UNIVERSITY OF CALIFORNIA
RIVERSIDE

Understanding How Tetrahydrofuran and Ethanol Solvent Pretreatments Impact Biological Deconstruction of Lignocellulosic Biomass

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

Doctor of Philosophy

in

Chemical and Environmental Engineering

by

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September 2021

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ACKNOWLEDGEMENTS

I would like to thank my advisor Dr.Charles E.Wyman from core of my heart. His continued encouragement and support during various challenging phases of research motivated constantly. He mentored me through entire degree with taking special care of me at times I was expecting my first child making sure I graduate with my Doctor of Philosophy in Chemical and Environmental Engineering.

I am thankful for funding by the Office of Biological and Environmental Research in the Department of Energy (DOE) Office of Science through the BioEnergy Science Center (BESC). I also express gratitude towards the Center for Environmental Research and Technology (CE-CERT) of the Bourns College of Engineering for providing the facilities and the Ford Motor Company for funding the Chair in Environmental Engineering that facilitates our projects.

I would like to acknowledge my entire family for supporting me through this endeavor. It would not have been possible if my husband, in-laws, parents, brother and sister-in law had not contributed to my achieving the first ever PHD in my entire family so I thank them from core of my heart for being present whenever needed.

DEDICATION

This thesis is dedicated to my kids Tanya and Navya.

ABSTRACT OF THE DISSERTATION

Understanding How Tetrahydrofuran and Ethanol Solvent Pretreatments Impact Biological Deconstruction of Lignocellulosic Biomass

by

Rachna Dhir

Doctor of Philosophy, Graduate Program in Chemical and Environmental Engineering
University of California, Riverside, September 2021
Dr. Charles E. Wyman, Chairperson

The abundance and low cost of cellulosic biomass such as wood, grasses, and agricultural and forestry residues make them very attractive sustainable resources for production of transportation fuels. However, low-cost bioethanol production is hindered largely by the recalcitrant nature of the biomass that results in high enzyme doses for enzymatic hydrolysis to realize high monomeric sugar yields. Biochemical conversion pathways typically apply a pretreatment step to make biomass accessible to enzymes during hydrolysis stage. This thesis focused on application of a new pretreatment technology that applies tetrahydrofuran (THF) in solution with very dilute sulfuric acid and water as a co-solvent pretreatment method to dramatically reduce the recalcitrance of poplar wood and make more sugars accessible to enzymes for hydrolysis along with high lignin separation. It is shown that this Co-solvent Enhanced Lignocellulosic Fractionation or CELF as we have called it is effective for various types of woody biomass for short pretreatment times and moderate temperatures in comparison to various other past pretreatments. Sugar yields from CELF coupled with subsequent 7-day enzymatic hydrolysis were compared with

results from dilute sulfuric acid, hydrothermal, and organosolv pretreatments. CELF was able to achieve 100% yields for an enzyme loading of 100mg/g glucan in raw biomass compared to 92% for dilute sulfuric acid pretreatment of the same poplar wood. More importantly, CELF realized much higher combined sugar yields at low enzyme (≤15mg/g glucan in raw biomass) compared to dilute sulfuric acid pretreatment. An important distinction that could account for the substantially better results by CELF from BESC standard poplar was its ability to remove a much greater fraction of lignin. However, CELF achieved higher total sugar yields than ethanol organosolv despite the latter's ability to also remove lignin. Furthermore, CELF performed better than ethanol organosolv at lower temperatures that resulted in negligible degradation of sugars. Removal of lignin by CELF resulted in an ability to mix higher glucan loadings that resulted in higher sugar titers. Preliminary results showed that a fed-batch strategy reduced hydrolysis times. In other experiments, hydrothermal pretreatment of BESC standard poplar and its variants in combination with consolidated biomass processing (CBP) showed how changes in biomass features could enhance performance. Finally, adding surfactants to enzymatic hydrolysis reactions was shown to reduce enzyme deactivation at the air-liquid.

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Chapter1

Introduction

1.1 Why Alternative Fuels?

World energy consumption has a constant upward trend for decades. Increasing energy demand all over the world poses a major threat to maintaining low costs for limited fossil fuels. The transportation sector is expected to witness a rise in use of petroleum and other liquid fuels to 121 million barrels per day in 2040 (Conti et al., 2016), and the transportation sector accounts for almost 62% of the total petroleum used presently. Some factors responsible for increase in energy demand are improved living standards, economic growth and development of countries. Worldwide growth in gross domestic product (GDP) is expected to average 3.3% /year from 2012-2040 (Conti et al., 2016). EIA reports the transportation and industrial sector are majorly responsible for growing liquid fuel consumption. Currently almost 87% of energy demands rely on non-renewable, fossil energy resources like coal, natural gas and oil (Conti et al., 2016; Gupta & Verma, 2015). Worldwide availability of oil, gas and natural gas reserves are reported to be 1688 Billion barrels (Bb), 6558 trillion cubic feet (TCF) and 891 billion tons (Bt), respectively, with corresponding consumption rates of 0.092 Bb, 0.329 TCF and 7.89 BT per day (Abas, Kalair, & Khan, 2015). At the current rate of consumption, the short life of the fossil reserves cannot be overlooked (Shafiee & Topal, 2009). Discoveries of oil reserves and recent drilling technologies shifted the King Hubert predicted oil peak plateau of 1995, but the higher rate of population growth and inflated standard of living will still overwhelm energy supplies. Peak oil production rates have been projected to decline from 105 million barrels per day (Mbpd) in 2100 to 40 Mbpd by 2400 (Trendlines, 2012). Although it is not certain when the world oil production will peak in

coming years, depletion of oil reserves is inevitable, and alternative sources of fuel are needed.

The rapid consumption of fossil fuels drives the need to switch the focus to renewable resources (Shafiee & Topal, 2009). In addition, emissions related to fossil fuels make it unattractive as a source of fuel. Climatic change resulting from carbon dioxide emissions particularly point out the need for renewable and affordable sources of energy. The United States has addressed air pollution for some time as evident by the first Federal legislation of 1955 provided air pollution research funding and the Clean Air Act of 1963 to control pollution. In 1980, concerns for fossil fuel combustion arose, and the world has been looking at alternative energy sources such as wind, solar and nuclear.

Lignocellulosic biomass is also a sustainable resource for fuel production. The abundance of lignocellulosic biomass like forest and agricultural residues, hardwoods, and grasses makes it a large lowcost resource for large-scale production of liquid transportation fuels. Biomass has a broad spectrum of energy applications ranging from fuels and chemicals to power production (Langholtz, Stokes, & Eaton, 2016).This inexpensive resource for fuel production would relieve the pressure on oil prices along with strengthening agricultural economies. Low greenhouse gas (GHG) emissions is a vital advantage of using cellulosic fuels. In particular over 12 billion tons of oil equivalent (BTOE) global annual energy demand results in emissions of 39.5 Giga tons of carbon dioxide (Gt-CO_2), and annual CO_2 emissions would increase to 75 Gt-CO_2 if future energy demand rises to 24–25 BTOE (Abas et al., 2015). In 2012, about 80% of the CO_2 emissions were attributed to transportation and electric power sectors (Conti et

al., 2016). The drop in global emissions from 43% in 1990 dropped to 36% in 2012 due to shifts in energy sources from coal to natural gas illustrates the advantage of investigating in other options (Conti et al., 2016). Fossil fuel accounted for 84% of worldwide energy consumption, but with increases in renewable and nuclear energy, the share of fossil fuel has dropped to 78%. Changes in the reduced share of energy from fossil sources and shifts in the fossil fuel mix resulted in reduced CO_2 emissions and could lead to almost a10% reduction by 2040 (Conti et al., 2016). Some projected changes could be partially facilitated by use of alternative sources of energy like biomass. Biomass and biomass-derived products are one of the most promising alternatives. These materials are made from atmospheric CO_2, and water through a process driven by sunlight called photosynthesis. These materials are considered to be the only source of organic carbon suitable for sustainable fuels production (Isikgor & Becer, 2015). Plants are mainly composed of three main components: cellulose, hemicellulose and lignin. Major component in biomass is typically cellulose that bears the load of the plant cell wall and is known to be the most abundant polymer on the planet (Sorek, Yeats, Szemenyei, Youngs, & Somerville, 2014). This glucan polymer consists of repeating units of the disaccharide cellobiose. Cellulose structure consists of extensive intermolecular and intramolecular hydrogen bonding tightly linking the glucose units (Isikgor & Becer, 2015). Cellulose microfibrils are insoluble and composed of β(1,4)-linked glucan molecules (Sorek et al., 2014). Endo- and exoglucanases depolymerizes cellulose into cellobiose and glucose. Hemicellulose is another important component of plant that mainly consists of β (1,4)-linked glucan, xylan, galactan, mannan, and

glucomannan branched with single or longer glycosyl residues (Sorek et al., 2014). All of these are fermentable sugars but vary widely in terms of composition in plant tissues and species during their growth, development, and maturation stages. Lignin is the third major component in plants and is an amorphous, irregular polymer of phenylpropanoid monomers in plant cell walls. This component hinders biomass saccharification during ethanol production by protecting cellulose fibers from depolymerization as well as interfering with enzymes. Various plant species differ in the amount of lignin and the lignin structure. Other components include pectins, ash, and some minerals that are present in relatively smaller amounts in plants and do not provide major source of sugars for biofuel production. The complex structure of plants presents various challenges to produce lignocellulosic fuels. In particular, the high cost of conventional biomass conversion technologies means more innovative and novel technologies are needed for biofuel production from lignocellulosic.

1.2 Biological Conversion of Cellulosic Biomass to Ethanol

This work focuses on biological conversion of lignocellulosic biomass to ethanol. The process starts with mechanical treatment of biomass to achieve the desired size for subsequent pretreatment that opens up the structure for enzymatic hydrolysis to sugars for fermentation to ethanol. Reducing cost remains a major challenge for the success of cellulosic ethanol as a transportation fuel. Presently the recalcitrant nature of biomass makes it difficult to deconstruct the biomass to sugars, as high amounts of costly enzymes are needed to realize high yields of sugars from the cellulose. Therefore, this

work focuses on biological pathways to understand factors and pretreatment conditions that maximize the total sugar yields at lower enzyme loadings.

Many physical, chemical, thermochemical and biological pretreatment methods have been reported in the literature to reduce the recalcitrance of biomass. Among these pretreatments, thermochemical methods have proven most favorable for achieving high yields in subsequent hydrolysis. Pretreatment disrupts the lignin and hemicellulose structure to make cellulose accessible to enzymes for further hydrolysis (Behera, Arora, Nandhagopal, & Kumar, 2014). Pretreatment changes the structure of lignocellulosic biomass by increasing surface area, enhancing porosity, relocating or removing lignin, partially depolymerizing or removing hemicellulose, and reducing cellulose crystallinity (Percival Zhang, Berson, Sarkanen, & Dale, 2009). Understanding deconstruction of biomass using various thermochemical pretreatments could lead to valuable insights to improve pretreatment. Pretreatments such as co-solvent-enhanced lignocellulosic fractionation (CELF), dilute acid, and ethanol organosolv remove varying amounts of lignin and hemicellulose to make biomass more susceptible to subsequent enzymatic hydrolysis to produce fermentable sugars. Pretreatment conditions should be defined based on maximizing sugar yields from pretreatment and hydrolysis combined. It is also important to close the mass balances of the sugars and track their fate during pretreatment and subsequent enzymatic hydrolysis to validate the yields. The fate of sugars during pretreatment is affected by temperature and time. The solids after pretreatments should be subjected to range of enzyme loadings to establish their susceptibility to sugar release.

The ability to release high sugar yields at low enzyme loading from solids recovered after pretreatment at low temperature and time will improve economics.

1.3 Thesis Organization

Poplar (*Populus trichocarpa*) was used as the lignocellulosic feedstock throughout this research. This biomass was provided by the BioEnergy Science Center (BESC). Chapter 2 discusses factors influencing the successful pretreatment of lignocellulosic biomass related to ethanol production and reviews previous research in this area. Chapter 3 establishes pretreatment conditions to maximize glucose and xylose yields from CELF and dilute acid pretreatments coupled with enzymatic hydrolysis over a range of enzyme loadings. Comparing these results over varying conditions provides valuable insights into routes to reduce enzyme loadings. Chapter 4 compares maximum total sugar yields from CELF and ethanol organosolv pretreatments followed by enzymatic hydrolysis. Comparison of lignin and hemicellulose removal by these two options with changing pretreatment temperatures and times provides additional insights into promising pathways to reduce the overall cost of ethanol production. Because, ethanol is widely used for biomass pretreatment, it provides a good reference point for this first study comparing the two solvent pretreatments. This study includes evaluation of biomass structural changes over enzymatic hydrolysis times and enzyme loadings required for complete hydrolysis. Chapter 5 presents results from hydrothermal pretreatment of lignocellulosic material followed by consolidated biomass processing (CBP) by *Clostridium therrmocellum*, a single organism that performs both enzyme production and fermentation. This study reveals the extent to which the hydrophilic capability of *C.*

thermocellum can significantly improve lignocellulosic biomass deconstruction. Chapter 6 reveals the effect of high glucan loadings of pretreated biomass on the effectiveness of enzymatic hydrolysis and resulting higher titers for fermentations. This approach can help identify pretreatment conditions to realize high ethanol yields. Chapter 7 presents the results from collaborative research to determine the impact of surfactants on hydrolysis of pretreated biomass. Chapter 8 is the final chapter to summarize the conclusions and provide recommendations from this dissertation.

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Chapter 2

Review of Factors Influencing Biological Pretreatment of Lignocellulosic Biomass

2.1 Abstract

Sustainable renewable transportation fuels are needed to slow down or replace fast growing petroleum consumption and its associated environmental hazards due to carbon dioxide emissions. Presently the cost of ethanol fuel production from cellulosic biomass prevents it from being a top contender in the fuel market. Biological conversion of lignocellulosic biomass involves use of expensive enzymes, but prior to hydrolysis a pretreatment step is required for biomass to be accessible to the enzymes for high sugar release. However, pretreatment by physical, chemical, thermal or microbial approach is expensive. This chapter reviews the factors affecting biological pretreatment to reduce recalcitrance of lignocellulosic biomass for ethanol production.

2.2 Introduction

Abundance of cellulosic biomass on earth along with advanced processing technologies developed at the industrial level to convert lignocellulosic biomass into ethanol have a promising future in the transportation sector (Tian, Fang, & Guo, 2012). Biodiesel and bioethanol are examples of alternative transportation fuels from biomass. Various feedstocks available for the bioethanol production are also part of the food chain, but non-edible plants have recently shown a good response to these technologies. Lignocellulosic biomass, that is carbohydrate rich, includes agricultural and forest residues and residues from production of wheat, corn, rice, and other herbaceous crops (Canam, Town, Iroba, Tabil, & Dumonceaux, 2013). However, bioconversion includes pretreatment step before the fermentation performed to increase the accessibility of

enzymes. Various pretreatment technologies include physical, chemical, physical-chemical and biological. Application of aqueous pretreatment started in the nineteenth century with acid catalyzed reactions mainly targeting hemicellulose, but pretreatment also restructures lignin. Lignin in biomass impedes degradation making pretreatment a necessary step to make the biomass accessible for breaking down complex sugars in the biochemical conversion process. Lignin disruption is favorable for reducing the recalcitrance of biomass for higher cellulose digestibility. Delignification is ameliorated with providing more surface area and increasing the median pore size since the biomass swells and helps break down lignin (Zhu, O'Dwyer, Chang, Granda, & Holtzapple, 2008). Because of these changes, there is an irreversible adsorption of enzymes on lignin with increasing accessibility to cellulose (Isroi et al., 2011). Different pretreatments involve different mechanisms and techniques, for example, physical pretreatments include milling, while physiochemical pretreatments include auto hydrolysis, steam explosion, SO_2 or CO_2 added steam explosion, and AFEX. Chemical pretreatments employ acid, alkaline, or solvents. Biological pretreatment involves the biodegradation of wood by microorganisms can be also favorable for lignin degradation.

Research on biological pretreatment based on decay causing fungi dates to 1870 (Blanchette, 1995). Although different microorganisms have the potential to degrade lignin, review of work in this area reveals that white-rot, brown-rot, and soft-rot are good candidates for effectively degrading lignin. Another aspect is bacterial decay based on morphological aspects of erosion, tunneling, and cavity-forming bacteria, but to date, not much seems to be as effective as fungi (Blanchette, 1995).

2.3 Cellulosic Biomass

Lignocellulosic/cellulosic biomass is composed of cellulose, hemicellulose and lignin. Being a linear mostly crystalline polymer of cellobiose (D-glucopyranosyl- β-1,4-D-glucopyranose), cellulose comprises over 50% of wood weight. Lignin is different since it is a three-dimensional network composed of dimethoxylated (syringyl, S), monomethoxylated (guaiacyl, G) and non-methoxylated (*p*-hydroxyphenyl, H) phenylpropanoid units, derived from the corresponding *p*-hydroxycinnamyl alcohols, plus a variety of subunits with different ether and C—C bonds. Resistance of lignin to chemical and biological degradation is mainly attributed to the middle lamella. Diverse H:G:S ratios occur in different vascular plant. Woody gymnosperms (softwoods) are composed of the highest lignin content, with most being G units. On the other hand, lignin of woody angiosperms (hardwoods) mainly contains S and G units, and non-woody angiosperms contain H units.

A third intermediate complex structural component, hemicelluloses (polyoses), is composed of different pentose and hexose residues often acetylated and generally forming branched chains. Typically, hemicelluloses in softwood are glucomannans, while those in hardwoods are mainly xylans together with variable percentages of galactose, arabinose, rhamnose, methylglucuronic acid units, and acetyl groups. Various other non-structural wood components are extractives polar (e.g., phenols and tannins) or nonpolar (e.g. fats and sterols) water-soluble compounds (e.g., sugars and starch), as well as proteins and ashes (Martínez et al., 2010).

The three main types of wood tissue, namely fibers, vessels, and parenchyma cells, are formed from the above-mentioned components. Structural differences between gymnosperms and angiosperms consist of 90–95% tracheid cells (softwood fibers) and low amounts of parenchyma, which includes the specialized resin channels in conifers. Radial arrangement exists in wood for parenchymatic ray that contains phenolic and lipophilic extractives and water-soluble compounds as storage materials. Vessels are large cells with a longitudinal arrangement, responsible for the transport of water and nutrients along the plant stem in hardwoods. Lastly, fibers are longitudinally arranged with thick cell walls to support the tree represent most of the wood volume (Martinez et al., 2010).

2.4 Polysaccharide Deconstruction

Cellulose and hemicellulose polymers that are either insoluble or closely associated with insoluble lignin make the deconstruction of sugars problematic (Dinis et al., 2009). Cell wall degradability is inefficient due to covalent bonding of hydroxycinnamic acids, mainly ferulic and p-coumaric acid. Pectins and polysaccharides (arabynoxylans, xyloglucans) linked through ester bonds to lignin hinder cell wall accessibility (Dinis et al., 2009). Biodegradation is easier in the presence of syringyl lignin in the cell wall than when coniferyl lignin is present (Isroi et al., 2011).

2.5 Impact of Various Factors on Pretreatment of Lignocellulosic Biomass

A wide range of microorganisms are known to degrade lignocellulosic biomass,. Enhanced yields have been reported with the application of pretreatments in combination

with microorganisms. Lignin mainly obstructs lignocellulosic biomass conversion. Microorganisms, namely white rot, brown rot and soft rot fungi, are known to degrade lignin at lower temperatures. Wood-decaying basidiomycete fungi produce multi-enzyme systems that help in recycling plants in nature. Degradation resulting from fungi makes them a potential alternative for processing biomass into fuels (Okamoto, Nitta, Maekawa, & Yanase, 2011).

Advantages and classification of wood rotting basidiomycetes based on macroscopic aspects as white-rot and brown rot fungi has been cited in the literature (Martínez et al., 2010). Wood decay is cumbersome due to its low nitrogen content and the presence of toxic and antibiotic compounds, which need to be overcome by the pretreatment. Extracellular oxidative enzymes (oxidoreductases) secreted by fungi favor cell wall degradation. White-rot basidiomycetes degrade lignin, hemicelluloses, and cellulose along with producing cellulose-enriched white material. Two white-rot patterns reviewed in literature in different types of wood are selective delignification, also called sequential decay, and simultaneous rot based on the white-rot basidiomycetes which are capable to degrade lignin selectively or simultaneously with cellulose (Martínez et al., 2010). Although it would be desirable for fungi to degrade lignin at a much faster rate than desirable carbohydrates like cellulose, most e white-rot fungi have low sugar recovery due to simultaneous lignin and cellulose degradation (F.-h. Sun, Li, Yuan, Yan, & Liu, 2011). Fungal hyphae massively colonize the ray parenchyma cells of the biomass feedstock, then penetrate through pits and form numerous boreholes and erosion troughs on the vessel beneath or around the hyphae in white-rot (Wan & Li, 2013). During further

stages of decay, feedstock becomes light colored, soft, and spongy. This group of fungi leads to various reactions within lignin which include side-chain oxidation, propyl side-chain cleavage, and demethylation (Chen et al., 1983). Polysaccharides attacked by white rot fungi include hemicellulose rather than cellulose during selective degradation providing delignified and cellulose rich biomass for subsequent processing (Blanchette, 1995).

2.5.1 Feedstocks

The variability of recalcitrance in different cellulosic materials impacts the efficiency of pretreatment. Various factors such as nutrients, porosity, and texture of the feedstock are found to increase the water retention capacity of the biomass. These factors also increase the surface area for the processing of the substrates such as wheat bran and rice straw. Previously the favorable texture of wheat bran and paddy straw even in moist conditions was shown to be a favorable feedstock characteristic (Das et al., 2013)**.** Research has shown that rice straw and wheat bran support different microorganisms to achieve high yields after biological pretreatment. (Jalc, Siroka, & Ceresnakova Zb, 1997; Knezevic et al., 2013; Kuhar, Nair, & Kuhad, 2008; Liers, Arnstadt, Ullrich, & Hofrichter, 2011; Niladevi, Sukumaran, & Prema, 2007; Okamoto et al., 2011; Shrivastava et al., 2011; Singh, Tyagi, Dutt, & Upadhyaya, 2009; Masayuki Taniguchi et al., 2005; Tiwari et al., 2013; Wicklow, Langie, Crabtree, & Detroy, 1984; Yamagishi, Kimura, & Watanabe, 2011) Less recalcitrant herbaceous biomass such as corn stover, cornstalks, switchgrass, soybean straw, lemon grass, citronella, residual maize stover, sugarcane residue bagasse,

and mustard stalks have been studied for sterile and non-sterile biological pretreatments due to their abundance (Chen, Fales, Varga, & Royse, 1996; Cui, Shi, Wan, & Li, 2012; Isikhuemhen, Mikiashvili, Adenipekun, Ohimain, & Shahbazi, 2012; Y. J. Lee, Chung, & Day, 2009; J. Liu et al., 2013; López, Elorrieta, Vargas-García, Suárez-Estrella, & Moreno, 2002; Lynch, O'Kiely, Murphy, & Doyle, 2013; Ma et al., 2011; Pal, Banik, & Khowala, 2013; Rolz, de Leon, de Arriola, & de Cabrera, 1986; Sasaki et al., 2011; L. Song, Ma, Zeng, Zhang, & Yu, 2013; Wan & Li, 2013; X. Yang, Zeng, Ma, Zhang, & Yu, 2010).

Hardwoods and their byproducts have also served as substrates for various experiments. Harwoods such as eucalyptus spent sulphite liquor (HSSL), a byproduct from acidic sulphite pulping, is specifically available in South Africa, Portugal and Spain. Toxic compounds produced are consumed by selected microorganisms to reduce inhibition of microbial pretreatment. Lignosulfonates and carbohydrates produced by spent sulphite liquor are also cited as substrates (Holmgren & Sellstedt, 2008). Kraft wood containing lignin has been pretreated for biological pretreatment studies (Kondo, Kurashiki, & Sakai, 1994). Hardwoods like eucalyptus, oak wood, various poplar varieties , bamboo clumps, sapwood of beech, aspen wood blocks, moso bamboo ,Chinese Willow, lolblolly pine chips and rubberwood (*Hervea Brasiliensis*) from Malaysia containing high cellulose content have been found to be suitable for biological pretreatment in different studies (Akhtar, Michael, & Gary, 1992; Blanchette, 1995; Giles, Galloway, Elliott, & Parrow, 2011; Itoh, Wada, Honda, Kuwahara, & Watanabe, 2003; Kang, Sung, & Kim, 2007; Liers et al., 2011; Mahajan, Jeremic, Goacher, & Master, 2012; Nazarpour,

Abdullah, Abdullah, & Zamiri, 2013; Otjen & Blanchette, 1985; Ian D. Reid, 1989; W. Wang, Yuan, Cui, & Dai, 2013; Zuchowski, Pecio, Jaszek, & Stochmal, 2013).

Other substrates widely available in a specific region have also been considered. Canola straw, due to high residue production, was used as substrate (Canam, Town, Tsang, McAllister, & Dumonceaux, 2011), but limited glucose availability with the application of alkali and acid pretreatment was reported. *Parthenium sp.,* a native weed in tropical America known as carrot grass, was tested with white rot fungi due to its wide growth rates and short life cycle along with greater reproductive cycles and prospective abilities, some of these traits making it potentially useful for ethanol production (Rana et al., 2013). A recent work cited the use of chestnut shells, a common food industry waste with high lignin content and the ability to produce monomers and oligomers (Dong, Dai, Xu, Cai, & Chen, 2013). Rapeseed meal is high in nutritional and fiber content but also contains anti-nutrients such as phytic acid and phenolic compounds. Norway spruce found in Northern and Central Europe have potential due to their abundance and degradation by the fungi (Maijala et al., 2008; Nagy et al., 2012; Zuchowski et al., 2013). Abundant cotton stalks in southern United States can also work as substrates (Kleman-Leyer, Agosin, Conner, & Kirk, 1992; Shi, Sharma-Shivappa, Chinn, & Howell, 2009). *Prosopis Julifora* weed in India could be biologically delignified by different fungi (Kuhar et al., 2008). Thinning of *Japanese Cedar* forests in Japan served as carbon source, but being a softwood, was resistant to hydrothermal and thermochemical pretreatments (Baba et al., 2011).

2.5.2 Organisms

Biological pretreatment could be tedious due microbial screening required since more than 10,000 species of white rot fungi are known to selectively degrade lignin in a process called the bio pulping (Kang et al., 2007). White rot fungi, the most suitable basidiomycetes, has two pathways for decay. Decaying patterns in different types of woods could be sequential decay or simultaneous rot (Martínez et al., 2010). Fungal growth and its enzyme activities of different strains of *Ceriporiopsis subvermispora* have been screened for the most effective lignin degrading pretreatments. High lignin selectivity makes this organism a potential candidate for biological processing (Nazarpour et al., 2013). Pretreatment with *Ceriporiopsis subvermispora* required lower energy consumption and achieved high pulp yields, but the effectiveness of different strains of the same microorganism could differ during bio-pulping (Akhtar et al., 1992). Among diverse white rot-fungi, *Phanerochaete chrysosporium* is capable of selective lignin degradation (Kondo et al., 1994; Kuhar et al., 2008; Nazarpour et al., 2013). However, during degradation, cellulose was consumed when fungi attacked the surface of the microfibrils in another kinetic study (Kleman-Leyer et al., 1992). Ongoing effort to improve fungi aims to use RNA interference to down regulate some specific genes and improve biological pretreatment (Canam et al., 2011; Matityahu, Hadar, Dosoretz, & Belinky, 2008). Lower recalcitrance resulting from hemicellulose and lignin removal was an important property of *Pleurotus ostreatus* (Itoh et al., 2003; Jalc et al., 1997; Knezevic et al., 2013; Lynch et al., 2013; Shrivastava et al., 2011; M. Taniguchi et al., 2010) (Taniguchi et al., 2005, Itoh et al., 2003, Eriksson et al., 1990, Xu et al., 2010). Many

lignin-carbohydrate structures were studied based on their availability with differing results (Shrivastava et al., 2011). Lower incubation times for *Trametes versicolor* strains are beneficial, but huge vessel sizes for industrial applications makes them less desirable (Canam et al., 2011; Shrivastava et al., 2011; W. Wang et al., 2013; X. Zhang, Yu, Huang, & Liu, 2007). Significant lignin extraction shown by different studies makes these organisms potentially attractive (Canam et al., 2011). *Trametes Hirsuta* strains screened for efficient production of ligninolytic enzymes (laccases) were capable of producing ethanol from hexoses sugar as well as various starch and lignocellulosic substrates (Mao, Zhang, Li, & Xu, 2013; Okamoto et al., 2011; Rana et al., 2013; F.h. Sun et al., 2011). *Irpex lacteus CD2* colonized well in non-sterile substrate conditions generating many by-products advantageous for hydrolysis (L. Song et al., 2013). Optimized enzyme dosage and producing value added products at the end of the overall process are some advantages of *Irpex lacteus,* but e long incubation times make it unfavorable for industrial use (López-Abelairas et al., 2013; Salvachúa, Prieto, Vaquero, Martínez, & Martínez, 2013; C. Xu, Ma, & Zhang, 2009; X. Yang, Dey Laskar, Ma, Zhang, & Chen, 2013; J. Yu, Zhang, He, Liu, & Yu, 2009; M. Zeng et al., 2012). Less popular lignolytic micromycete fungus *Myrothecium roridum LG7* isolated from decaying wood was attractive due to its high colonization rate and low cellulase activity (Tiwari et al., 2013). *Paecilomyces variotii NRRL-1115* was selected for its detoxification ability as this strain consumes phenol as a sole carbon source to make by-products for further bioprocessing (Pereira, Ivanuša, Evtuguin, Serafim, & Xavier, 2012). Biological pretreatment by *Echinodontium taxodii* 2538 enhanced hydrolysis of

hardwoods and softwoods with significant increase in yields with selective lignin degradation of native substrates (X. Yang et al., 2013; X. Yang et al., 2010; J. Yu et al., 2009; X. Zhang et al., 2007). *Heterobasidion parviporum* initiated detoxification with minimal cell wall degradation due to its long decay patterns and xylem defense mechanism based on the substrate selectivity (Nagy et al., 2012). *Pleurotus florida, Coriolopsis caperata RCK 2011,* and *Ganoderma sp. rckk-02* provided high sugar release and delignification (Deswal, Gupta, Nandal, & Kuhad, 2014). *Phlebia sp. MG-60* worked under aerobic conditions (Kamei, Hirota, & Meguro, 2012), *Coriolus versicolor* white rot fungi was also tested based on different factors of interest (Itoh et al., 2003; Kondo et al., 1994; López et al., 2002). *Termitomyces clypeatus* (Pal et al., 2013), *Pleurotus eryngii*, *Fomitopsis pinicola*, (Knezevic et al., 2013; López-Abelairas et al., 2013), *Lenzites betulinus*, *Pycnoporus Cinnabarinus* (Kuhar et al., 2008; Sharma et al., 2012), *Cyathus stercoreus (Yamagishi et al., 2011)*; *Phanerochaete chrysosporium(Chen et al., 1996)*; *Dichomitus squalens* (Dong et al., 2013; Itoh et al., 2003); *Trametes gibbosa* (TWS-TG) (Jalc, 2002); *Phanerochaete carnosa (Mahajan et al., 2012)*; *Coprinellus disseminates (Singh et al., 2009)*; *Ischnoderma resinosum*, *Poria medulla-panis*, and *Xylobolus frustulatus* (Otjen & Blanchette, 1985); *Streptomyces psammoticus (Niladevi et al., 2007)* are among those microorganisms that are being used for different biological pretreatments but have not gained momentum. Research using soft rot fungi has been reviewed either as monocultures or as co-cultures for increasing the effectiveness of biological pretreatment mostly for hardwoods (Holmgren & Sellstedt, 2008; Popescu, Popescu, & Vasile, 2010).

