UNIVERSITY OF CALIFORNIA
RIVERSIDE

Conversion of Poplar Carbohydrates and Lignin into Fuel Ethanol and Polyurethane Following Co-Solvent Pretreatment

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

Doctor of Philosophy

in

Chemical and Environmental Engineering

by

Priya Sengupta

September 2020

Dissertation Committee:
Dr. Charles E. Wyman, Chairperson
Dr. Ian R. Wheeldon
Dr. Charles M. Cai

The Dissertation of Priya Sengupta is approved:

_____

_____

_____

Committee Chairperson

University of California, Riverside

ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Charles E. Wyman for all the help, support and guidance during my years as a graduate student in his group. His unwavering enthusiasm for science has been a constant source of inspiration and encouragement for me. With his vast experience and knowledge in the field, Dr. Wyman has been like a "Google for Biofuels". I have thoroughly enjoyed working with him and I'll be forever indebted to him for all the wisdom and invaluable life lessons that he has imparted to me. I also take this opportunity to thank Dr. Charles Cai for his wisdom and guidance throughout my project. I would like to express my sincere gratitude towards Dr. Ian Wheeldon for mentoring me and for his insights and suggestions to help me with my thesis research. Additionally, I would like to thank Dr. Eugene A. Nothnagel, Dr. Arthur J. Ragauskas and Dr. David Kisailus for their assistance and suggestions throughout my project.

My appreciation also extends to the former lab members Dr. Abhishek Patri, Dr. Christian Alcaraz, Dr. Ninad Kothari and Dr. Rachna Dhir for teaching me the necessary skills to carry out my research. I would like to thank my collaborators Dr. Yun-Yan Wang and Ramya Mohan for all the help with the project milestone completion. A special mention goes to Priyanka Singh, the co-founder of the P.S. Squad, for her constant love and support during our time together in Wyman group. I would also like to thank my undergraduate students Alex Moore and Matthew Ealy who contributed greatly to various parts of my dissertation, especially with the lignin portion of my research.

I express my deepest gratitude to the CE-CERT staff members Carolyn Ray, Alta Vasquez, Kathy Vang, Valerie Thomas, Berenice Murillo-Quintana and Todd Ambriz for making life so much easy and fun at work.

I would like to thank my friends Isaac Afreh, Sooyoun Yu, Saba Baraghani, Chithra Asokan, Sushmit Poojary, Carola Acurio, Unnati Rao, Aakash Parikh for all the love and support that had made life so much fun away from home. I would also like to express my deepest gratitude to Dr. C. Balomajumder (Prof., IIT Roorkee) for always encouraging me and believing in me. A huge shout out to my family, my sister, Jaya Sengupta, my brother-in-law, Sujoy Sen, my niece Koel Sen and my parents, Jauhar Sen Gupta and Savita Sen Gupta for their constant love, support and encouragement and for pushing me farther than I thought I could go. Finally, a special mention for a special someone, I would like to thank Dr. Rajasekhar Anguluri for his wisdom, understanding, patience, enthusiasm, encouragement and for not letting me give up.

Your unconditional support and love throughout the past five years have been fundamental to my growth as a person and a researcher.

I would like to acknowledge the Department of Chemical and Environmental Engineering and the Center for Environmental Research and Technology (CE-CERT) in the Bourns College of Engineering at the University of California for allowing me to pursue my graduate research towards a Doctor of Philosophy degree. I would also like to thank The Biomass Research and Development Initiative (BRDI) program of the National Institute of Food and Agriculture (NIFA), United States Department of Agriculture, and the National Center for Sustainable Transportation (NCST) for their funding that allowed

me to pursue my research. Further, I acknowledge the Ford Motor Company for funding the Chair in Environmental Engineering that facilitates projects such as this one.

DEDICATION

To all the lives lost to COVID-19. May their souls rest in peace.

ABSTRACT OF THE DISSERTATION

Conversion of Poplar Carbohydrates and Lignin into Fuel Ethanol and Polyurethane Following Co-Solvent Pretreatment

by

Priya Sengupta

Doctor of Philosophy, Graduate Program in Chemical and Environmental Engineering
University of California, Riverside, September 2020
Dr. Charles E. Wyman, Chairperson

Lignocellulosic biomass provides a low cost and abundant resource for production of cellulosic ethanol for use as a fuel octane booster and a low-carbon standalone transportation fuel. However, native plant polysaccharides and lignin are recalcitrant to biological conversion due to plant's natural resistance to pathogen invasion. Numerous pretreatments have been developed to overcome biomass recalcitrance, but the solids produced require heavy doses of costly enzymes to breakdown polysaccharides into sugars. In addition, reaching industrial relevant ethanol titers in biological conversion processes has been challenging due product yield limitations at the associated high solids

concentrations. Co-Solvent Enhanced Lignocellulosic Fractionation (CELF) is a new pretreatment that applies a mixture of tetrahydrofuran (THF) with water and very dilute acid to extract most of the lignin and solubilize a high portion of the hemicellulose to leave a solid concentrated in glucan that is highly susceptible to enzymatic breakdown into fermentable sugars with low enzyme doses. In addition, extracted high quality lignin could be converted into valuable building blocks for biopolymer synthesis.

In this thesis, CELF pretreatment conditions were defined to maximize sugar yields and lignin recovery from hardwood poplar for the combined operations of CELF pretreatment and subsequent fungal enzyme hydrolysis. Fungal enzyme digestion of solids resulting from CELF pretreatment and characterization of the original poplar and those solids by FTIR, XRD, SEM, and Simon Staining revealed that the lignin left in CELF pretreated biomass profoundly impacted yields from enzymatic hydrolysis of CELF substrates. High solids Simultaneous Saccharification and Fermentation (SSF) of CELF pretreated poplar wood was carried out at a 15 mg protein/g glucan enzyme dose coupled with fermentation of the glucose released by *S. cerevisiae* variant D5A at 20 wt% solids loading to produce 87 g/L of ethanol in 7 days, 79% of the maximum possible theoretical yield. Fractal kinetic modeling of the enzymatic saccharification data revealed that CELF solids did not require an enzyme loading >15 mg protein/g glucan to improve the saccharification efficiency. Simultaneous Saccharification and Co-Fermentation (SSCF) using a recombinant yeast strain was employed at a 1 L volume to produce 72 g/L of ethanol (72% theoretical yield) at a 17 wt% solid loading in a 3 L bioreactor. In addition, polyurethanes were successfully synthesized from the CELF solubilized lignin recovered

when THF was removed from the CELF liquid hydrolysate. Finally, a technoeconomic model was developed to estimate process economics and identify opportunities for further improvements. These results indicate that ethanol production can be most cost-effective if all the sugars in the raw biomass are effectively utilized.

Table of Contents

List of Figures

List of Additional Figures

List of Tables

List of Additional Tables

Chapter 1 : Introduction

1.1 Lignocellulosic biomass: a pragmatic resource towards low carbon/ carbon neutral transportation fuel

Increasing human population and standards of living have rapidly increased energy demand, e.g., the BP Statistical Review of World Energy, 2019 reported that *"Primary energy consumption grew at a rate of 2.9% in 2018 that led to the growth of carbon emissions by 2.0%, the fastest growth since 2010."*(BP and Outlook 2019) Fossil-derived fuels, the key source of world energy, are not only non-renewable and insufficient to meet the ever increasing energy call but also present serious environmental concerns. (Pareto and Pareto 2011) Burning fossil fuel over the years has increased carbon levels in the atmosphere that cause global warming currently being experienced as worldwide climate change. In particular, carbon dioxide levels of 414 ppm in the air are at their highest in 650,000 years, the latest annual average temperature anomaly was recently 0.9 °C, the Earth's polar sheets are losing mass, and global average sea levels have risen by 178 mm over the past 100 years, as reported by NASA's Goddard Institute of Space Studies.(Anon n.d.)

The world is now facing a dual challenge of needing more energy to continue to improve global living standards and simultaneously reduce carbon emissions to curb global climate change and its aftermath. Hence, the worldwide energy system infrastructure crucially needs to capture and use greater amounts of renewable energy sources such as biomass, wind, solar, hydrothermal, and geothermal to take the burden off use of petroleum and lower greenhouse gas emissions. Alternative energy must also be implemented in all current petroleum end-use sectors: transportation, industrial, residential, commercial, and electric power. The transportation sector, however, is the largest consumer of petroleum

and the largest contributor to greenhouse gas emissions in United States. (OAR US EPA n.d.) Therefore, developing sustainable and carbon neutral (in terms of terrestrial-atmospheric carbon flux) alternative liquid transportation fuels is of utmost importance.

10% ethanol blended gasoline (E10) is an already available alternative that can be used in existing gasoline powered engines in the United States, and newer cars are mechanically compatible with up to 15% Ethanol blended gasoline (E15). A limited number of flexible fuel vehicles that can run on any combination of ethanol and gasoline up to 85% ethanol (E85) have been in operation for some time.(Anon n.d.) Currently, most ethanol in the U.S. is derived from the starch in corn or other high starch grains and is called first generation technology. Although corn-based ethanol reduces greenhouse gas emissions some compared to gasoline, it raises issues such as increased pressure on corn (food crop) prices and land-use change. (Naik et al. 2010) Additionally, corn production causes more soil erosion than many other crops and requires more herbicides, insecticides, and nitrogen fertilizers that cause groundwater and surface water pollution. (Pimentel, Patzek, and Cecil 2007; Tiffany 2009)

Lignocellulosic biomass i.e., plant dry matter in the form of agricultural, forestry, residential, and industrial residues and energy crops is plentiful, low in cost, and potentially plentiful resource to produce second generation cellulosic ethanol. (Demirbas 2009) Plants are a rich source of carbon in the form of cellulose and hemicellulose sugars that can be broken down into their monomers for fermentation into ethanol via various biological pathways. Second generation cellulosic ethanol avoids the food vs fuel issue for corn and

other food crops and contributes less GHG emissions due to a lower carbon footprint compared to the corn-based ethanol. (Tiffany 2009)

1.2 Lignin-based bioplastics: strengthening the bio based economy

Another environmental issue is pollution caused by use of petroleum based, single-use plastics. These polymers have a very long shelf-life and currently are major contributors to municipal solid waste within the United States and worldwide marine debris.(OLEM US EPA n.d.) They present both a physical (e.g., entanglement, gastrointestinal blockage, reef destruction), and chemical (e.g., bioaccumulation of chemicals from plastics) threat to wildlife and the marine ecosystems.(Hopewell, Dvorak, and Kosior n.d.; Thompson et al. n.d.; OW US EPA n.d.)

Lignin, one of the most abundant biopolymers on the planet, is a major component of plant cell walls that provides structural integrity to the plant and resistance to biological attack. It is a by-product of pulp and paper and existing biorefineries and has been historically undervalued. Being the largest available source of renewable aromatics, lignin becomes a promising resource for production of phenolic monomers now made from petroleum for conversion to plastics. Lignin based materials could not only serve as a sustainable and eco-friendly resource for the manufacture of renewable chemicals and biopolymers but it could also be used in the production of advanced biofuels. Additionally, value-added co-products from lignin could significantly allow the production of transportation fuels from biomass sugars more cost-competitive. (Calvo-Flores and Dobado 2010; Ragauskas et al. 2014)

1.3 Lignocellulosic biomass: an attractive but challenging substrate for biological conversion into cellulosic ethanol and synthesizing bio-plastics

Although there are many pathways for converting biomass into liquid fuels, this dissertation focused on biological conversion of the polysaccharides in lignocellulosic biomass into ethanol. Typically, this technology requires three major steps: 1) Disruption of the cell wall matrix to breakdown and expose polysaccharides, 2) Enzymatic deconstruction of exposed polysaccharides to monomeric sugars, and 3) Fermentation of the sugars into ethanol. The secondary plant cell wall is a complex structure made up of polysaccharides (polymers of pentose and hexose sugars) and lignin (a heterogeneous aromatic polymer). Together they contribute to plant recalcitrance, i.e., native defense against microbial attack, and thereby present the biggest obstacle to low cost biological conversion. (Luque et al. 2008) Hence, selective disintegration of plant cell walls via low cost physical or chemical means, called pretreatment, to release its components without extensive degradation becomes a prerequisite to increase the accessibility of polymeric sugars to cellulolytic fungal enzymes or other biological systems. (Kumar and Sharma 2017; Li, Pu, and Ragauskas 2016)

The polysaccharides exposed by pretreatment can be broken down into their respective monomers via enzymatic saccharification. The resulting monomers are then suitable to be consumed by ethanologenic micro-organisms to make ethanol. The enzymatic hydrolysis and fermentation process steps can be combined in a single vessel to reduce sugar feedback inhibition and improve ethanol yields via integrated technologies such as simultaneous saccharification and fermentation (SSF). (Mohagheghi et al. 1992)

The lignin left can either be used as a solid fuel or as a higher value building block for making bio-plastics. (Ragauskas et al. 2014)

Although there is an abundant supply, cellulosic ethanol production has to be cost-effective and cost-competitive with other fuels in order to partially or completely displace light-duty petroleum transportation fuels. Increased ethanol titers attainable via high solids SSF can reduce energy consumption and capital expenses for large-scale fermentation and downstream ethanol recovery, thereby achieving significant cost savings. (Mohagheghi et al. 1992; Nguyen et al. 2016a) Many pretreatment methods have been developed over the years, however, with few able to produce a substrate that lends itself to high commercially relevant ethanol yields at high solids fermentation loadings. Incomplete glucan digestion limits ethanol yields at low enzyme loadings needed to keep costs nearly competitive. Together these factors restrict ethanol production from being cost-effective for high solids configurations of SSF. (Anon n.d.; Nguyen et al. 2017)

To address these issues, the first goal of this dissertation was to attain high ethanol concentrations from SSF of glucan-rich solids produced by Co-Solvent Enhanced Lignocellulosic Fractionation (CELF) pretreatment at lower enzyme doses than possible by other pretreatments at high solids loadings. CELF uniquely employs tetrahydrofuran (THF) as a water-miscible co-solvent combined with a dilute acid catalyst, to rapidly encourage non-destructive dissociation and fractionation of plant cell walls to solubilize and recover a high proportion of the hemicellulose and lignin in the hydrolysate liquor as well as leaving much of the cellulose fibers intact. The result is extensive cell wall delignification and isolation of cellulose fibers suitable for enzymatic hydrolysis and

fermentation. The fractionated cellulose fibers are highly digestible glucan-rich that could be hydrolyzed with cellulolytic enzymes in doses as low as 2 mg-protein per g-glucan-in-raw biomass. (Nguyen et al. 2015, 2016b) This presents a major cost-saving advantage compared to other pretreatments by ensuring a high sugar yield while simultaneously reducing enzyme costs to support high solids fermentation. Since enzymes are expensive and a major contributor to operating costs, lowering enzyme requirements can improve the overall economy of the process and lower the minimum ethanol selling price. (Klein-Marcuschamer et al. 2012) CELF pretreatment conditions have been tuned to maximize sugar and lignin recovery. In addition, the impact of pretreatment operating conditions such as reaction temperature and duration on the physiochemical features of biomass were assessed understand features that ultimately impact the digestibility of the CELF cellulosic substrates. The digestibility of the CELF solids obtained at the optimized pretreatment condition was further explored at various enzyme loadings to understand the enzyme-substrate interaction and identify the optimal enzyme dose for high solids SSF experiments.

CELF pretreatment hydrolysates are highly acidic, contain >400 g/L of THF, and are rich in C-5 sugar monomers and lignin. Following neutralization and THF removal from this liquid, solubilized sugars can be separated from solubilized lignin, thereby producing a technical lignin stream and highly concentrated sugar syrup. The fermentability of the sugar is yet to be determined since it contains fermentation inhibitors such as furfural, acetic acid, and 1,4-butanediol that adversely impact yeast cell viability.(Mills, Sandoval, and Gill 2009; Rivard et al. 1996) In the second section of the

dissertation, the solubilized sugars in the hydrolysate were fermented to augment ethanol yields via simultaneous saccharification and co-fermentation (SSCF).

The last segment of this dissertation aimed at deriving value from the lignin recovered from the CELF hydrolysate. CELF lignin was characterized to determine its purity, molecular weight, and functional groups. Novel methods were developed to synthesize CELF Lignin-Polyurethanes, and the impact of lignin properties on polymer quality was analyzed. Lignin properties were further manipulated by changing pretreatment operating conditions to achieve desired polyurethane properties for downstream application.

1.4 Dissertation Organization

The overall goal of this dissertation is to increase the value derived from the major components in the cell wall matrix. The study was conducted entirely on Poplar (*Populus Trichocarpa*) as the lignocellulosic feedstock. Chapter 2 provides a comprehensive overview of high solids fermentations for cellulosic ethanol production. It points out the limitations of high gravity processes, identifies major and minor factors influencing ethanol yields, reviews remedies that have been suggested until now, and identifies prospects for investigation. Chapter 3 reports on the performance of CELF pretreatment applied to hardwood Poplar, looks at how pretreatment severity impacts physiochemical features of the biomass, and identifies features that possibly influence enzymatic digestibility of solids produced by CELF pretreatment of poplar. Chapter 4 focusses on applying SSF to solids produced by CELF pretreatment of Poplar at high insoluble solids loading with goal to achieve >80g/L ethanol titers while maintaining high yields. This chapter also includes a

fractal kinetic analysis of enzymatic saccharification data achieved over a range of enzyme doses to understand enzyme-substrate interactions and explain the high saccharification susceptibility of CELF substrates even at a low enzyme dose. Chapter 5 compares the fermentability of the *Saccharomyces cerevisiae* variant D5A to results with a novel *Kluveromyces marxianus* variant CBS 6556 for high solids SSF of CELF substrates. In this study, the ability of thermotolerant CBS 6556 to achieve high ethanol titers for high solids SSF at near saccharification temperatures was also assessed. Results from Chapters 3 and 5 prompted the studies in Chapter 6 to investigate the influence of THF washing prior to water washing of the pretreated substrates on its digestibility. Chapter 7 presents results from application of 1L SSCF using an engineered *Saccharomyces cerevisiae* strain, M11205, in a 3 L bioreactor to CELF pretreated solids containing mostly glucose mixed with CELF pretreatment liquids containing mostly xylose following CELF operation at an optimized pretreatment condition. This chapter also includes a techno-economic analysis of the co-fermentation process with and without lignin being considered as a valuable co-product. Chapter 8 reports on the impact of pretreatment temperature on the mass of lignin recovered, its physiochemical features, and the influence of those features on polyurethane that incorporates CELF-lignin. In this case, lignin characterization and polyurethane synthesis were performed by collaborators at the University of Tennessee Knoxville (UTK). Finally, Chapter 9 summarizes the key findings of this dissertation, presents concluding remarks, and points out opportunities for future research.

1.5 References

Anon. n.d. “Biofuels: Ethanol and Biodiesel - Energy Explained, Your Guide To Understanding Energy - Energy Information Administration.” Retrieved July 15, 2019a (https://www.eia.gov/energyexplained/index.php?page=biofuel_home).

Anon. n.d. “Doi_10.1016_j.Jbiotec.2009.11.001 _ Elsevier Enhanced Reader.Pdf.”

Anon. n.d. “NASA: Climate Change and Global Warming.” Retrieved July 12, 2019c (https://climate.nasa.gov/).

BP and B. P. Energy Outlook. 2019. “BP Energy Outlook 2019 Edition The Energy Outlook Explores the Forces Shaping the Global Energy Transition out to 2040 and the Key Uncertainties Surrounding That.” *BP Energy Outlook 2019*.

Calvo-Flores, Francisco García and José A. Dobado. 2010. “Lignin as Renewable Raw Material.” *ChemSusChem* 3(11):1227–35.

Demirbas, Ayhan. 2009. “Political, Economic and Environmental Impacts of Biofuels: A Review.” *Applied Energy* 86:S108–17.

Hopewell, Jefferson, Robert Dvorak, and Edward Kosior. n.d. “Plastics Recycling: Challenges and Opportunities.”

Klein-Marcuschamer, Daniel, Piotr Oleskowicz-Popiel, Blake A. Simmons, and Harvey W. Blanch. 2012. “The Challenge of Enzyme Cost in the Production of Lignocellulosic Biofuels.” *Biotechnology and Bioengineering* 109(4):1083–87.

Kumar, Adepu Kiran and Shaishav Sharma. 2017. “Recent Updates on Different Methods of Pretreatment of Lignocellulosic Feedstocks: A Review.” *Bioresources and Bioprocessing* 4(1):7.

Li, Mi, Yunqiao Pu, and Arthur J. Ragauskas. 2016. “Current Understanding of the Correlation of Lignin Structure with Biomass Recalcitrance.” *Frontiers in Chemistry* 4.

Luque, Rafael, Lorenzo Herrero-Davila, Juan M. Campelo, James H. Clark, Jose M. Hidalgo, Diego Luna, Jose M. Marinas, and Antonio A. Romero. 2008. “Biofuels: A Technological Perspective.” *Energy & Environmental Science* 1(5):542.

Mills, Tirzah Y., Nicholas R. Sandoval, and Ryan T. Gill. 2009. “Cellulosic Hydrolysate Toxicity and Tolerance Mechanisms in Escherichia Coli.” *Biotechnology for Biofuels* 2(1):26.

Mohagheghi, A., M. Tucker, K. Grohmann, and C. Wyman. 1992. “High Solids Simultaneous Saccharification and Fermentation of Pretreated Wheat Straw to Ethanol.” *Applied Biochemistry and Biotechnology* 33(2):67–81.

Naik, S. N., Vaibhav V. Goud, Prasant K. Rout, and Ajay K. Dalai. 2010. “Production of First and Second Generation Biofuels: A Comprehensive Review.” *Renewable and Sustainable Energy Reviews* 14(2):578–97.

Nguyen, Thanh Yen, Charles M. Cai, Rajeev Kumar, and Charles E. Wyman. 2015. "Co-Solvent Pretreatment Reduces Costly Enzyme Requirements for High Sugar and Ethanol Yields from Lignocellulosic Biomass." *ChemSusChem* 8(10):1716–25.

Nguyen, Thanh Yen, Charles M. Cai, Rajeev Kumar, and Charles E. Wyman. 2017. "Overcoming Factors Limiting High-Solids Fermentation of Lignocellulosic Biomass to Ethanol." *Proceedings of the National Academy of Sciences of the United States of America* 114(44):11673–78.

Nguyen, Thanh Yen, Charles M. Cai, Omar Osman, Rajeev Kumar, and Charles E. Wyman. 2016a. "CELF Pretreatment of Corn Stover Boosts Ethanol Titers and Yields from High Solids SSF with Low Enzyme Loadings." *Green Chemistry* 18(6):1581–89.

Nguyen, Thanh Yen, Charles M. Cai, Omar Osman, Rajeev Kumar, and Charles E. Wyman. 2016b. "CELF Pretreatment of Corn Stover Boosts Ethanol Titers and Yields from High Solids SSF with Low Enzyme Loadings." *Green Chemistry* 18(6):1581–89.

Pareto, V. E. and Marcos Pompeu Pareto. 2011. "The Urban Component of the Energy Crisis." *SSRN Electronic Journal* (August).

Pimentel, David, Tad Patzek, and Gerald Cecil. 2007. "Ethanol Production: Energy, Economic, and Environmental Losses." Pp. 25–41 in. Springer, New York, NY.

Ragauskas, Arthur J., Gregg T. Beckham, Mary J. Biddy, Richard Chandra, Fang Chen, Mark F. Davis, Brian H. Davison, Richard A. Dixon, Paul Gilna, Martin Keller, Paul Langan, Amit K. Naskar, Jack N. Saddler, Timothy J. Tschaplinski, Gerald A. Tuskan, and Charles E. Wyman. 2014. "Lignin Valorization: Improving Lignin Processing in the Biorefinery." *Science (New York, N.Y.)* 344(6185):1246843.

Rivard, Christopher J., Rebecca E. Engel, Tammy K. Hayward, Nicholas J. Nagle, Christos Hatzis, and George P. Philippidis. 1996. "Measurement of the Inhibitory Potential and Detoxification of Biomass Pretreatment Hydrolysate for Ethanol Production." *Applied Biochemistry and Biotechnology* 57–58(1):183–91.

Thompson, Richard C., Charles J. Moore, Frederick S. Vom Saal, and Shanna H. Swan. n.d. "Plastics, the Environment and Human Health: Current Consensus and Future Trends."

Tiffany, Douglas G. 2009. *Economic and Environmental Impacts of U.S. Corn Ethanol Production and Use*.

US EPA, OAR. n.d. "Sources of Greenhouse Gas Emissions."

US EPA, OW. n.d. "Toxicological Threats of Plastic."

Chapter 2 : Progress of high solids saccharification and fermentation of lignocellulosic biomass to fuel ethanol: a comprehensive overview

2.1 Abstract

The long-term goal of sustainable biofuel production to displace petroleum fuels relies on cost competitive production of renewable transportation fuels and chemicals from abundant and low-cost lignocellulosic biomass. Cellulosic ethanol has the potential to realize the greatest impact towards displacing petroleum consumption in gasoline and diesel markets as it has proven to be a high octane blendstock and versatile fuel intermediate for further transformation into diesel and jet range blendstocks. In order to realize lower cost ethanol fermentation of biomass sugars, high gravity or high solids fermentations must achieve sufficient ethanol titers to reduce ethanol recovery costs from water. However, several technical challenges have limited ethanol yields during high gravity or high solids fermentations that need to be overcome. Limiting factors such as inadequate heat and mass transfer in the bioreactor, exogenous or endogenous cellulolytic enzyme activity, and different toxic stress responses induced by high ethanol concentrations and inhibitory compounds such as furfurals, acetic acid etc. The current review focusses on the major, and minor roadblocks of high-gravity processes, and suggests remedies to overcome these limitations based on the current knowledge and projected technologies.

2.2 Introduction

Lignocellulosic biomass in the form of agricultural residues, forestry residues, municipal green waste, industrial green waste, and certain energy crops are a resource that is abundantly available and low enough cost to support the large-scale production of renewable transportation fuels. (Demirbas 2009) The bulk of the valuable components in

biomass are found within the plant cell wall. Fermentable sugars from the cellulose, and hemicellulose fractions and a poly-aromatic component lignin could be recovered from the biomass. In order to access the biomass sugars, chemical and biological processes must first overcome the plant's natural recalcitrance or resistance against microbial or chemical attack. In recent years, research focusing on the development of hydrothermal, chemical, and mechanical pretreatment processes to reduce biomass recalcitrance and promote biomass deconstruction to concentrated sugar streams has gained particular attention.(biotechnology and 2014 n.d.; Li, Pu, and Ragauskas 2016; Luque et al. 2008)

Some of the most-popular pretreatment technologies include:

- ***Uncatalyzed liquid hot water treatment/steam explosion*** are high temperature reactions (160- 230 °C) using water in liquid or vapor phase that mainly causes hemicellulose removal from the plant cell wall and releases them in the form of C-5 sugar monomers. Acetic acid is also released as a result of hemicellulose hydrolysis. Depending on the reaction severity, the C-5 monomers can further degrade to form furfural. The main advantages with this pretreatment type includes low cost and less use of chemicals.(Cara et al. 2007; Ko et al. 2015; Wang et al. 2012)
- ***Dilute Acid (DA)*** pretreatments, mostly using a low concentration of sulfuric acid, is one of the most widely investigated technologies. DA is an inexpensive method that has proven to be quite effective in causing hemicellulose removal and displacing lignin within the cell wall structure. The main drawbacks include the inability for this pretreatment to remove significant amounts of lignin, high reaction

severities leading to the formation of acetic acid from hemicellulose hydrolysis and sugar degradation products such as furfural and 5-HMF etc. High severity DA pretreatments also promote the formation of cross-condensed products such as "pseudo lignin" that negatively impacts the enzymatic digestibility of cellulose. (Geng et al. 2015; Humbird et al. 2002; Mcmillan et al. 1999; Ragauskas 2017; Stephen Glen Allen et al. 2001)

- ***Alkaline pretreatments*** using a suitable base such as sodium, ammonium or calcium hydroxide focusses on lignin removal that causes cell wall swelling and increases the substrate specific surface area. Alkaline treatments also reduce cellulose crystallinity. Pretreatment drawbacks are dependent on the base used. For example, calcium hydroxide leads to formation of salts that needs to be removed prior to fermentation. Ammonia based pretreatments such as Ammonia fiber explosion method (AFEX) are quite effective for pretreating agricultural biomass, but are not very economic due to the high cost of ammonia and the cost associated with recovery of ammonia and waste-treatment after pretreatment. (Bals et al. 2014; Kim et al. 2008; Kumar and Wyman 2009; Varga et al. 2004)
- ***Organosolv pretreatments*** use a mixture of organic solvent and water, catalyzed or uncatalyzed, to promote lignin removal and hemicellulose hydrolysis. Common solvents include methanol, ethanol, acetone etc. The pretreatment operating conditions are dependent on the type of solvent used. Expensive solvent recovery step and high severity reactions, however, are the most significant cost bottlenecks of the process. The high severity reactions also lead to potentially unwanted

degradation reactions and condensation of the lignin to reduce overall utility of the lignocellulosic fractions. (Jang et al. 2016; Smith et al. 2017)

- ***Sulfite Pretreatment to Overcome Recalcitrance of Lignocelluloses (SPORL)*** involves sulfite treatment of biomass using sodium bisulfite under acidic conditions maintained using sulfuric acid at temperature close to 180 °C. Developed using the concept of sulfite pulping, this technology focusses on hemicellulose removal and lignin sulfonation to improve cellulose digestibility. The main drawbacks include the cost of reagents needed and the potential production of fermentation inhibitors such as HMF and furfural at high severity reactions. (J. Y. Zhu et al. 2011; J.Y. Zhu et al. 2011; Zhu et al. 2009)
- ***Co-Solvent Enhanced Lignocellulosic Fractionation (CELF)*** is solvolysis-type pretreatment. CELF employs a mixture tetrahydrofuran and water in a 1:1 (weight basis) along with dilute acid as the catalyst to enhance both cell wall delignification and sugar hydrolysis at much milder reaction severities compared to organosolv (temperatures ranging from 140-160 °C), capable of producing highly digestible cellulose-rich solids and a hydrolysate liquor containing high yields of hemicellulose sugars and depolymerized lignin. (Nguyen et al. 2016a, 2017) The main advantages of CELF pretreatment include low-cost solvent recovery and simple isolation of dissolved lignin. (Wang et al. 2020) Disadvantages of this process involve additional costs associated with the recovery of THF and the need to recover all of the byproducts from the pretreatment to avoid high water-treatment costs.

Separate Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF) are the two most widely used biological conversion methods coupled with a suitable pretreatment to produce ethanol. SHF involves carrying out cellulose hydrolysis and glucose fermentation sequentially, and separately at the optimum conditions for each stage i.e. 50 °C, 150 rpm for enzymatic saccharification, and 30 ~ 35 °C, 130 rpm for fermentation. Whereas, SSF, proposed by Gauss *et al*, integrates the two processes in a single vessel at an optimum condition i.e. 37 °C, 130 rpm, for both saccharification, and fermentation to work together. The latter offers many benefits over the former, like reduction in end-product inhibition of enzymes, less contamination by unwanted micro-organisms, high yields of ethanol at low enzyme dose, and elimination of expensive equipment used otherwise. (Anon 1981; Gauss et al. n.d.; Mandels, J Kostick - US Patent 3, and 1973 n.d.; Nguyen et al. 2015a; Xu, Singh, and Himmel 2009) Simultaneous hydrolysis and co-fermentation (SSCF) is an extension of SSF that enables concurrent utilization of biomass derived hexose and pentose sugars present in both solid and liquid fraction achieved post pretreatment. SSCF offers several advantages including improved overall sugar utilization from biomass, reduced water requirement, and significant savings in operating costs.(Liu and Chen 2016)

High Solids Processes, with an insoluble solid loading > 10 wt%, are theoretically more economical at a large scale. Unlike dilute processes it does not require large volumes of water, thereby saving a valuable resource, and the capital cost associated with heating, and far along treating the water. Additionally, high ethanol concentration in the

fermentation broth reduces the energy expenses to recover ethanol from water via distillation. (Kim et al. 2008; Nguyen et al. n.d.)

Table 2-1 summarizes the results from SHF, SSF, SSF experiments with prehydrolysis and SSCF experiments carried out on a range of substrates generated using leading pretreatment technologies at a solid loading >5 wt%. As shown in Table 2-1, irrespective of the feedstock, ethanol titers increase with solid loading, however the ethanol yield follows a decreasing trend. Hence, although high solid processes seem ideally lucrative they are accompanied by multiple technical challenges that limit the ethanol yields. These limitations include mixing issues, inadequate heat and mass transfer, heavy requirement of fungal enzymes, toxicity to the yeast strain caused by increased ethanol titers etc. Due to these difficulties a tradeoff has to be made between a desirable ethanol titer at a lower yield and a reasonable ethanol titer at a higher yield. In this context, the current review helps to understands the issues with successful implementation of high solids processes, studies the major and minor factors influencing the ethanol yields, revises the remedies that have been suggested until date, and looks at the prospects still open for investigation.

This review is a three-part discussion; the first part lists the roadblocks limiting the product yields from a high solids process, followed by a section on the technical advances that help to overcome the process limitations and finally, a segment with the concluding remarks and the directions of future work.

Table 2-1 Literature Survey of ethanol titers and yields achieved from various biological conversion routes using the substrates generated from various leading biomass pretreatments

Pretreatment	Feedstock	Weight Insoluble Solids (%)	Enzyme (mg protein/ g substrate) approximate	Yeast Strain	Temp (°C)	Glucose Conc. (g/L)	Glucose Yield (%)	Reference
Enzymatic Hydrolysis (EH)								
Dilute Acid (DA)	Corn Stover	5	10	N/A	50	31.38	78.4	(Geng et al. 2015)
		15	17		45	79	49	(Hodge et al. 2008)
		15	10		50	99.3	73.2	(Geng et al. 2015)
		15 Fed-Batch				103.7	76.5	
Aqueous Ammonia (with washing)		10.36	100		50	55	80	(Qin et al. 2013)
		20	100			90	62	
Aqueous Ammonia (without washing)		16.3	100			35	52	
		24.45	100			50	50	
Ammonia fiber expansion, pelletized		18	20		50	60	68	(Bals et al. 2014)
Ammonia fiber expansion, unpelletized		18	20			54	61	
Steam Explosion		24 Fed-Batch	20		50	N/A	62	(Chen and Liu 2017)
Steam explosion (without washing)		27.9	40		50	85.1	70.9	(Lu et al. 2010)
Steam explosion (with washing)		30	40			103.3	72.5	
Phosphoric Acid Impregnated Steam explosion	Sugarcane Bagasse	20	62.5		50	76.8	69.2	(Ramos et al. 2015)
Liquid Hot water	Sweet Sorghum Bagasse	30	60		50	88.95	60.68	(Wang et al. 2012)
Liquid hot water		20	30		50	52	64	

	Olive tree pruning	30				61	49.9	(Cara et al. 2007)
Liquid hot water followed by alkaline peroxide delignification		20				63	56.8	
		30				73	43	
Steam Explosion		20				60	39.6	
		30				52	55.4	
Steam Explosion followed by alkaline peroxide delignification		20				63	50	
		30				70	37.7	
Unbleached Kraft Pulp	Poplar	20	45		50	144	76	(Zhang et al. 2009)
Ethanol-Organosolv		20				158	83	
Gamma Valerolactone (80:20) (Water Washed)	Hardwood	30	30		50	124	90	(Holwerda et al. 2014)
Gamma Valerolactone (80:20) (CO_2 - extracted)						124	87	
Gamma Valerolactone/ Water (60:40)	Reed Stover	15	20		50	130	N/A	(Zhou et al. 2016)
		20				184.3		
		25				231		
Pretreatment	**Feedstock**	**WIS (%)**	**Enzyme (mg protein/ g substrate) approximate**	**Yeast Strain**	**Temp (°C)**	**Ethanol Titer (g/L)**	**Ethanol Yield (%)**	**Reference**
Separate Hydrolysis and Fermentation (SHF)								
Dilute Acid (DA)	Rapeseed Straw	7.5	60	*S. cerevisiae*	35	15.5	60	(López-Linares et al. 2014)
		10	60		35	39.9	57.9	
		15	60		35	30.5	59.1	
Gamma Valerolactone/ Water (60:40)	Reed Stover	15	20	N/A	N/A	50	N/A	(Zhou et al. 2016)
Organosolv	Poplar	20	45	*S. cerevisiae*	30	63.1	83	(Zhang et al. 2009)

Unbleached Kraft Pulp						50.4	83	
Steam explosion (without washing)	Corn Stover	20 (Fed-Batch)	40	*S. cerevisiae*	30	20	68	(Lu et al. 2010)
Steam Explosion		20	60	*S. cerevisiae*	50 (EH), 30 (Ferm.)	50.4	67.6	(Liu et al. 2014)
Steam explosion (with washing)		30 (Fed-Batch)	40	*S. cerevisiae*	30	49.5	94	(Lu et al. 2010)
Liquid Hot water	Sweet Sorghum Bagasse	30	60	*S. cerevisiae*	30	43.36	54.62	(Wang et al. 2012)
Simultaneous Saccharification and Fermentation (SSF)								
Dilute Acid (DA)	Rapeseed Straw	7.5	60	*S. cerevisiae*	40	16.9	65.5	(López-Linares et al. 2014)
		10				34.1	49.5	
		15				31.6	61.2	
	Corn Stover	18.3	15	*S. cerevisiae*		47.8	73	(Nguyen et al. 2016b)
	Wheat Straw	12.1	80	*S. cerevisiae*	37	38	82	(Mohagheghi et al. 1992)
		12.1	40			36	76.5	
		16.1	80			48	75.4	
		16.1	40			47	72.2	
		20	80			57	68.5	
		20	40			52	62.4	
		24.2	80			57	54.5	
		24.2	40			55	49.9	
		28.2	80			40	33.2	
		28.2	40			35	27.6	
		32.3	80			34	23.9	
		32.3	40			32	21.5	
	Spruce	20	14	*S. cerevisiae*	35	9.5	13	(Koppram and Olsson 2014)
		20 (50:50)	15			27.9	37	
		20 (50:50)	15			33	44	

		20 (50:50)	15			40	53	
Gamma Valerolactone/ Water (60:40)	Reed Stover	15	20	N/A	N/A	54	N/A	(Zhou et al. 2016)
Steam Pretreated	Corn Stover	15	14	*S. cerevisiae*	37	24.7	76.5	(Zhang et al. 2009)
		20	14			31	68	(Zhang et al. 2010)
		25	14			39.3	64.8	
		30	14			40.6	52.1	
Co-Solvent Enhanced Lignocellulosic Fractionation (CELF)		15.5	15	*S. cerevisiae*	37	58.8	89.2	(Nguyen et al. 2016b)
		15.5	2			52.2	79.2	
		20	10			79.2	90.5	(Nguyen et al. 2017)
		21.5				81.3	86.1	
		23				85.6	80.8	
Sulfite Pretreatment to Overcome Recalcitrance of Lignocelluloses (SPORL) using Sodium Bisulfite	Aspen	12	20	*S. cerevisiae*	35	40.8	82.9	(J.Y. Zhu et al. 2011)
		15	22			52.5	83.2	
		18	12			37.7	46.8	
			14			43	54.3	
			24			59.3	76	
			30			60	77	
Steam Pretreated	Spruce	10	10	*S. cerevisiae*	37	17.5	77.4	(Hoyer, Galbe, and Zacchi 2010)
		10 Fed-Batch	30			16	68.9	
		14	30		37	14	51.8	
Simultaneous Saccharification and Fermentation with Prehydrolysis								
Dilute Acid (DA)	Rapeseed Straw	7.5	60	*S. cerevisiae*	50 -PH 40 (SSF)	17.4	67.3	(López-Linares et al. 2014)
		10				32.4	47.1	
		15				31.5	61.1	
Co-Solvent Enhanced Lignocellulosic Fractionation (CELF)	Corn Stover	15.5	15	*S. cerevisiae*	50 -PH 37 SSF	58	89	(Nguyen et al. 2016b)
Steam Explosion		20	60	*S. cerevisiae*	50 -PH 30 SSF	56.1	74.6	(Liu et al. 2014)
						59.8	77.2	

Simultaneous Saccharification and Co-fermentation (SSCF)								
Steam pretreated Using S. cerevisiae variants	Corn Cob	7.5	10	*KE6-12*	30	21.9	52.64	(Koppram et al. 2013)
				RHD-15		21.5	51.68	
				KE6-12		31.2	75	
		7.9	9	*KE6-12*		32	77	
		10.5	15	*KE6-12*		39.8	58	
Alkaline (NaOH)		15	120	*Z. mobilis*	30	49.2	85	(Su et al. 2013)
		25 Fed Batch				60.5	N/A	
Dilute Acid (DA)	Poplar	15	24	*Z. mobilis*	35	33~35	54	(Mcmillan et al. 1999)
			28			39~40	65	
Dilute Acid (DA)	Wheat Straw	20	20	*S. cerevisiae, KE6-12.A*	50 -PH 35 (SSCF)	50	53.84	(Westman et al. 2017)
		25 Fed-Batch			35	65	70	
Steam Exploded		12	150	*S. cerevisiae, KE6-12*	35	19	39	(Moreno et al. 2013)
		16				32.3	51	
Steam Exploded	Corn Stover	20	25	*S. cerevisiae (IPE003)*	30	60.8	75.3	(Liu and Chen 2016)
		20 Fed-Batch				57.7	69	
						64	79.3	
						54.1	67	
						65	80.5	
						61.3	75.9	
						62.8	77.9	
						59.5	73.6	
						56.1	69.5	

2.3 Factors limiting ethanol yields at high-solids loading

2.3.1 Rheological complexities, mixing issues, heat and mass transfer limitations

In theory, high insoluble solids content must increase the medium viscosity obstructing the freedom of solute particles in the suspension. According to Stokes-Einstein equation for spherical particles, the rate of diffusion of solute particles in a solution is inversely proportional to the absolute viscosity of the fluid medium.

$$D = \frac{k_B T}{6\pi\eta r}$$

Where k_B is the Boltzmann constant, T is the absolute temperature (298 K), η is the absolute viscosity of the fluid medium, and r is the hydrodynamic radius of the solute particle. (Cruickshank 1905; Roberts et al. 2011) However, this model equation was derived for pure sugar solutions, therefore is inapplicable here. Predicting the rheological behavior of highly concentrated suspensions of complex, and heterogeneous biomass, and their impacts on overall process yields is a lot more complicated.

Total amount of water present in the cellulose suspensions can be roughly categorized as 1) bound water (water trapped inside the porous, fibrous structure of pretreated biomass), 2) restricted bulk water (water obstructed by other pools of water), and 3) free water. (Roberts et al. 2011) High solid slurries have an almost negligible amount of free water that leads to an increase in the medium viscosity due to the friction caused by enhanced particle-particle, and particle-water interactions. (Modenbach and Nokes 2013) A considerable amount of research on biomass rheological studies have shown that slurries with high insoluble solid content are viscoelastic and exhibit a shear thinning behavior with a high yield stress. (Knutsen and Liberatore 2009; Lavenson et al. 2012; Roberts et al.

2011; Roche et al. 2009; Stickel et al. 2009; Stutzenberger and Lintz 1986) Physiochemical properties of the substrate like particle size, fiber-aspect ratio, water retention capacity etc. also have an influence on the yield stress. (Tozzi et al. 2014) These suspensions therefore are stiff, and act like a paste that is extremely hard to pump into or mix inside a bioreactor. (Modenbach and Nokes 2013) Inadequate mixing of reactants creates inhomogeneity and nutrient deficient pockets in the closed system causing improper heat and mass transfer within the bioreactor.

Free water in the broth is also vital for enzymatic hydrolysis being the medium for the enzymes to diffuse to and products to diffuse away from the reaction sites. (Roberts et al. 2011) Lack of free water in the broth restricts the movement of enzyme within the medium, hence decreases enzyme effectiveness that leads to a slow glucan digestion, thereby lowering the process yields. (Modenbach and Nokes 2013)

2.3.2 High reducing sugar concentrations impedes enzyme activity and induces osmotic stress on yeast cultures

Enzymatic hydrolysis of polysaccharides to monosaccharides is a mandatory, and rate limiting step for cellulosic ethanol production irrespective of the process configuration. Fungal enzymes are expensive and are a major contributor to the ethanol production cost ($0.35 gal^{-1} of ethanol). Hence, proper enzyme application becomes critical in realizing an economic cellulosic ethanol production. (Klein-Marcuschamer et al. 2010) High reducing sugar monomers and dimers accumulating in the fermentation broth, post enzymatic hydrolysis especially during a high solids SHF or a prehydrolysis for a certain time period prior to SSF, can negatively impact the overall process yields. Increased levels of glucose, and cellobiose are inhibitory to cellulase activity. Sugar monomers and dimers bind to the

enzyme active sites, reduce their access to cellulose and lower the saccharification rates. (Holtzapple et al. 1990; Stutzenberger and Lintz 1986; Xiao et al. 2004) The fungal enzymes used industrially is a cocktail of endoglucanses, exoglucanases, and cellobiases. Cellobiases are inhibited by glucose. An increased glucose concentration, subsequently in saccharification, results into a rise in cellobiose concentration. Since the exoglucanases are inhibited by cellobiose, a critical concentration of cellobiose can effectively shut down further saccharification. (Lee and Fan 1983; Montenecourt 1983)

Not only C-6 but C-5 sugars especially dimers have also been observed to impede enzyme action. Xylo-oligomers are a major component in the pretreatment liquor as an intermediate of hemicellulose hydrolysis. While co-fermenting the solid and liquid fraction, xylo-oligomers can be a major inhibitory component to cellulose hydrolysis by acting as a physical barrier and reducing the overall ethanol yields.(Zhang, Tang, and Viikari 2012). For example, Qing *et al*., studied the impact of xylo-oligomers on the Avicel^{lTM} cellulose hydrolysis. They observed that higher concentrations of xylo-oligomers, close to 12.5 mg/mL, could lower initial cellulose hydrolysis rates by 82%, and final yields by 38%. (Qing, Yang, and Wyman 2010) Another study by Kumar *et al.*, reported the impact of xylanase supplementation to cellulase enzyme cocktail on cellulose digestion from corn stover generated using various pretreatment technologies. It was reported that glucose yields increased with xylanase supplementation for all pretreatments albeit at a different rate. (Kumar and Wyman 2009) In the follow up study, the authors proposed a mechanism for cellulase inhibition by xylo-oligomers. They hypothesized that during thermochemical pretreatments at elevated temperatures hemicelluloses strongly

associates with the cellulose surface through hydrogen bonding, perhaps on the hydrophobic face. This reduces the accessibility of cellulose to fungal enzymes thereby slowing the saccharification process. (Kumar et al. 2018)

High sugar concentrations accumulating in the fermentation broth are also detrimental to the yeast cultures during fermentations. High-gravity sugar solutions create a hyperosmolar external condition that can be fatal to microorganism survival in the fermentation broth. With an increased external osmolarity, water effluxes are induced thereby causing cell shrinkage and increase in cytosolic ions, which are harmful for cell growth.

2.3.3 Lignin impedes enzyme activity during high solid processes

Lignin is one of the prime factors contributing to plant recalcitrance, hence, delignification of raw biomass becomes essential to increase polysaccharides accessibility. (DeMartini et al. 2013) While cellulosic substrates produced post pretreatment retains a small fraction of the initial lignin in the raw biomass, it is the major component of the pretreatment hydrolysate. However, the proportion of lignin in both solid and liquid fractions greatly depends on the type and severity of the pretreatment for a particular feedstock. Unfortunately, increase in solid loading also escalates the residual lignin content in the fermentation broth and the situation worsens further during a high solids SSCF.

Based on the results from a lignin-enzyme interaction study, Ooshima *et. al.*, reported that all the three components of cellulases i.e. exoglucanases, endoglucanases, and β-glycosidase adsorb equally on both cellulose, and lignacious residue and that these adsorptions can be characterized by Langmuir isotherm. (Ooshima, Burns, and Converse

1990) Rahikainen *et. al.*, also studied cellulase interactions with softwood lignin at both low and high temperatures of 4 and 45 °C for Avicel^{TM} cellulose, and steam pretreated spruce. They reported Braunner-Emmett- Teller (BET) surface area and Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) values that show that enzymes retain their activity at low temperatures but when close to hydrolysis temperature very strong interactions form between enzymes and lignin-rich residue that results in a loss of enzyme activity. (Rahikainen et al. 2011) Efficient fungal enzyme interaction only with polysaccharides is essential for quick and complete saccharification. Hence, unnecessary lignin-cellulase binding could potentially lower the saccharification rates, result into a longer hydrolysis time and reduce the overall productivity of the process.

2.3.4 High Concentrations of pretreatment toxins prove to be potentially harmful to both enzyme activity and the yeast culture

High severity pretreatment generates biomass derived toxins. Although the type and concentrations of the toxins released depends largely on the pretreatment type and severity but they mostly include sugar degradation products like 5-HMF, furfural, formic, levulinic, acetic acid etc., and phenolics released from lignin depolymerization and degradation such as coniferyl aldehyde, hydrobenzoic acid, vanillin etc. These degradation products are inhibitory to the both enzymes and microorganisms. (Almeida et al. 2007; Jönsson and Martín 2016; Mills, Sandoval, and Gill 2009; Palmqvist, Almeida, and Hahn-H�gerdal 1999; Palmqvist and Hahn-Hägerdal 2000b, 2000a)

Hodge *et al.*, observed acetic acid (15 g/L), released during hemicellulose hydrolysis prompted by dilute acid pretreatment of corn-stover, to be the next most important enzymatic hydrolysis inhibitor after sugars. (Hodge et al. n.d.) Jing *et al.*, studied

the inhibition performance of various lignocellulosic degradation products on fungal enzymes. They determined reaction rates of enzymatic hydrolysis of dilute acid pretreated corn stover in the presence of various degradation products and found that these deterrents inhibit hydrolysis by either reducing the enzyme activities or completely deactivating it. The order of inhibition strength to cellulase was found to be, lignin derivatives > furan derivatives > organic acids > ethanol. (Jing, Zhang, and Bao 2009)

In a similar study by Ximenes *et al.*, cellulases and β-glucosidases from different sources were incubated with phenolics like tannic acid, gallic acid, cinnamic acid, ferulic acid, p-coumaric acid, sinapic acid, vanillin, syringaldehyde, and 4-hydroxybenzoic acid for 24 h at 50 °C. These compounds caused inhibition of cellulase and deactivation of β-glucosidases, however, the strength of the inhibition or deactivation effect was dependent on the type and source of enzyme, and the type of phenolic compounds present. Glucosidase from *Aspergillus niger* was reported to be more resistant to inhibition and deactivation than the enzymes from *Trichoderma reesei*. In addition, out of the phenol molecules tested, tannic acid was the most damaging aromatic compound that caused both deactivation and reversible loss of all of enzyme activities tested. (Ximenes et al. n.d.)

The carryover toxins from pretreatment upset micro-organism growth and metabolism as well. Weak organic acids like acetic acid, formic acid etc., do not directly affect fermentation but they negatively impact the cell growth. (Mills et al. 2009) In undissociated form, weak acids have a pKa value lower than the intracellular pH of the cells, they can permeate the cell membrane and dissociate to release the anions and protons in the cytoplasmic area. This, in turn, decreases the pH of the cytoplasm and inhibits cell

growth. (O'Byrne et al. 2002; Schellhorn et al. 1992) The low intracellular pH resulting from weak acids inflow is neutralized by the action of the plasma membrane ATPase, which pumps protons out of the cell at the expense of ATP hydrolysis. (Stouthamer 1979; VERDUYN et al. 1990) The anion accumulation inside the cell can also affect the cell wall turgor pressure. (Roe et al. 1998)

Furfural and its derivatives, are a class of toxins derived from cellulose degradation. They have been shown to be cytotoxic to both bacteria and yeast. They also have a negative impact on the fermentative enzymes. (Zaldivar, Martinez, and Ingram 1999) These cyclic aldehydes denature polynucleotides and cause protein-protein crosslinking. (Sambrook and Russel 2001) In some studies, furfural has been shown to cause DNA damage by undergoing incubation inside the cell, triggering inactivation of cell replication. (Hadi, Letters, and 1989 n.d.; Palmqvist et al. 1999; Rahman, Toxicology, and 1991 n.d.) Cells with repair mechanism can maintain viability but only a few fermentative organisms are capable of such survival skills. (Khan et al. n.d.)

Phenolics, lignin degradation products, are the worst of all the toxins for the host micro-organism. They cause membrane destabilization, which leads to loss of membrane integrity, affecting their role as selective barriers. (Heipieper et al. n.d.) They have also shown inhibitory effects on fermentation. (Büchert, Puls, and Poutanen 1989; Clark and Mackie 2008) However, further investigation is required to completely understand the effects of low molecular weight phenolics and phenolic monomers on cell growth. The aforementioned different kinds of toxins, if present together in the fermentation broth especially during an SSCF, act synergistically causing more damage than when they are

exposed individually, thereby reducing the productivity of the yeast strain. (Myers, Montgomery, and Anderson-Cook 2016)

2.3.5 High ethanol concentrations proves fatal to the ethanalogenic organism

Ethanol tolerance of yeast organism is one of the major factors limiting product yields from high solids processes. Interestingly, there are many microorganisms capable of fermenting glucose into ethanol but only the ones with high ethanol tolerance (>4 %w/w) are preferred industrially. (Dien, Cotta, and Jeffries 2003) For instance, *Z. mobilis* has a higher specific glucose uptake rate than *S. cerevisiae*, however, the latter is the widely used strain for ethanol production due to its higher native ethanol tolerance. (D'amore et al. 1989; He et al. 2014) During continuous fermentations where ethanol is simultaneously removed from the broth, ethanol tolerance is not an issue, but high concentrations of ethanol in a batch culture is not beneficial for the cell growth or its metabolism. (Bai et al. n.d.) The cell functions influenced by high ethanol concentrations include inhibited cell viability and growth, impaired cell volume metabolism, lowered mRNA levels, protein denaturation, transformed vacuole morphology, loss of electrochemical gradients and proton-motive force, inhibition of transport processes, inhibition of H^+-ATPase activity, increased membrane fluidity etc. (Stanley et al. 2010)

2.3.6 Other System limitations

Simultaneous utilization of hexoses and pentoses, otherwise known as co-fermentation, is a beneficial trait in micro-organisms employed for high solids conversions. However, most microbes are usually unable to metabolize xylose in their natural form. Organisms that are transformed to ferment xylose, generally preferably ferment glucose

first, thereby exhibiting the diauxic effect. (C. Hu et al. 2011; Mahadevan, Edwards, and Doyle 2002; Zaldivar, Nielsen, and Olsson 2001) Many glucose consuming strains such as *S. cerevisiae, E. coli* etc. have been genetically engineered to ferment xylose. (Barbosa et al. 1992; Chandrakant and Bisaria 2000; Dien, Nichols, and Bothast 2002) Glucose and xylose enter the same metabolic pathway to ethanol conversion via common transporters, which arises a competitive inhibition of xylose uptake by glucose, causing a partial xylose uptake. (Olofsson, Bertilsson, and Lidén 2008) Also, fermentation of multiple sugar sources can increase by-product concentrations like acetate, which can affect the cell growth and impact ethanol production negatively. (Dumsday et al. 1999)

2.4 Known technological advances that help to overcome the limitations associated with high solid loading

2.4.1 Particle Size reduction

Particle size reduction by mechanical processing before pretreatment is one of the techniques to reduce slurry thickness that helps reducing the shear thinning behavior and dealing with the mixing issues. A study conducted by Viamajala *et al.* on dilute acid pretreated corn stover shows that pretreated slurries with smaller particle size, and a lower xylan content exhibit lower viscosity and yield stress. (Viamajala et al. 2009) In contrast Dibble *et al.*, did not observe particle size reduction to have any significant impact on yield stress or digestibility of high solid suspensions. (Dibble et al. 2011)

2.4.2 Selecting a suitable pretreatment type and tuning the operating conditions to generate an ideal substrate

The performance of any integrated biomass hydrolysis and fermentation process largely depends upon the fermentability of the cellulosic substrates. Favorable structural features such as an improved cellulose macro and micro accessibility, high substrate

surface area, reduced cellulose crystallinity, enhanced water retention capacity etc. are mainly influenced by the type of pretreatment used to generate the substrate. (Gonzales, Sivagurunathan, and Kim 2016; Jang et al. 2016; Um and Peter Van Walsum n.d.; Xiao et al. 2011) Although, several pretreatment techniques have been developed over the years to enhance the availability and digestibility of plant polysaccharides, however, only a few are able to generate a substrate ideal for a high solids process.

