeyer flasks made of borosilicate glass for 10 ml reaction volume from Kimble® Kimax® Valueware® (cat#5650025EMD), 125 ml polycarbonate flasks from Fisherbrand (cat# PBV125). These products from Acros Organics, Corning, and Kimble were purchased through distributor Fisher Scientific, Thermo Fisher Scientific Inc., Waltham, MA. Accellerase® 1500 (Batch# 1662334068, Genencor International, Inc. and made in Finland,) was a kind gift from DuPont Industrial Biosciences at Palo Alto, CA. To see the effect of surfactant and no shaking on enzymatic hydrolysis of lignocellulosic biomass, liquid hot water and dilute acid pretreated poplar hardwood solids were used. The two pretreatment conditions and the enzymatic deconstruction of resulting solids were thoroughly investigated in our previous work which is why these two materials were utilized in this study. Details of the pretreatment applied to poplar in this work were described previously (Bhagia et al., 2016a; Bhagia et al., 2016b). Briefly, liquid hot water (LHW) and 0.05 wt% sulfuric acid (extremely dilute acid or EDA) pretreatments of 2% solids loadings of poplar were carried out in a 1L Parr® reactor (Parr Instrument Co., Moline, IL) at 180°C for 12 minutes. The solids were thoroughly washed with DI water and not dried to avoid hardening. Avicel, cotton, LHW poplar, and EDA poplar had moisture contents of 4, 4.5, 60, and 56%, respectively, as determined by a halogen moisture analyzer (HB43-S; Mettler Toledo, Columbus, OH). Their glucan contents were 97, 100, 60, and 66%, respectively, as determined by the NREL standard procedure "Determination of Structural Carbohydrates and Lignin in Biomass" (Sluiter et al., 2008).

7.3.2 Reaction Vessel Preparation

All flasks were first thoroughly scrubbed and cleaned with lab detergent, followed by several washings with tap water and finally with deionized water, and dried at 105°C for 24 hours. For siliconized glass experiments, 125 ml Erlenmeyer flasks were siliconized with Aquasil® siliconizing fluid (cat# TS42799, Thermo Scientific, Thermo Fisher Scientific, Inc.). Freshly prepared 1% Aquasil solution in milli-Q water was added to flasks to reach the 125 ml mark and shaken for 15 seconds. The solution was drained and washed twice with 100% methanol (Fisher Scientific, Thermo Fisher Scientific Inc.) and dried at 105°C for 6 hours.

7.3.3 Additives

Due to its high foaming tendency, 1% stock solutions of BSA or Tween 20 were made by weighing 1 gram of BSA or Tween 20 into a tarred 125 ml conical flask, and the total mass was brought up to 100 grams by adding milli-Q water for accuracy and reproducibility. A 1% soy protein stock solution was prepared by weighing out 2.3 grams of soy flour (considering protein content of 52% and 85% dispersion) into a 125 ml conical flask followed by adding milli-Q water to bring the total mass to 100 grams.

For experiments with glass powder (high shear/high solid surface area), a 20 ml borosilicate glass serum vial (Wheaton® cat#223687) was crushed with a metal hammer until the particles would pass through a no. 60 sieve (W.S. Tyler ASTM E11-09). Although a portion of the powder was very fine, larger particles could be easily separated by gravity. 0.25 g each of large and fine sizes were added to an Erlenmeyer flask after Avicel addition.

7.3.4 Enzymatic Hydrolyses

Enzymatic hydrolysis was performed according to the NREL standard procedure "Enzymatic Saccharification of Lignocellulosic Biomass" (Selig, 2008) with only the following modifications. Most experiments were run at glucan loadings of 1% except those noted as run at a glucan loading of 15%. Reaction volumes were 50 ml for all Avicel and cotton experiments and 10 ml for never-dried pretreated poplar biomass experiments. Enzymatic hydrolysis runs, except those to show the effect of temperature (37 and 60°C) and the effect of not shaking, were carried out at 50°C at 150 rpm. Accellerase® 1500 with a measured BCA protein content of 82 mg/ml (Smith et al., 1985b) was diluted 20 times to 4.1 mg/ml in 50 mM citrate buffer in 100 ml glass volumetric flask to provide the stock solution for enzyme loading. Accellerase® 1500, BSA, Tween 20, and soy loadings were based on milligrams of protein or surfactant per gram glucan in the substrate to be hydrolyzed. The reaction medium was shaken gently after addition of enzyme for all experiments. For co-addition experiments, BSA, Tween, or Soy was added to an Erlenmeyer flask quickly after enzyme addition. Samples of flask contents for experiments with 5 mg cellulase, co-addition and sequential addition of 5 mg BSA and 5 mg cellulase, co-addition of 5 mg Tween and 5 mg cellulase, 30 mg cellulase, co-addition and sequential addition of 30 mg cellulase and 100 mg BSA, and 5 mg cellulase at 37 and 60°C were taken at 4, 24, 48, 72, 96, 120, 168, 216, 264, and 408 hours after enzyme addition. For all other conditions, samples were taken at 120, 216, and 408 hours. Three replicates were run of all enzymatic hydrolysis experiments with Avicel and cotton in Erlenmeyer flasks, and duplicate Erlenmeyer flasks were employed

for enzymatic hydrolysis of pretreated poplar. For no shaking experiments, separate flasks were kept for each time point (120, 216, and 408 hours) so as not to disturb the reaction medium, and samples were taken through withdrawing a 0.5 ml homogenous aliquot followed by centrifugation in a fixed-angle centrifuge (Eppendorf® Microcentrifuge Model 5424, Eppendorf North America, Hauppauge, NY) at 15000 rpm for 5 minutes. The supernatants were analyzed using a Waters® e2695 Separations Module equipped with a Waters® 2414 RI detector (Waters Corp., Milford, MA). Bio-Rad® Aminex® HPX-87H columns conditioned at 65°C were used for all HPLC separations with a 5 mM sulfuric acid mobile phase at a flow rate of 0.6 ml/min.

For enzymatic hydrolysis with a phosphate buffer, the 50 mM citrate buffer at pH 5 was replaced with a 50 mM phosphate buffer at pH 7. However, because the phosphate buffer peak interfered with the glucose peak in the HPLC analysis, the solids following 120 hours of hydrolysis with 5 mg cellulase and with co-addition of 5 mg cellulase and 5 mg BSA were recovered through centrifugation, washed three times, dried at 105°C, and weighed. The mass left after hydrolysis was used to calculate the percent cellulose saccharification in phosphate buffer at pH 7.