2.5.3 Pretreatment Strategies

Pretreatment strategies applicable for exploiting lignocellulosic biomass primarily affect the sugar yields. This section illustrates microbial-based or combination pretreatment strategies for biomass. Low cost and effectives of biological pretreatment improves it chances of application (Isroi et al., 2011; López-Abelairas et al., 2013). Incubating pretreated and unpretreated material with additives in distilled water before washing the incubated material impacts the biomass. Solid fractions are subjected to alkali treatment in order to improve the yields during enzymatic hydrolysis (Pal et al., 2013; Tiwari et al., 2013). Subjecting bio-pretreated residues to chlorite and dilute acid treatment to alter the chemical components of the residues does not cross affect hemicellulose and lignin. On the other hand, dilute acid pretreatment removed hemicellulose instead of lignin in this unique combination pretreatment strategy, showing alkali to be more favorable in combination with biological pretreatment (W. Wang et al., 2013). Drying of pretreated biomass to reach a constant weight was studied (Zhang et al., 2007; Kuhar et al., 2008; Sun et al., 2011; Yu et al., 2009) with subsequent application of biological pretreatment. Before hydroliquifaction and mechanical pulping, forced aeration was used to maintain biological activity throughout (Akhtar et al., 1992; Lemée, Kpogbemabou, Pinard, Beauchet, & Laduranty, 2012). Yet another approach is to disinfect biomass with lime for a day and then wash with DI water before treating the biomass under non-sterile conditions is reported to promote stable microbial growth (L. Song et al., 2013). A single batch pretreatment reactor and sequential pretreatment reactors have been applied for effective sugar recovery (Pereira et al., 2012). Biomass was dewaxed with toluene-

ethanol before a relatively long pretreatment was applied to the substrate (Mao et al., 2013). Suspending white rot-fungi during inoculum preparation in Tween-80 and adding nutrient broth like Kirk medium during pretreatment has been used to look at lignin degradation (Masayuki Taniguchi et al., 2005). Supplementing the culture bottles with nutrients did not prove to be very effective for the delignification (I. D. Reid, 1985). Addition of nutrients along with combined pretreatment had been followed for an effective bio-pretreatment (J. Yu et al., 2009)**.** Following ultrasonic or H_2O_2 pretreatment, the biomass was subjected to biological treatment. Co-culture strategy was also adopted to discover the effect on lignocellulolytic enzyme activity and degradation pattern (Ma et al., 2011)**.** Aerobic biological delignification was applied to the incubated biomass for inoculation in a sealed flask to look at the integrated delignification and saccharification (Kamei et al., 2012) . Steam explosion pretreated biomass was subject to submerged fermentation along with addition of Tween-80 (S. Zhang, Jiang, Zhou, Zhao, & Li, 2012). Combining steam explosion with subsequent fungal pretreatment had favorable effects on overall yields and pretreatment times (M. Taniguchi et al., 2010). Combinations such as applying biological-alkaline/oxidative pretreatment to the lignocellulosic material with fungal pretreatment followed by alkaline/oxidative pretreatment with low amount of chemicals at very low temperatures was compared to traditional physical and chemical pretreatments (H. Yu et al., 2010). Another combined pathway used microwave hydrothermolysis followed by fungal pretreatment to evaluate the effect on cell wall polysaccharides (Sasaki et al., 2011). A two stage fungal pretreatment for effective bio-pulping was applied in work conducted with different combinations of white and brown

rot fungi (Giles et al., 2011). Co-cultures were applied for 12 day pretreatments for the maximum activity with selected fungi strains (Dong et al., 2013). Another combination applied ethanolysis followed by separating biomass components with water, ethanol and fungi. A similar strategy applied solvolysis and cultivation with fungi to overcome softwood recalcitrance (Baba et al., 2011). Qi-he, Krügener, Hirth, Rupp, & Zibek (2011) by a co-cultivation to produce high activity enzymes but with the two white rot-fungi. Mixed culture was also applied to hardwoods (Nazarpour et al., 2013). Another co-culture of white and soft rot fungi was used to determine their ability to degrade lignocellulosic biomass (Holmgren & Sellstedt, 2008). Another pretreatment strategy includes lead supplementation during media preparation for the fungal pretreatment (Huang et al., 2010).

2.5.4 Temperature

Temperature affects the rate of delignification and alters selectivity due to differences in optimal conditions for different microorganism. Most fungi being mesophiles have been reported to be most effective between 22-30˚C (Ian D. Reid, 1989). Further literature cites growth medium incubated at 28˚C (Canam et al., 2011; Cui et al., 2012; López-Abelairas et al., 2013; Okamoto et al., 2011; Pereira et al., 2012; Salvachúa et al., 2013; Sasaki et al., 2011; Lili Song, Yu, Ma, & Zhang, 2013; W. Wang et al., 2013). Temperature for the medium was maintained between 25˚C and 30˚C for successful growth of microorganisms reported in various studies (Deswal et al., 2014; Ma et al., 2011; Rana et al., 2013; Tiwari et al., 2013; J. Yu et al., 2009**).** A low temperature of

20˚C has been reported in an earlier study (Bowen & Harper, 1990; Rolz et al., 1986). Overall, temperatures between 25 ˚C and 32.5˚C look favorable for delignification of biomass (I. D. Reid, 1985).

2.5.5 pH

Because pH plays an important role for fungal growth and cultivation, a brief review is presented in this section. pH values ranging from 4 to 6 were studied in a study exclusively with NaOH since the enzyme was highly stable during this range (Salvachúa et al., 2013). The fungal cultures were grown at a pH of 5.5 in most studies that achieved high concentrations of fermentable sugars (Deswal et al., 2014; Kuhar et al., 2008; X. Yang et al., 2013; X. Yang et al., 2010). pH 6 was used for the organisms used in an experiment (Das et al., 2013; Kamei et al., 2012). However, another study applied pH 5.0 in a two-step combination pretreatment during the experimental design for achieving high yields (Zeng, Yang, Yu, Zhang, & Ma, 2012). A fungi *Merulius tremellous* reported to acidify the wood below pH of 3 differs from other studies previously cited (I. D. Reid, 1985).

2.5.6 Moisture Content

In most of the experiments the moisture content was maintained either by replacing the lost water daily or by keeping the initial moisture content higher (Salvachúa et al., 2013). Increase or decrease in moisture levels in a fungal pretreatment makes up the environment for ineffective fungal pretreatment and might lead to more cellulose and hemicellulose removal from the biomass. Delignification is influenced by inappropriate

moisture levels during fungal pretreatments (Wan & Li, 2013). Also, long term storage of biomass needs suitable moisture levels for reduced cellulose degradation (Cui et al., 2012) . A moisture content between 60 and 77% was used in various studies (Giles et al., 2011; Kang et al., 2007; Lemée et al., 2012; López et al., 2002; Pointing, Parungao, & Hyde, 2003; L. Song et al., 2013). (Ian D. Reid, 1989) that reported lower or greater inter-particle space water clogs that resulted in spreading contaminating bacteria along with hindered gas circulation due to compaction of substrate.

2.5.7 Inoculum time

Inocula were incubated for 7 days prior to pretreatment in most studies (Bowen & Harper, 1990; Pereira et al., 2012; Qi-he et al., 2011; Salvachúa et al., 2013)**.** However, 5 days was found to be enough in some other reports (Costa, Gonçalves, & Esposito, 2002; Das et al., 2013; Mao et al., 2013; F.-h. Sun et al., 2011; W. Wang et al., 2013)**,** and a 10-day was employed in some experimental studies for selected substrates (J. Yu et al., 2009; X. Zhang et al., 2007). 12 week incubation was used in various other studies (Otjen & Blanchette, 1985; Pointing et al., 2003).

2.5.8 Particle Size

Particle size should be considered for the pretreatment as it improves pretreatment effectiveness by increasing surface area and impacting the interaction between particle and aeration (Ian D. Reid, 1989). Kang et al.(2007) used 20±1mm wood blocks for fungal pretreatment of biomass. Particle sizes of less than 0.5mm and less than 1mm used in most studies showed a significant improvement in digestibility (Salvachúa et al., 2013,

Rana et al., 2013). Similar particle sizes were found favorable by others (Tiwari et al., 2013, Lemee et al., 2012, Cui et al., 2012)**.** Particle size of 20 and 80 mesh was used in various studies (Kuhar et al., 2008; L. Song et al., 2013; F.-h. Sun et al., 2011; W. Wang et al., 2013; J. Yu et al., 2009)**.** Particle size ranges from1-3mm were also found favorable for yields (Das et al., 2013; Deswal et al., 2014; Dong et al., 2013)**.** Reducing particle size also assists enzymatic digestibility by reducing biomass crystallinity (Okamoto et al., 2011). A different technique applied a higher milling time of 120 min in order to disrupt the structure of the biomass. 0.18-0.25 mm particle size was preferred elsewhere (Mao et al., 2013) while (Rolz et al., 1986) used 2-3 cm particle size for the fungi screening process.

2.5.9 Pretreatment Time

Time for pretreatment is an important factor since longer times could impact the amount of lignin removal but also result in more carbohydrate degradation and higher costs (Ian D. Reid, 1989). A pretreatment time of 21-d (Salvachúa et al.,2013; Abelairas et al.,2013) was comparatively shorter than some who applied 8 weeks of pretreatment to some woods (Itoh et al., 2003; W. Wang et al., 2013). A 7-d pretreatment was sufficient for other fungal pretreatment studies (Canam et al., 2011; Kondo et al., 1994; Tiwari et al., 2013). An 18 and 35-d pretreatment span was applied to understand the effect of pretreatment on various biomasses (Wan & Li, 2013). A 15-d fungal pretreatment followed by alkali pretreatment was used to evaluate the delignification in another work (Y. Yu & Wu, 2010). Under non-sterile conditions, the pretreatment was carried for 56

days (Lili Song et al., 2013). Aerobic incubation for 56 days was required in another study for biologically treating biomass (Kamei et al., 2012). Increased pretreatment time showed increased fermentable sugar yield during the early pretreatment stages in some experiments leading to times as long as 120 days that consequently led to biomass loss and additional pretreatment costs (X. Zhang et al., 2007). A similar duration was chosen for the experimental set up elsewhere (Yu et al., 2009). An intermediate 3 week pretreatment was also applied to biomass (Lemee et al., 2012), and pretreatment times as short as 10-days were employed in another study (Kuhar et al., 2008). An incubation time of 25-days has been used in various studies (Deswal et al., 2014; Ma et al., 2011; Yamagishi et al., 2011). In a single batch pretreatment, a total of 16-day cycle has been reported to be beneficial (Pereira et al., 2012). Pretreating biomass for 3- 6 weeks to look at the effect of biological pretreatment on the delignification of biomass was applied in a few studies (Mao et al., 2013; F.-h. Sun et al., 2011). The effectiveness of 24, 48, and 72-days of pretreatment depended on the different fungi considered k (Masayuki Taniguchi et al., 2005). A 4 week pretreatment time before mechanical pulping process was applied too (Akhtar et al., 1992). A 30-day pretreatment was used of the delignification (Jalc et al., 1997; X. Yang et al., 2013; X. Yang et al., 2010). A 90-day period was selected by some other researchers (Cui et al., 2012; Nazarpour et al., 2013). A 60 -day period of fungal pretreatment for lignin degradation was preferred to screen the most effective fungi (Kang et al., 2007). On the other hand, 12 weeks cultivation is the highest among the work reviewed (Bowen & Harper, 1990; Mahajan et al., 2012).

2.5.10 Hydrolysis and Fermentation Methods

Different fermentation strategies are reported to be used for conversion of the pretreated material to ethanol or various value added products such as solid state fermentation to ethanol (Isikhuemhen et al., 2012; Rana et al., 2013; I. D. Reid, 1985; Salvachúa et al., 2013). Enzymatic hydrolysis was used by some to investigate the effect of biological pretreatment on sugar release from biomass (Canam et al., 2013; López-Abelairas et al., 2013; Lili Song et al., 2013; Y. Sun & Cheng, 2002)**.** Pyrolysis and liquefaction were involved in some of the experimental works (Lemée et al., 2012; Ma et al., 2011). Solid state cultivation was conducted for ethanol fermentation (Deswal et al., 2014; Kuhar et al., 2008; Lili Song et al., 2013). During the integrated saccharification and fermentation process, semi-aerobic conditions were maintained by plugging the silicon plugs during pretreatment (Kamei et al., 2012). Submerged fermentation (SmF) was conducted with one substrate as the sole carbon source for ethanol based on fungal pretreatment of the biomass (Dong et al., 2013; Rana et al., 2013). Simultaneous saccharification and fermentation (SSF) of biomass was the mode opted for fermenting pretreated material in another study (Itoh et al., 2003; Bak et al., 2010).

2.5.11 Yields and Delignification

Yields showed significant increase with biological pretreatment time due to decrease in lignin, increase in adsorption capacity of the pretreated material, and greater cellulose availability for sugar release (Deswal et al., 2014; X. Zhang et al., 2007). In contrast, another work reported the degradation of sugars with the biological pretreatment,

irreversible adsorption occurring on the biomass reduced along with decrease in the hydrolysis rate (J. Yu et al., 2009)**.** Diverse organisms released different amounts of cellulases and xylanases (Kuhar et al., 2008). Comparatively higher yields of 0.48g ethanol /g glucan were reported for the application of fungal pretreatment (Kuhar et al., 2008) whereas *P. striptis* showed lower ethanol yields (Nigam, 2001). Delignification under sterile and non-sterile pretreatment conditions suffered little delignification inhibition but more glucan was conserved for non-sterile conditions. Lower lignin content from operation at non-sterile conditions based on the incubation time selected for the study resulted in change of functional groups and favorable chemical decomposition (Lili Song et al., 2013). Ethanol yields were reported to be close for sterile and non-sterile conditions and were higher than from unpretreated biomass (L. Song et al., 2013).Yields were increased by applying fungal pretreatment to 57% as compared to just 11% of the raw biomass (Yamagishi et al., 2011). Maximum ethanol concentration of 2.4 g/l with a yield of 0.24 ethanol g /sugars after the detoxification of the biomass was observed (Pereira et al., 2012). During pretreatment, reportedly 12% of delignification occurred (Mao et al., 2013) whereas another study reported 71.49% lignin degradation after applying pretreatment on selected biomass but with high hemicellulose loss of 77.84% (F.-h. Sun et al., 2011). The overall sugar yields were even lower than the raw biomass after enzymatic hydrolysis in this work (Sun et al.,2011). Results from (Xu et al., 2009) also illustrated similar patterns as (Sun et al., 2011) since the most conveniently degrading component was hemicellulose during the overall process of pretreatment. A high lignin loss of 45.6±2% along with significant loss of 26.7±2% cellulose and

50±1.8% loss of hemicellulose over the entire pretreatment time showed a reduction in selectivity for lignin with increase in pretreatment time (H. Yu et al., 2010). Degradation of softwoods was more than 26% along with 15-19% hemicellulose degradation after 18 day biological pretreatment(Wan & Li, 2013). Glucose yields for different biomass were reported to be 2.5 to 3.0 fold higher than the raw biomass yields for selected fungi (Wan & Li, 2013). Enzymatic saccharification yields reported to be 3.5 fold more than unpretreated material (Yamagishi et al., 2011). 83%recovery of cellulose and 52% recovery of hemicellulose with about 41% Klason lignin degradation after pretreatment of rice straw showed some selective lignin degradation e is possible (Masayuki Taniguchi et al., 2005). Net sugar yields based on raw biomass were 33% for soluble sugar from holocellulose and 32% from glucose based on the raw biomass. Co-culture pretreatment technique showed degradation of all the carbohydrates with relatively lower lignin degradation in comparison to monocultures (Ma et al., 2011). 40.7% of initial lignin but no glucan degradation has been reported for pretreatment at semi aerobic conditions and produced 43.9% of theoretical ethanol yield at the end of 20d when all the steps were carried out biologically (Kamei et al., 2012). Lignin degradation in the range of 44-77% and little hemicellulose degradation occurred for chlorite pretreatment, but the cross effect was minimized for the dilute acid pretreatment (W. Wang et al., 2013) making it suitable in combination with fungal pretreatment. Enzymatic digestibility improved 2-3 fold in work testing fungal pretreated biomass for wet-storage (Cui et al., 2012). Maximum lignin removal of 64.25% was reported with application of additives and

submerged fermentation process for 28d (Zhang et al.,2012). Reid 1985 also reported 58% lignin removal and only 12% total wood weight reduction.

In a different study, two-step application of white rot-fungi followed by brown rot-fungi for biological delignification resulted in highest lignin removal and greater conversion by hydrolysis (Giles et al., 2011). Mixed cultures reported to be least effective with 37.68% delignification, although hemicellulose weight loss was similar for co-cultures and monocultures (Nazarpour et al., 2013). A 15-day fermentation resulted in 58.1% of theoretical glucose yields for the remaining glucan (Bak, Kim, Choi, & Kim, 2010). Results with straw showed weight loss of 20 to 45% after 84-d pretreatment. Combined pretreatment proved to be more effective than single pretreatment for 60-d period reducing the residence time and lowering carbohydrate losses (Bowen & Harper, 1990). Another result from combined pretreatment confirmed the results reported above**,** but the time was reduced from 60-d to 35-d for the same glucan yields as observed for a 60-d period (M. Taniguchi et al., 2010). Results from combined ethanolysis and fungal pretreatment gave 7-fold higher yield than just applying ethanolysis (Baba et al., 2011). Higher lignin than carbohydrates content was reported for a soft rot pretreated wood (Blanchette, 1995).

2.6 Conclusions

Feedstock, moisture level, pretreatment approach (single or combined), temperature, and pH are important factors influencing lignin degradation and successful pretreatment, as discussed in this chapter. Substrates have been an important factor since diverse composition of biomass influences microorganism selection for biological pretreatments.

Providing optimum temperature, pH, and other environmental conditions is important for the inoculum to grow and carry out biodegradation. Because microorganisms typically require several weeks to effectively degrade biomass, biological pretreatment may not be well suited for commercial operations. There are various challenges during biological pretreatment that need attention before this pretreatment could be applicable to wide variety of feedstocks. Therefore, selection of better fungal strains and understanding degradation of various wood components are needed to make biological pretreatment attractive. Improved understanding of the recalcitrant biomass would lead to developing pretreatment technologies favorable for feedstocks for providing high sugar titers for fermentations.

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Chapter 3

Maximum Yields from CELF or DA Pretreatment Combined with Enzymatic Hydrolysis

3.1 Abstract

The recalcitrance of lignocellulosic biomass poses a major obstacle to low cost conversion of lignocellulosic biomass to fuels. A pretreatment step is typically required to achieve hydrolysis of sugars from complex biomass structure. The emphasis of the present study is to determine conditions to realize maximum total sugar yields from dilute acid and THF assisted pretreatment. Analysis of solids produced by application of these two pretreatments to BESC standard *Populous* showed significantly different effects. Tetrahydrofuran co-solvent enhanced lignocellulosic fractionation (CELF) removed significant amounts of lignin and hemicellulose and achieved maximum sugar release by enzymatic hydrolysis. Furthermore, conditions to realize maximum total sugar yielding conditions for CELF pretreatment were less severe than those identified for dilute acid pretreatment. Close to 100 % of the glucan remained in solids from CELF pretreatment as compared to only 64% of the glucan in DA pretreated solids. Maximum sugar yielding conditions for CELF pretreatments at 150°C and 160°C resulted in 98-100% glucan release by enzyme loading of 100 mg/g glucan in raw biomass. Xylan removal by CELF pretreatment was somewhat more than for dilute acid pretreatment, but CELF removed 75- 85% of lignin vs only 3% removed by dilute acid (DA). Total glucan conversion reached 100% for CELF pretreatment within 5 days, while DA did not reach 100% yields even at 7 days of at the same enzyme loadings of 15 and 100 mg/g glucan in raw biomass. Even at low enzyme loadings of 5mg/g glucan in raw biomass, CELF reached glucan conversion close to 75% at the end of 21 days.

3.2 Introduction

The abundance and low cost of lignocellulosic biomass such as forest and agricultural residues, hardwoods or grasses makes it a viable resource for large-scale production of liquid transportation fuels (Charles E. Wyman, 2007). This inexpensive source would relieve pressure on oil prices while strengthening agricultural economies. Lower greenhouse gas (GHG) emissions are an added advantage of using cellulosic biomass (Charles E. Wyman, 1999). Biomass also has a broad spectrum of potential applications that range from fuels, chemicals and power production (Langholtz et al., 2016). Integrated cellulosic biorefineries that were constructed in 2013-2014 illustrate the U.S advancement to cellulosic ethanol that generates additional revenues for farmers. Almost 20,000 acres of land has been deployed for biomass crops since 2011 (Langholtz et al., 2016). Land used to grow energy crops such as hybrid poplar increased from 211 acres in August 2014 to 2,554 acres in November 2014 (Langholtz et al., 2016). Nevertheless, cost competitive sustainable energy fuel production from a variety of biomass is limited by the high cost of releasing sugars from their recalcitrant material (Davison, Parks, Davis, & Donohoe, 2013).

The structural carbohydrates cellulose and hemicellulose store the sun's energy for supporting plants during photosynthesis (Charles E Wyman & Wyman, 2013). They have crystalline regions due to strong hydrogen bonding among the long, linear chains of glucose sugar molecules called cellulose. Cellulose crystallinity makes biomass highly resistant to chemical and biological hydrolysis (Himmel et al., 2007). Hemicellulose is a major heterogeneous polymer with branched amorphous polymer chains in

lignocellulosic biomass and is easily hydrolysable into its monomer components such as sugars: xylose, mannose, galactose, glucose, and arabinose by acids (Fengel & Wegener, 1983; Taherzadeh & Karimi, 2007). Lignin is third major component in lignocellulosic material and is composed of phenylpropane units. Lignin plays a vital role in the growth and development of plants by strengthening the fibrous tissues. Lignin hinders the breakdown of polysaccharides during enzymatic hydrolysis of woods and grasses (Grabber, 2005). Further, hemicellulose-lignin limits access to cellulose. Therefore, a pretreatment step is needed to open up the biomass for enzyme action. Lignin and hemicellulose separation during pretreatment influences enzyme action during hydrolysis favorable for reduced enzyme cost (Argyros et al., 2011). Numerous aqueous pretreatment technologies have been applied over the past decades to disrupt the native cell wall structure and expose cellulose (Heitz et al., 1991). Solvent pretreatments apply various reaction temperatures, times and solvents to remove hemicellulose and lignin and achieve higher yields from biological or thermochemical processing (Charles E. Wyman, 2013).

3.3 Material and Methods

The National Renewable Energy Laboratory (NREL), Golden, Colorado, graciously provided BESC (Bioenergy Science Center, Oak Ridge National Laboratory, Oak Ridge TN) 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) at the University of California at Riverside. The moisture content of BESC STD was 7% as 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.

Compositional analysis of poplar wood was performed according to established NREL procedures (version 8-03-2012) in triplicate (Hames et al., 2008; A Sluiter et al., 2006). The resulting mass composition was 47.1% glucan,16.6% xylan, 19.1% K-lignin, and 18.0% other materials. These other materials are usually composed of ash (4–6 wt %), proteins (2–3 wt %), acetic acid (2–4 wt %), sugar acids (1–2 wt %), and extractives (2–8 wt %), but they were not quantified in this study. The reaction conditions for batch pretreatments to confirm the maximum sugar yielding conditions are summarized in Table 1. below. Batch pretreatments were carried out at a 10% 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 800 g. A solid loading of 10% was applied in laboratory studies because of the high amount of biomass needed for enzymatic hydrolysis at various conditions.

Co-solvent enhanced lignocellulosic fractionation (CELF) pretreatments were carried out with 1:1 volume of THF/H_2O solvent in the presence of 0.5 wt% dilute acid as a catalyst (Cai, Zhang, Kumar, & Wyman, 2013) to define conditions to achieve the highest total glucose+xylose yields for CELF and enzymatic hydrolysis. Dilute acid (DA) pretreatments were carried out with 0.5 wt% of sulfuric acid based on liquid mass with biomass moisture content included in the liquid mass (Bin Yang & Wyman, 2008). Milled biomass 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 by a K-type thermocouple (Omega Engineering Co., Stamford, CT) inserted in the reactor and maintained the targeted value by adjusting the level of the reactor vessel in contact with sand.

Table 3.1 Summary of Pretreatment conditions applied.

Pretreatment	Temperature (°C)	Time (min)	Catalyst
CELF	150	15	0.5 wt% H_2SO_4
		25	
		35	
	160	10	
		15	
		25	
DA	160	25	

Time zero for pretreatment was defined as when the temperature had recorded within 2 °C of the required pretreatment temperature. The variation 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. 2 - 3 min were required to heat-up the batch reactors to 150°C and 160 °C, respectively, and about the same time to cool them down to 40°C after reaction at the desired pretreatment time was over.

Following pretreatment, the liquid was removed from the solids by filtration through a Whatman® glass microfiber filter, and the solids were thoroughly washed with deionized water until neutral pH was achieved so as to be sure that no acid, soluble sugars, or other solubilized products were left in the pretreated solids. For compositional analysis, the wet solids were dried at 37°C for several days until the moisture content dropped to about 4–6%. With moisture content 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" (A Sluiter et al., 2006). All sugar analyses were conducted 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. Avicel control and washed pretreated solids were enzymatically hydrolyzed for 168 h with 5, 15 and100 mg of Accellerase® 1500 cellulase protein/g glucan in the unpretreated biomass. Enzymatic hydrolysis of never-dried solids was performed according to the standard NREL procedure, "Enzymatic Saccharification of Lignocellulosic Biomass" (M Selig, N Weiss, & Y Ji, 2008). Accellerase® 1500 enzyme with a 82 mg/mL protein content as determined by the standard BCA method (P K Smith et al., 1985) was generously provided by DuPont Industrial Biosciences, Palo Alto, CA. Statistical analyses and data plots were carried out using OriginPro® v. 2015 (OriginLab Corp., Northampton, MA) Statistics and Graphing software.

3.3.1 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 (e.g 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.

3.4 Results and Discussion

3.4.1 Conditions to Maximize Sugar Yields from CELF and DA Poplar

The emphasis of the present study is to define the CELF and DA pretreatment conditions to obtain maximum total sugar yields from the combined pretreatment and subsequent enzymatic hydrolysis. Thus, this study tracked glucose and xylose recovered after pretreatment (Stage 1) combined with those from subsequent enzymatic hydrolysis (Stage 2) as these are the major sugars in poplar wood. Dilute sulfuric acid (0.5 wt%) used for CELF and DA pretreatments achieved high reaction rates and xylose yields while maintaining high glucose yields from cellulose hydrolysis (P. Kumar, Barrett, Delwiche, & Stroeve, 2009).

Addition of tetrahydrofuran (THF) for CELF to an equivolume mixture of THF/water (1:1) achieved high lignin removal at all the pretreatment temperatures and time in the study (Cai et al., 2013).

CELF pretreatments were conducted at temperatures of 150°C or 160°C followed by enzymatic hydrolysis over a range of enzyme loadings. The temperatures and THF to water ratio were chosen based on previous work at a lowest favorable temperature of 150°C (Mostofian et al., 2016). THF/water volume ratio greater than 1:1 show higher biomass solubilization, but generation of undesirable degradation products makes it unattractive. All the pretreatment reactions were conducted with minimal heat up (<3 min) and cooling time (<2 min) to keep the reaction kinetics intact throughout the study. Monomeric sugar yield from enzymatic hydrolysis (stage 2) were determined at enzyme loading of 100mg, 15mg, and 5mg/g glucan performed using Accellerase 1500 enzyme cocktail. All the enzyme loadings calculations presented in the study were based on the glucan content measured during the composition analysis of raw biomass (before pretreatment). Using high glucan loading of 100mg/g glucan in raw biomass ensured CELF or DA pretreated biomass was completely accessible during enzymatic hydrolysis. Low enzyme loadings of 5 and 15mg/g glucan in raw biomass applied in the study to reach the maximum combined sugar yields at affordable enzyme loadings.

3.4.2 Glucose Yields for CELF Vs DA Pretreatment

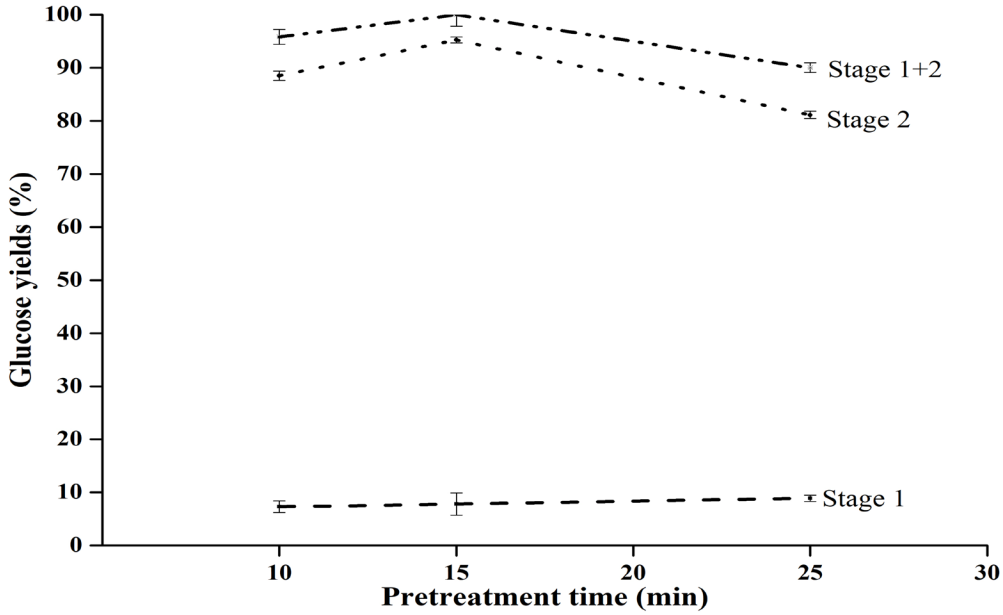
The total glucose yields from Stage 1 (Fig.3.1A) and Stage 2 (Fig.3.1B) at 5 or 15 mg enzyme loading were 100% for CELF pretreated solids at 160°C and 15min. These yields

decreased to 96% on further increasing the pretreatment time to 25 min, indicating some degradation with increasing time at temperature of 160°C. For CELF pretreated poplar at 150°C, maximum total glucose yields were achieved at 25 min reaction time. However, CELF pretreatment showed high glucose release from CELF pretreated solids Stage 2 at both temperatures (150°C or 160°C) over a range of pretreatment times. Because, CELF pretreatment had negligible degradation of monomeric sugars it can be a viable pretreatment option for enzymatic hydrolysis over a range of enzyme loadings. Total glucose yields at 150°C were higher at 15mg/g glucan in raw biomass enzyme loading than at 5 mg enzyme loading and the maximum total glucose was released at 150°C for 25min. Most of the glucose was released during Stage 2 for CELF pretreated poplar wood. This trend is visible at both enzyme loadings after 7 days of enzymatic hydrolysis for CELF pretreated BESC standard poplar wood.

Further, total glucose yields dropped substantially when DA-pretreated biomass was enzymatically hydrolyzed with 15mg enzyme loadings resulting in low combined glucose yields from DA pretreatments (J.-W. Lee & Jeffries, 2011; T. Zhang, Kumar, & Wyman, 2013). The maximum sugar yielding conditions for DA were determined to be 160°C for 25 min with 0.5 wt% sulfuric acid based on literature results for poplar wood and other pretreatments combined with some of initial experiments. DA pretreatment successfully released the monomeric sugars from hemicellulose in poplar wood in Stage 1, but most of the glucose was released in the presence of enzymes in Stage 2. DA pretreatment could only reach 91% glucose yield from Stage 1 and Stage 2 at 100mg/g glucan enzyme

loading and these yields showed a downward trend when the enzyme loading was lowered from 100 to 15 or 5mg/g.