The scatter plot shown in Figure 2-1, built upon the data from Table 2-1, helps to identify the pretreatment technologies that successfully accommodate high gravity conversion processes and help procuring >50 g/L of ethanol titers, the industrial benchmark. The analysis revealed that CELF, SPORL and DA are among the few technologies that support high solid SSF. The common trait in these technologies is the focus on hemicellulose removal from plant cell wall along with lignin structure modification to increase cellulose accessibility. (Nguyen et al. 2017; Shuai, Questell-Santiago, and Zhu et al. 2011) However, CELF, that was observed to outperform the ethanol titers from high solids SSF as compared to other technologies at the same solid loading, emphasized on lignin removal as a strategy to isolate the polysaccharides. (Nguyen et al. 2015b, 2017; Smith et al. 2016)

CELF pretreated corn stover has been reported to produce theoretical glucose yields even at an extremely low enzyme dose of 2 mg protein per g glucan in raw biomass albeit at a slow rate. Results from the fractal kinetic study conducted on the enzymatic hydrolysis data of CELF and DA pretreated corn stover by Nguyen *et al.,* have shown that when compared to DA pretreated version, CELF pretreated substrate exhibits a higher transient

rate coefficient that decreases very slowly with time, especially in the high glucan conversion region, showing the higher enzymatic activity of the latter. They attributed these results to the enhanced delignification that caused less cellulose surface blockage by lignin, as the extent of xylan removal was almost the same for both the pretreatment methods. (Nguyen et al. 2015b) In their follow up study, they reported very high ethanol yields of 90.5%, 86.1%, and 80.8% corresponding to ethanol titers of 79.2, 81.3, and 85.6 gL^{-1} at solids loadings of 20 wt %, 21.5 wt %, and 23 wt % from batch SSF experiments. (Nguyen et al. 2017)

Pretreatment operating conditions also play an important role in influencing biomass digestibility. Biomass liquefaction at high temperature and for longer residence times might decrease the viscosity of the slurry but it can also result in the production of sugars and lignin derived toxins. (Ehrhardt et al. 2010) Hence, selecting a pretreatment type that focusses more on lignin removal, along with hemicellulose hydrolysis and optimizing the operating conditions to maximize the sugar recovery without triggering any sugar and lignin degradation can generate an ideal candidate for high solid processes. A severity factor, log (R_0) can be used as a simple quantification method to estimate the reaction harshness. (Um and Peter Van Walsum n.d.)

$$\log R_0 = \log\left[t * exp\left\{\frac{(T-100)}{14.75}\right\}\right]$$

in which T is the temperature in °C and the time is in minutes. A combined severity factor, log (CS) can be used to take into account the impact of pH during acid pretreatments. (Jang et al. 2016)

$$\log(CS) = \log R_0 - pH$$

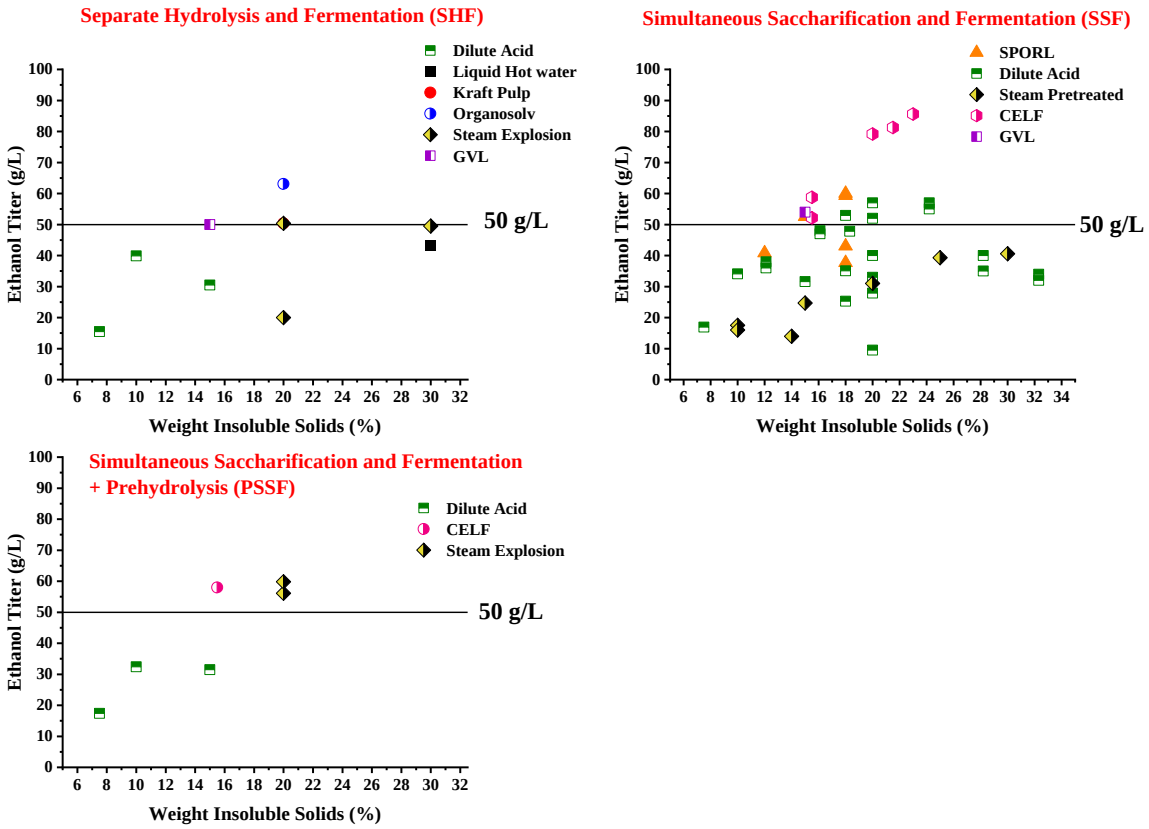


Figure 2-1 Scatter Plot showing the ethanol titers produced from different biochemical conversion routes at high solids using substrates generated from different pretreatment technologies. Data in Table 2-1.

2.4.3 SSF vs SHF

Figure 2-1, further reveals SSF as a superior alternative to SHF, especially at high solids. As mentioned earlier, SSF offers several benefits over SHF. However, the factor that significantly improves SSF yields is the immediate consumption of the sugar monomers when released in the fermentation broth, thus avoiding the accumulation of sugars to create a hyperosmolar high gravity solution and the technical issues that arises with it. The only drawback with SSF is the application of a compromised temperature at 37 °C that is below the optimum working temperature for enzymes, 50 °C which leads to

a slow sugar release. High temperature SSFs near ideal saccharification temperatures using a thermotolerant yeast strain can be an approach worth investigating to reap the benefits of SSF without compromising the enzyme activity. (Abdel-Banat et al. 2010)

2.4.4 Fed-batch feeding strategies

Fed-batch substrate feeding techniques have so far been the most studied and most effective approach in dealing with the mixing issues at high solid loadings. Introducing the substrate at certain intervals lowers the insoluble solids content in the solution, decreasing the viscosity and giving time to liquefy the slurry before adding another batch. For example, Gladis *et. al.*, conducted an SSF of steam pretreated corn stover at 20 wt% solid loading and an enzyme loading 10 FPU/g water-insoluble solids using both batch and fed-batch approach. They reported an increase in ethanol yield from 76% to 81% by using fed-batch feeding strategies. (Gladis et al. 2015) Another study conducted by Cardona *et al.* using cellulosic fibers (Solka, Floc C100 and 200EZ) observed that a total solid loading of 30.71% (w/w), at an enzyme dose of 4.28 FPU /g biomass, could be hydrolyzed by using a controlled fed-batch substrate feeding technique. They further monitored the rheological modifications in the biomass slurry and reported that introducing all the enzyme in the first batch (9.53 FPU/g biomass initially) resulted in faster liquefaction per batch and led to a higher glucose yields. (Cardona et al. 2015)

2.4.5 Use of customized enzyme cocktails

Commercially used enzyme cocktails are usually a mixture of exoglucanases, endoglucanases, and cellobiases in a certain ratio. Since, cellulosic substrates generated from different pretreatment types are unique, therefore, tuning the amount of individual

protein components based on the nature of the substrate can help improve saccharification efficiency. (Sant'Ana da Silva et al. 2016; Srivastava et al. 2019) Introducing enzyme supplements such as xylanses, pectinases, laccases etc. have also shown positive impacts on enzyme activity. (Cannella et al. 2012; J. Hu, Arantes, and Saddler 2011; Kumar and Wyman 2009; Reyes-Ortiz et al. 2013; Xu et al. 2019) Engineering enzymes to serve a particular purpose such as reducing unwanted lignin-cellulose interactions, have also been effective in enhancing hydrolysis efficiency. Nordwald *et al.* reported weakening of cellulase-lignin bond by engineering cellulase enzyme charge to create a highly negative surface charge density that helped in repelling lignin. (Nordwald et al. 2014)

2.4.6 Using an appropriate hydrolysate detoxification method

As mentioned already, pretreatment hydrolysates contain a range of biomass derived toxins that are extremely harmful to both the fermentative organism and cellulolytic enzyme action. Hence, the liquid fraction requires detoxification based on the pretreatment type and severity. Some of the known hydrolysate cleansing methods include biological, physical and chemical treatments.

Biological treatment involves the use of enzymes peroxidase and laccase to remove phenolic monomers from hemicellulose hydrolysate. (Jönsson et al. 1998) For instance, the fungus, *Trichoderma reesei*, has been reported to degrade inhibitors obtained after steam pretreatment of willow, resulting in increased ethanol productivity. (Palmqvist, Hahn-Hägerdal, …, et al. n.d.) Physical detoxification method like roto-evaporation of the volatiles does not improve the ethanol yields much, because the inhibitors are mostly the non-volatile phenolics. (Palmqvist, Hahn-Hägerdal, Technology, et al. n.d.) Another

detoxification method could be selective extraction of inhibitors causing the most damage but that would be a very tedious and time consuming process. (Wilson, Deschatelets, and Nishikawa 1989)

Chemical detoxification methods include overliming with $Ca(OH)_2$ and then readjusting the pH to 5~6 with a suitable acid. (Leonard and Hajny 1945) This has been the most effective detox method as it not only precipitates but also destabilizes some of the inhibitors, thereby drastically improving the ethanol yields. Hydrolysate treatments with sodium sulfite, (Larsson et al. 1999) sodium sulfite in combination with overliming, and adjustment with KOH and HCl have also proven to be effective detox methods. (Van Zyl, Prior, and Du Preez 1988) Although these treatments can remove the inhibitors, and improve fermentation performance, they also supplement an additional processing cost and considerable loss of fermentable sugars. (Rivard et al. 1996; von Sivers et al. 1994)

2.4.7 Selecting an ideal candidate for fermentations

The basic expectations from an organism to be used in ethanol production at high solid loading is that it should have a high productivity and the capability to withstand high sugar and ethanol concentrations to improve the process yields and reduce the distillation costs. Additional survival challenges present themselves when co-fermenting C-5 and C-6 sugars from both solid as well as the liquid fraction. Since, not many organisms are capable of living up to all the expectations, use of biotechnology tools such as promoting adaptive evolution, strain engineering etc. can prove extremely helpful in improving cell viability under stressful conditions.

Different fermentation strategies like using fed-batch substrate/enzyme feeding, increasing the cell density, cell flocculation, and cell recirculation have helped improving inhibitor tolerance and ethanol productivity. (Domingues et al. 2000; Ghose and Tyagi 1979; Hu, Bai, and An 2005; Verstrepen et al. 2003; Westman et al. n.d.; Xu, Zhao, and Bai 2005; Zhao, biotechnology, and 2009 n.d.) Some variants of S. *cerevisiae*, are known of being capable of naturally fermenting HMF to 2,5-bis-hydroxymethylfuran (HMF alcohol) and furfural to furfuryl alcohol under aerobic and anaerobic conditions rendering them less harmful. (Liu et al. 2004; Taherzadeh et al. 2000, n.d.)

All organisms have a natural survival instinct that motivates them to evolve and adapt to the environment they find themselves in. This natural resistance can be improved by acclimatizing the organism slowly to a more toxic environment via adaptive evolution. Yomano *et al.*, did serial transfers of E. *coli* in ethanol rich LB broths and after fourteen serial transfers they could ferment xylose to get close to 56 g/L of ethanol. (Yomano, York, and Ingram 1998) Dinh *et al.,* adapted *S. cerevisiae* to a very high ethanol concentration of 10%, producing a more tolerant strain. (Dinh et al. 2008) Heer *et al.,* adapted a *S. cerevisiae* strain to increasing furfural concentrations. They were able to grow the strain at high hydrolysate concentration with significantly reduced lag phases. (Heer and Sauer 2008) In a similar study by Gu *et al.,* the yeast was adapted to increasing corncob residue hydrolysate to achieve a high ethanol concentration of 62.68 g/L. (Gu, Zhang, and Bao 2014)

Genetically engineering the co-factor balance in the cell that is destabilized in the presence of inhibitors is also a powerful technique to create mutants capable of high

resistance. (Carmel-Harel, Microbiology, and 2000 n.d.; Gorsich et al. 2006) By identifying and overexpressing the genes and operons that could help improve stress tolerance, a desired phenotype could be conferred on the cells to help them survive a harsh environment. (Bonomo et al. n.d.; Gill et al. n.d.; Warnecke et al. n.d.) For example, Alper *et al.*, engineered the yeast transcription machinery, conferred three separate mutations in the SPT15 gene of S. *cerevisiae* to get the desired phenotype of higher glucose/ethanol tolerance. (Alper et al. 2006)

Studies have shown that metal ions are also capable of improving yeast ethanol tolerance. Dombek and Ingram, and Birch and Walker, have identified magnesium ion to be beneficial to fermentation, with the latter hypothesizing that the ions prevent increase in cell membrane permeability caused by ethanol. (Birch and Walker 2000; Dombek and Ingram 1986) Nabais *et al.,* showed the positive impact of calcium ion in augmenting ethanol tolerance. (Nabais et al. 1988) Tosun and Ergun, observed the impacts of zinc, sodium and potassium ions and found them to be also beneficial to yeast. (Tosun and Ergun 2007) Vanegas *et al.* studied the role of unsaturated lipids and ergosterol and observed that these components help the yeast strain uphold an optimal membrane thickness as ethanol concentration escalates during anaerobic fermentations. (Vanegas et al. 2012)

2.5 Concluding remarks and directions for future work

This review addresses the key obstacles and the existing solutions for effective execution of high solids processes. Several conclusive inferences concerning enzymatic hydrolysis and fermentation performed at high-solid loadings can be drawn and directions

for future research can be proposed following this comprehensive review of the available literature on this topic, including;

- The physical characteristics and rate of enzymatic digestibility of cellulosic substrates generated from different pretreatment technologies significantly affects the product yields and sets the upper bound on the allowable solid loading for high solids conversion processes for a particular feedstock. Hence, carefully selecting the pretreatment type and optimizing reaction conditions to generate the perfect substrate can definitely enhance process yields at high solid loading.
- In order to maximize overall sugar utilization from biomass via high solids SSCF, optimization of pretreatment method to minimize the formation of degradation products in the pretreatment hydrolysate, otherwise selecting a suitable hydrolysate detox method that is inexpensive and does not incur sugar losses is strongly recommended.
- Thoroughly investigating the impact of gradual substrate loading on the viscosity of biomass slurry via fed-batch feeding strategies is worth considering.
- Application of a customized enzyme cocktail with supplementation if necessary to meet the specific needs of a particular pretreated substrate would also improve saccharification efficiency.
- Selecting the ideal microbial candidate and using the modern genetic tools to modify the phenotypic traits of the strain to suit a particular process requirement is also worth the effort.

- Exploring native thermophilic ethnologenic organisms that can ferment both hexoses and pentoses should be deployed to fully realize the benefits of a high-solids SSFs.

2.6 References

Abdel-Banat, Babiker M. A., Hisashi Hoshida, Akihiko Ano, Sanom Nonklang, and Rinji Akada. 2010. "High-Temperature Fermentation: How Can Processes for Ethanol Production at High Temperatures Become Superior to the Traditional Process Using Mesophilic Yeast?" *Applied Microbiology and Biotechnology* 85(4):861–67.

Almeida, João RM, Tobias Modig, Anneli Petersson, Bärbel Hähn-Hägerdal, Gunnar Lidén, and Marie F. Gorwa-Grauslund. 2007. "Increased Tolerance and Conversion of Inhibitors in Lignocellulosic Hydrolysates BySaccharomyces Cerevisiae." *Journal of Chemical Technology & Biotechnology* 82(4):340–49.

Alper, Hal, Joel Moxley, Elke Nevoigt, Gerald R. Fink, and Gregory Stephanopoulos. 2006. "Engineering Yeast Transcription Machinery for Improved Ethanol Tolerance and Production." *Science (New York, N.Y.)* 314(5805):1565–68.

Anon. 1981. "Process for Producing Absolute Alcohol by Solvent Extraction and Vacuum Distillation."

Bai, FW, LJ Chen, Z. Zhang, … WA Anderson-Journal of, and undefined 2004. n.d. "Continuous Ethanol Production and Evaluation of Yeast Cell Lysis and Viability Loss under Very High Gravity Medium Conditions." *Elsevier*.

Bals, Bryan D., Christa Gunawan, Janette Moore, Farzaneh Teymouri, and Bruce E. Dale. 2014. "Enzymatic Hydrolysis of Pelletized AFEX^{TM}-Treated Corn Stover at High Solid Loadings." *Biotechnology and Bioengineering* 111(2):264–71.

Barbosa, M. F., M. J. Beck, J. E. Fein, D. Potts, and L. O. Ingram. 1992. "Efficient Fermentation of Pinus Sp. Acid Hydrolysates by an Ethanologenic Strain of Escherichia Coli." *Applied and Environmental Microbiology* 58(4):1382–84.

biotechnology, V. Balan-ISRN and undefined 2014. n.d. "Current Challenges in Commercially Producing Biofuels from Lignocellulosic Biomass." *Downloads.Hindawi.Com*.

Birch, Rosslyn M. and Graeme M. Walker. 2000. "Influence of Magnesium Ions on Heat Shock and Ethanol Stress Responses of Saccharomyces Cerevisiae." *Enzyme and Microbial Technology* 26(9–10):678–87.

Bonomo, J., T. Warnecke, P. Hume, … A. Marizcurrena-Metabolic, and undefined 2006. n.d. "A Comparative Study of Metabolic Engineering Anti-Metabolite Tolerance in Escherichia Coli." *Elsevier*.

Büchert, Johanna, Jürgen Puls, and Kaisa Poutanen. 1989. "The Use of Steamed Hemicellulose as Substrate in Microbial Conversions." *Applied Biochemistry and Biotechnology* 20–21(1):309–18.

Cannella, David, Chia Wen C. Hsieh, Claus Felby, and Henning Jørgensen. 2012. "Production and Effect of Aldonic Acids during Enzymatic Hydrolysis of Lignocellulose at High Dry Matter Content." *Biotechnology for Biofuels* 5(1):26.

Cara, Cristóbal, Manuel Moya, Ignacio Ballesteros, Ma José Negro, Alberto González, and Encarnación Ruiz. 2007. “Influence of Solid Loading on Enzymatic Hydrolysis of Steam Exploded or Liquid Hot Water Pretreated Olive Tree Biomass.” *Process Biochemistry* 42(6):1003–9.

Cardona, M. J., E. J. Tozzi, N. Karuna, T. Jeoh, R. L. Powell, and M. J. McCarthy. 2015. “A Process for Energy-Efficient High-Solids Fed-Batch Enzymatic Liquefaction of Cellulosic Biomass.” *Bioresource Technology* 198:488–96.

Carmel-Harel, O., G. Storz-Annual Reviews in Microbiology, and undefined 2000. n.d. “Roles of the Glutathione- and Thioredoxin-Dependent Reduction Systems in the Escherichia Coli and Saccharomyces Cerevisiae Responses to Oxidative Stress.” *Annualreviews.Org*.

Chandrakant, P. and V. S. Bisaria. 2000. “Simultaneous Bioconversion of Glucose and Xylose to Ethanol by Saccharomyces Cerevisiae in the Presence of Xylose Isomerase.” *Applied Microbiology and Biotechnology* 53(3):301–9.

Chen, Hong-Zhang and Zhi-Hua Liu. 2017. “Enzymatic Hydrolysis of Lignocellulosic Biomass from Low to High Solids Loading.” *Engineering in Life Sciences* 17(5):489–99.

Clark, Thomas A. and Keith L. Mackie. 2008. “Fermentation Inhibitors in Wood Hydrolysates Derived from the Softwood Pinus Radiata.” *Journal of Chemical Technology and Biotechnology. Biotechnology* 34(2):101–10.

Cruickshank, Christina. 1905. *The Stokes-Einstein Law Fo r Diffusion in Solution*. Vol. 17.

D’amore, Tony, Chandra J. Panchal, Inge Russell, and G. G. Stewart. 1989. “A Study of Ethanol Tolerance in Yeast.” *Critical Reviews in Biotechnology* 9(4):287–304.

DeMartini, Jaclyn D., Sivakumar Pattathil, Jeffrey S. Miller, Hongjia Li, Michael G. Hahn, and Charles E. Wyman. 2013. “Investigating Plant Cell Wall Components That Affect Biomass Recalcitrance in Poplar and Switchgrass.” *Energy & Environmental Science* 6(3):898.

Demirbas, Ayhan. 2009. “Political, Economic and Environmental Impacts of Biofuels: A Review.” *Applied Energy* 86:S108–17.

Dibble, Clare J., Tatyana A. Shatova, Jennie L. Jorgenson, and Jonathan J. Stickel. 2011. “Particle Morphology Characterization and Manipulation in Biomass Slurries and the Effect on Rheological Properties and Enzymatic Conversion.” *Biotechnology Progress* 27(6):1751–59.

Dien, B. S., M. A. Cotta, and T. W. Jeffries. 2003. “Bacteria Engineered for Fuel Ethanol Production: Current Status.” *Applied Microbiology and Biotechnology* 63(3):258–66.

Dien, B. S., N. N. Nichols, and R. J. Bothast. 2002. “Fermentation of Sugar Mixtures Using Escherichia Coli Catabolite Repression Mutants Engineered for Production of

L -Lactic Acid." *Journal of Industrial Microbiology and Biotechnology* 29(5):221–27.

Dinh, Thai Nho, Keisuke Nagahisa, Takashi Hirasawa, Chikara Furusawa, and Hiroshi Shimizu. 2008. "Adaptation of Saccharomyces Cerevisiae Cells to High Ethanol Concentration and Changes in Fatty Acid Composition of Membrane and Cell Size" edited by C. Herman. *PLoS ONE* 3(7):e2623.

Dombek, K. M. and L. O. Ingram. 1986. "Magnesium Limitation and Its Role in Apparent Toxicity of Ethanol during Yeast Fermentation." *Applied and Environmental Microbiology* 52(5):975–81.

Domingues, Lucília, António A. Vicente, Nelson Lima, and José A. Teixeira. 2000. "Applications of Yeast Flocculation in Biotechnological Processes." *Biotechnology and Bioprocess Engineering* 5(4):288–305.

Dumsday, G. J., B. Zhou, W. Yaqin, G. A. Stanley, and N. B. Pamment. 1999. "Comparative Stability of Ethanol Production by Escherichia Coli KO11 in Batch and Chemostat Culture." *Journal of Industrial Microbiology and Biotechnology* 23(1):701–8.

Ehrhardt, M. R., T. O. Monz, T. W. Root, R. K. Connelly, C. T. Scott, and D. J. Klingenberg. 2010. "Rheology of Dilute Acid Hydrolyzed Corn Stover at High Solids Concentration." *Applied Biochemistry and Biotechnology* 160(4):1102–15.

Gauss, WF, S. Suzuki, 990,944 M Takagi - US Patent 3, and undefined 1976. n.d. "Manufacture of Alcohol from Cellulosic Materials Using Plural Ferments." *Google Patents*.

Geng, Wenhui, Yongcan Jin, Hasan Jameel, and Sunkyu Park. 2015. "Strategies to Achieve High-Solids Enzymatic Hydrolysis of Dilute-Acid Pretreated Corn Stover." *Bioresource Technology* 187:43–48.

Ghose, T. K. and R. D. Tyagi. 1979. "Rapid Ethanol Fermentation of Cellulose Hydrolysate. I. Batch versus Continuous Systems." *Biotechnology and Bioengineering* 21(8):1387–1400.

Gill, RT, S. Wildt, YT Yang, … S. Ziesman-…. National Academy, and undefined 2002. n.d. "Genome-Wide Screening for Trait Conferring Genes Using DNA Microarrays." *National Acad Sciences*.

Gladis, Arne, Pia-Maria Bondesson, Mats Galbe, and Guido Zacchi. 2015. "Influence of Different SSF Conditions on Ethanol Production from Corn Stover at High Solids Loadings." *Energy Science & Engineering* 3(5):481–89.

Gonzales, Ralph Rolly, Periyasamy Sivagurunathan, and Sang Hyoun Kim. 2016. "Effect of Severity on Dilute Acid Pretreatment of Lignocellulosic Biomass and the Following Hydrogen Fermentation." *International Journal of Hydrogen Energy* 41(46):21678–84.

Gorsich, S. W., B. S. Dien, N. N. Nichols, P. J. Slininger, Z. L. Liu, and C. D. Skory.

2006. “Tolerance to Furfural-Induced Stress Is Associated with Pentose Phosphate Pathway Genes ZWF1, GND1, RPE1, and TKL1 in Saccharomyces Cerevisiae.” *Applied Microbiology and Biotechnology* 71(3):339–49.

Gu, Hanqi, Jian Zhang, and Jie Bao. 2014. “Inhibitor Analysis and Adaptive Evolution of Saccharomyces Cerevisiae for Simultaneous Saccharification and Ethanol Fermentation from Industrial Waste Corncob Residues.” *Bioresource Technology* 157:6–13.

Hadi, SM, A. Rehman-Mutation Research Letters, and undefined 1989. n.d. “Specificity of the Interaction of Furfural with DNA.” *Elsevier*.

He, Ming, Bo Wu, Han Qin, Zhi Ruan, Fu Tan, Jing Wang, Zong Shui, Li Dai, Qi Zhu, Ke Pan, Xiao Tang, Wen Wang, and Qi Hu. 2014. “Zymomonas Mobilis: A Novel Platform for Future Biorefineries.” *Biotechnology for Biofuels* 7(1):101.

Heer, Dominik and Uwe Sauer. 2008. “Identification of Furfural as a Key Toxin in Lignocellulosic Hydrolysates and Evolution of a Tolerant Yeast Strain.” *Microbial Biotechnology* 1(6):497–506.

Heipieper, HJ, FJ Weber, J. Sikkema, … H. Keweloh-Trends in, and undefined 1994. n.d. “Mechanisms of Resistance of Whole Cells to Toxic Organic Solvents.” *Elsevier*.

Hodge, David B., M. Nazmul Karim, Daniel J. Schell, and James D. McMillan. 2008. “Soluble and Insoluble Solids Contributions to High-Solids Enzymatic Hydrolysis of Lignocellulose.” *Bioresource Technology* 99(18):8940–48.

Hodge, DB, MN Karim, DJ Schell, JD McMillan-Bioresource Technology, and undefined 2008. n.d. “Soluble and Insoluble Solids Contributions to High-Solids Enzymatic Hydrolysis of Lignocellulose.” *Elsevier*.

Holtzapple, Mark, Mona Cognata, Yuancai Shu, and Christie Hendrickson. 1990. “Inhibition OfTrichoderma Reesei Cellulase by Sugars and Solvents.” *Biotechnology and Bioengineering* 36(3):275–87.

Holwerda, Evert K., Philip G. Thorne, Daniel G. Olson, Daniel Amador-Noguez, Nancy L. Engle, Timothy J. Tschaplinski, Johannes P. van Dijken, and Lee R. Lynd. 2014. “The Exometabolome of Clostridium Thermocellum Reveals Overflow Metabolism at High Cellulose Loading.” *Biotechnology for Biofuels* 7(1):155.

Hoyer, Kerstin, Mats Galbe, and Guido Zacchi. 2010. “Effects of Enzyme Feeding Strategy on Ethanol Yield in Fed-Batch Simultaneous Saccharification and Fermentation of Spruce at High Dry Matter.” *Biotechnology for Biofuels* 3(1):14.

Hu, Chun-Keng, Feng-Wu Bai, and Li-Jia An. 2005. “[Effect of Flocculence of a Self-Flocculating Yeast on Its Tolerance to Ethanol and the Mechanism].” *Sheng Wu Gong Cheng Xue Bao = Chinese Journal of Biotechnology* 21(1):123–28.

Hu, Cuimin, Siguo Wu, Qian Wang, Guojie Jin, Hongwei Shen, and Zongbao K. Zhao. 2011. “Simultaneous Utilization of Glucose and Xylose for Lipid Production by Trichosporon Cutaneum.” *Biotechnology for Biofuels* 4(1):25.

Hu, Jinguang, Valdeir Arantes, and Jack N. Saddler. 2011. “The Enhancement of Enzymatic Hydrolysis of Lignocellulosic Substrates by the Addition of Accessory Enzymes Such as Xylanase: Is It an Additive or Synergistic Effect?” *Biotechnology for Biofuels* 4:36.

Humbird, D., R. Davis, L. Tao, C. Kinchin, D. Hsu, A. Aden, P. Schoen, J. Lukas, B. Olthof, M. Worley, D. Sexton, and D. Dudgeon. 2002. *Process Design and Economics for Biochemical Conversion of Lignocellulosic Biomass to Ethanol: Dilute-Acid Pretreatment and Enzymatic Hydrolysis of Corn Stover*.

Jang, Soo Kyeong, Ho Yong Kim, Han Seob Jeong, Jae Young Kim, Hwanmyeong Yeo, and In Gyu Choi. 2016. “Effect of Ethanol Organosolv Pretreatment Factors on Enzymatic Digestibility and Ethanol Organosolv Lignin Structure from Liriodendron Tulipifera in Specific Combined Severity Factors.” *Renewable Energy* 87:599–606.

Jing, Xinyun, Xiaoxi Zhang, and Jie Bao. 2009. “Inhibition Performance of Lignocellulose Degradation Products on Industrial Cellulase Enzymes During Cellulose Hydrolysis.” *Applied Biochemistry and Biotechnology* 159(3):696–707.

Jönsson, L. J., E. Palmqvist, N. O. Nilvebrant, and B. Hahn-Hägerdal. 1998. “Detoxification of Wood Hydrolysates with Laccase and Peroxidase from the White-Rot Fungus Trametes Versicolor.” *Applied Microbiology and Biotechnology* 49(6):691–97.

Jönsson, Leif J. and Carlos Martín. 2016. “Pretreatment of Lignocellulose: Formation of Inhibitory by-Products and Strategies for Minimizing Their Effects.” *Bioresource Technology* 199:103–12.

Khan, QA, FA Shamsi, SM Hadi-Cancer letters, and undefined 1995. n.d. “Mutagenicity of Furfural in Plasmid DNA.” *Elsevier*.

Kim, Youngmi, Rick Hendrickson, Nathan S. Mosier, Michael R. Ladisch, Bryan Bals, Venkatesh Balan, and Bruce E. Dale. 2008. “Enzyme Hydrolysis and Ethanol Fermentation of Liquid Hot Water and AFEX Pretreated Distillers’ Grains at High-Solids Loadings.” *Bioresource Technology* 99(12):5206–15.

Klein-Marcuschamer, Daniel, Piotr Oleskowicz-Popiel, Blake A. Simmons, and Harvey W. Blanch. 2010. “Technoeconomic Analysis of Biofuels: A Wiki-Based Platform for Lignocellulosic Biorefineries.” *Biomass and Bioenergy* 34(12):1914–21.

Knutsen, Jeffrey S. and Matthew W. Liberatore. 2009. “Rheology of High-Solids Biomass Slurries for Biorefinery Applications.” *Journal of Rheology* 53(4):877–92.

Ko, Ja Kyong, Youngmi Kim, Eduardo Ximenes, and Michael R. Ladisch. 2015. “Effect of Liquid Hot Water Pretreatment Severity on Properties of Hardwood Lignin and Enzymatic Hydrolysis of Cellulose.” *Biotechnology and Bioengineering* 112(2):252–62.

Koppram, Rakesh, Fredrik Nielsen, Eva Albers, Annika Lambert, Sune Wännström, Lars

Welin, Guido Zacchi, and Lisbeth Olsson. 2013. “Simultaneous Saccharification and Co-Fermentation for Bioethanol Production Using Corncobs at Lab, PDU and Demo Scales.” *Biotechnology for Biofuels* 6(1):2.

Koppram, Rakesh and Lisbeth Olsson. 2014. “Combined Substrate, Enzyme and Yeast Feed in Simultaneous Saccharification and Fermentation Allow Bioethanol Production from Pretreated Spruce Biomass at High Solids Loadings.” *Biotechnology for Biofuels* 7(1):54.

Kumar, Rajeev, Samarthya Bhagia, Micholas Dean Smith, Loukas Petridis, Rebecca G. Ong, Charles M. Cai, Ashutosh Mittal, Michael H. Himmel, Venkatesh Balan, Bruce E. Dale, Arthur J. Ragauskas, Jeremy C. Smith, and Charles E. Wyman. 2018. “Cellulose–Hemicellulose Interactions at Elevated Temperatures Increase Cellulose Recalcitrance to Biological Conversion.” *Green Chemistry* 20(4):921–34.

Kumar, Rajeev and Charles E. Wyman. 2009. “Effect of Xylanase Supplementation of Cellulase on Digestion of Corn Stover Solids Prepared by Leading Pretreatment Technologies.” *Bioresource Technology* 100(18):4203–13.

Larsson, Simona, Anders Reimann, Nils-Olof Nilvebrant, and Leif J. Jönsson. 1999. “Comparison of Different Methods for the Detoxification of Lignocellulose Hydrolyzates of Spruce.” *Applied Biochemistry and Biotechnology* 77(1–3):91–104.

Lavenson, David M., Emilio J. Tozzi, Nardrapee Karuna, Tina Jeoh, Robert L. Powell, and Michael J. McCarthy. 2012. “The Effect of Mixing on the Liquefaction and Saccharification of Cellulosic Fibers.” *Bioresource Technology*.

Lee, Yong-Hyun -H and L. T. Fan. 1983. “Kinetic Studies of Enzymatic Hydrolysis of Insoluble Cellulose: (II). Analysis of Extended Hydrolysis Times.” *Biotechnology and Bioengineering* 25(4):939–66.

Leonard, R. H. and G. J. Hajny. 1945. “Fermentation of Wood Sugars to Ethyl Alcohol.” *Industrial & Engineering Chemistry* 37(4):390–95.

Li, Mi, Yunqiao Pu, and Arthur J. Ragauskas. 2016. “Current Understanding of the Correlation of Lignin Structure with Biomass Recalcitrance.” *Frontiers in Chemistry* 4.

Liu, Z. L., P. J. Slininger, B. S. Dien, M. A. Berhow, C. P. Kurtzman, and S. W. Gorsich. 2004. “Adaptive Response of Yeasts to Furfural and 5-Hydroxymethylfurfural and New Chemical Evidence for HMF Conversion to 2,5-Bis-Hydroxymethylfuran.” *Journal of Industrial Microbiology & Biotechnology* 31(8):345–52.

Liu, Zhi Hua and Hong Zhang Chen. 2016. “Simultaneous Saccharification and Co-Fermentation for Improving the Xylose Utilization of Steam Exploded Corn Stover at High Solid Loading.” *Bioresource Technology* 201:15–26.

Liu, Zhi Hua, Lei Qin, Jia Qing Zhu, Bing Zhi Li, and Ying Jin Yuan. 2014. “Simultaneous Saccharification and Fermentation of Steam-Exploded Corn Stover at High Glucan Loading and High Temperature.” *Biotechnology for Biofuels* 7(1):167.

López-Linares, Juan Carlos, Inmaculada Romero, Cristóbal Cara, Encarnación Ruiz, Manuel Moya, and Eulogio Castro. 2014. “Bioethanol Production from Rapeseed Straw at High Solids Loading with Different Process Configurations.” *Fuel* 122:112–18.

Lu, Yifeng, Yonghong Wang, Guoqian Xu, Ju Chu, Yingping Zhuang, and Siliang Zhang. 2010. “Influence of High Solid Concentration on Enzymatic Hydrolysis and Fermentation of Steam-Exploded Corn Stover Biomass.” *Applied Biochemistry and Biotechnology* 160(2):360–69.

Luque, Rafael, Lorenzo Herrero-Davila, Juan M. Campelo, James H. Clark, Jose M. Hidalgo, Diego Luna, Jose M. Marinas, and Antonio A. Romero. 2008. “Biofuels: A Technological Perspective.” *Energy & Environmental Science* 1(5):542.

Mahadevan, Radhakrishnan, Jeremy S. Edwards, and Francis J. Doyle. 2002. “Dynamic Flux Balance Analysis of Diauxic Growth in Escherichia Coli.” *Biophysical Journal* 83(3):1331–40.

Mandels, M., 764,475 J Kostick - US Patent 3, and undefined 1973. n.d. “Enzymatic Hydrolysis of Cellulose to Soluble Sugars.” *Google Patents*.

Mcmillan, James D., Mildred M. Newman, David W. Templeton, and Ali Mohagheghi. 1999. “Simultaneous Saccharification and Cofermentation of Dilute-Acid Pretreated Yellow Poplar Hardwood to Ethanol Using Xylose-Fermenting Zymomonas Mobilis.” Pp. 649–65 in *Twentieth Symposium on Biotechnology for Fuels and Chemicals*. Totowa, NJ: Humana Press.

Mills, Tirzah Y., Nicholas R. Sandoval, and Ryan T. Gill. 2009. “Cellulosic Hydrolysate Toxicity and Tolerance Mechanisms in Escherichia Coli.” *Biotechnology for Biofuels* 2(1):26.

Modenbach, Alicia A. and Sue E. Nokes. 2013. “Enzymatic Hydrolysis of Biomass at High-Solids Loadings - A Review.” *Biomass and Bioenergy* 56:526–44.

Mohagheghi, A., M. Tucker, K. Grohmann, and C. Wyman. 1992. “High Solids Simultaneous Saccharification and Fermentation of Pretreated Wheat Straw to Ethanol.” *Applied Biochemistry and Biotechnology* 33(2):67–81.

Montenecourt, S. 1983. *Trichoderma Reesei Cellulases*. Vol. 1.

Moreno, Antonio D., Elia Tomás-Pejó, David Ibarra, Mercedes Ballesteros, and Lisbeth Olsson. 2013. “Fed-Batch SSCF Using Steam-Exploded Wheat Straw at High Dry Matter Consistencies and a Xylose-Fermenting Saccharomyces Cerevisiae Strain: Effect of Laccase Supplementation.” *Biotechnology for Biofuels* 6(1):160.

Myers, RH, DC Montgomery, and CM Anderson-Cook. 2016. *Response Surface Methodology: Process and Product Optimization Using Designed Experiments*.

Nabais, R. C., I. Sá-Correia, C. A. Viegas, and J. M. Novais. 1988. “Influence of Calcium Ion on Ethanol Tolerance of Saccharomyces Bayanus and Alcoholic Fermentation by Yeasts.” *Applied and Environmental Microbiology* 54(10):2439–46.

Nguyen, Thanh Yen, Charles M. Cai, Rajeev Kumar, and Charles E. Wyman. 2015a. "Co-Solvent Pretreatment Reduces Costly Enzyme Requirements for High Sugar and Ethanol Yields from Lignocellulosic Biomass." *ChemSusChem* 8(10):1716–25.

Nguyen, Thanh Yen, Charles M. Cai, Rajeev Kumar, and Charles E. Wyman. 2015b. "Co-Solvent Pretreatment Reduces Costly Enzyme Requirements for High Sugar and Ethanol Yields from Lignocellulosic Biomass." *ChemSusChem* 8(10):1716–25.

Nguyen, Thanh Yen, Charles M. Cai, Rajeev Kumar, and Charles E. Wyman. 2017. "Overcoming Factors Limiting High-Solids Fermentation of Lignocellulosic Biomass to Ethanol." *Proceedings of the National Academy of Sciences of the United States of America* 114(44):11673–78.

Nguyen, Thanh Yen, Charles M. Cai, Omar Osman, Rajeev Kumar, and Charles E. Wyman. 2016a. "CELF Pretreatment of Corn Stover Boosts Ethanol Titers and Yields from High Solids SSF with Low Enzyme Loadings." *Green Chemistry* 18(6):1581–89.

Nguyen, Thanh Yen, Charles M. Cai, Omar Osman, Rajeev Kumar, and Charles E. Wyman. 2016b. "CELF Pretreatment of Corn Stover Boosts Ethanol Titers and Yields from High Solids SSF with Low Enzyme Loadings." *Green Chemistry* 18(6):1581–89.

Nguyen, TY, CM Cai, O. Osman, R. Kumar-Green Chemistry, and undefined 2016. n.d. "CELF Pretreatment of Corn Stover Boosts Ethanol Titers and Yields from High Solids SSF with Low Enzyme Loadings." *Pubs.Rsc.Org*.

Nordwald, Erik M., Roman Brunecky, Michael E. Himmel, Gregg T. Beckham, and Joel L. Kaar. 2014. "Charge Engineering of Cellulases Improves Ionic Liquid Tolerance and Reduces Lignin Inhibition." *Biotechnology and Bioengineering* 111(8):1541–49.

O'Byrne, Conor, Ian R. Booth, Andrew J. Roe, and Debra McLaggan. 2002. "Inhibition of Escherichia Coli Growth by Acetic Acid: A Problem with Methionine Biosynthesis and Homocysteine Toxicity." *Microbiology* 148(7):2215–22.

Olofsson, Kim, Magnus Bertilsson, and Gunnar Lidén. 2008. "A Short Review on SSF – an Interesting Process Option for Ethanol Production from Lignocellulosic Feedstocks." *Biotechnology for Biofuels* 1(1):7.

Ooshima, Hiroshi, Douglas S. Burns, and Alvin O. Converse. 1990. "Adsorption of Cellulase FromTrichoderma Reesei on Cellulose and Lignacious Residue in Wood Pretreated by Dilute Sulfuric Acid with Explosive Decompression." *Biotechnology and Bioengineering* 36(5):446–52.

Palmqvist, E., B. Hahn-Hägerdal, … Z. Szengyel-Enzyme and Microbial, and undefined 1997. n.d. "Simultaneous Detoxification and Enzyme Production of Hemicellulose Hydrolysates Obtained after Steam Pretreatment." *Elsevier*.

Palmqvist, E., B. Hahn-Hägerdal, M. Galbe-…. and Microbial Technology, and

undefined 1996. n.d. “The Effect of Water-Soluble Inhibitors from Steam-Pretreated Willow on Enzymatic Hydrolysis and Ethanol Fermentation.” *Elsevier*.

Palmqvist, Eva, Jonas S. Almeida, and B�rbel Hahn-H�gerdal. 1999. “Influence of Furfural on Anaerobic Glycolytic Kinetics OfSaccharomyces Cerevisiae in Batch Culture.” *Biotechnology and Bioengineering* 62(4):447–54.

Palmqvist, Eva and Bärbel Hahn-Hägerdal. 2000a. “Fermentation of Lignocellulosic Hydrolysates. I: Inhibition and Detoxification.” *Bioresource Technology* 74(1):17–24.

Palmqvist, Eva and Bärbel Hahn-Hägerdal. 2000b. “Fermentation of Lignocellulosic Hydrolysates. II: Inhibitors and Mechanisms of Inhibition.” *Bioresource Technology* 74(1):25–33.

Qin, Lei, Zhi-Hua Liu, Mingjie Jin, Bing-Zhi Li, and Ying-Jin Yuan. 2013. “High Temperature Aqueous Ammonia Pretreatment and Post-Washing Enhance the High Solids Enzymatic Hydrolysis of Corn Stover.” *Bioresource Technology* 146:504–11.

Qing, Qing, Bin Yang, and Charles E. Wyman. 2010. “Xylooligomers Are Strong Inhibitors of Cellulose Hydrolysis by Enzymes.” *Bioresource Technology* 101(24):9624–30.

Ragauskas, Arthur J. 2017. “Pseudo-Lignin Formation during Dilute Acid Pretreatment for Cellulosic Ethanol.” *Recent Advances in Petrochemical Science* 1(1):1–5.

Rahikainen, Jenni, Saara Mikander, Kaisa Marjamaa, Tarja Tamminen, Angelos Lappas, Liisa Viikari, and Kristiina Kruus. 2011. “Inhibition of Enzymatic Hydrolysis by Residual Lignins from Softwood-Study of Enzyme Binding and Inactivation on Lignin-Rich Surface.” *Biotechnology and Bioengineering* 108(12):2823–34.

Rahman, SA, SM Hadi-Food and Chemical Toxicology, and undefined 1991. n.d. “Reaction of Furfural and Methylfurfural with DNA: Use of Single-Strand-Specific Nucleases.” *Elsevier*.

Ramos, Luiz Pereira, Larissa da Silva, Annielly Comelli Ballem, Ana Paula Pitarelo, Luana Marcele Chiarello, and Marcos Henrique Luciano Silveira. 2015. “Enzymatic Hydrolysis of Steam-Exploded Sugarcane Bagasse Using High Total Solids and Low Enzyme Loadings.” *Bioresource Technology* 175:195–202.

Reyes-Ortiz, Vimalier, Richard A. Heins, Gang Cheng, Edward Y. Kim, Briana C. Vernon, Ryan B. Elandt, Paul D. Adams, Kenneth L. Sale, Masood Z. Hadi, Blake A. Simmons, Michael S. Kent, and Danielle Tullman-Ercek. 2013. “Addition of a Carbohydrate-Binding Module Enhances Cellulase Penetration into Cellulose Substrates.” *Biotechnology for Biofuels* 6(1):93.

Rivard, Christopher J., Rebecca E. Engel, Tammy K. Hayward, Nicholas J. Nagle, Christos Hatzis, and George P. Philippidis. 1996. “Measurement of the Inhibitory Potential and Detoxification of Biomass Pretreatment Hydrolysate for Ethanol Production.” *Applied Biochemistry and Biotechnology* 57–58(1):183–91.

Roberts, Katrina M., David M. Lavenson, Emilio J. Tozzi, Michael J. McCarthy, and Tina Jeoh. 2011. “The Effects of Water Interactions in Cellulose Suspensions on Mass Transfer and Saccharification Efficiency at High Solids Loadings.” *Cellulose*.

Roche, Christine M., Clare J. Dibble, Jeffrey S. Knutsen, Jonathan J. Stickel, and Matthew W. Liberatore. 2009. “Particle Concentration and Yield Stress of Biomass Slurries during Enzymatic Hydrolysis at High-Solids Loadings.” *Biotechnology and Bioengineering*.

Roe, Andrew J., Debra Mclaggan, Ian Davidson, Conor O’byrne, † And, and Ian R. Booth. 1998. *Perturbation of Anion Balance during Inhibition of Growth of Escherichia Coli by Weak Acids*. Vol. 180.

Sambrook, J. and DW Russel. 2001. “Molecular Cloning a Laboratory Manual. Vol. 3, Pp.”

Sant’Ana da Silva, Ayla, Marcella Fernandes de Souza, Ignacio Ballesteros, Paloma Manzanares, Mercedes Ballesteros, and Elba P. S. Bon. 2016. “High-Solids Content Enzymatic Hydrolysis of Hydrothermally Pretreated Sugarcane Bagasse Using a Laboratory-Made Enzyme Blend and Commercial Preparations.” *Process Biochemistry* 51(10):1561–67.

Schellhorn, H. E., V. L. Stones, D. N. Barstad, D. L. Blankenshorn, and J. L. Slonczewski. 1992. “Regulation of KatF and KatE in Escherichia Coli K-12 by Weak Acids.” *Journal of Bacteriology* 174(14):4769–76.

Shuai, Li, Ydna M. Questell-Santiago, and Jeremy S. Luterbacher. 2016. “A Mild Biomass Pretreatment Using γ-Valerolactone for Concentrated Sugar Production.” *Green Chemistry* 18(4):937–43.

von Sivers, Margareta, Guido Zacchi, Lisbeth Olsson, and Baerbel Hahn-Haegerdal. 1994. “Cost Analysis of Ethanol Production from Willow Using Recombinant Escherichia Coli.” *Biotechnology Progress* 10(5):555–60.

Smith, Micholas Dean, Xiaolin Cheng, Loukas Petridis, Barmak Mostofian, and Jeremy C. Smith. 2017. “Organosolv-Water Cosolvent Phase Separation on Cellulose and Its Influence on the Physical Deconstruction of Cellulose: A Molecular Dynamics Analysis.” *Scientific Reports* 7(1):1–9.

Smith, Micholas Dean, Barmak Mostofian, Xiaolin Cheng, Loukas Petridis, Charles M. Cai, Charles E. Wyman, and Jeremy C. Smith. 2016. “Cosolvent Pretreatment in Cellulosic Biofuel Production: Effect of Tetrahydrofuran-Water on Lignin Structure and Dynamics.” *Green Chemistry* 18(5):1268–77.

Srivastava, Neha, Rishabh Rathour, Sonam Jha, Karan Pandey, Manish Srivastava, Vijay Kumar Thakur, Rakesh Singh Sengar, Vijai K. Gupta, Pranab Behari Mazumder, Ahamad Faiz Khan, and Pradeep Kumar Mishra. 2019. “Microbial Beta Glucosidase Enzymes: Recent Advances in Biomass Conversation for Biofuels Application.” *Biomolecules* 9(6).

Stanley, D., A. Bandara, S. Fraser, P. J. Chambers, and G. A. Stanley. 2010. "The Ethanol Stress Response and Ethanol Tolerance of *Saccharomyces Cerevisiae*." *Journal of Applied Microbiology*.

Stephen Glen Allen, *, Deborah Schulman, and Joseph Lichwa, Jr. Michael Jerry Antal, Edward Jennings and, and Richard Elander. 2001. "A Comparison of Aqueous and Dilute-Acid Single-Temperature Pretreatment of Yellow Poplar Sawdust."

Stickel, Jonathan J., Jeffrey S. Knutsen, Matthew W. Liberatore, Wing Luu, Douglas W. Bousfield, Daniel J. Klingenberg, C. Tim Scott, Thatcher W. Root, Max R. Ehrhardt, and Thomas O. Monz. 2009. "Rheology Measurements of a Biomass Slurry: An Inter-Laboratory Study." *Rheologica Acta*.

Stouthamer, AH. 1979. "The Search for Correlation between Theoretical and Experimental Growth Yields Vol. 21."

Stutzenberger, Fred and Gillian Lintz. 1986. "Hydrolysis Products Inhibit Adsorption of Trichoderma Reesei C30 Cellulases to Protein-Extracted Lucerne Fibres." *Enzyme and Microbial Technology* 8(6):341–44.

Su, Rongxin, Yuanyuan Ma, Wei Qi, Mingjia Zhang, Fang Wang, Ruoyu Du, Jifeng Yang, Minhua Zhang, and Zhimin He. 2013. "Ethanol Production from High-Solid SSCF of Alkaline-Pretreated Corncob Using Recombinant Zymomonas Mobilis CP4." *BioEnergy Research* 6(1):292–99.

Taherzadeh, M. J., L. Gustafsson, C. Niklasson, and G. Lidén. 2000. "Physiological Effects of 5-Hydroxymethylfurfural on Saccharomyces Cerevisiae." *Applied Microbiology and Biotechnology* 53(6):701–8.

Taherzadeh, MJ, L. Gustafsson, … C. Niklasson-Journal of bioscience and, and undefined 1999. n.d. "Conversion of Furfural in Aerobic and Anaerobic Batch Fermentation of Glucose by Saccharomyces Cerevisiae." *Elsevier*.

Tosun, Ayşe and Mübeccel Ergun. 2007. "Use of Experimental Design Method to Investigate Metal Ion Effects in Yeast Fermentations." *Journal of Chemical Technology & Biotechnology* 82(1):11–15.

Tozzi, Emilio J., Michael J. McCarthy, David M. Lavenson, Maria Cardona, and Robert L. Powell, Nardrapee Karuna, and Tina Jeoh. 2014. "Effect of Fiber Structure on Yield Stress during Enzymatic Conversion of Cellulose." *AIChE Journal* 60(5):1582–90.

Um, Byung-Hwan and G. Peter Van Walsum. n.d. "Effect of Pretreatment Severity on Accumulation of Major Degradation Products from Dilute Acid Pretreated Corn Stover and Subsequent Inhibition of Enzymatic Hydrolysis of Cellulose."

Vanegas, Juan M., Maria F. Contreras, Roland Faller, and Marjorie L. Longo. 2012. "Role of Unsaturated Lipid and Ergosterol in Ethanol Tolerance of Model Yeast Biomembranes."

Varga, Enikõ, Helene B. Klinke, Kati Réczey, and Anne Belinda Thomsen. 2004. “High Solid Simultaneous Saccharification and Fermentation of Wet Oxidized Corn Stover to Ethanol.” *Biotechnology and Bioengineering*.

VERDUYN, C., E. POSTMA, W. A. SCHEFFERS, and J. P. VAN DIJKEN. 1990. “Energetics of Saccharomyces Cerevisiae in Anaerobic Glucose-Limited Chemostat Cultures.” *Journal of General Microbiology* 136(3):405–12.

Verstrepen, K. J., G. Derdelinckx, H. Verachtert, and F. R. Delvaux. 2003. “Yeast Flocculation: What Brewers Should Know.” *Applied Microbiology and Biotechnology* 61(3):197–205.

Viamajala, Sridhar, James D. McMillan, Daniel J. Schell, and Richard T. Elander. 2009. “Rheology of Corn Stover Slurries at High Solids Concentrations - Effects of Saccharification and Particle Size.” *Bioresource Technology* 100(2):925–34.

Wang, Wen, Xinshu Zhuang, Zhenhong Yuan, Qiang Yu, Wei Qi, Qiong Wang, and Xuesong Tan. 2012. “High Consistency Enzymatic Saccharification of Sweet Sorghum Bagasse Pretreated with Liquid Hot Water.” *Bioresource Technology* 108:252–57.

Wang, Yun-Yan, Priya Sengupta, Brent Scheidemantle, Yunqiao Pu, Charles E. Wyman, Charles M. Cai, and Arthur J. Ragauskas. 2020. “Effects of CELF Pretreatment Severity on Lignin Structure and the Lignin-Based Polyurethane Properties.” *Frontiers in Energy Research* 8:149.

Warnecke, TE, MD Lynch, A. Karimpour-Fard-…. engineering, and undefined 2008. n.d. “A Genomics Approach to Improve the Analysis and Design of Strain Selections.” *Elsevier*.

Westman, JO, V. Mapelli, … MJ Taherzadeh-Appl. Environ, and undefined 2014. n.d. “Flocculation Causes Inhibitor Tolerance in Saccharomyces Cerevisiae for Second-Generation Bioethanol Production.” *Am Soc Microbiol*.

Westman, Johan O., Ruifei Wang, Vera Novy, and Carl Johan Franzén. 2017. “Sustaining Fermentation in High-Gravity Ethanol Production by Feeding Yeast to a Temperature-Profiled Multifeed Simultaneous Saccharification and Co-Fermentation of Wheat Straw.” *Biotechnology for Biofuels* 10(1):213.

Wilson, J. Jeffrey, Lise Deschatelets, and Nora K. Nishikawa. 1989. “Comparative Fermentability of Enzymatic and Acid Hydrolysates of Steam-Pretreated Aspenwood Hemicellulose by Pichia Stipitis CBS 5776.” *Applied Microbiology and Biotechnology* 31–31(5–6):592–96.

Xiao, Ling Ping, Zhao Jun Sun, Zheng Jun Shi, Feng Xu, and Run Cang Sun. 2011. “Impact of Hot Compressed Water Pretreatment on the Structural Changes of Woody Biomass for Bioethanol Production.” *BioResources* 6(2):1576–98.

Xiao, Zhizhuang, Xiao Zhang, David J. Gregg, and John N. Saddler. 2004. “Effects of Sugar Inhibition on Cellulases and β-Glucosidase During Enzymatic Hydrolysis of

Softwood Substrates." Pp. 1115–26 in *Proceedings of the Twenty-Fifth Symposium on Biotechnology for Fuels and Chemicals Held May 4–7, 2003, in Breckenridge, CO*. Totowa, NJ: Humana Press.

Ximenes, E., Y. Kim, N. Mosier, B. Dien-…. and microbial technology, and undefined 2011. n.d. "Deactivation of Cellulases by Phenols." *Elsevier*.

Xu, Chao, Jun Zhang, Yu Zhang, Ying Guo, Huijuan Xu, Jingliang Xu, and Zhongming Wang. 2019. "Enhancement of High-Solids Enzymatic Hydrolysis Efficiency of Alkali Pretreated Sugarcane Bagasse at Low Cellulase Dosage by Fed-Batch Strategy Based on Optimized Accessory Enzymes and Additives." *Bioresource Technology* 292:121993.

Xu, Feng and Hanshu Ding. 2007. "A New Kinetic Model for Heterogeneous (or Spatially Confined) Enzymatic Catalysis: Contributions from the Fractal and Jamming (Overcrowding) Effects." *Applied Catalysis A: General* 317(1):70–81.

Xu, Qi, Arjun Singh, and Michael E. Himmel. 2009. "Perspectives and New Directions for the Production of Bioethanol Using Consolidated Bioprocessing of Lignocellulose." *Current Opinion in Biotechnology* 20(3):364–71.

Xu, T. J., X. Q. Zhao, and F. W. Bai. 2005. "Continuous Ethanol Production Using Self-Flocculating Yeast in a Cascade of Fermentors." *Enzyme and Microbial Technology* 37(6):634–40.

Yomano, L. P., S. W. York, and L. O. Ingram. 1998. "Isolation and Characterization of Ethanol-Tolerant Mutants of Escherichia Coli KO11 for Fuel Ethanol Production." *Journal of Industrial Microbiology and Biotechnology* 20(2):132–38.

Zaldivar, J., J. Nielsen, and L. Olsson. 2001. "Fuel Ethanol Production from Lignocellulose: A Challenge for Metabolic Engineering and Process Integration." *Applied Microbiology and Biotechnology* 56(1–2):17–34.