Soy flour blanks were kept along with other flasks that contained 5 mg soy flour and 5 mg Accellerase 1500 similar to other experiments with substrate and soy as additive. HPLC chromatogram showed no presence of carbohydrates or any other soy flour derived components.

7.4 Results

Figure 7.1A shows that enzyme loadings of 5 mg cellulase and co-added 5 mg cellulase and 5 mg BSA/g glucan in the substrate resulted in negligible differences in glucan yields (glucose represented in anhydrous form) for up to 24 hours of hydrolysis as initial rates were 11% in either case. Cellulose saccharification slowed down after 5 days for both cases, but BSA addition resulted in higher hydrolysis rates after 24 hours. The reaction was allowed to continue until 17 days at which point, no significant improvement in cellulose saccharification resulted. While cellulase alone produced a 48% yield after 5 days and a maximum yield of 60% after 17 days, adding BSA increased yields to 70% after 5 days and 92% after 17 days. A high dose of 100 mg BSA was also applied to compare our results to those from other studies that reported no change in Avicel saccharification throughout the reaction at high BSA loadings (Yang & Wyman, 2006). Figure 7.1B shows that the presence or absence of 100 mg BSA had no effect on yields for 30 mg cellulase loading at any time point. Thus, BSA only enhances cellulose saccharification at low ratios of enzyme to substrate.

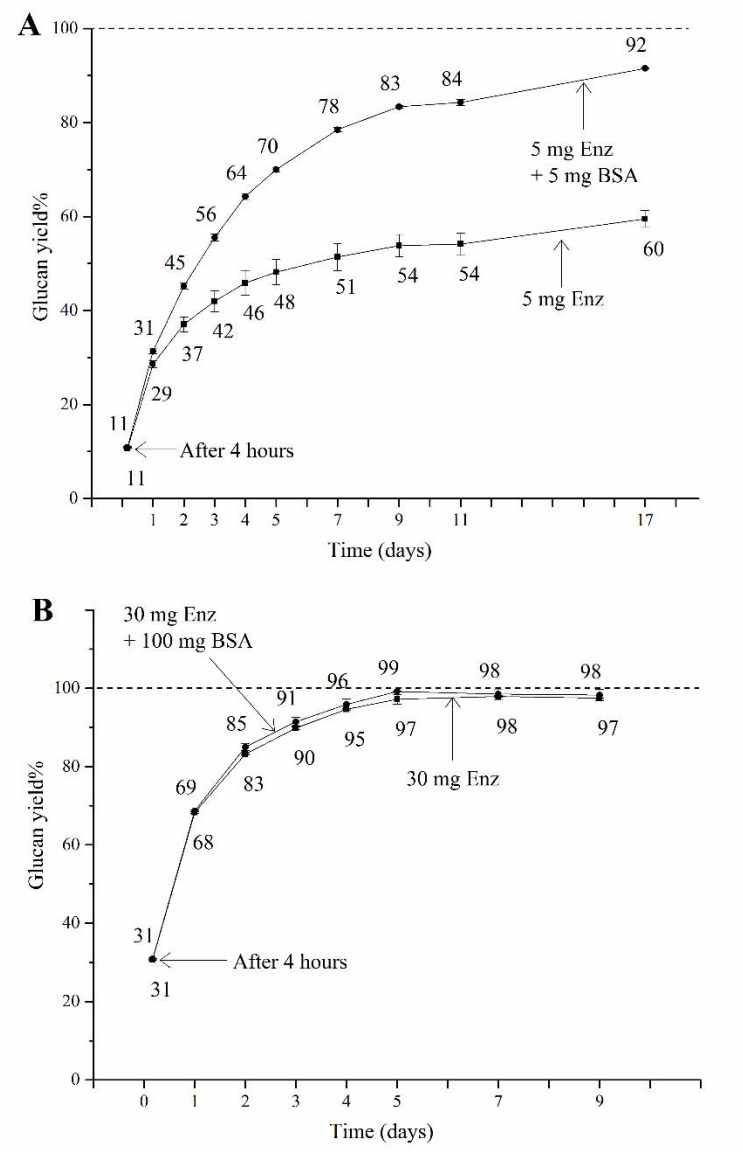

Figure 7.1 Glucan yields resulting from adding BSA to enzymatic saccharification of Avicel over time

(A): enzymatic hydrolysis with 5 mg cellulase protein (Accellerase® 1500)/g glucan in substrate (squares) and with co-addition of 5 mg enzyme and 5 mg BSA/ g glucan in substrate (circles) for up to 17 days of reaction. (B): enzymatic hydrolysis with 30 mg cellulase protein (Accellerase® 1500)/g glucan (squares) and with co-addition of 30 mg enzyme and 100 mg BSA/ g glucan in substrate (circles) for up to 9 days of reaction. Enzymatic hydrolysis was run at 50°C with shaking at 150 rpm for a 1% glucan loading of Avicel in a 50 ml slurry volume in 125 ml Erlenmeyer flasks made of borosilicate glass. Error bars represent standard deviation from three replicate flasks.

Next, BSA was added 30 hours prior to cellulase addition to determine if this strategy would enhance glucan deconstruction further. However, no significant change was seen for the yield data in Figure 7.2 from sequential and co-addition strategies for enzyme and BSA loadings of 5 mg cellulase with 5 mg BSA or 30 mg cellulase with 100 mg BSA. To determine if such large yield improvements by BSA could be due to more than its surfactant-like behavior, the nonionic surfactant Tween 20 was added in similar amounts but without any differential effect. Applying the hydrolysis procedure to soy protein revealed that it also increased yield above that for 5 mg cellulase alone, indicating that the beneficial effect was not restricted to BSA and Tween. However, adding soy protein could only achieve a maximum yield of 77% compared to 90 to 92% for BSA or Tween, respectively. It is important to note that the soy stock had to be shaken vigorously before addition as a large portion was insoluble. Figure 7.2 also shows that 30 mg cellulase with or without BSA or Tween was capable of yields of nearly 100% within 5 days of reaction.

