UNIVERSITY OF CALIFORNIA
RIVERSIDE

Biological Conversion of Alamo Switchgrass Carbohydrates 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

Abhishek S. Patri

June 2018

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

The Dissertation of Abhishek S. Patri is approved:

Committee Chairperson

University of California, Riverside

ACKNOWLEDGEMENTS

I would like to show my gratitude and appreciation to Dr. Charles E. Wyman for his continued support, advice and guidance during my years as a graduate student in his laboratory. The lessons and values that I have learned from my time working under him are ones that I will carry forward with me forever. I would also like to thank Dr. Eugene A. Nothnagel and Dr. Ian Wheeldon for their insight and suggestions to my thesis research. Additionally, I would like to thank Dr. Rajeev Kumar, Dr. Charles M. Cai and Dr. Bhogeswararao Seemala for their mentorship and collaborative thinking towards the completion of my research.

I would like to acknowledge Dr. Samarthya Bhagia, Dr. Thanh Yen Nguyen and Dr. Vanessa A. Thomas for taking me under their wings as a new graduate student and teaching me the necessary skills to carry out my research. I would also like to acknowledge all the members of Dr. Wyman's research team who were instrumental in maintaining to laboratory culture that I enjoyed working in: Christian Alcaraz, Rachna Dhir, Ninad Kothari, Aakash Parikh, May Ling Lu, Nikhil Nagane, Priya Sengupta, Priyanka Singh and Glen Svenningsen, as well as my undergraduate students Hamzah Alshatari and Laura McAlister who contributed greatly to various parts of my research.

Beyond the laboratory, I would like to thank my friends Peter Byrley, Michelle Chebeir, Pankaj Ramnani and Brandon Simmons-Rawls for our continued friendship that has been crucial to my overall positive experience during graduate school. Finally, I would like to thank my parents, Krishnamoorthy Seshachalam and Sundar Sheshu Seshachalam, my brother, Adarsh S. Patri, and my fiancée, Dr. Archana K. Reddy. 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 Office of Biological and Environmental Research in the Department of Energy (DOE) Office of Science through the BioEnergy Science Center at Oak Ridge National Laboratory (Contract DE-PS02-06ER64304) and the National Center for Sustainable Transportation 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 my parents, Krishnamoorthy Seshachalam and Sundar Sheshu Seshachalam.

ABSTRACT OF THE DISSERTATION

Biological Conversion of Alamo Switchgrass Carbohydrates Following Co-Solvent Pretreatment

by

Abhishek S. Patri

Doctor of Philosophy, Graduate Program in Chemical and Environmental Engineering
University of California, Riverside, June 2018
Dr. Charles E. Wyman, Chairperson

Lignocellulosic biomass is a renewable resource that can be converted to liquid fuels to reduce global dependence on fossil fuels and minimize greenhouse gas emissions. However, plants have evolved to protect their valuable cell wall polysaccharides through various mechanisms altogether termed recalcitrance. Pretreatment can overcome biomass recalcitrance and increase access to cellulose, although technologies require costly enzyme loadings to achieve high sugar yields after pretreatment, making biofuels unable to compete with petroleum-derived fuels. Recently, Co-solvent Enhanced Lignocellulosic Fractionation (CELF) has been developed as an advanced pretreatment technology to achieve high sugar yields from biomass at low enzyme loadings. In this thesis, the reasons behind increased digestibility of Alamo switchgrass after CELF were investigated in comparison to research benchmark dilute sulfuric acid (DSA) pretreatment. CELF was found to solubilize all of the hemicellulose and nearly 80% of lignin from switchgrass. Protein quantification revealed that residual lignin in CELF