Zaldivar, Jesus, Alfredo Martinez, and Lonnie O. Ingram. 1999. "Effect of Selected Aldehydes on the Growth and Fermentation of EthanologenicEscherichia Coli." *Biotechnology and Bioengineering* 65(1):24–33.

Zhang, Jian, Deqiang Chu, Juan Huang, Zhanchun Yu, Gance Dai, and Jie Bao. 2010. "Simultaneous Saccharification and Ethanol Fermentation at High Corn Stover Solids Loading in a Helical Stirring Bioreactor." *Biotechnology and Bioengineering* 105(4):718–28.

Zhang, Junhua, Ming Tang, and Liisa Viikari. 2012. "Xylans Inhibit Enzymatic Hydrolysis of Lignocellulosic Materials by Cellulases." *Bioresource Technology* 121:8–12.

Zhang, Xiao, Wenjuan Qin, Michael G. Paice, and John N. Saddler. 2009. "High Consistency Enzymatic Hydrolysis of Hardwood Substrates." *Bioresource Technology* 100(23):5890–97.

Zhao, XQ, FW Bai-Journal of biotechnology, and undefined 2009. n.d. "Mechanisms of

Yeast Stress Tolerance and Its Manipulation for Efficient Fuel Ethanol Production." *Elsevier*.

Zhou, Hua, Renli Zhang, Wang Zhan, Liuyang Wang, Lijun Guo, and Yun Liu. 2016. "High Biomass Loadings of 40 Wt% for Efficient Fractionation in Biorefineries with an Aqueous Solvent System without Adding Adscititious Catalyst." *Green Chemistry* 18(22):6108–14.

Zhu, J. Y., R. Gleisner, C. T. Scott, X. L. Luo, and S. Tian. 2011. "High Titer Ethanol Production from Simultaneous Enzymatic Saccharification and Fermentation of Aspen at High Solids: A Comparison between SPORL and Dilute Acid Pretreatments." *Bioresource Technology* 102(19):8921–29.

Zhu, J.Y., R. Gleisner, C. T. Scott, X. L. Luo, and S. Tian. 2011. "High Titer Ethanol Production from Simultaneous Enzymatic Saccharification and Fermentation of Aspen at High Solids: A Comparison between SPORL and Dilute Acid Pretreatments." *Bioresource Technology* 102(19):8921–29.

Zhu, J. Y., X. J. Pan, G. S. Wang, and R. Gleisner. 2009. "Sulfite Pretreatment (SPORL) for Robust Enzymatic Saccharification of Spruce and Red Pine." *Bioresource Technology* 100(8):2411–18.

Van Zyl, Carina, Bernard A. Prior, and James C. Du Preez. 1988. "Production of Ethanol from Sugar Cane Bagasse Hemicellulose Hydrolyzate ByPichia Stipitis." *Applied Biochemistry and Biotechnology* 17(1–3):357–69.

Chapter 3 : Influence of CELF pretreatment severity on physiochemical features impacting enzymatic deconstruction of Poplar*

*This chapter was completed in collaboration with Kisailus group at University of California Riverside. FTIR and XRD characterization experiments on biomass samples were performed by Ms. Ramya Mohan and Dr. David Kisailus.

3.1 Abstract

The influence of CELF pretreatment operating parameters on the yield of sugars, major degradation components, physiochemical features of the biomass, and enzymatic hydrolysis of cellulose were investigated for of hardwood Poplar. The combined effects of CELF pretreatment reaction time and temperature, grouped together as "reaction severity," were varied at a constant 0.5% sulfuric acid concentration. Although high severity reactions increased production of sugar monomers, sugar degradation products such as furfural and 5-hydroxymethylfurfural (5-HMF) also were enhanced. The biomass crystallinity index also increased with increasing pretreatment severity. "Pseudo-lignin" structures in the solid residues formed by degradation of polysaccharides and lignin also increased at harsher reaction conditions. Enzymatic hydrolysis yields could be correlated to changes in the physiochemical features of the biomass resulting operation at higher severities. It was found that although CELF pretreated Poplar was completely digestible irrespective of the reaction severity, substrates generated at the lowest and highest severities required the most time to hydrolyze completely. The results further revealed that this outcome could be due to lignin left in biomass at low severity and "pseudo lignin" formed at higher severities significantly impacted saccharification and that reducing "pseudo-lignin" formation is important to obtaining high cellulose digestibility. at higher severities.

3.2 Introduction

The native recalcitrance of lignocellulosic biomass is the biggest barrier to its enzymatic saccharification at competitive costs. The complex cell wall matrix formed by the interactions among cellulose, hemicellulose, and lignin restricts access of fungal

enzymes to the polysaccharides, making some type of treatment necessary to open up this complex structure.(Yang and Wyman 2008) Such pretreatment technologies, primarily removing or disrupting lignin and/or hemicellulose, alter the structural composition in such a way as to increase the macro and micro accessibility of cellulose and make it more amenable to enzymatic hydrolysis. (Brienzo et al. 2015, 2017)

Various pretreatment methods have been applied to lignocellulosic materials prior to enzymatic hydrolysis to release fermentable sugars. The most investigated methods involve application of steam, dilute acid, alkali, or solvents.(Kumar and Sharma 2017; Wyman et al. 2005) These pretreatments can be performed over a range of temperatures from 150 to 220 °C and for residence times between seconds to minutes to hours.(Yang and Wyman 2008) The combined effect of temperature and reaction duration can be conveniently combined in terms of "pretreatment severity."(Gonzales, Sivagurunathan, and Kim 2016; Kabel et al. 2007; Um and Peter Van Walsum n.d.) Every pretreatment method impacts the physiochemical features of the biomass and thereby influences its susceptibility to enzymatic saccharification.(Wyman et al. 2013) Increasing reaction severity further alters these features and changes the extent of biomass deconstruction, the degree of lignin and hemicellulose removal, and such cellulose morphological features as surface area, pore volume, and crystallinity. (Brienzo et al. 2015) Although the relationship between alteration of various structural and chemical traits of biomass by pretreatment and subsequent enzymatic hydrolysis have been the focus of considerable research, further understanding these fundamental aspects can facilitate pretreatment development and optimization.

Co-Solvent Enhanced Lignocellulosic Fractionation (CELF) employs a miscible solution of tetrahydrofuran (THF) and water in a 1:1 ratio by weight, along with very dilute sulfuric acid as a catalyst to disrupt the plant cell wall and fractionate the major components. It solubilizes most of the hemicellulose and Klason lignin along with some of the cellulose to generate a cellulose rich solid for further processing. The present work investigates the response of hardwood Poplar to CELF pretreatment conducted over a selected range of temperatures and times that can be combined in terms of a parameter known as severity. In this case, severity is defined as:

$$\log R_0 = \log\left[t * exp\left\{\frac{(T-100)}{14.75}\right\}\right]$$

in which T is the temperature in °C and the time is in minutes. Although acid concentration could be included to calculate a combined severity parameter, it is not used here in light of the acid concentration being held constant. The chemical compositions of solid and liquid streams were determined, and the physical properties of the raw biomass and pretreated solids were analyzed by Fourier transform infrared spectroscopy (FTIR) and X-ray powder diffraction (XRD). Finally, the influence of pretreatment severity on biomass digestibility was evaluated by correlating sugar yields from subsequent enzymatic hydrolysis of Poplar to the physiochemical changes caused by different CELF reaction conditions.

3.3 Experimental Section

3.3.1 Materials

Populus trichocarpa, a woody biomass, was generously provided by the BioEnergy Science Centre (BESC). The composition of the native material as determined by following

NREL LAP (version 08-03-2012) was 47.0 % glucan, 16.9 % xylan, and 21.2% acid-insoluble lignin.(Sluiter, Hames, Ruiz, et al. 2008) The biomass was air-dried, and knife milled by a laboratory mill (Model 4, Arthur H. Thomas Company, Philadelphia, PA) to pass through a 1mm internal sieve size. The enzyme cocktail used was Accellerase® 1500 generously provided by Dupont Industrial Biosciences (Palo Alto, CA) and had a protein content of 82 mg/ml as estimated using a Pierce BCA analysis kit.

3.3.2 Pretreatment

For CELF pretreatment of Poplar, milled wood chips were soaked overnight at 4 °C at a dry biomass loading of 7.5 wt% based on the total working mass of the reaction, in a 1:1 (weight basis) solution of THF to water, with 0.5 wt% H_2SO_4 based on the total solvent mass as the catalyst. The reactions were conducted in a 1 L Hastelloy Parr autoclave reactor (236HC Series, Parr Instruments Co., Moline, IL) equipped with a double stacked pitch blade impeller rotating at 200 rpm. Pretreatments were carried out at 150, 160, and 180 °C for 15 and 30 minutes at each temperature. All reactions were maintained at temperature (±2 °C) by convective heating using a 4 kW fluidized sand bath (Model SBL-2D, Techne, Princeton, NJ), with the temperature inside the reactor measured directly by an in-line thermocouple (Omega, K-type). At the end of the reaction, the reactor was cooled by submerging quickly in a large room temperature water bath. The solids were then separated from the reaction liquor by vacuum filtration at room temperature through glass fiber filter paper (Fisher Scientific, Pittsburgh, PA). The mass and density of the filtrates were measured to calculate yields and close mass balances. The solids collected were then washed with water until clear water ran through them. Compositional analysis was

performed on the solids following the NREL LAP (version 7-17-2005) to determine the sugars and K-lignin content followed by a total dissolved solids analysis of the pretreatment hydrolysate according to NREL LAP (version 03-31-2008). (Sluiter, Hames, Hyman, et al. 2008; Sluiter, Hames, Ruiz, et al. 2008)

3.3.3. CELF Lignin Extraction and Purification:

The filtrate collected from filtration of the pretreated solids was poured in a beaker and titrated to a pH ~ 7 using ammonium hydroxide. The neutralized hydrolysate was then poured in a rotary evaporator bulb that was placed in a water bath at 50 °C. The bulb was rotated at 100 rpm, and most of the THF was evaporated from the hydrolysate in ~ 30 min under vacuum (0.06-0.08 KPa). Almost 90% of the THF was recovered in the collection flask leaving behind precipitated lignin and a concentrated sugar solution in the evaporator bulb. The bulb contents were then filtered through a pre-weighed glass fiber filter paper. Lignin that had lined the interiors of the glass bulb was carefully removed on the filter paper and rinsed with water followed by diethyl ether and then again with water to remove soluble impurities. The washed lignin along with the filter paper was placed in a dark oven at 65 °C to dry overnight to a moisture content of <3%. The lignin along with the filter paper was then weighed to calculate the lignin recovered. CELF lignin was then removed from the filter paper and ground to a fine powder by a mortar and pestle.

3.3.4 Fourier Transform Infrared Spectroscopy (FTIR)

Cryo-ground samples in liquid N_2 were powdered together with KBr and pressed in pellets to perform conventional transmission FTIR experiments (FTIR, Nicolet 6700). For each spectrum registration, a total of 512 FTIR scans were made and averaged over a

wavenumbers region of 500 to 400 cm^{-1} at a 4cm^{-1} resolution corrected for ambient atmospheric conditions at ~30°C.

3.3.5 X-Ray Diffraction (XRD)

The pretreated samples were first ground under liquid nitrogen and subsequently characterized by powder X-ray diffraction (XRD, PANalytical Empyrean Series 2) using Cu Kα (λ = 0.1546 nm) radiation. Phase identification (crystal structure and % crystallinity of cellulose) and quantitative analyses were performed using PANalytical X'Pert Highscore Plus software. The Crystalline Index for the samples was calculated using Herman's method and Segal's method, Equations 1 and 2, respectively. The crystallite size was calculated using Scherrer Equation, 3

$$Crystalline\ Index\ (CrI) = \frac{A_{cryst}}{A_{total}} \times 100 \quad (1)$$

$$Crystalline\ Index\ (CrI) = \frac{I_{200} - I_{am}}{I_{am}} \times 100 \quad (2)$$

$$Crysatllite\ Size\ (L) = \frac{K \times \lambda}{\beta \times cos\theta} \quad (3)$$

In which A_{cryst} is the sum of the crystalline band areas, A_{total} is the total area under the diffractogram, I_{200} is the maximum intensity of the (200) lattice diffraction, I_{am} is the intensity diffraction of the amorphous band, K is a constant value 0.94, λ is the X-ray wavelength (0.1542 nm), β is the half-height width of the diffraction band, and Θ is the Bragg angle corresponding to the (200) plane.

3.3.6 Enzymatic Hydrolysis of CELF pretreated Poplar

Enzymatic hydrolysis followed the standard NREL protocol(Resch, Baker, and Decker 2015b) in 125 mL batch flasks with a total working volume of 50 mL containing

CELF pretreated biomass at a glucan loading of 1 wt%, 50 mM citrate buffer (pH 4.5) to reach the final pH of 4.8 in 50 mL, 0.02% sodium azide as an antimicrobial agent, and Accellerase® 1500 cocktail loaded at 30 mg protein loading per g-glucan in the flask. Triplicates at each condition were loaded with millipore water, citrate buffer, sodium azide, and the appropriate amount of substrate. The flasks were then placed in an incubator shaker at 50 °C at 150 rpm and equilibrated for 1 h. Appropriate amounts of enzyme cocktail were then added to the flasks, and they were placed again in the incubator shaker. 1 mL samples were taken out, centrifuged at 15000 rpm for 10 min, diluted, and analyzed to measure the sugar concentration in the broth. The percent glucan digestibility or the enzymatic hydrolysis sugar yields were calculated using Equation 4.

$$Percent\ glucan\ digestibility = Enzymatic\ hydrolysis\ yields = \frac{C_{glucose} \times WV}{M_{glucan} \times 1.11} \times 100 \quad (4)$$

Where $C_{glucose}$ is the glucose concentration in the flask at any time point, g/mL, WV is the working volume in the flask, 50 mL, M_{glucan} is the initial mass of glucan loaded in the flask, g.

3.3.7 Measuring sugar, furfural, and 5-HMF concentrations

Liquid samples along with appropriate calibration standards were analyzed using an HPLC (Waters Alliance 2695 system equipped with a Bio-Rad Aminex® HPX-87H column and Waters 2414 RI detector) at an eluent (5 mM sulfuric acid) flow rate of 0.6 ml min^{-1}. The chromatograms were integrated by using the Empower® 2 software package (Waters Co., Milford, MA).

3.4 Results and Discussion

3.4.1 Effects of CELF pretreatment severity on Poplar deconstruction

Acid based pretreatments, usually aim at breaking the polysaccharide-lignin complex interactions to retain the polysaccharides in the solid fraction and release sugar monomers and some lignin into the liquid. (Li et al. 2010) While lower (<160 °C) temperature pretreatments may disrupt polysaccharide-lignin linkages, it is likely that enough glycosidic bonds will be broken to produce a large portion of monomeric sugars. Harsher reaction conditions at high enough temperatures, however, can degrade monomeric sugars further into byproducts, such as 5-hydroxymethylfurfural (5-HMF), furfural, levulinic acid, and formic acid. These can inhibit fermentative organisms, resulting in less sugar consumption and lower fermentation productivity. (Anon n.d.) These degradation products can also undergo condensation reactions to form pseudo-lignin that impedes enzymatic hydrolysis through unproductive binding with enzymes or by blocking the active cellulose surface binding sites. (Aarum et al. 2018; Shinde et al. 2018) Therefore, pretreatment conditions should be optimized to maximize sugar and lignin recovery with minimal degradation.

Figures 3-1 (a) and (b) show the percent solubilization of poplar by CELF pretreatment over the reaction of times and temperatures chosen. Also shown are material balances for sugars and K-lignin in the biomass fed to CELF as well as in the solid and liquid fractions obtained after application of these conditions, all on the basis of 100 g of poplar fed to CELF pretreatment. Figure 3-1 (a) indicates that solid solubilization of Poplar increased with increasing pretreatment severity. While 44% of the raw biomass was solubilized at the lowest pretreatment severity, 56% solubilization was observed at the

medium severity of 160 °C, 30 min and escalated to 79% at the harshest reaction condition of 180 °C for 30 min. The material balance, Figure 3-1 (b), further illustrates that most of the glucan was retained in the solid fraction, with only a little glucose in the liquid up to the reaction at 160 °C, 15 min. The glucose monomer concentration in the hydrolysate increased with increasing pretreatment severity. However, at 180 °C, HMF, a glucan degradation product, was measured, and not all the glucan could be accounted for due to degradation to species that could not be found. Xylan, however, followed an opposite trend with most of it appearing as monomers in the hydrolysate and only a small amount remaining in the solid fraction even at the lowest severity reactions at 150 °C, 15 min. Furfural, a xylan degradation product, became measurable starting at 150 °C reaction for 30 min. However, unlike glucan, the amount of xylose and furfural accounted for most of the xylan originally in poplar for all reaction conditions. On the other hand, Klason lignin followed an irregular pattern in that K-lignin in the pretreated solids initially decreased with increasing pretreatment severity up to 160 °C, 30 min and then increased rapidly at harsher reaction conditions. The lignin recovered from the liquid fraction, however, continued to increase with increasing reaction severity. Total Klason lignin recovered from the solid and liquid fraction following 180 °C pretreatment was found to be greater than the amount of lignin in the poplar feed. This outcome suggested formation of pseudo lignin from degraded sugars. In fact, a correlation value of ~0.6-0.7 was calculated between the amount of unaccounted for glucan and the excess lignin formed for the high temperature reactions, Additional Figure 3-1 further shows the appearance of lignin-like structure that might result from acid-catalyzed condensation reactions of dehydrated/fragmented

cellulose. (Aarum et al. 2018; Shinde et al. 2018) Material balances indicated that operating CELF at 160 °C, 15 min gave the highest sugar and lignin recovery from Poplar with minimum degradation.

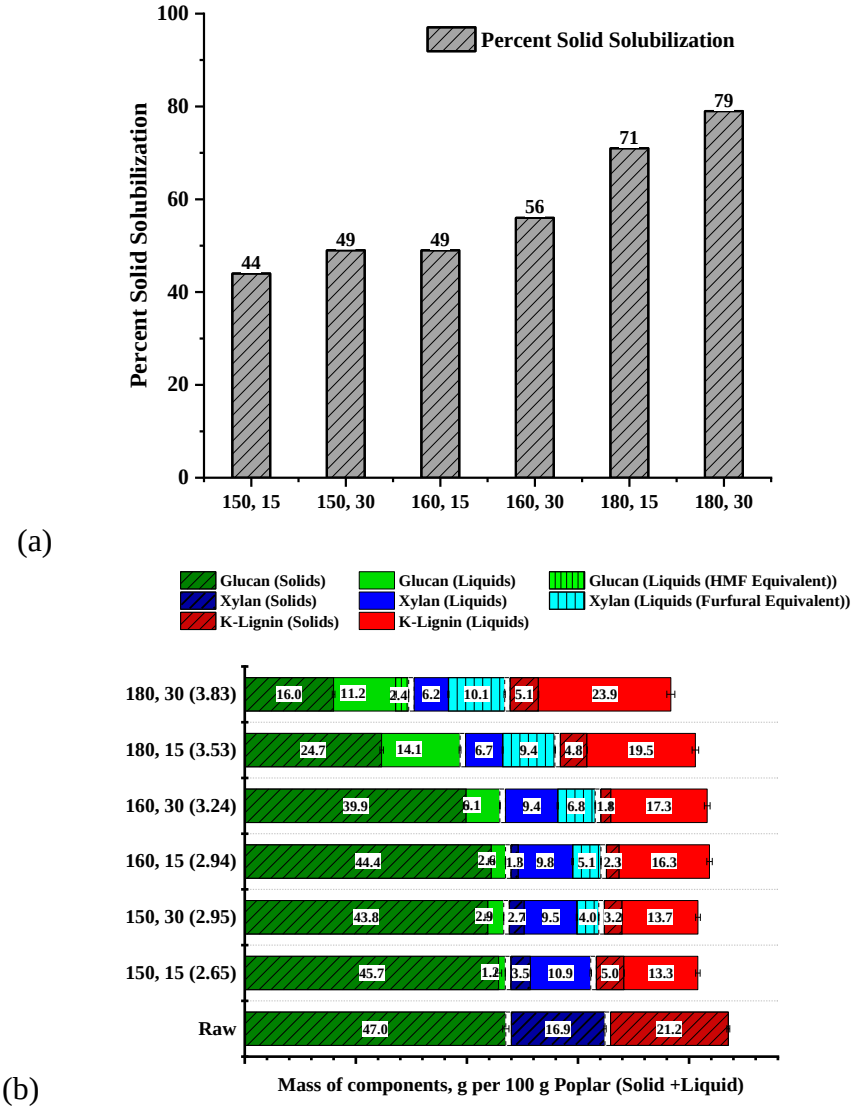


Figure 3-1 (a). Percent of raw biomass solubilized by CELF pretreatment with increasing reaction severity. (b). Mass of components (glucan, xylan and Klason lignin) in poplar fed as well as in the solid and liquid fractions resulting from CELF pretreatment at the stated reaction conditions. Entries refer to reaction temperature and time, e.g., 150, 15 (2.65), indicates CELF pretreatment of Poplar at 150 °C for 15 min at a severity of 2.65.

3.4.2 Influence of severity on the physiochemical traits of CELF solids

Lignocellulosic biomass undergoes physical and chemical changes during high temperature, low pH reactions. (Anon n.d.; Li et al. 2010) Understanding the physiochemical changes resulting from CELF pretreatment can reveal insights into underlying relationships between pretreatment severity and structural features that eventually impact biomass digestibility. In this case, FTIR spectroscopy and X-ray diffraction (XRD) results were coupled with chemical composition analyses (Additional Table 3-1) to determine how these factors might be related over the range of CELF pretreatment conditions applied.

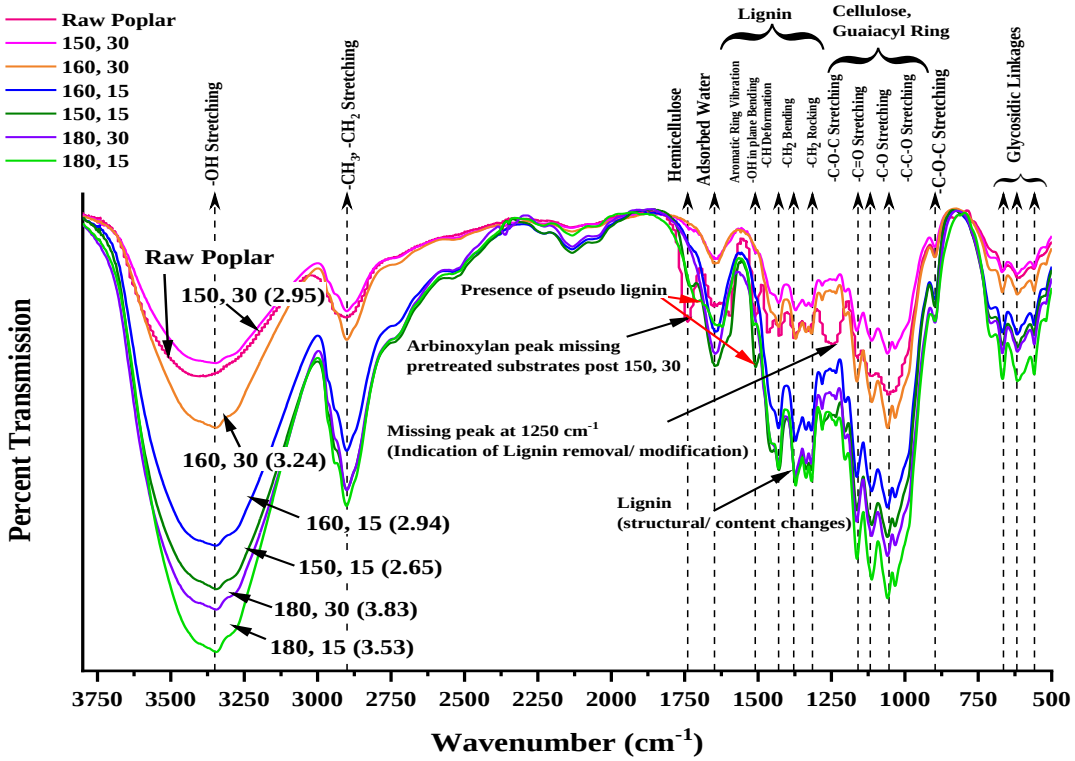


Figure 3-2. Fourier transform infrared spectroscopic (FTIR) results for raw and CELF pretreated Poplar. As before, entries refer to reaction temperature and time, e.g., 150, 15 (2.65), indicates CELF pretreatment of Poplar at 150 °C for 15 min at a severity of 2.65.

Figure 3-2 reports on the FTIR spectra transmission bands of different cellulosic samples, divided into a "diagnostic region" from 4000-2700 cm^{-1} and a "fingerprint region" from 1800-800 cm^{-1}. Figure 3-2 along with the known interpretations of specific transmission bands shown in Additional Table 3-2 were coupled to follow the chemical changes in Poplar post CELF pretreatment. The band between 3410-3350 cm^{-1} has been assigned to different –OH stretching modes. While a broad peak at 3400 cm^{-1} results from a strong intramolecular hydrogen bond, a sharp peak shifted towards the right to ~3350 cm^{-1} reveals hydrogen bonding due to the presence of free alcohols. (Poletto, Pistor, and Zattera 2013) The –OH stretching band was found to be stronger in pretreated substrates than in raw Poplar indicating a larger number of hydroxyl groups and more hydrogen bond formation. However, 150 °C, 15 min, 180 °C, 15 min, and 180 °C, 30 min residues with a higher lignin content exhibited more intense and sharper peaks than for other residues that are indicative of free alcohols in the form of lignin phenolics. (Xu et al. 2013) The bands at 2950 and 2835 cm^{-1} have been assigned to the methoxy groups ($–OCH_3$) that are recognized as common functional groups associated with lignin, such as coniferyl alcohol and sinapyl alcohol. (Guo et al. 2009) These bands are also more intense for samples with a higher lignin content. The 1750 cm^{-1} peak due to –C=O stretch in ketone/aldehyde groups in hemicellulose sugars is prominent in raw Poplar and the 150 °C, 15 min sample; a shoulder peak is observed in the 150 °C, 30 min sample; and the peak is missing in residues produced at higher temperatures. This trend could be attributed to partial followed by complete removal of xylan with increasing reaction severity. (Poletto et al. 2013) A water adsorption peak was also observed at 1650 cm^{-1} for all pretreated substrates that could be

credited to cellulose increasing as a result of xylan and lignin removal. (Poletto et al. 2013) The peak at 1500 cm^{-1} associated with aromatic ring vibration of lignin was observed prominently in raw Poplar and solids from CELF pretreatment at 150 °C, 15 min, with shoulders appearing in higher severity samples indicating lignin removal or a modified lignin structure. (Xu et al. 2013, 2015) The bands at 1710 and 1510 cm^{-1} in the residue from 180 °C reactions could indicate the presence of pseudo-lignin structures formed by condensation reaction between glucose and 5-HMF. (Aarum et al. 2018) Peaks at 1375 and 1336 cm^{-1}, associated with –C-H vibration, –C-H bending, and -OH in plane bending in cellulose also deepen in residues obtained at harsher reaction conditions. (Li et al. 2010) There was no peak for all pretreated residues at 1250 cm^{-1} that would be associated with –C-O stretch of guaicyl lignin showing delignification/ lignin modification. (Brienzo et al. 2017) The bands at 1430 and 894 cm^{-1} are sensitive to crystalline and amorphous cellulose. (Oh et al. 2005; Poletto et al. 2013) Intensity of crystalline peak increased with severity, while the amorphous peak dropped, indicating increase in crystallinity of the residue with increasing severity. The bands at 1164, 1118, 1064, and 1027 cm^{-1} are assigned to asymmetric –C-O-C bridge stretching, anhydroglucose ring asymmetric stretching, -CO stretching, in-plane C-H deformation and C-H deformation of cellulose, respectively. (Poletto et al. 2013) Overall, the FTIR analysis is consistent with the compositional analysis data (Additional Table 3-1) in that the absence of arabinoxylan bands in high severity reaction residues confirms xylan removal from the solids, the intensified water adsorption peaks point towards a glucan-rich residue, and the bands corresponding to

pseudo-lignin structures confirm the rationale that lignin was generated for the 180 °C pretreatment.

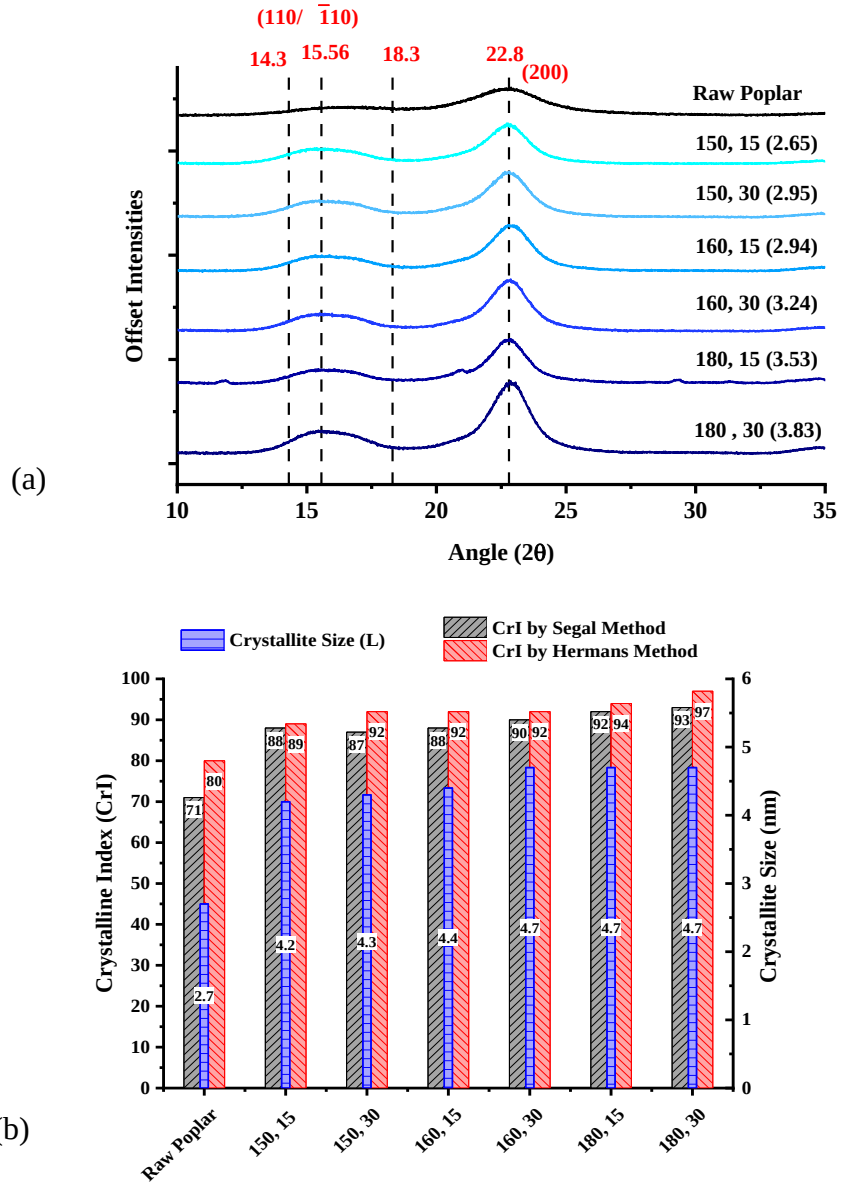


Figure 3-3. (a) XRD patterns of raw and CELF pretreated Poplar for CELF pretreatment conditions employed. (b) Crystalline Index (CrI) calculated by Segal's and Herman's methods along with crystallite size calculated by Scherrer's equation of raw and CELF pretreated solids at different reaction conditions. As before, entries refer to reaction temperature and time, e.g., 150, 15 (2.65), indicates CELF pretreatment of Poplar at 150 °C for 15 min at a severity of 2.65.

The crystalline nature of pretreated residues as determined by Crystalline Index (CrI) and crystallite size (L) has also been considered as an important factor that influences the digestibility of cellulosic substrates. Hence, CrI was calculated using Segal's method and Herman's method and crystallite size (L) was calculated using Scherrer's equation from X-ray diffractograms for both native as well as CELF pretreated poplar solids. (Poletto et al. 2013) Figure 3-3 (a), (b) shows XRD patterns and CrI and L values for different cellulosic samples, respectively. The peak intensities and broadening differs from one sample to another with the major differences occurring at 22.8°, 15.6° and 18.3° 2Ɵ reflections that correspond to the crystallographic plane of (200) and (110), and the amorphous phase of cellulose, respectively. Figure 3-3 (b) reveals that CrI and crystallite size increased with CELF pretreatment and continued to rise with pretreatment severity. This behavior can be attributed to the enrichment of pretreated material with cellulose due to removal of the amorphous components xylan and K-lignin. On the contrary, samples obtained at reaction conditions of 180 °C for 30 min had the highest crystallinity index despite having a high reported K-lignin value, supporting the theory of pseudo lignin formation from the chemical composition and FTIR analysis. These results along with Additional Figure 3-1 further suggest that pseudo lignin formed was crystalline in nature, hence, probably a glucan condensation product.

3.4.2 Correlation between physiochemical changes and enzymatic digestibility of CELF pretreated Poplar

Understanding how changes in physiochemical features by CELF pretreatment impact enzymatic saccharification of the resulting solids can help target methods and conditions to apply to realize high yields for subsequent biological conversion to ethanol.

Hence, CELF pretreated solids were subjected to enzymatic hydrolysis at a glucan loading of 1 wt% and an enzyme dose of 30 mg protein per g glucan following NREL LAP (version 02-04-2015). (Resch, Baker, and Decker 2015a) Figure 3-4 shows the percent glucan hydrolyzed estimated from the amount of glucose released during the course of saccharification as a function of hydrolysis time. Although, the extent of overall saccharification was similar for all the cellulosic substrates irrespective of the pretreatment harshness, reaction severity impacted the rate of saccharification. In particular, solids generated at the lowest severity of 150 °C, 15 min exhibited the slowest initial hydrolysis rate and took the longest time for complete digestion. But, at the other extreme, solids produced at the highest severity of 180 °C, 30 min reaction experienced the next slowest rate followed by solids produced at 150 °C, 30 min. The latter started at a faster initial rate than for the 180 °C, 15 min sample but slowed enough that the two had similar extents of digestion from 12 to 48 hours.

While the two extreme severity cases took almost 48 h to digest completely, the digestibility of the medium severity samples from CELF pretreatment at 160 °C, 15 min and 160 °C, 30 min were consistently higher throughout the course of saccharification and were completely digested in less than 24 h. The result that samples with a higher Klason lignin content and a higher crystalline index exhibited a lower saccharification rate suggested that these factors negatively impact the digestibility of CELF pretreated substrates. This result is consistent with some previous findings that crystallinity adversely affects the efficacy of enzymatic hydrolysis since highly crystalline cellulose is less amenable to cellulase attack than amorphous cellulose. (Zhu et al. 2008) Furthermore,

residual lignin in biomass has been known to impose various structural obstructions to enzyme action or unproductively bind with enzymes to compromise their activity, thereof, leading to slow digestion. (Brienzo et al. 2017; Rahikainen et al. 2011) Pseudo-lignin formation that results from CELF operation at the higher severity conditions also had an undesirable effect on enzymatic saccharification as indicated by the decline in rate of hydrolysis for these samples. Similar to our observations, Hu *et al.* suggested that pseudo-lignin formed by dilute acid pretreatment of Poplar holocellulose slowed hydrolysis and reduced in enzymatic conversion due to enzyme adsorption on pseudo-lignin. (Hu, Jung, and Ragauskas 2012)

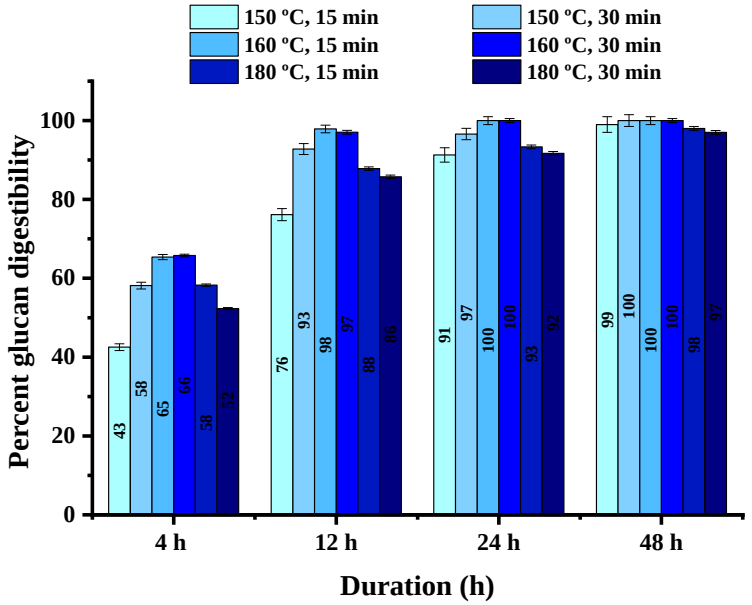


Figure 3-4. Percent enzymatic hydrolysis of 1 wt% glucan loadings of solids produced by CELF pretreatment at the stated condition for an enzyme dose of 30 mg protein per g glucan.

3.5 Conclusions

Subjecting hardwood Poplar to CELF pretreatment over a range of reaction temperatures and times demonstrated that CELF was capable of breaking polysaccharide-lignin linkages and hydrolyzing xylan and lignin even at the lower reaction severities applied to produce a glucan-rich substrate. FTIR and XRD analysis of the pretreated solids revealed that increasing pretreatment severity resulted in sugars degradation and formation of pseudo lignin that could be a condensation product of dehydrated cellulose that is crystalline in nature. Application of a medium severity condition of 160 °C, 15 min maximized both sugar yields and lignin recovery from Poplar. Enzymatic hydrolysis of these solids suggested that pretreatment severity may not have affected the extent of hydrolysis but it had impacted the rate of enzymatic saccharification. Substrates generated at the lowest and highest severities with a high lignin or pseudo-lignin content, respectively, required the longest times to digest completely, suggesting that these components play a key role in determining the rate of enzymatic hydrolysis and a high lignin or pseudo lignin content is detrimental to the saccharification process.

3.6 References

Aarum, Ida, Hanne Devle, Dag Ekeberg, Svein J. Horn, and Yngve Stenstrøm. 2018. "Characterization of Pseudo-Lignin from Steam Exploded Birch."

Anon. n.d. "Effect of Severity on Dilute Acid Pretreatment of Lignocellulosic Biomass and the Following Hydrogen Fermentation | Elsevier Enhanced Reader." Retrieved May 21, 2020 (https://reader.elsevier.com/reader/sd/pii/S036031991631179X?token=9917359BB03478E885EAA647C39A6C537818F1701269211E93534E7BEAD2CDBF9854951520FBCE71D64BDFD24D2D5D77).

Brienzo, Michel, Simphiwe Fikizolo, Yuda Benjamin, Luvuyo Tyhoda, and Johann Görgens. 2017. "Influence of Pretreatment Severity on Structural Changes, Lignin Content and Enzymatic Hydrolysis of Sugarcane Bagasse Samples." *Renewable Energy* 104:271–80.

Brienzo, Michel, Luvuyo Tyhoda, Yuda Benjamin, and Johann Görgens. 2015. "Relationship between Physicochemical Properties and Enzymatic Hydrolysis of Sugarcane Bagasse Varieties for Bioethanol Production." *New Biotechnology* 32(2):253–62.

Gonzales, Ralph Rolly, Periyasamy Sivagurunathan, and Sang Hyoun Kim. 2016. "Effect of Severity on Dilute Acid Pretreatment of Lignocellulosic Biomass and the Following Hydrogen Fermentation." *International Journal of Hydrogen Energy* 41(46):21678–84.

Guo, Gia Luen, Deng Chieh Hsu, Wen Hua Chen, Wei Hsi Chen, and Wen Song Hwang. 2009. "Characterization of Enzymatic Saccharification for Acid-Pretreated Lignocellulosic Materials with Different Lignin Composition." *Enzyme and Microbial Technology* 45(2):80–87.

Hu, Fan, Seokwon Jung, and Arthur Ragauskas. 2012. "Pseudo-Lignin Formation and Its Impact on Enzymatic Hydrolysis." *Bioresource Technology* 117:7–12.

Kabel, Mirjam A., Gijs Bos, Jan Zeevalking, Alphons G. J. Voragen, and Henk A. Schols. 2007. "Effect of Pretreatment Severity on Xylan Solubility and Enzymatic Breakdown of the Remaining Cellulose from Wheat Straw." *Bioresource Technology* 98(10):2034–42.

Kumar, Adepu Kiran and Shaishav Sharma. 2017. "Recent Updates on Different Methods of Pretreatment of Lignocellulosic Feedstocks: A Review." *Bioresources and Bioprocessing* 4(1):7.

Li, Chenlin, Bernhard Knierim, Chithra Manisseri, Rohit Arora, Henrik V. Scheller, Manfred Auer, Kenneth P. Vogel, Blake A. Simmons, and Seema Singh. 2010. "Comparison of Dilute Acid and Ionic Liquid Pretreatment of Switchgrass: Biomass Recalcitrance, Delignification and Enzymatic Saccharification." *Bioresource*

Technology 101(13):4900–4906.

Oh, Sang Youn, Il Yoo Dong, Younsook Shin, Chul Kim Hwan, Yong Kim Hak, Sik Chung Yong, Ho Park Won, and Ho Youk Ji. 2005. "Crystalline Structure Analysis of Cellulose Treated with Sodium Hydroxide and Carbon Dioxide by Means of X-Ray Diffraction and FTIR Spectroscopy." *Carbohydrate Research* 340(15):2376–91.

Poletto, Matheus, Vinícios Pistor, and Ademir J. Zattera. 2013. "Structural Characteristics and Thermal Properties of Native Cellulose."

Rahikainen, Jenni, Saara Mikander, Kaisa Marjamaa, Tarja Tamminen, Angelos Lappas, Liisa Viikari, and Kristiina Kruus. 2011. "Inhibition of Enzymatic Hydrolysis by Residual Lignins from Softwood-Study of Enzyme Binding and Inactivation on Lignin-Rich Surface." *Biotechnology and Bioengineering* 108(12):2823–34.

Resch, M. G., J. O. Baker, and S. R. Decker. 2015a. *Low Solids Enzymatic Saccharification of Lignocellulosic Biomass: Laboratory Analytical Procedure (LAP), Issue Date: February 4, 2015*.

Resch, M. G., J. O. Baker, and S. R. Decker. 2015b. *Low Solids Enzymatic Saccharification of Lignocellulosic Biomass: Laboratory Analytical Procedure (LAP), Issue Date: February 4, 2015*.

Shinde, Somnath D., Xianzhi Meng, Rajeev Kumar, and Arthur J. Ragauskas. 2018. "Recent Advances in Understanding the Pseudo-Lignin Formation in a Lignocellulosic Biorefinery." *Green Chemistry* 20(10):2192–2205.

Sluiter, A., B. Hames, D. Hyman, C. Payne, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, and J. Wolfe. 2008. *Determination of Total Solids in Biomass and Total Dissolved Solids in Liquid Process Samples Laboratory Analytical Procedure (LAP) Issue Date: 3/31/2008*.

Sluiter, A., B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, and D. Crocker. 2008. *Determination of Structural Carbohydrates and Lignin in Biomass: Laboratory Analytical Procedure (LAP); Issue Date: 7/17/2005*.

Um, Byung-Hwan and G. Peter Van Walsum. n.d. "Effect of Pretreatment Severity on Accumulation of Major Degradation Products from Dilute Acid Pretreated Corn Stover and Subsequent Inhibition of Enzymatic Hydrolysis of Cellulose."

Wyman, Charles E., Bruce E. Dale, Venkatesh Balan, Richard T. Elander, Mark T. Holtzapple, Rocío Sierra Ramirez, Michael R. Ladisch, Nathan S. Mosier, Y. Y. Lee, Rajesh Gupta, Steven R. Thomas, Bonnie R. Hames, Ryan Warner, and Rajeev Kumar. 2013. "Comparative Performance of Leading Pretreatment Technologies for Biological Conversion of Corn Stover, Poplar Wood, and Switchgrass to Sugars." Pp. 239–59 in *Aqueous Pretreatment of Plant Biomass for Biological and Chemical Conversion to Fuels and Chemicals*. Chichester, UK: John Wiley & Sons, Ltd.

Wyman, Charles E., Bruce E. Dale, Richard T. Elander, Mark Holtzapple, Michael R. Ladisch, and Y. Y. Lee. 2005. "Coordinated Development of Leading Biomass Pretreatment Technologies." *Bioresource Technology* 96(18 SPEC. ISS.):1959–66.

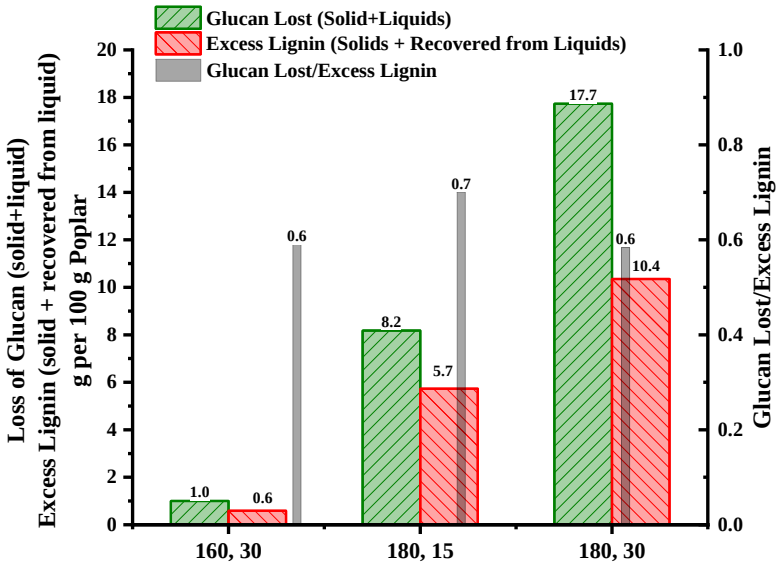
Xu, Feng, Jianming Yu, Tesfaye Tesso, Floyd Dowell, and Donghai Wang. 2013. "Qualitative and Quantitative Analysis of Lignocellulosic Biomass Using Infrared Techniques: A Mini-Review." *Applied Energy* 104:801–9.

Xu, Huanfei, Guang Yu, Xindong Mu, Chunyan Zhang, Paul DeRoussel, Chao Liu, Bin Li, and Haisong Wang. 2015. "Effect and Characterization of Sodium Lignosulfonate on Alkali Pretreatment for Enhancing Enzymatic Saccharification of Corn Stover." *Industrial Crops and Products* 76:638–46.

Yang, Bin and Charles E. Wyman. 2008. "Pretreatment: The Key to Unlocking Low-Cost Cellulosic Ethanol." *Biofuels, Bioproducts and Biorefining* 2(1):26–40.

Zhu, Li, Jonathan P. O'Dwyer, Vincent S. Chang, Cesar B. Granda, and Mark T. Holtzapple. 2008. "Structural Features Affecting Biomass Enzymatic Digestibility." *Bioresource Technology* 99(9):3817–28.

3.7 Additional Information



Additional Figure 3-1 Amount of raw glucan missing in the pretreated slurry solid +liquid fractions, excess lignin recovered post pretreatment from the solid + liquid fractions, and ratio of glucan lost to excess lignin measured.

$$Glucan\ lost\ (Solid + Liquid) = m_{glucan,initial} - (m_{glucan,solid} + m_{glucan,liquid}) \quad (1)$$

$$Excess\ Lignin\ (Solid + Liquid) = m_{lignin,initial} - (m_{lignin,solid} + m_{lignin,liquid}) \quad (2)$$

Where, $m_{glucan,initial}$ is the mass of glucan in raw biomass introduced during pretreatment, g per 100 g of Poplar, $m_{glucan,solid}$ is the mass of glucan recovered from the solid fraction post pretreatment, g per 100 g of raw Poplar, $m_{glucan,liquid}$ is the total mass of glucan hydrolyzed in the liquid fraction including glucose monomers and glucan equivalent to the mass of 5-HMF produced post pretreatment, g per 100 g of raw Poplar, $m_{lignin,initial}$ is the mass of lignin in raw biomass introduced during pretreatment, g per 100 g of Poplar, $m_{lignin,solid}$ is the mass of residual lignin in the solid fraction post

pretreatment, g per 100 g of raw Poplar, $m_{lignin,liquid}$ is the total mass of lignin recovered from the pretreatment hydrolysate, g per 100 g of raw Poplar.

Additional Table 3-1 Compositional analysis of raw and CELF pretreated Poplar at reaction conditions applied.

Sample	% Glucan	% Xylan	% K-lignin
Raw Poplar	47	16.9	21.2
CELF Pretreated Poplar			
150 °C, 15 min	81.6	6.3	8.9
150 °C, 30 min	86.9	5.3	6.3
160 °C, 15 min	88.5	2.5	4.6
160 °C, 30 min	90.6	0	4.2
180 °C, 15 min	85.2	0	14.8
180 °C, 30 min	75.8	0	24

Additional Table 3-2 Functional groups associated with polymer types assigned to FTIR transmittance wavenumber bands. (Brienzo et al. 2017; Guo et al. 2009; Li et al. 2010; Oh et al. 2005; Poletto, Pistor, and Zattera 2013; Xu et al. 2013)

Wavenumber (cm^{-1})	Assignment/ Functional Group	Polymer
3410 (Strong, Broad)	-OH Stretching, Hydrogen Bonding	
3350 (Strong, Sharp)	-OH Stretching, Free alcohols, phenols (lignin)	Lignin
2835, 2950	-CH Stretching, Alkanes, Aldehydes, Methoxy groups in lignin	Lignin
1750	-C=O stretching in ketones, ester group of Xylan acetates	Hemicellulose
1650	Adsorbed water	Cellulose, Hemicellulose
1595	Aromatic ring vibration, C=O Stretch	Lignin
1511	Aromatic ring vibration	Lignin
1460	-CH deformation	Lignin
1425, 1440	-C-H_2 Bending, -C-H deformation, O-H in-plane bending	Lignin
1375, 1336	-CH Bending, C-H Vibration, O-H in plane Bending	Cellulose, Hemicellulose, Lignin
1327	-C-O Syringyl Ring	Lignin
1317	-CH_2 Rocking	Cellulose, Hemicellulose
1250	-C=O, Esters, Aromatic Ring Vibration	Guaicyl Lignin
1207	-O-H Bending	Cellulose, Hemicellulose
1166	-C-O-C- Asymmetrical Stretching	Cellulose, Hemicellulose
1118	Anhydroglucose Ring Asymmetric Stretching	Cellulose
1064	-CO stretching	Cellulose, Hemicellulose,
1027	In Plane –CH deformation	Cellulose, Hemicellulose, Lignin
711, 671, 620, 559	Glycosidic Linkages	Cellulose, Hemicellulose

3.7.1 References

Brienzo, Michel, Simphiwe Fikizolo, Yuda Benjamin, Luvuyo Tyhoda, and Johann Görgens. 2017. "Influence of Pretreatment Severity on Structural Changes, Lignin Content and Enzymatic Hydrolysis of Sugarcane Bagasse Samples." *Renewable Energy* 104:271–80.

Guo, Gia Luen, Deng Chieh Hsu, Wen Hua Chen, Wei Hsi Chen, and Wen Song Hwang. 2009. "Characterization of Enzymatic Saccharification for Acid-Pretreated Lignocellulosic Materials with Different Lignin Composition." *Enzyme and Microbial Technology* 45(2):80–87.

Li, Chenlin, Bernhard Knierim, Chithra Manisseri, Rohit Arora, Henrik V. Scheller, Manfred Auer, Kenneth P. Vogel, Blake A. Simmons, and Seema Singh. 2010. "Comparison of Dilute Acid and Ionic Liquid Pretreatment of Switchgrass: Biomass Recalcitrance, Delignification and Enzymatic Saccharification." *Bioresource Technology* 101(13):4900–4906.

Oh, Sang Youn, Il Yoo Dong, Younsook Shin, Chul Kim Hwan, Yong Kim Hak, Sik Chung Yong, Ho Park Won, and Ho Youk Ji. 2005. "Crystalline Structure Analysis of Cellulose Treated with Sodium Hydroxide and Carbon Dioxide by Means of X-Ray Diffraction and FTIR Spectroscopy." *Carbohydrate Research* 340(15):2376–91.

Poletto, Matheus, Vinícios Pistor, and Ademir J. Zattera. 2013. "Structural Characteristics and Thermal Properties of Native Cellulose."

Xu, Feng, Jianming Yu, Tesfaye Tesso, Floyd Dowell, and Donghai Wang. 2013. "Qualitative and Quantitative Analysis of Lignocellulosic Biomass Using Infrared Techniques: A Mini-Review." *Applied Energy* 104:801–9.

Chapter 4 : CELF pretreatment of hardwood poplar improves enzymatic digestibility and subsequent ethanol yields from high solids SSF

4.1 Abstract

High solids loadings in bioreactors are needed to achieve economically attractive ethanol titers from simultaneous saccharification and fermentation (SSF) of lignocellulosic biomass. Although woody biomass species contain higher glucan content than other biomass feedstock, it's also more recalcitrance to enzymatic breakdown, resulting in limited SSF performance at high solids loadings. Aqueous dilute acid pretreatment of woody biomass can offer improved enzymatic digestibility of cellulose, but the presence of lignin in the solids leads to increased enzyme deactivation and severe pretreatment conditions lead to loss of total sugars resulting in poor ethanol yields and low ethanol titers. Here, we unlock the potential ethanol production from woody biomass by optimizing CELF pretreatment with SSF. Using this combination, we report ethanol titers of 60, 78, and 87 g/L from SSF using solids loadings of 13, 17, and 20wt%, resulting in ethanol yields 87, 84, and 79% of theoretical, respectively. *S. cerevisiae* D5A was used in combination with Cellic® CTec2 cellulase at a modest enzyme loading of 15 mg-protein/ g-glucan in raw Poplar. Sugar release during fermentation was sustained throughout the fermentation, irrespective of solids loading, suggesting that this combined process was not limited by glucan digestibility but by the strain's ethanol tolerance and metabolic capability. We further developed a fractal kinetic model for the fermentation data to reveal that the CELF pretreated solids are able to sustain high enzyme activity with cellulases over the entire course of hydrolysis with minimal enzyme deactivation. The model parameters also suggest that increasing the enzyme loading beyond 15 mg protein/g glucan in raw Poplar would not improve the saccharification performance of CELF pretreated solids.

4.2 Introduction

The transportation sector is among the greatest contributors to greenhouse gas emissions within the United States and the rest of the world. (US EPA n.d.) Lignocellulosic biomass is a non-food plant material that can provide an abundant, low cost resource for producing clean, sustainable transportation fuels, at large enough scale to impact energy demands and reduce greenhouse gas emissions. (Demirbas 2009; Luque et al. 2008) Fast growing trees such as Poplar can serve as a sustainable energy crop providing a rich source of cellulose and hemicellulose sugars with many existing and potential applications including production of pulp and paper, biofuels, and bio based products such as chemicals and adhesives. (Sannigrahi, Ragauskas, and Tuskan 2010) Biological conversion of plant based sugars into fuel typically involves three primary steps 1) fractionation of the plant cell wall via pretreatment to increase the availability of polysaccharides for downstream deconstruction, 2) breakdown of the polysaccharides into fermentable sugars via enzymatic hydrolysis, and 3) fermentation of these sugars into desired products such as ethanol. (Kumar and Sharma 2017; Lee 1997; Yang and Wyman 2008) Three primary biological conversion pathways for steps 2 and 3 are to follow application of a suitable pretreatment to cellulosic biomass by Separate Hydrolysis and Fermentation (SHF), Simultaneous Saccharification and Fermentation (SSF), or Consolidated Bioprocessing (CBP). (Gauss et al. n.d.; Mohagheghi et al. 1992; Tian et al. 2016)

SSF combines enzymatic saccharification of pretreated biomass with fermentation of the sugars released in a single vessel. Increased ethanol titers attainable via high solids SSF lower energy requirements and equipment size for downstream ethanol recovery,

thereby achieving significant savings in operating as well as capital costs. (Kim et al. 2008; Nguyen et al. n.d.) However, increased insoluble solid content interferes with proper mixing and causes insufficient heat and mass transfer within the system. (Modenbach and Nokes 2013; Roberts et al. 2011) In addition, accumulation of sugar oligomers and monomers inhibits enzyme activity, lignin left in the pretreated solids unproductively binds enzymes and blocks cellulose accessibility to enzymes, and dissolved lignin and higher ethanol concentrations inhibit growth and metabolism of the fermentative microorganisms. (Attfield 1997; Holtzapple et al. 1990; Mills, Sandoval, and Gill 2009; Montenecourt 1983; Ooshima, Burns, and Converse 1990; Rahikainen et al. 2011; Saito et al. 2012; Xiao et al. 2004) Thus, end-product inhibition and insoluble lignin can constraint polysaccharide digestion and resulting ethanol concentrations, while dissolved lignin and ethanol can limit viability of the fermentative organism to the extent that fermentable sugars are left in the broth. (Stanley et al. 2010)

Co-solvent Enhanced Lignocellulosic Fractionation (CELF) is a novel pretreatment technology that involves the use of THF-water based co-solvent in its miscible range along with very dilute sulfuric acid as catalyst to disrupt biomass cell walls and solubilize the majority of hemicellulose sugars and much of the lignin to generate a glucan-rich substrate with low lignin content. (Nguyen et al. 2015a) In previous studies, high solids SSF of CELF pretreated corn stover solids has achieved ethanol concentrations of 85.6 g/L that correspond to an 80.8% of theoretical yield at an insoluble solid loading of 23wt% with an enzyme loading of 10 mg protein per g glucan in raw biomass. (Nguyen et al. 2017) However, hardwood poplar is more recalcitrant to enzymatic breakdown than corn stover,

and hence, far more difficult to achieve high ethanol titers with SSF. (Kumar et al. 2009) Here, we show that combination of CELF pretreatment with subsequent SSF can successfully overcome woody recalcitrance to realize high ethanol titers and yields comparable to processing agricultural feedstock such as corn stover.