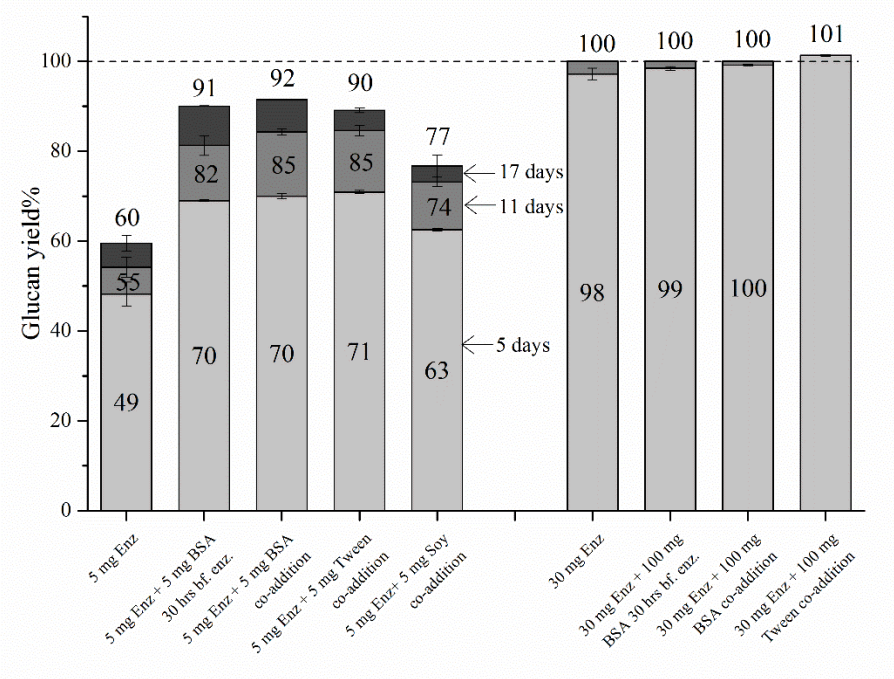

Figure 7.2 Effect of adding BSA, Tween, and soy flour on enzymatic hydrolysis of Avicel over time

The five bars on the left show glucan yields from enzymatic hydrolysis of Avicel with 5 mg of Accellerase® 1500 enzyme/g glucan in substrate, its supplementation with 5 mg of BSA 30 hours before enzyme addition, and co-addition of BSA, Tween 20, and soy flour with the enzyme. The four bars on the right show glucan yields from enzymatic hydrolysis of Avicel with 30 mg Accellerase® 1500 alone/g glucan in substrate and its supplementation with 100 mg of BSA 30 hours before enzyme addition, and co-addition of BSA, Tween 20, or soy flour. All enzyme and additive loadings are based on one gram of glucan in the substrate for a 1% glucan loading of Avicel in a 50 ml slurry volume contained in 125 ml Erlenmeyer flasks made of borosilicate glass and carried out at 50°C and shaking at 150 rpm. Glucan yields are shown after 5, 11, and 17 days of hydrolysis. Error bars represent standard deviation from three replicate flasks.

To determine if BSA enhancement of hydrolysis at low enzyme loadings could be associated with vessel surface effects, experiments were carried out in siliconized glass, in polycarbonate flasks, and in the presence of borosilicate glass powder. As presented in Figure 7.3, yields with 5 mg enzyme and with 5 mg BSA in siliconized glass were very similar, and Tween and Soy still realized large yield improvements in siliconized glass

flasks. Yields were slightly higher when carried out in polycarbonate flasks instead of borosilicate glass flasks. However, addition of glass powder to the flasks gave the most striking results in that the yield increased to 74% and further increased to 94% by addition of 5 mg of Tween while yields were near 60% with 5 mg cellulase in borosilicate glass flasks.

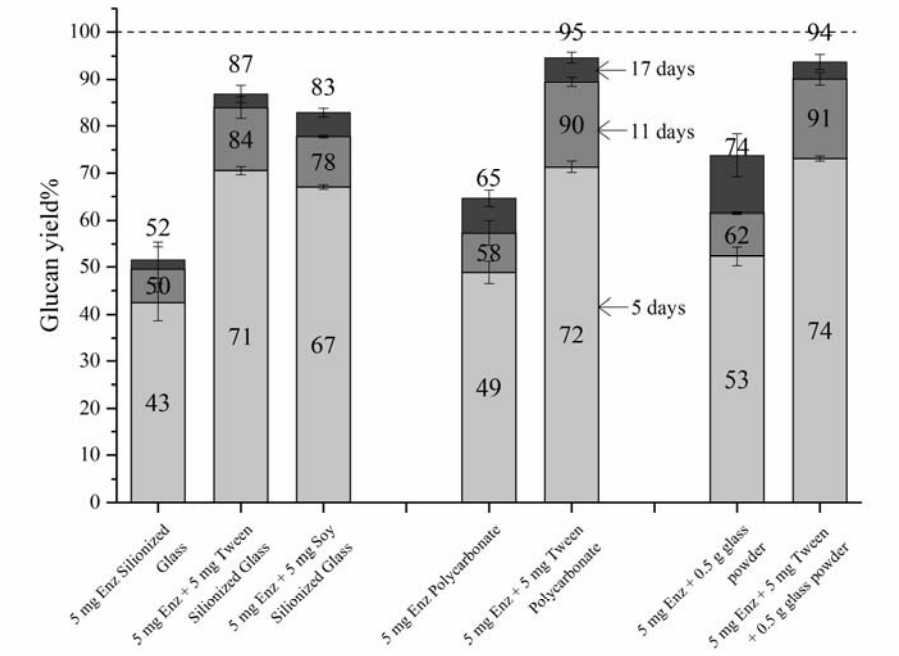

Figure 7.3 Effect of vessel surface and shear on enzymatic hydrolysis of Avicel over time

Glucan yields from enzymatic hydrolysis of Avicel in siliconized glass flasks by 5 mg Accellerase® 1500 enzyme/g glucan alone, co-addition with 5 mg of Tween 20, and co-addition with 5 mg of soy flour. Also shown are yields in polycarbonate flasks for 5 mg Accellerase® 1500 enzyme/g glucan alone and co-addition with 5 mg of Tween 20. The final two bars on the right show how adding glass powder to borosilicate glass flasks impacts glucan yields for 5 mg Accellerase® 1500 enzyme/g glucan alone and co-addition with 5 mg of Tween 20. All enzyme and additive loadings are based on one gram of glucan in the substrate for a 1% glucan loading of Avicel in a 50 ml slurry volume contained in 125 ml Erlenmeyer flasks made of borosilicate glass and carried out at 50°C and shaking at 150 rpm. Glucan yields are shown after 5, 11, and 17 days of hydrolysis. Error bars represent standard deviation from three replicate flasks.