A)


Glucose yields (%)
Pretreatment time (min)
Stage 1+2
Stage 2
Stage 1

B)

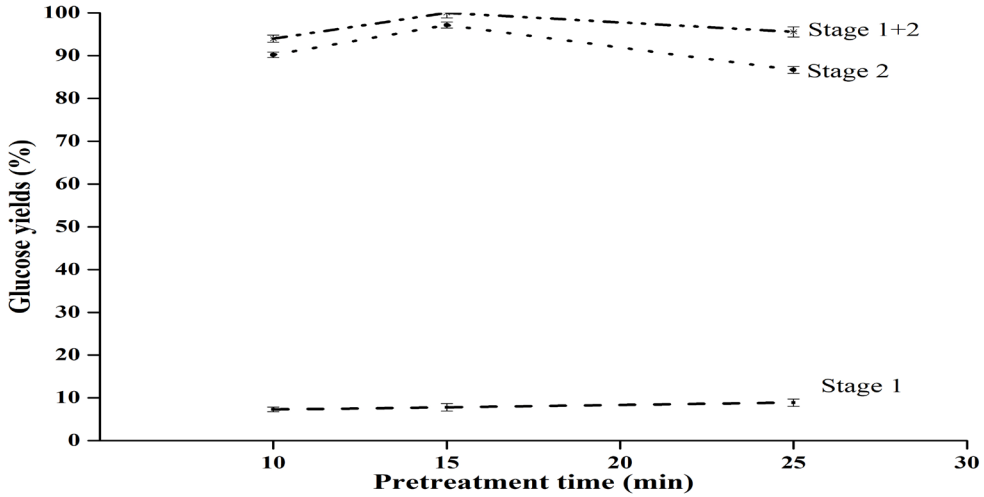
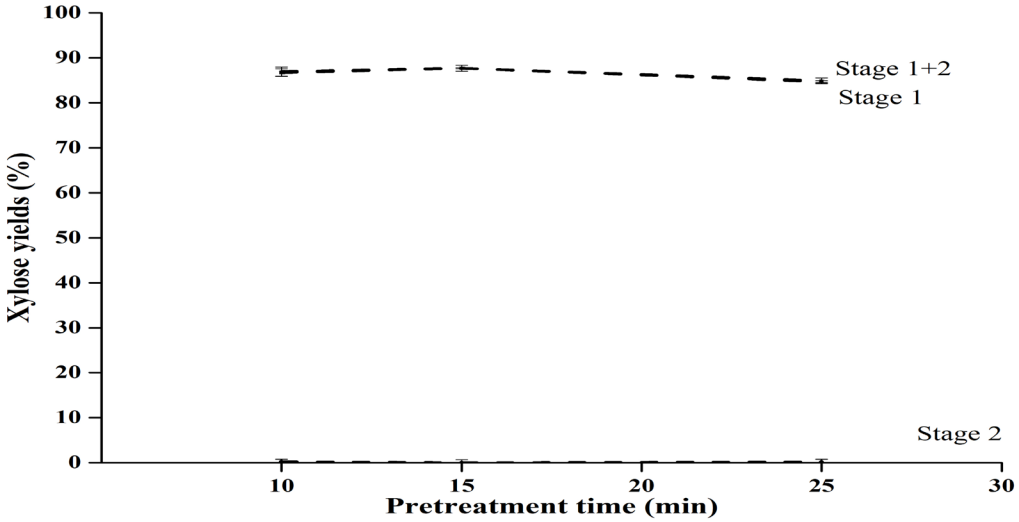

Glucose yields (%)
Pretreatment time (min)
Stage 1+2
Stage 2
Stage 1

Figure 3.1 Glucose yields over a range of pretreatment times for CELF pretreated BESC standard poplar from pretreatment (Stage 1) and enzymatic hydrolysis (Stage 2) at enzyme loadings of: A) 5mg/g glucan in raw biomass, B) 15mg/g glucan in raw biomass. Reaction conditions: 10 wt % poplar wood, 160°C,0.5% H2SO4, and 1:1 THF/water volume ratio. Stage 2 performed for 7 days using Accellerase 1500 enzyme based on glucan in raw poplar wood. Error bars in the graph are standard deviations from three replicate flasks.

3.4.3 Xylose yields for CELF VS DA pretreatment

The total xylose yields achieved from Stage 1 (Fig.3.2A) did not change over the range of pretreatment time conditions applied as shown below. Most of the xylose was released during Stage 1, and the remaining xylose in pretreated solids was released during Stage 2 (Fig.3.2B) during subsequent enzymatic hydrolysis. The maximum total xylose yield from Stage 1 and Stage 2 was constant as most xylose was released during Stage 1 for CELF and DA pretreatments. It is interesting to observe that the maximum glucose yielding conditions coincide with the highest total xylose yielding conditions at 150°C 25min and 160°C 15min as 87% and 88% respectively. Thus, trends for total xylose yields from Stage 1 and Stage 2 at 5 or 15 mg/g enzyme loading at 160°C heavily influenced to identify the maximum sugar yielding conditions. Results for total xylose yields at all the enzyme loadings reported in Figure 3.2. The DA pretreatment shows the maximum xylose recovery during stage1 as 82% lower than reported at the maximum sugar yielding conditions for CELF pretreated material. DA pretreatment conditions are 160°C-25 min based on literature review, which is comparatively higher than identified for CELF pretreatments as 150°C for 25min or 160°C for 15min.

A)



B)

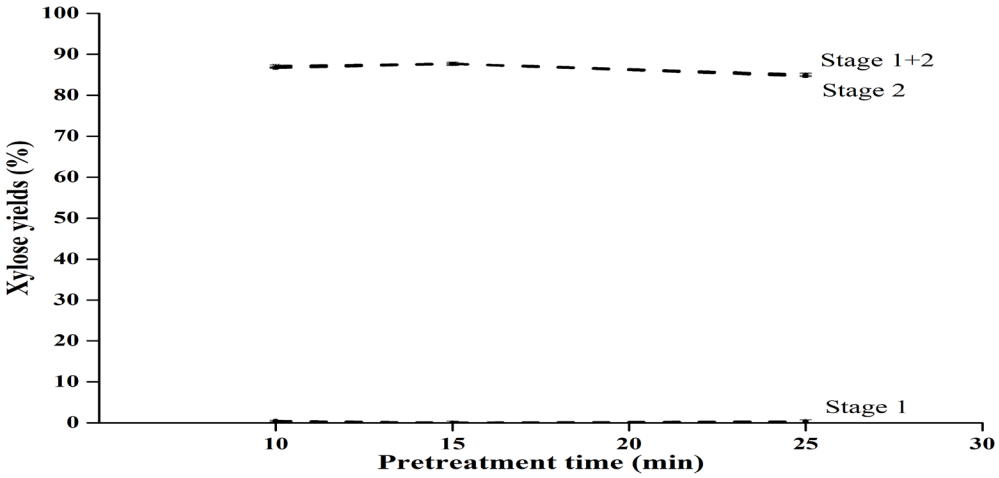
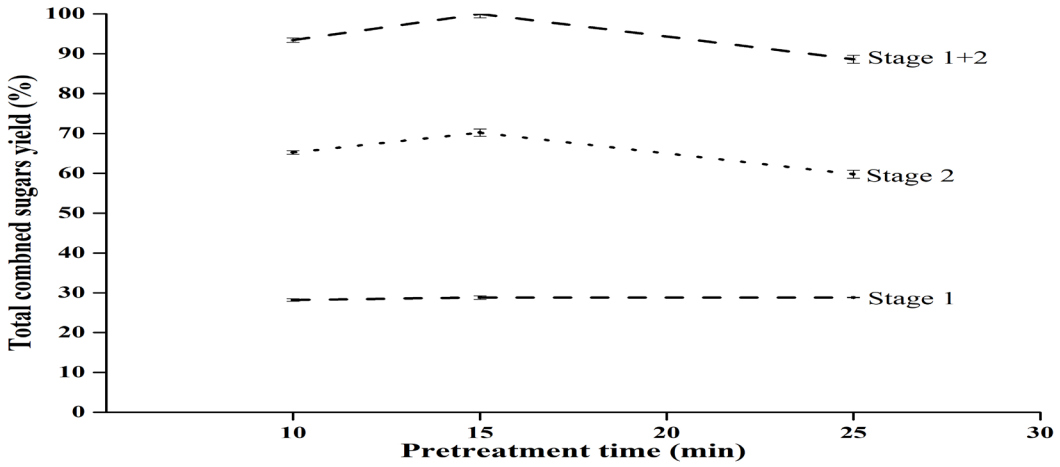


Figure 3.2 Total xylose yields for CELF pretreated BESC standard poplar from pretreatment (Stage 1) and enzymatic hydrolysis (Stage 2) combined: A) 5mg/g glucan in raw biomass, B) 15mg/g glucan in raw biomass for CELF. Reaction conditions: 10 wt% poplar wood, 160°C,0.5% H2SO4, and 1:1 THF/water volume ratio. Stage 2 results are for 7 days of hydrolysis Accellerase 1500 at enzyme loading based on glucan in raw poplar. Error bars in the graph are standard deviations from three replicate flasks.

3.4.4 Total Combined Sugar Yields

CELF pretreated solids followed by subsequent enzymatic hydrolysis at 15mg/g enzyme loading achieved 99% glucose + xylose yields at 150°C and reaction time 25 min whereas CELF reaction at 160°C for 15 min resulted in 100% yields. These results indicate that CELF at 150°C and 160°C are close at low enzyme loading of 15mg/g. Figure 3.3 shows the total combined glucose + xylose yields from Stage (1+2) at both the enzyme loadings of 5 and 15 mg/g glucan in raw at 160°C for CELF pretreatments. Solids produced after pretreatments at 150°C and 160°C were subjected to very high enzyme loading of 100 mg/g glucan in raw biomass to ensure that enzymes were able to access all the polysaccharides present in the pretreated material (Lloyd & Wyman, 2005). Results show close to 100% total sugar release from Stage (1+2) for most conditions in less than 7 days for results collected at the end of 7 days to keep data consistent with yields at other enzyme loadings for CELF pretreated solids.

A)


Total combned sugars yield (%)
100
90
80
70
60
50
40
30
20
10
0
10
15
20
25
30
Pretreatment time (min)
Stage 1+2
Stage 2
Stage 1

B)

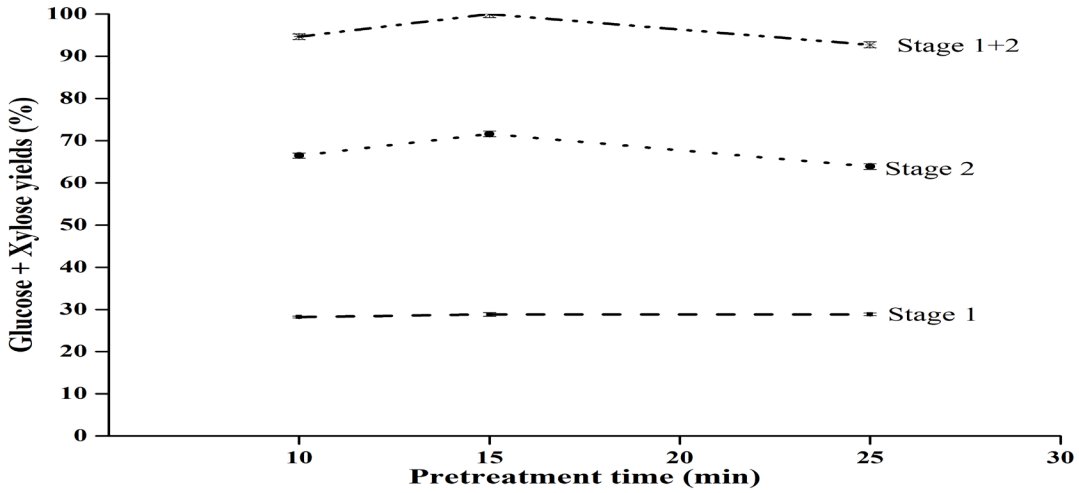

Glucose + Xylose yields (%)
100
90
80
70
60
50
40
30
20
10
0
10
15
20
25
30
Pretreatment time (min)
Stage 1+2
Stage 2
Stage 1

Figure 3.3 Glucose+xylose yields vs pretreatment times for CELF pretreated BESC standard poplar wood based on combined yields from pretreatment (Stage 1) and enzymatic hydrolysis (Stage 2) for 7 days: A) 5mg/g glucan in raw biomass, B) 15mg/g glucan in raw biomass. CELF Reaction conditions: 10 wt % poplar wood, 160°C,0.5% H_2SO_4, and 1:1 THF/water volume ratio. Stage 2 performed using Accellerase 1500 enzyme loading based on glucan in raw poplar. Error bars in the graph are standard deviations from three replicate flasks.

3.4.5 Hydrolysis Yields VS Lignin Removal for CELF Pretreated Poplar

Table 3.2 below shows the effect of lignin removal on the hydrolysis yields obtained during CELF and DA pretreatments. Overall results show increased pretreatment time and temperature increased the lignin removal. Higher pretreatment times evaluated 150°C and 160°C for CELF pretreatment removed more lignin achieved the higher hydrolysis yields at most conditions. However, a downward trend in yields was observed for CELF after 150°C reaction time of 25 min and 160°C with reaction time higher than 15 min because the fermentable sugars degraded; however, the degradation may not be as high as observed for other solvent pretreatments. Hydrolysis yields from enzymatic hydrolysis of CELF pretreated poplar solids are very high in comparison to DA pretreated solids. CELF pretreatment of poplar at 160°C for 15min had lignin removal above 90% whereas the lignin removal was lower for CELF at 150°C for 25min. However, hydrolysis yields were still higher for CELF pretreated material in comparison to those observed for dilute acid pretreated poplar. It is known that solublization followed by precipitation of solubilized lignin are more pronounced during stronger acid pretreatments, decreasing yields for DA can be accounted for by the condensation and precipitation of solubilized lignin components (Hendriks & Zeeman, 2009). It can be observed here that using lower enzyme loadings did not influence yields in case of CELF but did for DA pretreatment, as the hydrolysis yields remain high. Therefore, lowering the enzyme loading is one of the objectives of this study so as to have a low cost of production for fermentable sugars during the overall process of ethanol production. While hydrolysis yields for DA pretreatment at160°C for a reaction time of 25 min removed a high portion of the lignin,

hydrolysis yields were limited to 40 % at 5 mg/g glucan in raw biomass enzyme loading and achieving only 55% for an enzyme loading of 15mg/g glucan in raw biomass. On the other hand, CELF results for poplar at 160°C reaction time of 15min achieved high hydrolysis yields close to 95% for 5mg enzyme loading and 97% for 15mg enzyme loading based on glucan in raw biomass. These results strengthen the case greater lignin removal does increase hydrolysis of pretreated biomass consistent with various literature articles. However, this study reinforces the trends for lower enzyme loadings (Yoshida et al., 2008). Increased hydrolysis of biomass with higher lignin removal is hypothesized to result from more sites being accessible to enzymes for hydrolysis of polysaccharides (Mooney, Mansfield, Touhy, & Saddler, 1998; Mussatto, Fernandes, Milagres, & Roberto, 2008; Z. Yu, Jameel, Chang, & Park, 2011).

Table 3.2 Glucan yields from enzymatic hydrolysis (Stage 2) of poplar at 5and 15 mg/g glucan in raw biomass enzyme loading after 7 days and lignin removal for CELF and DA pretreated poplar

Pretreatment condition	Lignin content (%)	Lignin removal (%)	Xylose (%)	Xylan removal (%)	Glucan yield (5mg EL) (%)	Glucan yield (15mg EL) (%)
CELF-150°C-25 min	13.90	63.77	3.19	89.24	79.22	91.20
CELF- 160°C-15 min	4.05	90.27	3.30	89.92	95.26	97.15
DA-160°C-25 min	28.10	10.15	1.80	92.68	40.03	55.00

3.5 Conclusions

Total sugar yields were achieved with high lignin removal for CELF pretreated poplar at 160°C reaction time 15min and 160°C and reaction time of 25 min for DA pretreatments. At moderate pretreatment conditions, complete deconstruction of sugars by enzymes was possible by CELF pretreatment at 150°C and 160°C followed by application enzyme loadings as low as 5mg/g glucan in raw biomass. CELF pretreatment realized higher sugar yields from pretreatment and subsequent enzymatic hydrolysis compared to DA pretreatment at otherwise identical conditions. It appears that the catalytic effect of dilute acid is much more favorable in combination with THF used for CELF pretreatment than pretreatment by dilute acid alone. Enzymatic hydrolysis improved sugar yields were greater from solids produced by CELF pretreatment than those from DA apparently due to lower lignin content in CELF than DA solids. Overall, CELF is very effective in

enhancing sugar yields at lower enzyme loadings apparently by reducing the non-productive binding of the enzymes during hydrolysis to lignin.

3.6 Acknowledgments

We are grateful for funding by the Office of Biological and Environmental Research in the Department of Energy (DOE) Office of Science through the BioEnergy Science Center (BESC) at Oak Ridge National Laboratory (Contract DE-PS02-06ER64304). We also, acknowledge the Center for Environmental Research and Technology (CE-CERT) of the Bourns College of Engineering for providing the facilities and the Ford Motor Company for funding the Chair in Environmental Engineering that facilitates projects such as this one.

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Chapter 4

Maximum Total Sugar Yields from Ethanol Organosolv and CELF Pretreatments Combined with Subsequent Enzymatic Hydrolysis

4.1 Abstract

Lignocellulosic biomass consists of strongly interlinked components cellulose, hemicellulose and lignin as well as other compounds generally present in lower amounts. Pretreatment is an important step to alter these structural components from lignocellulosic biomass structure for their higher accessibility for enzymatic hydrolysis. Various pretreatments can disrupt these complex lignocellulosic structures. In this chapter, combined sugar yields from pretreatment (Stage 1) coupled with subsequent enzymatic hydrolysis (Stage 2) at the end of 7 days were used to identify the maximum total glucose plus xylose yields from ethanol organosolv pretreated poplar and THF co-solvent enhanced lignocellulosic fractionated (CELF) pretreatment. Ethanol organosolv pretreatment resulted in maximum combined total sugar yields of 78.2% when pretreatment was operated at 185°C for 15min while CELF pretreatment at 160°C-15min achieved 100% yields for the same enzyme loading of 15mg/g glucan in raw biomass enzyme loading used for hydrolysis. Ethanol organosolv pretreatment removed less than 85% of the lignin compared to CELF removing over 90%.

4.2 Introduction

Depleting fossil fuels with increased global energy demands and need to lower greenhouse gas emissions point to the need for alternative energy sources. The abundance of lignocellulosic biomass such as grasses, wood, or agricultural residues without interference with the food production makes them sustainable for biofuels production (Teramura et al., 2016). Higher yields during enzymatic hydrolysis are required for

biological ethanol production to be economical, but pretreatment needs to make crystalline cellulose available to enzymes during hydrolysis (P. Alvira, E. Tomás-Pejó, M. Ballesteros, & M. J. Negro, 2010; Mosier et al., 2005a). Hence, pretreatment is an essential step. Various pretreatment methods that have been applied to increase the digestibility of biomass can be grouped as physical, chemical, biological or their combination (X. Zhao, Li, Wu, & Liu, 2017). Aqueous pretreatments such as methanol organosolv dilute acid require high temperatures and times that degrade sugars. This study compares experimentally determined total combined sugar yields from organosolv pretreatments to those of CELF pretreatment of poplar wood.

4.3 Material and Methods

4.3.1 Materials

The National Renewable Energy Laboratory (NREL, Golden, CO) kindly provided air-dried *Populus trichocarpa*. The poplar was knife milled to pass through a 1mm particle size interior sieve using a laboratory mill (model 4, Arthur H. Thomas Company, Philadelphia, PA). The moisture content of the biomass after milling was between 4-6 % as determined by a halogen moisture analyzer (HB43-S; Mettler Toledo, Columbus, OH). Moisture content was used for calculation of sugar yields on a dry biomass basis. The composition analysis was performed according to the NREL procedures (version 08-03-2012) in triplicate. Enzyme cocktail Accelerase 1500® was generously provided by DuPont industrial Biosciences (Palo Alto, CA).

4.3.2 Composition Analysis

Compositional analysis of BESC STD poplar was performed according to the established NREL procedure (version 8-03-2012) in triplicate. The resulting mass composition was 47.1% glucan, 16.6 %xylan, 19.1 % K-lignin, and 18.0% other materials. Other materials are usually composed of ash, proteins, acetic acid (2–4 wt %), sugar acids, and extractives not quantified in this study.

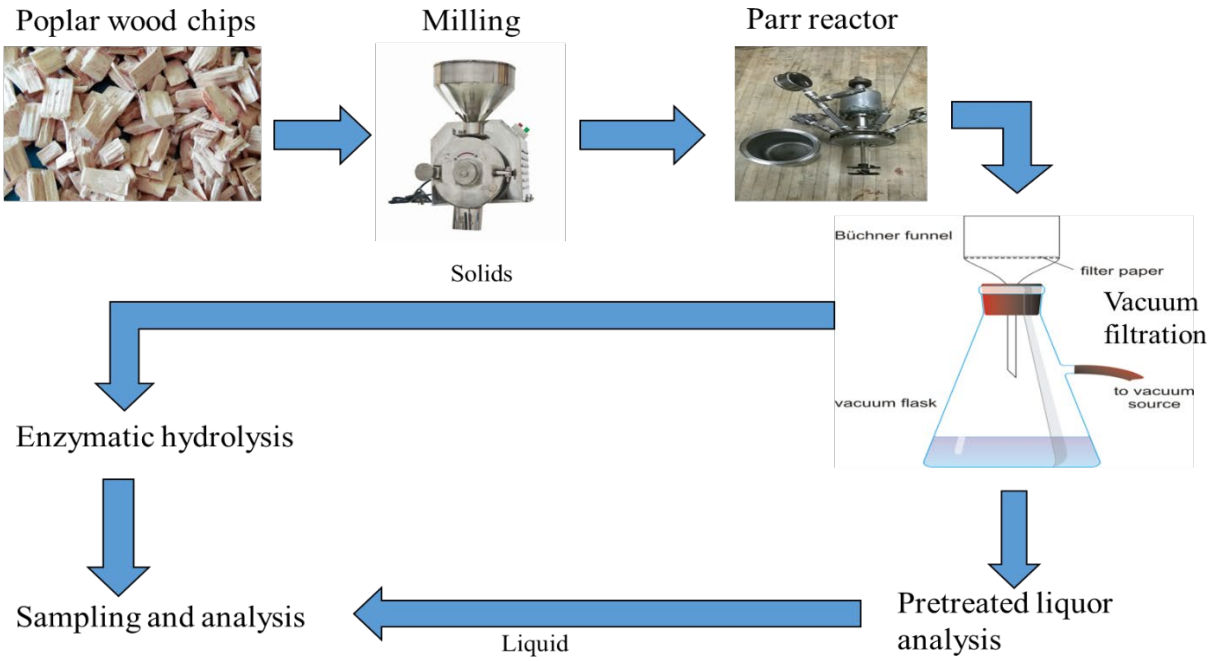


Figure 4.1 Process flow diagram for pretreatment and enzymatic hydrolysis of lignocellulosic biomass.

4.3.3 Analytical Procedures

All chemical analyses were based on Laboratory Analytical Procedures (LAPs) documented by NREL (A. Sluiter & J. Sluiter, 2005). Liquid samples and appropriate

calibration standards were analyzed by HPLC (Waters Alliance 2695 system equipped with a Bio-Rad Aminex HPX-87H column and Waters 2414 refractive index (RI) detector) with an eluent (5 mM sulfuric acid) flow rate of 0.6 mL min^{-1}. The chromatograms were integrated by Empower 2 software (Waters Co., Milford, MA). Combined total sugar yields from each pretreatment alone were determined as the sum of the total mass of soluble glucose and xylose released by pretreatment (Stage 1) plus the total mass of these two sugars released by saccharification of the washed pretreated solids with enzymes (Stage 2). Details of the calculation of sugar yields are outlined in calculation section. The total lignin recovered was calculated from the mass of total unwashed K-lignin precipitated upon recovery of ethanol or THF, whereas delignification was calculated from the percentage of K-lignin that remained in the pretreated material compared to the initial K-lignin content of the raw BESC STD poplar.

4.3.4 Pretreatment of BESC STD Poplar

Pretreatment reactions were performed in a 1 L Hastelloy Parr autoclave reactor (236HC Series, Parr Instruments Co., Moline, IL) equipped with a double stacked pitch blade impeller rotated at 200 rpm. The ethanol organosolv (EO) pretreatment used a 1:1 volume ratio of ethanol (>99 % purity, Fisher Scientific, Pittsburgh, PA) to water. 0.5 wt % sulfuric acid (Ricca Chemical Company, Arlington, TX) concentration was determined elsewhere to achieve the highest total glucose + xylose yields from DA alone or CELF pretreatment coupled with subsequent enzymatic hydrolysis (T. Zhang et al., 2013). The THF co-solvent for CELF pretreatment used a 1:1 volume ratio of THF (>99 % purity,

Fisher Scientific, Pittsburgh, PA) and water. A 0.5 wt % sulfuric acid (Ricca Chemical Company, Arlington, TX) concentration was found to achieve the highest total glucose + xylose yields from EO alone or CELF pretreatment coupled with subsequent enzymatic hydrolysis. These pretreatment conditions were applied to obtain Stage 1 yields. Before each reaction, BESC STD poplar was soaked overnight at 4 °C. BESC STD poplar solid loadings were 10 wt % for both EO and CELF pretreatments based on the total working mass of liquids and solids in the reaction. All reactions were maintained at temperature (±2 °C) by convective heating by a 4 kW fluidized sand bath (Model SBL-2D, Techne, Princeton, NJ), and the reactor temperature was measured directly by using an in-line thermocouple (Omega, K-type). The sand bath temperature was set to 340 °C to reduce the heat-up time to under 3 min. At the conclusion of each reaction, the reactor was cooled by submerging quickly it in a large room temperature water bath at RT. The solids were then separated from the reaction liquor by vacuum filtration at RT through glass fiber filter paper (Fisher Scientific, Pittsburgh, PA). The mass and density of the liquid fractions were measured to complete accurate yield calculations. As a result final densities were determined by weighing 10 mL of the reacted liquid in a teared volumetric flask after each reaction. Liquid samples were analyzed by HPLC as described in the analytical procedures section. Pretreatment conditions for ethanol organosolv are summarized in Table 4.1 below.

Table 4.1 Ethanol organosolv pretreatment conditions applied to BESC STD poplar.

Pretreatment	Catalyst/Solvent ratio	Temperature	Time
Ethanol organosolv	(1:1 ethanol/water), H2SO4 (0.5 wt%)	185°C	7
			15
			25
			40
			60
	(1:1 ethanol/water), H2SO4 (0.5 wt%)	160°C	7
			15
			25
			40
			60

4.3.5 Enzymatic Hydrolysis of Pretreated BESC STD Poplar

As noted in the NREL standard protocol, enzymatic hydrolysis of pretreated materials was performed in triplicate in 125 mL Erlenmeyer flasks with a 50 g total working mass that contained 50 mM citrate buffer (pH 4.8) to maintain pH, 0.02 % sodium azide to prevent microbial growth, and approximately 1 wt % glucan from pretreated solids or Avicel PH-101 cellulose (Sigma Aldrich, St. Louis, MO) (Resch, Baker, Decker, & National Renewable Energy, 2015). The cellulase enzyme (Accellerase1500, DuPont Industrial Biosciences, Palo Alto, CA) loadings were 15 and 100 mg/g glucan in raw biomass. So a pretreatment is not biased for releasing sugars before enzymatic hydrolysis. Maximum total combined sugar yields obtained from both Stage 1 (pretreatment) and Stage 2 (enzymatic hydrolysis) were used to determine the maximum sugar yield conditions for pretreatment combined with enzymatic hydrolysis. The flasks containing

biomass slurry were placed in a Multitron orbital shaker (Infors HT, Laurel, MD) set at 150 rpm and 50 °C. Samples of approximately 1 mL were taken periodically into 2 mL centrifuge vials (Fisher Scientific, Pittsburgh, PA) from each flask and centrifuged at 15000 rpm for 5 min to determine yields during enzymatic hydrolysis. The supernatant was then transferred into 500 µL HPLC vials (Grace Davison, Deerfield, IL) for HPLC analysis. Three replicates were run for each sample for all standard biomass procedures.

4.3.6 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 compensate for the mass of water added to each sugar during hydrolysis. Enzymatic hydrolysis loading was based on mg of enzyme per gram glucan in the unpretreated biomass to allow fair comparison as mentioned in the previous section. Mass units were in grams, volumes in liters, and concentrations in grams per liter.

Mass of polymeric sugar in solids before pretreatment

= Dry weight of biomass in pretreatment reactor * fraction of polymeric sugars calculated in unpretreated biomass

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

3. Solid yield % = Wet solids recovered from pretreatment ∗ (1 -moisture content) ∗100

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

6. Stage1 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. Enzyme loading = $\frac{\text{mg of protein per gram glucan in enzymatic hydrolysis flask}}{\text{glucan yield fraction after pretreatment}}$

8. Enzymatic saccharification efficiency % = $\frac{\text{Concentration of monomeric sugar from HPLC} * \text{anhydrous correction factor} * \text{total reaction volume of enzymatic hydrolysis flask} * 100}{\text{mass of glucan or xylan in enzymatic hydrolysis flask}}$

9. Stage 2 sugar yield = Enzymatic saccharification efficiency (%) *

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

10. Total Stage 1+2 glucan or xylan yield = Stage 1 yield% + Stage 2 yield%

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

4.4 Results and Discussion

4.4.1 Composition of EO Pretreated BESC STD Poplar

Focus of this study was to track the maximum glucan and xylan from pretreatment and enzymatic hydrolysis steps combined. The change in composition of EO pretreated BESC STD poplar at 185°C is shown in Figure 4.2.

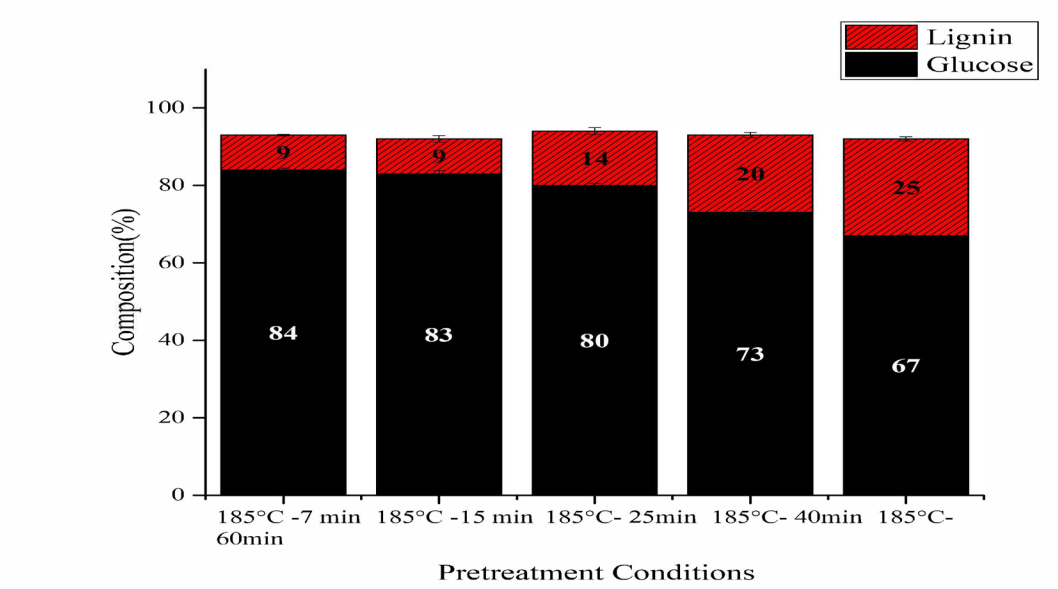


Figure 4.2 Composition of solids recovered from ethanol organosolv pretreated BESC STD poplar at 185°C. Error bars in the graph are standard deviations from three replicate flasks.

The amount of solids recovered dropped with increase in pretreatment time also seen prior studies (Pan et al., 2005). Drop in glucose in ethanol organosolv pretreated BESC STD poplar is visible in the Figure 4.2 above. This drop in glucose composition of solids after EO pretreatment can be explained by generation of glucose degradation products with increase in pretreatment time. The lignin content drops initially at 185°C for 7 min until 40 min after which we do see the pseudo lignin increase at 185°C for 60 min. There was no xylose found in solids after EO organosolv pretreatment. The temperature and time combinations chosen for the EO pretreatment were those that achieved the complete removal of xylose from the solids. Lignin removal demonstrated below in Figure 4.3 drops with increase in pretreatment time however, lignin removal increased at 185°C for 15 min after which it dropped. The maximum lignin removal occurred at 185°C reaction

time of 15 min. Because lignin hinders the hydrolysis, it is desirable to reduce lignin in biomass to enable enzymatic attack for the release of fermentable sugars.

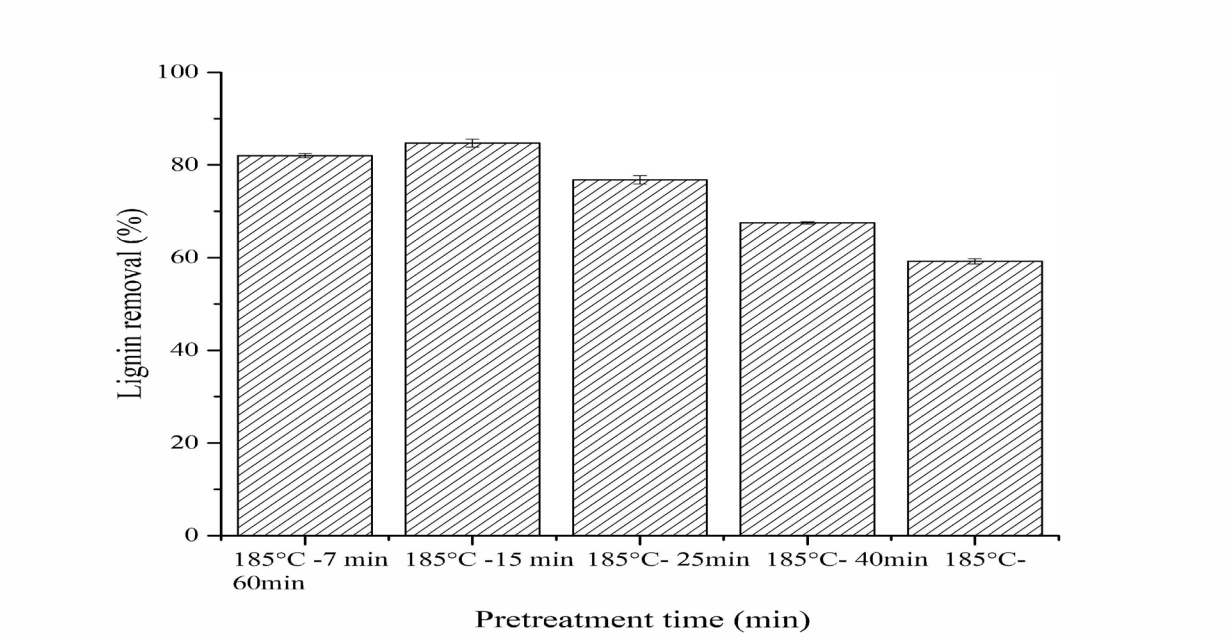


Figure 4.3 Total lignin removal during ethanol organosolv pretreatment of BESC STD poplar at selected pretreatment conditions at 185°C. Error bars in the graph are standard deviations from three replicate flasks.