4.3 Experimental Section

4.3.1 Materials

This study was conducted on *Populus trichocarpa* woody biomass generously provided by the BioEnergy Science Center (BESC). The composition of the raw Poplar as determined by following NREL LAP (version 08-03-2012) was 47.0 % glucan, 16.9 % xylan, and 21.2% acid-insoluble lignin. (Sluiter et al. 2008) The biomass was air-dried, knife milled using a laboratory mill (Model 4, Arthur H. Thomas Company, Philadelphia, PA), and passed through a 1mm internal sieve size. The enzyme cocktail used for the study was Cellic® CTec 2 generously provided by Novozymes®. The protein content of the enzyme mixture as estimated using Pierce BCA analysis kit was 250 mg/ml. The yeast strain used for fermentation was D5A, a variant of *Saccharomyces cerevisiae*, generously provided by the National Renewable Energy Laboratory (NREL).

4.3.2 Pretreatment

Prior to CELF pretreatment, milled Poplar wood chips were soaked overnight at 4 °C in a 1:1 (weight basis) solution of THF to water containing 0.5 wt% H_2SO_4 based on total solvent. The dry biomass loading was 7.5 wt% of the total working mass for reaction, Reactions were conducted in a 1 L Hastelloy Parr autoclave reactor (236HC Series, Parr Instruments Co., Moline, IL) equipped with a double stacked pitch blade impeller rotating at 200 rpm. Based on the results from Chapter 3, a series of pretreatments were carried out

at 160 °C for 15 minutes, i.e., conditions that resulted in maximum sugar recovery for CELF followed by enzymatic hydrolysis. Temperature inside the reactor were measured by an in-line thermocouple (Omega, K-type) and all reactions were maintained within ±2 °C using a 4 kW fluidized sand bath (Model SBL-2D, Techne, Princeton, NJ). At the end of each reaction, the reactor was cooled by submerging quickly in a large water bath at room temperature. The solids were then separated from the liquor by vacuum filtration at room temperature through glass fiber filter paper (Fisher Scientific, Pittsburgh, PA). The mass and density of the liquid fractions were measured to calculate yields and close mass balances. The solids collected were then washed with water until clear water ran through the solids. The solids were then hydraulically pressed to reduce the moisture content to 61%.

4.3.3 Enzymatic hydrolysis of CELF pretreated Poplar

Batch enzymatic hydrolysis was performed by following the standard NREL protocol.(Resch, Baker, and Decker 2015) using 125 mL flasks with a total working volume of 50 mL. For each, CELF pretreated biomass was loaded to result in a 1 wt%, glucan loading. In addition, 50 mM citrate buffer (pH 4.5) was added to reach a final pH of 4.8 in 50 mL, along with Millipore water and 0.02% sodium azide as an antimicrobial agent. Triplicates were run with the appropriate amount of substrate. The flasks were then placed in a 50 °C incubator shaker and allowed to equilibrate for 1 h at 150 rpm. Appropriate amounts of Cellic® CTec2 enzyme cocktail were then added to the flasks at various protein loadings per g-glucan-in-raw poplar, and the flasks were then returned to

the incubator shaker. 1 mL samples were taken at the times reported, centrifuged at 15000 rpm for 10 min, diluted, and analyzed for sugar concentration in the broth.

4.3.4 Fractal modeling of enzymatic hydrolysis kinetics

A fractal model based on first-order breakdown of glucan to form glucose was used to describe cellulose hydrolysis with a time-dependent rate coefficient k_t related to the hydrolysis time raised to the fractal exponent h.

$$\frac{dC}{dt} = -k_t C, \text{ where } k_t = kt^{-h} \quad (1)$$

The k, k_t, and h parameters in equation 1 were determined simultaneously by MATLAB.

4.3.5 Seed inoculum preparation

Saccharomyces cerevisiae (D5A) cells were grown in 10 mg/mL yeast extract (Becton, Dickinson and Company, Redlands, CA), 20 mg/mL peptone (Becton, Dickinson and Company, Redlands CA) and 50 mg/mL glucose to the exponential phase and then stored in (~14 wt%) glycerol. When needed, the frozen stock was thawed and grown overnight in 10 mg/mL yeast extract, 20 mg/mL peptone, and 50 mg/mL glucose in a 250 mL baffled flask in a shaking incubator maintained at 37 °C for a speed of 130 rpm. The inoculum was then centrifuged and re-suspended in sterile deionized (DI) water, and an inoculation was prepared at an optical density (O.D.) of 0.5 determined at 600 nm.

4.3.6 Pure sugar fermentations and growth curve

Pure sugar fermentations by D5A were carried out in 125 mL flasks at specified glucose concentrations. Glucose was dissolved in Millipore water and added to a flask and bubble trap assembly. Duplicates of those and a substrate blank were sterilized at 121 °C for 35 min in an autoclave and cooled in a laminar flow hood to prevent contamination

followed by adding water to adjust for losses. 50 mM citrate buffer (pH 4.5) was added to reach a final pH of 4.8 in 50 mL, and 40 mg/L of tetracycline along with the seed inoculum were used in 48 h fermentations shaking at 130 rpm and 37 °C. 0.75 mL samples were taken every 2 h until the stationary phase was reached. These samples were centrifuged at 15000 rpm for 10 min, diluted, and analyzed for ethanol and sugar concentrations.

4.3.7 Simultaneous Saccharification and Fermentation (SSF)

Batch SSF experiments were performed in 125 mL flasks with a total working volume of 25 mL containing CELF pretreated biomass corresponding to desired glucan loadings, 50 mM citrate buffer (pH 4.5) was added to reach a final pH of 4.8 in 25 mL, followed by 40 mg/L tetracycline (Sigma Aldrich, St. Louis, MO) as an antimicrobial agent. Cellic® CTec2 cocktail was loaded at 15 mg-protein per g-glucan-in-raw poplar, and the yeast inoculum was added next. Flasks with attached bubble traps were loaded with Millipore water and the appropriate amount of substrate. Duplicates with substrate along with substrate blanks were sterilized at 121 °C for 35 min. The flasks were cooled in a laminar flow hood (Baker and Baker Ruskinn, Sanford, ME) to prevent contamination and reweighed to allow appropriate water replenishment. After adding the buffer, antimicrobial agent, enzyme cocktail, and yeast inoculum, SSF was carried out for 10 days at 37 °C at 130 rpm. 1 mL samples were taken periodically, centrifuged at 15000 rpm for 10 min, diluted, and analyzed for sugar and metabolite concentrations in the liquid.

4.3.8 Measuring sugar and ethanol concentrations

Liquid samples along with appropriate calibration standards were analyzed by HPLC (Waters Alliance 2695 system equipped with a Bio-Rad Aminex® HPX-87H

column and Waters 2414 RI detector) with a 5 mM sulfuric acid eluent flow rate of 0.6 ml min^{-1}. The chromatograms were integrated by the Empower® 2 software package (Waters Co., Milford, MA).

4.3.9 Model Equations used

Percent glucan conversion to glucose via enzymatic hydrolysis of a 1 wt% glucan loading was calculated as follows:

$$Percent\ glucan\ conversion\ to\ glucose = \frac{C_{Glucose} \times 0.9 \times WV}{M_G} \times 100 \quad (2)$$

In which, $C_{Glucose}$ is the concentration of glucose in the enzymatic hydrolysis liquid at a given time, mg/ml, WV is the working volume in the flask, ml (50 ml), M_G is the mass of glucan initially added, g.

At lower solid loadings, i.e., <5 wt%, the density of the solvent phase can be assumed to be same as density of water. As the insoluble solid fraction increases, the density of the liquid fraction first increases due to increase in sugar concentration and then drops slightly due to the increasing ethanol concentration. The fluid density was measured directly.

$$Percent\ Theoretical\ Ethanol\ Yield = \left(C_{Eth} \times V_L \times 0.9 \times 100\right) \Big/ \left(0.511 \times M_G\right) \quad (3)$$

with

$$V_L = (M_W + M_{DS})/\rho \quad (4)$$

$$V_L = (M_W + V_W \times \left(C_{Eth} + C_{Glucose} + C_{Glycerol} + etc.\right))/\rho \quad (5)$$

In which C_{Eth} is the ethanol concentration in the fermentation broth, mg/ml, $C_{Glucose}$ is the glucose concentration in the fermentation broth, mg/ml, $C_{Glycerol}$ is the

glycerol concentration in the fermentation broth, mg/ml, V_L is the volume of liquid fraction in the fermentation medium, ml, M_G is the mass of glucan initially added, g, M_W is the mass of water initially added, g, M_{DS} is the mass of dissolved solids in the fermentation medium at any given time point, g, ρ is the density of the medium, g/ml.

4.4 Results and Discussion

4.4.1 CELF pretreatment reduces solids loadings at high SSF glucan levels

High insoluble content in biomass such as lignin and inorganics reduces the amount of accessible sugars for fermentation resulting in limited sugar loading as high solids slurries result in significant mixing and mass transfer limitations. Improper mixing leads to uneven heat and mass transfer as well as limited water contact for enzymes and microbes to interact with the substrate and sugars in the system, respectively. (Modenbach and Nokes 2013; Roberts et al. 2011) Pretreatments that efficiently remove lignin from biomass produce solids with high glucan content that are far better suited for high solids fermentations, as glucan concentration in the substrate is substantially increased, resulting higher sugars per solids ratio. Furthermore, delignified biomass substrates increases the specific accessibility of fungal enzymes to glucan helping to improve enzyme effectiveness and minimizes non-productive binding of enzymes to non-sugar components. (Nguyen et al. 2015b; Ooshima et al. 1990; Wyman et al. 2013) As shown in Figure 4-1, CELF pretreated poplar solids are comprised of 89% glucan, 3% xylan, and 5 % lignin, obtained from mild pretreatment reaction conditions of 160 °C with 15 min residence time. It was previously shown that these pretreatment conditions were optimal for total sugar digestibility when CELF pretreatment of poplar was combined with consolidated

bioprocessing (CBP) using *Clostridium thermocellum*. (Thomas et al. 2017) Furthermore, close to 96% of the initial glucan in the raw biomass was recovered in the solid fraction, while 4% of the glucan remaining and 60% of the xylan (C5 sugars) from the raw biomass was recovered as soluble sugars the liquid fraction following CELF pretreatment. Thus, the glucan-rich solids and low residual lignin hold the promise of not only supporting high solids fermentations but also improving total sugar utilization from biomass with co-fermentation strategies. Furthermore, 89% of the lignin extracted from poplar was isolated in an independent stream as a highly pure lignin powder suitable for lignin valorization. In prior studies, CELF lignin from poplar has been successfully incorporated into lignin-based polyurethanes with high strength and good mechanical properties. (Wang et al. 2020)

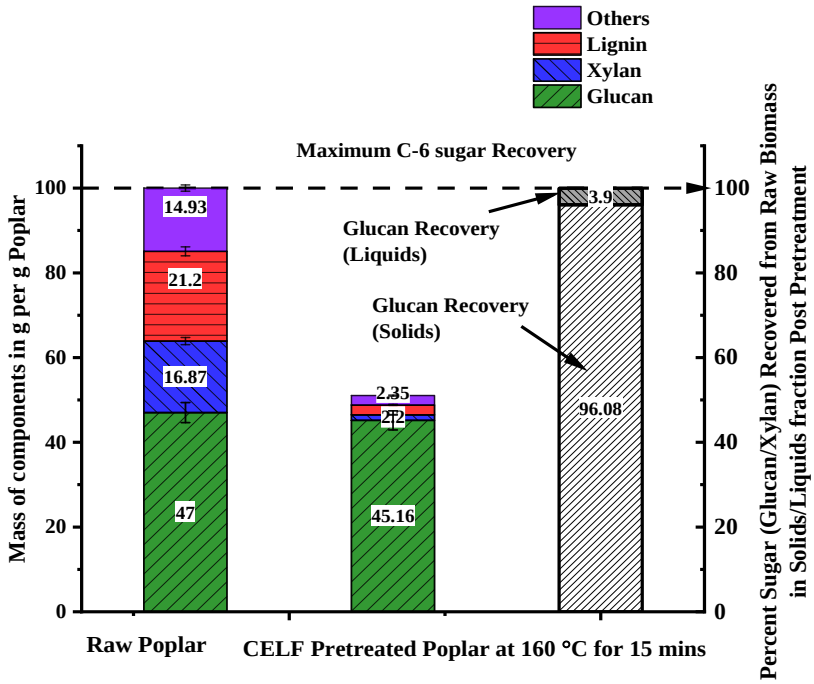


Figure 4-1 Compositional analysis of raw and CELF pretreated Poplar and glucan recovered in solid and liquid fractions post pretreatment at 160 °C and 15 min.

4.4.2 CELF pretreated Poplar solids are highly susceptible to saccharification at a low enzyme dose

The high cost of cellulolytic enzymes contributes to a significant cost (up to $1.2/gal ethanol) in 2nd generation biorefinery models. Thus, minimizing enzyme consumption during SSF is paramount to reduce the cost of cellulosic ethanol. (Klein-Marcuschamer et al. 2012) In particular, SSF fermentations typically require substantially higher enzyme loadings at higher solids loadings to overcome end-product inhibition and enzyme deactivation by non-productive binding. (Kristensen, Felby, and Jørgensen 2009; Liu et al. 2014; Nguyen et al. 2017) Therefore, we investigated the enzymatic digestibility CELF pretreated poplar solids to optimize for the lowest enzyme dosage needed to achieve the highest glucose yield. Enzymatic hydrolysis optimizations were conducted on CELF solids at lower 1 wt% glucan loading with Cellic® CTec2 as the enzyme cocktail at various protein doses to avoid the influence of mass transfer limitations. The glucan digestibility results, presented in Figure 4-2, show that the rate of sugar release was highest at 65 mg protein per g glucan in raw biomass followed by 30 mg protein per g glucan. However, 96 % of the glucan in the solids was hydrolyzed to glucose in just 24 h at a lower enzyme dose of 15 mg protein per g glucan. Furthermore, almost all the glucan was converted in 4 days by 7.5 mg protein and in 8 days at a very low enzyme dose of 5 mg protein per g glucan in raw poplar.

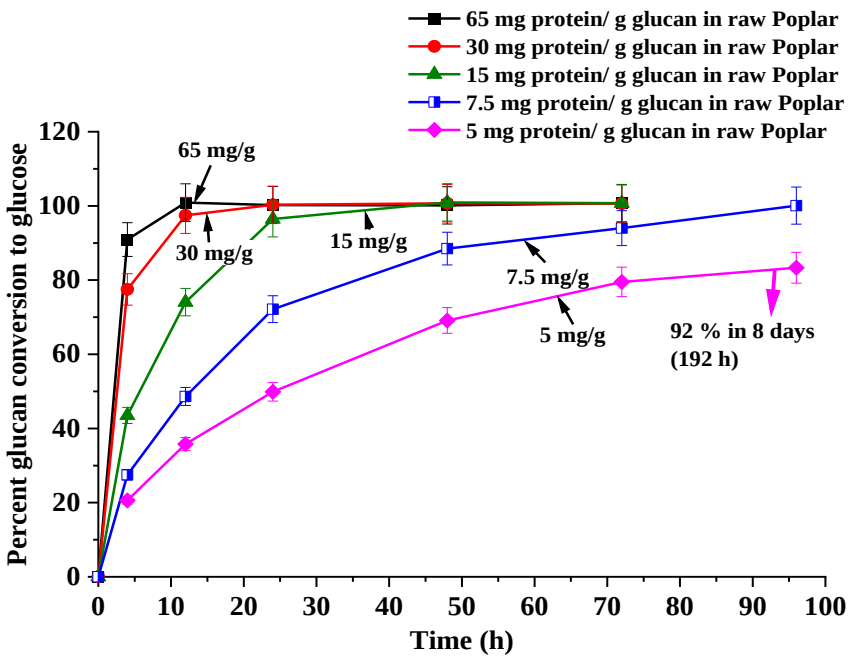


Figure 4-2 Percent glucan conversion from enzymatic hydrolysis of CELF pretreated Poplar at 160 °C, 15 min at 1 wt% glucan loading and Cellic® CTec2 cellulase cocktail at different enzyme dose (mg protein per g glucan in raw Poplar)

4.4.3 Insights into substrate-enzyme interactions by fractal kinetic modeling of hydrolysis at various enzyme doses

Heterogeneous enzyme catalyzed systems differ from homogeneous counterparts in the interaction between the substrate and catalyst is primarily due to diffusion and collision processes prior to reaction in two- or one-dimension space. (Xu and Ding 2007) Application of a fractal kinetics model to the glucan hydrolysis results provide additional insights into substrate-enzyme interactions at different enzyme loadings. A time-dependent rate coefficient "k_t" used in the reaction fractal rate equation with a fractal exponent "h" gives an indication of how the rate decays with time, and the correlation of "k_t/k" with glucan conversion shows the time-dependency of the enzymatic hydrolysis for a particular enzyme dose over the course of reaction. A high "k_t/k" and low "h" indicate a better enzyme-substrate interaction and a faster hydrolysis. (Aguiar et al. 2013) Figure 4-3 shows

the trend in k_t/k values over the course of hydrolysis for various enzyme doses. For the highest enzyme dose of 30 mg protein per g glucan in raw Poplar, the starting correlation value of 1 shows that the initial rate of reaction is not limited by diffusion. The drop in initial values with lower enzyme doses of 15, 7.5, and 5 mg per g glucan show a proportional relationship between enzyme dose and initial reaction rate. However, the steep drop in k_t/k values with increasing glucan conversion at the highest enzyme dose of 30 mg protein/ g glucan compared to that for lower enzyme loadings could be due to the greater amount of enzyme restricting the path of hydrolysis. The correlation values at the lower enzyme loadings drop more slowly, apparently because the reaction is more limited by diffusion as saccharification proceeds.

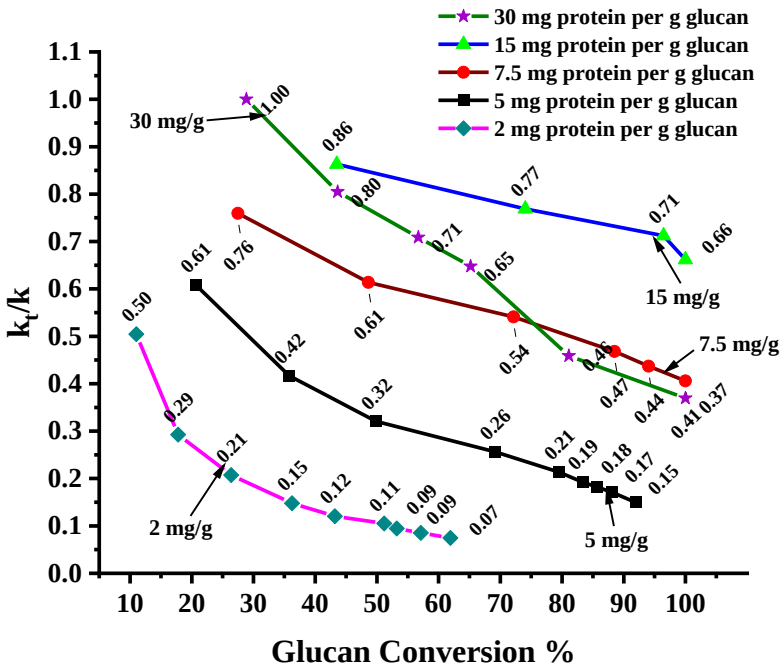


Figure 4-3 Change in fractal kinetic kt/k correlation values with glucan conversion for enzymatic saccharification of CELF pretreated Poplar at different enzyme doses.

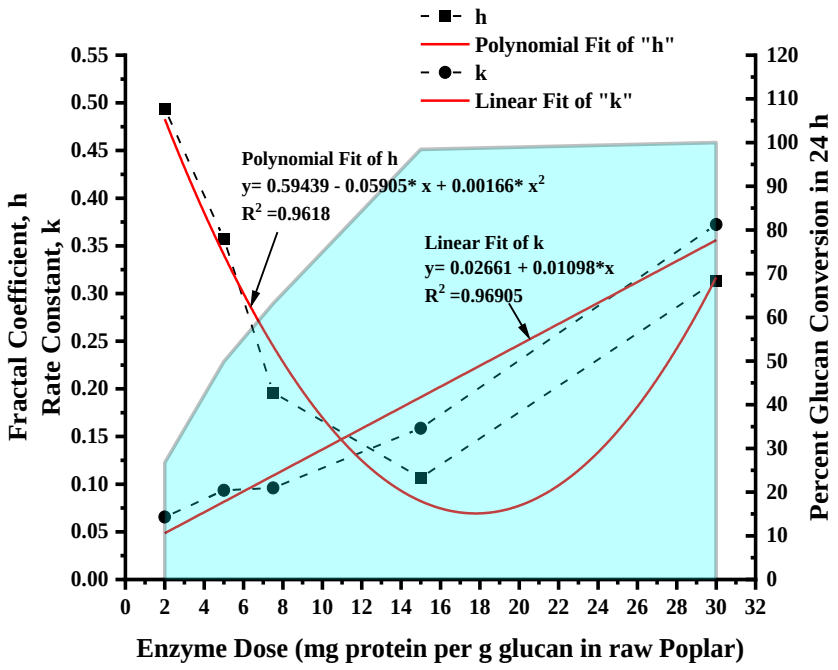


Figure 4-4 Change in fractal exponent "h" and rate constant "k" of the enzymatic hydrolysis reaction with enzyme dose at a glucan loading of 1 wt%. The black line and the area under the line (blue trapezium) indicate the percent glucan conversion achieved in 24 h.

The "h" values have been attributed to characterizing structural features and accessibility of the cellulosic substrate. Furthermore, a lower "h" value has been interpreted to indicate greater substrate-enzyme interaction and correlate with a higher rate of reaction. (Aguiar et al. 2013) The interesting observation in Figure 4-4 is that although the rate constant increased linearly with enzyme dose, the fractal exponent "h" followed a polynomial (parabolic) pattern: it first dropped with increasing enzyme dose and then drastically increased. The high "h" value at the lowest enzyme dose suggests low substrate-enzyme interaction, possibly due to cellulase inhibition or slow hydrolysis rates due to the extra time spent for enzyme reallocation from one active site to another. The dropping trend in "h" values with increased enzyme dose up to 15 mg protein per g glucan indicates

improved glucan hydrolysis and better substrate-enzyme interactions at higher cellulase content. The dramatic increase in the value of "h" at 30 mg protein per g glucan could be a result of a "jamming-effect" due to a high amount of enzyme remaining in the broth compared substrate availability, especially towards the end of the reaction. (Xu and Ding 2007) The combined data in Figures 4-2, 4-3, and 4-4 shows that a higher enzyme loading definitely improved the reaction rate but does not necessarily improve their hydrolysis effectiveness or efficiency. Moreover, a jamming effect near hydrolysis completion indicates sustained enzyme activity throughout the reaction, possibly as a result of the low lignin content in the solids reducing unproductive cellulase binding.

Similar to these observations, Nguyen *et.al.* compared first-order fractal kinetics of enzymatic hydrolysis of CELF and Dilute Acid (DA) pretreated corn stover and hypothesized that extensive delignification by CELF pretreatment reduced unproductive cellulase-lignin binding and thereby kept the rate of reaction from dropping much throughout the saccharification. (Nguyen et al. 2015c) Overall, the fractal kinetic results reported here show that solids produced by CELF pretreatment of Poplar exhibit such good substrate-enzyme reactivity that glucan can be hydrolyzed completely even at enzyme loadings below 15 mg protein per g glucan enzyme. Based on the enzymatic saccharification data and fractal kinetic model results, an enzyme loading of 15 mg protein per g glucan in raw Poplar was selected for SSF at high solids loadings.

4.4.4 Ethanol tolerance of host microorganism restraints ethanol yields from SSF

High ethanol concentrations in the fermentation broth negatively impacts cell viability, growth, and metabolism (Stanley et al. 2010), thus ethanol tolerance of the host

microorganism could limit ethanol yields in a high solids SSF. In order to estimate the ethanol tolerance of D5A, the *Saccharomyces cerevisiae* variant used in this study, fermentations were conducted at glucose concentrations of 180 and 200 g/L at 37 °C as control experiments. Figure 4-5 shows that D5A reached an ethanol titer close to 87 g/L from 180 g/L of glucose, a yield of 90% of theoretical, in about 48 hours. However, when the organism was fed 200 g/L, the titers and the percent theoretical ethanol yield dropped to 81 g/L and 78%, respectively, with residual sugars left. This result indicated that the maximum attainable ethanol titer using D5A was close to 87 g/L.

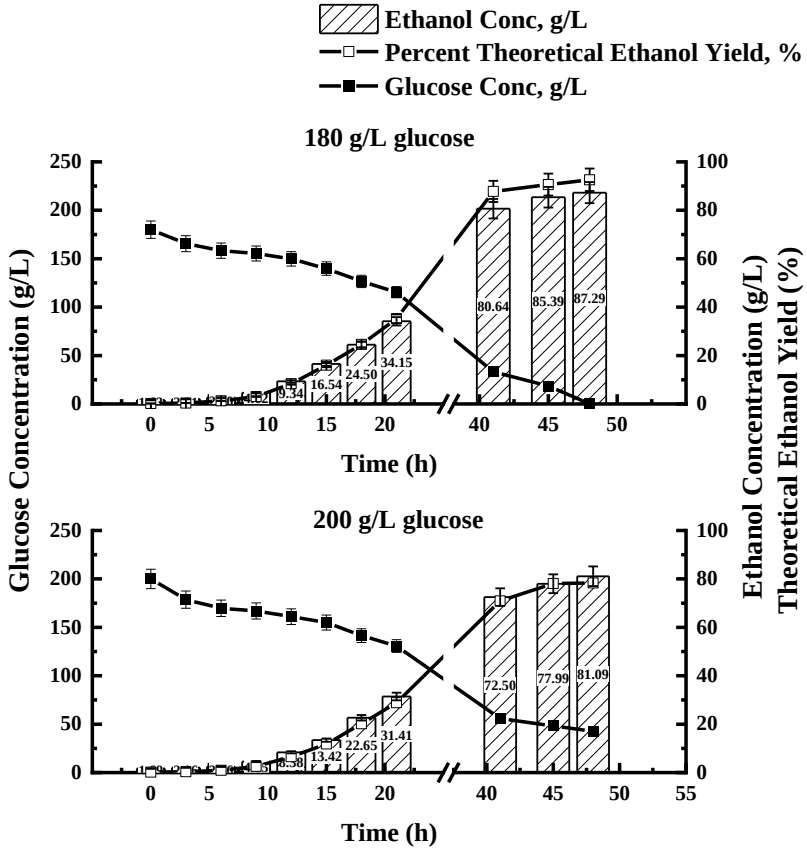


Figure 4-5 Glucose consumption, ethanol formation, and theoretical ethanol yields for fermentations of 180 g/L and 200 g/ glucose L by S. cerevisiae variant D5A at 37 °C.

4.4.5 Ethanol titers from high solids SSF of CELF pretreated Poplar were similar to those from glucose control experiments

In order to increase the insoluble solid content to 20 wt% in shake flasks, CELF pretreated solids were compressed with a hydraulic jack to reduce the moisture content from 72 to 61%. SSF experiments using Cellic® CTec2 enzyme at a dose of 15 mg protein per g glucan in raw Poplar were then conducted on the pressed substrate at solids loading of 13, 17, and 20 wt% that corresponded to glucan loadings of 11, 15, and 18 wt%, respectively. As shown in Figure 4-6, the result was ethanol titers of 60 g/L, 78 g/L, and 87 g/L in just 7 days with no residual sugars left in the broth. These yields corresponded to ethanol yields of 87%, 84%, and 79%, respectively, from the increasing progression in glucan loadings. Figure 4-6 (a) shows that at the lowest solid loading of 13 wt%, glucan was completely hydrolyzed and all the sugars were totally consumed in 7 days. Almost complete hydrolysis of glucan was also observed for the next higher solid loading of 17 wt% in 10 days, but now glucose began to accumulate on day 8, at which point ethanol production slowed down substantially, as shown in Figure 4-6 (b). At the highest solid loading of 20 wt%, Figure 4-6 (c) shows that close to 92% of the glucan was completely hydrolyzed in 10 days. However, the ethanol concentration plateaued at 87 g/L at 6 days despite continued glucose release, consistent with pure glucose fermentations also stopping at this ethanol concentration.

It has been demonstrated that achieving greater than 50 g/L ethanol titers while sustaining high yields at low enzyme dosages can help reduce the cost of biological processing of cellulosic biomass to ethanol. (Nguyen et al. 2017) The results reported here show that nearly identical high saccharification extents can be achieved for enzymatic

hydrolysis of poplar at 13, 17, and 20 wt% loadings of CELF pretreated solids. This outcome is distinctive from results for solids produced by other pretreatments of cellulosic biomass. (Zhang et al. 2010) However, even though high conversion of glucan in CELF solids was achieved for all solids levels, ethanol concentrations were limited to about 87 g/L, beyond which glucose accumulated. The latter result points towards ethanol toxicity being the roadblock to attaining higher ethanol yields with the particular D5A yeast employed. Hence, ethanol yields from SSF of CELF solids was not limited by substrate digestibility but by yeast strain's ethanol tolerance. Thus, a higher yield is likely if future research could improve ethanol tolerance when employed in the SSF environment.

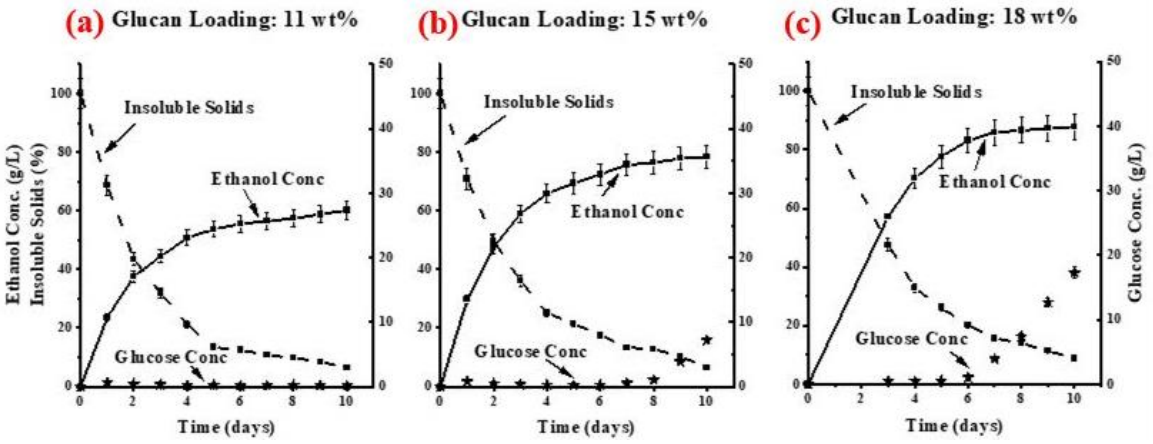


Figure 4-6 Ethanol concentration, glucose concentration, and percent insoluble solids remaining from application of SSF to CELF pretreated Poplar solids at loadings of 13, 17, and 20 wt% that correspond to 11, 15, and 18 wt% glucan loadings, respectively. SSFs were conducted with an enzyme dose of 15 mg protein per g glucan in raw Poplar and using the D5A strain of S. cerevisiae yeast.

4.5 Conclusions

In a previous study of high solids SSF of CELF pretreated corn stover, ethanol titers close to 86 g/L were reported to be achieved in 7 days from a 23 wt% solids loading using Accellerase® 1500 enzyme cocktail at a dose of 10 mg protein per g glucan in raw corn

stover, and *S. cerevisiae* strain D5A. (Nguyen et al. 2017) In this study, nearly identical ethanol titers of 87 g/L were realized from SSF of a 20 wt% solid loading of CELF pretreated Poplar solids with an enzyme dose of 15 mg protein per g glucan in raw biomass and using the D5A yeast strain in just 6 days. However, although the CELF solids were almost completely digestible at this high solid loading, the ethanol concentration leveled off at 87 g/L, showing that the strain's ethanol tolerance and not the digestibility of CELF solids limited product yields. Application of a fractal kinetic model to saccharification data revealed that although increasing the enzyme loadings for SSF of CELF pretreated Poplar might enhance the rate of saccharification, it would not improve enzyme effectiveness or substrate-enzyme interactions. Overall, this study further shows that through fractionation of a range of cellulosic biomass materials, CELF pretreatment technology can produce highly digestible solids that are amenable to high yield breakdown of glucan at high solids concentrations in SSF. However, it also points out the challenge in developing or identifying fermentative organisms the can tolerate the resulting high ethanol concentrations.

4.6 References

Aguiar, Rodrigo Souza, Marcos Henrique Luciano Silveira, Ana Paula Pitarelo, Marcos Lucio Corazza, and Luiz Pereira Ramos. 2013. "Kinetics of Enzyme-Catalyzed Hydrolysis of Steam-Exploded Sugarcane Bagasse." *Bioresource Technology* 147:416–23.

Attfield, Paul V. 1997. "Stress Tolerance: The Key to Effective Strains of Industrial Baker's Yeast." *Nature Biotechnology* 15(13):1351–57.

Demirbas, Ayhan. 2009. "Political, Economic and Environmental Impacts of Biofuels: A Review." *Applied Energy* 86:S108–17.

Gauss, WF, S. Suzuki, 990,944 M Takagi - US Patent 3, and undefined 1976. n.d. "Manufacture of Alcohol from Cellulosic Materials Using Plural Ferments." *Google Patents*.

Holtzapple, Mark, Mona Cognata, Yuancai Shu, and Christie Hendrickson. 1990. "Inhibition OfTrichoderma Reesei Cellulase by Sugars and Solvents." *Biotechnology and Bioengineering* 36(3):275–87.

Kim, Youngmi, Rick Hendrickson, Nathan S. Mosier, Michael R. Ladisch, Bryan Bals, Venkatesh Balan, and Bruce E. Dale. 2008. "Enzyme Hydrolysis and Ethanol Fermentation of Liquid Hot Water and AFEX Pretreated Distillers' Grains at High-Solids Loadings." *Bioresource Technology* 99(12):5206–15.

Klein-Marcuschamer, Daniel, Piotr Oleskowicz-Popiel, Blake A. Simmons, and Harvey W. Blanch. 2012. "The Challenge of Enzyme Cost in the Production of Lignocellulosic Biofuels." *Biotechnology and Bioengineering* 109(4):1083–87.

Kristensen, Jan B., Claus Felby, and Henning Jørgensen. 2009. "Yield-Determining Factors in High-Solids Enzymatic Hydrolysis of Lignocellulose." *Biotechnology for Biofuels*.

Kumar, Adepu Kiran and Shaishav Sharma. 2017. "Recent Updates on Different Methods of Pretreatment of Lignocellulosic Feedstocks: A Review." *Bioresources and Bioprocessing* 4(1):7.

Kumar, Rajeev, Gaurav Mago, Venkatesh Balan, and Charles E. Wyman. 2009. "Physical and Chemical Characterizations of Corn Stover and Poplar Solids Resulting from Leading Pretreatment Technologies." *Bioresource Technology* 100(17):3948–62.

Lee, Jeewon. 1997. "Biological Conversion of Lignocellulosic Biomass to Ethanol." *Journal of Biotechnology* 56(1):1–24.

Liu, Zhi-Hua, Lei Qin, Jia-Qing Zhu, Bing-Zhi Li, and Ying-Jin Yuan. 2014. "Simultaneous Saccharification and Fermentation of Steam-Exploded Corn Stover at High Glucan Loading and High Temperature." *Biotechnology for Biofuels* 7(1):167.

Luque, Rafael, Lorenzo Herrero-Davila, Juan M. Campelo, James H. Clark, Jose M. Hidalgo, Diego Luna, Jose M. Marinas, and Antonio A. Romero. 2008. "Biofuels: A Technological Perspective." *Energy & Environmental Science* 1(5):542.

Mills, Tirzah Y., Nicholas R. Sandoval, and Ryan T. Gill. 2009. "Cellulosic Hydrolysate Toxicity and Tolerance Mechanisms in Escherichia Coli." *Biotechnology for Biofuels* 2(1):26.

Modenbach, Alicia A. and Sue E. Nokes. 2013. "Enzymatic Hydrolysis of Biomass at High-Solids Loadings - A Review." *Biomass and Bioenergy* 56:526–44.

Mohagheghi, A., M. Tucker, K. Grohmann, and C. Wyman. 1992. "High Solids Simultaneous Saccharification and Fermentation of Pretreated Wheat Straw to Ethanol." *Applied Biochemistry and Biotechnology* 33(2):67–81.

Montenecourt, S. 1983. *Trichoderma Reesei Cellulases*. Vol. 1.

Nguyen, Thanh Yen, Charles M. Cai, Rajeev Kumar, and Charles E. Wyman. 2015a. "Co-Solvent Pretreatment Reduces Costly Enzyme Requirements for High Sugar and Ethanol Yields from Lignocellulosic Biomass." *ChemSusChem* 8(10):1716–25.

Nguyen, Thanh Yen, Charles M. Cai, Rajeev Kumar, and Charles E. Wyman. 2015b. "Co-Solvent Pretreatment Reduces Costly Enzyme Requirements for High Sugar and Ethanol Yields from Lignocellulosic Biomass." *ChemSusChem* 8(10):1716–25.

Nguyen, Thanh Yen, Charles M. Cai, Rajeev Kumar, and Charles E. Wyman. 2015c. "Co-Solvent Pretreatment Reduces Costly Enzyme Requirements for High Sugar and Ethanol Yields from Lignocellulosic Biomass." *ChemSusChem* 8(10):1716–25.

Nguyen, Thanh Yen, Charles M. Cai, Rajeev Kumar, and Charles E. Wyman. 2017. "Overcoming Factors Limiting High-Solids Fermentation of Lignocellulosic Biomass to Ethanol." *Proceedings of the National Academy of Sciences of the United States of America* 114(44):11673–78.

Nguyen, TY, CM Cai, O. Osman, R. Kumar-Green Chemistry, and undefined 2016. n.d. "CELF Pretreatment of Corn Stover Boosts Ethanol Titers and Yields from High Solids SSF with Low Enzyme Loadings." *Pubs.Rsc.Org*.

Ooshima, Hiroshi, Douglas S. Burns, and Alvin O. Converse. 1990. "Adsorption of Cellulase FromTrichoderma Reesei on Cellulose and Lignacious Residue in Wood Pretreated by Dilute Sulfuric Acid with Explosive Decompression." *Biotechnology and Bioengineering* 36(5):446–52.

Rahikainen, Jenni, Saara Mikander, Kaisa Marjamaa, Tarja Tamminen, Angelos Lappas, Liisa Viikari, and Kristiina Kruus. 2011. "Inhibition of Enzymatic Hydrolysis by Residual Lignins from Softwood-Study of Enzyme Binding and Inactivation on Lignin-Rich Surface." *Biotechnology and Bioengineering* 108(12):2823–34.

Resch, M. G., J. O. Baker, and S. R. Decker. 2015. *Low Solids Enzymatic*

Saccharification of Lignocellulosic Biomass: Laboratory Analytical Procedure (LAP), Issue Date: February 4, 2015.

Roberts, Katrina M., David M. Lavenson, Emilio J. Tozzi, Michael J. McCarthy, and Tina Jeoh. 2011. "The Effects of Water Interactions in Cellulose Suspensions on Mass Transfer and Saccharification Efficiency at High Solids Loadings." *Cellulose*.

Saito, Haruo, Francesc Posas, J. Horecka, R. A. DePinho, G. F. Sprague, M. Tyers, and S. J. Elledge. 2012. "Response to Hyperosmotic Stress." *Genetics* 192(2):289–318.

Sannigrahi, Poulomi, Arthur J. Ragauskas, and Gerald A. Tuskan. 2010. "Poplar as a Feedstock for Biofuels: A Review of Compositional Characteristics." *Biofuels, Bioproducts and Biorefining* 4(2):209–26.

Sluiter, A., B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, and D. Crocker. 2008. *Determination of Structural Carbohydrates and Lignin in Biomass: Laboratory Analytical Procedure (LAP); Issue Date: 7/17/2005*.

Stanley, D., A. Bandara, S. Fraser, P. J. Chambers, and G. A. Stanley. 2010. "The Ethanol Stress Response and Ethanol Tolerance of *Saccharomyces Cerevisiae*." *Journal of Applied Microbiology*.

Thomas, Vanessa A., Bryon S. Donohoe, Mi Li, Yunqiao Pu, Arthur J. Ragauskas, Rajeev Kumar, Thanh Yen Nguyen, Charles M. Cai, and Charles E. Wyman. 2017. "Adding Tetrahydrofuran to Dilute Acid Pretreatment Provides New Insights into Substrate Changes That Greatly Enhance Biomass Deconstruction by Clostridium Thermocellum and Fungal Enzymes." *Biotechnology for Biofuels* 10(1):252.

Tian, Liang, Beth Papanek, Daniel G. Olson, Thomas Rydzak, Evert K. Holwerda, Tianyong Zheng, Jilai Zhou, Marybeth Maloney, Nannan Jiang, Richard J. Giannone, Robert L. Hettich, Adam M. Guss, and Lee R. Lynd. 2016. "Simultaneous Achievement of High Ethanol Yield and Titer in Clostridium Thermocellum." *Biotechnology for Biofuels* 9(1):116.

US EPA, OAR. n.d. "Sources of Greenhouse Gas Emissions."

Wang, Yun-Yan, Priya Sengupta, Brent Scheidemantle, Yunqiao Pu, Charles E. Wyman, Charles M. Cai, and Arthur J. Ragauskas. 2020. "Effects of CELF Pretreatment Severity on Lignin Structure and the Lignin-Based Polyurethane Properties." *Frontiers in Energy Research* 8:149.

Wyman, Charles E., Bruce E. Dale, Venkatesh Balan, Richard T. Elander, Mark T. Holtzapple, Rocío Sierra Ramirez, Michael R. Ladisch, Nathan S. Mosier, Y. Y. Lee, Rajesh Gupta, Steven R. Thomas, Bonnie R. Hames, Ryan Warner, and Rajeev Kumar. 2013. "Comparative Performance of Leading Pretreatment Technologies for Biological Conversion of Corn Stover, Poplar Wood, and Switchgrass to Sugars." Pp. 239–59 in *Aqueous Pretreatment of Plant Biomass for Biological and Chemical Conversion to Fuels and Chemicals*. Chichester, UK: John Wiley & Sons, Ltd.

Xiao, Zhizhuang, Xiao Zhang, David J. Gregg, and John N. Saddler. 2004. “Effects of Sugar Inhibition on Cellulases and β-Glucosidase During Enzymatic Hydrolysis of Softwood Substrates.” Pp. 1115–26 in *Proceedings of the Twenty-Fifth Symposium on Biotechnology for Fuels and Chemicals Held May 4–7, 2003, in Breckenridge, CO*. Totowa, NJ: Humana Press.

Xu, Feng and Hanshu Ding. 2007. “A New Kinetic Model for Heterogeneous (or Spatially Confined) Enzymatic Catalysis: Contributions from the Fractal and Jamming (Overcrowding) Effects.” *Applied Catalysis A: General* 317(1):70–81.

Yang, Bin and Charles E. Wyman. 2008. “Pretreatment: The Key to Unlocking Low-Cost Cellulosic Ethanol.” *Biofuels, Bioproducts and Biorefining* 2(1):26–40.

Zhang, Jian, Deqiang Chu, Juan Huang, Zhanchun Yu, Gance Dai, and Jie Bao. 2010. “Simultaneous Saccharification and Ethanol Fermentation at High Corn Stover Solids Loading in a Helical Stirring Bioreactor.” *Biotechnology and Bioengineering* 105(4):718–28.

Chapter 5 Prospects of higher temperature tolerance of Kluveromyces marxianus enhancing ethanol yields from high solids SSF of CELF pretreated poplar*

*This chapter was completed in collaboration with Wheeldon group and Kisailus group at University of California Riverside. The *K. marxianus* yeast strain CBS 6556 was provided by Dr. Ian Wheeldon. SEM imaging of various samples was performed by Ms. Ramya Mohan and Dr. David Kisailus.

5.1 Abstract

Biological conversion of lignocellulosic biomass to ethanol via Simultaneous saccharification and fermentation (SSF) has been frequently applied to enhance ethanol yields by reducing end-product inhibition by sugars. However, fungal enzymes perform best at temperatures of 50-60 °C while conventional yeast such as *Saccharomyces cerevisiae* do not perform well at temperatures beyond 37 °C. Conversely, the CBS 6556 strain of *Kluveromyces marxianus* is a thermophilic eukaryote that grows and ferments well at 43 °C, thereby offering an opportunity to improve SSF production rates and yields. Therefore, this study compares SSF results with CBS 6556 to those with the D5A strain of *S. cerevisiae* to evaluate if the higher temperature tolerance of CBS 6556 could enhance high solids SSF of the glucan-rich solids produced by Co-Solvent Enhanced Lignocellulosic Fractionation (CELF) pretreatment of poplar. Our results show that although CBS 6556 had a higher initial ethanol productivity than D5A for SSF, ethanol yields were lower due to an early fermentation arrest, especially when subjected to the combined stresses of high ethanol concentrations and temperature. The insights presented here highlight the potential for *K. marxianus* to produce ethanol in SSF configuration and help guide future genetic engineering efforts to improve its ethanol tolerance.

5.2 Introduction

Plants store carbon in their secondary cell walls in the form of polysaccharides *viz.* cellulose, hemicellulose, and the aromatic polymer, lignin. These cell wall components can be converted via various biological and/or thermochemical routes into fuel ethanol, fuel additives, and/or specialty chemicals, or can be used as building blocks for synthesizing biopolymers. (Demirbas 2009b) Biological conversion of the sugars that make up the

polysaccharides in plants via Simultaneous saccharification and fermentation (SSF) that combines enzymatic hydrolysis of cellulosic substrates to sugars with fermentation of those sugars to ethanol promises low fuel cost as it has the potential to realize nearly theoretical yields and can take advantage of powerful existing and future biotechnological tools to facilitate improvement. (Anon n.d.)

Despite recent progress in the development of lignocellulosic pretreatments and in enzymatic hydrolysis of cellulose, few studies have demonstrated high ethanol yields at the high solids loadings needed to also realize high titers. Titers above 4 wt% ethanol are particularly important to reduce energy and capital requirements for product separation from the fermentation broth, via distillation, to levels that are more economically attractive. (Humbird et al. 2010; Roberts et al. 2011; Wyman, Spindler, and Grohmann 1992) These high ethanol titers are only achievable for SSF of polysaccharide loadings at 9 wt% or higher. However, the high viscosity of the 12 wt% loadings required to reach these polysaccharide levels for solids produced by many pretreatment systems results in inadequate mixing of the fermentation broth. This, in turn, leads to poor heat and mass transfer, while the build up of sugars, ethanol, and lignin in the broth adversely impacts both enzyme activity and microorganism survival. (Nguyen et al. 2017; Samaniuk et al. 2012; Viamajala et al. 2009)

Lignocellulosic biomass must be pretreated to achieve high ethanol yields at an economical enzyme dose for commercial success in biological conversion. (Wyman et al. 2005) Co-Solvent Enhanced Lignocellulosic Fractionation (CELF) is a pretreatment that uses a THF-water mixture in its miscible range along with very dilute sulfuric acid as a

catalyst to enhance cell wall breakdown by solubilizing much of the lignin and hemicellulose. The result is that CELF pretreatment can produce glucan-rich solids, soluble hemicellulose sugars, and isolated lignin. The extensively delignified solids thus produced are enzymatically digestible with high yields and are susceptible to nearly complete saccharification even at a low enzyme dose of 2 mg protein per g glucan in raw biomass. (Nguyen et al. 2015b, 2016a) The high glucan content of CELF solids has proven to facilitate high solids SSF as the higher glucan loadings for a given solids loading reduce mixing limitations to increasing ethanol titers while maintaining yields. (Nguyen et al. 2016a, 2017)

Since commercial fungal enzymes prefer a working range of 50-60 °C, while conventional yeast have an optimal growth range of 30-35 °C, SSF is typically conducted at an intermediate temperature of 37 °C to allow enzymes and yeast to both work effectively. (Anon 1975) Thus, although SSF offers a major benefit of lower inhibition of cellulase enzymes by sugar oligomers and monomers, the reduced enzyme activity at the reduced temperature leads to a slower rate of sugar release than the rate of sugar consumption by the organism, eventually resulting in cell death by starvation. (Mohagheghi et al. 1992; Nguyen et al. 2017) Thermotolerant organisms capable of fermenting sugars from a range of cellulosic substrates at temperatures close to 50 °C would offer two major benefits: 1) increased enzyme activity resulting in a faster rate of hydrolysis and fermentation and 2) reduced bacterial contamination due to the presence of ethanol, thereby saving additional costs for antibiotics. (Anderson, McNeil, and Watson

1986; Bai, Anderson, and Moo-Young 2008; Ghose, Roychoudhury, and Ghosh 1984; Lee et al. 1981; Nguyen et al. 2009; Zhang et al. 2010)

Saccharomyces cerevisiae, an ethanologen commonly used for industrial ethanol production, is a robust yeast that has a high ethanol tolerance and exhibits good survival in hyper-osmotic conditions. (Hohmann 2002) However, because growth of *S. cerevisiae* is limited to about 37 °C, using this yeast in SSF requires use of lower temperatures than those preferred by fungal enzymes. (Radecka et al. 2015) On the other hand, *Kluveromyces marxianus* is a rather newly isolated, non-model, thermotolerant, fast growing yeast that can ferment sugars at up to 45 °C. These qualities suggest it could allow higher temperature SSF operation than *S. cerevisiae*, (Fonseca, de Carvalho, and Gombert 2013; Húngaro et al. 2013; Lane et al. 2011; Zhang et al. 2010)

In light of the ability of CELF to facilitate high solids SSF and operability of *K. marxianus* at higher temperatures, the objective of this work is to evaluate the performance of *K. marxianus* for high solids SSF of CELF pretreated solids at near optimal saccharification temperatures. In particular, the performances of D5A (a *S. cerevisiae* variant often used for SSF) and CBS 6556 (a *K. marxianus* variant) were compared at conventional SSF conditions and at a near optimal saccharification temperature of 43 °C to determine how each impacts the titers and yields of cellulosic ethanol from CELF solids in a high solids SSF environment. CELF pretreatment lignin removal increases cellulose accessibility to fungal enzymes, minimizes phenolic based toxicity to the yeast cells, lowers broth viscosity at a given cellulose loading, and reduces nonproductive binding of enzymes to lignin. (Palmqvist and Hahn-Hägerdal 2000; Rahikainen et al. 2011) Thus, an

extra low temperature delignification step was applied to CELF solids to produce an almost pure cellulosic substrate with an extremely low lignin content with the goal to further improve ethanol yields.

5.3 Experimental Section

5.3.1 Materials

The woody biomass, *Populus trichocarpa*, also known as California Poplar, was generously provided by the BioEnergy Science Centre (BESC). The composition of the raw biomass as determined by following NREL LAP (version 08-03-2012) was 47.0 % glucan, 16.9% xylan, and 21.2% acid-insoluble lignin. (Sluiter et al. 2008a) The biomass was air-dried, knife milled using a laboratory mill (Model 4, Arthur H. Thomas Company, Philadelphia, PA), and passed through a 1mm internal sieve size. The enzyme cocktail used for the study was Cellic® CTec 2 generously provided by Novozymes®. Its protein content, as estimated using Pierce BCA analysis kit, was 250 mg/ml. The yeast strains used for fermentation were D5A, a variant of *Saccharomyces cerevisiae*, generously provided by the National Renewable Energy Laboratory (NREL), and CBS 6556, a *Kluveromyces marxianus* strain obtained from the American Type Culture Collection (ATCC).

5.3.2 CELF Pretreatment

For CELF pretreatment of poplar wood chips, milled raw biomass was soaked overnight at 4 °C at a dry biomass loading of 7.5 wt% based on the total working mass in a 1:1 (weight basis) solution of THF: water, with a 0.5 wt% loading of H_2SO_4 based on the total solvent mass. The reactions were conducted in a 1 L Hastelloy Parr autoclave reactor (236HC Series, Parr Instruments Co., Moline, IL) equipped with a double stacked pitch blade impeller rotating at 200 rpm. A series of CELF pretreatments were carried out at 160

°C for 15 minutes, i.e., conditions optimized for maximum total sugar recovery. All reactions were maintained at temperature (±2 °C) by convective heating using a 4 kW fluidized sand bath (Model SBL-2D, Techne, Princeton, NJ), and the temperature inside the reactor was measured directly by an in-line thermocouple (Omega, K-type). At the end of the reaction, the reactor was cooled by submerging it quickly in a large water bath at room temperature. The solids were then separated from the reaction liquor by vacuum filtration at room temperature through glass fiber filter paper (Fisher Scientific, Pittsburgh, PA). The mass and density of the liquid fractions were measured to calculate yields and close mass balances. The solids collected were then washed with (~150 mL) THF to remove residual lignin, followed by water washing until clear water ran through the solids. The solids were then hydraulically pressed to reduce the moisture content to 51.82%.

5.3.3.Seed inoculum preparation

K. marxianus (CBS 6556) and *S. cerevisiae* (D5A) were both grown in 10 mg/mL yeast extract (Becton, Dickinson and Company, Redlands, CA), 20 mg/mL peptone (Becton, Dickinson and Company, Redlands, CA), and 50 mg/mL glucose to the exponential phase and then stored in ~14 wt% glycerol. When needed for SSF, a frozen stock was thawed and grown overnight in 10 mg/mL yeast extract, 20 mg/mL peptone, and 50 mg/mL glucose in a 250 mL baffled flask shaking at 130 rpm in an incubator maintained at 37 °C. The inoculum was then centrifuged and re-suspended in sterile deionized (DI) water, and an inoculation was prepared at an optical density (O.D.) of 0.5 as determined at 600 nm.

5.3.4 Pure sugar fermentations and growth curve

Pure sugar fermentations were carried out in 125 mL flasks at specified glucose concentrations. Glucose was dissolved in Millipore water and added to the flask and bubble trap assembly. Duplicates of those and a substrate blank were sterilized at 121 °C for 35 min in an autoclave and cooled in a laminar flow hood to prevent contamination followed by adding water to adjust for losses. 50 mM citrate buffer (pH 4.8) and 40 mg/L of tetracycline along with the seed inoculum were used in for 48 h fermentations shaking at 130 rpm and 37 °C for D5A and CBS 6556 and at 43 °C for CBS 6556. A 0.75 mL sample was taken every 2 h until stationary phase was reached, centrifuged at 15000 rpm for 10 min, diluted, and analyzed to measure ethanol and sugar concentrations. Growth of the organisms was monitored by measuring the optical density of the broth for fermentations at both aerobic and anaerobic conditions. Growth rate, α (min^{-1}) was measured by calculating the slope of the plot of ln (O.D.) versus time, t, using Equation 1.

$$\ln\left(\frac{O.D.at\ time\ (t+dt)}{O.D.at\ time\ t}\right) = \alpha[(t+dt)) - t] \quad (1)$$

5.3.5 Simultaneous saccharification and fermentation (SSF)

Batch SSF experiments were performed in 125 mL flasks with a 25 mL total working volume containing CELF pretreated biomass corresponding to a desired glucan loading, 50 mM citrate buffer (pH 4.8), 40 mg/L tetracycline (Sigma Aldrich, St. Louis, MO) as an antimicrobial agent, Cellic® Ctec2 cocktail loaded at 15 mg-protein per g-glucan-in-raw poplar, and yeast inoculum. An assembly made with the flask and attached bubble trap was loaded with millipore water and the appropriate amount of substrate (Table 5-1). Duplicates with substrate along with a substrate blank assembly were sterilized at 121

°C for 35 min. The flasks were cooled in a laminar flow hood (Baker and Baker Ruskinn, Sanford, ME) to prevent contamination, and reweighed to allow appropriate water replenishment. After adding the buffer, antimicrobial agent, enzyme cocktail, and yeast inoculum, SSF was carried out in flasks shaken at 130 rpm for 7 days at 37 °C for both D5A and CBS 6556 and at 43 °C only for CBS 6556. 1 mL samples were taken periodically, centrifuged at 15000 rpm for 10 min, diluted, and analyzed to measure the sugar and metabolite concentration in the broth.