From Figure 7.4 reports the effect of BSA on glucan yields for enzymatic hydrolysis at 37°C and 60°C instead of the optimum temperature of 50°C. For operation at 37ºC with 5 mg cellulase, BSA increased yields from 53% to 82% after 17 days, while BSA increased yields for enzymatic hydrolysis at 60°C from a maximum of 33% yield with 5 mg cellulase to 49%. Thus, yields were severely impacted by increasing the temperature to 60°C, but adding BSA resulted in yields being only 6 to 10% lower for reaction at 37°C than at 50°C. As also shown in Figure 7.4, altering the pH from 5, the optimum for the cellulase used, to 7 through replacing 50 mM citrate buffer with 50 mM phosphate buffer severely hurt glucan yields. Furthermore, BSA had a negligible effect on glucan yields at pH 7 as a 19% yield resulted after 5 days of reaction for a 5 mg cellulase protein loading and 20% for co-addition of 5 mg cellulase and 5 mg BSA.

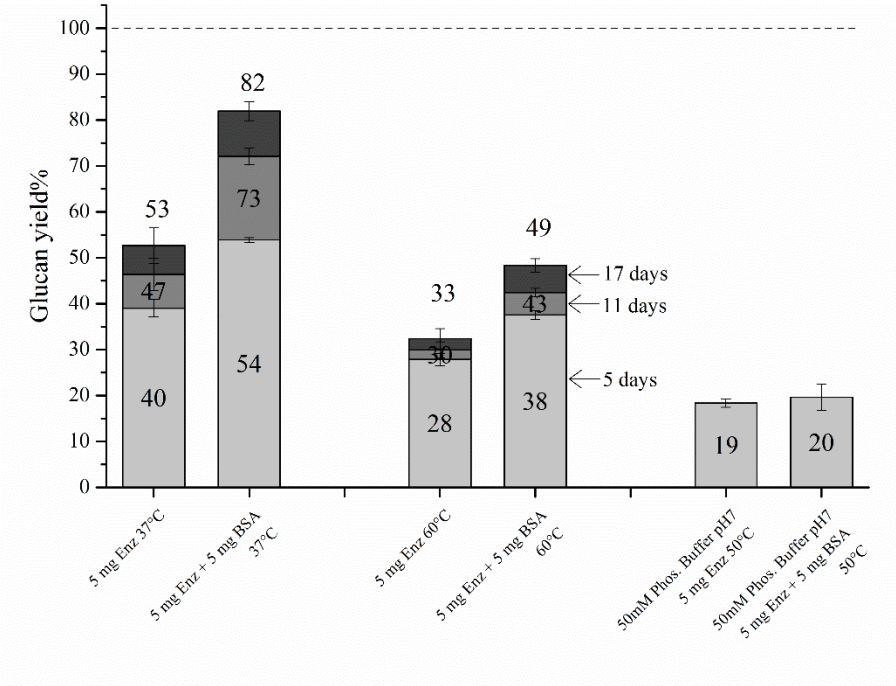

Figure 7.4 Effect of surfactant on enzymatic hydrolysis of Avicel with temperature and pH

Effect of surfactant on sensitivity of glucan yields from enzymatic hydrolysis of Avicel by 5 mg Accellerase® 1500/g glucan in substrate to temperatures of 37 and 60°C and pH 5 and 7 for 5, 11, and 17 days of reaction. All enzyme and additive loadings are based on one gram of glucan in the substrate for a 1% glucan loading of Avicel in a 50 ml slurry volume contained in 125 ml Erlenmeyer flasks made of borosilicate glass and carried out at 50°C and shaking at 150 rpm. Error bars represent standard deviation from three replicate flasks.

To determine if BSA lowered enzyme deactivation at the air-liquid interface, experiments were carried out without shaking and with and without adding Tween. At the 1% loading of Avicel glucan in Figure 7.5, it was surprising that yields for a loading of 5 mg cellulase protein were higher without shaking than with shaking at 150 rpm. However, when Tween was added to cellulase and the reaction flask was not shaken, there was no improvement in yields. This result was true for all samples taken at 4 hours and then every 24 hours (data no shown). While the yield with shaking in presence of

Tween was 90%, the value was 3% higher with 5 mg cellulase alone without shaking. However, the yield after 5 days with shaking at 150 rpm in the presence of Tween was higher (71%) than without shaking with cellulase alone (61%).