solids bound negligible amounts of enzyme compared to those from DSA pretreatment. Further analysis, in conjunction with scanning electron microscopy, suggested that unlike DSA, CELF prevented lignin redeposition during pretreatment. Molecular dynamics simulations and chemical characterization techniques showed that the tetrahydrofuran (THF):water co-solvent used during CELF was responsible for unraveling of lignin structure during pretreatment and facilitating acid-catalyzed hydrolysis of lignin inter-unit linkages. CELF was also found to be capable of achieving near identical sugar yields from unmilled and milled switchgrass, unlike DSA, thus potentially eliminating an entire processing step. In subsequent work, the liquid hydrolyzate after CELF pretreatment was found to contain fermentable sugars, lignin-derived compounds and a unique sugar-derived surfactant. The surfactant was found to be highly stimulatory to aerobic and anaerobic performance of *Saccharomyces cerevisiae*. Lignin-derived phenolics in CELF hydrolyzate were found to be the major source of inhibition to fermenting microorganisms after removal of THF. Immiscible organic solvents were successful in extracting the majority of lignin-derived phenolics to overcome inhibition. Additionally, *Saccharomyces cerevisiae* was capable of acclimatizing to CELF hydrolyzate inhibitors by exposure to incremental concentrations of hydrolyzate and whole cell recycle in successive fermentation runs.

Table of Contents

List of Figures

List of Tables

Chapter 1

Introduction

1.1 Global Energy Climate and Need for Alternative Energy

Global economic growth in recent decades has strongly correlated with the increasing use of fossil-derived fuels, more particularly oil (Murphy and Hall 2011). Global consumption has increased 10-fold in the last century (Bilgen 2014). The U.S. Energy Information Administration's latest predictions project a 28% increase in world energy use by the year 2040 with a steady increase in oil consumption and most of this growth expected to come from countries where demand is driven by strong economic growth, such as China and India (EIA 2017). Peak Oil, first expressed by Marion King Hubbert, refers to a point in time when the global society reaches the maximum possible rate of extraction of petroleum, i.e., barrels of oil per day (Hubbert 1956). After this point, it is predicted that demand for oil will begin to overtake supply and extraction of oil will become more difficult and expensive (Kerschner, Prell et al. 2013). Some experts believe that the world is currently entering the era of peak oil (Campbell 1998, Deffeyes 2004). Cheap crude oil sources are expected to be depleted in the next 20-30 years at current consumption rates and increasing oil supply will require a higher price oil from high-cost sources, such as oil sands and ultra-deep reservoirs (Murphy and Hall 2011). This situation creates an economic growth paradox where increasing oil supply to maintain economic growth will require high oil prices that will, in turn, undermine that economic growth (Murphy and Hall 2011). Further, oil reserves are unequally distributed around the world and the industry is wholly globalized with 60% of global oil supply internationally traded (Gupta 2008). As oil reserves are depleted, the increasing demand will cause political tensions to escalate, as the majority of proven reserves are in

countries characterized with high degrees of political instability (Gupta 2008). Increased fossil fuel use also contributes to increased greenhouse gas (GHG) emissions, resulting in global climate change (Ramanathan and Feng 2008, Schellnhuber 2008), which can cause irreversible damage to the climate (Solomon, Plattner et al. 2009). Up to 30% of carbon dioxide can also remain in the atmosphere for 2-20 centuries after its release (Archer, Eby et al. 2009) causing the greenhouse effect on the Earth. Global surface temperature has steadily increased in the past 130 years (Jones, Wigley et al. 1986) with a rapid increase of ~0.2 °C per decade in the past 30 years (Hansen, Sato et al. 2006). The impact of global climate change is expected to cause a reduction in overall agricultural yields, extinction of vulnerable species, and profound implications on economic and social systems that depend on them (Fischer, Frohberg et al. 1994, Thomas, Cameron et al. 2004, Harley, Hughes et al. 2006).

The outlined concerns of depleting oil resources, potential political instability and environmental concerns have intensified the need for alternative energy sources. Wind, solar, and nuclear power are leading candidates for alternative energy production. Solar power, in particular, is attractive as the sun supplies the Earth with 86,000 trillion Watts of energy at all times, which is 6600 times the amount of energy that all humans use every year (Kerr and Service 2005). The use and application of materials that exhibit photovoltaic properties to convert sunlight into electricity has made significant strides in recent years albeit with hurdles that need to be overcome to provide widespread cost-effective solar energy (Kannan and Vakeesan 2016). Future developments in nanotechnology, materials and physical sciences may enable step-change approaches

towards cost-effective and globally scalable systems for solar energy use (Lewis 2007). However, with the demand for energy currently pressing, currently available sources of effective solar energy capture are required.