4.4.2 Mass Balance for Glucan and Xylan

Figure 4.4 shows total mass balance of glucan and xylan recovered in the solids and liquids with change in the pretreatment times. Glucan in solution increased from 8g recovered in pretreated liquid to 16 g over times changing from 7 min to 60 min for ethanol organosolv at 185°C. At this pretreatment temperature, the amount of glucan in solids dropped with the increase in pretreatment time from 7 min to 60 min. With increase in pretreatment time glucan and xylan degradation to 5-HMF, furfurals or

levulinic acid, acetic acid and formic acid as well as humins occurs (Yan, Zhang, & Yang, 2014). The increase in degradation products with the increase in pretreatment time leading to lower total mass recovery reduces our interest in higher pretreatment times. During ethanol organosolv pretreatment time of 7 min the mass recovery was 100% but with lower xylan recovery from the solids lead to peruse further pretreatment conditions. The maximum xylan recovery occurred at 185°C- 15 min. The change in pretreatment time also increased in the production of degradation products. Hence, enzymatic hydrolysis was not applied on biomass pretreated beyond 25 min. Results from enzymatic hydrolysis at various enzyme loadings are reported in the next section.

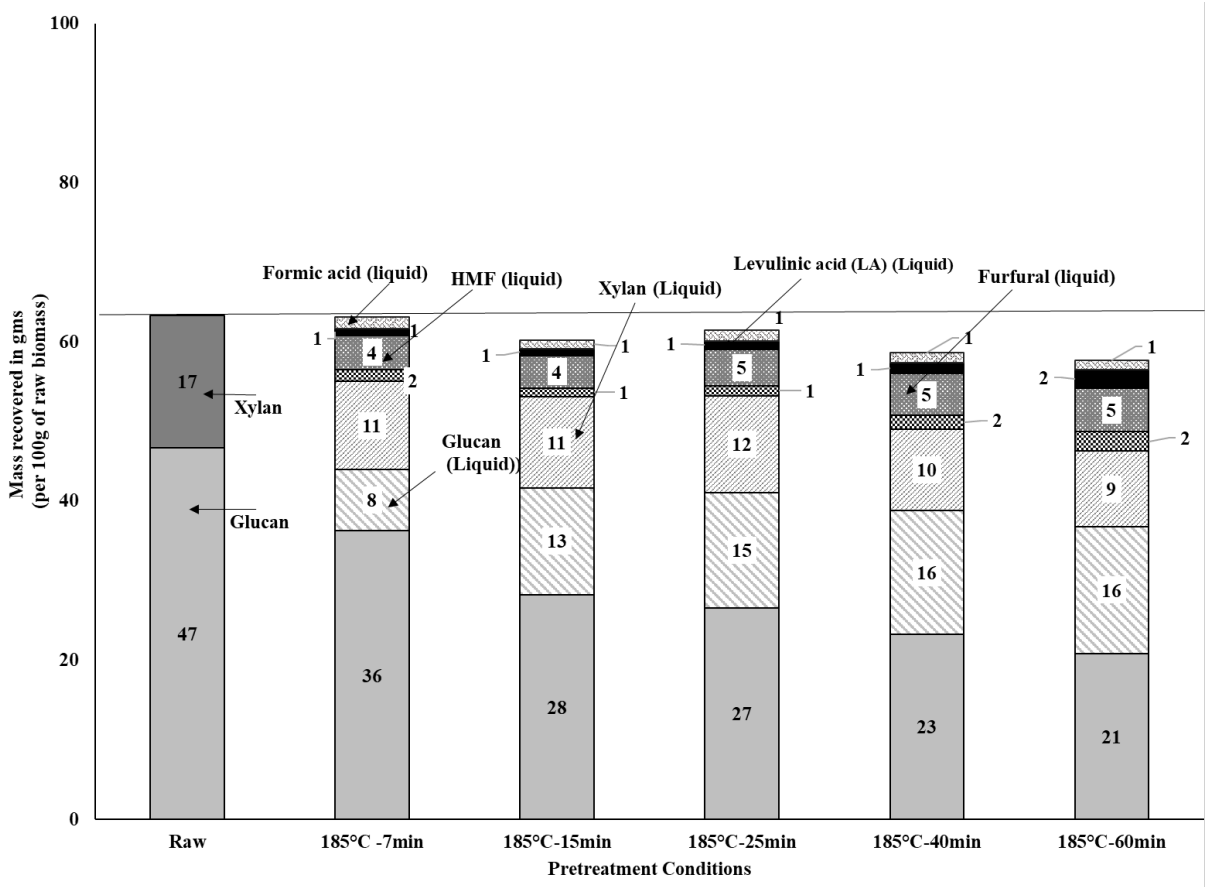


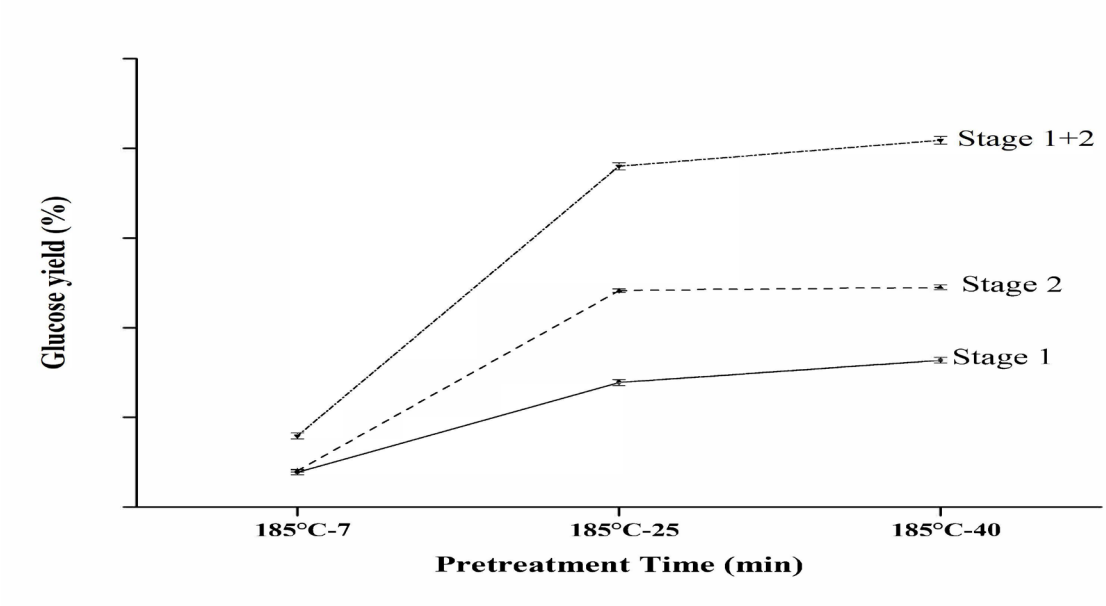
Figure 4.4 Mass balance of glucan and xylan recovered in solids after ethanol organosolv pretreatment and dissolved in the liquid on the basis of 100 grams of initial BESC STD poplar for EO pretreatment at 185°C for selected pretreatment times with 0.5 % sulfuric acid as a catalyst.

4.4.3 Maximum Total Sugar Yielding Conditions for Ethanol Organosolv Pretreated Poplar

Influence of temperature and time on the BESC STD poplar during ethanol organosolv (EO) pretreatment was used to study the change in sugar yields during enzymatic hydrolysis. Results from EO pretreatment at 185°C with pretreatment times ranging from 7, 15 and 25 min are reported in Figure 4.5. Total combined glucan yield from

pretreatment and enzymatic hydrolysis were highest at 185°C and 15 min as 78.8 % (Figure 4.5 (A)) with enzyme loading of 15 mg/g glucan in raw biomass respectively. To know the maximum total sugar yielding conditions for the ethanol organosolv pretreated (Stage 1) BESC STD poplar wood and subsequent enzymatic hydrolysis (Stage 2), total glucose and xylose yields from Stage 1 and Stage 2 at enzyme loading of 15 mg /g glucan in raw biomass are 78.2 % (Figure 4.5 (B)).

A)



B)

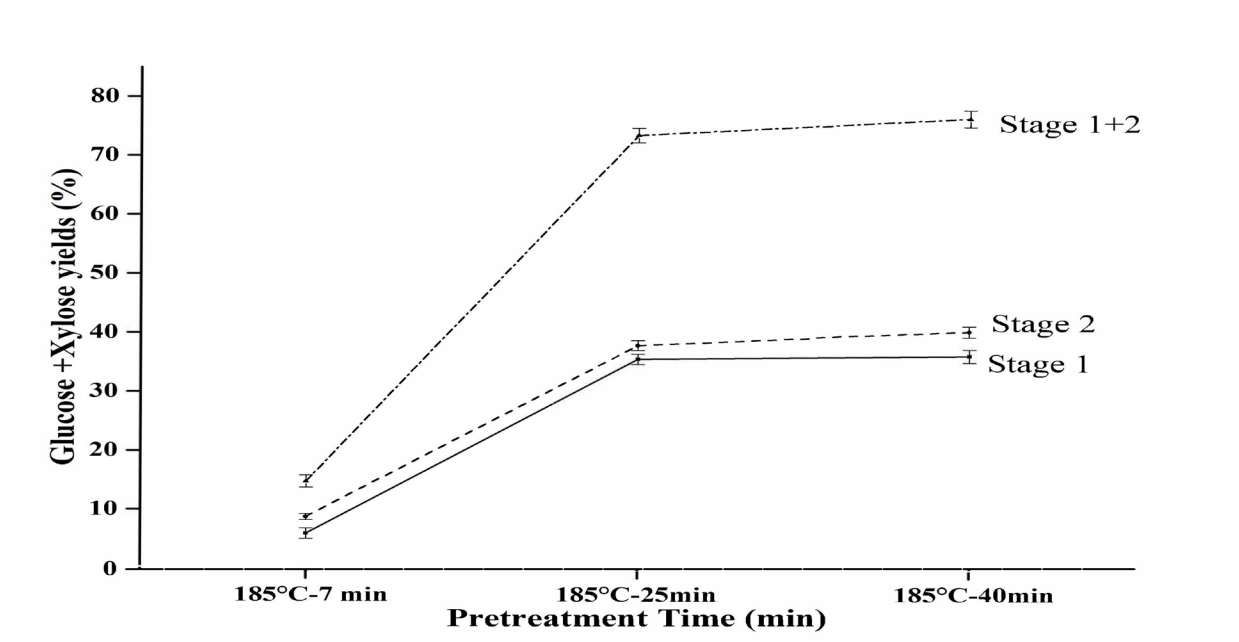


Figure 4.5 Total yields from Stage 1 (Ethanol Organosolv) and Stage 2 (Enzymatic Hydrolysis) using BESC STD poplar wood using enzyme loading of 15 mg/g glucan in raw biomass in a shaker at 150 rpm for 7 days at 50°C A) Total glucan yield Stage (1+2) and B) Total glucan + xylan yields from Stage 1 + Stage 2). **Error bars in the graph are standard deviations from three replicate flasks.**

4.4.4 Comparing EO and CELF pretreatment conditions

In a previous chapter (Chapter 3) the maximum sugar yielding conditions were determined for CELF pretreatment of BESC STD poplar. As shown above pretreatment conditions for EO for highest total sugar yields were 185°C – 15 min whereas CELF realized at 160°C -15 min achieving glucan rich biomass. CELF removed more than 90% of the lignin while lignin removed was close to 85% for EO. Application of higher temperatures and EO pretreatment times also resulted in production of degradation products like 5-HMF, furfural, acetic acid, levulinic acid and formic acid but there was virtually no degradation of fermentable sugars at CELF pretreatment conditions. The composition of solids produced after EO and CELF are 83% and 90% glucose respectively. The lignin content is 9% in EO solids but only 4% in CELF solids making the biomass more accessible to enzymatic hydrolysis. Lower pretreatment temperature and times will conserve more glucan in the solids from pretreatment, while the high temperatures and times applied for EO pretreatment result in degradation products shown in Figure 4.4. Table 4.2 below compares the composition of pretreated solids from EO and CELF.

Table 4.2 Comparing the composition of solids from EO and CELF pretreatments of BESC STD Poplar at optimal conditions.

Pretreatment conditions	Glucose (%)	Xylose (%)	Lignin (%)
EO-185°C-15 min (1:1 ethanol/water), H_2SO_4 (0.5 wt%)	83	0	9
CELF- 160°C-15 min (1:1 THF/water), H2SO4 (0.5 wt%)	90.32	3.3	4.05
EO- 160°C -15 min (1:1 ethanol/water), H2SO4 (0.5 wt%)	84.11	4.4	10.25

The Table 4.2 above also includes the results from the composition of solids produced by ethanol organosolv pretreatment at 160°C and 15 min. The glucose and lignin composition for EO at 160°C and 185°C are very similar but differ in complete removal of xylan component, the temperatures need to be higher during EO pretreatments. Solids from CELF pretreated biomass have highest glucose content of 90.32% at a temperature of 160°C.

4.4.5 Comparing EO and CELF Pretreated BESC STD Poplar Sugar Yields

The total glucan yields from Stage (1+2) for EO pretreated BESC STD poplar are 92.1% at 185°C – 15min, 23.3% at 160°C -15 min and for CELF pretreated poplar the yields are 100% at low enzyme loading of 15 mg/g glucan in raw biomass after 7 days of hydrolysis. Total glucan and xylan yields for EO at 185°C were 88%, EO at 160°C were

very low 20.4% whereas using CELF pretreatment at 160°C -15 min resulted in yields close to 100% at an enzyme loading 15mg/g glucan in raw biomass.

4.5 Conclusions

Maximum total sugar yielding conditions were determined for ethanol organosolv and co-solvent enhanced lignocellulosic fractionation (CELF) for BESC STD poplar. Pretreatment and enzymatic hydrolysis of pretreated solids from ethanol organosolv pretreatment results in lower yields than those from CELF pretreatments. The maximum total sugar yielding conditions obtained from EO is 185°C -15 min but CELF resulted in higher yields at 160°C – 15 min. The yields are close to 100 % for CELF in comparison to EO resulting in comparatively lower yields of 88% at 15 mg/g glucan in raw biomass enzyme loading. The lower amount of lignin and high glucan in BESC STD poplar pretreated using CELF could be the possible reasons for the higher yields observed compared to EO pretreatment. The absence of degradation products during CELF pretreatment is an added advantage not possible during EO pretreatments due to high temperatures required for the xylan and lignin removal. Future detailed characterization of the pretreated and hydrolyzed BESC standard poplar obtained from the EO and CELF pretreatment could provide insights into structural changes that enhanced yields.

4.6 Acknowledgements

We are grateful for funding by the Office of Biological and Environmental Research in the Department of Energy (DOE) Office of Science through the BioEnergy Science Center (BESC) at Oak Ridge National Laboratory (Contract DE-PS02-06ER64304). We

also, acknowledge the Center for Environmental Research and Technology (CE-CERT) of the Bourns College of Engineering for providing the facilities and the Ford Motor Company for funding the Chair in Environmental Engineering that facilitates projects such as this one.

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Chapter 5

Enzymatic Hydrolysis of CELF Pretreated Poplar Wood Solids at High Glucan Loadings

5.1 Abstract

Batch enzymatic hydrolysis of THF Co-Solvent Enhanced Lignocellulosic Fractionation (CELF) pretreated poplar wood was carried out at solid loadings up to 20 wt% to produce high concentrations of fermentable sugars to reduce product recovery costs. The solids from CELF fractionation of poplar wood were hydrolyzed by a fed batch process at an enzyme loading of 30mg/g glucan in raw biomass. Optimal conditions were first defined for CELF and dilute acid pretreatment of poplar wood, with the resulting solids fed to enzymatic hydrolysis at final glucan loadings of 15,18 and 20% in a total working volume of 100 ml, complete hydrolysis able to produce a maximum theoretical sugar concentrations of 167, 200 and 222 g/l, respectively. Enzymatic hydrolysis was initiated at a 5% glucan loading with an enzyme dose of 30mg/g glucan in raw biomass. Additional 5% glucan loadings were fed based on solublization curve to achieve maximum sugar concentrations, and data were analyzed over 25 days. CELF pretreated biomass achieved maximum possible glucan concentrations of 63 g/l, 70g/L, and 75g/l in 2 days, while dilute acid concentrations were limited to 45, 53, and 60g/l at 15, 18 and 20% glucan loadings, respectively. These results improved over a period of 25 days when the glucan loading for CELF was 15%, 18% and 20% (w/v), while the low conversion of dilute acid solids limited the ability to feed more to the enzyme conversion system. The maximum sugar concentrations was 143g/l at a glucan loading of 20% (w/v) for CELF pretreated biomass with an enzyme loading of 30mg/g glucan in raw biomass.

5.2 Introduction

Lignocellulosic biomass structures are naturally recalcitrant leading to resistance to enzymatic attack for generating fermentable sugars. An important step for improving the accessibility of biomass is treating the biomass during pretreatment step. Various pretreatments such as dilute acid, organosolv, and other solvent pretreatments (Amiri, Karimi, & Zilouei, 2014; Larsson et al., 1999; K. Zhang, Pei, & Wang, 2016) open up biomass structures for enzymatic hydrolysis but also lead to the degradation of the fermentable sugars. At higher temperatures and times, the pretreatment step can convert the acetyl groups on hemicellulose backbone into acetic acid which inhibit fermentation organisms. Degradation products also inhibit fermentations. As a result, a conditioning step is typically required that further adds to the cost of ethanol production.

To support commercialization of production of biofuels such as ethanol various pilot scale or demonstration plants have been run. Some examples include a plant in Denmark that produced cellulose based ethanol from lignocellulosic biomass starting in 2004, Danish power producer Elsam A/S since 2002, and other plants under integrated biomass utilization project focus mainly on pretreatment, enzymatic hydrolysis and fermentation of lignocellulosic biomass (Henning, Jakob, Jan, & Claus, 2007). An important factor to improve process economics and energy use is to feed high glucan concentrations for enzymatic hydrolysis to reduce the cost of ethanol recovery (Thomsen et al., 2006). As shown in Table 5.1, sugar concentrations reported in the literature for enzymatic hydrolysis at high solid loadings have limited ethanol concentrations to less than 64 g/L.

Table 5.1 Summary of results at high solid loadings for different feedstocks.

Biomass	Configuration process	Solid loading (%)	Sugar concentration (g/l)	Ethanol concentration (g/l)	Refrence
Agave bagasse	SHF	20	225	64	Caspeta et al. 2014
Citrus Peel waste	SHF	15	62.2	29.5	Choi et al. 2015
Prosopis Juliflora	Fed batch SHF	20	127	52.83	Gupta et al. 2012
Olive tree pruining	Batch	20	64.5	-	Cara et al. 2007
Hardwood chips	Batch	20	121	-	Xue et al. 2012
Poplar wood	Batch	20	158	63.1	Zhang et al. 2009

The main focus of this study was to identify glucan loading and biomass feeding strategies to realize high concentrations of fermentable sugars from poplar. CELF pretreatment resulted in a glucan rich biomass that was applied at an enzyme dose based on literature review. Several feeding strategies were tested to find that most suitable for achieving high concentrations of sugars from enzymatic hydrolysis. This study further evaluated the results for maximum ethanol titers that could be obtained from the higher sugar concentrations achieved after hydrolysis.

5.3 Material and Methods

5.3.1 Biomass

Bioenergy Science Center standard poplar (BESC STD) was provided by the National Renewable Energy Laboratory (NREL), Golden, CO, in the form of wood chips. This biomass was knife milled through a 20-mesh screen (Model No. 3383- L20, Thomas Scientific, Swedesboro, NJ, USA). Milling of chips in this size range was based on literature review since large particle sizes or chips may increase mass transfer resistance and impact yields (Archambault-Leger, Shao, & Lynd, 2012). The compositions of raw and pretreated poplar variants were determined by following the standard NREL LAP procedure. Three replicates were run for each substrate variant used in the study. The low extractives in all the varieties resulted in no need for extraction. The moisture content of the prepared poplar samples was determined with a laboratory moisture analyzer (Mettler Toledo, Model: HB43 Halogen Moisture Analyzer, Columbus, OH). Klason lignin, glucan, and xylan contents were determined following the modified NREL Laboratory Analytical Procedure (Technical Report NREL/TP-510-42618) that employed two-step acid hydrolysis: (1) about 0.3 g substrate was placed into a vial and hydrolyzed in 72 % (w/w) sulfuric acid at 30 °C for 1 h and (2) the slurry was further hydrolyzed in 4% (w/w) sulfuric acid at 121 °C for 1 h. The sugars in the liquid were determined by HPLC (2695, Waters corporation, Milford, MA). The mass of each sugar was converted to the mass of its corresponding anhydrous form by multiplying glucose values by 0.90 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. Sugar monomers in the liquid portion were analyzed quantitatively using a Waters Alliance HPLC (model 2695) equipped with a 2414 refractive detector and a Waters 2695 auto sampler using Empower 2 software (Waters Co., Milford, MA). The mobile phase was 0.005 mol/l sulfuric acid in water for the HPX-87H column at a flow rate of 0.6 ml/min.

Cellulolytic enzyme cocktail Accellerase® 1500 was generously provided by DuPont Industrial Biosciences (Palo Alto, CA). The BCA protein concentration was about 82 mg/ml as reported previously (Rajeev et al., 2013). The non-xylose fermenting *Saccharomyces cerevisiae* D_5A yeast strain used for fermentation was kindly supplied by NREL in plate monocultures. A frozen culture stock was prepared as previously described (Dowe, McMillan, & National Renewable Energy, 2008). The seed inoculum was prepared by thawing, transferring, and growing the frozen stock using a shaker incubator at 130 rpm and 37 °C for 12 h in 250 mL baffled flasks using sterilized YPD medium (Nguyen, Cai, Kumar, & Wyman, 2017) before starting fermentations during separate hydrolysis and fermentation (SHF). The inoculum was then centrifuged and re-suspended in sterile deionized (DI) water for washing and prepared for inoculation (Nguyen, Cai, Kumar, & Wyman, 2017).

5.3.2 Co-Solvent Enhanced Lignocellulosic Fractionation (CELF) Pretreatment

Pretreatments were performed in a 1L Hastelloy Parr® autoclave reactor (236HC Series, Parr Instruments Co., Moline, IL) equipped with a double stacked pitch blade impeller rotated at 200 rpm. The reaction solutions, temperatures, and times for Cosolvent-Enhanced Lignocellulosic Fractionation (CELF) pretreatment were selected based on conditions that achieved maximum total sugar yields, as reported in previous Chapter 3. Thus, the CELF reaction solution was loaded with 0.5 wt% (based on liquid mass) sulfuric acid (Ricca Chemical Company, Arlington, TX), while in CELF reactions THF (>99% purity, Fisher Scientific, Pittsburgh, PA) was added at a 1 : 1 (v : v) ratio to water based on adding equal volumes of each. Temperatures for CELF was 160 °C, while the reaction time was 15 min. Prior to each reaction, poplar (10 wt%) was soaked overnight in the solution at 4 °C. All reactions were maintained at temperature (±2 °C) by convective heating with a 4 kW fluidized sand bath (Model SBL-2D, Techne, Princeton, NJ) as previously described, with the reactor temperature directly measured by an in-line K-type thermocouple (Omega Engineering, Inc., Stamford, CT). After the reactions, the solids were separated from the reaction liquor by vacuum filtration at room temperature through glass fiber filter paper (Fisher Scientific, Pittsburgh, PA) . The solids were washed with room temperature DI water until neutral pH. Considering the limited pretreatment vessel capacity, the pretreatment reactions were conducted four times to generate enough solids for enzymatic hydrolysis. All the pretreated solids recovered after a set of pretreatments at the same conditions were mixed, and the composition was determined via the NREL procedure (Arnie Sluiter & National Renewable Energy, 2012).

After separating and washing, the solids were pressed to a moisture content of 61 % with a hydraulic press (Westward; model 3ZC62G). The glucan composition of the pretreated BESC STD poplar solids as determined according to the established NREL procedure (version 8-03-2012) was 91.2 %. Reducing moisture in pretreated solids by conventional air-drying was avoided as that reduced saccharification yields. The low moisture content in the biomass from pressing provided more head space in the reaction bottles during hydrolysis and better mass transfer.

5.3.3 Separate Hydrolysis and Fermentation (SHF)

Separate hydrolysis and fermentation was conducted according to the NREL protocol (Dowe et al., 2008). Each fermentation was performed in triplicate in 250 mL flasks with a 100 g working mass containing 100 mM citrate buffer (pH 4.8), 20 g /L yeast extract (Becton, Dickinson and Company, Redlands, CA), 40 g /L peptone (Becton, Dickinson and Company, Redlands, CA), 40 mg /L tetracycline (Sigma Aldrich, St. Louis, MO) as an antimicrobial agent, and D_5A yeast inoculum (from Materials section). Millipore water along with the sugars from enzymatic hydrolysis were loaded into flasks with attached bubble traps to achieve 15, 18, or 20 wt% glucan loadings of CELF pretreated solids and 15, 18, or 20 wt% glucan loadings for Avicel as the control. The mass of the whole flask assembly was recorded before and after autoclaving at 121 °C for 30 min. The flasks were then cooled, reweighed, and placed into a laminar flow hood (Baker and Baker Ruskinn, Sanford, ME) for aseptic addition of pre-sterilized Millipore water (to replenish lost water), yeast extract, peptone citrate buffer, tetracycline, and yeast. For SHF

experiments, the flasks were placed in an orbital shaker incubator at 130 rpm and 37 °C. Samples were drawn every 2 days as needed to effectively track the entire time course for short and long fermentations and centrifuged at 15000 rpm for 10 min so that the supernatant could be withdrawn for HPLC analysis for sugars, ethanol, lactic acid, and acetic acid (Dowe et al., 2008). Ethanol yield calculations were as explained elsewhere (Nguyen et al., 2017).

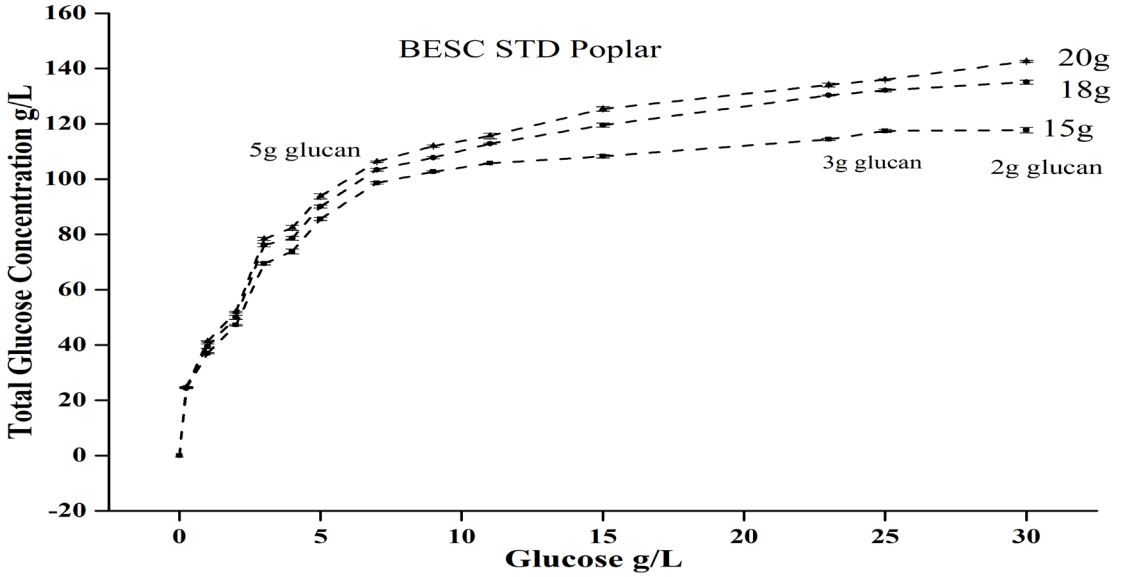
5.4 Experimental Results

5.4.1 Impact of Glucan Loadings on Sugar Concentrations

CELF pretreatment of poplar was carried out at 160ºC for 15min consistent with previously identified conditions to maximize combined total sugar yields from coupled CELF pretreatment and enzymatic hydrolysis (Chapter 3). CELF pretreatment produced solids highly enriched in glucan. Negligible degradation products were generated by CELF pretreatment. A fed-batch strategy was applied for enzymatic hydrolysis to lower the initial viscosity and overcome the mixing and diffusion problems encountered for batch hydrolysis at high solid loadings (Modenbach & Nokes, 2013). Total glucan loadings of 15g, 18g, and 20g produced maximum sugar concentrations of 124.5 g/L, 135.8 g/L, and 140.7 g/L on day 30 over a period of 35 days, as shown in Figure 5.1. After day 30, the concentration began a downward trend. Avicel control concentrations were 117.7 g/L, 135.1 g/L, and 142.7 g/L at 15g, 18g, and 20g glucan loadings, respectively. A delay was applied between additions to let the biomass liquefy before the next addition. This strategy kept the free water available for the reaction and diffusion of inhibitory sugars generated by enzymatic hydrolysis. Longer hydrolysis times are

reported in the literature with the increase in the solids loading for hydrolysis to achieve higher sugar yields (David B. Hodge, Karim, Schell, & McMillan, 2008). These results were utilized later for further experimental work to lower hydrolysis time by testing other feeding strategies and choosing an appropriate enzyme cocktail.

A)



B)

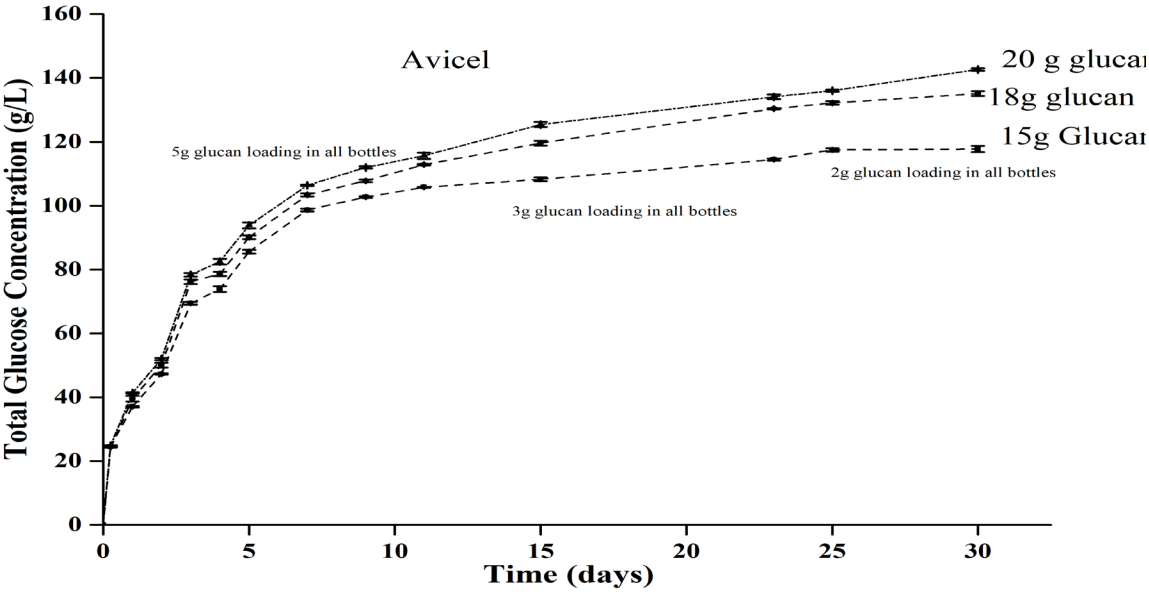


Figure 5.1 Total sugar concentrations for different glucan loadings from A) CELF pretreated BESC STD poplar wood and B) Avicel

5.4.2 Impact of Enzyme Cocktails on Sugar Production

Because the choice of enzymes can greatly affect performance for high solid loadings, different cellulases enzymes in combination with β-glucosidase were applied to identify the best combination at high solid loadings. Accellerase®1500 and Novozymes CTec2 and HTec2 Cellic CTec cellulase preparations were applied. Accellerase®1500 has high levels of β-glucosidase to more quickly convert cellobiose into glucose. On the other hand CTec2 contained high amount of β-glucosidase but also hemicellulases to ensure complete conversion of cellulose and residual hemicellulose into fermentable sugars over a wide of lignocellulosic biomass types. These enzymes are said to achieve high conversions and stability for high solid loadings. HTec2 is also known to enhance cellulose conversion with highly specific to hemicelluloses. Because HTec2 has been reported to be synergistic with CTec2, this combination was applied. Hence, two different combinations of CTec2 and HTec2 were applied, and Accellerase 1500 was applied separately to determine its impact on sugar production. The enzyme loading of Accellerase®1500, CTech2:HTec 2::2:3.2 and CTec2 and Htec2 1::1 was 30mg total protein/g glucan in raw biomass for a fair comparison of concentrations in Figure 5.2. Avicel was used a s control. The data were collected until the concentrations stopped increasing at about day 15. The results show maximum total sugar concentration of 161 g/L, 139 g/ L, and 145 g/L for the different ratios of Accellerase®1500, CTech2:HTec2::2:3.2 and CTec2 and Htec2 1::1 applied in this study. The maximum concentrations for Avicel were 146 g/L, 153g/L, and 142g/L for Accellerase®1500, CTech2:HTec2::2:3.2 and CTec2 and Htec2 1::1, respectively. Based on these results,

Accellerase®1500 was determined to be the enzyme complex favorable for high glucan loadings of pretreated poplar wood in this study.