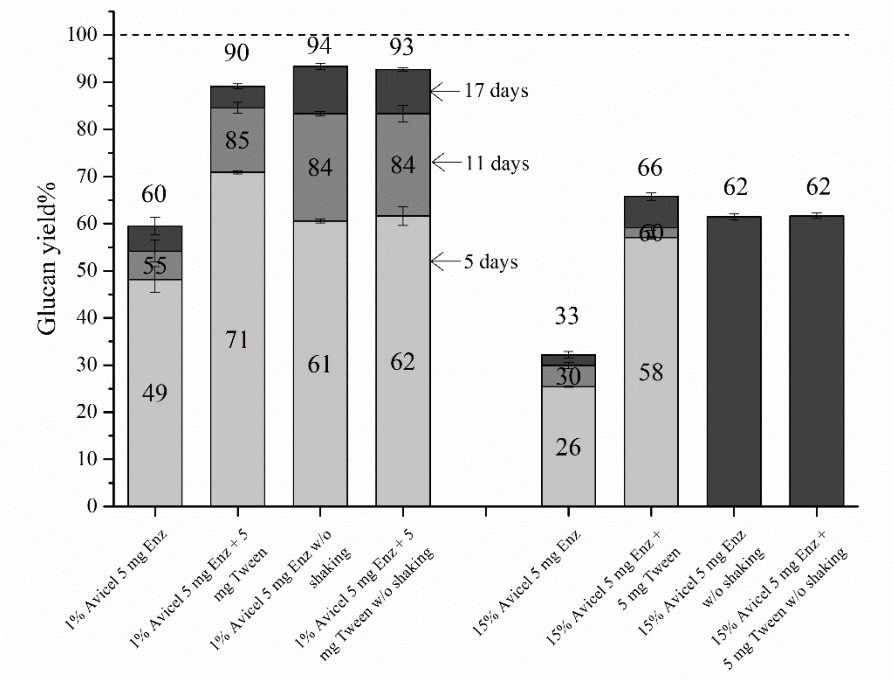

Figure 7.5 Effect of surfactant on enzymatic hydrolysis of Avicel with and without shaking.
Glucan yields from enzymatic hydrolysis of 1 and 15 % glucan loadings in Avicel by 5 mg of Accellerase® 1500 enzyme protein with shaking, co-addition of 5 mg of Tween to 5 mg enzyme at glucan loading with shaking, 5 mg of Accellerase® 1500 enzyme protein without shaking, and co-addition of 5 mg of Tween to 5 mg enzyme at glucan loading without shaking. The reaction volume was 50 ml performed in 125 ml Erlenmeyer flasks made of borosilicate glass at 50°C. Error bars represent standard deviation from three replicate flasks.

Next hydrolysis of 15% Avicel glucan loadings were performed to determine if accumulation of glucose due to lower mass transfer without shaking significantly reduced cellulose saccharification. In this case with shaking at 150 rpm, although the yield was only 33% for 5 mg cellulase/g glucan, adding Tween increased the yield to 66% (Figure

7.5). However, without shaking, 5 mg cellulase alone achieved a surprisingly high 62% yield, but similar to results for a low solids loading, adding Tween had no effect on cellulose saccharification in the absence of shaking at high solids loading. Supplemental File 1 shows that yields from cotton were enhanced from 41 to 53% through supplementation of 5 mg Tween to 5 mg enzyme at 1% glucan loading, and shaking at 150 rpm at 50°C.

Figure 7.6 summarizes the effects of Tween, Soy, and no shaking on glucan yields for a 5 mg enzyme loading applied to solids from liquid hot water (LHW) and extremely dilute acid (EDA) batch pretreatments of poplar at the conditions outlined in the Materials section. For LHW solids, addition of 5 mg Tween or Soy to 5 mg cellulase had little effect on yields, and glucan yields were virtually the same 10% with or without shaking at an enzyme loading of 5 mg/g glucan in LHW pretreated poplar solids. However, adding 5 mg Tween or soy to 5 mg enzyme/g glucan in EDA solids slightly enhanced yields, with the highest being about 26% for no shaking or surfactant. By comparison, the yield was about 22% for shaking and addition of 5 mg of Tween or soy. For both pretreated poplar substrates, 5 mg Tween had no effect when the Erlenmeyer flasks were not shaken.

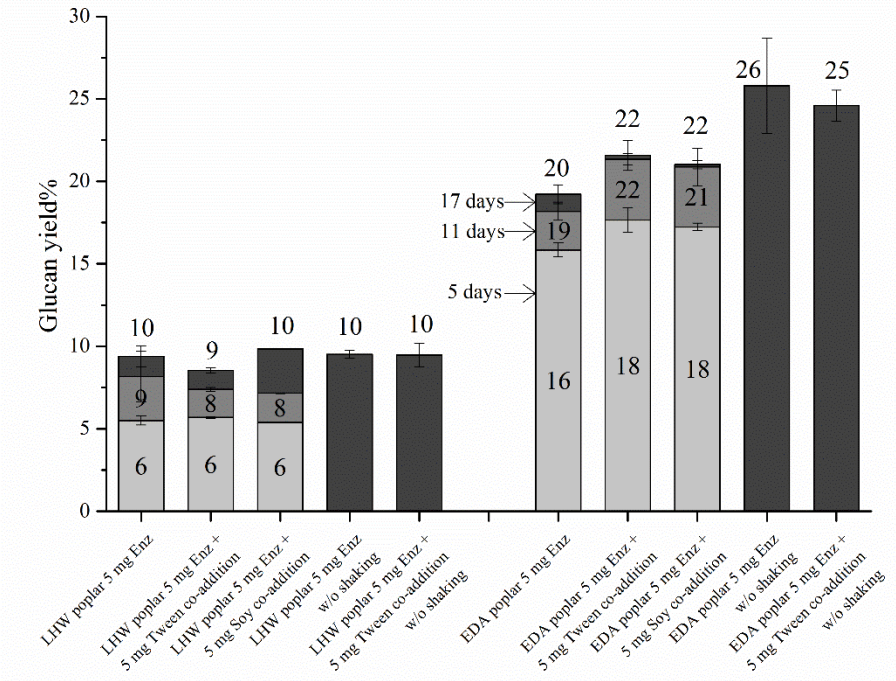

Figure 7.6 Effect of surfactant on enzymatic hydrolysis of batch pretreated poplar with and without shaking

Effect of surfactant and flask shaking on time course of glucan yields from enzymatic hydrolysis of solids produced by batch liquid hot water (LHW) and extremely dilute acid (EDA, 0.05 wt% sulfuric acid) pretreatments of BESC standard poplar. Enzymatic hydrolysis was at a loading of 5 mg of Accellerase® 1500 protein/g glucan alone and with co-addition of 5 mg of Tween or 5 mg of soy flour for a 1% glucan loading with and without shaking at 150 rpm. The reaction volume was 50 ml performed in 125 ml Erlenmeyer flasks made of borosilicate glass at 50°C. Error bars represent standard deviation from three replicate flasks.

7.5 Discussion

The similarity in the effects of BSA and Tween indicate that BSA works as a surfactant in improving cellulose saccharification. Soy flour contains lecithin (a mixture of phospholipids), protein, and saponin (Xu et al., 2011) that can have corresponding properties. Many other amphiphilic substances such as glycolipids, amphoteric, and cationic surfactants have been shown to improve cellulose saccharification, but with varying degrees (Tanaka et al., 1986) likely due to differences in their hydrophilic lipophilic balance (HLB) (Park et al., 1992). The large improvements in cellulose saccharification by BSA, Tween, and soy were apparent at low but not high enzyme loadings. The positive effects of BSA had been discussed by D.R. Whitaker in 1952 (Whitaker, 1952). However, a low ratio of inactive to active enzyme at high loadings might mask the effects of BSA and explain why some studies did not see any impact of BSA or Tween on Avicel (Eriksson et al., 2002; Yang & Wyman, 2006; Zheng et al., 2008).