Table 5-1 Substrate loadings employed in SSF experiments

Case	Insoluble Solid Loading (wt%)	Corresponding Glucan Loading (wt%)	Enzyme Dosage
1	13	11	15 mg protein per g raw glucan in raw poplar
2	17	15	
3	20	18	

5.3.6 Measuring sugar and ethanol concentrations

Liquid samples along with appropriate calibration standards were analyzed by High performance liquid chromatography **(**HPLC) (Waters Alliance 2695 system equipped with a Bio-Rad Aminex® HPX-87H column and Waters 2414 RI detector) with a 5 mM sulfuric acid eluent flow rate of 0.6 ml min^{-1}. The chromatograms were integrated using the Empower® 2 software package (Waters Co., Milford, MA).

5.3.7 Model equations

At lower solid loadings, i.e., <5 wt%, the density of the solvent phase was assumed to be the same as for just water. As the insoluble solid fraction increased, the density of the liquid fraction first increased due to increased sugar concentration and then slightly

dropped due to the increasing ethanol concentration. Here, the modified version of the equations from Roche et al. (Roche, Dibble, and Stickel 2009) were employed to calculate the density of liquid fraction. (Wang, Templer, and Murphy 2012)

$$Yield_{Glucose} = \frac{C_g \times V_l / 1.11}{M_g} \times 100 \quad (2)$$

$$\%\ conversion_{Glucan} = \frac{(\frac{C_g}{1.11} + \frac{C_{cb}}{1.056} + \frac{C_{Gly}}{1.135} + \frac{C_{Ac}}{1.111} + \frac{C_{Eth}}{0.567}) \times V_l}{M_g} \times 100 \quad (3)$$

$$V_l = \frac{M \times (1 - S_i)}{\rho_l} \quad (4)$$

$$S_i = \frac{S_{i0} - (\frac{\Delta C_g}{1.11} + \frac{\Delta C_{cb}}{1.056} + \frac{\Delta C_x}{1.36} + \frac{\Delta C_{Gly}}{1.135} + \frac{\Delta C_{Ac}}{1.11} + \frac{\Delta C_{Eth}}{0.567}) / \rho_l}{1 - (\frac{\Delta C_g}{1.11} + \frac{\Delta C_{cb}}{1.056} + \frac{\Delta C_x}{1.36} + \frac{\Delta C_{Gly}}{1.135} + \frac{\Delta C_{Ac}}{1.11} + \frac{\Delta C_{Eth}}{0.567}) / \rho_l} \quad (5)$$

$$\rho_l = 0.456(C_g + C_{cb} + C_x) + 0.97 \quad (6)$$

$$Theoretical\ Ethanol\ yield\ (\%) = M_{Eth,g} / M_g \times 100 = (C_{Eth} \times V_{l1} \times 0.9) / (0.51 \times M_g) \quad (7)$$

Where C_g is the glucose concentration, g/mL, C_{cb} is the cellobiose concentration, g/mL, C_x is the xylose concentration, g/mL, C_{Gly} is the glycerol concentration, g/mL, C_{Ac} is the acetic acid concentration, g/mL, C_{Eth} is the ethanol concentration, g/mL, M is the initial mass of the system (solids +liquids), M_g is the initial mass of glucan, g, V_l is the volume of liquid phase, mL, S_{i0} is the initial insoluble solid fraction, S_i is the insoluble solid fraction at time t, ρ_l is the density of the liquid phase, g/cc, $M_{Eth,G}$ is the mass of ethanol in glucan equivalents, g.

5.3.8 SEM sample preparation

Approximately 2 mL of SSF broth was centrifuged at 2400 rpm for 5 min to concentrate yeast cells. The cells were then suspended in saline phosphate buffer to remove

any residual media. Next the cells were fixed in 2.5% glutaraldehyde in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer for at least 48 h followed by a serial dehydration (i.e., exposure to a series of ethanol concentrations: 50, 75, 80, 85, 90, 95, 99, and 100% for 10 minutes at each step).

The dehydrated cells were then carefully mounted onto an aluminum SEM stub coated with conductive carbon tape and air dried. The cells were then sputter coated with Pt/Pd for 90 seconds using a Cressington 108 auto sputter coater.

5.3.9 Scanning electron microscopy (SEM)

Samples were examined using scanning electron microscopy (NNS450 (FEI, USA) under high vacuum over a voltage range of ~ 2 to 5 kV. Images were collected at 10,000x magnification.

5.3.10 Image analysis

Cell diameters were measured by using the line tool and analyze/ measure function of the Image J software package. (Abramoff, Magalhães, and Ram 2004) Length measurements were calibrated using the scale bars on the image and the scale function of the software. Yeast cells were assumed to be prolate ellipsoids, and their total surface areas and volumes were estimated using the following equations:

$$Surface\ area\ of\ a\ prolate\ spheroid = 2\pi\left(a^2 + \frac{ab\alpha}{sin(\alpha)}\right) \quad 8$$

In which a is the horizontal radius, b is the vertical radius, and α is the angular eccentricity calculated as

$$\alpha = arccos\left(\frac{a}{b}\right) \quad 9$$

$$Volume\ of\ prolate\ ellopsoid = \frac{4}{3}\pi ab^2 \quad 10$$

5.4 Results and Discussion

5.4.1 Growth, productivity, and sugar tolerance of *K. marxianus* and *S. cerevisiae* grown on glucose

If the activity of fungal enzymes is maintained over the entire course of SSF and the fermentation organism is unable to ferment the sugars released to ethanol fast enough, the sugars and their oligomers can reach high concentrations, particularly at high solids loadings. The resulting high sugar levels can in turn create hyperosmotic stress on the cells. (Hohmann 2002) Coupling this stress with the need to operate at higher than optimal growth temperatures to foster sufficient enzyme action and ethanol accumulation results in osmotic, temperature, and ethanol stresses. (Pratt, Bryce, and Stewart 2003; Rothschild and Mancinelli 2001) To understand how these factors could impact D5A and CBS 6556, their anaerobic growth and ethanol production were first evaluated with pure glucose when subjected to (i) a higher temperature, (ii) a high osmolarity, and (iii) evaluation of the combined effect of (i) and (ii). First, glucose concentrations of 50 and 150 g/L were fermented by both strains at 37 and 43 °C to determine how temperature and glucose concentration impacted performance. The optical density results in Figure 5-1 show that at 37 °C, CBS 6556 grew almost twice as fast as D5A for both 50 g/L and 150 g/L glucose concentrations. Thus, although both strains grew on either glucose concentration, *K. marxianus* outperformed at 37 °C temperature typically employed to achieve reasonable enzyme activity in SSF. It is important to note that the growth of both CBS 6556 and D5A were hindered in the presence of higher glucose concentrations at the higher temperature. However, the performance of D5A suffered much more under the combined stresses of temperature and higher glucose concentrations. This data also reveals that *K. marxianus*

maintained high growth rates at glucose concentrations of 50 g/L and 150 g/L at 43 °C, while *S. cerevisiae* failed to grow at either concentration at this temperature. These results suggest that CBS 6556 should be more compatible than D5A at 37°C (typically employed for SSF enzymes to be sufficiently active) and could also enable the use of higher temperatures that are more favorable for fungal enzymes.

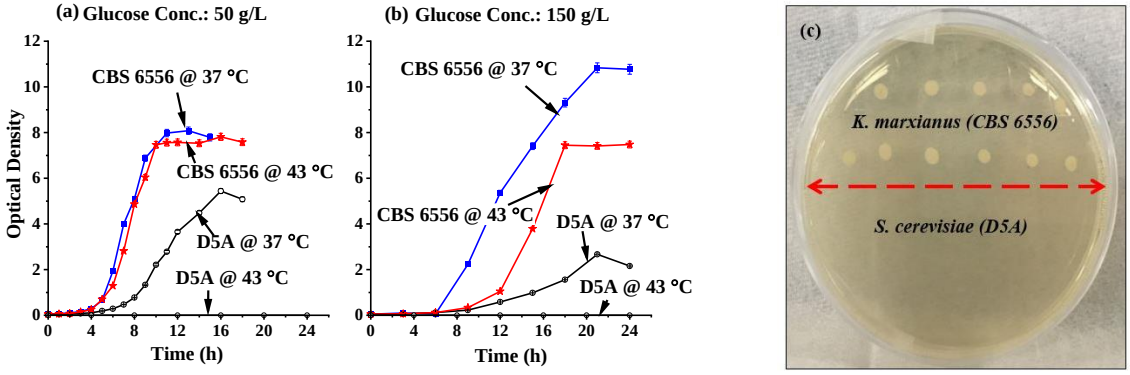
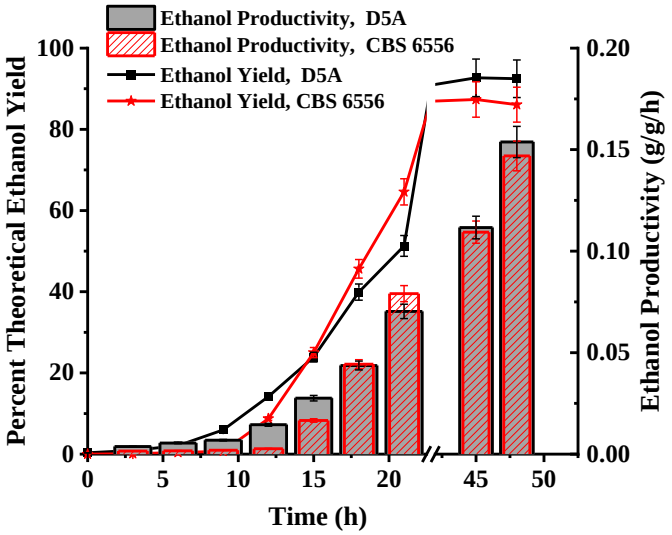


Figure 5-1 Anaerobic growth over time as measured by optical density for the CBS 6556 of *Kluveromyces marxianus* and D5A strain of *Saccharomyces cerevisiae* cultured on glucose concentrations of (a) 50 g/L and (b) 150 g/L at 37 and 43 °C. Rich media (YPD) plate of D5A and CBS 6556 cultures inoculated and incubated at 43 °C in (c) shows that only *K. marxianus* grew at this temperature.

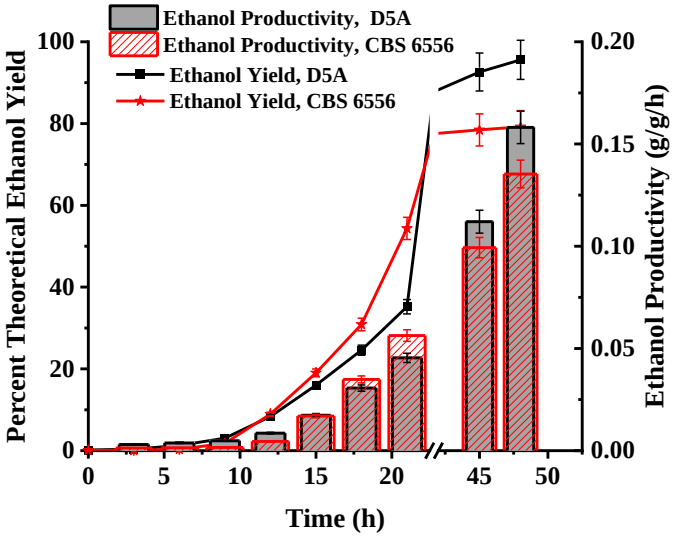
Next, the effect of glucose concentration on ethanol production by each organism was evaluated by fermenting glucose concentrations of 150, 180, and 200 g/L. As shown in Figure 5-2, D5A and CBS 6556 both performed well for all glucose concentrations at 37 °C. This data also showed that CBS 6556 had a higher initial ethanol productivity at the larger glucose concentration, but performed similarly to D5A at other glucose concentrations. It is interesting to note that despite the slower growth rates for D5A shown in Figure 5-1, D5A was able to produce ethanol at a similar rate to the faster growing CBS 6556. Furthermore, after 2 days of glucose fermentation by both yeasts, concentrations of ethanol and glucose (Additional Table 5-1) indicate that CBS 6556 left more glucose in

solution than D5A for the two lower starting concentrations of glucose, while residual glucose reached almost 50 g/L for both strains when grown on 200 g/L glucose. The ethanol concentration from both yeasts did not increase significantly when the glucose concentration was raised from 180 to 200 g/L, as observed by the significant increase in residual glucose shown in Additional Table 5-1, suggests that these yeasts were reaching an ethanol tolerance limit of about 80 g/L.

(a)



(b)



(c)

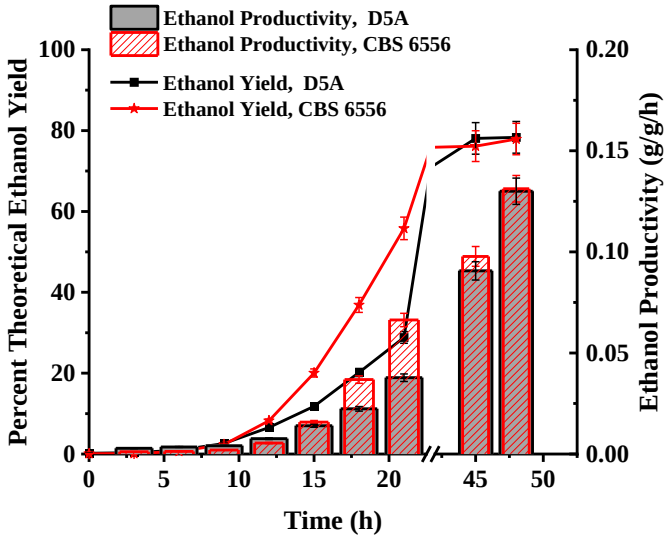


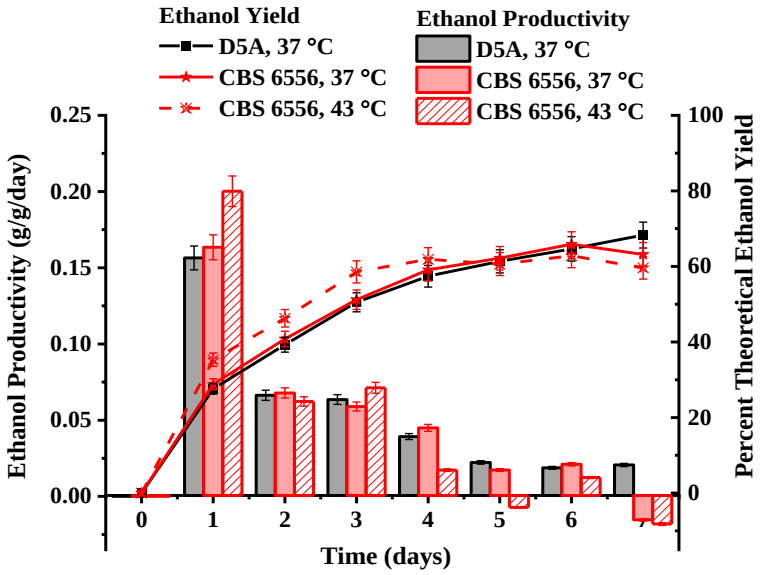
Figure 5-2 Percent of theoretical ethanol yields and ethanol productivities (g/g/h) for growth of *K. marxianus* (CBS 6556) and *S. cerevisiae* (D5A) at 37 °C on glucose concentrations of (a) 150, (b) 180, and (c) 200 g/L in a shake flask with a 50 mL working volume, in triplicates. Error bars indicated in the figure are standard deviation error bars among the triplicates.

5.4.2 Ethanol productivity and yields for high solids SSF of CELF pretreated poplar

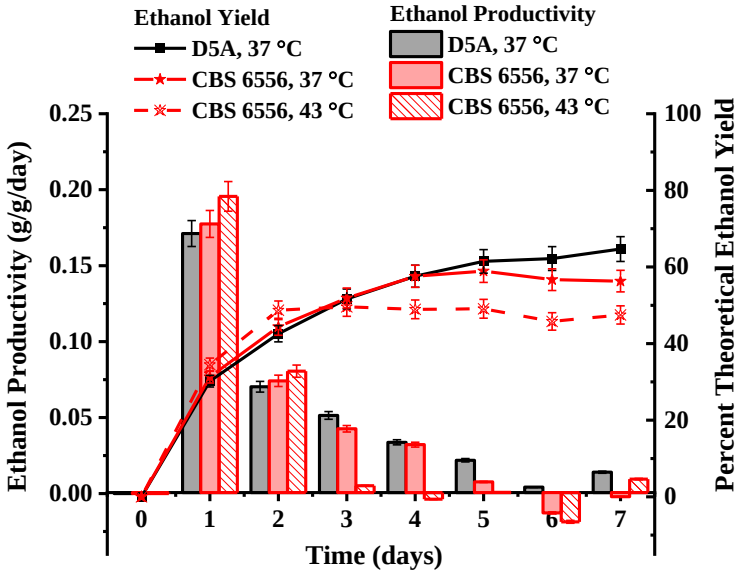
In light of the fermentation results with pure glucose, CBS 6556 and D5A would be expected to have similar ethanol tolerance and productivity and not be inhibited by the glucose concentrations expected in SSF. However, these results, albeit on glucose, indicated that CBS 6556 should be more suitable than D5A for SSF due to its ability to grow and perform effectively at higher temperatures. To test whether these attributes would enhance SSF performance, each organism was employed for SSF of solids that contained 88.5 % glucan, 3.0 % xylan, and 2.3% acid-insoluble lignin following CELF pretreatment of poplar. Furthermore, SSF experiments were carried out at 13, 17, and 20 wt% insoluble solids produced by CELF pretreatment that corresponded to 11, 15, and 18 wt% glucan loadings. Both D5A and CBS 6556 were run at 37 °C, while CBS 6556 was also used in

SSF at 43 °C to take advantage of the higher temperature tolerance displayed for glucose fermentations. A Cellic® CTec 2 enzyme cocktail was employed for each fermentation at a dosage of 15 mg protein per g glucan in raw poplar.

Operation of CBS 6556 at 43 °C initially resulted in higher ethanol productivities (Figure 5-3 (a)), but 5 day yields for all three experiments were approximately the same (63%) and did not significantly increase at longer times. At a higher initial glucan concentration of 15%, the productivity of CBS 6556 at 43 °C was greater at an even shorter period of time (Figure 5-3(b)). However, when operated at 37 °C, this rate dropped below that observed for both D5A and CBS 6556. The latter two had similar productivities up to day 5, after which D5A increased slightly while CBS 6556 leveled off. The final yields for D5A at both 11 and 15 wt% glucan were about the same, while the yields dropped with increased glucan for CBS 6556, particularly for operation at 43 °C. For application of SSF at 18 wt% glucan, D5A demonstrated similar productivities and yields to those for both 11 and 15% glucan. On the other hand, although CBS 6556 run at 37 °C shadowed yields and productivities of D5A for the first 3 days, it virtually stopped fermentations thereafter. The results show that the yield did not exceed 60% of the theoretical maximum and ethanol production ceased. Thus, these results show that operation of CBS 6556 at 43 °C exhibited the highest initial fermentation rates for 11 and 15 wt% glucan, apparently due to higher sugar release by cellulase operated nearer to its optimum temperature. However, CBS 6556 also suffered from a much earlier fermentation arrest, likley due to the combined effects of higher ethanol concentrations and temperature.


Ethanol Yield
D5A, 37 °C
CBS 6556, 37 °C
CBS 6556, 43 °C
Ethanol Productivity
D5A, 37 °C
CBS 6556, 37 °C
CBS 6556, 43 °C
Ethanol Productivity (g/g/day)
Percent Theoretical Ethanol Yield
Time (days)
0.25
0.20
0.15
0.10
0.05
0.00
100
80
60
40
20
0
0
1
2
3
4
5
6

(a)


Ethanol Yield
D5A, 37 °C
CBS 6556, 37 °C
CBS 6556, 43 °C
Ethanol Productivity
D5A, 37 °C
CBS 6556, 37 °C
CBS 6556, 43 °C
Ethanol Productivity (g/g/day)
Percent Theoretical Ethanol Yield
Time (days)
0.25
0.20
0.15
0.10
0.05
0.00
100
80
60
40
20
0
0
1
2
3
4
5
6
7

(b)

(c)

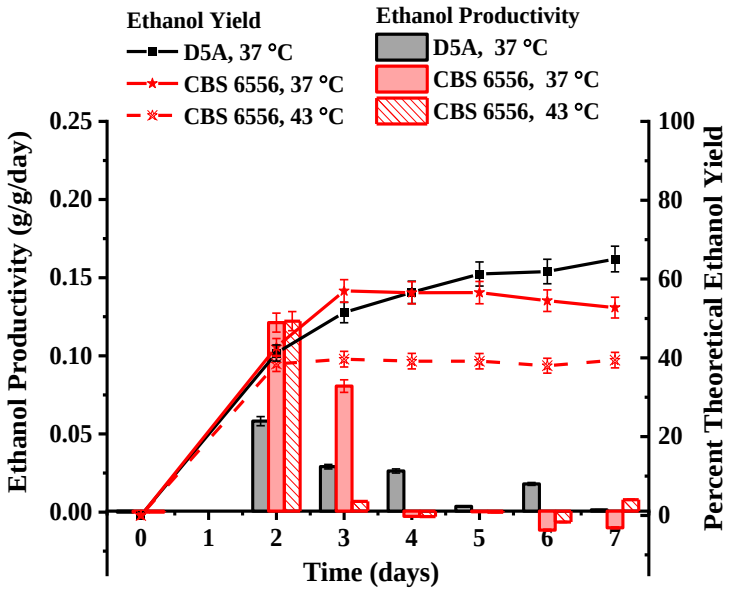


Figure 5-3 Ethanol productivities (g/g/day) and percent of theoretical ethanol yields produced by *S. cerevisiae* (D5A) at 37 °C and *K. marxinaus* (CBS 6556) at 37 and 43 °C when used for SSF of (a) 11, (b) 15, and (c) 18 wt% glucan loadings in solids produced by CELF pretreatment of poplar. The SSF enzyme dose was 15 mg protein per g glucan for all cases. All the experiments were conducted in a shake flask with a 25 mL working volume, in duplicates. Error bars indicated in the figure are standard deviation error bars among the duplicates.

The results presented in Additional Figure 5-1 shed additional light on factors that impacted the results in Figure 5-3. As shown, D5A completely converted glucose released by the enzymes at 11 and 15 wt% glucan loadings and left only a little glucose in solution at the end of the 18% glucan run. On the other hand, when CBS 6556 was operated at the same temperature as D5A (37 ^{o}C), glucose accumulation progressively increased with glucan loading to reach about 30 g/L at the two highest loadings. Furthermore, because ethanol production virtually stopped at the point glucose started building up, the greater amount of ethanol appeared to stop fermentation at these points. However, it is noteworthy that the final ethanol concentration increased with glucan loading, suggesting that faster

glucose release from more glucan allowed more ethanol to form before the fermentations stopped. Increasing the temperature to 43 °C resulted in glucose buildup starting sooner and ethanol production stopping at lower concentrations.

Overall, these results show that operation of CBS 6556 at 43 °C exhibited the highest initial fermentation rates for 11 and 15 wt% glucan, apparently due to higher sugar release by cellulase operated nearer to its optimum temperature. However, CBS 6556 also suffered from a much earlier fermentation arrest due to the combined effects of higher ethanol concentrations and temperature. This outcome is consistent with results with *marxianus* strains capable of fermenting glucose and cane syrup at high temperatures of up to 47 °C that showed that although fermentation was rapid initially, the organism suffered from a rapid rate of cell death at higher temperatures in high gravity fermentations. (Anderson et al. 1986) Other studies also observed a high temperature later-stage ethanol fermentation arrest by *K. marxianus*. (Fu et al. n.d.; Li et al. n.d.)

5.4.3 Impact of glucose, ethanol, and temperature on yeast

Yeasts, in general, are polymorphic organisms and can take many sizes and shapes such as ellipsoidal, spherical, or elongated cylinders, depending on the environment to which they are exposed. (O'shea and Walsh n.d.; Walker and O'Neill 2007) Hyperosmotic stress, due to increased glucose concentration, results in rapid water diffusion from the yeast cells into the surrounding medium, thereby leading to loss of cell wall turgor pressure and cells shrinkage. Higher ethanol concentrations act adversely on the integrity of the cell membrane by increasing membrane fluidity and permeability that result in cellular ion leakage. (Birch and Walker 2000) Ethanol also negatively impacts cell metabolism and

inhibits cell growth and cell division. (Stanley et al. 2010) In response to hyper osmolarity and ethanol shock, the cells can accumulate glycerol or other polyols such as arabitol, mannitol, meso-erythritol, and/or xylitol to alter the equilibrium between the intracellular and extracellular environments and reduce diffusion of intracellular water. (Hohmann 2002; Pratt et al. 2003; Scanes, Hohrnann, and Prior 2017; Siderius et al. 2002) The result can be an increase in cell volume due to swelling. Heat shock, however, not only increases cell membrane fluidity but also causes protein damage, practically killing the organism unless it possesses heat-shock proteins (HSP), i.e., proteins that enhance thermotolerance of unicellular organisms like yeasts and bacteria. HSPs usually protect thermally damaged proteins from accumulation, unfold aggregated proteins, and refold damaged proteins or efficiently degrade them. (Kregel 2002; Rothschild and Mancinelli 2001)

Figure 5-4 shows that while D5A produced some glycerol initially for SSF of 18% glucan, the amount did not change much as ethanol production continued at 37 °C. At the same temperature, CBS 6556 coproduced glycerol along with ethanol, and glycerol plateaued at about a 50% higher level than for D5A when ethanol production ceased. Figure 5-4 also reveals that glycerol production similarly followed ethanol build up for SSF by CBS 6556 at 43 °C and again leveled off when ethanol production stopped. However, the concentrations of ethanol and glycerol stopped building up at somewhat lower concentrations than for operation at 37 °C. Additional Figure 5-2 reports that for SSF by D5A at 37 °C, glycerol concentrations increased by about 50% when glucan loadings were increased from 11 to 18 wt%. However, this figure also shows that although glycerol levels reached a similar high value for SSF of 11 wt% glucan for CBS 6556 at 37

°C, the amount rose with increasing glucan loadings to reach about 250% of that amount at 18% glucan. Increasing the temperature to 43 °C for SSF by CBS 6556 resulted in about a 50% increase in maximum glycerol at 11 wt% glucan, but that value only increased modestly as the glucan loading increased. Overall, the somewhat higher glycerol concentrations produced by CBS 6556 suggest it was more stressed by the coupling of ethanol and temperature than D5A.

In order to further comprehend the impact of temperature and ethanol concentration on CBS 6556 and D5A performance, electron micrographs were taken of both strains following fermentation of pure glucose and SSF of CELF pretreated poplar. As shown in Figure 5-5(a-h), both D5A and CBS 6556 cells maintained ellipsoidal or yeast-like morphologies when grown in an anaerobic environment. Therefore, we assumed the cells to be prolate ellipsoids and estimated their total surface areas and volumes (Figure 5-6) based on their vertical and horizontal dimensions. Although, the presence of fibrous biomass in the SSF broth made it difficult to obtain meaningful images, it appears that the oval structures highlighted in the yellow boxes (Figures 5-5 c, g, and h) are similar in shape to the native ellipsoidal yeast. The cell volume estimations (Figure 5-6) are therefore calculated based on an elliptical geometry. Figure 5-5 (f) also revealed that CBS 6556 cells suffered substantial surface damage including shrinking and wrinkling, likely due to greater shock at 43 °C compared to the behavior of this yeast (Figure 5-5 d) and D5A (Figure 5-5 b) under similar stresses at 37 °C. Figure 5-6 further indicates that when subjected to a 150 g/L glucose concentration at 37 °C, the cell volumes of D5A and CBS 6556 increased by 66.0% and 46.64%, respectively, as compared to their sizes at seed

culture conditions. However, when subjected to higher ethanol concentrations at 43 °C, the average volume of CBS 6556 cells dramatically shrunk by almost 64%. These observations further indicate that CBS 6556 was more stressed by high concentrations of ethanol than D5A and the adverse impact was more pronounced at a higher temperature resulting in shrinking of CBS 6556 cells to an abnormally small size with quite noticeable surface damage.

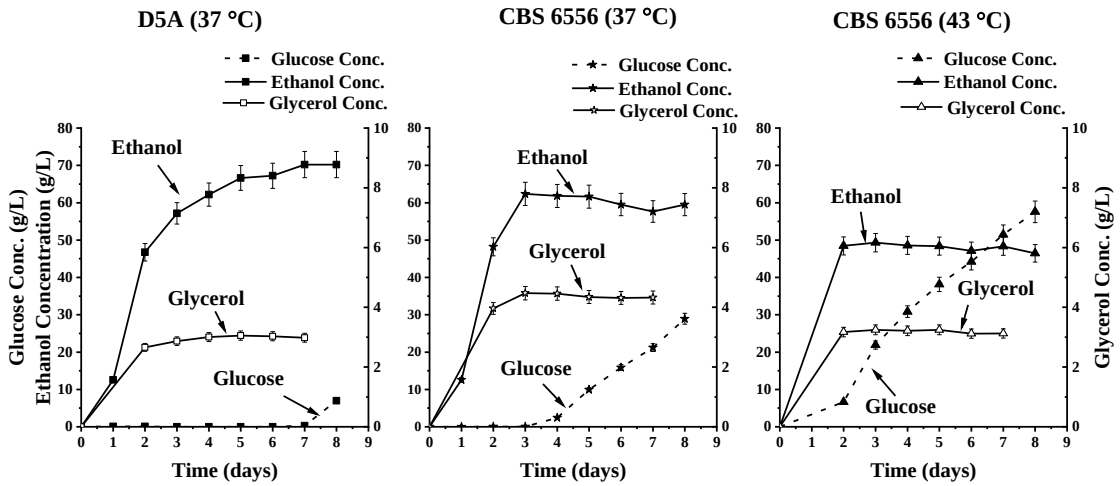


Figure 5-4 Glucose, ethanol, and glycerol concentrations in broths following SSF of 18 wt% glucan loadings of CELF solids by *S. cerevisiae* (D5A) at 37 °C and *K. marxianus* (CBC 6556) at 37 °C and 43 °C. All the experiments were conducted in a shake flask with a 25 mL working volume, in duplicates. Error bars indicated in the figure are standard deviation error bars among the duplicates.

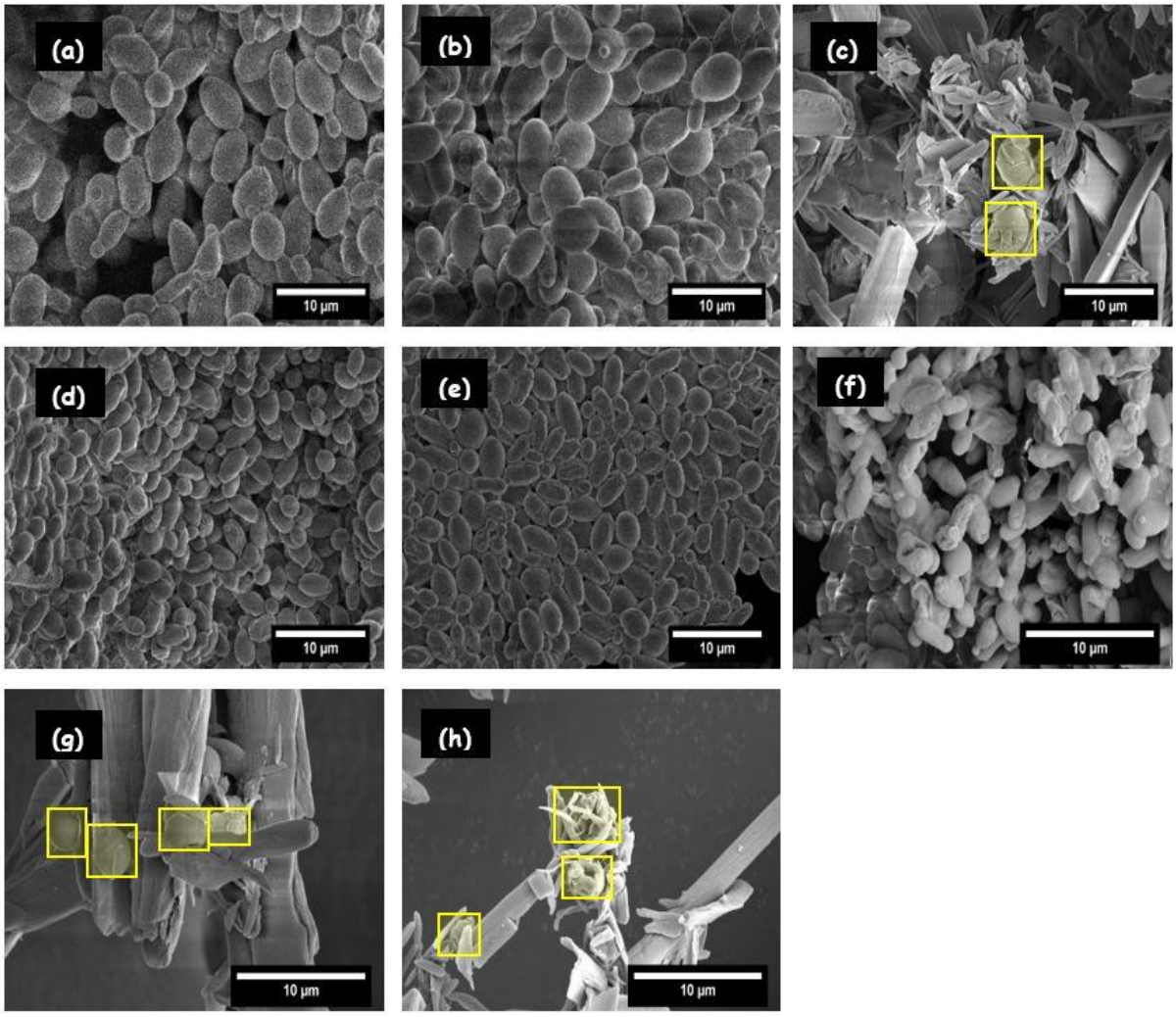


Figure 5-5 Scanning electron micrographs for anaerobic growth of *S. cerevisiae* D5A on (a) 50 g/L glucose at 37 °C, (b) 150 g/L glucose at 37 °C, (c) in SSF of CELF pretreated poplar with 18 wt% glucan loading at 37 °C; and of *K. marxianus* CBS 6556 on (d) 50 g/L glucose at 37 °C, (e) 150 g/L glucose at 37 °C, (f) 150 g/L glucose at 43 °C, (g) in SSF of CELF pretreated poplar at 18 wt% glucan loading at 37 °C and (h) SSF of CELF pretreated poplar at 18 wt% glucan loading at 43 °C. (Magnification 10,000x at a voltage range of 2kV-5kV.)

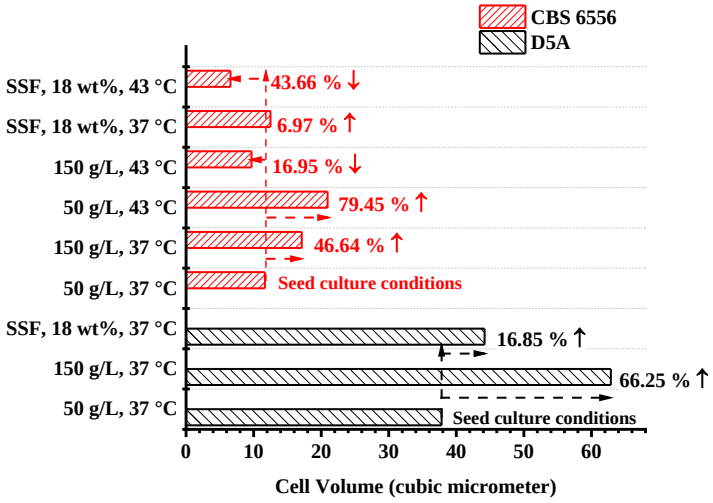


Figure 5-6 Calculated cell volume of *S. cerevisiae* D5A and *K. marxianus* CBS 6556 cells following pure glucose fermentations and SSF of CELF pretreated poplar, based on data collected from SEM images using ImageJ software.

Figure 5-6 shows similar observations from SSF of 18 wt% glucan at the end of 5-day glucose fermentations in that D5A and CBS 6556 volumes expanded by 16.8 % and 6.97 %, respectively, at 37 °C, while CBS 6556 contracted by 43.66% at 43 °C. However, as shown in Figure 5-4 and Additional Table 5-2, the glucose concentration remaining at the end of 5 days of SSF at 43 °C was less than 50 g/L, a value within the tolerance limit of CBS 6556. This outcome indicated that the lower ethanol productivity could be attributed to reduced ethanol tolerance of CBS 6556 cells at higher temperatures. (Hacking, Taylor, and Hanas 1984)

Overall, these results suggest that CBS 6556 cells suffered major cell damage due to the combined effects of ethanol and heat shock. Because the cells were unable to make sufficient glycerol and/or maintain the turgor pressure of the cell wall, they shrunk to an abnormally small size. In addition, yeast cells need a critical size that is characteristic for the growth medium to initiate budding, and extremely small cells are incapable of budding,

thereby arresting the cell cycle. The atypically small cell size at high temperature and higher ethanol concentrations appeared to limit growth and metabolism of CBS 6556, thereby causing premature cessation of sugar uptake and fermentation at elevated temperature.

These observations are consistent with an analysis by Li *et al*. (Li et al. n.d.) of protein samples collected during *K. marxianus* fermentations at 45 °C that revealed some biochemical and enzymatic modifications triggered by stress conditions. They observed that some of the proteins related to gene transcription and translation, along with some of the proteins involved in oxidative phosphorylation, were down-regulated in *K. marxianus* after fermentation arrest. They attributed the repression of transcription and translation to a yeast self-defense mechanism to cope with stress condition during the late fermentation stage. They also reported up-regulation of some molecular chaperones and proteasome proteins involved in the protein quality control (PQC) system after fermentation arrest. The interactions of the proteins in the PQC system are responsible for the folding of proteins, refolding of misfolded proteins, and degradation of misfolded and damaged proteins. These observations provide some explanation for the observed fermentation halt and offer possible opportunities for metabolic engineering towards improvement of the stress tolerance in *K. marxianus*.

5.5 Conclusion

The ability of CELF pretreatment to realize higher glucan concentrations at a given solids level in SSF was coupled with the greater thermotolerance of *K. marxianus* than for *S. cerevisiae* when grown on glucose with the goal of enhancing ethanol concentrations,

rates, and yields from SSF of poplar. For SSF at 37 °C, application of the CBS 6556 strain of *marxianus* to solids produced by CELF pretreatment of poplar achieved similar yields and concentrations as for application of the D5A strain of *S. cerevisiae* to the same solids and but did not attain high ethanol yields at a near optimal saccharification temperature for enzymes. CBS 6556 cells experienced an early fermentation arrest and underwent shrinkage, apparently due to the combined stresses of high ethanol concentrations and elevated temperature. Further insights into factors responsible for its inability to tolerate these conditions could help guide strain engineering that would allow it to survive these combined stresses and make it better suited for SSF ethanol fermentations.

5.6 References:

Abramoff, M. D., Paulo J. Magalhães, and Sunanda J. Ram. 2004. "Biophotonics International." *Biophotonics International* 11(7):36–42.

Anderson, P. J., K. McNeil, and K. Watson. 1986. "High-Efficiency Carbohydrate Fermentation to Ethanol at Temperatures above 40 Degrees C by Kluyveromyces Marxianus Var. Marxianus Isolated from Sugar Mills." *Applied and Environmental Microbiology* 51(6):1314–20.

Anon. 1975. "Manufacture of Alcohol from Cellulosic Materials Using Plural Ferments."

Anon. n.d. "Biomass Conversion - an Overview | ScienceDirect Topics." Retrieved February 2, 2020 (https://www.sciencedirect.com/topics/chemical-engineering/biomass-conversion).

Bai, F. W., W. A. Anderson, and M. Moo-Young. 2008. "Ethanol Fermentation Technologies from Sugar and Starch Feedstocks." *Biotechnology Advances* 26(1):89–105.

Birch, Rosslyn M. and Graeme M. Walker. 2000. "Influence of Magnesium Ions on Heat Shock and Ethanol Stress Responses of Saccharomyces Cerevisiae." *Enzyme and Microbial Technology* 26(9–10):678–87.

Demirbas, Ayhan. 2009. "Political, Economic and Environmental Impacts of Biofuels: A Review." *Applied Energy* 86:S108–17.

Fonseca, Gustavo Graciano, Nuno Miguel Barbosa de Carvalho, and Andreas Karoly Gombert. 2013. "Growth of the Yeast Kluyveromyces Marxianus CBS 6556 on Different Sugar Combinations as Sole Carbon and Energy Source." *Applied Microbiology and Biotechnology* 97(11):5055–67.

Fu, Xiaofen, Pengsong Li, Lei Zhang, and Shizhong Li. n.d. "Understanding the Stress Responses of Kluyveromyces Marxianus after an Arrest during High-Temperature Ethanol Fermentation Based on Integration of RNA-Seq and Metabolite Data."

Ghose, T. K., P. K. Roychoudhury, and P. Ghosh. 1984. "Simultaneous Saccharification and Fermentation (SSF) of Lignocellulosics to Ethanol under Vacuum Cycling and Step Feeding." *Biotechnology and Bioengineering* 26(4):377–81.

Hacking, A. J., I. W. F. Taylor, and C. M. Hanas. 1984. "Selection of Yeast Able to Produce Ethanol from Glucose at 40  C." *Applied Microbiology and Biotechnology* 19(5):361–63.

Hohmann, Stefan. 2002. "Osmotic Stress Signaling and Osmoadaptation in Yeasts." *Microbiology and Molecular Biology Reviews : MMBR* 66(2):300–372.

Humbird, David, Ali Mohagheghi, Nancy Dowe, and Daniel J. Schell. 2010. "Economic Impact of Total Solids Loading on Enzymatic Hydrolysis of Dilute Acid Pretreated

Corn Stover." *Biotechnology Progress* 26(5):1245–51.

Húngaro, Humberto Moreira, Natalia Oliveira Calil, Aline Siqueira Ferreira, Anuj Kumar Chandel, and Silvio Silvério da Silva. 2013. "Fermentative Production of Ribonucleotides from Whey by Kluyveromyces Marxianus: Effect of Temperature and PH." *Journal of Food Science and Technology* 50(5):958–64.

Kregel, Kevin C. 2002. "Invited Review: Heat Shock Proteins: Modifying Factors in Physiological Stress Responses and Acquired Thermotolerance." *Journal of Applied Physiology* 92(5):2177–86.

Lane, Melanie M., Niall Burke, Rob Karreman, Kenneth H. Wolfe, Conor P. O'Byrne, and John P. Morrissey. 2011. "Physiological and Metabolic Diversity in the Yeast Kluyveromyces Marxianus." *Antonie van Leeuwenhoek* 100(4):507–19.

Lee, J. H., J. C. Woodard, R. J. Pagan, and P. L. Rogers. 1981. "Vacuum Fermentation for Ethanol Production Using Strains of Zymomonas Mobilis." *Biotechnology Letters* 3(4).

Li, Pengsong, Xiaofen Fu, Ming Chen, Lei Zhang, and Shizhong Li. n.d. "Proteomic Profiling and Integrated Analysis with Transcriptomic Data Bring New Insights in the Stress Responses of Kluyveromyces Marxianus after an Arrest during High-Temperature Ethanol Fermentation."

Mohagheghi, A., M. Tucker, K. Grohmann, and C. Wyman. 1992. "High Solids Simultaneous Saccharification and Fermentation of Pretreated Wheat Straw to Ethanol." *Applied Biochemistry and Biotechnology* 33(2):67–81.

Nguyen, Thanh Yen, Charles M. Cai, Rajeev Kumar, and Charles E. Wyman. 2015. "Co-Solvent Pretreatment Reduces Costly Enzyme Requirements for High Sugar and Ethanol Yields from Lignocellulosic Biomass." *ChemSusChem* 8(10):1716–25.

Nguyen, Thanh Yen, Charles M. Cai, Rajeev Kumar, and Charles E. Wyman. 2017. "Overcoming Factors Limiting High-Solids Fermentation of Lignocellulosic Biomass to Ethanol." *Proceedings of the National Academy of Sciences of the United States of America* 114(44):11673–78.

Nguyen, Thanh Yen, Charles M. Cai, Omar Osman, Rajeev Kumar, and Charles E. Wyman. 2016. "CELF Pretreatment of Corn Stover Boosts Ethanol Titers and Yields from High Solids SSF with Low Enzyme Loadings." *Green Chemistry* 18(6):1581–89.

Nguyen, Viet D., H. Kosuge, J. Auresenia, R. Tan, and Y. Brondial. 2009. "Effect of Vacuum Pressure on Ethanol Fermentation." *Journal of Applied Sciences* 9(17):3020–26.

O'shea, D. G. and P. K. Walsh. n.d. *The Effect of Culture Conditions on the Morphology of the Dimorphic Yeast Kluyveromyces Marxianus Var. Marxianus NRRLy2415: A*

Study Incorporating Image Analysis.

Palmqvist, Eva and Bärbel Hahn-Hägerdal. 2000. "Fermentation of Lignocellulosic Hydrolysates. I: Inhibition and Detoxification." *Bioresource Technology* 74(1):17–24.

Pratt, Patricia L., James H. Bryce, and Graham G. Stewart. 2003. "The Effects of Osmotic Pressure and Ethanol on Yeast Viability and Morphology." *Journal of the Institute of Brewing* 109(3):218–28.

Radecka, Dorota, Vaskar Mukherjee, Raquel Quintilla Mateo, Marija Stojiljkovic, María R. Foulquí E-Moreno, and Johan M. Thevelein. 2015. "Looking beyond Saccharomyces: The Potential of Non-Conventional Yeast Species for Desirable Traits in Bioethanol Fermentation." *FEMS Yeast Research* 15(6).

Rahikainen, Jenni, Saara Mikander, Kaisa Marjamaa, Tarja Tamminen, Angelos Lappas, Liisa Viikari, and Kristiina Kruus. 2011. "Inhibition of Enzymatic Hydrolysis by Residual Lignins from Softwood-Study of Enzyme Binding and Inactivation on Lignin-Rich Surface." *Biotechnology and Bioengineering* 108(12):2823–34.

Roberts, Katrina M., David M. Lavenson, Emilio J. Tozzi, Michael J. McCarthy, and Tina Jeoh. 2011. "The Effects of Water Interactions in Cellulose Suspensions on Mass Transfer and Saccharification Efficiency at High Solids Loadings." *Cellulose*.

Roche, Christine M., Clare J. Dibble, and Jonathan J. Stickel. 2009. "Laboratory-Scale Method for Enzymatic Saccharification of Lignocellulosic Biomass at High-Solids Loadings." *Biotechnology for Biofuels* 2(1):28.

Rothschild, Lynn J. and Rocco L. Mancinelli. 2001. "Life in Extreme Environments." *Nature* 409(6823):1092–1101.

Samaniuk, Joseph R., C. Tim Scott, Thatcher W. Root, and Daniel J. Klingenberg. 2012. "Rheological Modification of Corn Stover Biomass at High Solids Concentrations." *Journal of Rheology* 56(3):649–65.

Scanes, K. T., S. Hohrnann, and B. A. Prior. 2017. "Glycerol Production by the Yeast Saccharomyces Cerevisiae and Its Relevance to Wine: A Review." *South African Journal of Enology & Viticulture* 19(1).

Siderius, Marco, Olivier Van Wuytswinkel, Karin A. Reijenga, Marco Kelders, and Willem H. Mager. 2002. "The Control of Intracellular Glycerol in Saccharomyces Cerevisiae Influences Osmotic Stress Response and Resistance to Increased Temperature." *Molecular Microbiology* 36(6):1381–90.

Sluiter, A., B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, and D. Crocker. 2008. *Determination of Structural Carbohydrates and Lignin in Biomass: Laboratory Analytical Procedure (LAP); Issue Date: 7/17/2005*.

Stanley, D., A. Bandara, S. Fraser, P. J. Chambers, and G. A. Stanley. 2010. "The

Ethanol Stress Response and Ethanol Tolerance of *Saccharomyces Cerevisiae*." *Journal of Applied Microbiology*.

Viamajala, Sridhar, James D. McMillan, Daniel J. Schell, and Richard T. Elander. 2009. "Rheology of Corn Stover Slurries at High Solids Concentrations – Effects of Saccharification and Particle Size." *Bioresource Technology* 100(2):925–34.

Walker, Graeme M. and Joseph D. O'Neill. 2007. "Morphological and Metabolic Changes in the Yeast Kluyveromyces Marxianus Var. Marxianus NRRLy2415 during Fermentation of Lactose." *Journal of Chemical Technology & Biotechnology* 49(1):75–89.

Wang, Lei, Richard Templer, and Richard J. Murphy. 2012. "High-Solids Loading Enzymatic Hydrolysis of Waste Papers for Biofuel Production." *Applied Energy* 99:23–31.

Wyman, Charles E., Bruce E. Dale, Richard T. Elander, Mark Holtzapple, Michael R. Ladisch, and Y. Y. Lee. 2005. "Coordinated Development of Leading Biomass Pretreatment Technologies." *Bioresource Technology* 96(18 SPEC. ISS.):1959–66.

Wyman, Charles E., Diane D. Spindler, and Karel Grohmann. 1992. "Simultaneous Saccharification and Fermentation of Several Lignocellulosic Feedstocks to Fuel Ethanol." *Biomass and Bioenergy* 3(5):301–7.

Zhang, Meng, Pratyoosh Shukla, Manimaran Ayyachamy, Kugen Permaul, and Suren Singh. 2010. "Improved Bioethanol Production through Simultaneous Saccharification and Fermentation of Lignocellulosic Agricultural Wastes by Kluyveromyces Marxianus 6556." *World Journal of Microbiology and Biotechnology* 26(6):1041–46.

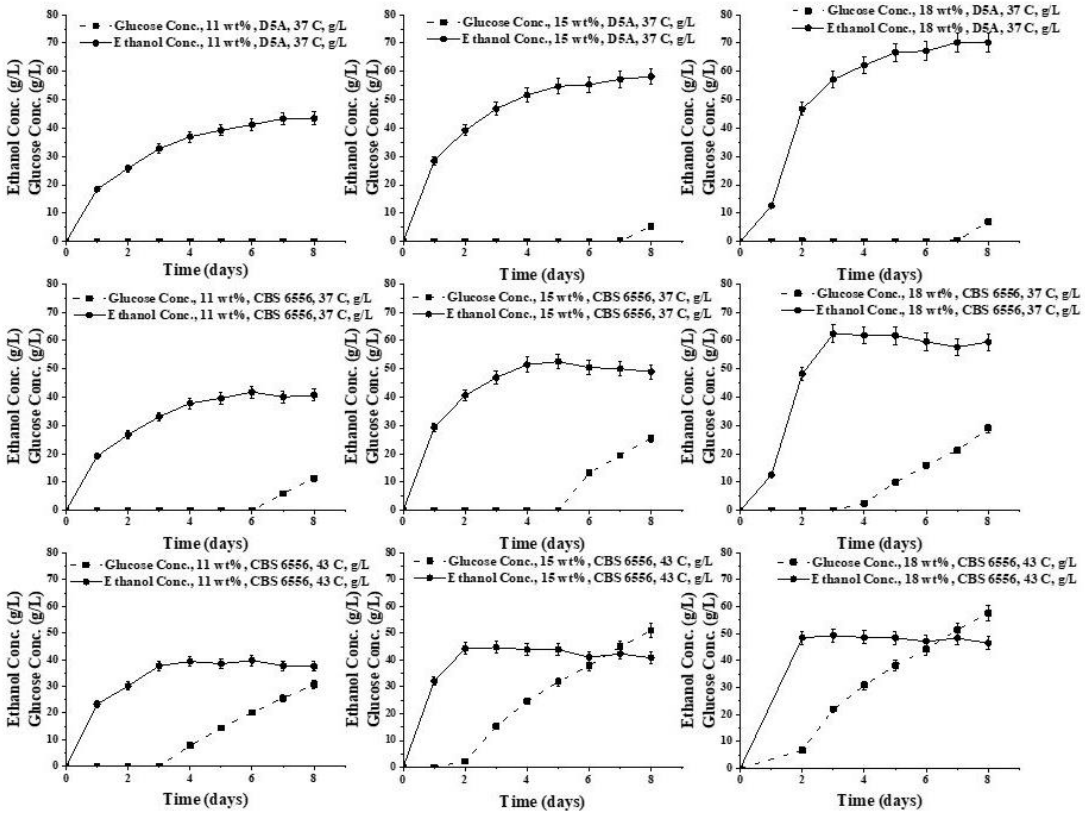
5.7 Additional Information

Additional Table 5-1 Glucose and ethanol concentrations and corresponding percent of theoretical ethanol yields after 2 days of 37 °C fermentations of 150, 180, and 200 g/L glucose by S. cerevisiae D5A and K. marxianus CBS 6556.

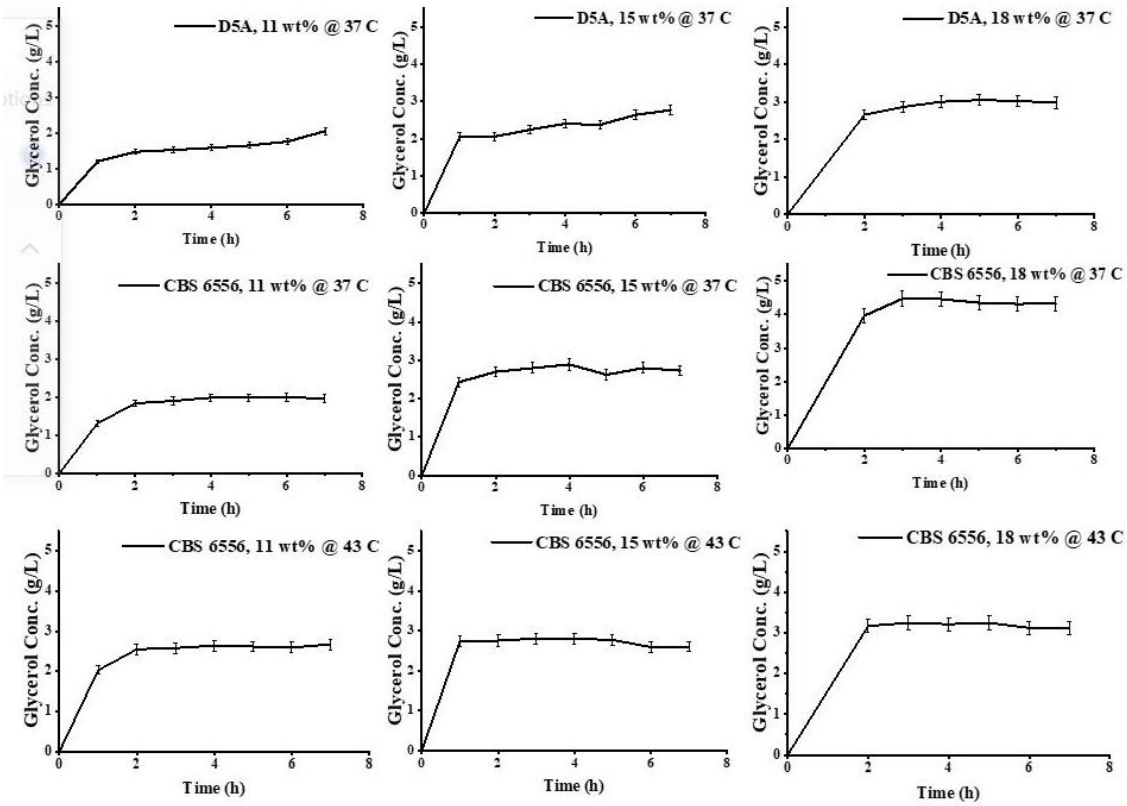
Glucose concentration (g/L)	Yeast strain	Ethanol concentration (g/L)	Percent of theoretical ethanol yield	Glucose remaining (g/L)
150	D5A	70.63	90	0.15
	CBS 6556	66.23	86.08	13.63
180	D5A	84.39	90	0.15
	CBS 6556	73.51	79.20	37.33
200	D5A	81.09	78.33	42.62
	CBS 6556	79.98	77.91	48.35

Additional Table 5-2 Ethanol concentrations and yields and glucose concentrations resulting from application of D5A at 37 °C and CBS 6556 at 37 and 43 °C to SSF of CELF pretreated solids at 13, 17, and 20 wt% solids loadings and corresponding glucan levels using Cellic® CTec2 enzyme at a loading of 15 mg protein/g glucan in raw poplar.

Insoluble solid loading (wt%)	Glucan loading (wt%)	Yeast	Temp. (°C)	Ethanol concentration (g/L)	Percent theoretical ethanol yield (%)	Fermentation time (days)	Glucose remaining (g/L) after fermentation
13	11	D5A	37	43.00	69	7	0
		CBS 6556	37	41.80	66	6	11.27
			43	40.00	62	4	14.35
17	15	D5A	37	58.21	66	7	5.26
		CBS 6556	37	53.00	59	5	13.21
			43	44.70	50	2	15.39
20	18	D5A	37	70.00	65	7	6.96
		CBS 6556	37	62.00	57	3	9.95
			43	49.00	40	1	21.90



Additional Figure 5-1 Ethanol and glucose concentrations (g/L) produced by S. *cerevisiae (D5A)* at 37 °C and *K. marxianus* (CBS 6556) at 43 °C when used in SSF of CELF pretreated poplar solids at glucan loadings of 11, 15, and 18 wt% with an enzyme loading of 15 mg protein/g glucan.