Photosynthetic organisms, including purple bacteria, cyanobacteria, green-sulfur bacteria, green algae and plants, have evolved a variety of light-harvesting proteins that allow them to capture incident solar energy and combine carbon dioxide and water molecules to form sugars, such as glucose. These sugars serve as intermediates for synthesis of complex organic compounds found in plant matter. One form of sugar storage is starch, which can be hydrolyzed and converted to free sugars and fermented to ethanol. The U.S. has produced nearly 5 billion gallons of corn ethanol in the year 2005 (Wang, Hong et al. 2007). However, current corn ethanol production technologies, while reducing petroleum use significantly, have GHG emissions similar to those of gasoline (Farrell, Plevin et al. 2006). Therefore, alternative source of plant sugars are required to be explored. A second form of sugar storage in plants is the production of plant biomass, also known as lignocellulosic biomass, which is primarily made up of three major components by mass – cellulose, hemicellulose and lignin. Cellulose and hemicellulose are complex polysaccharides, comprised of hexose and mostly pentose sugars, respectively. Lignin is a complex heterogeneous aromatic polymer that is crucial to the structural integrity of plant cell walls (Grabber 2005). The sugars present in polysaccharides make up nearly 70% of plant matter and have the potential to be converted to fuels using fermenting microorganisms to produce ethanol fuel (Lynd, Wyman et al. 1999). Lignocellulosic biomass can be made available as perennial plants

grown on degraded lands abandoned from agricultural use, crop residues, sustainably harvested wood and forest residues, energy crops grown along with food crops, and municipal and industrial solid wastes (Tilman, Socolow et al. 2009). The U.S. Department of Energy (DOE) projects a potential of over 1 billion dry tons of biomass per year to be available by 2030 (U.S. Department of Energy. 2016). Assuming the achievement of 100 gallons of ethanol from one ton of dry biomass (Wyman 2007), there is potential to produce 100 billion gallons of ethanol per year from lignocellulosic feedstocks. Ethanol from lignocellulosic sources has been projected to decrease GHG emissions by 88% in comparison to gasoline (Farrell, Plevin et al. 2006). Ethanol's energy density is one-third that of gasoline. However, ethanol possesses a higher average octane number than gasoline, allowing for higher engine compression ratios and an estimated a 15% increase in power from a spark ignition engine (Bailey 1996). Technological advances in automobile engineering have been met with the majority of automobiles in Brazil running on high blends of ethanol with gasoline (Potter 2008). In the U.S., ethanol usage is currently restricted by the E10 "blend wall", a 10% blend of ethanol with gasoline. The barrier to increasing ethanol consumption up to an 85% blend with gasoline (E85) is an economic one that requires a 15% reduction in ethanol costs (Babcock and Pouliot 2013), thus highlighting the need for cost-effective sustainably-derived ethanol.

1.2 Biological Conversion of Lignocellulosic Biomass to Ethanol

In this thesis, conversion of lignocellulosic biomass to ethanol was explored using biological methods of conversion. In summary, lignocellulosic biomass is mechanically

processed to appropriate particle size, before being fed to a pretreatment stage followed by enzymatic saccharification of polysaccharides to monomeric sugars, and finally, fermentation of the released sugars to ethanol. Pretreatment is typically required to overcome plants' natural ability to resist biological breakdown termed recalcitrance (Yang and Wyman 2008). The goal of pretreatment is to release cellulose from the cell wall matrix for greater enzymatic access while preserving sugars for further downstream fermentation (Mosier, Wyman et al. 2005). Numerous pretreatment methods have been developed, however, without any being able to achieve commercially relevant processing costs, thus stifling successful industrial adoption of biofuels. Dilute sulfuric acid (DSA) pretreatment utilizes small concentrations of sulfuric acid to solubilize hemicellulose and disrupt the cell wall matrix to increase cellulose accessibility to enzymatic digestion (Lloyd and Wyman 2005). DSA is a research and commercial benchmark pretreatment, which is the basis for many techno-economic models for the production of cellulosic fuels (Kazi, Fortman et al. 2010, Klein-Marcuschamer, Oleskowicz-Popiel et al. 2012). In particular, enzyme costs can be extremely expensive, with estimates placing the cost as high as $1.47/gal of ethanol produced at recently reported yields (Klein-Marcuschamer, Oleskowicz-Popiel et al. 2012). Recently, Co-solvent Enhanced Lignocellulosic Fractionation (CELF) has been developed as an advanced pretreatment that is capable of achieving high sugar yields while reducing enzyme loadings by one order of magnitude (Nguyen, Cai et al. 2015). CELF utilizes a mixture of tetrahydrofuran (THF) and water with an acid catalyst to solubilize the majority of hemicellulose and lignin, thus leaving behind highly digestible glucan-rich solids. However, the underlying reasons behind