A) B)

Figure 5.2 The impact of enzyme cocktails on total sugar yields from A) CELF pretreated BESC STD poplar wood and B) Avicel.

5.4.3 Impact of Feeding Strategies on Sugar Concentrations

Three feeding strategies were tested for their impact on sugar concentrations and time needed for hydrolysis: 1) batch, 2) fed-batch addition of enzyme, and 3) fed-batch feeding of biomass and enzyme combined. As shown in Figure 5.3, sugar concentrations were followed over a period of 10 days, with the maximum concentrations being 150 g/L, 149 g/L, and 145 g/L on day 7 for batch, fed-batch enzyme addition, and fed-batch feeding of both, respectively. Concentrations for the Avicel control were 148 g/L, 141 g/L, and 157 g/L for fed-batch enzyme addition, and fed-batch feeding of both, respectively. The latter results differ from those with CELF pretreated BESC STD poplar, possibly due to differences in structural properties. Another possibility could result from lignin and other components left in CELF pretreated poplar that would not be in Avicel. Figure 5.3 shows concentrations are so similar for batch and fed-batch enzyme addition for pretreated BESC STD poplar that it is difficult to choose between them. High cellulose conversions are reported in fed batch studies with various lignocellulosic biomasses, and the ability to maintain a constant substrate to enzyme ratio over longer hydrolysis times makes the fed-batch enzyme strategy appealing for this study (David B Hodge, Karim, Schell, & McMillan, 2009; Modenbach & Nokes, 2013; J. Zhang & Bao, 2012).

5.4.4 Ethanol Yields with High Sugar Titers

As shown in Figure 5.4, the Mascoma yeast achieved ethanol titers of 68.7 g/L from CELF solids and 66.7 g/L from Avicel in 3 days, corresponding to yields of about 85% and 88%. The time for ethanol production was relatively low, possibly due to the low recalcitrance of the solids resulting from CELF pretreatment of BESC STD poplar. Imaging of the pretreated biomass not shown hear showed structural differences in the raw and pretreated BESC STD poplar wood with high hemicellulose removal that made it well suited for high glucan loading.

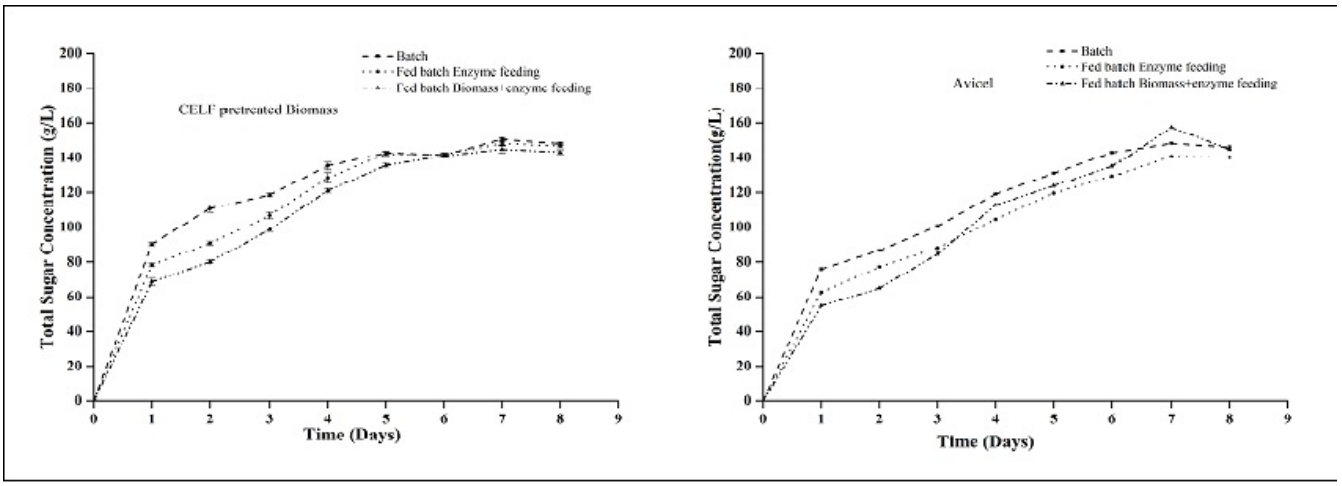


Figure 5.3 Total sugar concentrations from application of different feeding strategies to A) CELF pretreated BESC STD poplar wood and B) Avicel.

A) B)

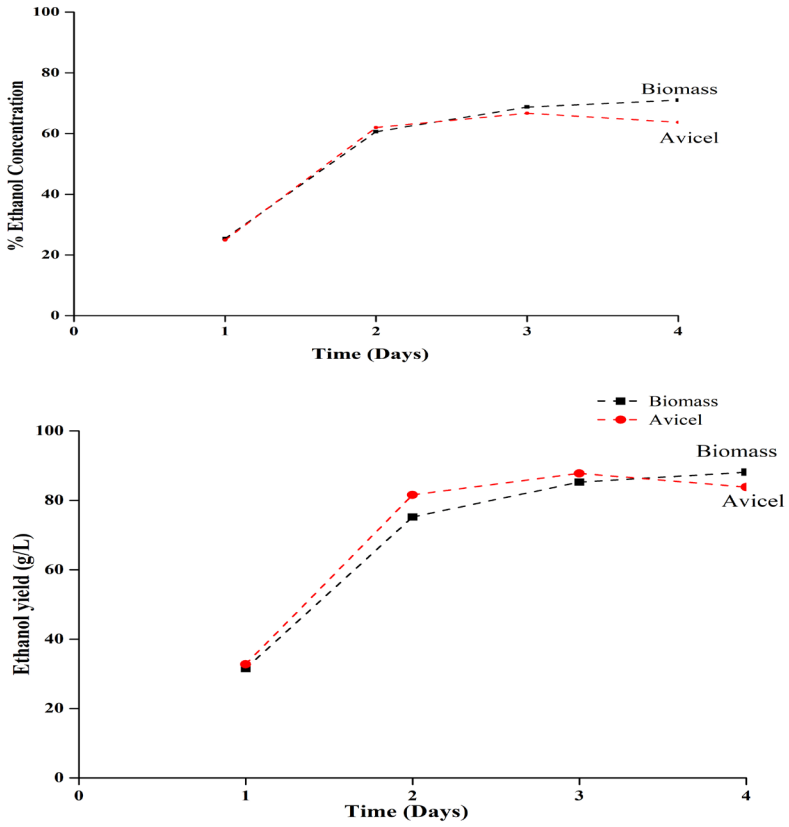


Figure 5.4 A) Ethanol concentrations and B) ethanol yields for CELF pretreated BESC STD poplar wood and Avicel control.

5.5 Conclusions

CELF pretreated poplar wood solids achieved high amounts of fermentable sugars from 20 gms of glucan loaded for enzymatic hydrolysis using a fed-batch enzyme loading of 30 mg/g glucan in raw biomass. The solids loadings are higher than reported in the literature, and sugar concentrations were highest for highest solids loadings used in this study. Accelerase 1500® resulted in the higher sugar concentrations than the combination of CTec2 and HTec2 enzymes. In addition, a fed-batch of biomass reduced hydrolysis times.

5.6 Acknowledgements

We are grateful for funding by the Office of Biological and Environmental Research in the Department of Energy (DOE) Office of Science through the BioEnergy Science Center (BESC) at Oak Ridge National Laboratory (Contract DE-PS02-06ER64304). We also wish to recognize Ford Motor Company for funding the Chair in Environmental Engineering for facilitates projects such as this one. We are also thankful to the Center for Environmental Research and Technology (CE-CERT) and Bourns College of Engineering for providing facilities.

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Chapter 6

Consolidated Bioprocessing of Solids Produced by Hydrothermal Pretreatment of Advanced Poplar Lines

6.1 Abstract

Hydrothermal pretreatment is a low cost, catalyst-free, organic solvent-free method for disrupting the highly recalcitrant structure of lignocellulosic biomass in order to improve cellulose accessibility to hydrolysis by microbes or enzymes. In this study, we combined hydrothermal pretreatment with subsequent consolidated bioprocessing (CBP) to produce ethanol from biomass. CBP uses microbes producing cellulolytic enzymes on their own avoiding the supplementation with expensive fungal cellulases. First, hydrothermal pretreatment conditions were optimized to maximize total sugar yields from hydrothermal pretreatment of a native poplar wood (BESC standard) and advanced transgenic poplar lines provided by Oak Ridge National Laboratory (ORNL). Then the advanced CBP organism *Clostridium thermocellum* was used to solubilize and consume the available glucan left in solids from the poplar lines. Pretreatment conditions were determined to maximize glucose yields from pretreatment (Stage 1) and CBP (Stage 2) as 83.1% and xylose yields from pretreatment (Stage 1) and CBP (Stage 2) as 98.3%, whereas, the combined total sugar yield from glucose and xylose Stage (1+2) at these conditions were estimated at 88.6%, at the end of 7 days. These hydrothermal pretreatment conditions with highest glucose and xylose yields were further applied to several advanced varieties of poplar. Solids from pretreated poplar varieties were subsequently subjected to CBP for comparing their performance to that of BESC standard poplar.

6.2 Introduction

"Fuel is in every bit of the vegetable matter that can be fermented," Henry Ford once said. Presently, the potential to use a wide variety of lignocellulosic biomass such as agricultural residues, woods, and grasses for ethanol fuel production affirms this statement (Kalyani, Zamanzadeh, Müller, & Horn, 2017; Moreno, Alvira, Ibarra, & Tomás-Pejó, 2017). Composition of plant cell walls differ in proportion of cellulose, hemicellulose, and lignin (Ximenes, Farinas, Kim, & Ladisch, 2017). Cellulose, an insoluble polymer of glucose linked in anhydrocellobiose dimers forms the major component of biomass. Cellulose mostly consists of crystalline regions. On the other hand, hemicellulose, generally the second most important component in lignocellulosic biomass, is a heteropolysaccharide typically consisting of the pentose sugars xylose and arabinose along with the hexoses glucose, mannose and galactose (Ximenes et al., 2017). A third significant component of lignocellulosic biomass is the aromatic macromolecule lignin that glues the plant structure together and also helps impede microbial attack of cell walls (Marriott, Gómez, & McQueen-Mason, 2016). Biomass heterogeneity makes it difficult for microorganisms to access the fermentable sugars. Thus, fuel ethanol production requires pretreatment of lignocellulosic biomass prior to hydrolysis and fermentation. The large amount of polysaccharides in lignocellulosic biomass can be converted to monosaccharides for fermentation (Whitney, Gothard, Mitchell, & Gidley, 1999). Various factors must be considered in developing an effective pretreatment for lignocellulosic biomass including recovery of pentoses, minimizing formation of toxic compounds during pretreatment, and keeping energy demand low (Y. Sun & Cheng,

2002). Physical, chemical, and biological pretreatments have been explored to alter biomass structure. However, few pretreatment technologies are cost effective to be commercially viable for converting biomass to fuels (P. Alvira, E. Tomás-Pejó, M. Ballesteros, & M. Negro, 2010). Hydrothermal pretreatment introduced during 1920's breaks down most of the hemicellulose to open up the biomass structure to facilitate enzyme attack (Brunner, 2009). Most hydrothermal pretreatments are effective at high temperatures due to a drop in dielectric constant of water making it a good solvent for non-polar substances. Ionic product, density, miscibility, and transport properties change with temperature and pressure during reaction (Pavlovič, Knez, & Škerget, 2013). Water, being environmentally friendly, non-toxic, and non-corrosive, makes it an attractive reaction medium for biomass pretreatment (Yedro, Cantero, Pascual, García-Serna, & Cocero, 2015). However, pretreatment conditions must be optimized to maximize recovery of fermentable sugars with negligible formation of degradation products. Hydrothermal pretreatment is effective between 160°C-200°C for most lignocellulosic biomass (Mosier et al., 2005b). The self-buffering nature of biomass maintains the pH of this pretreatment between 4-5 without addition of any base or buffer (Y. Kim, Hendrickson, Mosier, & Ladisch, 2009; Mosier et al., 2005b). An added advantage of hydrothermal pretreatment is that up to 90 % of the hemicellulose is removed and the accessibility of enzymes is enhanced for hydrolysis (Petal Alvira et al., 2010; Mosier et al., 2005b). This pretreatment is facilitated by hydronium ions produced at higher temperatures acting as a catalyst along with production of acetic and other organic acids assisting hydrolysis and solublization at these temperatures (Weil et al., 1998). However,

limited delignification results from hydrothermal pretreatment due to recondensation of soluble components originating from lignin as the lignin cycles between phase transition, reaction, solublization, and reaction in solution to form insoluble products (Donohoe, Decker, Tucker, Himmel, & Vinzant, 2008). However, the harsh pretreatment conditions that must be applied for hydrothermal pretreatment to promote high sugar yields from fungal cellulases result in loss of sugars to degradation products that hurt yields and inhibit subsequent fermentations. On the other hand, consolidated biomass processing (CBP) in which a single organism produces cellulolytic enzymes, hydrolyses cellulose, and ferments the sugars released to desired products has been shown to be more effective in deconstructing biomass than fungal enzymes (Thomas et al., 2017;Lee R. Lynd, Paul J. Weimer, Willem H. van Zyl, & Isak S. Pretorius, 2002; Lynd, Zyl, McBride, & Laser, 2005). Thus, this chapter focuses on optimizing hydrothermal pretreatment (HT) conditions when followed by consolidated biomass processing (CBP) with the objective to determine if more mild hydrothermal conditions can result in high glucose and xylose yields from poplar wood while avoiding high loss of sugars to degradation.

6.3 Material and Methods

6.3.1 Biomass

Bioenergy Science Center standard poplar (BESC STD) wood chips provided by National Renewable Energy Laboratory (NREL), Golden, CO, were knife milled through a 20-mesh screen (Model No. 3383- L20, Thomas Scientific, Swedesboro, NJ, USA) as large particle sizes or chips may increase mass transfer resistance and impact yields (Archambault-Leger et al., 2012; DeMartini et al., 2015). Poplar varieties BESC 215 and

BESC 218 were provided as large logs chipped using a wood chipper (Patriot Products CSV-3100B 10 HP Briggs & Stratton Gas-Powered Wood Chipper/Leaf Shredder) and then milled to a consistent size for composition analysis, pretreatment, CBP, and hydrolysis. The moisture content after milling was 7.24% for the BESC standard poplar, 5.59% for BESC 215 and 5.70% for BESC 218, as determined by a halogen moisture analyzer (Model HB43-S, Mettler Toledo, Columbus, OH). The BESC standard poplar (BESC STD) was used as a reference. DuPont Industrial Biosciences (Palo Alto, CA) graciously supplied Accellerase® 1500 cellulase (Batch# 4901298419 Genencor®, DuPont Industrial Biosciences, Palo Alto, California, United States). The protein content of this commercial preparation was 82 mg/ml for Accellerase® 1500 as determined by the standard BCA method (P. K. Smith et al., 1985a).

6.3.2 Composition Analysis

The compositions of raw and pretreated poplar variants were determined by the standard NREL LAP procedure (A. Sluiter & J. Sluiter, 2005). Three replicates were run for each substrate variant. Solvent extraction was not applied in light of the low extractives in all the varieties. The moisture content of the prepared poplar samples was determined by a laboratory moisture analyzer (Mettler Toledo, Model: HB43 Halogen Moisture Analyzer, Columbus, OH). Klason lignin, glucan, and xylan contents were determined following the modified NREL Laboratory Analytical Procedure (Technical Report NREL/TP-510-42618) that employed two-step acid hydrolysis: (1) about 0.3 g substrate was placed into a vial and hydrolyzed in 72% (w/w) sulfuric acid at 30 °C for 1 h and (2) the slurry was further

hydrolyzed in 4% (w/w) sulfuric acid at 121 °C for 1 h. The sugars in the liquid were determined by a HPLC (model 2695, Waters Corporation, Milford, MA) as described in the sugar analysis section.

6.3.3 Determination of Sugars

Total sugars in the liquid, which included monomers and oligomers, were measured by post-hydrolysis with 4 wt% sulfuric acid at 121°C for 1 h. The total oligomer amount was determined as the difference between the amount of monomers measured after post-hydrolysis after correcting for losses in post-hydrolysis and the amount measured before post-hydrolysis according to the following calculation (A. Sluiter & J. Sluiter, 2006):

> Oligomers (g) = total xylose (g) in the hydrolysate corrected for degradation after post hydrolysis − monomers (g) in the hydrolysate liquid before post hydrolysis
>
> 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 steps, respectively. Detailed calculations for the study are given elsewhere (Thomas et al., 2017).

6.3.4 Product Analysis

Sugar monomers in the liquid portion were analyzed quantitatively using a Waters Alliance HPLC system (model 2695) equipped with a 2414 refractive detector and a Waters 2695 auto sampler using Empower 2 software (Waters Co., Milford, MA). BioRadAminex HPX-87P (Bio-Rad Laboratories, Hercules, CA) was used to analyze sugars and other products. The mobile phase for the column was 0.005 mol/l sulfuric acid in water at a flow rate of 0.6 ml/min.

Table 6.1 Hydrothermal pretreatment conditions applied to BESC STD poplar and its variants.

Number	Pretreatment	Biomass type	Temperature	Time
1	Hydrothermal	BESC STD	190	15
2	Hydrothermal	BESC STD	190	25
3	Hydrothermal	BESC STD	190	35
4	Hydrothermal	BESC 215	190	25
5	Hydrothermal	BESC 218	190	25

Table 6.1 summarizes the pretreatment conditions applied to BESC STD poplar to identify the maximum total sugar yields achieved after pretreatment and CBP. The identified conditions of 190°C for a reaction time of 25 min was found to be appropriate for the advanced poplar varieties BESC 215 and BESC 218 used in this study.

6.3.5 Pretreatment

Batch pretreatments were carried out at a 10% 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 800 g. 10% solids loading was chosen due to large volume of biomass required for optimization. Hydrothermal pretreatments (HT) were carried out on 713.76 g including biomass moisture content 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 370 °C with the temperature maintained at target values as measured by a K-type thermocouple (Omega Engineering Co., Stamford, CT) by adjusting the level of the reactor vessel in contact with sand. Pretreatment time zero was defined as when the temperature was within 2 °C of the target pretreatment temperature. The variation 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 10 °C water tank (C. Liu & Wyman, 2004; Lloyd & Wyman, 2005). It took 2 to 3 min to heat-up the batch reactors to 190 °C and around the same time to cool down to 40 °C after the reaction was over.

Following pretreatment, the liquid was removed from the solids by filtration through a Whatman® glass microfiber filter, and the solids were thoroughly washed with deionized water until neutral pH was achieved, to be sure no acid, soluble sugars, or other solubilized products were left in the pretreated solids. For compositional analysis, the wet

solids were dried at 37 °C for several days until the moisture content dropped to about 4–7%. This moisture content was accounted for in the dry weight calculation. Biomass composition and determination of oligomeric sugars were determined according to the standard NREL procedure "Determination of Structural Carbohydrates and Lignin in Biomass" (A. Sluiter & J. Sluiter, 2005) and "Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples"(A. Sluiter & J. Sluiter, 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-87 H column conditioned with a 5 mM sulfuric acid mobile phase at 65 °C. Washed pretreated solids were enzymatically hydrolyzed for 168 h with 100 mg of Accellerase® 1500 cellulase protein/g glucan in the unpretreated biomass. Enzymatic hydrolysis of never-dried pretreated solids was performed according to the standard NREL procedure "Enzymatic Saccharification of Lignocellulosic Biomass" (M. Selig, 2008). Accellerase® 1500 enzyme with an 82 mg/mL protein content as determined by the standard BCA method (P. K. Smith et al., 1985a) was generously provided by DuPont Industrial Biosciences, Palo Alto, CA. Graphs and statistical analyses were by OriginPro® v. 2015 (OriginLab Corp., Northampton, MA) statistics and graphing software.

6.3.6 Consolidated Biomass Processing

Dr. Lynd's laboratory at Dartmouth College (Hanover, NH) provided *Clostridium thermocellum* strain DSM 1313 . All chemicals were reagent grade from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) unless indicated otherwise. Seed inoculum was prepared by a single batch of a mono-colony isolate of exponential phase *C. thermocellum* cultured in MTC medium on Avicel ® PH-101 at 160°C and 180 rpm (A. L. Hogsett, 2018). Storage protocols were strictly followed for seed inoculum that was divided into 4 mL aliquots and stored at − 80 °C. A 2 v/v % inoculum was obtained from the frozen stocks cultured on 5 g Avicel® PH-101 glucan/L using MTC medium. 5 g glucan/L of pretreated biomass was transferred with freezer stock cultures in a 50 mL working volume. Avicel® controls were run at identical conditions to be sure the inoculum continued to reach ~ 90% glucan release in 24 h. To calculate glucan release, glucan weight in solution after 24 h relative to the glucan weight loaded initially was recorded. Entire fermentation contents were collected, washed (via vortexing), and oven dried at 105 °C overnight for calculating the dry weight of solids after 24h. Cultures and media were stored in serum bottles plugged with butyl rubber stoppers (Chemglass Life Sciences, Vineland, NJ) sealed by aluminum crimps. Anaerobic conditions were created by flushing the headspace with nitrogen gas and then evacuating by a compressor (model ABF63 4B 7RQ, ATB, Vienna, Austria) for 45 s. This flush/evacuation cycle was repeated 15 times. Biomass, media, and substrates were autoclaved at 121 °C for 30 min or filter sterilized (0.22 μm filter, Millipore, Billerica, MA) for heat sensitive compounds. Bottle fermentations were at pH 7.0 with MOPS

buffer. All the anaerobic digestion experiments were run in triplicate and sampled at 12 or 24 h intervals for 7 days. All the reactor contents were centrifuged at 2800 rpm to remove liquid for HPLC analysis, and residual solids were washed three times, each with 50 mL of DI water after vortexing the combined solids and water in-between washings. Drying of residual solids and weighing to determine total mass loss was followed by polysaccharide and lignin quantification (Thomas et al., 2017).

6.3.7 Quantifying Structural Sugars during Consolidated Biomass Processing

Raw biomass and solid residues from pretreatment and CBP were analyzed for structural sugar and lignin contents according to the NREL procedure "Determination of Structural Carbohydrates and Lignin in Biomass" (A. Sluiter & J. Sluiter, 2005). Wheat straw (RM 8494) from the National Institute of Standards and Technology (NIST, Gaithersburg, MD) with known composition was used as a standard with each composition analysis. Because recovered solids could be less than 300 mg after fermentation, the composition analysis was scaled down based on the available sample weight. Soluble sugar monomers and oligomers analysis by HPLC was conducted for liquid samples from the pretreatment liquor, enzymatic hydrolysis reaction solution, and CBP broth. The Waters HPLC, separations module e2695 with refractive index detector 2414 (Milford, MA) was operated with a 50 mM sulfuric acid solution eluent and an Aminex HPX-87H column (Bio-Rad, Hercules, CA) for separation of cellobiose, glucose, xylose. A minimum of three replicates was run for each analysis. Prior to monomer sugar analysis, a 30 μL of 10% (w/w) sulfuric acid solution was added to 1 mL samples of enzymatic hydrolysis or

CBP liquid to stop reactions, and the liquids were vortexed and centrifuged to remove solids and cell debris. To quantify soluble oligomers, post-hydrolysis was performed as outlined in the NREL procedure, "Determination of Structural Carbohydrates and Lignin in Biomass" (A. Sluiter & J. Sluiter, 2006). The sugar release calculations and Stage 1 material balance calculations were performed as described in detail elsewhere (Thomas et al., 2017).

6.4 Results and Discussion

6.4.1 Tracking Glucan and Xylan Mass in Raw and Hydrothermally Pretreated BESC Standard Poplar

The composition of raw poplar referred to as BESC STD poplar was 46.5% glucan, 16.6% xylan, and 21.1 % lignin. Figure 6.1 shows the change in composition of BESC STD poplar as a function of hydrothermal pretreatment time. Solid yields were calculated based on solids recovered after pretreatment. Cellulose comprises most of the glucan in most of the lignocellulosic materials whereas a small amount can be as xyloglucans in hardwoods such as poplar (Shu Lai Mok & Antal, 1992). Xylose was from hemicellulose (Fan & Art, 2012). Based on the trends in poplar composition, cellulose rich biomass is available for further consolidated bioprocessing along with hemicellulose that was not removed. The hydrothermal pretreatment conditions used in the study were selected with the aim of enhancing fermentable sugar recovery during pretreatment (Garrote, Domínguez, & Parajó, 1999; Torget & Teh-An, 1994). A temperature of 190°C was chosen based on literature results for various hardwoods and was applied to BESC standard poplar over a range of pretreatment times.

Hydrothermal pretreatment at 190°C over a range of pretreatment times showed the xylan content decreased by 7% in the pretreated solids at 190°C -15 min and dropped further as pretreatment time increased from 25 min to 35 min with the temperature remaining constant.

Because xylan solublization increased with increasing pretreatment time, the resulting solids were cellulose rich, with the glucan content increasing from 46.5% in raw poplar to 60.04% in poplar pretreated at 190°C and 35 min as shown in Figure 6.1. Fractionation of solubilized lignin depends on the conditions and biomass type (Bouchard, Nguyen, Chornet, & Overend, 1991; Shimizu, Sudo, Ono, Ishihara, & Fujii, 1994). The lignin increase in Figure 6.1 is attributed to its repolymerization in the presence of organic acids released during hydrothermal pretreatment coupled with formation of pseudolignin produced by degradation of sugars and other components. as shown (Aoyama, Seki, & Saito, 1995; R. Dekker, 2016; R. F. H. Dekker & Wallis, 1983; Heitz et al., 1991; Montané, Salvadó, Farriol, Jollez, & Chornet, 1994). Literature reports that up to 60% of the lignin can repolymerize at severe hydrothermal pretreatments along with degrading a significant amount of cellulose (Biermann, Schultz, & McGinnia, 1984). Also, the lignin redeposited on the solid residue after pretreatment is lower in molecular weight but does not result in any difference in Klason lignin content compared to the actual lignin in the biomass. This recalcitrant lignin could be removed by further application of organic solvents (Lora & Wayman, 1980).

As shown in Figure 6.2, the mass of xylan left in biomass solids that were pretreated at 190°C dropped with increasing pretreatment time from 7.03 g for15 min to 3.91 g for 35

min compared to 16.6 g available in the raw BES STD poplar. This trend of lower xylan in the pretreated poplar could have further continued with increasing time but would have led to production of more degradation products.

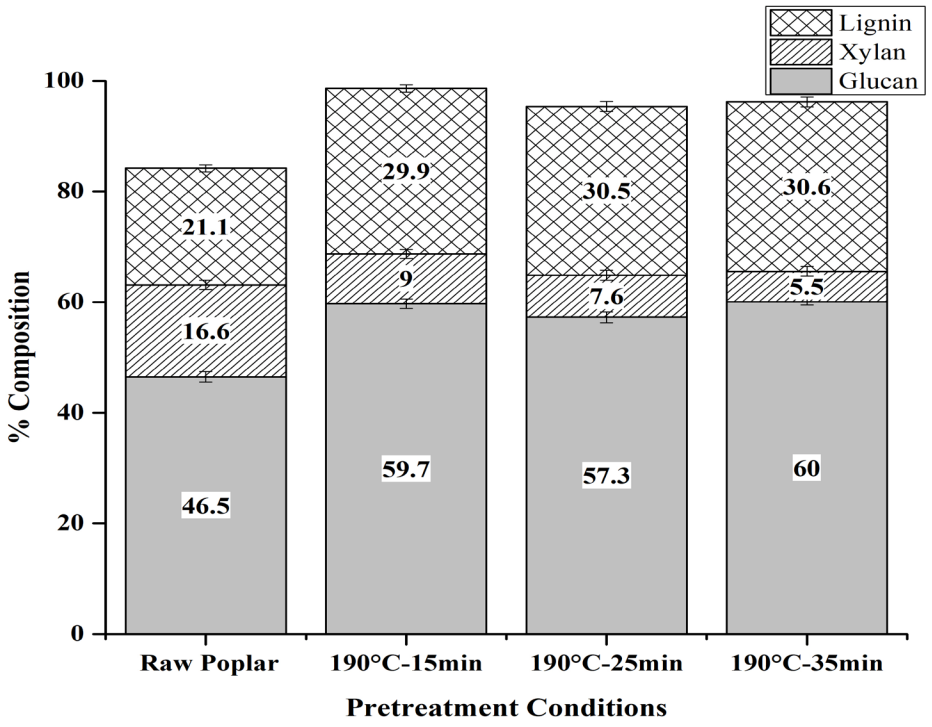


Figure 6.1. Change in composition of BESC STD poplar solids with time of 190°C hydrothermal pretreatment**. Error bars in the graph are standard deviations from three replicate flasks.**

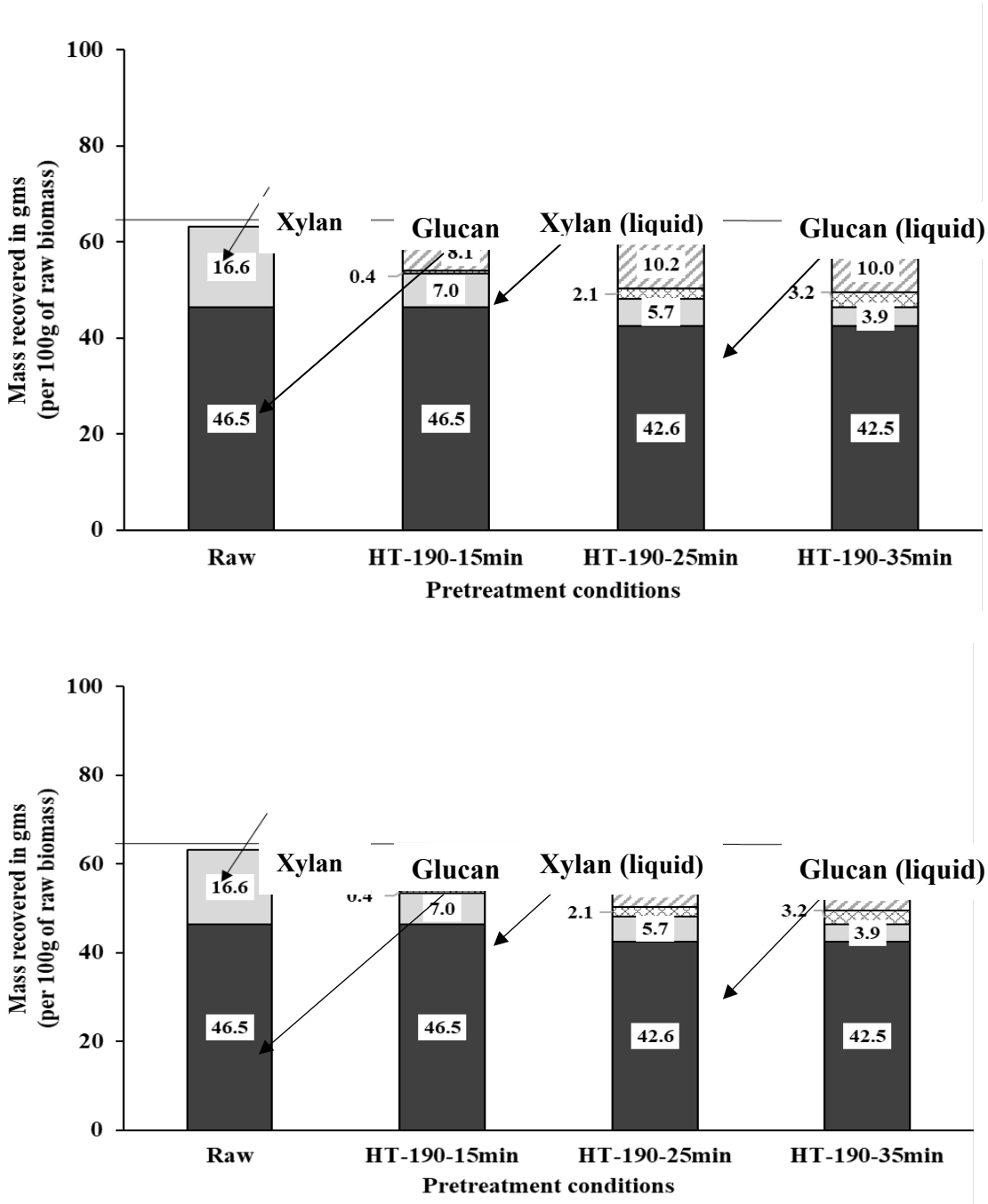


Figure 6.2 Tracking the mass of glucan and xylan before and after hydrothermal pretreatment of BESC STD poplar at 190°C for times of 15, 25 and 35 min.