Additional Figure 5-2 Glycerol concentrations resulting from use of *S. cerevisiae* (D5A) at 37 °C and *K. marxianus* (CBS 6556) at 37 and 43 °C in SSF at glucan loadings of 11, 15, and 18 wt% of CELF pretreated poplar solids for an enzyme loading of 15 mg protein per g glucan.

Chapter 6 : THF washing of CELF pretreated poplar reduces enzymatic digestibility*

*This chapter was completed in collaboration with Kisailus group at University of California Riverside. FTIR, XRD and SEM characterization experiments on the biomass samples were performed by Ms. Ramya Mohan and Dr. David Kisailus. Sample preparation for the SEM imaging of Water washed CELF pretreated poplar were performed by Dr. Rachna Dhir.

6.1 Abstract

In this study we show the effect of washing CELF pretreated poplar with tetrahydrofuran (THF) on its structural features and enzymatic digestibility. Two varieties of poplar were produced using solids produced by CELF pretreatment at optimal conditions by either washing with THF followed by water or just water. THF rinsing of biomass removed ~40% of the residual lignin from the solid fraction, resulting in an overall lignin removal of ~94%, while pretreatment along with water washing alone removed ~90% of the total. Parallel enzymatic saccharification experiments were carried out on the solids produced from both variants. Despite promoting biomass delignification, THF washing adversely impacted digestibility of CELF poplar, contrary to expectations. Characterization of solids produced by FTIR, XRD, SEM, and Simon's straining revealed that THF washing especially removed lignin redeposited as droplets on the biomass surface during pretreatment. These techniques also showed that the THF washing reduced the substrate specific surface area, perhaps by excessive lignin removal causing cellulose pore collapse and formation of dense structures with a lower enzyme accessibility.

6.2 Introduction

Efficient saccharification of plant polysaccharides to fermentable sugars is crucial for economic bioethanol production. (Aden et al. 2002; Gubicza et al. 2016) However, the structural complexities of the cell wall due to the intricate network of polysaccharides and lignin contribute to the native recalcitrance that restricts enzyme access to cellulose and becomes the primary barrier to cost-competitive ethanol production. (Kothari et al. 2019; Yang and Wyman 2008) Hence, biomass pretreatment becomes essential to deconstruct the basic cell wall matrix and increase cellulose accessibility. (Wyman et al. 2005)

Lignin has been identified as a major deterrent to enzymatic hydrolysis due to two major reasons: 1) its close association with cellulose microfibrils acts as a shield that restricts enzyme accessibility and 2) its unproductive binding of enzymes reduces enzyme availability for hydrolysis. (Li, Pu, and Ragauskas 2016; Rahikainen et al. 2011) Biomass delignification can swell cell walls, disrupt the matrix structure, and enhance internal surface area and pore volume, thus increasing cellulose availability to enzymes. (Zhu et al. 2008) However, although considerable research has shown that lignin removal improves cell wall saccharification susceptibility, the extent of delignification required to enhance digestibility is not fully understood and may differ depending on biomass sources. (Ishizawa et al. 2009; Li et al. 2016)

Co-Solvent Enhanced Lignocellulosic Fractionation (CELF) pretreatment of lignocellulosic biomass promotes cell wall delignification to expose polysaccharides for further processing. Pretreatment operating conditions of 160 °C for 15 minutes has been shown to maximize sugar and lignin recovery from hardwood poplar without incurring much degradation. The cellulosic substrate generated at this condition was not only found to be susceptible to saccharification at a 1 wt% glucan loading with a low enzyme dose of 5 mg protein/g glucan, but also ~92% digestible at a higher glucan loading of 18wt% with an enzyme dose of 15 mg protein/g glucan to produce 87 g/L of ethanol via Simultaneous saccharification and fermentation (SSF) in just 7 days. Enhanced saccharification rates of solids produced by CELF pretreatment of poplar and corn stover without heavy doses of fungal enzymes is largely credited to the low lignin content of CELF solids. (Nguyen et al. 2015) Moreover, a low lignin content minimizes phenolic based toxicity to the yeast cells

during fermentation and reduces unnecessary enzyme-lignin interactions. (Palmqvist and Hahn-Hägerdal 2000; Rahikainen et al. 2011)

Hence, in this study, we endeavored to enhance cell wall delignification of poplar by subjecting it to additional tetrahydrofuran (THF) washing of solids produced by CELF pretreatment at optimal conditions. The solids were then subjected to subsequent enzymatic saccharification to generate fermentable sugars. Next, the digestibility of the THF washed substrate was compared to that for CELF pretreated substrate that was washed with just water. THF washed and water washed solids were then characterized by Scanning Electron Microscopy (SEM), Fourier Transform Infrared Spectroscopy (FTIR), X-ray Powder Diffraction (XRD), and Simons's Staining to understand the impact of extra delignification by THF washing on the structural features of delignified CELF poplar and their relationship to solids digestion by enzymes.

6.3 Experimental Section

6.3.1 Materials

This study was conducted on woody biomass, *Populus trichocarpa*, generously provided by BioEnergy Science Centre (BESC). The composition of the raw biomass as determined by following NREL LAP (version 08-03-2012) is 47.0 % glucan, 16.9 % xylan, and 21.2% acid-insoluble lignin. (Sluiter, Hames, Ruiz, et al. 2008) The biomass was air-dried, knife milled using a laboratory mill (Model 4, Arthur H. Thomas Company, Philadelphia, PA), and passed through a 1mm internal sieve size. The enzyme cocktail used for the study was Accellerase® 1500 generously provided by Dupont Industrial Biosciences (Palo Alto, CA). The protein content of the concoction, as estimated using Pierce BCA analysis kit, was 82 mg/ml. The dyes used for the Simon's Staining study were

DB (Pontamine Fast Sky Blue 6BX) and DO (Pontamine Fast Orange 6RN) dyes, obtained from Pylam Products (Garden City, NY).

6.3.2 Pretreatment

For CELF pretreatment of Poplar wood chips, milled raw biomass was soaked overnight at 4 °C at a dry biomass loading of 7.5 wt% based on the total working mass of the reaction, in a 1:1 (weight basis) solution of THF to water, with H_2SO_4 as the catalyst at a 0.5 wt% loading based on the total solvent mass. The reactions were conducted in a 1 L Hastelloy Parr autoclave reactor (236HC Series, Parr Instruments Co., Moline, IL) equipped with a double stacked pitch blade impeller rotating at a rpm of 200. Multiple CELF pretreatments were carried out at 160 °C for 15 minutes. All reactions were maintained at temperature (±2 °C) by convective heating using a 4 kW fluidized sand bath (Model SBL-2D, Techne, Princeton, NJ), and the temperature inside the reactor was measured directly by using an in-line thermocouple (Omega, K-type). At the end of the reaction, the reactor was cooled by submerging quickly in a large water bath at room temperature. The solids were then separated from the reaction liquor by vacuum filtration at room temperature through glass fiber filter paper (Fisher Scientific, Pittsburgh, PA). The mass and density of the filtrates were measured to calculate yields and close mass balances. Water washed substrates were produced by washing the solids collected from the reactions with water until clear water runs through them. Similarly, THF washed substrates were produced from parallel pretreatments at the optimal conditions by rinsing the solid fraction from pretreatment with 150 mL of THF prior to water washing. Compositional analysis was then performed on both water washed and THF washed solid fraction following the

NREL LAP (version 7-17-2005) to determine the sugar, and K-lignin content. (Sluiter, Hames, Hyman, et al. 2008; Sluiter, Hames, Ruiz, et al. 2008)

6.3.3 Enzymatic Hydrolysis of CELF pretreated Poplar

Enzymatic hydrolysis experiments were performed by following the standard NREL protocol. (Resch, Baker, and Decker 2015) The experiments were carried out in 125 mL flasks in a batch configuration with a total working volume of 50 mL containing CELF pretreated biomass corresponding to a glucan loading of 1 wt%, 50 mM citrate buffer (pH 4.5) added to reach the final pH of 4.8 in 50 mL, 0.02% sodium azide as an antimicrobial agent, and Accellerase® 1500 cocktail loaded at various protein loadings per g glucan in raw poplar. Triplicates at each condition were loaded with Millipore water, citrate buffer, sodium azide and the appropriate amount of substrate. The flasks were then placed, and equilibrated for 1 h in an incubator shaker at 50 °C at 150 rpm. Appropriate amount of enzyme cocktail was then added to the flasks and they were placed again in the incubator shaker. 1 mL sample was taken out, centrifuged at 15000 rpm for 10 min, diluted, and analyzed to measure the sugar concentration in the broth. The percent glucan digestibility or the enzymatic hydrolysis sugar yields were calculated using Equation 1.

$$Percent\ glucan\ digestibility = Enzymatic\ hydrolysis\ yields = \frac{C_{glucose} \times WV}{M_{glucan} \times 1.11} \times 100 \qquad 1$$

Where $C_{glucose}$ is the glucose concentration in the flask at any time point, g/mL, WV is the working volume in the flask, 50 mL and M_{glucan} is the initial mass of glucan loaded in the flask, g.

6.3.4 Measuring sugar, furfural and 5-HMF concentrations

Liquid samples along with appropriate calibration standards were analyzed by HPLC (Waters Alliance 2695 system equipped with a Bio-Rad Aminex® HPX-87H column and Waters 2414 RI detector) with an eluent (5 mM sulfuric acid) flow rate of 0.6 ml min^{-1}. The chromatograms were integrated by using the Empower® 2 software package (Waters Co., Milford, MA).

6.3.5 Scanning electron microscopy (SEM)

Pretreated poplar samples were freeze dried in a FreeZone 4.5 Liter Benchtop Freeze Dry System (Labconco, Kansas City, MO) for 24 hours. Samples were sputter-coated with Pt/Pd (Cressington 108 Auto) for 90 seconds to form a conductive coating (~ 10-15 nanometer thickness). Samples for Figure 6-3 (a) and (b) were examined with a Tescan MIRA3 GMU scanning electron microscope, while, samples for Figure 6-3 (c) were examined using scanning electron microscopy (NNS450 (FEI, USA).

6.3.6 Fourier Transform Infrared Spectroscopy (FTIR)

Cryo-ground samples (in liquid N_2) were powdered together with KBr and pressed in pellets, to perform conventional transmission FTIR experiments (FTIR, Nicolet 6700). For each spectrum registration, a total of 512 FTIR scans were collected, and averaged, over a region of 500 to 4000 cm^{-1} wavenumber, at a resolution of 4 cm^{-1}, corrected for ambient atmospheric conditions at ~30°C.

6.3.7 X-Ray Diffraction (XRD)

The pretreated samples were first ground under liquid nitrogen, and subsequently characterized using powder X-ray diffraction (XRD, PANalytical Empyrean Series 2) using Cu Kα (λ = 0.1546 nm) radiation. Phase identification (crystal structure and %

crystallinity of cellulose) and quantitative analyses were performed using PANalytical X'Pert Highscore Plus software. The Crystalline Index for the samples were calculated using Herman's method, Equation 2. The crystallite size was calculated using Scherrer equation, Equation 3

$$Crystalline\ Index\ (CrI) = \frac{A_{cryst}}{A_{total}} \times 100 \quad (2)$$

$$Crysatllite\ Size\ (L) = \frac{K \times \lambda}{\beta \times cos\theta} \quad (3)$$

Where, A_{cryst} is the sum of the crystalline band areas, A_{total} is the total area under the diffractogram, K is a constant value 0.94, λ is the X-ray wavelength (0.1542 nm), β is the half-height width of the diffraction band, and Θ is the Bragg angle corresponding to the (200) plane.

6.3.8 HMW DO and DB dye preparations for Simon's Staining Experiments

1% (10 mg/mL) Direct Orange (DO) and Direct Blue (DB) dyes were prepared. The DO solution was poured in 4 to 6 ultrafiltration tubes (100 KDa membrane, EMD Millipore Amicon™ Ultra-15 Centrifugal Filter Units) and ultrafiltration was carried out based on the company specific instructions. The upper portion in the tube that did not pass through the membrane (>100 KDa) was used as the High Molecular Weight (HMW) fraction. The HMW dye concentration was calculated by pouring 0.5 to 1 mL dye in a pre-weighed aluminum pan, followed by drying it for 48 h at 105 °C and reweighing it. After the concentration of HMW Direct Orange (HMW DO) was determined, a 0.66% (6.6 mg/mL) stock solution was prepared for further analysis.

6.3.8 Modified Simon's Staining Experiments

Modified Simon Staining experiments were carried out using the protocol in Chandra *et al.* (Chandra et al. 2008) 100 mg (dry weight) fiber samples were added into six 15 mL Corning polypropylene centrifuge tubes. To each tube 1.0 mL of PBS (phosphate- buffered saline solution) (pH 7, 0.3M sodium phosphate buffer, 1.4 mM NaCl) was added. The HMW DO solution (6.6 mg/mL) and DB (10 mg/mL) were then added in a series of increasing volumes (0.25, 0.50, 0.75, 1.0, 1.5, 2.0 mL) each to the tubes containing biomass and buffer. Distilled water was added to these tubes to reach a volume of 10.0 mL. These tubes were then placed in an incubator shaker at 65 °C and 200 rpm for at least 8 h. After the incubation period, the tubes were centrifuged at 10,000 rpm for 5 min. 1 mL of the supernatant was placed in a cuvette and the absorbance was read on a UV–vis spectrophotometer at 624 and 455 nm. The amount of dye adsorbed onto the fiber was determined using the difference in the concentration of the initial dye added and the concentration of the dye in the supernatant.

For all tests, the concentrations of HMW DO and DB dyes ($C_{HMW\,O}$ and C_B) were determined by solving Equations 4 and 5 simultaneously.

$$A_{455nm} = \varepsilon_{HMW\,O}{}_{/_{455}} LC_{HMW\,O} + \varepsilon_{B}{}_{/_{455}} LC_B \quad (4)$$

$$A_{624nm} = \varepsilon_{HMW\,O}{}_{/_{624}} LC_{HMW\,O} + \varepsilon_{B}{}_{/_{624}} LC_B \quad (5)$$

where A is the absorption of the mixture at 450 or 624 nm, ε is the extinction coefficient of each component at the respective wavelength, and L is the path length, 1 cm. The extinction coefficients were calculated by measuring the slope of the absorbance at 455 and 624 nm from the standard curves of each dye. The values calculated and used in this

study were $\varepsilon_{HMW\,O}/_{455}$ = 45.83, $\varepsilon_{B}/_{455}$ = 2.38, $\varepsilon_{HMW\,O}/_{624}$ = -0.11, and $\varepsilon_{B}/_{624}$ = 11.09 L g^{-1} cm^{-1}.

6.4 Results and Discussions

6.4.1 Compositional changes and solid solubilization induced by THF washing

CELF pretreatments were carried out at previously optimized conditions of 160 °C for 15 minutes on milled hardwood poplar. The solid fraction retained from the batch pretreatments, post vacuum filtration, were washed with water until clear water ran through them, those solids henceforth noted as "water washed solids." Other solids produced by CELF pretreatments at the same conditions were washed with 150 mL of THF prior to water washing, referred to as "THF washed solids." Figure 6-1 provides the amounts of glucan, xylan, and K-lignin left in these solids relative to a basis of 100 mass units total for these components in the poplar fed to CELF, all analyzed as described by NREL LAP (version 7-17-2005). (Sluiter, Hames, Ruiz, et al. 2008) As shown in the figure, CELF pretreatment solubilized 49% of the raw biomass, retained almost 94% of the initial glucan in the solid fraction, and hydrolyzed 92% of the xylan and 90% of the K-lignin in the liquid fraction. Application of THF washing further removed 2% of the remaining solids (mostly as fine particles) including 1.7% of the glucan, 0.6% of the xylan, and almost 40% of the residual lignin. The solids resulting from the extra THF wash were almost pure cellulose with < 3% lignin, while those from just water washing contained ~5% lignin. THF washed and water washed variants of CELF poplar could be easily physically distinguished as the former was lighter in color and appeared to be more dense than the latter, as shown in Additional Figure 6-1.

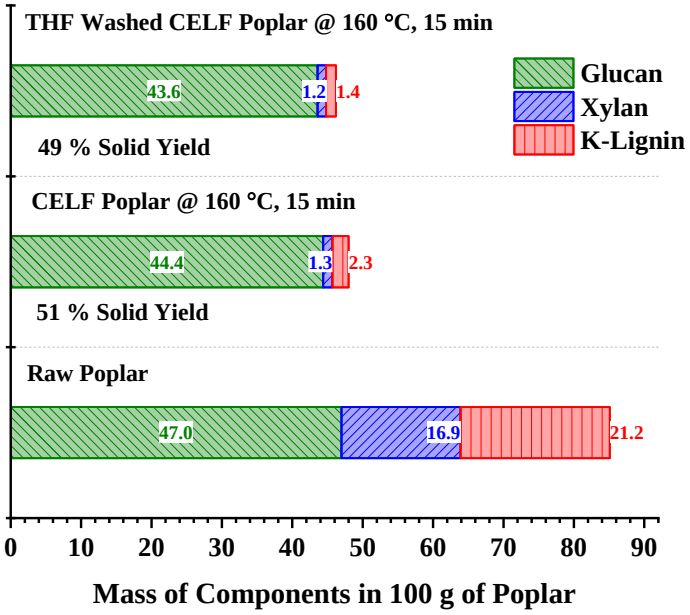


Figure 6-1 Solid yield following pretreatment and washing step along with the mass of components (glucan, xylan and K-lignin) in raw biomass as well as the solid fraction obtained post pretreatment for both the CELF alternates.

6.4.2 Impact of THF washing on enzymatic digestibility of CELF pretreated Poplar

Complex networks of covalently bonded hemicellulose and lignin restrict the access of enzymes to cellulose, hence, delignification of biomass can improve cellulose macro and micro accessibility, thereby enhancing its enzymatic digestibility. (Bhalla et al. 2019; Kabel et al. 2007; Xu et al. 2015; Zhu et al. 2008) Hence, an additional delignification step of THF rinsing of pretreated biomass was expected to further enhance poplar digestibility. (Hu, Jung, and Ragauskas 2012) However, the enzymatic hydrolysis results in Figure 6-2 were counter to this anticipation as the digestibility of CELF solids dropped post THF washing. In particular, it took almost twice as long to entirely digest THF washed substrate as it did for solids only washed with water for enzyme doses of 65,

30, and 15 mg protein/ g glucan in raw poplar, and THF washed solids could not be hydrolyzed completely at low enzyme doses of 7.5 and 5 mg protein/ g glucan.

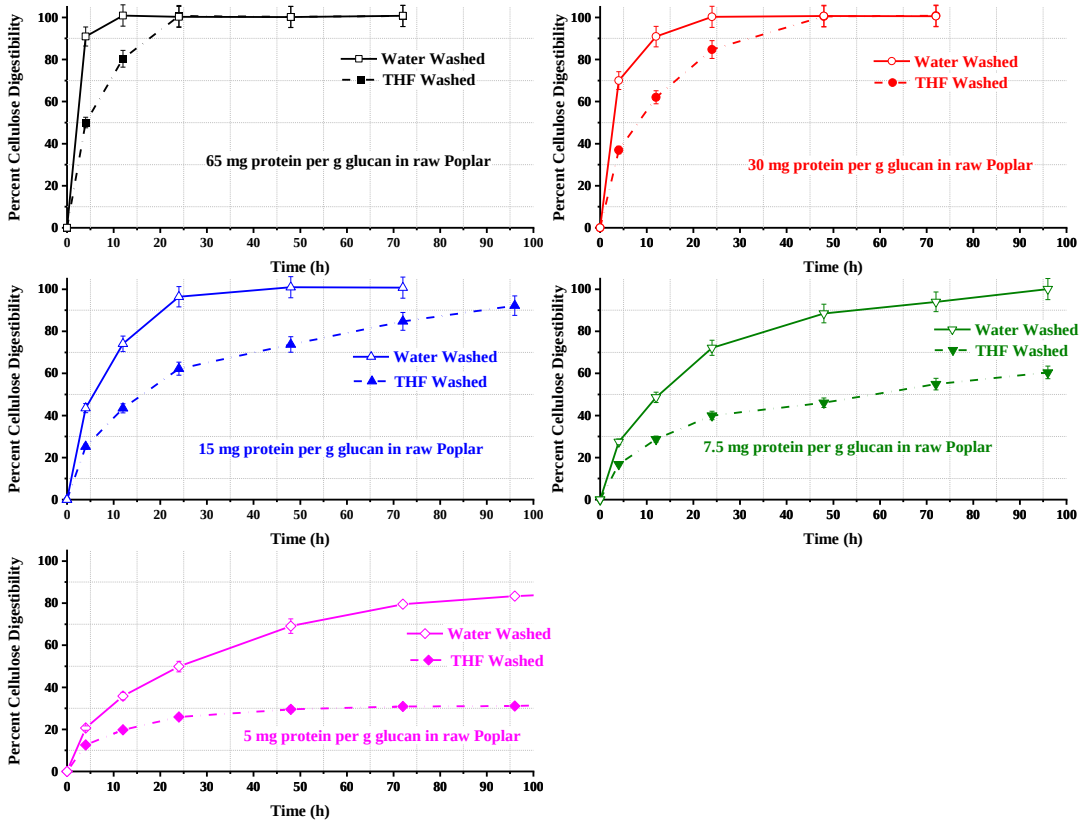


Figure 6-2 Percent cellulose digestibility of Water washed and THF washed CELF pretreated solids obtained from enzymatic hydrolysis carried out at 1 wt% glucan loading and various enzyme loadings using Accellerase® 1500 as the enzyme cocktail at 50 °C.

6.4.3 Characterization of CELF poplar variants

Previous results from fractal kinetic modeling of hydrolysis of CELF poplar for enzyme doses greater than 15 mg protein/ g glucan and a 1wt% glucan loading indicated that hydrolysis was not quite limited by diffusion or end product inhibition. Hence, the rate of hydrolysis and glucose yields from CELF solids, especially at a low substrate loading and higher enzyme dose, is expected to primarily depend on cellulose accessibility, which is inseparably linked to biomass structural features. Thus, scanning electron micrographs

(SEM) images were taken for both water and THF washed solids to understand differences in micro-scale surface alterations resulting from THF washing. As shown in Figure 6-3 (a) for water washed CELF pretreated poplar, drop-like structures can be seen deposited all over the solid surface. Further magnification of the sample section enclosed in the yellow box illustrates a clearer picture of the droplets, as shown in Figure 6-3 (b). These structures resemble the spherical redeposited lignin spotted on the surface of corn stover by high temperature acid pretreatment, as reported by Selig *et. al*. The authors proposed that these droplets were lignin structures which were removed from the cell wall matrix and then deposited back onto the cell wall surface during pretreatment. (Selig et al. 2007) Meng *et. al*. also observed similar structures that they described as redeposited pseudo-lignin on poplar holocellulose by dilute acid pretreatment. (Ragauskas 2017) Thus, these drop-like structures in Figure 6-3 (a) and (b) could be redeposited poplar lignin that was initially removed from the cell wall network and then accumulated back on the biomass surface. Such lignin droplets, however, could not be found on the THF washed substrate shown in Figure 6-3 (c), indicating that THF rinsing washed away redeposited lignin from the surface of pretreated biomass. Although Figures 6-3 (a) and (c) are images taken of different materials, their structural features are representative of SEM images of CELF samples taken at multiple locations.

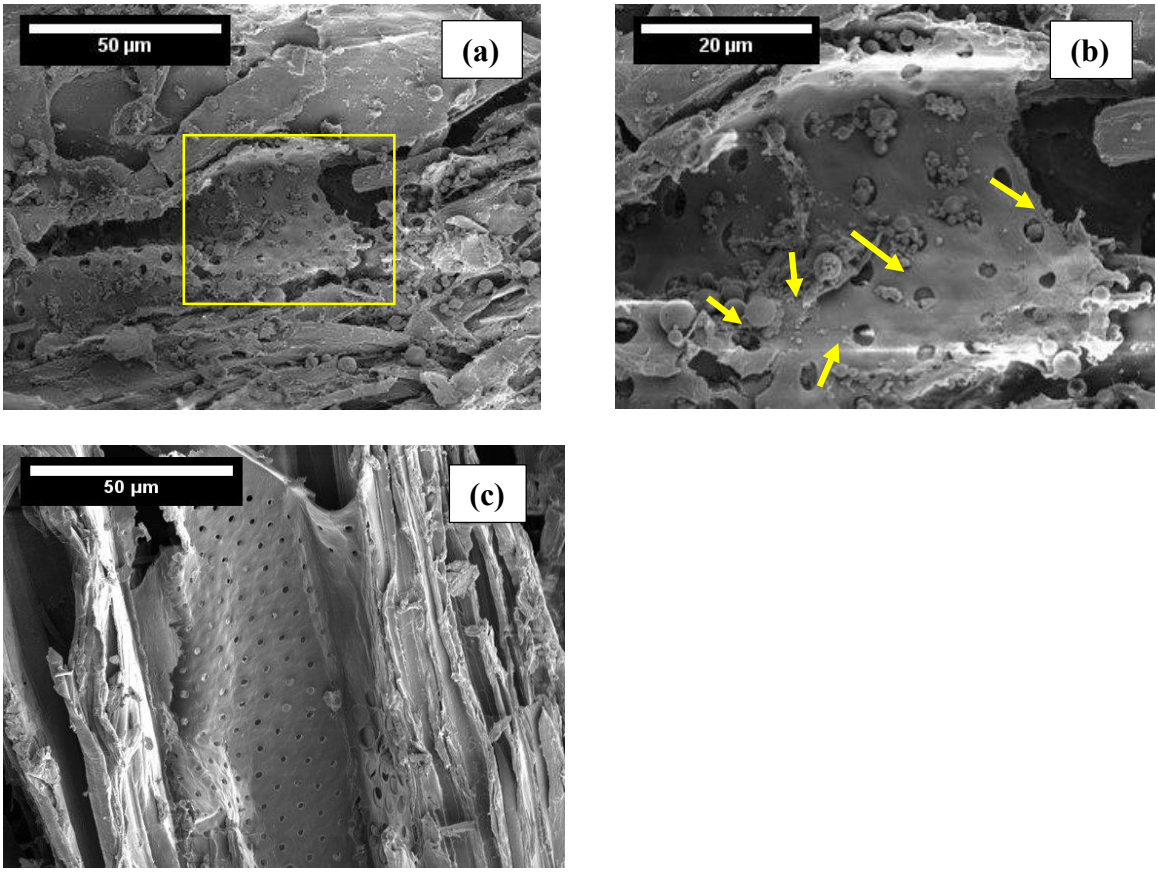


Figure 6-3 SEM images of (a) CELF pretreated water washed biomass, (b) Magnified view of the yellow box in (a), and (c) CELF pretreated THF washed biomass

Figure 6-4, illustrates the FTIR spectra of raw, water washed and THF washed CELF pretreated poplar in the form of transmission bands. A strong sharp peak at ~3500 cm^{-1} in woody biomass is known to indicate the presence of hydrogen bonding due to free alcohols present in lignin. (Poletto, Pistor, and Zattera 2013; Xu et al. 2013) The strong transmission band at 3500 cm^{-1} for the water washed substrate appears to weaken with THF washing, demonstrating the impact of lignin removal. The strength of the bands occurring at 2950 and 2835 cm^{-1} associated with the methoxy groups ($–OCH_3$) related to the coniferyl and sinapyl alcohol components of lignin structure were also reduced for the THF washed samples. (Guo et al. 2009) An interesting observation, however, was the missing water

adsorption band at 1650 cm^{-1} for THF washed substrates which was present in its counterpart indicating towards a reduced water retention capacity of the CELF substrate as a result of an extra solvent washing step. (Poletto et al. 2013) Other differences between the two pretreated substrate variations were observed at 1500, 1375, 1336 and 1329 cm^{-1} transmission bands associated with aromatic ring vibration of lignin, –C-H vibration, –C-H bending, -OH in plane bending in cellulose, hemicellulose, syringyl and guaiacyl condensed lignin, respectively, indicating towards a modified biomass structure. (Li et al. 2010; Xu et al. 2013, 2015) The most interesting observation, however, was the band shift and aggregation of the transmission bands from 900 to 500 cm^{-1}, representative of the glycosidic linkages in cellulosic substrates, to the right at a lower wavenumber in THF washed substrates, possibly indicating towards the presence of a huge condensed cellulose structures due to a likely pore collapse. (Vitas et al. 2019; Xu et al. 2013)

The effect of the solvent washing step on the crystallinity of CELF pretreated Poplar was next evaluated. Crystalline Index (CrI) and crystallite size (L) were calculated for both water and THF washed CELF pretreated poplar from the XRD patterns shown in Figure 6-5, using Herman's method and Scherrer's equation, respectively. (Poletto et al. 2013) As shown in Additional Table 6-1, CrI value increased from 88% to 92% with THF washing, however, the crystallite size (L) did not change much. This increase in CrI was possibly a result of cellulose concentration in the pretreated material due to an additional amorphous K-lignin removal.

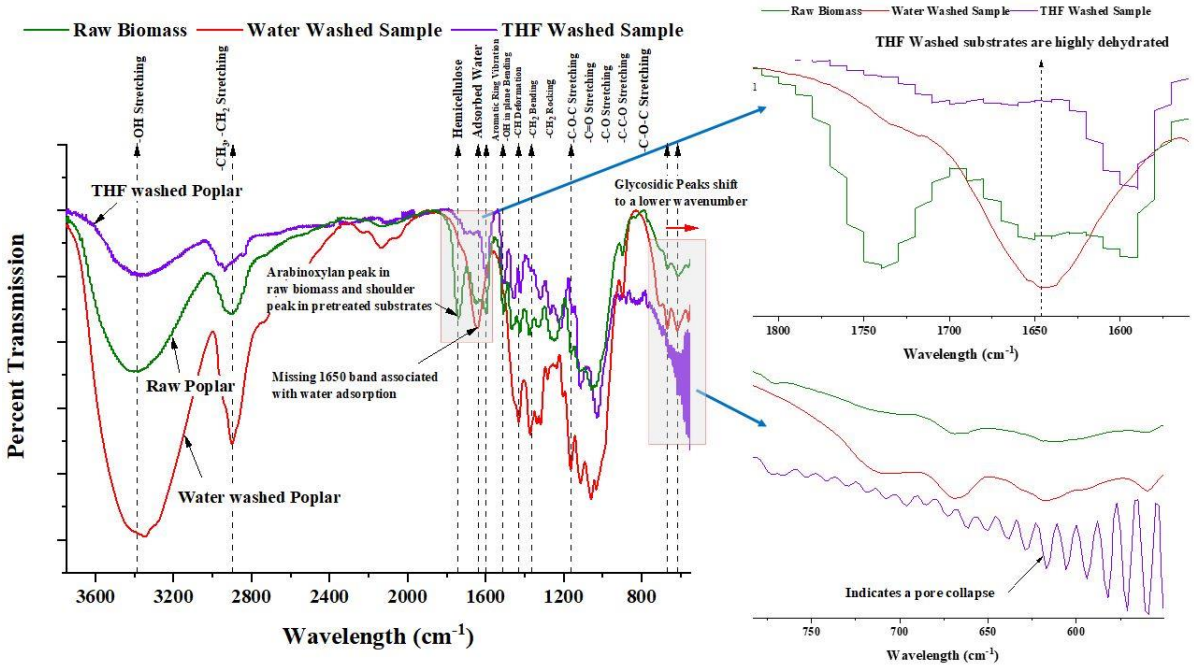


Figure 6-4 FTIR spectra of Raw Poplar, Water washed and THF washed CELF pretreated substrate along with zoomed in segments of the bands corresponding to 1650 cm^{-1} and 900 to 500 cm^{-1} wavelength range.

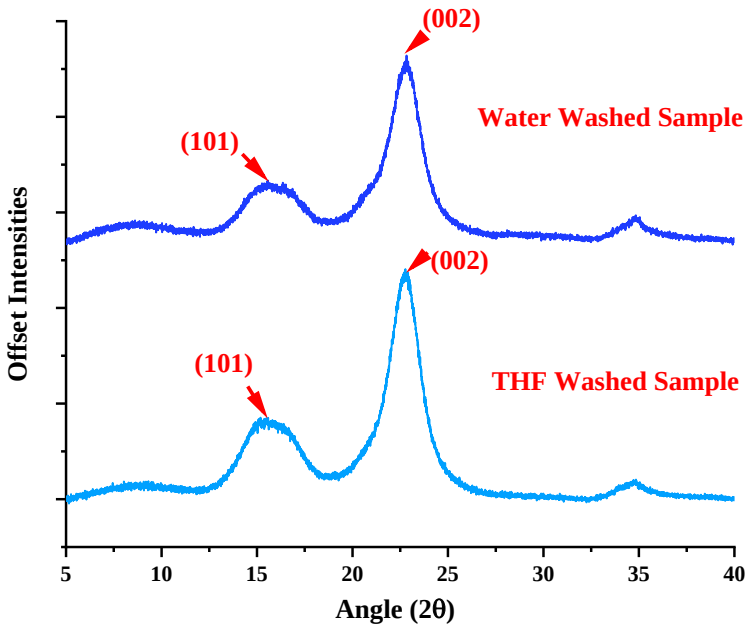


Figure 6-5 XRD pattern of Water washed and THF washed substrate

Modified Simon's staining combines high molecular weight (>100 Kda) orange dye (HMW DO) with a lower molecular weight blue dye (DB) to estimate substrate specific surface area and pore volume distribution of lignocellulosic substrates. (Chandra et al. 2008; Kothari et al. 2019) The response to Simon's staining is dependent on the accessibility of the interior surface of the fibers to the dyes and has often been used to estimate the susceptibility of cellulosic substrates to enzymatic hydrolysis. (Esteghlalian et al. 2001) Because the larger molecular size HMW DO dye can only penetrate larger cellulose pores for adsorption, smaller pores are left for DB dye to access once it seeps into the fiber interior. (Yu, Minor, and Atalla 1995) The maximum amounts of HMW DO and DB dye absorbed and the ratio of maximum adsorbed HMW DO to DB dye, therefore, was used to qualitatively compare the accessible surface area and pore volume distribution for CELF poplar solids washed by the two methods. As shown in Figure 6-6, HMW DO and DB dye adsorption dropped by 25% and 18%, respectively, after additional THF rinsing of CELF pretreated poplar solids. However, the ratio of adsorbed HMW DO to DB dye remained at a value of ~2. These observations indicated that although the pore volume distribution was not substantially affected, THF washing significantly reduced accessible substrate specific surface area.

As shown in Figure 6-6, the relative apparent density defined as the dry mass of solids divided by their total wet volume, was observed to be 44% higher for THF washed substrate compared to that for water washed solids. It was also observed that THF washed CELF solids settled completely to the bottom of a flask containing water while a fraction

(mostly fines) of the water washed solids remained suspended, as shown in Additional Figure 6-1.

Additionally, the moisture content of the THF washed CELF solids following vacuum filtration was 24% less than that for the water washed solids. Moreover, when hydraulically pressed to further reduce the water content and accommodate a higher solid loading for high solids SSF, the moisture content of THF washed solids could be easily reduced to only 50%, while that for water washed solids could not be reduced below about 64%. These observations along with the lack of a FTIR spectra water adsorption band revealed that THF washing of CELF poplar dehydrated biomass and reduced the moisture holding capability. Overall, the apparent density calculated from Simon's staining and band shifting and aggregation to the right in the FTIR spectrum suggest formation of a dense, condensed cellulose structure of CELF poplar as a result of a possible pore collapse due to enhanced lignin removal by THF washing of CELF solids.

Extreme delignification, together with the pronounced removal of hemicelluloses might have strongly enfeebled the cell wall material of poplar which likely led to a breakdown, and stimulation of a "compact" cellulose structure. This compact structure perhaps had a reduced micro-accessibility to enzymes which possibly led to a drop in rate of saccharification at higher enzyme loadings and inability of the substrate to be fully digested at a lower enzyme dose.

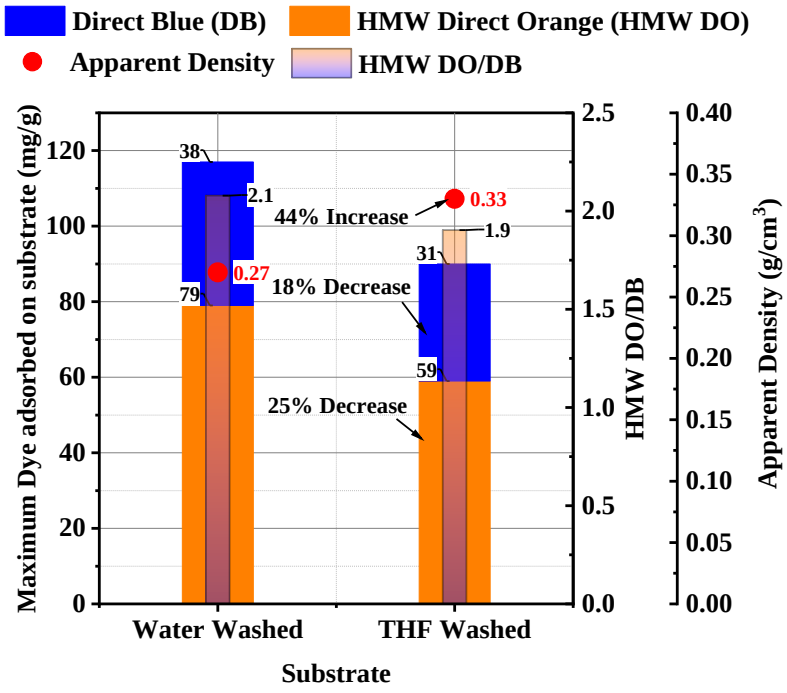


Figure 6-6 Maximum Dye Adsorption of High Molecular Weight Direct Orange (HMW DO) and Direct Blue (DB) dye onto water washed and THF washed substrate. Ratio of HMW DO to DB adsorption along with the apparent density of water washed and THF washed substrates in g/cm^3.

These observations are consistent with the results from the analysis of the impact of delignification on biomass porosity and pore size distribution by Vitas *et al.* When these authors delignified beechwood using acetic acid and hydrogen peroxide at mild (60 °C) to harsh (80 °C) conditions, they found biomass discoloration and compacting of the cellulose structure. (Vitas et al. 2019) Our results also bear similarity to the drop in cellulose digestibility of dilute acid pretreated corn stover reported by Ishizawa *et al.* when they applied an additional delignification step after pretreatment using acidified sodium chlorite to reduce the lignin content below 5%. The authors also attributed the reduced digestibility to lower enzyme accessibility due to aggregation of cellulose microfibrils because of excessive lignin and xylan removal. (Ishizawa et al. 2009)

The SEM images in Figure 6-3 (a, b, and c) suggest that redeposited lignin that was removed by THF washing contributed to maintaining the integrity of cellulose structures in CELF poplar. However, the formation mechanism of these droplets and their impact on cell wall accessibility by enzymes needs to be further explored to understand their significance. Nevertheless, these observations suggest there is a lignin removal threshold beyond which further removal negatively impacts the digestibility of cellulosic substrates.

6.5 Conclusion

Here, the enzymatic digestibility of solids produced by CELF pretreatment of poplar was shown to drop with additional delignification by THF washing. Characterization results indicated a drop in substrate specific surface area and micro-accessibility of CELF poplar due to possible cellulose pore collapse caused by extreme lignin removal. Hence, we conclude that lignin removal beyond about 90% or lowering the lignin content to less than 5% hurt enzymatic saccharification of poplar. However, this limit might differ with choice of lignocellulosic feedstock. Staining of pretreated biomass solids with a suitable lignin binding dye prior to THF washing along with the use of suitable microscopic imaging, therefore, is recommended to better understand the impact of delignification.

6.6 References

Aden, a, M. Ruth, K. Ibsen, J. Jechura, K. Neeves, J. Sheehan, B. Wallace, L. Montague, A. Slayton, and J. Lukas. 2002. "Lignocellulosic Biomass to Ethanol Process Design and Economics Utilizing Co-Current Dilute Acid Prehydrolysis and Enzymatic Hydrolysis for Corn Stover." *National Renewable Energy Laboratory-NREL* (June):Medium: ED; Size: 154 pages.

Bhalla, Aditya, Charles M. Cai, Feng Xu, Sandip K. Singh, Namita Bansal, Thanaphong Phongpreecha, Tanmoy Dutta, Cliff E. Foster, Rajeev Kumar, Blake A. Simmons, Seema Singh, Charles E. Wyman, Eric L. Hegg, and David B. Hodge. 2019. "Performance of Three Delignifying Pretreatments on Hardwoods: Hydrolysis Yields, Comprehensive Mass Balances, and Lignin Properties." *Biotechnology for Biofuels* 12(1):1–15.

Chandra, Richard, Shannon Ewanick, Carmen Hsieh, and Jack N. Saddler. 2008. "The Characterization of Pretreated Lignocellulosic Substrates Prior to Enzymatic Hydrolysis, Part 1: A Modified Simons' Staining Technique." *Biotechnology Progress* 24(5):1178–85.

Esteghlalian, A. R., M. Bilodeau, S. D. Mansfield, and J. N. Saddler. 2001. "Do Enzymatic Hydrolyzability and Simons' Stain Reflect the Changes in the Accessibility of Lignocellulosic Substrates to Cellulase Enzymes?" *Biotechnology Progress* 17(6):1049–54.

Gubicza, Krisztina, Ismael U. Nieves, William J. Sagues, Zsolt Barta, K. T. Shanmugam, and Lonnie O. Ingram. 2016. "Techno-Economic Analysis of Ethanol Production from Sugarcane Bagasse Using a Liquefaction plus Simultaneous Saccharification and Co-Fermentation Process." *Bioresource Technology* 208:42–48.

Guo, Gia Luen, Deng Chieh Hsu, Wen Hua Chen, Wei Hsi Chen, and Wen Song Hwang. 2009. "Characterization of Enzymatic Saccharification for Acid-Pretreated Lignocellulosic Materials with Different Lignin Composition." *Enzyme and Microbial Technology* 45(2):80–87.

Hu, Fan, Seokwon Jung, and Arthur Ragauskas. 2012. "Pseudo-Lignin Formation and Its Impact on Enzymatic Hydrolysis." *Bioresource Technology* 117:7–12.

Ishizawa, Claudia I, A. E. Tina, Jeoh Ae, William S. Adney Ae, Michael E. Himmel Ae, David K. Johnson Ae, Mark F Davis, C I Ishizawa, Á. T. Jeoh, Á. W. S. Adney, Á. M. E. Himmel, Á. D. K. Johnson, W. S. Adney, M. E. Himmel, M F Davis, and T. Jeoh. 2009. "Can Delignification Decrease Cellulose Digestibility in Acid Pretreated Corn Stover?" *Cellulose* 16:677–86.

Kabel, Mirjam A., Gijs Bos, Jan Zeevalking, Alphons G. J. Voragen, and Henk A. Schols. 2007. "Effect of Pretreatment Severity on Xylan Solubility and Enzymatic Breakdown of the Remaining Cellulose from Wheat Straw." *Bioresource Technology* 98(10):2034–42.

Kothari, Ninad, Samarthya Bhagia, Maher Zaher, Yunqiao Pu, Ashutosh Mittal, Chang Geun Yoo, Michael E. Himmel, Arthur J. Ragauskas, Rajeev Kumar, and Charles E. Wyman. 2019. "Cellulose Hydrolysis by *Clostridium Thermocellum* Is Agnostic to Substrate Structural Properties in Contrast to Fungal Cellulases." *Green Chemistry* 21(10):2810–22.

Li, Chenlin, Bernhard Knierim, Chithra Manisseri, Rohit Arora, Henrik V. Scheller, Manfred Auer, Kenneth P. Vogel, Blake A. Simmons, and Seema Singh. 2010. "Comparison of Dilute Acid and Ionic Liquid Pretreatment of Switchgrass: Biomass Recalcitrance, Delignification and Enzymatic Saccharification." *Bioresource Technology* 101(13):4900–4906.

Li, Mi, Yunqiao Pu, and Arthur J. Ragauskas. 2016. "Current Understanding of the Correlation of Lignin Structure with Biomass Recalcitrance." *Frontiers in Chemistry* 4.

Nguyen, Thanh Yen, Charles M. Cai, Rajeev Kumar, and Charles E. Wyman. 2015. "Co-Solvent Pretreatment Reduces Costly Enzyme Requirements for High Sugar and Ethanol Yields from Lignocellulosic Biomass." *ChemSusChem* 8(10):1716–25.

Palmqvist, Eva and Bärbel Hahn-Hägerdal. 2000. "Fermentation of Lignocellulosic Hydrolysates. I: Inhibition and Detoxification." *Bioresource Technology* 74(1):17–24.

Poletto, Matheus, Vinícios Pistor, and Ademir J. Zattera. 2013. "Structural Characteristics and Thermal Properties of Native Cellulose."

Ragauskas, Arthur J. 2017. "Pseudo-Lignin Formation during Dilute Acid Pretreatment for Cellulosic Ethanol." *Recent Advances in Petrochemical Science* 1(1):1–5.

Rahikainen, Jenni, Saara Mikander, Kaisa Marjamaa, Tarja Tamminen, Angelos Lappas, Liisa Viikari, and Kristiina Kruus. 2011. "Inhibition of Enzymatic Hydrolysis by Residual Lignins from Softwood-Study of Enzyme Binding and Inactivation on Lignin-Rich Surface." *Biotechnology and Bioengineering* 108(12):2823–34.

Resch, M. G., J. O. Baker, and S. R. Decker. 2015. *Low Solids Enzymatic Saccharification of Lignocellulosic Biomass: Laboratory Analytical Procedure (LAP), Issue Date: February 4, 2015*.

Selig, Michael J., Sridhar Viamajala, Stephen R. Decker, Melvin P. Tucker, Michael E. Himmel, and Todd B. Vinzant. 2007. "Deposition of Lignin Droplets Produced during Dilute Acid Pretreatment of Maize Stems Retards Enzymatic Hydrolysis of Cellulose." *Biotechnology Progress* 23(6):1333–39.

Sluiter, A., B. Hames, D. Hyman, C. Payne, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, and J. Wolfe. 2008. *Determination of Total Solids in Biomass and Total Dissolved Solids in Liquid Process Samples Laboratory Analytical Procedure (LAP) Issue Date: 3/31/2008*.

Sluiter, A., B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, and D. Crocker.

2008. *Determination of Structural Carbohydrates and Lignin in Biomass: Laboratory Analytical Procedure (LAP); Issue Date: 7/17/2005*.

Vitas, Selin, Jana S. Segmehl, Ingo Burgert, and Etienne Cabane. 2019. "Porosity and Pore Size Distribution of Native and Delignified Beech Wood Determined by Mercury Intrusion Porosimetry." *Materials* 12(3):1–13.

Wyman, Charles E., Bruce E. Dale, Richard T. Elander, Mark Holtzapple, Michael R. Ladisch, and Y. Y. Lee. 2005. "Coordinated Development of Leading Biomass Pretreatment Technologies." *Bioresource Technology* 96(18 SPEC. ISS.):1959–66.

Xu, Feng, Jianming Yu, Tesfaye Tesso, Floyd Dowell, and Donghai Wang. 2013. "Qualitative and Quantitative Analysis of Lignocellulosic Biomass Using Infrared Techniques: A Mini-Review." *Applied Energy* 104:801–9.

Xu, Huanfei, Guang Yu, Xindong Mu, Chunyan Zhang, Paul DeRoussel, Chao Liu, Bin Li, and Haisong Wang. 2015. "Effect and Characterization of Sodium Lignosulfonate on Alkali Pretreatment for Enhancing Enzymatic Saccharification of Corn Stover." *Industrial Crops and Products* 76:638–46.

Yang, Bin and Charles E. Wyman. 2008. "Pretreatment: The Key to Unlocking Low-Cost Cellulosic Ethanol." *Biofuels, Bioproducts and Biorefining* 2(1):26–40.

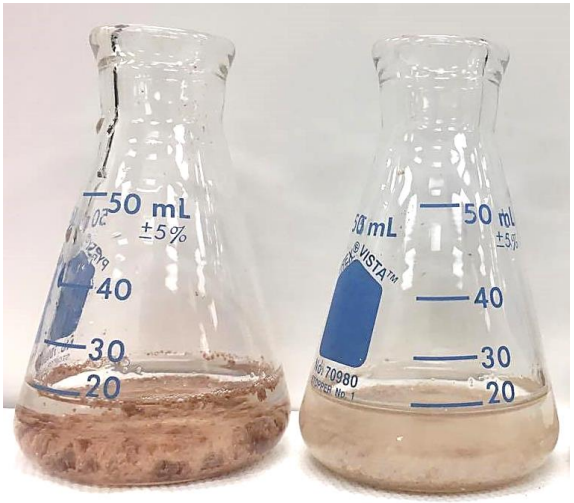
Yu, Xiaochun, James L. Minor, and Rajai H. Atalla. 1995. *Mechanism of Action of Simons' Stain*.

Zhu, Li, Jonathan P. O'Dwyer, Vincent S. Chang, Cesar B. Granda, and Mark T. Holtzapple. 2008. "Structural Features Affecting Biomass Enzymatic Digestibility." *Bioresource Technology* 99(9):3817–28.

6.7 Additional Information:

Additional Table 6-1 Crystalline Index (CrI) and Crystallite Size (L) of water washed and THF washed substrate calculated from XRD patterns

Sample	Crystalline Index % (CrI)	Crystallite Size, nm (L)
Water Washed	88	4.3
THF Washed	92	4.4



Additional Figure 6-1 Water washed CELF pretreated poplar (left) and THF washed CELF pretreated poplar (right) added to ~20 mL of water. THF washed substrate is one shade lighter and denser than water washed substrate.

Chapter 7 : Co-fermentation of CELF pretreated poplar increased total sugar utilization

7.1 Abstract

Co-Solvent Enhanced Lignocellulosic Fractionation (CELF) pretreatment removes a large fraction of lignin and hemicellulose from lignocellulosic biomass to produce a glucan-rich solid and a liquid hydrolysate containing most of the hemicellulose sugars. In this study, Simultaneous Saccharification and Co-Fermentation (SSCF) was applied to a 15 wt% glucan loading of CELF pretreated poplar solids combined with CELF hydrolysate from which most of the lignin was removed to utilize sugars from both solid and liquid fractions. Ethanol titers of 72 g/L corresponding to a 72% theoretical yield were achieved in 13 days using the engineered *S. cerevisiae* strain M11205 in a 3L benchtop fermenter. The minimum ethanol selling price (MESP) calculated by a technoeconomic analysis for a base case CELF-SSCF process with an ethanol yield of 0.33 g/g of raw poplar was predicted to be 2.24 $/gal ethanol while increasing the overall ethanol yield to 0.38 g/g of raw poplar would reduce the MESP to 2.00 $/gal. Uncertainty analysis revealed that MESP is most sensitive to the extent of total sugar utilization of biomass followed by the co-valorization of the extracted lignin as an organic polyol source. The findings from profitability analysis further suggested that augmenting overall ethanol yields per g of biomass and utilizing lignin as a valuable co-product is significant for enhancing the commercial feasibility of second generation of cellulosic ethanol.

7.2 Introduction

Lignocellulosic biomass provides an excellent natural resource from which to produce cellulosic ethanol that can reduce the dependence of the transportation sector on fossil derived fuels. It can be low cost, abundant, and grown domestically to meet the increasing energy demand without an extra burden on the existing automobile

infrastructure. (BP and Outlook 2019; Demirbas 2009b) Because second generation ethanol produced from agricultural, industrial, and forestry residues also has a much lower carbon footprint than petroleum based fuels and even much lower than its predecessor, corn-based ethanol, it contributes much less greenhouse gas emissions. (Tiffany 2009) However, in order to be a reasonable substitute for fossil-derived fuels, cellulosic ethanol production must be cost-competitive. (Klein-Marcuschamer et al. 2010)

Biomass recalcitrance restricts cellulose accessibility to fungal enzymes, it presents the primary barrier to economic biological conversion of biomass to ethanol. As a result, pretreatment of biomass to overcome its recalcitrance is generally required. (Demirbas 2009a; Naik et al. 2010) Co-solvent Enhanced Lignocellulosic Fractionation (CELF) that employs tetrahydrofuran (THF) as a solvent miscible with water along with dilute acid has recently emerged as an efficient biomass pretreatment technology that effectively deconstructs cell walls to produce a solid fraction highly enriched in glucan and a liquid fraction containing most of the hydrolyzed C-5 and C-6 sugar monomers and a large portion of lignin along with lesser amounts of other cell wall components. (Nguyen et al. 2015, 2016, 2017, n.d.)

CELF pretreatment of hardwood poplar at 160 °C for 15 min produces a highly digestible glucan-rich solid fraction. Simultaneous Saccharification and Fermentation (SSF) of these solids realized 79 % of the theoretical maximum yield and ethanol titers of ~87 g/L in 7 days at a 20 wt% loading of CELF pretreated insoluble solids with an enzyme dose of 15 mg protein per g glucan in raw poplar coupled with fermentation by D5A, a *Saccharomyces cerevisiae* variant. CELF pretreatment at these conditions also co-

produced a liquid stream that contained sugars and lignin breakdown products solubilized by CELF. Simultaneous Saccharification and Co-Fermentation (SSCF) of the liquid and solid fractions produced by CELF pretreatment of poplar could utilize all sugars in one step, augment the volumetric ethanol productivity, and reduce production costs. (Öhgren et al. 2006; Yen and Nguyen 2016) Unfortunately, the sugars in the liquid are also accompanied by fermentation inhibitors such as furfural and acetic acid that are generally formed or released by acid-based pretreatments and reduce cell viability and limit ethanol yields from co-fermentations. (Mills, Sandoval, and Gill 2009) The pretreatment hydrolysates, therefore, are generally conditioned by expensive extra steps such as liquid-liquid extraction, activated carbon, or overlimining to reduce the toxin concentration and increase fermentation viability. (Anon n.d.; Rivard et al. 1996)

In this context, the present study aimed at maximizing sugar utilization post CELF pretreatment of poplar via SSCF of both solids and liquid fractions using an engineered *Saccharomyces cerevisiae* strain, M11205. SSF of just C-6 sugars in the solid fraction was performed prior to SSCF to provide a baseline for comparison of enzyme activity, sugar consumption, ethanol and glycerol production patterns, and enhanced ethanol production by SSCF at the same solids loading. In addition, a technoeconomic analysis (TEA) of a hypothetical biorefinery was applied to estimate the minimum ethanol selling price (MESP) for CELF pretreatment of poplar followed by SSCF as a measure of commercial feasibility. Finally, a sensitivity analysis of the TEA model to key variables in the process model was employed to identify factors that most profoundly impact the ethanol production cost.

7.3 Experimental Section

7.3.1 Materials

This study was conducted on *Populus trichocarpa* woody biomass generously provided by the BioEnergy Science Center (BESC). The composition of the raw Poplar as determined by following NREL LAP (version 08-03-2012) was 47.0 % glucan, 16.9 % xylan, and 21.2% acid-insoluble lignin. (Sluiter et al. 2008) The biomass was air-dried, knife milled using a laboratory mill (Model 4, Arthur H. Thomas Company, Philadelphia, PA), and passed through a 1mm internal sieve size. The enzyme cocktails used for the study were Cellic® CTec 2 and HTec2 generously provided by Novozymes®. The protein content of the Cellic® CTec 2 and HTec2 enzyme cocktails as estimated using Pierce BCA analysis kit was 250 mg/ml and 230 mg/ml, respectively. The yeast strain used for fermentation was M11205, an engineered strain of *Saccharomyces cerevisiae,* generously provided by the Mascoma LLC, a Lallemand company.

7.3.2 Pretreatment

Prior to CELF pretreatment, milled Poplar wood chips were soaked overnight at 4 °C in a 1:1 (weight basis) solution of THF to water containing 0.5 wt% H_2SO_4 based on total solvent. The dry biomass loading was 7.5 wt% of the total working mass for reaction. Reactions were conducted in a 1 L Hastelloy Parr autoclave reactor (236HC Series, Parr Instruments Co., Moline, IL) equipped with a double stacked pitch blade impeller rotating at 200 rpm. A series of pretreatments were carried out at 160 °C for 15 minutes, i.e., conditions that resulted in maximum sugar recovery for CELF followed by enzymatic hydrolysis. Temperature inside the reactor were measured by an in-line thermocouple

(Omega, K-type) and all reactions were maintained within ±2 °C using a 4 kW fluidized sand bath (Model SBL-2D, Techne, Princeton, NJ). At the end of each reaction, the reactor was cooled by submerging quickly in a large water bath at room temperature. The solids were then separated from the liquor by vacuum filtration at room temperature through glass fiber filter paper (Fisher Scientific, Pittsburgh, PA). The mass and density of the liquid fractions were measured to calculate yields and close mass balances. The solids collected were then washed with water until clear water ran through the solids. For SSCF experiments, the solids were hydraulically pressed to reduce the moisture content to 61%.

7.3.3 CELF hydrolysate processing

The filtrate collected from filtration of the pretreated solids was poured in a beaker and titrated to a pH ~ 7 using ammonium hydroxide. THF was then boiled out of solution at 80 °C under a hot plate with continuous stirring at 130 rpm for about 5 h. The beaker along with its contents was then allowed to cool to room temperature overnight and the liquor was filtered through a pre-weighed glass fiber filter paper. The processed hydrolysate was then diluted and analyzed for concentrations of sugars, acetic acid, furfural, 5-HMF, THF, BDO and other minor components.