greater sugar yields and prolonged enzymatic activity on CELF solids have not been fully understood. Further, the molecular principles behind effective co-solvent pretreatment have only been addressed with computational simulations (Smith, Cheng et al. 2015, Mostofian, Cai et al. 2016, Smith, Mostofian et al. 2016) and require further experimental analysis. The fermentability of solubilized sugars in CELF liquid hydrolyzate has also not been explored. Numerous compounds are released during pretreatment, many of which can be inhibitory to fermentation microorganisms (Palmqvist and Hahn-Hagerdal 2000), however, the inhibition caused by compounds solubilized and generated during CELF has not been identified. The results of CELF solids digestibility and CELF hydrolyzate fermentability are compared to those from DSA, thus assessing the how the incorporation of tetrahydrofuran during acidic pretreatments can affect biological conversion of pretreatment solids and liquids by enzymatic and microbial means.

1.3 Thesis Organization

Senescent switchgrass (*Panicum virgatum*), a perennial grass species, of Alamo variety was used almost exclusively in this work as the source of lignocellulosic biomass. Some experimental results were compared with those achieved with hardwoods, poplar (*Populus trichocarpa*) and maple (*Acer*). Chapter 2 discusses characteristics of lignocellulosic biomass, pretreatments, fermenting microorganisms, and potential inhibitors to fermentation. Chapter 3 compares the enzymatic hydrolysis sugar yields from DSA and CELF pretreated switchgrass at varying enzyme loadings and quantifies the amount of enzyme that is uncompetitively bound to lignin during hydrolysis of the two substrates. Additionally, it also investigates the removal and redeposition of lignin

during the two pretreatments, and quantifies the impact of lignin redeposition on enzymatic hydrolysis from DSA pretreated switchgrass. Chapter 4 elucidates molecular principles behind co-solvent pretreatment with the aid of molecular dynamics simulations, nano infra-red imaging, enzymatic kinetics and chemical characterization experiments. This work was performed with the aid of collaborators from Oak Ridge National Laboratory (ORNL), University of Central Florida (UCF), and University of Tennessee Knoxville (UTK), and the underlying principles behind effective biomass pretreatment are illustrated and a template for future pretreatment design is proposed. Chapter 5 investigates the effect of switchgrass pre-soaking and particle size reduction prior to DSA and CELF on pretreated solids compositions and enzymatic digestibility. Chapter 6 identifies components in CELF liquid hydrolyzate and assesses their effect on fermentability of solubilized sugars by an engineered strain of *Saccharomyces cerevisiae*. Further, a solvent extraction technique is proposed that can remove inhibitors and increase fermentation ethanol yields from CELF hydrolyzate. Chapter 7 discusses a compound that dramatically enhances aerobic and anaerobic performance of *S. cerevisiae* and can be produced from glucose, and is produced during CELF of various lignocellulosic feedstocks and model compounds. This compound is the first of its kind to show a striking improvement in fermentation performance at very low concentrations and far outperforms commercially available fermentation enhancing compounds, such as the surfactant Tween 20. Chapter 8 investigates acclimatization strategies of *S. cerevisiae* to overcome inhibition by dissolved compounds and ferment sugars present in CELF hydrolyzate at high ethanol yields and fermentation rates. Finally, chapter 9 summarizes

main findings and conclusions from the dissertation and looks at future research potential.

1.4 References

Archer, D., M. Eby, V. Brovkin, A. Ridgwell, L. Cao, U. Mikolajewicz, K. Caldeira, K. Matsumoto, G. Munhoven, A. Montenegro and K. Tokos (2009). "Atmospheric lifetime of fossil fuel carbon dioxide." Annual Review of Earth and Planetary Sciences **37**: 117-134.