6.4.2 Combined Sugar Yields for Pretreatment of BESC STD Poplar

The highest combined sugar yields from pretreatment and CBP would give the most suitable pretreatment conditions for BESC STD poplar. The pretreated solids (Step1) were washed after pretreatment and subjected to consolidated biomass processing (Step 2) using *C. thermocellum* with a glucan loading of 5g glucan/L for up to 5 days. The results from the combined sugar yields from Stage 1+2 are presented in Figure 6.3. The maximum total glucose yield of 83.1% from Stage 1+2 was for 25 min at the hydrothermal pretreatment temperature of 190°C, at which condition, the combined Stage 1+2 xylose yields for BESC STD poplar was 98.3%. The latter was a small drop from xylose yields at 15 min since we know that increased pretreatment time leads to degradation at these high temperatures. The total combined Stage1+2 glucose plus xylose yields were also highest at 88.6% for hydrothermal pretreatment for 25 min at 190°C, while the yields dropped to 86.2% and 86.5% for pretreatment times of 15 and 35 min, respectively, at the end of 7 days of CBP digestion. Based on these results, a time of 25 min was employed for 190°C hydrothermal pretreatment of the poplar varieties used in this study.

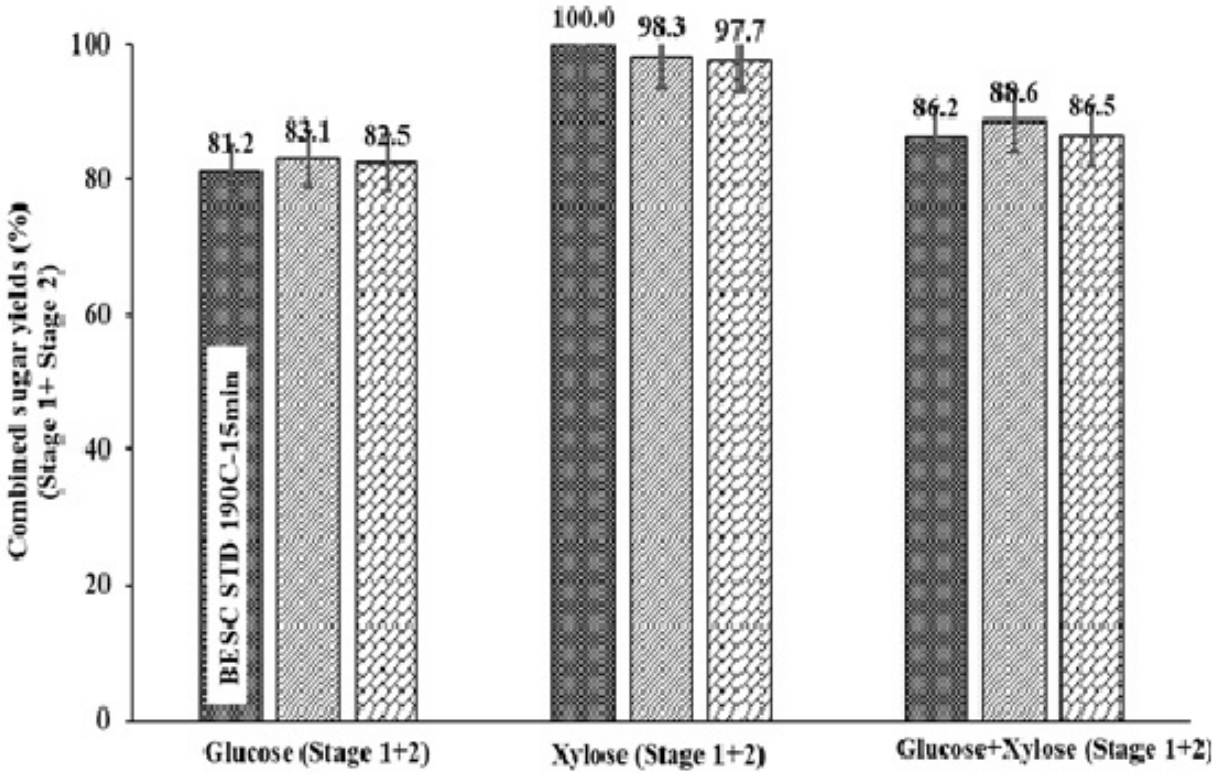


Figure 6.3. Stage 1+2 yields of glucose and xylose monosaccharides and their total after 190ºC hydrothermal pretreatment followed by *C. thermocellum* consolidated biomass processing for 7 days for BESC STD poplar wood. Error bars in the graph are standard deviations from three replicate flasks.

6.4.3 Tracking Xylose from Unpretreated and Pretreated Poplar Varieties

Figure 6.4 reports the composition of the poplar varieties and the solids produced following pretreatment at 190ºC for 25 min. The initial glucan compiosition of BESC215 was similar to that of BESC STD poplar, but BESC 218 had a higher glucan content than BESC STD. In addtion, the xylan content was highest for BESC 218 at 19.6%. Hydrothermal pretreatment of BESC 215 increased the glucan content from 46.3% to 62.4% and reduced the xylan portion from 16.3% to 5.1 %. The lignin content increased from 20.8% to 30.1%. For BESC 218, hydrothermal pretreatment at these conditions

increased the glucan content from 58.04% to 68 %, xylan from 19.6% to 6.1%, and lignin from 22.4 % to 26%.

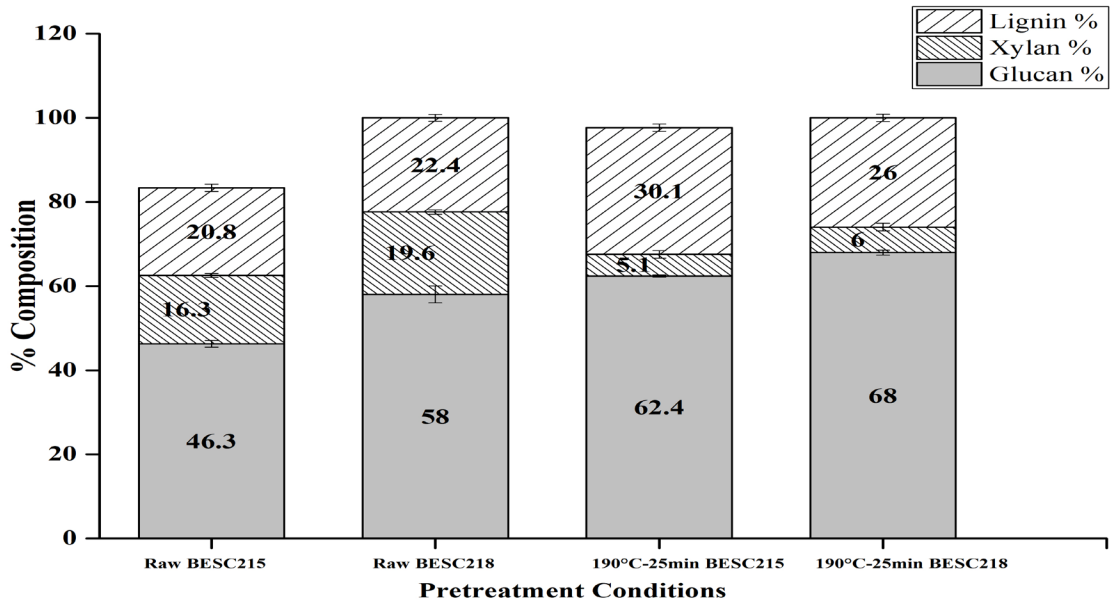


Figure 6.4. Composition of poplar prior to pretreatment and solids produced after hydrothermal pretreatment (HT) at 190°C for 25 min of BESC 215 and BESC 218. Error bars in the graph are standard deviations from three replicate flasks.

The change in the xylan mass in BESC 215 and BESC 218 solids is shown in Figure 6.5. As discussed in earlier sections, if less xylan is left in the solids, the biomass will be more glucan rich and suitable for CBP or enzymatic hydrolysis (EH). The trends for BESC 215 and BESC 218 were similar to those for pretreated BESC standard poplar. The amount of xylan recovered in BESC 215 is similar to BESC STD poplar at the chosen pretreatment conditions while more xylan is recovered for pretreatment of BESC218. The reason

behind higher recovery of xylan from pretreated BESC 218 could be attributed to the higher xylan content present in this variety to start with and the lower recalcitrance of this variety at the hydrothermal pretreatment conditions applied.

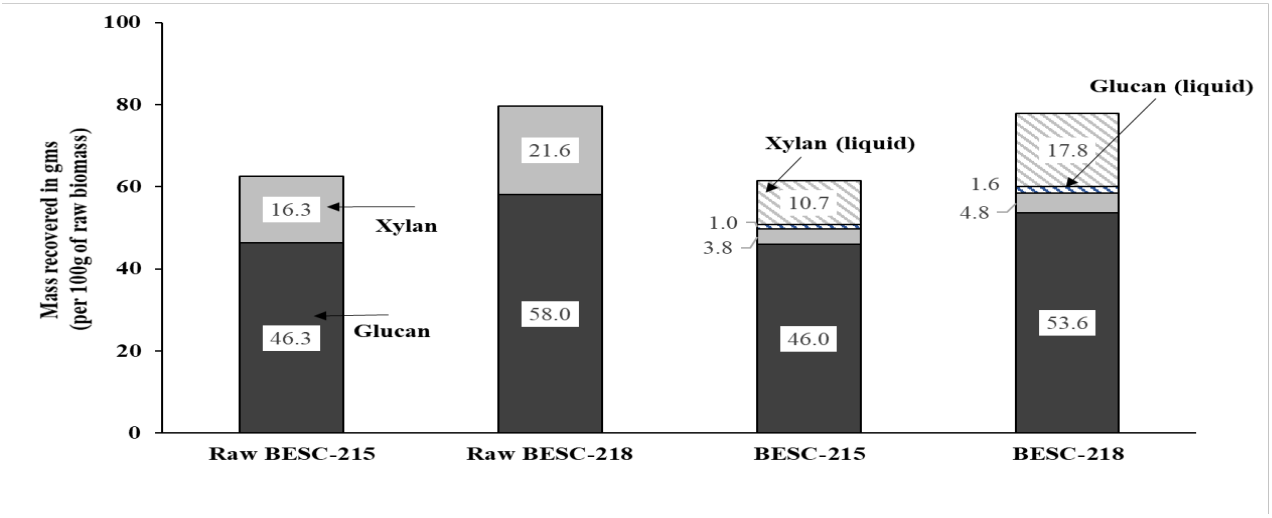


Figure 6.5. Tracking the mass of Glucan and Xylan before and after hydrothermal pretreatment of BESC 215 and BESC 218 poplar varieties at 190ºC for 25 minutes.

6.5 Comparing Pretreatment and CBP Yields from BESC STD Poplar to BESC 215 and BESC 218

Combined total sugar yields from hydrothermal pretreatment and CBP for the poplar varieties BESC 215 and BESC 218 were compared to yields from BESC STD poplar. The hydrothermal pretreatment conditions for BESC 215 and BESC 214 were established as 25 min for 190ºC based on maximizing total sugar yield from BESC STD pretreatment combined with CBP. The solids produced by hydrothermal pretreatment of BESC 215 and BESC 218 were subjected to CBP using *C. thermocellum*, with results after 7 days of digestion reported in Figure 6.6. The total glucan yields from Stage1 and Stage 2 combined were highest for BESC 215 as 87.9 % followed by BESC STD and BESC 218,

while the total xylan yields from Stage 1 plus Stage 2 reached 100 % closely followed by BESC STD as 98.3 %. The results from total glucan and xylan yields from Stage1 and Stage 2 shows BESC 215 has the highest yield of 92.4 %. The combined glucan and xylan yields from Stage1 and Stage 2 for BESC 218 and BESC STD were 86.0 and 88.6%, respectively

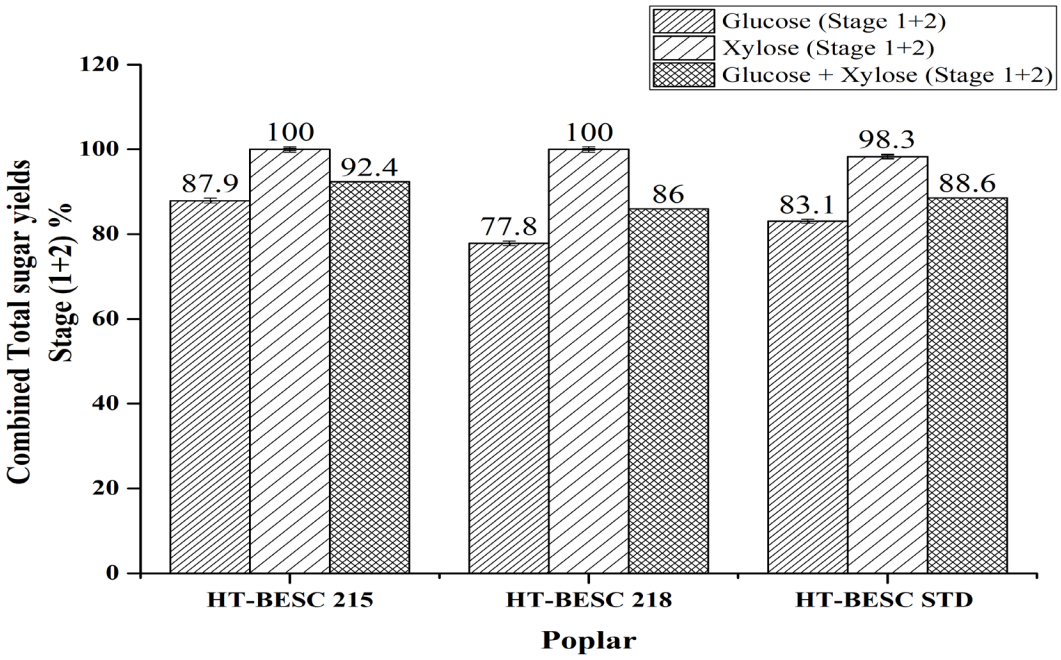


Figure 6.6. Total sugar yields from Stage 1 (hydrothermal pretreatment at 190oC for 25 minutes) and Stage 2 (C.thermocellum CBP) after 7 days of digestion of the pretreated solids by CBP. Error bars in the graph are standard deviations from three replicate flasks.

6.5.1 Comparing Enzymatic Hydrolysis Yields of Raw and Pretreated Poplar Varieties

The solids from the hydrothermal pretreatments were also subjected to enzymatic hydrolysis at enzyme loadings of 100 and 15 mg/g glucan in raw biomass. The high enzyme loading was applied to determine if the pretreated poplar was highly accessible to

enzymes, while the lower enzyme loading was closer, although still too high, to an economically viable loading. BESC STD poplar total Stage1 and Stage 2 glucan yields were very low at the end of 5 days of hydrolysis even for the very high enzyme loading, as shown in Figure 6.7A. This figure also shows that the higher total glucan yields for BESC 215 compared to BESC 218 with fungal enzymes are consistent with the trend for the combined yields from pretreatment followed by CBP. However, the results shown in Figure 6.7A for enzymatic hydrolysis at the high enzyme loading of 100 mg/g glucan in raw biomass were lower, varying from 67.2 %, 76.4 %, and 83.5 % for BESC 215, BESC 218, and BESC STD poplar, respectively. The results from combined Stage1 and Stage 2 sugar yields were highest for BESC 215 as 92.1% at an enzyme loading of 100 mg/g glucan in raw biomass, while yields dropped to 87.0 % for BESC 218 and much lower yet to 47.8% for BESC standard.

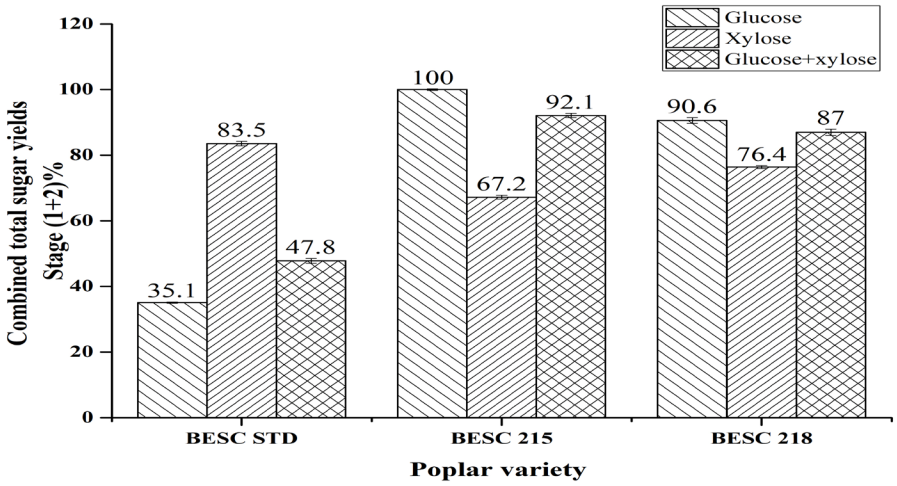
As shown in Figure 6.7B for a lower, albeit still too costly, enzyme loading of 15 mg/g glucan in raw biomass, the combined sugar yields were very low for BESC 215, BESC 218, and BESC STD poplar. The highest combined total sugar yields from Stage 1 plus Stage 2 were 68.1 % for BESC 215. Thus, it appears that the lower amount of enzymes could not access the biomass completely after hydrothermal pretreatments.

6.6 Conclusions

Hydrothermal pretreatment provides digestible solids from a variety of biomasses without application of any catalyst. The maximum total sugar yielding conditions for hydrothermal pretreatment of BESC STD poplar with subsequent CBP to digest polysaccharides in the resulting solids were established as 190ºC for 25 min. These

conditions were applied to hydrothermally pretreated BESC poplar varieties BESC 215 and BESC 218 with variable compositions. BESC 215 with higher glucan content after hydrothermal pretreatment realized the highest combined yields from pretreatment combined with CBP. Hydrothermally pretreated BESC 215 also achieved higher yields than for the other two substrates when fungal enzymes were applied to the same pretreated solids, but the yields were considerably lower than those from hydrothermal pretreatment followed by CBP and particularly poor at the lower enzyme loading of 15 mg/g.

A)



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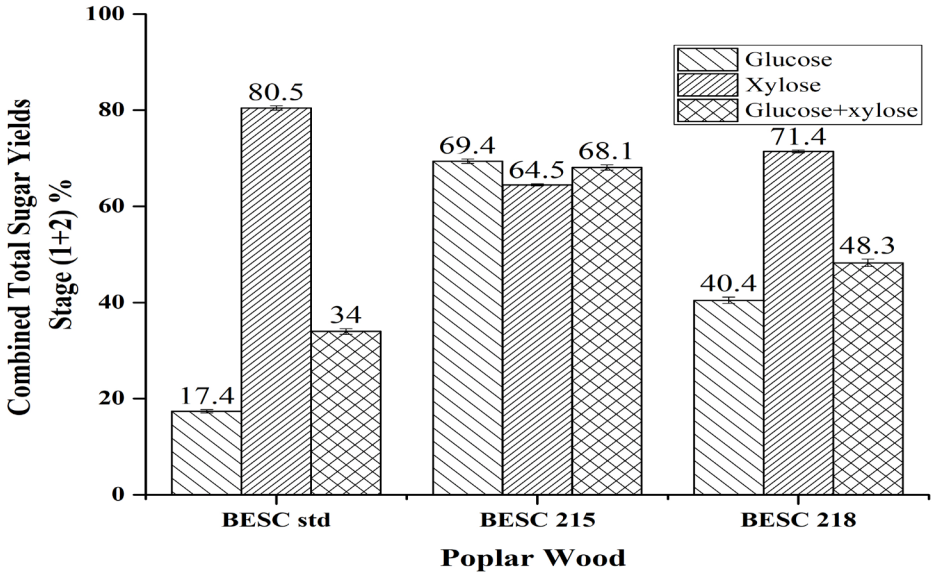

Figure 6.7. Total sugar yields from Stage 1 (Hydrothermal pretreatment) and Stage 2 (enzymatic hydrolysis at A) 100mg /g glucan in raw biomass and B) 15 mg/g glucan in raw biomass. Error bars in the graph are standard deviations from three replicate flasks.

6.7 Abbreviations

HT; hydrothermal BESC; Bioenergy Science Center BESC STD; BESC standard poplar CBP: Consolidated Biomass Processing EH; Enzymatic hydrolysis

6.8 Acknowledgements

We are grateful for funding by the Office of Biological and Environmental Research in the Department of Energy (DOE) Office of Science through the BioEnergy Science Center (BESC) at Oak Ridge National Laboratory (Contract DE-PS02-06ER64304). We also wish to recognize Ford Motor Company for funding the Chair in Environmental Engineering for facilitates projects such as this one. We are also thankful to the Center for Environmental Research and Technology (CE-CERT) and Bourns College of Engineering for providing the facilities.

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Chapter 7

Deactivation of Cellulase at the Air-Liquid Interface Is the Main Cause of Incomplete Cellulose Conversion at Low Enzyme Loadings

This chapter was published as follows: Bhagia S, Dhir R, Kumar R, Wyman CE. 2018. "Deactivation of Cellulase at the Air-Liquid Interface Is the Main Cause of Incomplete Cellulose Conversion at Low Enzyme Loadings," *Scientific Reports* **8**:1350.

SB, RK, and CEW designed the experiments. SB carried out all experiments except pretreatments of poplar and their compositional analysis, and wrote the first draft of the manuscript. RD carried out pretreatments of poplar and their compositional analysis, and assisted in delayed Avicel substrate addition and effect of varying interfacial area experiments, as well as HPLC quantification. SB, RK, and CEW wrote and edited the manuscript. All authors read and approved the final manuscript.

7.1 Abstract

Amphiphilic additives such as bovine serum albumin (BSA) and Tween have been used to improve cellulose hydrolysis by cellulases. However, there has been a lack of clarity to explain their mechanism of action in enzymatic hydrolysis of pure or low-lignin cellulosic substrates. In this work, a commercial *Trichoderma reesei* enzyme preparation and the amphiphilic additives BSA and Tween 20 were applied for hydrolysis of pure Avicel cellulose. The results showed that these additives only had large effects on cellulose conversion at low enzyme to substrate ratios when the reaction flasks were shaken. Furthermore, changes in the air-liquid interfacial area profoundly affected cellulose conversion, but surfactants reduced or prevented cellulase deactivation at the air-liquid interface. Not shaking the flasks or adding low amounts of surfactant resulted in near theoretical cellulose conversion at low enzyme loadings given enough reaction time. At low enzyme loadings, hydrolysis of cellulose in lignocellulosic biomass with low lignin content suffered from enhanced enzyme deactivation at the air-liquid interface.

7.2 Introduction

Cellulases, such as those secreted extracellularly by the industrial strain of *Trichoderma reesei*, a soft-rot fungus, find applications in cellulosic ethanol, pulp and paper, textiles, food, and agriculture industries due to their ability to cleave glycosidic bonds in cellulose at low temperatures at environmentally safe conditions(Ryu & Mandels, 1980). However, these enzymes are relatively expensive, lose their activity over time of reaction, and cannot be recycled efficiently from the solid-liquid reaction medium(C. E. Wyman,

2007). A typical example is that with 5 mg protein per gram cellulose loading of commercial preparation of DuPont Accellerase® 1500 at 50°C and at pH 5.0 after 5 days of reaction, only 50% conversion of pure Avicel cellulose (PH-101 grade) to glucose could be realized(R. Kumar & Wyman, 2014). Cellulose conversions at the low cellulase loadings required for commercial application, however, are even lower for real lignocellulosic biomass(Bhagia, Kumar, & Wyman, 2017). Therefore, there is an acute need to improve cellulose conversions at low enzyme loadings to improve the return on products derived through reactions catalyzed by cellulase. Non-ionic surfactants such as polyethoxylated sorbitan ester type surfactants, commonly known as Tween® (Kaar & Holtzapple, 1998) and non-catalytic proteins like bovine serum albumin (BSA)(Kawamoto, Nakatsubo, & Murakami, 1992; R. Kumar & Wyman, 2009c) are known to improve cellulose conversions from enzymatic hydrolysis of pretreated lignocellulosic biomass. Two key studies, one by Eriksson et al.(Eriksson, Börjesson, & Tjerneld, 2002) on surfactants and BSA in 2002 and the other by Yang and Wyman(B. Yang & Wyman, 2006) on BSA in 2006, showed that the mechanism by which surfactants or BSA improve cellulose conversions is through reducing or preventing adsorption of enzyme to lignin. This important discovery came from the learning that while large increases in cellulose conversions and rates were seen in enzymatic hydrolysis of lignocellulosic biomass with surfactant or BSA addition, these additives did not increase cellulose conversions and rates in substrates devoid of lignin (Avicel, or delignified lignocellulosic biomass). It was also seen that surfactants or BSA had no effect on adsorption of cellulase to Avicel while they reduced adsorption of cellulase onto

lignin in lignocellulosic biomass. Now, while these studies found no effect of surfactants or BSA on lignin-free cellulosic substrates such as Avicel, several articles from the past century show large effect of surfactants on hydrolysis of pure cellulosic substrates such as Avicel, cotton, and newspaper by cellulases recovered from *T. reesei* and other wood-rot fungi (Castanon & Wilke, 1981; Helle, Duff, & Cooper, 1993; R. Kumar & Wyman, 2009a; Park, Takahata, Kajiuchi, & Akehata, 1992; Elwyn T. Reese, 1980). Despite extensive research, it has not been clear why some studies showed no effect, while others showed significant effect of surfactants on enzymatic hydrolysis of pure cellulosic substrates. Thus, the purpose of this study was to better understand the mechanism of surfactants and BSA in enhancing hydrolysis of pure cellulosic substrates by cellulases. Improving our knowledge of their impact on enzymatic hydrolysis is of extreme importance to help define paths to reduce cellulase costs for industries that employ cellulases, especially ones targeting production of renewable fuels and chemicals from renewable lignocellulosic biomass.

Several mechanisms have been put forward to explain the positive effect of surfactants on hydrolysis of lignin-free (pure) cellulosic substrates by enzymes from wood-rotting fungi. Earliest records date to 1952 when D. R. Whitaker (Whitaker, 1952) proposed that this was due to stimulation of cellulase adsorbed onto insoluble cellulose substrates by BSA. Elwyn T. Reese (Elwyn T. Reese, 1980) in 1980 proposed that shear stress caused deactivation of cellulase due to shaking as enzymes were stable in absence of shaking. He proposed that in presence of cellulose, cellulases expose their hydrophobic groups and denature due to aggregation through hydrophobic binding at shaken conditions. He

further noted that formation of protein-surfactant complexes reduces this aggregation, thus protecting the enzyme. Castanon and Wilke (Castanon & Wilke, 1981) in 1981 proposed that surfactants alleviate immobilization of enzymes on the surface of insoluble cellulosic substrates to prevent them getting jammed or stuck. In a symposium in 1980 (Elwyn T. Reese, 1982), Reese and co-workers hypothesized that certain compounds may protect enzymes from shear or air-liquid interface inactivation under shaking conditions. In 1982, they (M. H. Kim, Lee, Ryu, & Reese, 1982) found that deactivation of enzymes was severe at the air-liquid interface along with shear stress rather than shear stress alone. Ooshima et al. in 1986 (Ooshima, Sakata, & Harano, 1986) proposed that surfactants perturb the exoglucanase-endoglucanase adsorption ratio on the solid substrate surface to favor enzymatic hydrolysis and simultaneous saccharification and fermentation (SSF). Helle et al.(Helle et al., 1993) in 1993 suggested that surfactants possibly modify the surface of cellulose substrates and reduce inactivation of adsorbed enzyme (enzyme immobilization). Apart from these mechanisms, it is also known that BSA(Claesson, Blomberg, Fröberg, Nylander, & Arnebrant, 1995) and non-ionic surfactants(Duncan, Lee, & Warchol, 1995) are used to reduce nonspecific binding of proteins or peptides on solid vessel surface. It is also possible that BSA or surfactants shield enzymes from product inhibition by glucose or protect against heat deactivation due to long time reactions at 50°C. Thus, a total of ten possible mechanisms can be proposed to explain how surfactants could increase cellulose conversion: stimulation, lower shear stress, protection through enzyme-surfactant complex, lower enzyme immobilization on cellulose, reduced loss at the air-liquid interface, altered exoglucanase-endoglucanase

adsorption ratio, substrate modification, reduced loss of enzyme on reaction vessel walls, lower glucose inhibition, and reduced heat deactivation.

Our investigation involved Avicel as a model cellulosic substrate that contained 97% cellulose and 3% xylan by weight as determined by compositional analysis of biomass (Bhagia, Nunez, Wyman, & Kumar, 2016; A. Sluiter et al., 2008). The DuPont's Accellerase® 1500 cellulase enzyme preparation employed had a protein content of 82 mg/ml as determined by bicinchoninic acid (BCA) assay (P. K. Smith et al., 1985b). The filter paper activity (FPU) of this preparation was 0.5 FPU/mg, reported elsewhere (Alvira, Negro, & Ballesteros, 2011; R. Kumar et al., 2012). First, the enzyme to substrate ratio was varied in the presence of surfactant to define their effect on cellulose conversion. Then, the effects of BSA, Tween 20, and defatted soybean flour were compared to determine if proteins and surfactants acted differently. The possibility of amphiphilic additives working through prevention of nonspecific binding on glass surface was investigated by comparing cellulose conversions in borosilicate glass, polycarbonate, and siliconized Erlenmeyer glassware. Experiments without shaking were performed and substrate addition delayed to determine the relevance of these effects in altering conversion. Then the air-liquid interfacial area of the reaction vessel was varied while keeping the volume constant to understand the effect of air on cellulose conversion. Lastly, solids with low and high lignin content produced by poplar pretreatment were hydrolyzed at low enzyme loading with surfactant to determine the impact of surface deactivation on enzymatic conversion of lignocellulosic biomass into fermentable sugars. Nearly 130 combinations that included other cellulosic substrates, biomass substrates,

another commercial enzyme preparation (Novozymes Cellic® CTec2), different substrate and surfactant loadings, and several environmental conditions were carried out. This first report on this subject reveals that surface tension at the air-liquid interface was primarily responsible for cellulase deactivation resulting in low conversions at low cellulase loadings. Additional findings on this work beyond what can be covered here will be published later.