One possibility for lowering of enzyme effectiveness can be due denaturation at the reaction vessel surface due to hydrophilic or hydrophobic functional groups and electrostatic charges (Duncan et al., 1995). Another study reported that shear stress can be an important contributor to enzyme deactivation (Reese & Ryu, 1980), and rough solid surfaces can deactivate protein due to shear (Biddlecombe et al., 2009). In addition, modification of solid surfaces might reduce protein losses. Siliconization of glass renders the glass surface hydrophobic through reacting with hydroxyl bonds on glass surface. Surfactants can reduce protein losses to the solid surface (Chawla et al., 1985). However,

this study showed that cellulose saccharification in siliconized glass flasks and polycarbonate resulted in similar results to those for borosilicate glass. Moreover, cellulose saccharification was not lower than for the conventional use of glass flasks for enzymatic hydrolysis with glass powder added to attenuate the surface area and shear due to sharp surfaces. Lee and Jones (Jones & Lee, 1988) found that enzyme deactivation was not impacted by shear in a high shear attrition reactor, similar to our results with high shear/high surface glass powder. Yields improved with surfactant addition by similar amounts in siliconized flasks, polycarbonate flasks, and in presence of glass powder in borosilicate glass flasks as well. Therefore, interaction with solid surfaces and shear stress were deduced not to influence loss of enzyme activity in these experiments.

The positive surfactant effect at low enzyme loadings might not due to stimulation of enzyme by BSA as hypothesized in some studies (Ooshima et al., 1986; Whitaker, 1952). Yields were the same at all time points in high ratio of enzyme to substrate. If surfactant improved catalytic rates through stimulation, than hydrolysis rates would have been faster when flasks were not shaken in presence of BSA or Tween. Rather, surfactants only had a positive impact when flasks were shaken. This outcome leaves open the possibility that denaturation at the air-liquid interface was responsible for loss of enzyme activity (Converse et al., 1988; Tanaka et al., 1978). In shaken flasks, the air-liquid interface is continuously renewed and can cause a significant fraction of the total enzyme at low concentration to become inactive. Kim et al. (Kim et al., 1982) found that excess surface in Gibbs' adsorption isotherm was constant between enzyme concentrations of 0.02 and 8 mg/ml, meaning that as enzyme loadings increase, the ratio

of surface enzyme to bulk decreases, explaining why this effect is seen only at low enzyme loadings. On the other hand, surfactants can arrange at the air-liquid interface to reduce deactivation. Kim et al. (Kim et al., 1982) showed that the surface excess of enzyme became negligible at 0.05 mg/ml of Zonyl FSN (non-ionic surfactant). The concentration of Tween 20 in our work was also 0.05 mg/ml. Through covering the attrition reactor vessel to eliminate teh air-liquid interface, Jones and Lee reduced enzyme deactivation from 50% to 5% after 20 hours despite using a very high enzyme loading of 0.4 g enzyme (125 FPU/g) per g newspaper. In our study, enzyme did not lose effectiveness due to shaking at high enzyme concentration. However, because their reactor employed ball milling and impeller mixing, their results may not be comparable with those from shaken flasks.

Increasing the temperature from 50 to 60°C lowered glucan yields much more than reducing the temperature from 50 to 37°C. The beneficial effect of BSA seemed to decline slightly with increasing temperature, as relative increases in yields were 55% at 37°C, 52% at 50°C and 48.5% at 60°C. This outcome indicates a protective effect of surfactant on enzyme especially at 10°C higher temperature that can severely deactivate enzymes.

Enzymes rapidly adsorb initially and then gradually desorb from the substrate over the course of hydrolysis, and it has been proposed previously that activity of adsorbed enzyme may decline over time (Converse et al., 1988; Fan & Lee, 1983). Cellulose aberrations can cause the enzyme substrate complex to become inactive and immobile (Igarashi et al., 2011) and result in irreversible activity losses (Rao et al.,

1983). Surfactant might reduce surface inactivation by reducing immobilization (Castanon & Wilke, 1981). Exoglucanase binds on the hydrophobic face (Liu et al., 2011) and travels on the surface of cellulose and exfoliates layers for saccharification. Hydrophobic tails of surfactant may bind to the hydrophobic face of the cellulose crystal and mediate exoglucanase transport to the substrate (Helle et al., 1993; Kim et al., 1997). Evidence that supports this mechanism is that free enzyme concentration has been found to be higher in the presence of surfactant (Castanon & Wilke, 1981; Helle et al., 1993). For example, Ooshima et al. (Ooshima et al., 1985) showed that Tween 20 did not affect the specific activity of endoglucanase (CMCase) and β-glucosidase and that free endoglucanase concentrations were higher in the presence of Tween 20 due to higher endoglucanase activity in the liquid phase. However, Kim et al. (Kim et al., 1997) showed that surfactant did not affect the Langmuir adsorption constant of endoglucanase but lowered them for Cellobiohydrolase II (CBH II). Another work determined that shaking speeds from 0 to 100 to 180 rpm reduced filter paper and Avicelase activity but not CMCase or CBU activity (Yang et al., 2011).

Overall, these results suggest that surfactant might reduce formation of inactive enzyme substrate complexes that form only in shaken flasks. While shaking coupled with surfactants can enhance hydrolysis rates by improving mass transfer, similar yields can be achieved without surfactant or shaking when the reaction is allowed to continue for a long period of time. At relatively high enzyme to substrate ratios, the surface regions that cause such inactivation might be modified or solubilized before formation of non-productive complexes. The impact of surfactant may be dependent on the architecture of

the cellulosic substrate surface because the yield improvement with Tween 20 was much less for cotton than Avicel. Gama and Mota (Gama & Mota, 1997) showed that the increase in yield by surfactants at low enzyme loadings was greater for Sigmacell than Avicel and negligible for Whatman filter paper. This trend is consistent with Sigmacell having lower crystallinity than Avicel or Whatman filter paper and Whatman filter paper having the lowest surface area among the three (Gama et al., 1993). Dependence of a non-productive complex on substrate features is supported here by the result that yields with 5 mg of cellulase alone increased from 60 to 74% by adding glass powder possibility due to substrate grinding changing the cellulose structure or reducing its crystallinity. When both glass powder and surfactant were added, enzymatic hydrolysis rates were faster than without glass powder. Moreover, in one study, enzyme recovery from digested residue was 90% when grinding with glass powder and Tween 80 were combined (Rao et al., 1983).