7.3.4 Seed inoculum preparation

Saccharomyces cerevisiae (M11205) cells were grown in 10 mg/mL yeast extract (Becton, Dickinson and Company, Redlands, CA), 20 mg/mL peptone (Becton, Dickinson and Company, Redlands CA) and 50 mg/mL glucose to the exponential phase and then stored in (~14 wt%) glycerol. When needed, the frozen stock was thawed and grown overnight in 10 mg/mL yeast extract, 20 mg/mL peptone, and 50 mg/mL glucose in a 250

mL baffled flask in a shaking incubator maintained at 37 °C for a speed of 130 rpm. The inoculum was then centrifuged and re-suspended in sterile deionized (DI) water, and an inoculation was prepared at an optical density (O.D.) of 0.05 determined at 600 nm.

7.3.5 Pure sugar co-fermentations (PSCF)

Pure sugar co-fermentations (PSCF) by M11205 were carried out in a 3 L Sartorius benchtop fermenter with a working volume of 1 L using 75 g/L concentrations of glucose and xylose each. Sugars along with Millipore water were added to the bioreactor vessel. The fermenter was assembled and sealed along with the pH probe. The fermenter assembly was then sterilized at 121 °C for 35 min in an autoclave and cooled in a laminar flow hood (Baker and Baker Ruskinn, Sanford, ME) to prevent contamination. 50 mM citrate buffer (pH 4.5), 40 mg/L of tetracycline (Sigma-Aldrich, St. Louis, MO) as an antimicrobial agent along with the yeast extract and peptone media were then added in the fermenter. The fermenter assembly was then connected to the control tower. The temperature and rpm were maintained at 37 °C and 130 respectively. Yeast inoculum was then introduced at a O.D. of 0.05. Initial pH was recorded at 5.16. pH measurements were recorded every 24 h. 5 mL samples were taken every 24 h until ethanol production plateaued. 1 mL of these samples were used for O.D. measurements at 600 nm. The remaining sample volume was centrifuged at 15000 rpm for 10 min, diluted, and analyzed for ethanol and sugar concentrations.

7.3.6 Simultaneous Saccharification and Fermentation (SSF)

Batch SSF experiments by M11205 were carried out in a 3 L Sartorius benchtop fermenter with a working volume of 1 L using CELF pretreated poplar at a 15wt% glucan

loading. Appropriate amount of substrate along with Millipore water was added to the bioreactor vessel. The fermenter was assembled and sealed along with the pH probe. The fermenter assembly was then sterilized at 121 °C for 35 min in an autoclave and cooled in a laminar flow hood (Baker and Baker Ruskinn, Sanford, ME) to prevent contamination. 50 mM citrate buffer (pH 4.5), 40 mg/L of tetracycline (Sigma-Aldrich, St. Louis, MO) as an antimicrobial agent along with the yeast extract and peptone media were then added in the fermenter. Cellic® CTec2 cocktail was loaded at 15 mg-protein per g-glucan-in-raw poplar. The fermenter assembly was then connected to the control tower. The temperature and rpm were maintained at 37 °C and 130 respectively. Yeast inoculum was then introduced at a O.D. of 0.05. Initial pH was recorded as 4.74. pH measurements were recorded every 24 h. 5 mL samples were taken every 24 h until ethanol production plateaued. The sample was centrifuged at 15000 rpm for 10 min, diluted, and analyzed for ethanol and sugar concentrations.

7.3.7 Simultaneous Saccharification and Co-fermentation (SSCF)

Batch SSCF experiments by M11205 were carried out in a 3 L Sartorius benchtop fermenter with a working volume of 1 L using CELF pretreated poplar at a 15wt% glucan loading. Appropriate amount of substrate along with Millipore water was added to the bioreactor vessel. The bioreactor was then assembled and sealed along with the pH probe. The fermenter assembly was then sealed and sterilized at 121 °C for 35 min in an autoclave and cooled in a laminar flow hood (Baker and Baker Ruskinn, Sanford, ME) to prevent contamination. 50 mM citrate buffer (pH 4.5), 40 mg/L of tetracycline (Sigma-Aldrich, St. Louis, MO) as an antimicrobial agent along with the yeast extract and peptone media were

then added in the fermenter. Appropriate amount of processed CELF hydrolysate along with Cellic® CTec2 and HTec2 enzyme cocktails were loaded at 15 mg-protein per g-glucan-in-raw poplar and 2 mg protein per g xylan loaded in the flask. The fermenter assembly was then connected to the control tower. The temperature and rpm were controlled at 50 °C and 130, respectively for 72 h. The temperature was then dropped to 37 °C and yeast inoculum was introduced at a O.D. of 0.05. Initial pH was recorded as 5.17 which dropped to 5.00 at the time of yeast inoculation after 72h. pH measurements were recorded every 24 h. 5 mL samples were taken every 24 h until ethanol production plateaued. The sample was centrifuged at 15000 rpm for 10 min, diluted, and analyzed for ethanol and sugar concentrations.

7.3.8 Measuring sugar and ethanol concentrations

Liquid samples along with appropriate calibration standards were analyzed by HPLC (Waters Alliance 2695 system equipped with a Bio-Rad Aminex® HPX-87H column and Waters 2414 RI detector) with a 5 mM sulfuric acid eluent flow rate of 0.6 ml min^{-1}. The chromatograms were integrated by the Empower® 2 software package (Waters Co., Milford, MA).

7.3.9 Model Equations used

Percent theoretical ethanol yield was calculated as follows:

$$Percent\ Theoretical\ Ethanol\ Yield = {(C_{Eth} \times V_L \times 0.9 \times 100)}\Big/{(0.511 \times M_G)} \quad (1)$$

$$V_L = (M_W + M_{DS})/\rho \quad (2)$$

$$M_{DS} = V_W \times \left(C_{Eth} + C_{Glucose} + C_{Glycerol} + etc.\right) \quad (3)$$

In which C_{Eth} is the ethanol concentration in the fermentation broth, mg/mL, $C_{Glucose}$ is the glucose concentration in the fermentation broth, mg/mL, $C_{Glycerol}$ is the glycerol concentration in the fermentation broth, mg/mL, V_L is the volume of liquid fraction in the fermentation medium, mL, M_G is the mass of glucan initially added, g, M_W is the mass of water initially added, g, M_{DS} is the mass of dissolved solids in the fermentation medium at any given time point, g, and ρ is the density of the medium, g/mL.

At lower solid loadings, i.e., <5 wt%, the density of the solvent phase can be assumed to be same as density of water. As the insoluble solid fraction increases, the density of the liquid fraction first increases due to increase in sugar concentration and then drops slightly due to the increasing ethanol concentration. The fluid density was measured directly by weighing the mass of a known fluid volume.

7.3.10 Technoeconomic Analysis (TEA)

A list of technical assumptions for the base case includes the following:

- Utilization of a n^{th}- plant economics i.e. based on a mature technology. (Tao et al. 2012)
- Poplar is the primary source of carbohydrates and lignin available at 60 $/dry tonne.
- Plant capacity is 2000 tonne/day of poplar.
- Annual Operating hours is 7446.
- Total sugar content in poplar (glucan +xylan) is 0.64 g of sugar per g biomass.
- CELF pretreatment of poplar combined with SSCF as the biological conversion route to produce ethanol.
- A 10% xylan loss during CELF hydrolysate processing.

- Maximum sugar available for ethanol production is 0.62 g/g dry biomass
- Enzyme contribution is 0.25 $/gal based on the literature estimates inspired by 15 mg protein per g glucan required for SSCF of CELF solids. (Humbird et al. 2002)
- For SSF, the glucose and xylose in the processed liquid fraction goes to a waste stream.
- Percent theoretical ethanol yield is 72% for the SSCF process.
- Overall ethanol yield is ethanol production (L/h) divided by the biomass capacity (kg/h)
- Furfural production is 0.015 g/dry biomass.
- Furfural price is 1600 $/tonne. (Krishna et al. 2018)
- Lignin generation is 0.15 g/g dry biomass.
- Lignin recovered from the raw biomass is utilized as a solid fuel to generate electricity.
- Solid Fuel (Lignin) to power efficiency is 0.35.
- Equipment, chemical, and labor cost indexed to 2018 dollars.
- Total Direct Cost (TDC) consists of:
    - Power generation
    - Feedstock handling
    - Pretreatment
    - Separation and filtration
    - Fermentation process
    - Warehouse

    - Site development
    - Additional Piping
- Annual Operating Cost (AOC) consists of
    - Power generation
    - Feedstock handling
    - Chemicals cost
    - Cost of enzymes
    - Employees (Labor Cost)
    - Equipment replacement costs
- Working Capital (WC) is 25% of TDC of the plant.
- Total Capital Investment (TCI) is the total of TDC, WC, Land Cost and Site Overhead Costs
- The investment is 100% equity financed.
- Internal rate of return is 10%.
- The plant life is 20 years.
- Income tax rate is 45%.
- Income tax paid and depreciation is annually computed.

The Total Capital Investment (TCI), along with the plant operating expenses, is used in a discounted cash flow rate of return (DCFROR) analysis. Plant Depreciation was calculated by using the Double Declining Balance Method, Equation 4.

$$Depreciation = 2 \times \frac{(TCI - Accumulated\ Depriciation)}{Useful\ plant\ life} \quad 4$$

Total Annual Revenue (TAR), $/year consisted of the revenue generated by ethanol, furfural and lignin. Annual Profit was the difference of TAR and Total Annual Cost.

Net Present Value (NPV) and Rate of Return (ROR) were calculated using Equation 5 and 6.

$$Net\ Present\ Value\ (NPV) = \sum_{t=0}^{N} \frac{Net\ Cash\ Flow_{time,t}}{(1+Discount\ Rate)^t} \quad 5$$

$$Rate\ of\ Return\ (ROR) = \frac{\frac{Sum\ of\ Discounted\ Cash\ Flow}{TCI}}{Total\ Life\ Span} \quad 6$$

Minimum ethanol selling price (MESP, in $/gal) was determined as the selling price required to obtain a NPV of zero for a 10% discount rate/ IRR after taxes. Payback period is the time taken to cover the cost of investment.

7.4 Results and Discussion

7.4.1 SSCF of poplar to maximize sugar utilization from poplar

Cellulosic ethanol production can only be economic if all the sugars available in biomass can be effectively converted into products. (Gubicza et al. 2016; Öhgren et al. 2006) SSF has been recognized as a promising technology for ethanol production, however, it only utilizes the six carbon fraction of the total sugars in raw biomass recovered in the solid fraction post pretreatment. As shown in Figure 7-1, CELF pretreatment of poplar generates a solid fraction that retains ~94.4% of the glucan and ~7.7% of the xylan from raw poplar. The resulting liquid fraction/CELF hydrolysate is comprised of ~5.5% of the glucan and ~57.9% of the xylan, in the form of monomers along with ~30% of the

xylan as furfural.

In addition to containing > 400 g/L of tetrahydrofuran (THF), 4.5 g/L of 1,4-Butanediol (BDO), 3.2 g/L of glucose, 7.2 g/L of xylose, and ~90 % of the total K-lignin originally in raw poplar, CELF hydrolysates were highly acidic, and the liquid fraction, therefore, was titrated using ammonium hydroxide prior to THF removal by boiling the hydrolysate on a hot plate to reduce the THF concentration to < 5 g/L. Most of the solubilized lignin in the hydrolysate precipitated during THF removal was considered as a potentially valuable source of organic polyol. (Wang et al. 2020) The filtered liquor volume was less than half of its initial value and contained ~3.6 g/L of THF, ~23.3 g/L of BDO, ~14.6 g/L of glucose, and ~15.7 g/L of xylose.

Figure 7-1 indicates that although titers of ~87 g/L of ethanol resulted from a SSF of poplar at 18wt% glucan loading, only ~79% of the total sugars released from pretreatment were utilized in that the five carbon sugars in the hydrolysate were not included. However, SSCF enabled utilization of ~88% of the total sugars in the raw biomass, with ~20% of the xylose in the liquid fraction being lost during hydrolysate processing. Co-fermentations, especially at a high solid loading, reduce water requirement, avoid carbon losses, and lower ethanol production costs as it enhances the overall process yield by making the most out of the sugars in the raw biomass without adding an extra requirement of costly enzymes. By enhancing ethanol titers, co-fermentations also reduce energy requirements to separate ethanol from the fermentation broth via distillation. (Koppram et al. 2013)

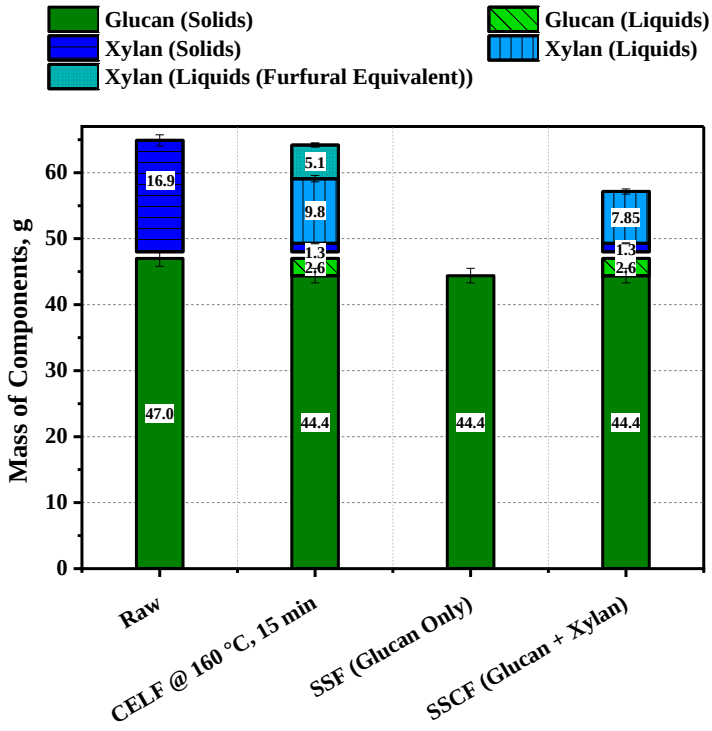


Figure 7-1 Material balance of sugars in raw poplar as well as the solid and liquid fraction obtained post CELF pretreatment at 160 °C, 15 min along with the total sugars utilized during SSF and SSCF processes.

7.4.2 Xylose co-consumption and osmotic tolerance of the engineered *Saccharomyces cerevisiae* strain, M11205

Since evaporation of water along with THF increased the concentrations of the fermentation inhibitors, especially BDO, prehydrolysis at 50 °C became desirable before the yeast inoculation for co-fermentation at 37 °C to dilute the inhibitor concentration and make the fermentation broth more amenable to yeast. However, the considerate portion of glucose released into the broth by prehydrolysis along with the sugars already present in the hydrolysate created a very concentrated hyperosmotic stressful environment for yeast sustenance. (Hohmann 2002) In this study, an engineered *Saccharomyces cerevisiae* strain designed to co-ferment xylose along with glucose, M11205, was employed to produce ethanol via SSCF. In order to understand the xylose consumption and pattern of uptake in

the presence of glucose along with the ability of the strain to adapt to osmotic stress, co-fermentation experiments were performed using 75 g/L of glucose and xylose each at 37 °C for 72 h in a 3 L benchtop fermenter. 150 g/L of sugars was completely consumed, and ethanol titers of ~68 g/L corresponding to a theoretical yield of ~84% were reached in 50 h, as shown in Figure 7-2. The key inferences drawn from these results were twofold: 1) M11205 can survive high gravity sugar solutions by producing ~9 g/L of glycerol to survive the osmotic shock and 2) the M11205 yeast strain prefers glucose to xylose as evidenced by xylose fermentation not starting until most of the glucose was consumed. Pure sugar fermentations carried out using >150 g/L total sugar concentrations at a 1:1 glucose to xylose ratio established the maximum achievable ethanol concentration by M11205 to be ~72 g/L.

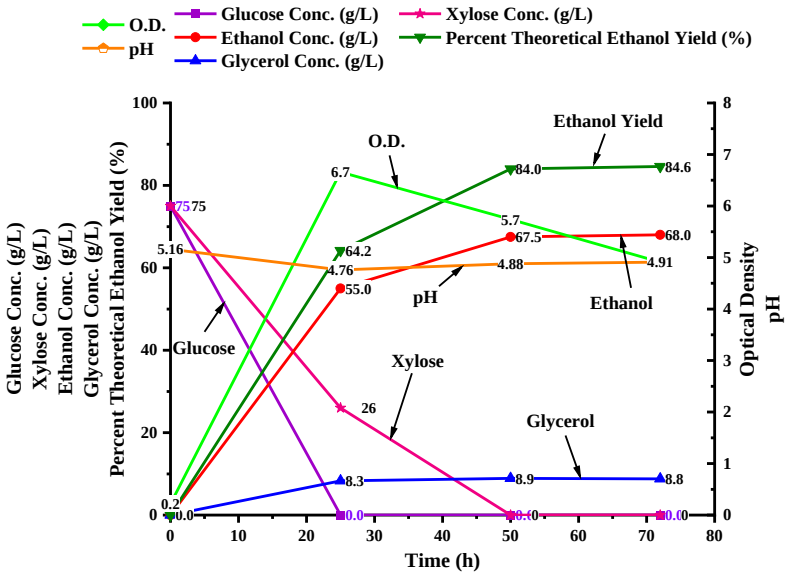


Figure 7-2 Glucose, xylose consumption, and ethanol, glycerol production pattern along with pH and O.D. measurements from pure sugar co-fermentations (PSCF) at 150 g/L total sugar, using M11205 at 37 °C at a 1L scale in a 3L benchtop fermenter.

7.4.3 SSF of CELF pretreated poplar by M11205

Excess lignin and pretreatment inhibitors in the fermentation broth can potentially limit the ethanol yields by affecting enzyme activity leading to slow sugar release, by reducing the viability of the yeast cells, or by stimulating their combined effect. (Ooshima, Burns, and Converse 1990; Rahikainen et al. 2011; Ximenes et al. n.d.) Because processing CELF hydrolysate increases the concentration of these deterrents, SSF experiments were conducted at the same solid loading to provide a control on enzyme and yeast activities and ethanol yields for the co-fermentation experiment. In light of the ethanol tolerance of M11205, targeting glucan loadings above 15 wt% would not have been productive. Hence, SSF experiments using M11205 at 37 °C were carried out at a solid loading of 17 wt% (glucan loading of 15 wt%) using Cellic® CTec2 enzyme cocktail at a dose of 15 mg protein per g glucan in raw poplar at a 1L scale in a 3L fermenter. As shown in Figure 7-3, an ethanol concentration of 67 g/L corresponding to a theoretical yield of 72% was attained in 10 days with 4.7 g/L of residual glucose left in the broth. Continuing the experiment for another 4 days increased the glucose accumulation to 13 g/L while the ethanol concentration had plateaued. An observed glycerol concentration of 6.7 g/L was 25% less than the amount produced during PSCF, probably because the immediate glucose consumption during SSF did not increase the osmolarity of the medium.

7.4.4 SSCF of CELF pretreated poplar using M11205

In order to increase the hydrolysate concentration, CELF pretreated solids were pressed via a hydraulic jack to reduce the moisture content from 72 to 61% (Materials and Methods). SSCF was then applied to CELF polar at a 17 wt% solids loading (15 wt% glucan) along with an extra 10 g of sugar included in the processed hydrolysate. A Cellic®

CTec2 and HTec2 enzyme cocktail at loadings of 15 mg protein per g glucan in raw poplar and 2 mg protein per g xylan loaded in the solids, respectively, was employed along with the genetically modified M11205 yeast to a 1L volume in a 3L benchtop fermenter. Prehydrolysis for 72 h was applied to reduce the BDO concentration to <10 g/L prior to yeast inoculation.

As shown in Figure 7-3, M11205 exhibited a longer lag phase (~24 h) to acclimate to the challenging SSCF surroundings compared to SSF at the same solids loading, with the result that initial ethanol production rates were slower. Yet, glycerol concentration only reached 5 g/L, 25% and 44% less than that for SSF and PSCF, respectively. The enzyme activity, however, was found to be unaffected by excess lignin content as evident by continued glucose release at a similar pace as observed for SSF. A final ethanol concentration of 72 g/L, corresponding to a theoretical yield of 72%, achieved on day 13 was 5 g/L higher than the ethanol titers produced by SSF. That fermentation lasted for 10 days after yeast inoculation, with 10.6 g/L of residual glucose left in the broth. The runs were continued for another day, but ethanol plateaued despite continued glucose release to 16.4 g/L. All the xylose in the hydrolysate was fully consumed in just 48 h, and that released by hydrolysis of the xylan in the solids was also completely consumed within the fermentation span.

Overall, SSCF of glucose and xylose in the solids and liquid produced by CELF pretreatment of poplar reached high yields at a high solid loading without requiring extensive hydrolysate conditioning and reached 7.4% more ethanol than SSF at the same solids loading. Almost 90% of the C-5 and C-6sugars were accounted for in the material

balance for PSCF, SSF, and SSCF, as shown in Additional Figure 7-1. The remaining 10% is expected to have been utilized by the yeast for metabolism and growth, in line with sugar use for such anaerobic fermentations.

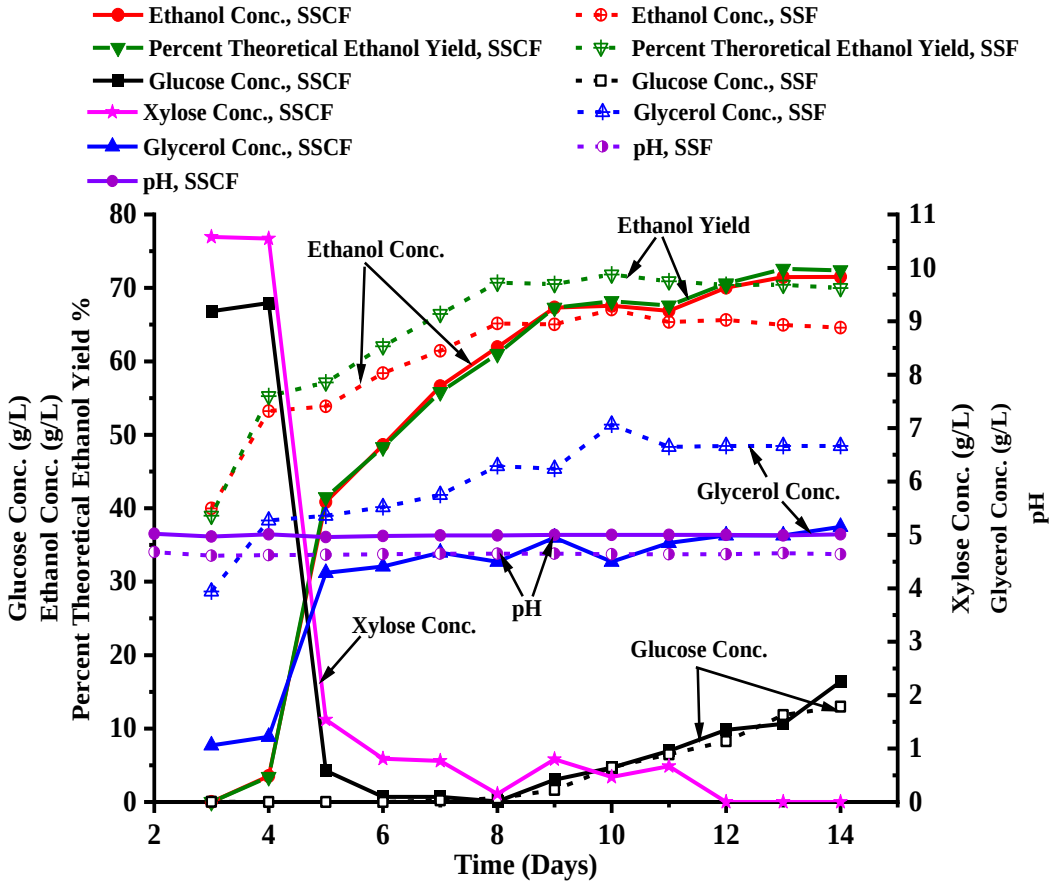


Figure 7-3 Glucose, xylose, ethanol, glycerol concentrations, ethanol yields, O.D. and pH measured during SSF and SSCF conducted with CELF solids at 17 wt% substrate loading corresponding to a 15 wt% glucan loading using M11205 at 37 °C.

7.4.5 The impact of sugar utilization on the cost of ethanol production

The ASPEN Plus™ Process Simulator was used for a technoeconomic analysis (TEA) of an nth-plant design for the process outlined in Figure 7-4. Capital and operating

costs for a hypothetical biorefinery were estimated based on the data presented above to determine the commercial feasibility of a CELF-SSCF process.

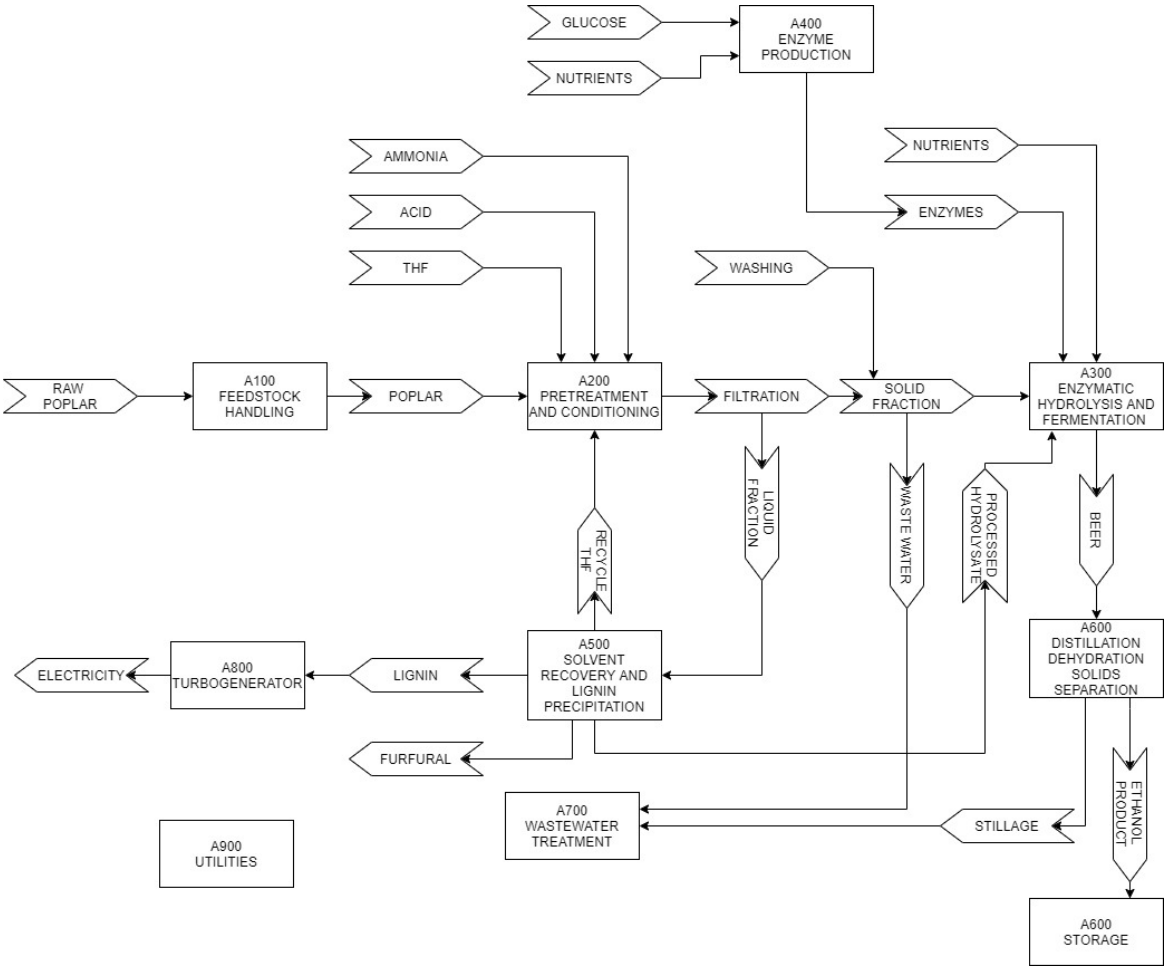


Figure 7-4 Simplified process scheme

The base case was based on a 72% theoretical ethanol yield from SSCF of poplar containing 0.63 g sugars per g (Experimental Section). The TCI and the AOC were estimated to be $320 million and $74 million, respectively. As shown in Figure 7-5, the majority of the AOC is to cover feedstock, chemicals, and enzymes. The feedstock cost

dominated the AOC, followed by the cost of chemicals. The enzyme cost was assumed to be $0.25 per gal of ethanol, ~12% of the total AOC.

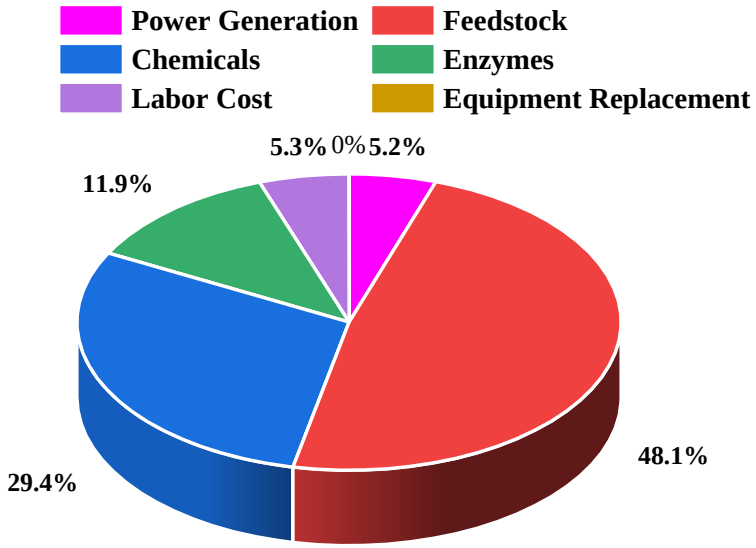


Figure 7-5 Components of the Annual Operating Cost (AOC) for the base case

Table 7-1 Table showing various biological conversion routes, sugar utilization for each conversion route, percent of theoretical ethanol yield, overall ethanol yield achieved per g of biomass and the MESP for each case calculated from the hypothetical biorefinery using a particular yeast strain and CELF pretreated poplar

Scenario	Process	Sugar Utilization (g per g dry biomass)	Percent of theoretical ethanol yield (%)	Yeast Strain	Overall Ethanol Yield (g per g dry biomass)	MESP ($/gal)
Total Sugar (Glucan + Xylan) in Poplar = 0.64 g per g dry biomass						
A (Base Case)	SSCF	0.63	72	M11205	0.33	2.24
B	SSF	0.44	72		0.23	2.97
C	SSF	0.44	84	D5A	0.27	2.63
D (Assumption)	SSCF	0.63	84	N/A	0.38	2.00

The MESP for the base case was estimated at 2.24 $/gal, equivalent to gasoline at ~3.30 $/gal. The tornado plot in Figure 7-6 shows how the MESP was affected by the following process parameters in order of increasing impact: sugar utilization > considering 70% of the lignin recovered as a potential polyol source > plant biomass capacity > enzyme

contribution > xylan losses during hydrolysate processing > furfural price. Compared to the base case, the MESP increased by 73 cents and 39 cents per gal ethanol for a 30% and 18% drop in overall ethanol yield for Scenarios B and C, respectively, as shown in Table 7-1, if only 0.44 g of sugars per g of biomass was fermented via SSF. However, MESP dropped by 24 cents/gal ethanol for scenario D when the overall ethanol yield increased by 15% to reach an 84% theoretical ethanol yield from the SSCF process, producing 0.38 g ethanol from 0.63 g of sugars per g of dry poplar. Figure 7-7 shows MESP was linearly proportional to the overall ethanol yield which is dependent on the amount of total sugars (glucan and xylan) in the raw biomass effectively utilized to produce ethanol.

A significant drop of the MESP to 1.82 $/gal, as compared to the base case, was observed when 70% of the lignin generated was sold as an organic polyol source at 500 $/tonne. MESP was also sensitive to the plant capacity. While a 25% decrease in biomass capacity to 1500 tonne/day increased the ethanol selling price by 35 cents/gal, a 25% increase to 2500 tonne/day, dropped the MESP by 21 cents/gal. Reducing the enzyme cost contribution to $0.11/gal, corresponding to a requirement of 10 mg protein per g glucan-in raw poplar reduced MESP by 10 cents/gal. However, an enzyme contribution of 0.50 $/gal, corresponding to an enzyme dose of 30 mg protein per g glucan in raw poplar, increased the ethanol selling price by 18 cents/gal. We assumed a 10% additional xylan loss for the base case occurred during hydrolysate processing. However, an increase in xylan loss to 30% could increase MESP by 15 cents/gal. An optimistic furfural market value of 2.7 $/kg reduced the MESP by 19 cents/gal, while a lower price of 0.8 $/kg increased MESP by 14 cents/gal.

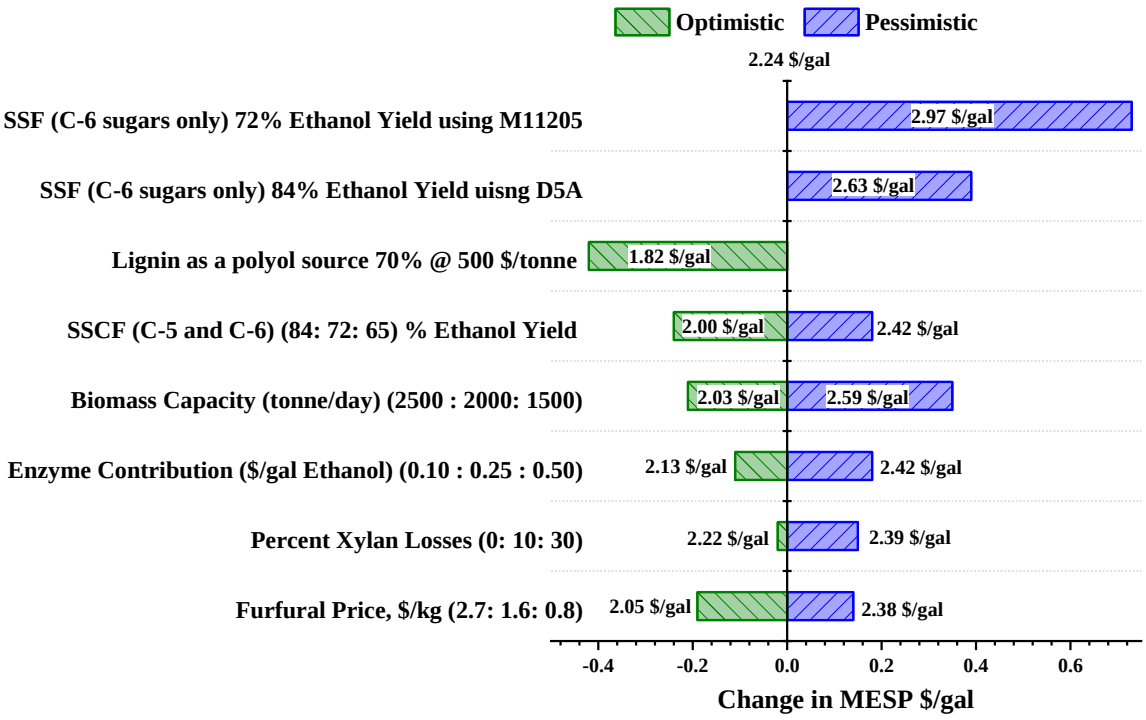


Figure 7-6 Sensitivity Analysis conducted on MESP estimated from the SSF/SSCF process of CELF poplar.

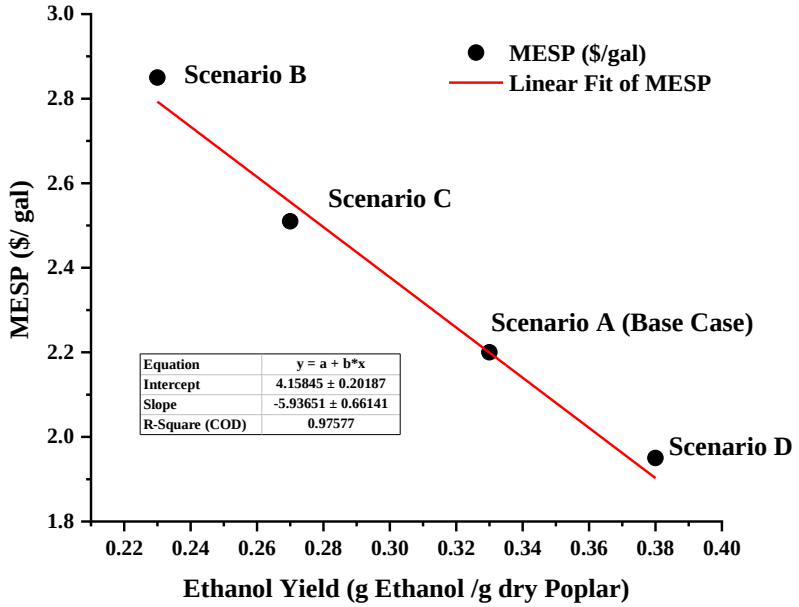


Figure 7-7 Relationship between MESP $/gal and ethanol yield (g ethanol/g dry poplar)

7.4.6 Profitability evaluation

Second generation of cellulosic ethanol is more expensive than the first generation starch based ethanol due to the additional operating costs including the cost of pretreatment and enzymatic hydrolysis. In order to determine the profitability of the process, the Total Annual Revenue (TAR), Rate of Return (ROR) or the profit on the investment, and the Payback Period i.e. the amount of time taken to cover the cost of investment, were calculated for various scenarios with varying ethanol selling prices ($/gal) and the fate of the lignin recovered while keeping furfural market value constant at 1.6 $/kg. As shown in Table 7-2, the TAR for the base case estimated using an ethanol selling price of 2.24 $/gal was $185 million. The investment had a Payback Period of 4 years and a 7.59% ROR. With an additional co-valorizing of 70% of the lignin recovered as a polyol source, sold at 500 $/tonne, the TAR and ROR increased to $229 million and 11.07%, respectively. Using the selling price of first generation cellulosic ethanol, 2.7 $/gal, estimated by adding the ethanol market price to the D3 Renewable Identification Number (RIN) credit in compliance with Renewable Fuel Standards (RFS), without lignin valorization increased the TAR to $219 million, while Payback Period remained constant at 4 years and the ROR increased to 11.44%. However, using the ethanol selling price of 2.7 $/gal along with lignin co-valorization significantly increased the TAR to $264 million and raised the ROR to a lucrative value of 14.98%.

The results from the TEA analysis reveal that maximizing utilization of the total available sugars in the biomass while maintaining high process yields at a reasonable enzyme loading and lignin valorization as a high value co-product along with government

subsidy policies are the key factors that can help enhance commercialization of second generation of biofuels to be more competitive to the first generation of starch based cellulosic ethanol.

Table 7-2 Total Annual Revenue (TAR), Rate of Return (ROR) and Payback Period calculated for various scenarios

Ethanol Selling Price $/gal	Scenario	Total Annual Revenue, (TAR)	Rate of Return % (ROR)	Payback Period
2.24	MESP for the Base Case	$185 million	7.59	4 years
2.24	MESP for the Base Case + 30% lignin burned +70% lignin sold at 500 $/tonne	$229 million	11.07	4 years
2.7	Ethanol market price (1.2 $/gal) + D3 RIN credit (1.5 $/gal) + lignin burned	$219 million	11.44	4 years
2.7	Ethanol market price (1.2 $/gal) + D3 RIN credit (1.5 $/gal) + 30% lignin burned +70% lignin sold at 500 $/tonne	$264 million	14.98	4 years

7.5 Conclusion

CELF pretreatment of poplar enabled efficient utilization of the glucan and xylan components in poplar. Not only were CELF solids glucan-rich and highly digestible to enhance ethanol yields via high solids SSF, CELF hydrolysate containing monomeric sugars were completely fermentable without requiring an extensive acclimation. In particular, an ethanol titer of 72 g/L corresponding to a theoretical yield of 72% was achieved by SSCF of CELF pretreated poplar at a 15 wt% glucan loading in 13 days using M11205 yeast. A 72 h prehydrolysis successfully diluted inhibitors in the fermentation broth prior to the co-fermentation to improve yeast performance. A preliminary TEA analysis projected a minimum ethanol selling price of 2.24 $/gal for CELF pretreatment followed by SSCF, and a sensitivity analysis showed that maximizing sugar utilization and

enhancing ethanol yields at a low enzyme dose via SSCF would further reduce the minimum ethanol selling price to 2.00 $/gal. The profitability evaluation results revealed that co-valorizing lignin along with maximizing total sugar utilization to produce ethanol is the key to unlocking greater revenues and to enable cost-effective production of second generation of cellulosic ethanol.

7.6 References

Anon. n.d. “Biofuel Production: Recent Developments and Prospects - Google Books.” Retrieved July 12, 2020 (https://books.google.com/books?hl=en&lr=&id=R3WfDwAAQBAJ&oi=fnd&pg=PA225&dq=pretreatment+hydrolysate+detoxification&ots=8_EbgWWYEO&sig=br5fI4-Px4HMhP0lWyJlMa1JrYM#v=onepage&q=pretreatment hydrolysate detoxification&f=false).

BP and B. P. Energy Outlook. 2019. “BP Energy Outlook 2019 Edition The Energy Outlook Explores the Forces Shaping the Global Energy Transition out to 2040 and the Key Uncertainties Surrounding That.” *BP Energy Outlook 2019*.

Davis, R., C. Kinchin, J. Markham, E. C. D. Tan, L. M. L. Laurens, D. Sexton, D. Knorr, P. Schoen, and J. Lukas. 2013. *Process Design and Economics for the Conversion of Algal Biomass to Biofuels: Algal Biomass Fractionation to Lipid- and Carbohydrate-Derived Fuel Products*.

Demirbas, Ayhan. 2009a. “Biofuels Securing the Planet’s Future Energy Needs.” *Energy Conversion and Management* 50(9):2239–49.

Demirbas, Ayhan. 2009b. “Political, Economic and Environmental Impacts of Biofuels: A Review.” *Applied Energy* 86:S108–17.

Gubicza, Krisztina, Ismael U. Nieves, William J. Sagues, Zsolt Barta, K. T. Shanmugam, and Lonnie O. Ingram. 2016. “Techno-Economic Analysis of Ethanol Production from Sugarcane Bagasse Using a Liquefaction plus Simultaneous Saccharification and Co-Fermentation Process.” *Bioresource Technology* 208:42–48.

Hohmann, Stefan. 2002. “Osmotic Stress Signaling and Osmoadaptation in Yeasts.” *Microbiology and Molecular Biology Reviews : MMBR* 66(2):300–372.

Humbird, D., R. Davis, L. Tao, C. Kinchin, D. Hsu, A. Aden, P. Schoen, J. Lukas, B. Olthof, M. Worley, D. Sexton, and D. Dudgeon. 2002. *Process Design and Economics for Biochemical Conversion of Lignocellulosic Biomass to Ethanol: Dilute-Acid Pretreatment and Enzymatic Hydrolysis of Corn Stover*.

Klein-Marcuschamer, Daniel, Piotr Oleskowicz-Popiel, Blake A. Simmons, and Harvey W. Blanch. 2010. “Technoeconomic Analysis of Biofuels: A Wiki-Based Platform for Lignocellulosic Biorefineries.” *Biomass and Bioenergy* 34(12):1914–21.

Koppram, Rakesh, Fredrik Nielsen, Eva Albers, Annika Lambert, Sune Wännström, Lars Welin, Guido Zacchi, and Lisbeth Olsson. 2013. “Simultaneous Saccharification and Co-Fermentation for Bioethanol Production Using Corncobs at Lab, PDU and Demo Scales.” *Biotechnology for Biofuels* 6(1):2.

Krishna, Siddarth H., Kefeng Huang, Kevin J. Barnett, Jiayue He, Christos T. Maravelias, James A. Dumesic, George W. Huber, Mario De bruyn, and Bert M.

Weckhuysen. 2018. “Oxygenated Commodity Chemicals from Chemo-Catalytic Conversion of Biomass Derived Heterocycles.” *AIChE Journal* 64(6):1910–22.

Ludmila, Hodásová, Jablonský Michal, Škulcova Andrea, and Ház Aleš. 2015. “Lignin, Potential Products and Their Market Value.” *Wood Research* 60(6):973–86.

Mills, Tirzah Y., Nicholas R. Sandoval, and Ryan T. Gill. 2009. “Cellulosic Hydrolysate Toxicity and Tolerance Mechanisms in Escherichia Coli.” *Biotechnology for Biofuels* 2(1):26.

Naik, S. N., Vaibhav V. Goud, Prasant K. Rout, and Ajay K. Dalai. 2010. “Production of First and Second Generation Biofuels: A Comprehensive Review.” *Renewable and Sustainable Energy Reviews* 14(2):578–97.

Nguyen, Thanh Yen, Charles M. Cai, Rajeev Kumar, and Charles E. Wyman. 2015. “Co-Solvent Pretreatment Reduces Costly Enzyme Requirements for High Sugar and Ethanol Yields from Lignocellulosic Biomass.” *ChemSusChem* 8(10):1716–25.

Nguyen, Thanh Yen, Charles M. Cai, Rajeev Kumar, and Charles E. Wyman. 2017. “Overcoming Factors Limiting High-Solids Fermentation of Lignocellulosic Biomass to Ethanol.” *Proceedings of the National Academy of Sciences of the United States of America* 114(44):11673–78.

Nguyen, Thanh Yen, Charles M. Cai, Omar Osman, Rajeev Kumar, and Charles E. Wyman. 2016. “CELF Pretreatment of Corn Stover Boosts Ethanol Titers and Yields from High Solids SSF with Low Enzyme Loadings.” *Green Chemistry* 18(6):1581–89.

Nguyen, TY, CM Cai, O. Osman, R. Kumar-Green Chemistry, and undefined 2016. n.d. “CELF Pretreatment of Corn Stover Boosts Ethanol Titers and Yields from High Solids SSF with Low Enzyme Loadings.” *Pubs.Rsc.Org*.

Öhgren, Karin, Oskar Bengtsson, Marie F. Gorwa-Grauslund, Mats Galbe, Bärbel Hahn-Hägerdal, and Guido Zacchi. 2006. “Simultaneous Saccharification and Co-Fermentation of Glucose and Xylose in Steam-Pretreated Corn Stover at High Fiber Content with Saccharomyces Cerevisiae TMB3400.” *Journal of Biotechnology* 126(4):488–98.

Ooshima, Hiroshi, Douglas S. Burns, and Alvin O. Converse. 1990. “Adsorption of Cellulase FromTrichoderma Reesei on Cellulose and Lignacious Residue in Wood Pretreated by Dilute Sulfuric Acid with Explosive Decompression.” *Biotechnology and Bioengineering* 36(5):446–52.

Rahikainen, Jenni, Saara Mikander, Kaisa Marjamaa, Tarja Tamminen, Angelos Lappas, Liisa Viikari, and Kristiina Kruus. 2011. “Inhibition of Enzymatic Hydrolysis by Residual Lignins from Softwood-Study of Enzyme Binding and Inactivation on Lignin-Rich Surface.” *Biotechnology and Bioengineering* 108(12):2823–34.

Rivard, Christopher J., Rebecca E. Engel, Tammu K. Hayward, Nicholas J. Nagle, Christos Hatzis, and George P. Philippidis. 1996. *Measurement of the Inhibitory Potential and Detoxification of Biomass Pretreatment Hydrolysate for Ethanol Production*. Vol. 183.

Sluiter, A., B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, and D. Crocker. 2008. *Determination of Structural Carbohydrates and Lignin in Biomass: Laboratory Analytical Procedure (LAP); Issue Date: 7/17/2005*.

Tao, Ling, Dan Schell, Ryan Davis, Eric Tan, Rick Elander, and Adam Bratis. 2012. *NREL 2012 Achievement of Ethanol Cost Targets: Biochemical Ethanol Fermentation via Dilute-Acid Pretreatment and Enzymatic Hydrolysis of Corn Stover*.

Tiffany, Douglas G. 2009. *Economic and Environmental Impacts of U.S. Corn Ethanol Production and Use*.

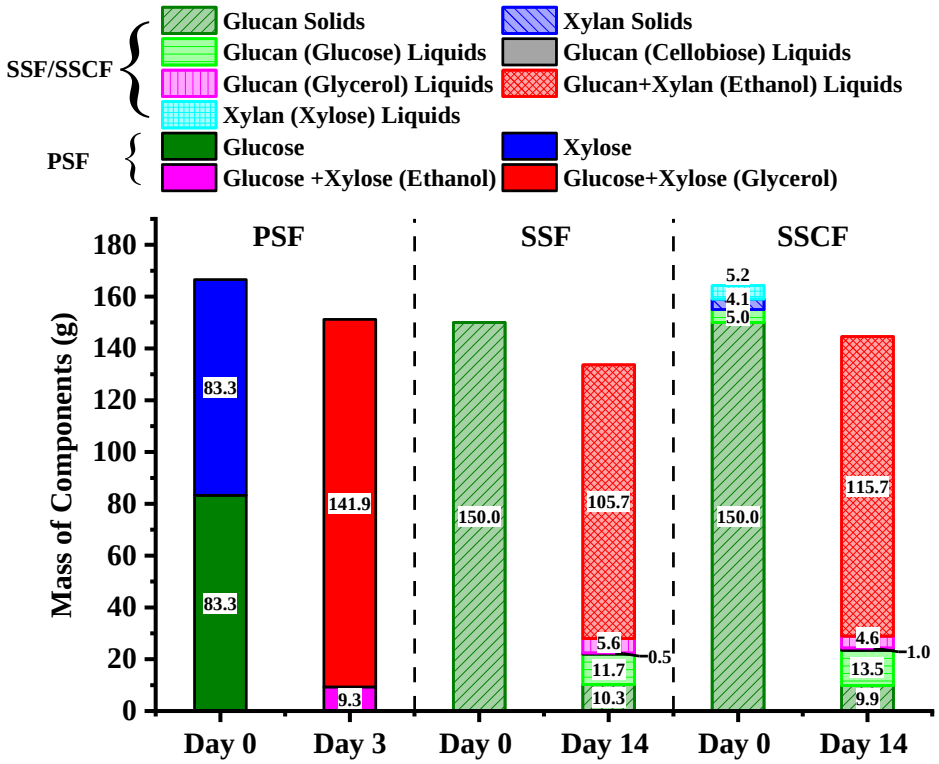
Wang, Yun-Yan, Priya Sengupta, Brent Scheidemantle, Yunqiao Pu, Charles E. Wyman, Charles M. Cai, and Arthur J. Ragauskas. 2020. "Effects of CELF Pretreatment Severity on Lignin Structure and the Lignin-Based Polyurethane Properties." *Frontiers in Energy Research* 8:149.

Wang, Yun Yan, Mi Li, Charles E. Wyman, Charles M. Cai, and Arthur J. Ragauskas. 2018. "Fast Fractionation of Technical Lignins by Organic Cosolvents." *ACS Sustainable Chemistry and Engineering* 6(5):6064–72.

Ximenes, E., Y. Kim, N. Mosier, B. Dien-…. and microbial technology, and undefined 2011. n.d. "Deactivation of Cellulases by Phenols." *Elsevier*.

Yen, Thanh and Vuong Nguyen. 2016. *UNIVERSITY OF CALIFORNIA RIVERSIDE Integration of a Novel Co-Solvent Enhanced Lignocellulosic Fractionation (CELF) Pretreatment with Biological Conversion to Ethanol*.

7.7 Additional Information



Additional Figure 7-1 Material balance of sugars during the pure sugar co-fermentations (PSCF), simultaneous saccharification and fermentation (SSF) and simultaneous saccharification and co-fermentation (SSCF)

Chapter 8 : Effects of CELF pretreatment severity on lignin structure and resulting impact towards lignin-polyurethane properties*

*This chapter was published in its entirety in *Frontiers in Energy Research* (2020) Wang YY, Sengupta P, Scheidemantle B, Pu Y, Wyman CE, Cai CM and Ragauskas AJ. 2020. "Effects of CELF Pretreatment Severity on Lignin Structure and Lignin-Based Polyurethane Properties." *Frontiers in Energy Research* 8:149.
Lignin extraction protocol was developed and optimized by Priya Sengupta. CELF lignin samples were generated by Priya Sengupta and Brent Scheidemantle. Lignin characterization, polyurethane synthesis and characterization experiments were conducted by Dr. Yun-Yan Wang.

8.1 Abstract

Conversion of technical lignin into performance biopolymers such as polyurethane offers environmental and economic advantages when combined with production of biofuels from biomass sugars, presenting significant interest towards studying the role of pretreatment on lignin structure and functionality. Co-solvent enhanced lignocellulosic fractionation (CELF) pretreatment, employing acidic aqueous tetrahydrofuran (THF) mixtures, was recently developed to effectively break down the lignin-carbohydrate matrix and promote extraction of lignin from lignocellulosic biomass with desirable purity and yield. In this study, we report on the effects of CELF pretreatment reaction severity on the molecular structure of CELF-extracted lignin and its impact towards the mechanical properties of resulting lignin-polyurethanes. Reaction temperature was found to play the most significant role, compared to reaction time and acidity, in manipulating structural features such as molecular weight, functionality and intra-polymer structure. At more severe 180 °C reaction conditions, the order of reactivity for primary lignin interlinkages characterized by semiquantitative HSQC NMR analysis were found to be β-ether > phenylcoumaran (β–5') > resinol (β–β') facilitating a high degree of depolymerization yielding a high frequency of free phenolics and reduced aliphatic hydroxyl groups. All side-chain interlinkages were depleted converting guaiacyl subunits into condensed forms, while retaining more uncondensed syringyl subunits. Under milder 150 °C temperature reaction, CELF lignin was more native-like with higher molecular weight retaining more flexible β-ether interlinkages. These results were then applied to optimizing the polyurethanes synthesized from CELF lignin.

8.2 Introduction

Lignin found in lignocellulosic biomass is a class of heterogeneous biopolymers typically derived from three types of methoxylated phenylpropanoid subunits: guaiacyl (G), syringyl (S), and *p*–hydroxylphenyl (H). (HIGUCHI 2003) Angiosperm poplar lignin is composed of S, G with S/G ratio ranging from 0.65 to 2.19 depending on the species, and a small amount of H subunits which are attached by six predominant interlinkages: β–*O*–4', β–β', β–5', 5–5', β–1' and 4–*O*–5'. (Sannigrahi, Ragauskas, and Tuskan 2010) In the plant cell wall, about 3% of the subunits are covalently bonded with hemicelluloses to form lignin-hemicellulose matrix that provides drought-resistance and a protective barrier against pathogen invasion. (Balakshin, Capanema, and Chang 2007; Giummarella et al. 2019) The recalcitrance of plant cell wall is designed by nature to be resistant to biological and chemical degradation; therefore to reduce the costs associated with processing lignocellulosic biomass to biofuels and biochemical, pretreatment is often employed to modify the plant cell wall to improve accessibility of cellulolytic enzymes to the crystalline cellulose domains from which fermentable glucose can be released. (Smith et al. 2016)

In order to improve upon conventional aqueous biomass pretreatment methods, the addition of miscible co-solvents greatly improves the dissolution of lignin that is critical in maximizing utilization of all major biomass fractions by subsequent catalytic and biological conversion methods. Novel co-solvent-based pretreatment technologies employing tetrahydrofuran (THF), γ-valerolactone (GVL), and ionic liquids in aqueous solutions have been shown to provide significant functional advantages over other co-solvents in improving microbial and enzymatic accessibility of cellulose while also

achieving clean extraction of lignin and high total sugar recovery, merits that are important towards improving the competitiveness of liquid fuels from biomass. (Liu et al. 2018; Petridis and Smith 2018; Shuai, Questell-Santiago, and Luterbacher 2016; Smith et al. 2017) The pretreatment method that employs THF as a co-solvent is known as Co-solvent enhanced lignocellulosic fractionation (CELF). THF is uniquely lower boiling than the other advanced co-solvents so that it can be simply boiled out of the solution after pretreatment in order to induce the precipitation lignin out of solution and to recover THF. This avoids potentially more complicated and energy-intensive solvent recovery methods, such as CO_2-induced phase modification or anti-solvent extraction, that have been proposed for the recovery of high boiling co-solvents. (Meng et al. 2019) In previous studies, CELF has demonstrated wide operating flexibility in terms of reaction conditions such as temperature, solvent ratio, duration, and acid loading to finely control the extent of cellulose and lignin dissolution independently to support sugar hydrolysis at lower severities and to support tandem sugar hydrolysis and dehydration to furfurals at higher severities. (Cai et al. 2013; Nguyen et al. 2016c; Seemala et al. 2018) THF is non-pernicious and is considered a toxicologically safer alternative to dioxane and can be classified as a green chemical if produced from furfural by catalytic decarbonylation followed by hydrogenation. (Cai et al. 2013; Fowles et al. 2013; Seemala et al. 2018) In recent studies, all-atom molecular-dynamics (MD) simulation studies have probed the functionality of THF-water mixtures to “relax” native lignin globules into non-aggregated random-coils under the CELF pretreatment reaction environment to facilitate both lignin solvation and depolymerization, offering a wider operating range to alter the structure and

degree of polymerization of lignin during pretreatment. (Mostofian et al. 2016; Smith et al. 2016) This high degree of lignin tunability opens a broad range of potential pathways for upgrading lignin such as biopolymers, carbon substrates, antioxidants, resins, and hydrocarbon fuels.(Arthur J. Ragauskas et al. 2014; Ragauskas et al. 2006) While structural characterization of CELF lignin resulting from reaction conditions identified for achieving optimal total sugar recovery or high furfural yields have been reported previously, (Meng et al. 2018, 2019; Wang, Li, and Wyman 2018) a systematic study focused on elucidating the precise impact of pretreatment temperature, reaction time, and acid loading on lignin structure is needed to understand the potential spectrum of chemical moieties and inter-unit components that would be available to serve future lignin valorization efforts. Herein, the correlation between CELF pretreatment severity and resultant CELF lignin characteristics from hardwood poplar was established quantitatively by ^{31}P NMR, 2D HSQC, GPC, TGA and DSC. To improve our understanding of lignin fragmentation by acidolysis under CELF conditions, we tracked potential side-reactions such as lignin condensation and loss of monosaccharides as well as the primary acidolysis reaction on the lignin β–*O*–4′ interlinkages. Lignin has been considered as a sustainable and low-cost replacement for petrochemical polyols in the production of commercial polyurethanes products. In the study of Kraft lignin-based polyurethanes, it was found that the mechanical strength of the polyurethane network was dependent on the molecular weight of Kraft lignin cuts prepared by sequential precipitation, and the presence of long-chain polyethylene glycol was able to improve the ductility of the materials.(Wang et al. 2019) The understanding of CELF lignin molecular features, in return, facilitated the screening

of lignin species for producing CELF lignin-based polyurethane (CL-PU) products such as adhesives.