7.3 Methods

7.3.1 Materials

Avicel (Avicel® PH-101, Fluka, Cat. No. 11365-1KG, Lot No. BCBN7864V), bovine serum albumin (BSA) (Cat. No. A7906-500G, Batch No. 078K0730), and defatted soybean flour (Cat. No. S9633, Lot No. SLBL7333V, 52% approximate protein content and 85+% dispersible and 1% fat as noted by the manufacturer) were purchased from Sigma-Aldrich Corp. at St. Louis, MO. Tween® 20 was purchased from Acros Organics (Lot No. A0226412). Corning® Pyrex® 125 ml Erlenmeyer flasks made of borosilicate glass (Cat. No. 4985-125), 25 ml Erlenmeyer flasks made of borosilicate glass, Kimble® Kimax® Valueware®, (Cat. No. 5650025EMD), 125 ml Erlenmeyer flasks made of polycarbonate material Fisherbrand (Cat. No. PBV125), Corning® Pyrex® 500 ml Erlenmeyer flasks made of borosilicate glass (Cat. No. 4995-500), tetrahydrofuran (certified) and 72 w/w% sulfuric acid (Ricca Chemical) were all purchased through Fisher Scientific, Thermo Fisher Scientific Inc. at Waltham, MA. Accellerase® 1500 (Batch No. 1662334068), was a kind gift from DuPont Industrial Biosciences at Palo Alto, CA. Due to their high foaming tendency, 1% stock solutions of BSA or Tween 20

were made by weighing 1 gram of BSA or Tween 20 in a tared 125 ml conical flask followed by addition of milli-Q water to reach a total mass of 100 grams for accuracy and reproducibility. For 1% soy protein stock solution, 2.3 grams of soy flour (considering protein content of 52% and 85% dispersion) was weighed in a similar conical flask and brought to a total mass of 100 grams by adding milli-Q water. For experiments with glass powder (enhanced shear/high solid surface area), a 20 ml borosilicate glass serum vial (Wheaton® Cat. No. 223687) was crushed with a metal hammer until the particle size was about 0.250 mm as determined by passing through a no. 60 sieve (W.S. Tyler ASTM E11-09). Although some of the powder was very fine, the larger and small particles were easily separated by gravity. 0.25 g each of the large and fine sizes were added to Erlenmeyer flask after addition of Avicel.

BESC (Bioenergy Science Center) standard poplar was provided by the National Renewable Energy Laboratory (NREL), Golden, CO as chips and knife milled through a 1 mm screen (Model 4 Wiley Mill Thomas Scientific, Swedesboro, NJ) at the University of California Riverside. Batch dilute acid (DA) and co-solvent enhanced lignin fractionation (CELF) pretreatments were applied to 10% poplar solids loading in a 1L Parr® reactor (Parr Instruments, Moline, IL, USA) with a total reaction mass of 800 g. Dilute acid pretreatment of biomass was carried out with 0.5 wt% sulfuric acid in water at 160 °C for 25 minutes. CELF pretreatment of the same biomass was performed at 160 °C for 15 minutes in a mixture of equal volumes of 0.5 wt% sulfuric acid in water and tetrahydrofuran solvent. Pretreated solids were vacuum filtered with Whatman® glass microfiber filter and thoroughly washed with DI water. An elaborate description of the

pretreatment procedure has been previously documented (Bhagia, Li, Gao, Kumar, & Wyman, 2016).

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. No. TS42799, Thermo Scientific, Thermo Fisher Scientific Inc.). Freshly prepared 1% Aquasil solution in Milli-Q water was added to flasks up to 125 ml mark and shaken for 15 seconds. The solution was drained and the flasks were washed twice with 100% methanol (Fisher Scientific, Thermo Fisher Scientific Inc.) and dried at 105°C for 6 hours.

7.3.3 Compositional Analyses

Composition of Avicel cellulose as determined by the standard NREL procedure "Determination of Structural Carbohydrates and Lignin in Biomass" was 97% glucan and 3% xylan by mass (A. Sluiter et al., 2008). Avicel had an average 4 wt. % moisture content as determined by a halogen moisture analyzer (HB43-S; Mettler-Toledo, Columbus, OH). Wet pretreated solids were dried at 40±1 °C in an incubator for several days until the moisture content dropped to about 5% before compositional analysis.

7.3.4 Enzymatic Hydrolyses

All enzymatic hydrolysis runs were according to the NREL standard procedure "Enzymatic Saccharification of Lignocellulosic Biomass" (M. Selig, Weiss N., Ji Y., 2008) with only the following modifications. All experiments were done at 1 w/v% glucan loading of Avicel or pretreated poplar. Never-dried pretreated poplar solids were used for enzymatic hydrolysis to avoid drying possibly collapsing pores and thereby lowering cellulose accessibility. The reaction volume was 50 ml performed in 125 ml Erlenmeyer flasks in all experiments except those evaluating the effect of interfacial area (Figure7.5) for which 10 ml of reaction volume was carried out in 25, 125, or 500 ml Erlenmeyer flasks. The static interfacial areas measured with a ruler or Vernier calipers of 10 ml reaction volume for 25, 125, and 500 ml flasks were 7.9 cm^2, 25.6 cm^2, and 45.6 cm^2, respectively. All experiments were carried out in reaction vessels made of borosilicate glass except those evaluating the effect of reaction vessel surface or shear (Figure 7.3) for which siliconized borosilicate glass flasks, polycarbonate flasks, and borosilicate glass flasks containing 0.5 grams of glass powder were used along with borosilicate glass flasks. All enzymatic hydrolyses were carried out in 50 mM sodium citrate buffer at pH 5.0, 50°C, and 150 rpm for shaking experiments and 0 rpm for those without shaking. Orbital shaking was in a Multitron Standard (Infors® HT Biotech, Laurel, MD) that held the flasks firmly with sticky pads. In addition to the temperature reading visible in the LCD panel, a K-type thermocouple (Omega Engineering Co., Stamford, CT) was inserted inside the shaker in several locations as a secondary measure to assure temperature stability. Accellerase® 1500 with a BCA protein content of 82

mg/ml (P. K. Smith et al., 1985b) was diluted 20 times to 4.1 mg/ml in 50 mM citrate buffer in 100 ml borosilicate glass volumetric flasks. Accellerase® 1500, BSA, Tween 20, and soy loadings were based on milligrams of protein or surfactant per gram glucan in the substrate. In all experiments including no shaking experiments, the reaction medium was shaken gently after addition of enzyme. In the co-addition experiments, BSA, Tween, or soy was added to the Erlenmeyer flask quickly after the enzyme of (co-addition). Loading of Accellerase® 1500 was either 5 or 30 mg protein per gram glucan in Avicel. 5 mg or 100 mg BSA or Tween 20 was also added based on grams of glucan in Avicel. Loading of Accellerase® 1500 was 5 mg protein per gram glucan in unpretreated poplar for lignocellulosic biomass experiments (Figure 7.6). Glucan yields from the pretreatment process were 96% and 100% for DA and CELF pretreatments, respectively, making enzyme loading in enzymatic hydrolysis flask as 5.2 mg for DA and 5 mg for CELF on per gram glucan basis. This enzyme loading method involving a pretreatment process have been documented previously (Bhagia, Li, et al., 2016). 5 mg or 100 mg Tween 20 was added based on grams of glucan in pretreated poplar. In cellulose conversion vs. time experiments (Figure 7.1), sampling for 5 mg cellulase, co-addition of 5 mg BSA and 5 mg cellulase, 30 mg cellulase, co-addition of 30 mg cellulase and 100 mg BSA, were at 4, 24, 48, 72, 96, 120, 168, 216, 264, and 408 hours after enzyme addition. For all other conditions, sampling was at 120, 264, and 408 hours.

Enzymatic hydrolysis results for all conditions were determined from three replicates in Erlenmeyer flasks. Error bars in all figures represent sample standard deviation from three replicate flasks. For no shaking experiments, separate flasks were kept for each time

point (120, 264, and 408 hours) to not to disturb the reaction medium. Sampling was by 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 on a Waters® e2695 Separations Module with detection on Waters® 2414 RI detector (Waters Corp., Milford MA) equipped with a Bio-Rad® Aminex® HPX-87H column conditioned at 65°C using 5 mM sulfuric acid mobile phase at a flow rate of 0.6 ml/min for all separations. Cellulose conversion was calculated by:

Cellulose conversion (glucan yield%) =

$$\frac{\left(\text{Glucose}\ \left(\frac{\text{mg}}{\text{ml}}\right) + \left(\text{Cellobiose}\left(\frac{\text{mg}}{\text{ml}}\right) * 1.053\right)\right) * \text{Reaction volume (ml)} * 0.9 * 100}{\text{Glucan in cellulosic substrate (mg)}}$$

where 0.9 accounts for the mass of water added to cellulose (glucan) during enzymatic hydrolysis and 1.053 accounts for the addition of water to form glucose from cellobiose. A soy flour blank was also 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. An HPLC chromatogram showed no carbohydrates or any other soy flour derived components to be present. In the experiments that studied effect of interfacial area (Figure 7.5), aliquots from 10 ml reaction volume in 500 ml flask in presence of surfactant after 11 days of reaction could not be analyzed. Thus, only cellulose conversion after 5 and 17 days of reaction are shown for this condition. Sampling could not be done for no surfactant and no shaking condition of CELF pretreated poplar (Figure

7.6) after 17 days of reaction, and only conversions after 5 and 11 days of reaction are shown.

7.4 Results and Discussion

Conditions similar to those applied by Yang and Wyman (B. Yang & Wyman, 2006) of high enzyme and BSA loading were applied first to Avicel cellulose to confirm the effect of BSA on conversion. Figure 7.1 shows that 30 mg of enzyme achieved nearly complete cellulose conversion to glucose and minor amounts of cellobiose, with both represented in their anhydrous forms. Throughout this paper, all loadings of enzyme preparations or additives (BSA or Tween 20) are expressed in milligrams per gram glucan in the substrate. Furthermore, the term enzyme refers to the commercial Accellerase® 1500 cellulase preparation. Addition of 100 mg BSA added simultaneously to 30 mg enzyme had no effect on rates or final cellulose conversion. However, addition of 5 mg of BSA had a large positive effect on conversion of Avicel at low enzyme loading of 5 mg. For this low cellulase loading, although BSA had no effect on initial reaction rates, beneficial effects started to appear after 24 hours of reaction, with an ultimate 40 percent absolute increase in cellulose conversion after 17 days. The fact that its impact was only apparent at low enzyme loadings and not seen until after 24 hours of reaction explains why it was missed in prior studies with high enzyme loadings or experimentation at shorter durations. Because Figure 2 shows that BSA and Tween 20, a non-ionic surfactant, had the same effect, the amphiphilic nature of BSA appears important to enhancing performance. Since BSA and Tween 20 have a different hydrophilic-hydrophobic balance (HLB), their equivalent effect on cellulose conversion suggests that a stimulation

mechanism is unlikely. Moreover, stimulation would possibly need an allosteric enzyme site that is inconsistent with the very different structures of BSA and Tween 20.

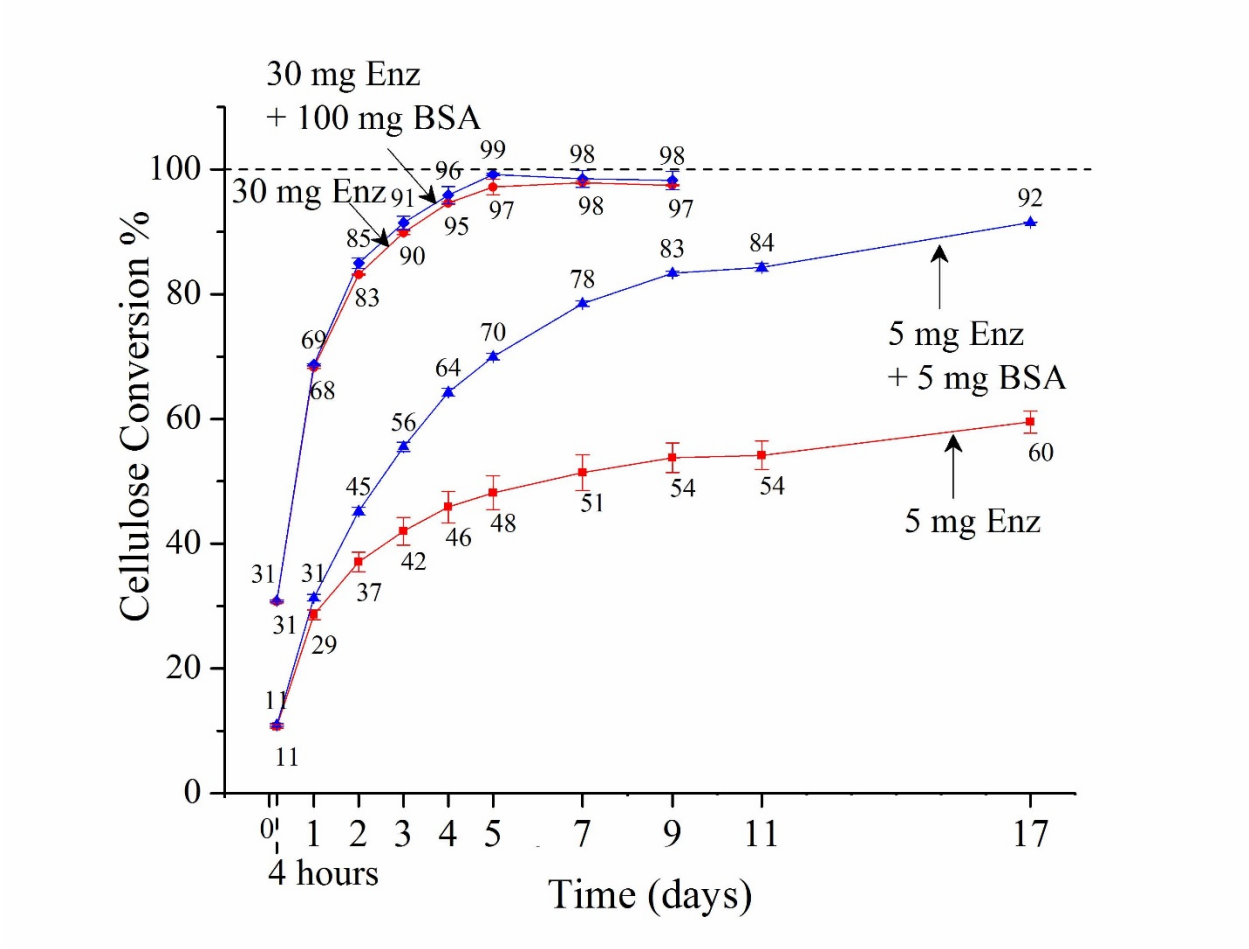

Figure7.1 Effect of BSA at low and high cellulase loadings on Avicel cellulose conversion. Low enzyme loading curves show cellulose conversion for up to 17 days of enzymatic hydrolysis of Avicel (1% glucan loading) with 5 mg enzyme (Accellerase® 1500) (square) and 5 mg enzyme with co-addition of 5 mg BSA (triangle). High enzyme loading curves show cellulose conversion for up to 9 days of enzymatic hydrolysis of Avicel (1% glucan loading) with 30 mg enzyme (Accellerase® 1500) (circle) and 30 mg enzyme with co-addition of 100 mg BSA (diamond). Enzyme and BSA loadings were based on mg per gram glucan in substrate. Error bars represent standard deviation from three replicate flasks.

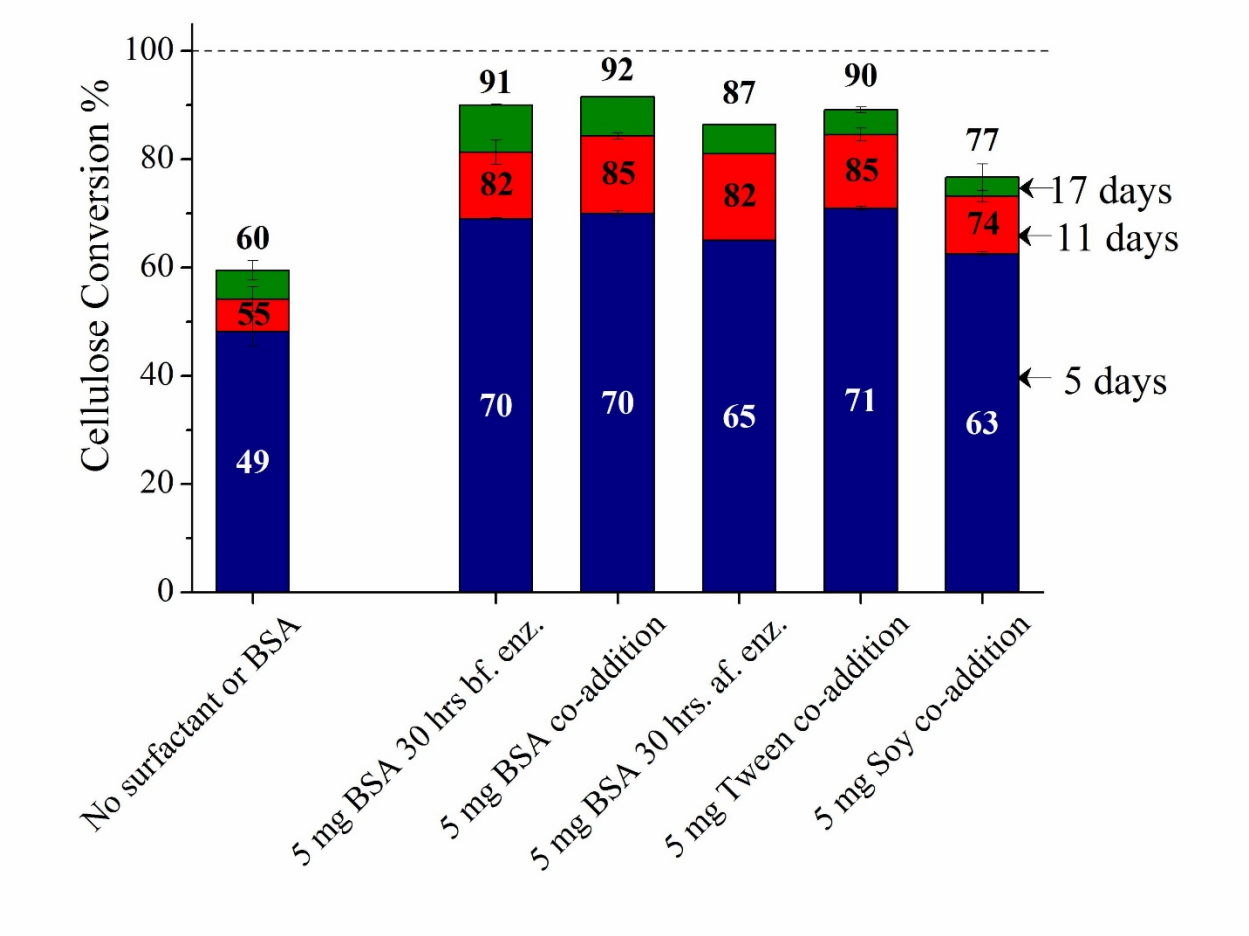

Figure 7.2 Comparison of BSA, Tween, and soy flour on enzymatic hydrolysis of Avicel. From left to right, columns show cellulose conversion after 5, 11, and 17 days of enzymatic hydrolysis of Avicel (1% glucan loading) with 5 mg enzyme (Accellerase® 1500), with supplementation of 5 mg BSA 30 hours before enzyme addition, co-addition of 5 mg BSA, with supplementation of 5 mg of BSA 30 hours after enzyme addition, with co-addition of 5 mg Tween 20, and with co-addition of 5 mg soy flour, added based on per gram glucan in the substrate. Error bars represent standard deviation from three replicate flasks.

Soy flour was also employed to take advantage of the lower cost of high protein soybean meal of $324 to $490 per metric ton over the last three years, as reported by the October 2016 USDA Feed Outlook (USDA, 2016). It is also compatible with microbes in subsequent ethanol fermentations (H. Zhao, Wan, Zhao, Lei, & Mo, 2014). Soy flour

addition also increased cellulose conversion at the low enzyme loading. Soy flour contains soy lecithin (mixture of phospholipids), soy protein, and soy saponin that exhibit surfactant behavior (Q. Xu, Nakajima, Shiina, & Liu, 2011). However, although the exact soluble surfactant concentration in the complex mixture was unknown, the low solubility of a large portion of soy flour in water likely resulted in the smaller increase in cellulose conversion by enzymes than seen with addition of BSA or Tween 20. Overall, we expect any additive with amphiphilic properties that does not denature enzyme should be able to cause this effect, as evidenced by similar observations of enhanced enzymatic hydrolysis of cellulose by polyethylene glycols (Hsieh, Cannella, Jørgensen, Felby, & Thygesen, 2015), β-lactoglobulin, pepsin, lysozyme, gelatin, peptone (Whitaker, 1952), Tweens (Ooshima et al., 1986), Pluronics, Zonyl, polypropylene glycol, polyvinyl pyrrolidone, Triton, Digitonin, methocyl, quarternary ammonium compounds (Elwyn T. Reese, 1980), sophorolipid, rhamnolipid, bacitracin (Helle et al., 1993), lecithins, phospholipids, and even cattle saliva (Seki et al., 2015) that contains muocproteins and saponins (Mangan, 1959). This diverse range includes cationic, anionic, zwitterionic, and non-ionic surfactants, as well as compounds that cannot be strictly classified as surfactants as they do not form association colloids, and are hence termed as amphiphilic additives (Davis, Bodet, Scriven, & Miller, 1987). This study focused on finding the underlying cause of cellulase deactivation using the most popular additives Tween and BSA rather than comparing different classes of these additives. Due to their strong electrostatic interactions with proteins, charged surfactants like sodium dodecyl sulfate (SDS) can denature proteins at low concentrations. On the other hand, nonionic and zwitterionic

surfactants with overall neutral charge, in most cases, do not denature proteins (Otzen, 2011). Ooshima et al.(Ooshima et al., 1986) reported that cationic Q-86W and anionic Neopelex F-25 surfactants denatured cellulase above 0.008% and 0.001%, respectively, while zwitterionic Anhitol 20BS and nonionic Tween 20 did not.

Figure 7.2 shows that there were little differences in cellulose conversion through addition of BSA 30 hours before addition of enzyme, co-addition of enzyme and BSA, or BSA addition 30 hours after enzyme. Since BSA and Tween 20 show similar effects, further experiments were carried out with Tween 20 as it is less expensive for commercial use. It is also compatible with ethanologenic organisms like *Saccharomyces cerevisiae*(Alkasrawi et al., 2003) and *Zymomonas mobilis* (Zikmanis, Shakirova, Auzina, & Andersone, 2007). Figure 3 shows that changing the reaction vessel from glass (hydrophilic, water contact angle: 16º) (Ikada et al., 1981) to polycarbonate (fairly hydrophobic, water contact angle: 70º) ("Bio-MicroElectroMechanical Systems (BioMEMS)," 2008) increased cellulose conversion by 5 percentage points at the end of reaction, whereas siliconized glass (hydrophobic water contact angle: 90º) reduced it by 8 percentage points (Ikada et al., 1981). This small variation in cellulose conversions with change of reaction vessel material indicated that avoiding enzyme denaturation at the solid surface could not account for the benefits seen with the additives. Interestingly, Tween 20 addition did not reduce these variations. These data point to an enzyme binding mechanism not solely dependent on the hydrophobic or hydrophilic character of the vessel material but that could be influenced by differences in electrostatic charge (Goebel-Stengel, Stengel, Taché, & Reeve, 2011) or microscopic surface roughness

(Preedy, Perni, Nipic, Bohinc, & Prokopovich, 2014). In any case, since the change in cellulose conversion due to nonspecific binding of enzyme on the solid vessel surface was relatively small, it cannot account for large increase caused by amphiphilic additives as shown in Figure 7.3.

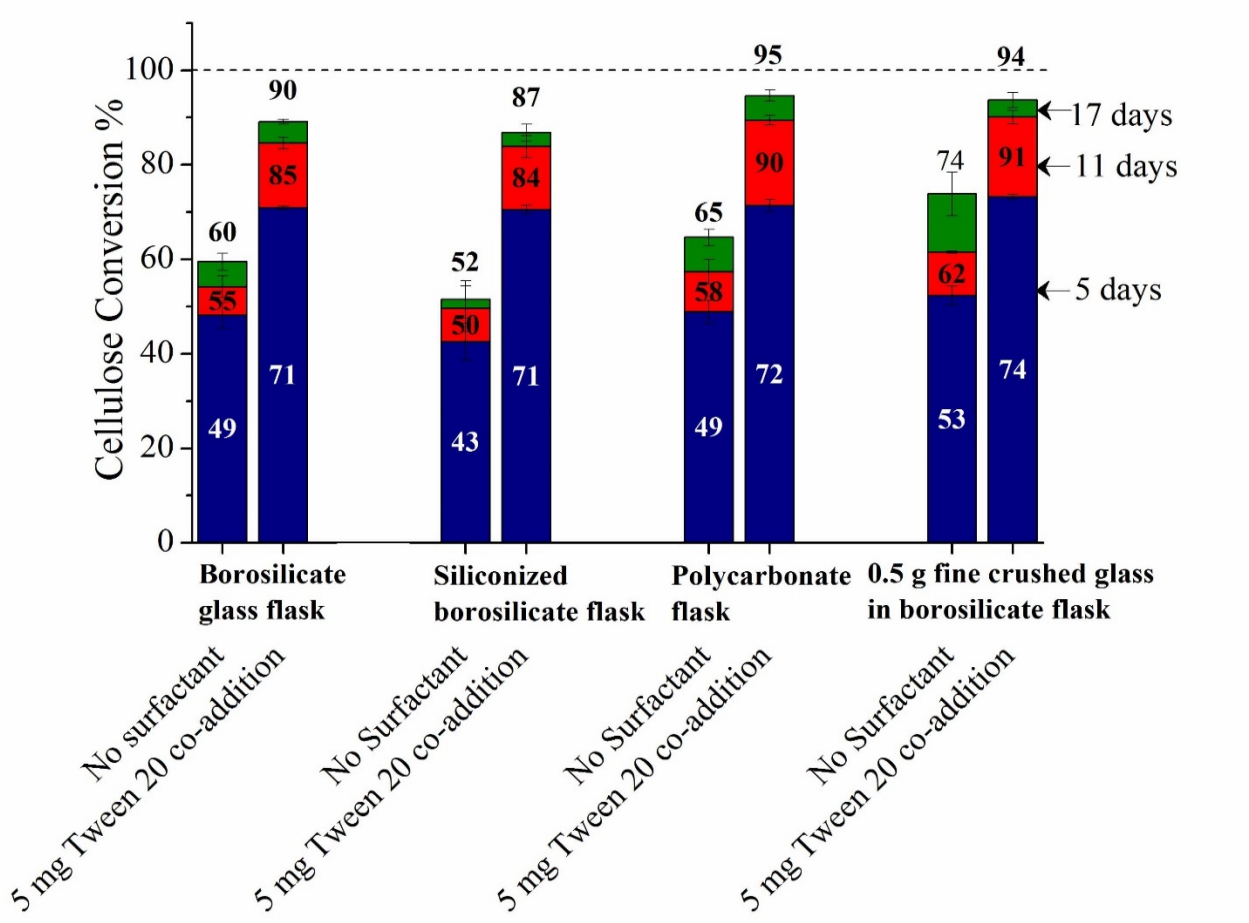

Figure 7.3 Effect of reaction vessel surface and shear on enzymatic hydrolysis of Avicel. Columns show cellulose conversion after 5, 11, and 17 days of enzymatic hydrolysis of Avicel (1% glucan loading) with 5 mg enzyme (Accellerase® 1500) and with co-addition of 5 mg Tween 20 added based on per gram glucan in substrate, in borosilicate glass flasks, siliconized glass flasks, polycarbonate flasks, and borosilicate glass flasks containing 0.5 grams of glass powder. Error bars represent standard deviation from three replicate flasks.

To evaluate the role of shear stress might, 0.5 g of fine glass powder, made by crushing a 20 ml glass serum vial with a hammer in a plastic bag, was added to the glass flasks before hydrolysis. Apart from increasing the shear stress, the powered glass greatly increased the contact area between enzyme and glass. Although it was expected that the greater shear stress resulting from shaking the glass powder would severely reduce cellulose conversion, conversion surprisingly increased from 60 to 74%. Furthermore, in the presence of Tween 20 with crushed glass, conversion increased even more to 94%. This outcome ruled out that Tween 20 reduced enzyme deactivation due to shear stress but showed these enzymes were robust even in the presence of sharp surfaces. Thus, it was hypothesized that the increase in cellulose conversions in the presence of crushed glass resulted from particle size reduction and consequently increased Avicel surface area. Reese and co-workers (E. T. Reese & Ryu, 1980), on the other hand, observed a large effect of shear stress, as their experimentation involved a flowthrough-type apparatus in which flow rate was changed to affect shear stress in glass capillary tubing so that shear rate could be estimated by the Hagen-Poiseuille equation(Sutera & Skalak, 1993). However, Erlenmeyer shaken flasks do not produce such high shear rates. Furthermore, the glass powder experiments above reveal these cellulases are robust even in the presence of moving sharp glass surfaces.

Since surfactants are surface-active compounds, experiments were performed without shaking. Figure 7.4 shows that cellulose conversion with surfactant and shaking was similar to cellulose conversion without shaking and without surfactant after 17 days, although the reaction rate was slower for the latter case. There was no effect of

surfactants on cellulose conversion without shaking. This result strongly indicated an air-liquid interface mechanism. The National Renewable Energy Laboratory (NREL) standard procedure "Enzymatic Saccharification of Lignocellulosic Biomass" (M. Selig, N. Weiss, & Y. Ji, 2008) mentions that the shaking rate (rpm) of the reaction flasks should be chosen to maintain solids suspension. Because we observed that Avicel at 1% loading in 50 ml reaction volume was suspended at around 125 rpm in a 125 ml Erlenmeyer flask, all of our shaking experiments were carried out at 150 rpm. However, the fact that cellulose conversions were higher without shaking than with shaking at 150 rpm at low enzyme loading strongly suggested that maintaining a suspension is not always beneficial for achieving high cellulose conversions. Flasks are shaken to improve mass-transfer and reduce localized sugar concentrations that otherwise can be inhibitory to enzymes. However, while shaking increases reaction rates, it also deactivates enzyme. Furthermore, activity loss by shaking should become more significant at low enzyme loadings, resulting in lower cellulose conversion at the longer reaction times needed at low loadings. On the other hand, this observation is not consistent with earlier proposed mechanisms that attribute activity loss to formation of a protein-surfactant complex (Elwyn T. Reese, 1980), protection against thermal deactivation, or less product inhibition as these possibilities should not be influenced by whether the reaction medium was shaken or not. Likewise, as hypothesized in substrate dependent mechanisms, enzyme immobilization due to jamming of cellulase (Castanon & Wilke, 1981), cellulose modification by change in pore size (Helle et al., 1993), or change in exoglucanase-endoglucanase adsorption ratio on cellulose (Ooshima et al., 1986) should affect cellulose

conversions in the presence of surfactants even if the flasks were not shaken. Based on the results that deactivation was much more severe when air-bubbles were entrapped in a flowthrough capillary column than by shear stress alone, Reese and co-workers (M. H. Kim et al., 1982) hypothesized that the air-liquid interface plays a critical role in cellulases deactivation.

Our results with conventional hydrolysis in Erlenmeyer flasks are consistent with the hypothesis that the air-liquid interface alone was decisive in cellulase deactivation. However, to further rule out substrate dependent mechanisms, Avicel cellulose was added to the reaction flasks five days after shaking was started with just enzymes present. Figure 7.4 shows that cellulose conversions with or without surfactants were lower for shaking flasks to which substrate was added after 5 days. This result is consistent with a significant fraction of enzymes losing activity due to environmental factors when there was no substrate present initially. On the other hand, if Avicel and enzymes are added together as in conventional shaking experiments, cellulase adsorption onto cellulose due to affinity of their carbohydrate binding module (CBM) reduces their bulk concentration, and hence less enzyme is deactivated at the air-liquid interface. Devoid of substrate, shaking exposes more enzyme to the surface where they deactivate, and even 5 mg of Tween 20 was unable to completely avoid such deactivation in absence of substrate.