The nature of adsorption of surfactant on pure cellulosic materials has been reported to be different than lignocellulosic materials due to affinity of lignin for surfactant (Eriksson et al., 2002; Yang & Wyman, 2006). In case of lignocellulosic materials, a large quantity of surfactant is employed to block the surface of lignin to prevent unproductive loss of enzyme (Brethauer et al., 2011; Yang & Wyman, 2006). It has been recently shown that BSA had a greater effect on yields from solids produced by extremely dilute acid (EDA) pretreatment of poplar than solids from liquid hot water (LHW) pretreatment. Also, LHW poplar was more recalcitrant than EDA poplar (Bhagia et al., 2016a). The slight improvement in yields from EDA poplar solids by surfactant

addition could be due to lower recalcitrance coupled with the protective surfactant mechanism hypothesized above and reduction of on enzyme loss to lignin. The surprising result that yields were either similar or better without shaking for both of these pretreated lignocellulosic materials suggests loss of enzyme activity due to shaking for enzymatic hydrolysis of pretreated biomass at low solids loading. Although mixing could be necessary to improve mass transfer at high solids loadings, shaking also reduced glucan yields by nearly half for Avicel hydrolysis at high solids loadings (15%). However, cellulose solubilization was likely lower at high solids than at low solids loadings (62 to 66% compared to >90%) due to inhibition by high glucose and cellobiose concentrations (Xiao et al., 2004). Overall, shaking reduced yield at low solid loading (1% glucan) of Avicel and pretreated poplar lignocellulosic biomass, and high solid loading (15% glucan) of Avicel.

7.6 Conclusions

Surfactant only enhanced glucan released from nearly pure cellulose substrates at low enzyme loadings and only then if the reaction medium was shaken. Surprisingly similar yields were also achieved at low enzyme loadings but without shaking. Because surfactant addition did not enhance yields for the no shaking condition, its positive effects may be due to protection of enzyme from activity loss and not stimulation. The benefits were also not caused by loss of enzyme on vessel solid surfaces or shear stress. Overall, these results support a hypothesis that surfactants lower enzyme activity loss due to denaturation at the air-liquid interface that shaking promotes. However, another

possibility is that the surfactant lowers shaking induced formation of inactive enzyme complexes at the surface of the cellulosic substrate.

7.7 Abbreviations

BSA: Bovine Serum Albumin **CMCase:** Carboxymethylcellulose activity **FPU:** Filter Paper Unit **CBU:** cellobiose unit **LHW:** Liquid hot water **EDA:** Extremely dilute acid **BESC:** Bioenergy Science Center **BESC STD:** BESC standard poplar

7.8 Acknowledgements

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7.10 Supplemental Files

Supplemental File 1

File Format: .xlsx

Title of data: Glucan yields from all enzymatic hydrolysis conditions

Description of data: Additional file 1 shows glucan yields and their standard deviations for all conditions after 5, 11 and 17 days of enzymatic hydrolysis.

Chapter 8

Conclusions and Recommendations

8.1 Summary of Key Developments and Findings

Factors that contribute to recalcitrance of poplar lignocellulosic biomass to deconstruction by pretreatment and enzymatic hydrolysis were investigated.

Chapter 2 provided an overview of various pretreatment options for reducing recalcitrance of lignocellulosic biomass. Thermochemical pretreatments have been applied more extensively to lignocellulosic biomass and are more promising than mechanical and biological pretreatments. The thermochemical pretreatments that use organic solvents or expensive chemicals need recycling for a low cost pretreatment process. Aqueous based pretreatments are promising but currently need moderate to high enzyme doses to realize high sugar yields from enzymatic hydrolysis of cellulose in pretreated biomass.

In chapter 3 it was discussed that lignin content and structure may affect recalcitrance of poplar. Reduced lignin content was found in a few poplar varieties that carried a natural mutation in a novel EPSP synthase-like transcriptional regulator of the phenylpropanoid pathway. High throughput pretreatment and co-hydrolysis (HTPH) showed that these rare varieties had lower recalcitrance and showed high sugar yield compared to standard poplar. Sugar yields were significantly improved with pretreatment at 180°C compared to 140 and 160°C but at a constant pretreatment severity factor. Lignin content strongly affected glucan yields but had limited effect on xylan yields. Also, this work showed that plant ranks varied greatly with processing condition. Thus, picking a feedstock for cellulosic ethanol production should be based on sugar yields found through tests that mimic expected industrial processing conditions.

In Chapter 4, sugar yields and mass balances were discussed for liquid hot water and extremely dilute acid flowthrough and batch pretreatment of poplar. Flowthrough pretreatment showed high lignin removal than batch pretreatment in both liquid hot water and dilute acid conditions. Lignin removal led to better enzymatic digestibility of flowthrough pretreated solids than batch pretreated solids. However, between flowthrough pretreatment temperatures, enzymatic digestibility of 140°C solids was much lower than 180°C solids in spite of high lignin removal, indicating that higher temperatures play an important role in reducing recalcitrance through modification of lignin or other barriers to deconstruction. It seems that the leftover 30 to 35% lignin is difficult to remove by pretreatment and this fraction might contribute significantly to recalcitrance of biomass to enzymatic deconstruction.

In Chapter 5, solids generated through aqueous flowthrough and batch pretreatments in the previous study (Chapter 4) were subjected to varying dose of cellulase, and supplemented with xylanase. Xylanase supplementation resulted in some improvement in xylan yield in liquid hot water pretreated solids but not in dilute acid pretreated solids. Bovine serum albumin (BSA) was also utilized to block the surface of lignin from causing unproductive loss of enzymes. 180°C extremely dilute acid batch pretreated solids had a large impact on BSA supplementation to cellulase that was better than found through xylanase supplementation to cellulase. In all other solids, xylanase supplementation to cellulase had a similar or better effect than BSA supplementation to cellulase. Moreover, BSA supplementation to cellulase showed better effect on flowthrough pretreated solids than batch pretreated solids in most cases. This indicates

that BSA might have other roles than preventing loss of enzyme to lignin. Interestingly, BSA supplementation to cellulase affected only glucan yields but not xylan yields.