8.3 Experimental Section

8.3.1 Materials

The poplar wood chips used for this study is known as BESC standard poplar. It was determined through compositional analysis (NREL protocol TP-510-42618) to contain 21.2% acid-insoluble lignin. (Sluiter et al. 2008b) Before pretreatment, the poplar chips were knife-milled and passed through a 1 mm particle screen. Chemicals reagents such as THF, sulfuric acid, poly[(phenyl isocyanate)-co-formaldehyde] (PMDI, M_n~340) and dibutyltin dilaurate were purchased from Sigma-Aldrich and Fisher Scientific.

8.3.2 CELF Pretreatment

Poplar wood chips were loaded into a 1 L Hastelloy Parr autoclave reactor (236HC Series, Parr Instruments Co., Moline, IL) equipped with twin pitched-blade Rushton impellers at a solid to liquid loading of 7.5 wt%. The chips were soaked overnight at 4 °C in a 1:1 (w/w) THF-water solution containing dilute mixtures of sulfuric acid (0.025M to 0.1M or 0.25% to 1% in liquid). The pretreatment reactions were carried out at temperatures of 150, 160, and 180 °C for durations of 15 min and 30 min. All reactions were maintained at target temperature (± 1 °C) by convective heating by using a 4 kW fluidized sand bath (Model SBL-2D, Techne, Princeton, NJ), and the reactor temperature was measured directly by using an internally fixed thermocouple (Omega, K-type). To arrest the reaction after the allotted duration, the reactor was submerged in a large room-temperature water bath. The pretreated solids were then vacuum filtered and separated from

the pretreatment liquor at room temperature through a paper filter. Finally, the dry mass of the solids and the mass and density of the liquor was recorded.

8.3.3 CELF Lignin Recovery and Purification

The liquid fraction collected from post filtration was poured in a beaker and titrated to pH ~ 7 using ammonium hydroxide. THF was then boiled out of solution at 80 °C under a hot plate with continuous stirring at 130 rpm for about 4 h. The beaker and contents were then allowed to cool to room temperature overnight and the liquor was then poured out. Lignin that had precipitated onto the beaker after the removal of THF and liquor was rinsed with water and then placed in a dark oven at 65 °C to dry overnight. The resulting lignin was collected and placed onto a glass fiber filter paper. The lignin was then washed with diethyl ether followed by a water wash to remove soluble impurities and placed in an oven at 65 °C to dry overnight to a moisture content of <3%. The lignin was then ground to a fine powder by a mortar and pestle.

8.3.4 Structural Characterization of CELF Lignin

Quantitative ^{31}P NMR and the heteronuclear single quantum coherence (HSQC) NMR spectra were acquired on a Bruker Avance III HD 500-MHz spectrometer according to a previously published literature.(Wang et al. 2018) In the quantitative ^{31}P NMR experiments, a 90° pulse width, 1.2 s acquisition time, 25 s pulse delay were used in collecting 64 scans. 20~30 mg (accurately weighed) CELF lignin sample was dissolved in 700 μL pyridine/$CDCl_3$ (1.6:1, v/v) with 1mg/mL chromium(III) acetylacetonate and 2.5 mg/mL *N*-hydroxy-5-norbornene-2,3-dicarboximide (internal standard). The lignin sample was subjected to NMR analysis promptly after phosphitylating with 60 μL 2-chloro-4,4,5,5

tetramethyl-1,3,2-dixoaphospholane (TMDP). The obtained ^{31}P NMR spectra were calibrated by using the TMDP-water phosphitylation product (δ132.2 ppm) as the internal reference. The HSQC NMR spectra were processed and analyzed by using TopSpin software (version 3.5pl7, Bruker).

8.3.5 Gel Permeation Chromatography (GPC)

Dried CELF lignin sample (~2 mg) was acetylated and processed according to a previous literature.(Wang et al. 2018) The acetylated CELF lignin was dissolved and then incubated in tetrahydrofuran for 24 h. The molecular weight analysis was performed on an Agilent GPC SECurity 1200 system equipped with several Waters Styragel columns (Water Corporation, Milford, MA), an Agilent UV detector (λ=280nm) at a flow rate of 1.0 mL/min at 30 °C.

8.3.6 Thermal Analyses

The thermal gravimetric analysis (TGA) of lignin was operated on a TA Q50 thermogravimetric analyzer (TA Instruments) heating in a nitrogen atmosphere. The sample (~10 mg) was initially incubated at 105 °C for 15 min to remove the last trace of moisture and THF. Then, the temperature was raised from 105 to 900 °C at 10 °C/min. The differential scanning calorimetry (DSC) measurements were performed in heat-cool-heat mode on a TA Q2000 DSC (TA Instruments) with a heating/cooling rate of 20 °C/min.

8.3.7 CL-PU Synthesis and Characterization

The CL-PUs were synthesized by polycondensation as described in a previous literature, and they were denoted according to the corresponding CELF lignin samples. In this work, the selected CELF lignin samples, CELF2, CELF3 and CELF4, were dissolved

in THF with or without poly(ethylene glycol) (PEG, MW = 4000, Alfa Aesar) (1:1, w/w). The polyol/THF mixture was incubated in a thermal shaker (Alkali Scientific Inc.) at 140 rpm, 60 °C for 1 h, and then it was combined with a THF solution containing PMDI with NCO/OH ratio at 1:1 and 1.5% dibutyltin dilaurate. After 3-day curing at room temperature, the CL-PU samples were kept at 150 °C for 3 h. The tensile testing was carried out on a dual column Instron 5567 universal testing system equipped with a 500 N static load cell. For each CL-PU sample, three dog-bone specimens were tested according to ASTM D638 standard (Type V) at a strain rate of 0.1 mm/min.

8.4 Results and Discussion

8.4.1 Delignification in Acidic CELF Pretreatment

Poplar wood meal was pretreated under five CELF pretreatment conditions varying in catalyst dosage, temperature and duration time as summarized in Table 8-1. The THF-water content was fixed at 1:1 (w/w) which has been determined to be the minimum THF needed to achieve high delignification. (Cai et al. 2013) The resultant CELF lignin samples were denoted "CELF1 – CELF5" referring to the degree of pretreatment severity. During the CELF pretreatment, the macromolecular lignin was degraded into fragments and dissolved in the THF-water mixture. Below 180 °C, the removal of lignin increased steadily when the poplar biomass was pretreated at elevated temperature or with higher catalyst dosage. However, total CELF lignin recovered after 180 °C reaction was significantly more for CELF5 (142.1% of total lignin in poplar biomass) as compared to CELF4 (94.4% of total lignin in poplar biomass). The mass in excess of 100% for the CELF5 sample was likely due to cross-polymerization reactions between soluble sugars and lignin during

pretreatment and the formation pseudo-lignin, a polyphenolic compounds derived from carbohydrates subjected to dilute acid reaction. (Hu, Jung, and Ragauskas 2012; Sannigrahi et al. 2011; Shinde et al. 2018) For example, Fu *et al*. found that up to 87% of the holocellulose was converted into acid insoluble pseudo-lignin including approximately 30% aqueous-dioxane-soluble pseudo-lignin after a severe two-step dilute acid pretreatment at 180 °C. (Hu et al. 2012) Pseudo-lignin preferentially forms *via* polymerization or polycondensation of carbohydrate degradation products at high temperature in the presence of oxygen during acid pretreatment.(Hu and Ragauskas 2014) It consumes valuable fuel precursors such as furfural, 5-hydxoylmethylfurfural, and levulinic acid; moreover, similar to lignin, pseudo-lignin absorbs on the surface of biomass and creates a recalcitrant barrier against cellulosic enzymatic hydrolysis.(Hu et al. 2012) Therefore, the generation of pseudo-lignin should be suppressed during CELF pretreatment to provide higher yields of reactive intermediates from both the sugars and lignin in biomass.

Another index to evaluate the efficiency of CELF pretreatment is the molecular weight of the CELF lignin. The GPC profiles presented in Figure 8-1 showed the typical bimodal molecular weight distribution pattern for all five CELF lignin samples, and the impact of pretreatment severity on lignin degradation can be visualized by the intensity changes of high- and low-molecular weight peaks. At low pretreatment temperature (150 °C), the high molecular-weight peaks were found to be predominant for CELF1 and CELF2, and mild degradation of lignin occurred as its M_w was reduced by 20~25% compared with the reported value of poplar cellulolytic enzyme lignin (CEL) (M_w ~

12,000). (Meng et al. 2018) The most dramatic changes in molecular weights and polydispersity (PDI) were observed between CELF2 and CELF3: more than 50% reduction of M_w was achieved by increasing the pretreatment temperature from 150 to 160 °C while other variables remained the same. However, M_w of CELF4 obtained at 180 °C was decreased by an additional 17% compared with CELF3 (160 °C). At 180 °C, the reduction in molecular weight was caused by β–*O*–4' acidolysis which was, however, partially compensated by the repolymerization of degraded lignin fragments *via* C_α condensation (see NMR lignin section).

Table 8-1 CELF pretreatment conditions and mass yield (%) of CELF lignin in total poplar lignin.

Lignin sample	H_2SO_4 (wt %)	Temperature (°C)	Duration (min)	Lignin yield (%)
CELF1	0.25	150	15	65.2
CELF2	0.5	150	15	69
CELF3	0.5	160	15	75.5
CELF4	0.5	180	15	94.4
CELF5	1	180	30	142.1

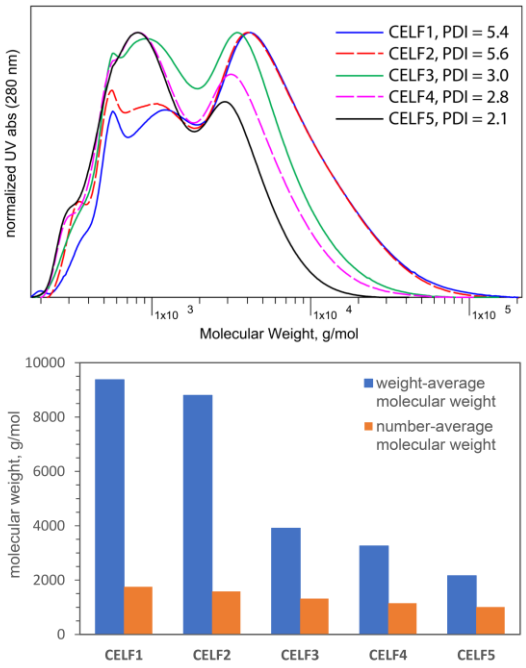


Figure 8-1 GPC profiles, weight-average and number average molecular weights of CELF lignin obtained under different pretreatment conditions.

8.4.1 Impacts of Pretreatment Severity on the Structural Features of CELF Lignin

Acid-catalyzed delignification preferentially starts from β–*O*–4' linked alkyl aryl ethers with a free phenolic end, and the cleavage reaction proceeds along the polymer chain until reaching more recalcitrant bonds.(Sturgeon et al. 2014) Under the acidic condition, the C_α position of beta ether loses a water molecule and forms a benzylic carbocation for the subsequent electrophilic substitution. The beta ether cleavage involves two pathways giving two end products: phenylacetaldehyde and Hibbert ketone (Scheme 1 (A)), when sulfuric acid is used as the catalyst, forming Hibbert ketone is thermodynamically favored.(Imai, Yokoyama, and Matsumoto 2011; Sturgeon et al. 2014) In addition to lignin depolymerization at low pH, C–C crosslinking between lignin components occurs via C_α condensation as depicted in Scheme 1(B).(Liu et al. 2018)

The multifunctionality of lignin macromolecules includes aliphatic, phenolic and carboxylic --OH groups. The phenolic -OH group can be classified into guaiacyl, C_5-substituted and *p*-hydroxyphenyl. The hydroxyl contents of CELF1~5 determined by ^{31}P NMR analysis and the corresponding spectra are shown in Table 8-2 and Figure 8-2(A), respectively. As the CELF pretreatment adopts more severe conditions, more free phenolic hydroxyl groups were released as a result of acid-catalyzed β–*O*–4' cleavage, and they grew to be the major functional groups (~70%) found in CELF lignin when the pretreatment temperature was raised to 180 ℃ (Figure 8-2(B)). Meanwhile, the relative content of aliphatic hydroxyl groups decreased from 74% to 26%. In the predominant lignin substructure, β–*O*–4' alkyl aryl ether, the loss of aliphatic -OH groups arises from several factors including the cleavage of the monomeric components, oxidation of hydroxyl groups, and dehydration of side chain C_α and C_γ leading to C_α condensation or formation of stilbene structures. (Hallac, Pu, and Ragauskas 2010; Meng et al. 2018) The contents of syringyl and guaiacyl phenolic -OH groups grew comparably with the release of free phenolic ones (Table 8-2 and Figure 8-2 (C)). However, unlike the former two, the *p*-hydroxyphenyl end units derived from esterified *p*-coumaric acid were found to be more vulnerable to cleavage at low pH as its corresponding hydroxyl content decreased from ~20 % to ~5% of the phenolic hydroxyl content.

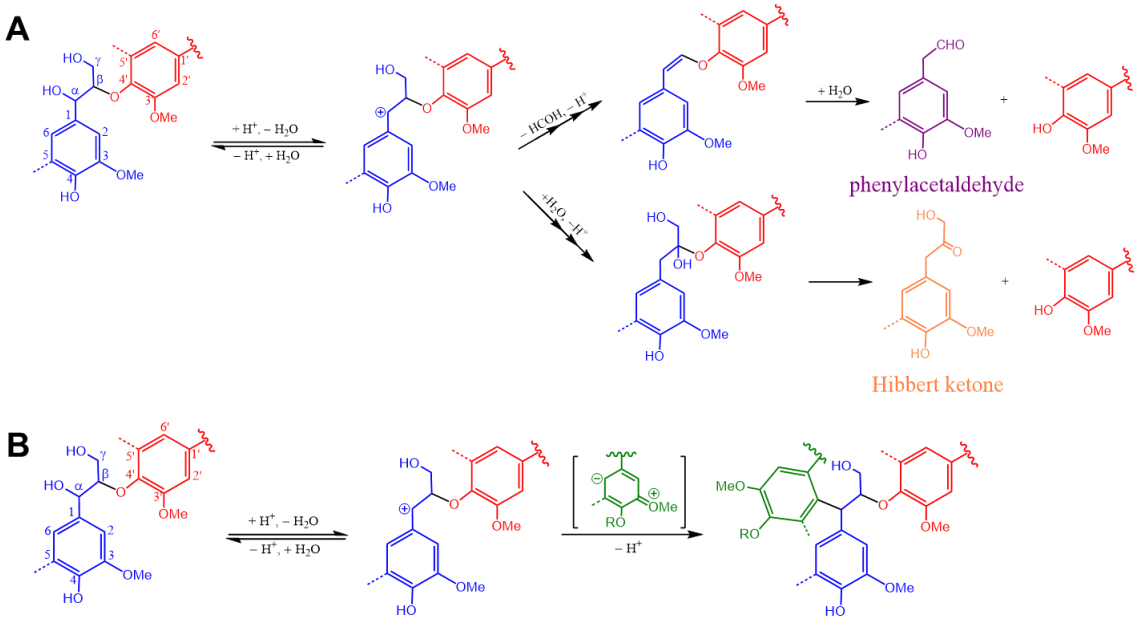
Detailed structural evolution of CELF lignin in relation to the pretreatment severity can be mapped by semiquantitative HSQC NMR analysis. As shown in Figure 8-3, the 2D HSQC spectra of CELF1, 3 and 5 prepared under mild, medium and harsh pretreatment conditions, were distinguishably different based upon the appearance and disappearance of

some specific structural features. In the aliphatic region, CELF1 resembled the poplar CEL, it possessed clear and intensive characteristic cross-peaks such as β–*O*–4′ alkyl aryl ether (A), β–β′ resinol (B) and β–5′ phenylcoumaran (C) substructures, but it showed little trace of carbohydrate signals compared with the poplar CEL. (Meng et al. 2018) In the aromatic region, in addition to those well-defined cross-peaks of S and G subunits, and *p*-hydroxybenzoate substructure (PB), new cross-peaks of $S_{condensed}$ and $G_{2,\ condensed}$ representing condensed S and G subunits, can be found around δ105.7~106.9/δ6.46~6.53 ppm and δ112.8/δ6.78 ppm, respectively. In the G subunits, condensation reactions can occur on open aromatic C_5 or C_6 and cause chemical shift migration of C_2–H_2 in the HSQC spectrum. In the spectrum of CELF3, the peak areas of $S_{condensed}$ and $G_{2,\ condensed}$ expanded, and a weak cross-peak of C_γ–H_γ in lignin-bound Hibbert ketone (HK) end group can be observed. Under extreme pretreatment condition (1 wt% H_2SO_4, 180 °C and 30 min duration time), CELF5 lost all side-chain interlinkages. The missing G_2 and G_6, remaining G_5 cross-peaks indicated that all G subunits were in a condensed form and substitution on C_6 was preferred at 180 °C. Moreover, 5-hydroxymethylfurfural, a dehydration product of glucose, was found in CELF lignin, given that the cross-peaks of its C_3–H_3 (δ122.8~124.2/δ7.50~7.55 ppm), C_4–H_4 (δ109.6~110.1/δ6.43~6.62 ppm) and C_6–H_6 (δ55.8/δ4.55 ppm) can be clearly observed in the spectra of CELF4 and CELF5.(Constant et al. 2016)

The quantified impacts of pretreatment severity on lignin structure is summarized in Figure 8-4. Compared with other CELF lignin samples, CELF1 underwent minimal

structural modification and persevered most of the native lignin structural features such as high frequency of β–*O*–4′ interlinkages (41 per 100 (S+G) units) and high molecular weight (Figure 8-4(A)). The content of β–*O*–4′ decreased rapidly from CELF1 to CELF5; on the other hand, β–β′ and β–5′ interlinkages were more resistant to acidolysis, but they were eventually cleaved or transformed at 180 °C, and β–5′ that can only be formed from G subunits was removed more rapidly. Due to the presence of C_5-methoxyl group, S subunits are favorably linked through β–*O*–4′. It was found that the transgenic poplar lignin composed of ~98% S subunits possessed similar β–*O*–4′, but higher β–β′ content compares with wild poplar species. (Stewart et al. 2009) Interestingly, in this work, the change of S/G with increasing pretreatment severity indicated that only at 180 °C the loss of S subunits started to surpass the G ones accompanying with the removal of β–β′ (Figure 8-4(B)). Below 180 °C, $S_{2,6condensed}$/S was higher than $G_{2condensed}$/G, but under harsh pretreatment conditions (180 °C), the trend was reversed, and less than 80% of the S subunits were in condensed form in virtue of steric hindrance created by the bulk C_5–methoxyl group. In the HSQC spectra of CELF lignin samples (Figure 8-3), the cross-peak at δ106.3/δ7.25 ppm is assigned to $C_{2,6}$ in the oxidized S subunits, and S_{ox}/S was hardly affected by pretreatment severity (Figure 8-4(B)). It has been reported that oxidation of C_α or C_γ–OH in β–*O*–4′ substructure can lower the

C–*O*–aryl bond strength, and consequently facilitate lignin depolymerization. (Guo et al. 2018) Therefore, the oxidized S subunits were presumably located at the end of the CELF lignin polymer chain.



Scheme 8-1 Mechanisms of acid-catalyzed β–O–4' cleavage (**A**) and C_α condensation (**B**).

Table 8-2 CELF lignin hydroxyl contents determined by quantitative ^{31}P NMR analysis. (The error values were obtained from standard deviation of duplicate results.)

OH content, mmol/g lignin	CELF1	CELF2	CELF3	CELF4	CELF5
aliphatic	4.55 ± 0.04	3.94 ± 0.01	2.45 ± 0.05	1.11 ± 0.02	1.07 ± 0.02
phenolic	1.56 ± 0.05	1.71 ± 0.05	2.18 ± 0.03	3.05 ± 0.01	2.81 ± 0.05
carboxylic	0.08 ± 0.01	0.07 ± 0.01	0.10 ± 0.01	0.15 ± 0.00	0.17 ± 0.02
total	6.19 ± 0.08	5.72 ± 0.05	4.72 ± 0.08	4.31 ± 0.03	4.04 ± 0.09
C_5-substituted	0.76 ± 0.03	0.9 ± 0.02	1.30 ± 0.01	1.71 ± 0.01	1.71 ± 0.02
guaiacyl	0.50 ± 0.02	0.54 ± 0.03	0.67 ± 0.02	1.02 ± 0.00	0.97 ± 0.00
***p*-hydroxyphenyl**	0.30 ± 0.01	0.28 ± 0.01	0.21 ± 0.00	0.22 ± 0.00	0.13 ± 0.01

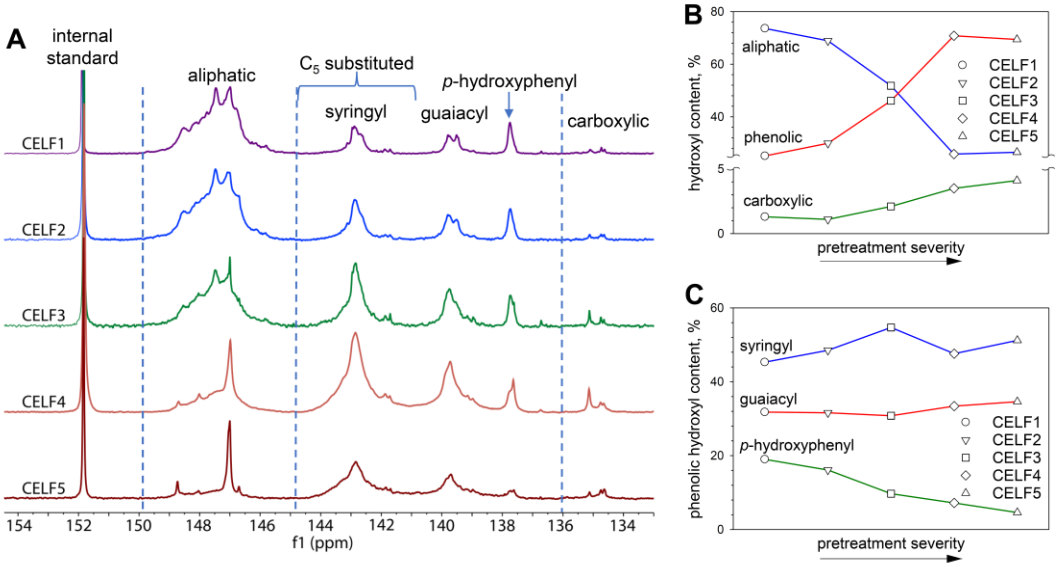


Figure 8-2 (**A**) Quantitative ^{31}P NMR spectra of TMDP phosphitylated CELF lignin samples. (**B**) The relative quantities (%) of aliphatic, phenolic and carboxylic hydroxyl groups in total lignin hydroxyl groups. (**C**) The relative contents (%) of syringyl, guaiacyl and *p*-hydroxyphenyl hydroxyl groups in total lignin phenolic hydroxyl groups.

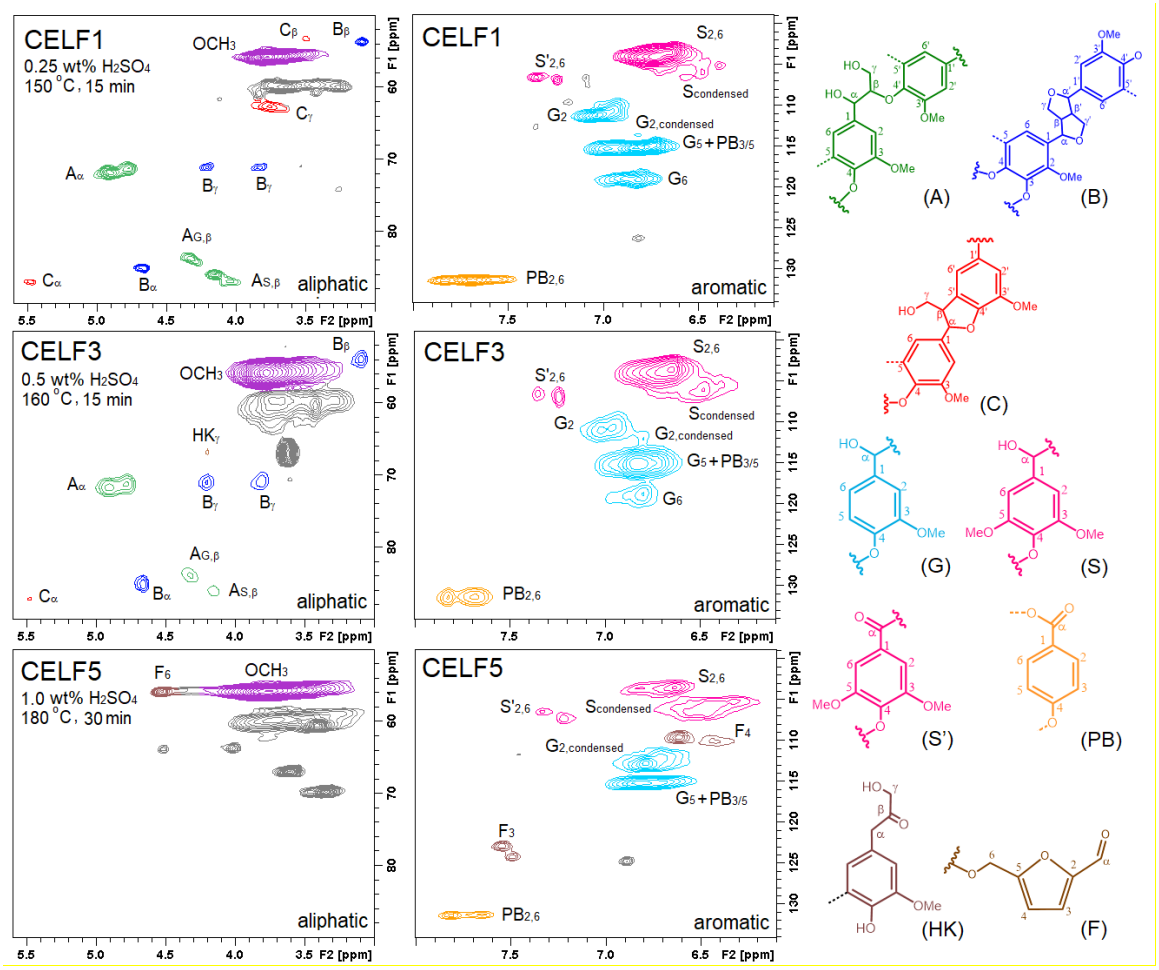


Figure 8-3 HSQC spectra of CELF1, 3 and 5. Structure (A) β–*O*–4′ linked alkyl aryl ether substructure; (B) β–β′ linked resinol substructure; (C) β–5′ and α–*O*–4′ linked phenylcoumaran substructure; (G) guaiacyl unit; (S) syringyl unit; (S′) oxidized syringyl unit; (PB) *p*–hydroxybenzoate substructure; (HK) Hibbert ketone; (F) etherified 5-(hydroxylmethyl) furfural.

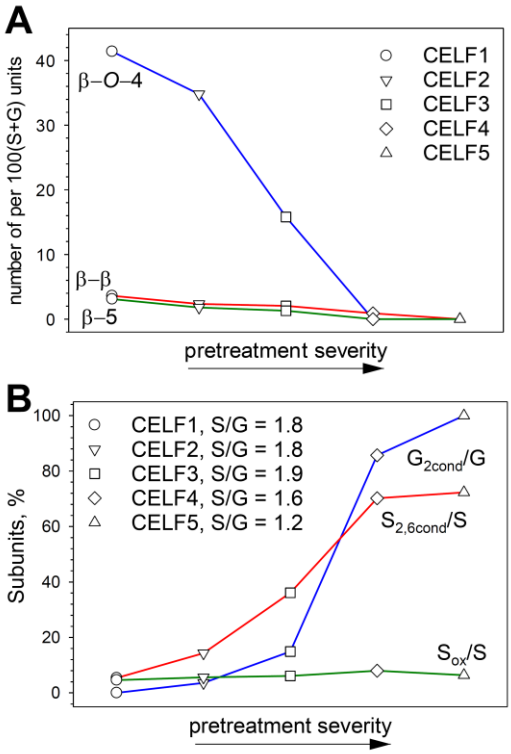


Figure 8-4 Semiquantitative HSQC analyses of CELF lignin interunit linkages and subunits. (A) Changes of β–O–4′, β–β′ and β–5′ interunit linkage contents (per 100 (S+G) units) with increasing pretreatment severity; (B) Changes of condensed guaiacyl (G_{2cond}), condensed syringyl ($S_{2,6cond}$) and oxidized syringyl (S_{ox}) subunit contents with increasing pretreatment severity.

8.4.2 Correlation between Thermal Behaviors and Molecular Structure of CELF lignin

One of the pathways to the valorization of lignin co-products isolated from CELF process is to incorporate them into polymeric materials; therefore, it is essential to have a deep fundamental understanding of their thermal behaviors. The DSC profiles in Figure 8-5 (A) exhibited two distinct glass transition patterns for CELF lignin depending on the molecular structure that can be tuned by pretreatment severity. Below 180 °C (CELE1~3), glass transition temperature (T_g) of CELF lignin is reversely proportional to molecular weight. The CELF lignin samples obtained at 180 °C are highly condensed and crosslinked

through rigid C–C bonds rather than C–O bonds. Although their molecular weights were significantly lower than CELF1~3, no clear glass transition state can be observed for CELF4 and CELF5 within the experimental temperature range. In Figure 8-5 (B), the TGA thermograms indicated that the CELF lignin samples underwent three degradation steps. The most prominent peak arising from breaking C–C interlinkages and demethoxylation of aromatic rings at 350~400 °C can be observed for all CELF lignin samples.(Wang et al. 2018) However, the peak was shrinking as the pretreatment becomes harsher, and such phenomenon is consistent with the decreasing S/G ratio caused by demethoxylation. The peak around 280 °C is mainly caused by the bond rupture of ether interlinkages and aliphatic side chains, which release phenolic compounds, aldehydes, and carboxylic acids.(Zhao et al. 2014) Its decay reflected lignin molecular structure evolving from flexible and native-like to a rigid and highly condensed under elevated pretreatment severity. The mass loss around 150 °C is attributed to dehydration of aliphatic hydroxyl groups. (Hirose et al. 1998)

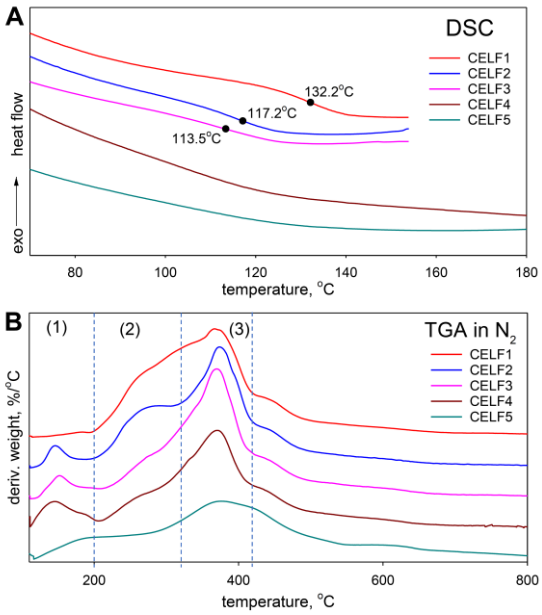


Figure 8-5 (**A**) DSC and (**B**) TGA analyses of CELF lignin samples obtained under different pretreatment conditions.

8.4.3 Screening CELF Lignin for CL-PUs

The CELF lignin samples, CELF2, CELF3 and CELF4 prepared under 150 °C, 160 °C and 180 °C pretreatment temperatures, were selected for producing CL-PUs. As shown in Table 8-3, the CL-PUs (CL2, CL3 and CL4) using lignin as the solo polyol were brittle materials with elongation at break (ε_b) hardly exceeded 5%, and their Young's modulus (E), ultimate stress (σ_{max}) increased as higher pretreatment temperature was employed. The aliphatic -OH groups in lignin are found more reactive in polyurethane synthesis, and urethane formation on the aromatic ring are less favorable due to steric hindrance effect and acidic character of phenolic -OH groups. However, in this work, the mechanical properties of CL-PUs were determined by the miscibility between CELF lignin and PMDI in THF. In the sequential precipitation study, it was found that CELF lignin cuts with higher molecular weight inclined to precipitate out from the THF-methanol co-solvent as the

solvent polarity decreased. (Wang et al. 2018) Similarly, in this work, the solvation behavior of CELF lignin was manipulated by its molecular weight. CELF2 (M_w = 8800 g/mol) and CELF3 (M_w = 3900 g/mol) were not completely soluble in THF at 60 °C, and further precipitation occurred when they were mixing with PMDI in THF. Compared to CELF3, CELF 4 (M_w = 3250 g/mol) possessed higher proportion of lower-molecular-weight lignin species as shown in the GPC profiles (Figure 8-1). CELF4 was fully soluble in THF at room temperature, and as a result, CL4 exhibited better E and σ_{max} given the fact that CELF4 is structurally highly condensed and rigid. It was reported that PEG was able to form strong hydrogen bonds with lignin aliphatic and phenolic -OH groups, and thus disrupt the noncovalent intermolecular interaction between macromolecular lignin species. (Kadla and Kubo 2003; Wang, Chen, and Sarkanen 2017) Herein, 50% (w/w) PEG was pre-mixed with CELF lignin samples aiming to promote the solvation of the latter ones in THF. In general, the soft segments formed by PEG reduced the brittleness and improved the ductility of the CL-PEG PUs (Table 8-3). In consistent with the control set, the variation of ε_b for CL-PEG PUs indicated that the efficacy of PEG depended on the solvation behavior of CELF lignin.

Table 8-3 The tensile properties of CL-PUs: Young's modulus (E), ultimate stress (σ_{max}) and elongation at break (ε_b). (The error values were obtained from standard deviation of triplicate results.)

CL-PU[a]	E, GPa	σ_{max}, MPa	ε_b, %	lignin, %
CL2	0.80 ± 0.16	22.01 ± 6.35	3.46 ± 0.80	59.6
CL3	0.97 ± 0.09	27.85 ± 13.19	4.50 ± 0.46	61.7
CL4	1.04 ± 0.10	39.92 ± 8.92	4.47 ± 1.06	62.9
CL2-PEG[b]	0.21 ± 0.03	9.23 ± 2.04	7.13 ± 1.96	36.5
CL3-PEG[b]	0.21 ± 0.01	13.20 ± 1.18	24.23 ± 5.05	37.4
CL4-PEG[b]	0.07 ± 0.00	8.87 ± 1.90	89.77 ± 26.3	37.9

8.5 Conclusion

Poplar biomass was pretreated in the CELF process under different conditions, in which lignin was depolymerized and extracted with acidic aqueous THF. The pretreatment severity strongly influenced the molecular weight, multifunctionality and intra-polymer structure characteristics of the co-product lignin. Mild CELF pretreatment at low temperature was conducted to reduce the changes on lignin chemical structure and preserve high molecular weight, high β–*O*–4′ , and aliphatic hydroxyl contents. When the pretreatment temperature was increased from 150 °C to 180 °C, the content of aliphatic hydroxyl groups was reduced 4-fold, which had a negative impact on the multifunctionality of CELF lignin. The studies of CELF lignin thermal behaviors confirmed that CELF lignin isolated from high-severity pretreatment was composed of hetero-oligomers born with rigid and highly condensed molecular structure. Considering the efficiency of CELF process, high temperature (180 °C) should be avoided given that monosaccharides can be

wasted on the massive side-reactions forming pseudo-lignin and etherification between lignin and 5-hydroxymethylfurfural during the pretreatment. On the other hand, the synthesis of CL-PU indicated that the tensile properties depended on the miscibility of CELF lignin with other components such as PMDI, and the presence of PEG would disrupt the strong hydrogen bonding in between lignin macromolecules and improve the dispersion of CELF lignin in the PU network. Therefore, for CELF lignin prepared under mild pretreatment conditions such as at 150 and 160 °C, fractionation to separate out high-molecular-weight cuts or suitable compatibilizers will be required to improve its dispersion in the CELF lignin-based polyurethanes.

8.6 References

Balakshin, Mikhail Yu, Ewellyn A. Capanema, and Hou Min Chang. 2007. "MWL Fraction with a High Concentration of Lignin-Carbohydrate Linkages: Isolation and 2D NMR Spectroscopic Analysis." *Holzforschung* 61(1):1–7.

Cai, Charles M., Taiying Zhang, Rajeev Kumar, and Charles E. Wyman. 2013. "THF Co-Solvent Enhances Hydrocarbon Fuel Precursor Yields from Lignocellulosic Biomass." *Green Chemistry* 15(11):3140–45.

Constant, Sandra, Hans L. J. Wienk, Augustinus E. Frissen, Peter De Peinder, Rolf Boelens, Daan S. Van Es, Ruud J. H. Grisel, Bert M. Weckhuysen, Wouter J. J. Huijgen, Richard J. A. Gosselink, and Pieter C. A. Bruijnincx. 2016. "New Insights into the Structure and Composition of Technical Lignins: A Comparative Characterisation Study." *Green Chemistry* 18(9):2651–65.

Fowles, Jefferson, Rodney Boatman, Jim Bootman, Chris Lewis, David Morgott, Erik Rushton, Joost Van Rooij, and Marcy Banton. 2013. "A Review of the Toxicological and Environmental Hazards and Risks of Tetrahydrofuran." *Critical Reviews in Toxicology* 43(10):811–28.

Giummarella, Nicola, Yunqiao Pu, Arthur J. Ragauskas, and Martin Lawoko. 2019. "A Critical Review on the Analysis of Lignin Carbohydrate Bonds." *Green Chemistry* 21(7):1573–95.

Guo, Haiwei, Daniel M. Miles-Barrett, Andrew R. Neal, Tao Zhang, Changzhi Li, and Nicholas J. Westwood. 2018. "Unravelling the Enigma of LigninOX: Can the Oxidation of Lignin Be Controlled?" *Chemical Science* 9(3):702–11.

Hallac, Bassem B., Yunqiao Pu, and Arthur J. Ragauskas. 2010. "Chemical Transformations of Buddleja Davidii Lignin during Ethanol Organosolv Pretreatment." Pp. 2723–32 in *Energy and Fuels*. Vol. 24. American Chemical Society.

HIGUCHI, Takayoshi. 2003. "Pathways for Monolignol Biosynthesis via Metabolic Grids: Coniferyl Aldehyde 5-Hydroxylase, a Possible Key Enzyme in Angiosperm Syringyl Lignin Biosynthesis." *Proceedings of the Japan Academy, Series B* 79B(8):227–36.

Hirose, Shigeo, Ken Kobashigawa, Yoshinobu Izuta, and Hyoe Hatakeyama. 1998. "Thermal Degradation of Polyurethanes Containing Lignin Studied by TG-FTIR." *Polymer International* 47(3):247–56.

Hu, Fan, Seokwon Jung, and Arthur Ragauskas. 2012. "Pseudo-Lignin Formation and Its Impact on Enzymatic Hydrolysis." *Bioresource Technology* 117:7–12.

Hu, Fan and Arthur Ragauskas. 2014. "Suppression of Pseudo-Lignin Formation under Dilute Acid Pretreatment Conditions." *RSC Advances* 4(9):4317–23.

Imai, Takaaki, Tomoya Yokoyama, and Yuji Matsumoto. 2011. "Revisiting the Mechanism of β-O-4 Bond Cleavage during Acidolysis of Lignin IV: Dependence

of Acidolysis Reaction on the Type of Acid." *Journal of Wood Science* 57(3):219–25.

Kadla, John F. and Satoshi Kubo. 2003. "Miscibility and Hydrogen Bonding in Blends of Poly(Ethylene Oxide) and Kraft Lignin." *Macromolecules* 36(20):7803–11.

Liu, Enshi, Mi Li, Lalitendu Das, Yunqiao Pu, Taylor Frazier, Bingyu Zhao, Mark Crocker, Arthur J. Ragauskas, and Jian Shi. 2018. "Understanding Lignin Fractionation and Characterization from Engineered Switchgrass Treated by an Aqueous Ionic Liquid." *ACS Sustainable Chemistry and Engineering* 6(5):6612–23.

Meng, Xianzhi, Aakash Parikh, Bhogeswararao Seemala, Rajeev Kumar, Yunqiao Pu, Phillip Christopher, Charles E. Wyman, Charles M. Cai, and Arthur J. Ragauskas. 2018. "Chemical Transformations of Poplar Lignin during Cosolvent Enhanced Lignocellulosic Fractionation Process." *ACS Sustainable Chemistry and Engineering* 6(7):8711–18.

Meng, Xianzhi, Aakash Parikh, Bhogeswararao Seemala, Rajeev Kumar, Yunqiao Pu, Charles E. Wyman, Charles M. Cai, and Arthur J. Ragauskas. 2019. "Characterization of Fractional Cuts of Co-Solvent Enhanced Lignocellulosic Fractionation Lignin Isolated by Sequential Precipitation." *Bioresource Technology* 272:202–8.

Mostofian, Barmak, Charles M. Cai, Micholas Dean Smith, Loukas Petridis, Xiaolin Cheng, Charles E. Wyman, and Jeremy C. Smith. 2016. "Local Phase Separation of Co-Solvents Enhances Pretreatment of Biomass for Bioenergy Applications." *Journal of the American Chemical Society* 138(34):10869–78.

Nguyen, Thanh Yen, Charles M. Cai, Omar Osman, Rajeev Kumar, and Charles E. Wyman. 2016. "CELF Pretreatment of Corn Stover Boosts Ethanol Titers and Yields from High Solids SSF with Low Enzyme Loadings." *Green Chemistry* 18(6):1581–89.

Petridis, Loukas and Jeremy C. Smith. 2018. "Molecular-Level Driving Forces in Lignocellulosic Biomass Deconstruction for Bioenergy." *Nature Reviews Chemistry* 2(11):382–89.

Ragauskas, Arthur J., Gregg T. Beckham, Mary J. Biddy, Richard Chandra, Fang Chen, Mark F. Davis, Brian H. Davison, Richard A. Dixon, Paul Gilna, Martin Keller, Paul Langan, Amit K. Naskar, Jack N. Saddler, Timothy J. Tschaplinski, Gerald A. Tuskan, and Charles E. Wyman. 2014. "Lignin Valorization: Improving Lignin Processing in the Biorefinery." *Science* 344(6185).

Ragauskas, Arthur J., Charlotte K. Williams, Brian H. Davison, George Britovsek, John Cairney, Charles A. Eckert, William J. Frederick, Jason P. Hallett, David J. Leak, Charles L. Liotta, Jonathan R. Mielenz, Richard Murphy, Richard Templer, and Timothy Tschaplinski. 2006. "The Path Forward for Biofuels and Biomaterials." *Science* 311(5760):484–89.

Sannigrahi, Poulomi, Dong Ho Kim, Seokwon Jung, and Arthur Ragauskas. 2011.

"Pseudo-Lignin and Pretreatment Chemistry." *Energy and Environmental Science* 4(4):1306–10.

Sannigrahi, Poulomi, Arthur J. Ragauskas, and Gerald A. Tuskan. 2010. "Poplar as a Feedstock for Biofuels: A Review of Compositional Characteristics." *Biofuels, Bioproducts and Biorefining* 4(2):209–26.

Seemala, Bhogeswararao, Xianzhi Meng, Aakash Parikh, Nikhil Nagane, Rajeev Kumar, Charles E. Wyman, Arthur Ragauskas, Phillip Christopher, and Charles M. Cai. 2018. "Hybrid Catalytic Biorefining of Hardwood Biomass to Methylated Furans and Depolymerized Technical Lignin." *ACS Sustainable Chemistry and Engineering* 6(8):10587–94.

Shinde, Somnath D., Xianzhi Meng, Rajeev Kumar, and Arthur J. Ragauskas. 2018. "Recent Advances in Understanding the Pseudo-Lignin Formation in a Lignocellulosic Biorefinery." *Green Chemistry* 20(10):2192–2205.

Shuai, Li, Ydna M. Questell-Santiago, and Jeremy S. Luterbacher. 2016. "A Mild Biomass Pretreatment Using γ-Valerolactone for Concentrated Sugar Production." Pp. 937–43 in *Green Chemistry*. Vol. 18. Royal Society of Chemistry.

Sluiter, A., B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, and D. Crocker. 2008. *Determination of Structural Carbohydrates and Lignin in Biomass: Laboratory Analytical Procedure (LAP) (Revised July 2011)*.

Smith, Micholas Dean, Xiaolin Cheng, Loukas Petridis, Barmak Mostofian, and Jeremy C. Smith. 2017. "Organosolv-Water Cosolvent Phase Separation on Cellulose and Its Influence on the Physical Deconstruction of Cellulose: A Molecular Dynamics Analysis." *Scientific Reports* 7(1):1–9.

Smith, Micholas Dean, Barmak Mostofian, Xiaolin Cheng, Loukas Petridis, Charles M. Cai, Charles E. Wyman, and Jeremy C. Smith. 2016. "Cosolvent Pretreatment in Cellulosic Biofuel Production: Effect of Tetrahydrofuran-Water on Lignin Structure and Dynamics." *Green Chemistry* 18(5):1268–77.

Stewart, Jaclyn J., Takuya Akiyama, Clint Chapple, John Ralph, and Shawn D. Mansfield. 2009. "The Effects on Lignin Structure of Overexpression of Ferulate 5-Hydroxylase in Hybrid Poplar 1[W]." *Plant Physiology* 150(2):621–35.

Sturgeon, Matthew R., Seonah Kim, Kelsey Lawrence, Robert S. Paton, Stephen C. Chmely, Mark Nimlos, Thomas D. Foust, and Gregg T. Beckham. 2014. "A Mechanistic Investigation of Acid-Catalyzed Cleavage of Aryl-Ether Linkages: Implications for Lignin Depolymerization in Acidic Environments." *ACS Sustainable Chemistry and Engineering* 2(3):472–85.

Wang, Yun-Yan, Mi Li, and Charles E. Wyman. 2018. "UC Riverside 2018 Publications Title Fast Fractionation of Technical Lignins by Organic Cosolvents Publication Date."

Wang, Yun-Yan, Charles E. Wyman, Charles M. Cai, and Arthur J. Ragauskas. 2019.

"Lignin-Based Polyurethanes from Unmodified Kraft Lignin Fractionated by Sequential Precipitation." *ACS Applied Polymer Materials* 1(7):1672–79.

Wang, Yun Yan, Yi Ru Chen, and Simo Sarkanen. 2017. "Blend Configuration in Functional Polymeric Materials with a High Lignin Content." *Faraday Discussions* 202(0):43–59.

Zhao, Jing, Wang Xiuwen, Jun Hu, Qian Liu, Dekui Shen, and Rui Xiao. 2014. "Thermal Degradation of Softwood Lignin and Hardwood Lignin by TG-FTIR and Py-GC/MS." *Polymer Degradation and Stability* 108:133–38.

Chapter 9 : Conclusion and Future Recommendations

9.1 Summary of Findings

This research showed that Co-Solvent Enhanced Lignocellulosic Fractionation (CELF) pretreatment efficiently breaks polysaccharide-lignin linkages in poplar and solubilizes hemicellulose sugars and lignin from the plant cell wall matrix to allow greater access to cellulose. While low to moderate severity reactions partially break glycosidic linkages to release a fraction of sugars as monomers, higher severity pretreatments degrade sugars released and form pseudo-lignin. CELF pretreatment at 160 °C for 15 minutes was found to maximize sugar and lignin release from poplar with minimal degradation. At these conditions, CELF was found to remove ~90% of the lignin and ~92% of the hemicellulose sugars from poplar cell walls while preserving ~94% of the glucan in the solid fraction. The glucan in the solids produced by CELF pretreatment were fully digestible, irrespective of pretreatment severity. However, solids generated at the mildest and the harshest CELF reaction conditions, with higher lignin or more pseudo-lignin contents, respectively, required the longest times to digest completely. These results suggest that maximizing lignin removal without generating much pseudo-lignin is a key to improving the enzymatic digestibility of cellulosic biomass. However, the results from Chapter 6 revealed that extra delignification of already extensively delignified CELF poplar solids via THF washing resulted in cellulose pore collapse, dehydration of the biomass, and reduced substrate specific surface area for subsequent enzymatic hydrolysis, thereby negatively impacting saccharification rates.

Solids produced at optimized CELF pretreatment conditions contained nearly 89% glucan, 92% of which were hydrolyzed by subsequent enzymatic hydrolysis even at an

enzyme loading as low as 5 mg protein per g glucan in 8 days. A fractal kinetic analysis of the enzymatic saccharification data indicated strong bonding and reactivity between CELF substrates and cellulolytic fungal enzymes. Another interesting interpretation from the fractal kinetic analysis was a "jamming-effect" at high enzyme loadings (>15 mg protein per g glucan in raw poplar) due to the high amount of active enzymes remaining in the broth compared to the available substrate, especially towards the end of reaction. These results indicated that a higher protein dose may increase the rate of saccharification of CELF solids but not improve enzyme effectiveness. The sustained enzyme activity observed throughout the course of hydrolysis was mainly attributed to the low lignin content in the CELF substrate reducing unproductive enzyme-lignin binding.

Ethanol titers of 60 g/L, 78 g/L, and 87 g/L were achieved from the Simultaneous Saccharification and Fermentation (SSF) of CELF solids at 37 °C in 7 days for insoluble solid loadings of 13, 17, and 20 wt%, respectively, using a Cellic® CTec2 enzyme cocktail at 15 mg protein per g glucan in raw poplar in combination with *Saccharomyces cerevisiae* variant D5A. Despite a considerable amount of residual sugars in the fermentation broth, ethanol concentration leveled off at 87 g/L, the same as the maximum ethanol concentration achievable from pure glucose fermentations using D5A. These results suggested that SSF yields in this study were not limited by digestibility of the cellulosic substrates but by ethanol tolerance of the yeast strain. A thermotolerant *Kluveromyces marxianus* strain CBS 6556 reached higher ethanol concentrations sooner in high solids SSF at the temperatures near optimum for enzymatic hydrolysis of the highly digestible CELF solids. For SSF at 37 °C, CBS 6556 achieved similar yields and concentrations of

ethanol as D5A on the same solids. However, the yeast strain experienced an early fermentation arrest and did not attain high ethanol yields at 43 °C. SEM images further revealed that *K. marxianus* cells experienced cell volume shrinkage to an abnormally small size and cell wall wrinkling under the combined stresses of high ethanol concentrations and elevated temperature.

CELF hydrolysates were highly acidic and contained > 400 g/L THF, cellulose and hemicellulose-derived monomers, acetic acid, 1,4-butanediol, and lignin-derived phenolics. Hence, the hydrolysate was first neutralized followed by either THF removal by boiling it off or THF recovery by vacuum mediated extraction in a rotary evaporator. Either step produced a stream of pure technical lignin and highly concentrated monomeric sugar solutions with elevated levels of 1,4-butanediol, acetic acid, and water-soluble lignin based phenolics. Simultaneous Saccharification and Co-Fermentation (SSCF) of the C-6 sugars from CELF solids along with the C-5 and C-6 monomers from conditioned CELF hydrolysate were also conducted at an enzyme dose of 15 mg protein per g glucan in raw poplar in combination with an engineered *Saccharomyces cerevisiae* variant M11205. To achieve good performance, these experiments required 72 h prehydrolysis before fermentation to dilute THF and 1,4-butanediol concentrations below 5 g/L and 10 g/L, respectively, so they could no longer inhibit fermentation of sugars by M11205. SSCF reactions scaled up to a 1L working volume in a 3L bioreactor produced 72 g/L ethanol titers, corresponding to a theoretical yield of 72%, with ~10 g/L residual glucose left in the broth at the end of 13 days at a 17 wt% insoluble solids loading.

A technoeconomic analysis was conducted for a hypothetical biorefinery using the CELF-SSCF process as the base case, to estimate the minimum ethanol selling price per gallon ethanol (MESP). This analysis calculated the MESP to be 2.24 $/gal and 1.82 $/gal for ethanol production via SSCF of CELF pretreated poplar, assuming the entire lignin stream to be burned to generate energy for the former and 70% of the lignin stream being sold as a polyol source at 500 $/tonne, for the latter. MESP was found to be greatly influenced by the overall ethanol yield achieved per g biomass, the enzyme contribution to the cost of production and the fate of lignin recovered from the pretreatment step. Overall, the TEA analysis of the plant design revealed that maximizing utilization of total sugars available in the raw biomass towards ethanol production along with use of lignin as a value-added co-product is essential to ensure profitable ethanol production competitive to the first generation of starch based cellulosic ethanol.

Finally, a high purity, depolymerized technical lignin was recovered from the CELF hydrolysate. Reaction temperature was found to play a more significant role than reaction time and acidity in influencing structural features of CELF lignin such as molecular weight, functionality, and intra-polymer structure. At harsh reaction conditions of 180 °C, CELF lignin exhibited a high degree of depolymerization to produce free phenolics and reducing aliphatic hydroxyl groups. These reaction conditions were not considered suitable for lignin extraction, as monosaccharides reacted to form pseudo-lignin and underwent etherification between lignin and 5-hydroxymethylfurfural during pretreatment. CELF demonstrated a wide flexibility in terms of manipulating lignin structural features to fit desired applications, hence, opening up a wide range of potential

products for lignin valorization such as biopolymers, carbon substrates, and resins. CELF lignin polyurethanes (CL-PU) were synthesized by polycondensation with lignin as the organic polyol source. Mechanical testing of the CL-PUs indicated that the tensile properties depended on CELF lignin miscibility with other components such as poly[(phenyl isocyanate)-co-formaldehyde] (PMDI). Poly(ethylene glycol) (PEG) was shown to disrupt strong hydrogen bonding between lignin macromolecules and improved CELF lignin dispersion in the PU network. Hence, high-molecular-weight cuts fractionated from CELF lignin prepared under mild reaction conditions of 150 and 160 °C are expected to improve dispersion in the CELF lignin-based polyurethanes.

9.2 Concluding Remarks and Future Recommendations

Lignocellulosic biomass is the only sustainable resource capable of supporting large-scale production of liquid transportation fuels or organic chemicals in the near future. However, cellulosic ethanol production must be cost competitive in order to emerge as an alternative to gasoline. This work showed CELF pretreatment can reduce loadings of costly enzymes, increase ethanol concentrations in fermentation broths, and valorize hemicellulose sugars and lignin. CELF pretreatment has also proven to be effective on a range of feedstock including switchgrass, corn stover, and hardwood poplar. Not only are CELF solids highly digestible and suitable for high solids SSF with a comparatively lower enzyme dose than other leading pretreatment methods, but the sugars in the CELF hydrolysate are also completely fermentable without requiring extensive expensive and complicated conditioning, thereby enabling more full utilization of major cell wall sugars. CELF lignin has also proven to be amenable to use in bioplastics. A technoeconomic

analysis shows that deriving value from lignin can significantly improve the overall process economics of ethanol production. Additionally, lignin based biopolymers are also beneficial for the environment. Hence, continued research to better understand CELF lignin and upgrade its quality for a wide range of applications is highly recommended.

This study suggests many new opportunities for research. Pseudo lignin was formed at the highest severity CELF reactions, but the mechanism behind pseudo lignin formation and its properties are not clear. Delignification of biomass improved its digestibility, but a deeper understanding is needed of the threshold of lignin removal beyond which further removal negatively impacts digestibility. Although *Kluveromyces marxianus* is a promising strain for high temperature fermentations, fermentation arrest and cell shrinkage due to the combined influence of elevated temperatures and high ethanol concentrations in an SSF environment prompts the need for further research to understand how to overcome its response to stressful conditions and guide metabolic engineering to create a better and more tolerant version for future applications. Improving the ethanol tolerance of *Saccharomyces cerevisiae* strain M11205 or exploring other organisms with a higher ethanol tolerance and the capability to co-ferment glucose and xylose is also recommended to further enhance ethanol titers and yields.