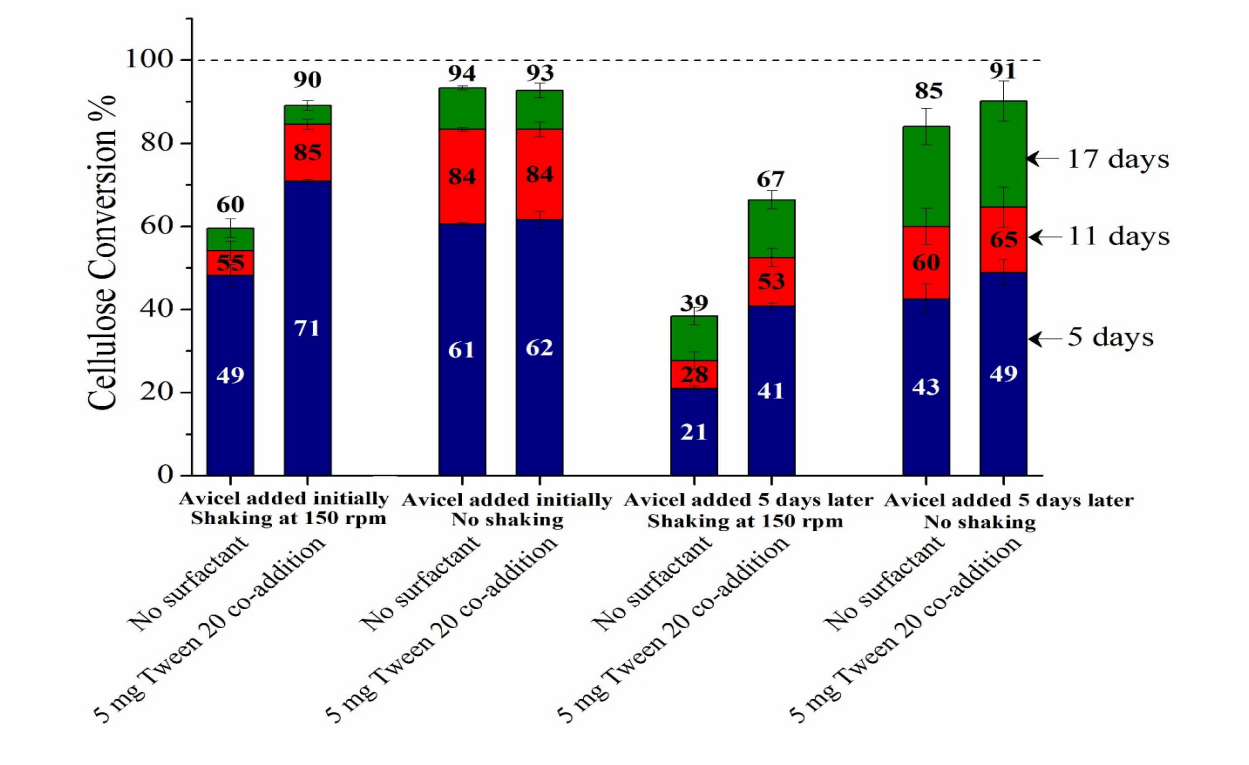

Figure 7.4 Effect of surfactant on enzymatic hydrolysis of Avicel with and without shaking. Columns show cellulose conversion after 5, 11, and 17 days of enzymatic hydrolysis of Avicel (1% glucan loading) with 5 mg enzyme (Accellerase® 1500) and with co-addition of 5 mg of Tween 20 added based on per gram glucan in the substrate, with and without shaking at 150 rpm. Avicel was either present initially or added 5 days after incubation of enzyme or enzyme with surfactant in buffer solution. For Avicel added 5 days later, days were counted after Avicel was added to the flask. Error bars represent standard deviation from three replicate flasks.

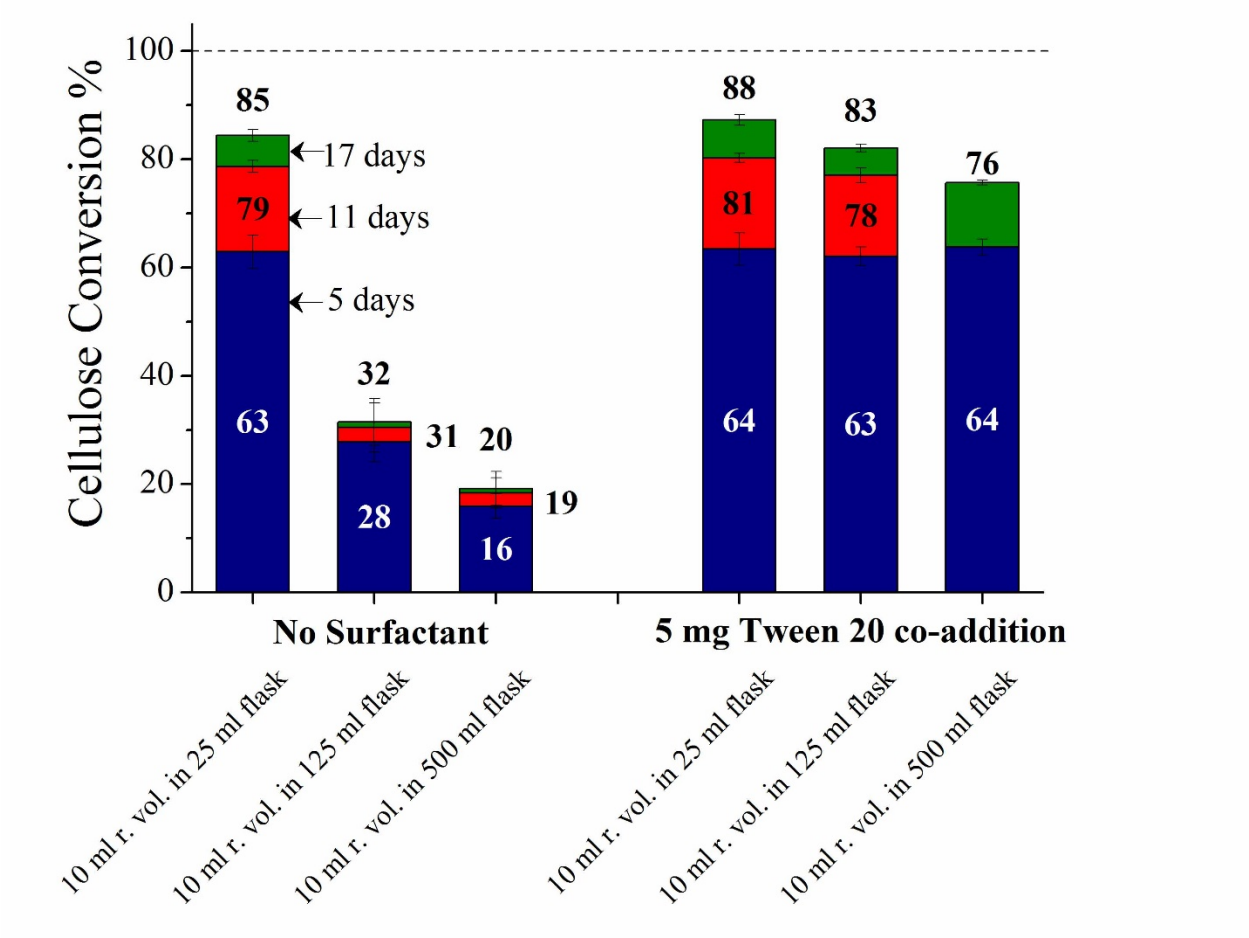

Figure 7.5 Effect of air-liquid interfacial area on enzymatic hydrolysis of Avicel with and without surfactant. Columns show cellulose conversion after 5, 11, and 17 days of enzymatic hydrolysis of Avicel (1% glucan loading) with 5 mg enzyme (Accellerase® 1500) and with co-addition of 5 mg of Tween 20 added based on per gram glucan in substrate. Reaction volume was kept constant at 10 ml, and reactions were performed in 25, 125, and 500 ml Erlenmeyer flasks. Error bars represent standard deviation from three replicate flasks.

Because changing the interfacial area should affect cellulose conversions if the air-liquid interface causes deactivation, straightforward experiments were run with different interfacial surface areas by keeping the reaction volume constant but changing the size of reaction vessel. The approximate ratio of static interfacial area for 10 ml reaction volume in Erlenmeyer flasks of capacity 25:125:500 ml was 1:3.2:5.8. Figure 5 shows that high

cellulose conversions were realized with low interfacial area and shaking (10 ml in 25 ml flask). Furthermore, when surfactant was added to a 10 ml reaction volume in a 500 ml reaction flask where the interfacial area was very high, cellulose conversions jumped up from 20 to 76%, clearly indicating that air-liquid interfacial area is the dominant cause of cellulase deactivation at low enzyme loadings. Also, considerable precipitation of solids was observed on walls of the 125 and 500 ml flasks that looked like a white ring at the highest point the liquid could reach in shaken flasks.

Why cellulase enzymes at low enzyme to substrate ratios cannot efficiently solubilize crystalline cellulose is a long-standing question. Based on published results including those by Whitaker et al.(Whitaker, 1952), Reese (Elwyn T. Reese, 1980), Ooshima et al.(Ooshima et al., 1986), Castanon and Wilke (Castanon & Wilke, 1981), and Helle et al.,(Helle et al., 1993) it wasn't clear which mechanism is the prime driver for lowering of cellulase deactivation by surfactant. In light of current results, it is worthwhile to delve into the past work by Reese and co-workers on interfacial deactivation of cellulase. Reese and Mandels first introduced shaking induced cellulase inactivation in a May 1979 paper (Elwyn T. Reese & Mandels, 1980) followed shortly by another paper(E. T. Reese & Ryu, 1980) by Reese and Ryu in October 1979 suggesting that shear may play the most important role in cellulase deactivation. In a March 1980 paper,(Elwyn T. Reese, 1980) Reese proposed that shear and surface denaturation was minimized by a protein-surfactant complex that resists changes in protein confirmations. This paper was followed by a conference proceeding (June-July 1980) chapter (Elwyn T. Reese, 1982) that hypothesized three possibilities: 1) surfactants prevented shear-induced conformational

change or aided in refolding of cellulase to native state; 2) shaking made it easier for proteases to change the cellulase confirmation; and 3) inactivation due to shaking is a surface related phenomenon. In their last article in June 1981 (M. H. Kim et al., 1982), Reese and co-workers showed far more severe cellulase deactivation in the presence of air bubbles than from shear stress alone in capillary tubing. However, the issue of cellulase deactivation and the role of surfactants took another turn when a few years later Sakata, Ooshima, and Harano (1985) challenged Reese's results by showing no difference in rate of reaction or final conversion of Avicel cellulose by *Trichoderma viride* cellulase contained in 75 ml to 150 ml reaction volumes while stirring at 500 rpm at pH 4.8 and 40 °C in a cylindrical reactor. However, because the 5 wt% Avicel with 1 mg/ml cellulase used was equivalent to a high enzyme loading of 20 mg enzyme per g cellulose, their experiment did not show any difference in cellulose conversion with changing reaction volume. Next, Ooshima et al. in 1986 hypothesized that surfactants change the exoglucanase-endoglucanase ratio, while Castanon and Wilke (Castanon & Wilke, 1981) in 1981 and Helle et al.(Helle et al., 1993) in 1993 indicated that surfactants reduce enzyme immobilization to cellulose. Thus, further research was needed to build upon these findings for better understanding of cellulase deactivation. In this work, the use of experimental approaches far different from those applied by Reese in combination with conventional laboratory reactors (Erlenmeyer flasks) leave little doubt that deactivation of enzymes at the air-liquid interface are the primary cause of incomplete cellulose conversion at low enzyme loadings, consistent with the possible hypothesis first proposed by Reese and Mandels in 1979.

Proteins are known to deactivate at the air-liquid interface to minimize surface excess (James & Augenstein, 1966). They can unfold and expose their hydrophobic groups to the gas phase that were previously buried inside their tertiary structure in bulk aqueous solution. Exclusion of hydrophobic regions from the aqueous phase decreases the free energy that drives their adsorption in the interfacial layer (James & Augenstein, 1966; Narsimhan & Uraizee, 1992). Because cellulases have a catalytic core and a carbohydrate binding module (CBM) joined together by a linker region (Van Tilbeurgh, Tomme, Claeyssens, Bhikhabhai, & Pettersson, 1986), it is possible for hydrophobic platform of CBM that binds to cellulose (Boraston, Bolam, Gilbert, & Davies, 2004) to orient itself toward the air phase and catalytic core toward the aqueous phase at the air-water interface. In this case, reorientation of the enzyme may make cellulase deactivation reversible. On the other hand, partial unfolding the cellulase that exposes the hydrophobic region in the active site of the catalytic core to the gas phase may be irreversible. Precipitation seen in large flasks at the low reaction volume suggests enzymes underwent aggregation in the interfacial layer. Such aggregations occur due to intermolecular hydrophobic interactions after their unfolding and is known to be irreversible for many proteins (Treuheit, Kosky, & Brems, 2002). Dr. Reese also showed that cellulase deactivation was irreversible and found precipitation of protein in shaken flasks (Elwyn T. Reese, 1982). Therefore, it is more likely that the catalytic core undergoes unfolding rather than reorientation of CBM towards the gas phase.

Without shaking at an enzyme loading of 5 mg, the amount of enzyme exposed to the interfacial surface where it can be deactivated is too low to drop cellulose solubilization.

However, shaking the flask at the same enzyme loading allows displacement of deactivated enzyme in this layer by active enzyme arriving from bulk solution, eventually making the ratio of deactivated to active enzyme large enough to affect cellulose conversion and reaction rate. Surfactants, due to their higher surface activity than cellulases can form a network of surface domains at the interface (Gunning et al., 2004), thereby reducing the amount of surface available for enzymes and the accompanying deactivation. This network was apparently stable in shaken flasks at conditions tested in this study. At high enzyme loadings, the amount of active enzyme is so large that most of the cellulose is hydrolyzed before enough enzyme can be deactivated to affect conversion. On the other hand, it is possible that unfolded enzymes initially adsorbed in the interfacial layer form a barrier (Lad, Birembaut, Matthew, Frazier, & Green, 2006) and that prevents further enzyme deactivation while leaving enough enzymes in solutions at high loadings to still realize high conversions.

To further understand the responsible mechanism, enzymatic hydrolysis of lignocellulosic biomass was studied at low enzyme loading relevant to achieve low-cost sugar production for conversion to biofuels and renewable chemicals. As stated earlier, lignin can adsorb enzyme, and surfactants or proteins such as BSA block the lignin surface from adsorbing enzyme. Therefore, dilute acid (DA) and CELF pretreatments were applied to BESC standard poplar solids to leave high and low amounts of lignin in the pretreated solids, respectively. In particular, dilute acid pretreated poplar solids contained 64% glucan, 2% xylan, and 28% Klason lignin (acid insoluble lignin) while solids from CELF pretreatment of poplar contained 90% glucan, 3% xylan and 4%

Klason lignin. A significant increase in enzymatic hydrolysis of the cellulose in DA pretreated poplar was only evident when a large amount of surfactant (100 mg Tween 20) was added, consistent with surfactant blocking the lignin surface from adsorbing enzyme. However, in the CELF pretreated poplar solids containing much less lignin, addition of just a small amount of surfactant (5 mg Tween 20) increased the yield from 55 to 85%. It is important to note that hydrolysis yields after 11 days of reaction without shaking and without surfactant were similar to those from shaking combined with a low amount of surfactant for low-lignin lignocellulosic biomass but with the drawback of slower reaction rate, similar to the results seen with Avicel cellulose. Enzyme added to a mixture of lignocellulosic biomass can bind to cellulose, lignin, or hemicellulose but to a lesser extent, remain in bulk solution, reside at the interface to lower the free energy of the gas-liquid interface, and bind in limited quantities to the solid-liquid interface. Furthermore, the concentration of enzyme on the cellulose surface, in the bulk solution, and at the interface change with time reaction. When low amounts of a surface-active additive are added to biomass solids with high hydrophobic lignin content such as that from dilute acid pretreatment of poplar for hydrolysis at low enzyme concentrations, most of the surfactant is bound to lignin, with little left at the interface. As a result, enzymes still deactivate at the gas-liquid interfacial phase boundary, and the slight reduction in unproductive binding of enzyme to lignin has a miniscule effect on cellulose conversion due to the fractional blocking of lignin by surfactant. On the other hand, biomass solids with low lignin content produced by CELF pretreatment offer a large number of cellulose binding sites while limiting the amount of lignin available for unproductive enzyme

binding. Thus, at low enzyme concentrations, addition of surfactant or absence of shaking reduces interfacial deactivation of enzymes and increases active enzyme concentrations that allow large increase in cellulose conversion, similar to that seen for Avicel cellulose. Moreover, this mechanism explains why BSA supplementation generally increased sugar yields from enzymatic hydrolysis of flowthrough pretreated poplar solids with lower lignin content than batch pretreated poplar solids with higher lignin content,(Bhagia et al., 2017) contrary to the expectation that blocking of lignin by BSA would have had a larger effect on cellulose conversion of batch pretreated poplar.

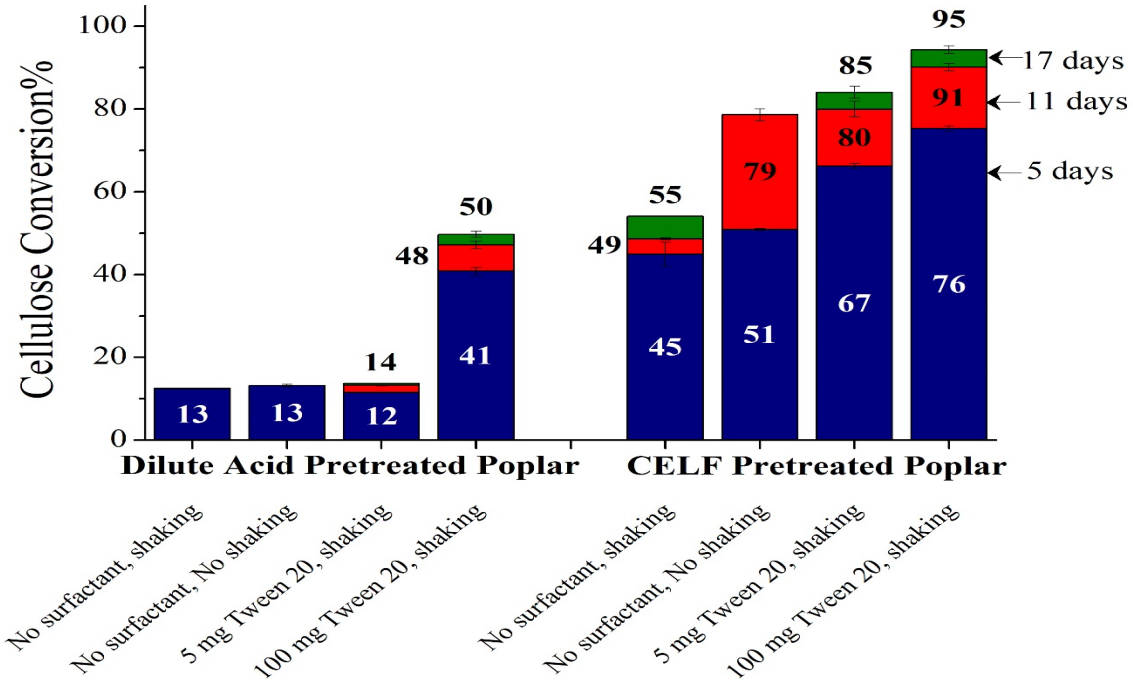

Figure 7.6 Effect of surfactant and shaking on enzymatic hydrolysis of dilute acid and CELF pretreated poplar. Columns show cellulose conversion after 5, 11, and 17 days of enzymatic hydrolysis of dilute acid (0.5% H_2SO_4) and CELF (0.5% H_2SO_4 in 1:1 H_2O:THF) pretreated poplar (1% glucan loading) with 5 mg enzyme (Accellerase® 1500) with and without shaking at 150 rpm, and with co-addition of 5 mg or 100 mg of Tween 20 added based on per gram glucan in substrate. Error bars represent standard deviation from three replicate flasks.

The high foaming of globular protein BSA (Graham & Phillips, 1979) results in strikingly similar effects to the surfactant Tween 20. BSA lowers the surface tension of water from 72.5 to about 50 ergs/cm^2 (Absolom, Van Oss, Zingg, & Neumann, 1981). Like BSA, human serum albumin (HSA) is also highly surface active, and accumulates at the air-liquid interface in a denatured but reversible state. These albumins and surfactants orient their hydrophobic moieties towards the air (gas) phase to reduce solution surface tension (Docoslis, Giese, & van Oss, 2000). Additives such as polyethylene glycols and polypropylene glycols lower surface tension of water, and their surface activity increases with concentration and degree of polymerization (Schwuger, 1973). The earlier use of term "amphiphilic additive" in this work could alternatively be labeled as "surface-active additives" or "surface-active agents" as they reduce cellulase deactivation at the air-liquid interface.

In the natural environment of wood-rot fungi, small concentrations of extracellular cellulases are capable of slowly degrading cellulose in wood over the course of several weeks without shaking as applied in laboratory practice (Wilcox, 1970). On the other hand, commercial enzyme preparations consist of *T. reesei* secretome supplemented with *β*-glucosidase as the fungus does not naturally produce enough *β*-glucosidase to convert cellobiose to glucose fast enough to prevent cellulose inhibition (García-Aparicio et al., 2006; R. Kumar & Wyman, 2009b, 2009c). None of our extensive experiments showed increased cellobiose concentrations that would indicate *β*-glucosidase deactivation at the interface. However, proteomic analysis of the commercial cellulase preparations used for deconstruction of lignocellulosic biomass show a broad array of enzyme activities:

roughly 40% cellobiohydrolases, 20% endoglucanases, 15% β-glucosidases, 5% endoxylanases, 6 to 10% xyloglucanases, less than 2% each of acetyl xylan esterases, α-arabinofuranosidases, α-glucuronosidase, and 10 to 15% of non-cellulolytic enzymes and low-abundance glycosyl hydrolases (Chundawat et al., 2011). Two studies indicated that cellobiohydrolase II (CBHII; also known as exoglucanase II or Cel6A), maybe susceptible to deactivation. A 1997 article (D. Kim, Jang, Jeong, & Son, 1997) suggested that CBH II immobilization on cellulose is reduced by Tween 20, but their preparation had 90% CBH II from *T. viride*. Another recent article (Okino, Ikeo, Ueno, & Taneda, 2013) indicated that CBH II is only stabilized by Tween 80 at 30°C under agitation conditions, but despite finding agitation to be a decisive factor, the authors did not offer a mechanism that could explain this effect. These two studies provide preliminary evidence that CBH II might be the component in enzyme cocktail that is affected, but, in any case, the reason for its deactivation is unfolding at the interface to minimize surface tension for achieving thermodynamic stability. However, commercial enzyme preparations contain higher amounts of CBH I than CBH II, and a relatively high amount of endoglucanase I (EG I) (Chundawat et al., 2011). Since cellulose conversions were at least 40 percentage points lower after 17 days when enzymes could liberally move to the interfacial layer and deactivate in the absence of surfactant, this level of loss in cellulose conversion indicates CBH I and EG I, and not just CBH II, may also be susceptible to deactivation. At this point, it is uncertain which component is deactivated most at the interface as it is difficult to obtain pure enzymes in the amounts needed for such a study. A further improvement can be made in targeting mass loadings of surfactant by determining its critical micelle

concentration (CMC) in the hydrolysis environment. CMC can be affected by substrate and enzyme concentration, pH, ionic strength due to buffer and antibiotic, temperature, and time-dependent glucose concentration in the liquid from reaction of solid substrate. A concentration that realizes a minimum in surface tension is required, beyond which, the it will only accumulate as micelles in the bulk solution and increase the cost of hydrolysis of lignin-free cellulosic substrate at low enzyme loading.

Cellulases appear to fall in the category of air-sensitive proteins like lysozyme and BSA and peptides such as insulin (Sluzky, Klibanov, & Langer, 1992) and recombinant human growth hormone (rhGH) (Y.-F. Maa, Nguyen, & Hsu, 1998; Y. F. Maa & Hsu, 1997) and need to be further explored. Since enzymes stock solutions are almost always shaken to evenly distribute them before taking aliquots for experiments, adding a small quantity of non-ionic or biological surface-active agent to current commercial enzyme preparations might improve enzyme shelf-life. These experiments show that cellulases deactivate at the air-liquid interface to lower free energy, resulting in incomplete Avicel hydrolysis at low enzyme loading. Thus, the results in this paper showed that of ten possible mechanisms, the one first proposed by Reese and co-workers in 1980s is primarily responsible for cellulase deactivation at low enzyme loadings. Moreover, cellulase deactivation at the air-liquid interface significantly reduced hydrolysis of cellulose in solids with low lignin content at low enzyme loadings in addition to pure cellulose. As a result, simply reducing the air-liquid interface of the cellulose-cellulase system through selection of the proper size glassware for shaking can dramatically improve cellulose conversion at low enzyme loadings. And adding small amounts of surface-active agents

that do not denature the enzyme and are compatible with downstream fermentation microbes could significantly lower activity loss by reducing cellulase accumulation at the air-liquid interface.

7.5 Acknowledgements

We are grateful for funding by the Office of Biological and Environmental Research in the Department of Energy (DOE) Office of Science through the BioEnergy Science Center (BESC) at Oak Ridge National Laboratory (Contract DE-PS02-06ER64304). We also acknowledge the Ford Motor Company for funding the Chair in Environmental Engineering that facilitates projects such as this one and the Center for Environmental Research and Technology (CE-CERT) of the Bourns College of Engineering for providing the facilities.

7.6 Competing Financial Interests

CEW is a cofounder of Mascoma Corporation and former chair of their Scientific Advisory Board. CEW is also founding Editor-in-Chief of *Biotechnology for Biofuels*. The other authors declare that they have no competing interests.

Data Availability

All data generated or analyzed during this study are included in this published article.

7.7 References

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Chapter 8

Conclusions and Recommendations

8.1 Summary of Key Developments and Findings

Pretreatments followed by enzymatic hydrolysis and fermentations or consolidated biomass processing were applied to BESC STD poplar. Chapter 2 reviewed factors influencing biological pretreatment of lignocellulosic biomass that could be used alone or in combination with other pretreatments. Conditions for biological pretreatment to be successful include the incubation time for microorganisms, which could result in delay in delignification of the biomass (Isroi et al., 2011). An appropriate moisture content for fungal growth during pretreatment is very important and assists in lignin degradation as well as the activity of white rot fungi (Fujian, Hongzhang, & Zuohu, 2001; Shi, Chinn, & Sharma-Shivappa, 2008). Other factors discussed in this chapter are pH and particle size influencing fungal cultivation and limiting the penetration of fungi, respectively (van Kuijk, Sonnenberg, Baars, Hendriks, & Cone, 2015). A combination of pretreatments could be beneficial, due to longer time intervals of needed for pretreatment to be effective for enzymatic hydrolysis (Wei Wang, Yuan, Wang, Cui, & Dai, 2012).

Chapter 3 defines CELF pretreatment conditions to maximize total sugar yields from BESC STD poplar. Reducing the lignin content with conservation of fermentable sugars is shown to be desirable. Co-solvent enhanced lignocellulosic biomass fractionation (CELF) pretreatment uses THF as a biomass sourced green solvent to open up poplar wood for enzyme accessibility. Application of CELF pretreatment over a range of pretreatment times followed by subsequent enzymatic hydrolysis was used to identify the maximum total sugar yielding conditions for BESC STD poplar. CELF pretreatment conditions at 160°C for 15 min resulted in total sugar yields of 100 % at enzyme loading

of 15 mg/g glucan in raw biomass while dilute acid pretreatment could not reach a 100 % yield even at a very high enzyme dosage of 100 mg/g glucan in raw biomass. It can be concluded that high lignin removal of about 90 % at the identified conditions for CELF pretreatment were very beneficial to realizing high yields. Another insight from this study was ability of CELF pretreated biomass to provide high sugar concentrations desired for ethanol production at reduced enzyme dosages on recalcitrant woods.

Chapter 4 compares maximum total sugar yielding conditions for CELF pretreatment defined in Chapter 3 to the more traditional ethanol organosolv (EO) pretreatment of BESC STD poplar. The maximum total sugar yielding conditions using ethanol organosolv pretreatment were identified as 185°C for a reaction time of 15 min that resulted in 100% hemicellulose removal. Thus, EO required higher temperature than CELF pretreatment (160°C 15 min) to be as effective as possible. A major drawback of ethanol organosolv pretreatment was the high loss of solids during pretreatment in comparison to CELF pretreatment. Generation of degradation products including 5-HMF, furfurals, and organic acids not seen in CELF pretreatment with EO resulted in loss of fermentable sugars. The composition of solids produced after CELF pretreatment had 3-4 % lignin, whereas the solids produced after EO pretreatment had 9-10 % lignin, reducing accessibility of biomass for hydrolysis. Success of any pretreatment is highly dependent on glucan conservation of biomass. EO had lower sugar yields and the lower lignin removal from EO pretreated biomass hindered hydrolysis more than CELF pretreatment.

Chapter 5 demonstrated the ability of CELF pretreated BESC STD poplar solids to effectively process high loadings of glucan for enzymatic hydrolysis. Lowering the cost

of ethanol production involves production of high sugar titers (Jin et al., 2017). The key driving factor in this study was the glucan rich BESC STD poplar produced by co-solvent enhanced lignocellulosic fractionation (CELF) with total high yields observed in the previous chapter (Chapter 3) at comparatively lower enzyme loadings. This study was successful in achieving high ethanol titers when appropriate feeding strategy was applied for a 20 wt% glucan loading (20 g glucan dry basis) along with reducing the hydrolysis time to 7 days. The selection of an appropriate feeding strategy to overcome mixing limitations at high glucan loadings was important for effective enzymatic hydrolysis that is generally limited by the mass transfer issues and high viscosity (Modenbach & Nokes, 2013). This study concluded fed-batch feeding of CELF pretreated BESC STD poplar resulted in high ethanol titers using separate hydrolysis and fermentation (SHF). This study could provide a foundation for further investigating and optimizing conditions to reach high ethanol titers by microorganism capable of glucan and xylan fermentations. In Chapter 6, hydrothermal pretreatment of BESC STD poplar is applied in combination with CBP. Hydrothermal pretreatment has the advantage of not requiring addition of chemicals, but yields from coupled pretreatment and enzymatic hydrolysis are too low, particularly at economically viable enzyme loadings. Consolidated bioprocessing by the organism *C. thermocellum* that deconstructs cellulosic biomass in one step without addition of external enzymes has been shown to be more effective than fungal enzymes (Olson, McBride, Joe Shaw, & Lynd, 2012). And genetic tools are being developed to enhance the yields of ethanol (Lu, Zhang, & Lynd, 2006).Thus, CBP was applied to hydrothermally pretreated BESC STD poplar to define maximum total sugar yielding

pretreatment conditions. The study revealed that hydrothermal pretreatment of BESC STD poplar at 190°C for 25 min in combination with CBP resulted in the highest total sugar release of close to 88%. The pretreatmnet conditions were further applied to BESC varieties to determine yields using CBP. Compariosn of hydrothermal pretreatment and CBP as well as HT pretreatment and enzymatic hydrolysis yields at low enzyme loading show much higher yields from CBP that should be attractive for low cost ethanol production (L. R. Lynd, P. J. Weimer, W. H. van Zyl, & I. S. Pretorius, 2002).

In Chapter 7, pretreated BESC STD poplar was supplied for investigation of the deactivation of the cellulases at the air-liquid interface leading to incomplete cellulose hydrolysis. CELF and DA pretreated BESC STD poplar were provided to support determination of the effect of lignin after pretreatment on hydrolysis. This study showed that large amounts of surfactant additions were needed to enhance enzymatic hydrolysis of DA pretreated poplar.

8.2 Novelty and Broader Impact

Organic solvents have long been applied to lignocellulosic biomass to break lignin and hemicellulose bonds, separate the components, and produce high sugar yields. Fundamentally the process involves the use of high temperatures wih long pretreatment times to achive the desired glucan rich biomass for further steps. These high temperatures result in degradation of the fermentable sugars to undesired furfurals and acids, making the overall process less economical. Pretreatment needs to be less cumbersome and able to recover maximum fermentable sugars for further processing steps. Achieving low lignin content after pretreatment of lignocellulosic biomass using CELF pretreatment

addresses a major challenge in the area of ethanol fuel production significantly impacting the cost of biomass processing. The next study compared the results using THF as a solvent for pretreatment with subsequent enzymatic hydrolysis to the commonly used ethanol pretreatment where CELF outperformed EO in various aspects, further strengthening the novelty of CELF pretreatment. Application of CELF at high loadings during pretreatment and subsequent enzymatic hydrolysis resulted in high sugar and ethanol titers that could have broad impacts on ethanol production. Another chapter of this dissertation showed consolidated biomass processing (CBP) with *C. thermocellum* was highly effective on hydrothermally pretreated poplar and performed much better than fungal enzymes, particularly at lower enzyme loadings. This study gives an insight on the CBP method as one step solution without addition of enzymes for producing high sugar titers using microbe that can hydrolyze C5 and C6 sugars at the same time.

8.3 Closing comments

Overcoming challenges to ethanol production essentially requires use of lower enzyme loadings during hydrolysis. Selecting appropriate pretreatment conditions are important to conserve the sugars and improve their concentration to lower the overall cost of ethanol fuel production. The selection of most pretreatment condition depends on various factors such as the composution of biomass and total sugar yields possible after pretreatment and subsequent hydrolysis or CBP. Different feedstock have different structures leading to difference in yields but choosing a feedstock widely available in nature and reproducing the expected industrial conditions. It would be valuable to further test pretreatment conditions on various poplar varities grown under different

environmental conditions or genitically modified to enhance yields. The impact of lignin in the biomass on performance was confirmed where dilute acid pretreated poplar with high lignin content when subjected surfactant addition resulted in higher yields in comparison to low lignin CELF pretreated poplar. These results enforce the idea of lower lignin favoring higher yields from enzymatic hydrolysis due to less enzyme binding to lignin. Hence, producing glucan rich biomass with lower lignin by pretreatment can assist in lowering enzyme dosage and increasing sugar titers from enzymatic hydrolysis. In future, more work is needed to develop pretreatments that remove lignin from biomass and understand how pretreatment and enzymatic hydrolysis enhance performance.

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