Babcock, B. A. and S. Pouliot (2013). "Price it and they will buy: how E85 can break the blend wall." CARD Policy Briefs **13**

Bailey, B. K. (1996). "Performance of ethanol as a transportation fuel." Handbook on Bioethanol: Production and Utilization: 37-60.

Bilgen, S. (2014). "Structure and environmental impact of global energy consumption." Renewable & Sustainable Energy Reviews **38**: 890-902.

Campbell, C. J. (1998). "The end of cheap oil." Scientific American **278**(6): 8a-8a.

Deffeyes, K. S. (2004). "Hubbert's peak: the impending world oil shortage." Abstracts of Papers of the American Chemical Society **228**: U670-U670.

EIA (2017). "International Energy Outlook 2017." U.S. Energy Information Administration **DOE/EIA-0484(2017)**.

Farrell, A. E., R. J. Plevin, B. T. Turner, A. D. Jones, M. O'Hare and D. M. Kammen (2006). "Ethanol can contribute to energy and environmental goals." Science **311**(5760): 506-508.

Fischer, G., K. Frohberg, M. L. Parry and C. Rosenzweig (1994). "Climate-change and world food-supply, demand and trade - who benefits, who loses." Global Environmental Change-Human and Policy Dimensions **4**(1): 7-23.

Grabber, J. H. (2005). "How do lignin composition, structure, and cross-linking affect degradability? A review of cell wall model studies." Crop Science **45**(3): 820-831.

Gupta, E. (2008). "Oil vulnerability index of oil-importing countries." Energy Policy **36**(3): 1195-1211.

Hansen, J., M. Sato, R. Ruedy, K. Lo, D. W. Lea and M. Medina-Elizade (2006). "Global temperature change." Proceedings of the National Academy of Sciences of the United States of America **103**(39): 14288-14293.

Harley, C. D. G., A. R. Hughes, K. M. Hultgren, B. G. Miner, C. J. B. Sorte, C. S. Thornber, L. F. Rodriguez, L. Tomanek and S. L. Williams (2006). "The impacts of climate change in coastal marine systems." Ecology Letters **9**(2): 228-241.

Hubbert, M. K. (1956). "Nuclear energy and the fossil fuels." Drilling and Production Practice **95**: 1-40.

Jones, P. D., T. M. L. Wigley and P. B. Wright (1986). "Global temperature-variations between 1861 and 1984." Nature **322**(6078): 430-434.

Kannan, N. and D. Vakeesan (2016). "Solar energy for future world: - a review." Renewable & Sustainable Energy Reviews **62**: 1092-1105.

Kazi, F. K., J. A. Fortman, R. P. Anex, D. D. Hsu, A. Aden, A. Dutta and G. Kothandaraman (2010). "Techno-economic comparison of process technologies for biochemical ethanol production from corn stover." Fuel **89**: S20-S28.

Kerr, R. A. and R. F. Service (2005). "What can replace cheap oil - and when." Science **309**(5731): 101-101.

Kerschner, C., C. Prell, K. S. Peng and K. Hubacek (2013). "Economic vulnerability to peak oil." Global Environmental Change-Human and Policy Dimensions **23**(6): 1424-1433.

Klein-Marcuschamer, D., P. Oleskowicz-Popiel, B. A. Simmons and H. W. Blanch (2012). "The challenge of enzyme cost in the production of lignocellulosic biofuels." Biotechnology and Bioengineering **109**(4): 1083-1087.

Lewis, N. S. (2007). "Toward cost-effective solar energy use." Science **315**(5813): 798-801.

Lloyd, T. A. and C. E. Wyman (2005). "Combined sugar yields for dilute sulfuric acid pretreatment of corn stover followed by enzymatic hydrolysis of the remaining solids." Bioresource Technology **96**(18): 1967-1977.

Lynd, L. R., C. E. Wyman and T. U. Gerngross (1999). "Biocommodity engineering." Biotechnology Progress **15**(5): 777-793.

Mosier, N., C. Wyman, B. Dale, R. Elander, Y. Y. Lee, M. Holtzapple and M. Ladisch (2005). "Features of promising technologies for pretreatment of lignocellulosic biomass." Bioresource Technology **96**(6): 673-686.

Mostofian, B., C. M. Cai, M. D. Smith, L. Petridis, X. L. Cheng, C. E. Wyman and J. C. Smith (2016). "Local phase separation of co-solvents enhances pretreatment of biomass for bioenergy applications." Journal of the American Chemical Society **138**(34): 10869-10878.