In Chapter 6, the standard acid hydrolysis procedure by National Renewable Energy Laboratory "Determination of Structural Carbohydrates and Lignin in Biomass" was studied for its robustness. It has two steps: primary hydrolysis in 72% wt sulfuric acid at 30°C for 1 h followed by secondary hydrolysis of the slurry in 4 wt% acid at 121°C for 1 h. Particle sizes of poplar from lower than 0.250 mm significantly lowered glucan content and increased acid insoluble lignin content. However, xylan, acetate, and acid soluble lignin were not affected through variation in particle size 1 mm and lower. Interestingly, results were the same when primary hydrolysis time was varied between 30 and 90 minutes than standard time of 60 minutes. Moreover, through changing the filtration strategy it was found that there were no lignin carbohydrate complexes remaining after primary hydrolysis and secondary hydrolysis procedure converted sugar oligomers to sugar monomers.

Chapter 7 showed that cellulose deconstruction from microcrystalline cellulose (Avicel) could be improved from 60 to 95% after 17 days of enzymatic hydrolysis with 5 mg cellulase in shaken flasks through addition of 5 mg of additives with surfactant properties such as bovine serum albumin, Tween, and soy flour. This positive effect is only seen at low enzyme dose and only when flasks are shaken. Without shaking, 95% yields can also be achieved from 5 mg enzyme after 17 days but without addition of surfactant. Also, similar or better glucan yields can be achieved from enzymatic hydrolysis of pretreated lignocellulosic biomass in dilute solutions without shaking. This

work indicates a possible role of air-liquid interface on deactivation of enzyme when reaction flasks are shaken. However, this can be also be due formation of inactive complex on surface of cellulosic substrate.

8.2 Novelty of this Dissertation and Bridging the Gaps

Prior studies have shown that genetic modification of lignin in plants can reduce recalcitrance leading to improvement in sugar yields, however, such studies were carried out using enzymatic hydrolysis without pretreatment that leaves a large portion of sugars unrecovered from biomass solids. In commercial biofuel plants most of the sugars need to be recovered from biomass and this requires pretreatment followed by enzymatic hydrolysis. It was not known how pretreatment affects sugar yields in low recalcitrance plants. The research in this dissertation was the first to discuss sugar yields found through pretreatment in various conditions coupled with enzymatic hydrolysis from feedstocks with reduced lignin content such as rare natural variants. The second study bridged the gap by studying sugar and lignin mass balances for flowthrough pretreatment of poplar in dilute acid conditions that had not been thoroughly investigated previously. This comparative study was one of the few studies that discussed lower temperature pretreatments of hardwood biomass and enzymatic hydrolysis of solids produced by them. Moving on, it was shown for the first time how BSA and xylanases affect enzymatic hydrolysis of moderately pretreated biomass by cellulases, and the only study to show their effect on flowthrough pretreated biomass. Further, study of robustness of two-step acid hydrolysis procedure found that only glucan and acid insoluble lignin were affected by particle size. Significant variation in time of primary hydrolysis did not affect

results. Moreover, this was the first study that showed that linkages between lignin and carbohydrates are completely cleaved in the primary strong acid hydrolysis step. In the end, effect of additives such as BSA and Tween 20 non-ionic surfactant on enzymatic hydrolysis were studied as previous work in this dissertation indicated that BSA may have a role greater than preventing unproductive binding of enzyme to lignin. In some prior studies, it was shown that there was no effect of non-ionic surfactants on hydrolysis of Avicel by enzymes, but they were carried out at relatively high enzyme doses and high amount of surfactant. However, the research in this dissertation led to the finding that at low enzyme doses (5 mg enzyme per gram cellulose), saccharification of Avicel can be improved greatly by addition of equally low amount of non-ionic surfactant, or additives with surfactant like properties. The novelty of this work was in finding that enzymes are inhibited due to shaking, and non-ionic surfactants reduce this inhibition due to shaking that leads to near complete saccharification of cellulose at low enzyme loading.

8.3 Closing Remarks and Suggestions for Future Work

Lowering of enzyme dose requires overcoming recalcitrance to reduce the overall cost of producing cellulosic ethanol. Through selection of feedstocks with reduced lignin content and/or architecture, high sugar yields can be achieved at lower enzyme dose. However, the top candidate at one process condition may not be the top candidate in another process condition. Since lignocellulosic biomass has a large heterogeneity that is dependent on several inherent factors as well as environmental factors, best benefit of biomass as raw material depends on picking a candidate based on sugar yield found through reproducing expected industrial conditions. It would also be worthwhile to test

plants for sugar yields that are stressed due to environmental factors such as climate, nutrients in soil, and water scarcity. From the other work, it seems that there may be a 30 to 35% higher recalcitrant lignin that is difficult to solubilize, and might be one of the most significant factors that affects enzymatic deconstruction of pretreated biomass. The results with xylanase supplementation to cellulase needs further investigation to understand why co-addition of the two enzymes is better than sequential addition of xylanase to cellulase carried out for the same time after addition of cellulase. This requires adsorption studies of xylanases and cellulase on flowthrough and batch pretreated solids, as well as on pure crystalline cellulose. While it was shown in this work that BSA reduces or prevents enzyme deactivation, and not only loss of enzyme to lignin, BSA adsorption to lignin in flowthrough solids needs to be studied in detail. This can be helpful in understanding adsorption of enzyme by residual lignin from flowthrough pretreatment. In all of this work with pretreated poplar, sugar yields were much lower with pretreatment at 140°C than 180°C. This might be linked to stronger grip of lignin on cellulose at lower temperature and/or its surface area. Knowing the exact nature of this grip can lead to development of strategies to achieve high sugar yields but at lower pretreatment temperatures which can ultimately lower cost of sugar recovery. Also, experiments need to be carried out to see whether air-liquid interface or inactive complex on substrate or both are the reasons for loss of enzyme in shaken condition. One way to figure this out can be through eliminating the air-liquid interface by filling the reaction volume to the top of the reaction vessel. It also needs to be determined which component of the cellulase cocktail, i.e., exoglucanase or endoglucanase, is deactivated more than

the other due to shaking. This can help in improving stability of this enzyme for improving the process of enzymatic hydrolysis.