Murphy, D. J. and C. A. S. Hall (2011). "Energy return on investment, peak oil, and the end of economic growth." Ecological Economics Reviews **1219**: 52-72.

Nguyen, T. Y., C. M. Cai, R. Kumar and C. E. Wyman (2015). "Co-solvent pretreatment reduces costly enzyme requirements for high sugar and ethanol yields from lignocellulosic biomass." ChemSusChem **8**(10): 1716-1725.

Palmqvist, E. and B. Hahn-Hagerdal (2000). "Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition." Bioresource Technology **74**(1): 25-33.

Potter, N. I. (2008). "How Brazil achieved energy independence and the lessons the United States should learn from Brazil's experience." Wash. U. Global Stud. L. Rev. **7**: 331.

Ramanathan, V. and Y. Feng (2008). "On avoiding dangerous anthropogenic interference with the climate system: Formidable challenges ahead." Proceedings of the National Academy of Sciences of the United States of America **105**(38): 14245-14250.

Schellnhuber, H. J. (2008). "Global warming: stop worrying, start panicking?" Proceedings of the National Academy of Sciences of the United States of America **105**(38): 14239-14240.

Smith, M., X. L. Cheng, L. Petridis, B. Mostofian and J. Smith (2015). "Modifications to the structure and dynamics of lignin under THF-water co-solvent systems." Abstracts of Papers of the American Chemical Society **249**.

Smith, M. D., B. Mostofian, X. L. Cheng, L. Petridis, C. M. Cai, C. E. Wyman and J. C. Smith (2016). "Cosolvent pretreatment in cellulosic biofuel production: effect of tetrahydrofuran-water on lignin structure and dynamics." Green Chemistry **18**(5): 1268-1277.

Solomon, S., G. K. Plattner, R. Knutti and P. Friedlingstein (2009). "Irreversible climate change due to carbon dioxide emissions." Proceedings of the National Academy of Sciences of the United States of America **106**(6): 1704-1709.

Thomas, C. D., A. Cameron, R. E. Green, M. Bakkenes, L. J. Beaumont, Y. C. Collingham, B. F. N. Erasmus, M. F. de Siqueira, A. Grainger, L. Hannah, L. Hughes, B. Huntley, A. S. van Jaarsveld, G. F. Midgley, L. Miles, M. A. Ortega-Huerta, A. T. Peterson, O. L. Phillips and S. E. Williams (2004). "Extinction risk from climate change." Nature **427**(6970): 145-148.

Tilman, D., R. Socolow, J. A. Foley, J. Hill, E. Larson, L. Lynd, S. Pacala, J. Reilly, T. Searchinger, C. Somerville and R. Williams (2009). "Beneficial biofuels-the food, energy, and environment trilemma." Science **325**(5938): 270-271.

Wang, M., M. W. Hong and H. Huo (2007). "Life-cycle energy and greenhouse gas emission impacts of different corn ethanol plant types." Environmental Research Letters **2**(2).

Wyman, C. E. (2007). "What is (and is not) vital to advancing cellulosic ethanol." Trends in Biotechnology **25**(4): 153-157.

Yang, B. and C. E. Wyman (2008). "Pretreatment: the key to unlocking low-cost cellulosic ethanol." Biofuels Bioproducts & Biorefining **2**(1): 26-40.

Chapter 2

Fermentation Inhibitors Arising From Pretreatment Products and By-Products

2.1 Abstract

Lignocellulosic biomass is a promising renewable resource for the production of sustainable fuels to displace traditional fossil-derived fuels. Production of biomass-derived fuels relies on fermentation of sugars present in plant cell wall polysaccharides. Biomass pretreatment is performed to release polysaccharides and sugars from the cell wall and allow for access by enzymes and fermenting microorganisms. Following thermochemical pretreatment, a liquid hydrolyzate is produced, which contains fermentable sugars. However, pretreatment also generates numerous other compounds that are inhibitory to fermenting microorganisms. These inhibitors are generated from sugars, lignin, and other compounds that are present in pretreatment reactors. Methods of overcoming inhibition have been studied and developed. In particular, detoxification of hydrolyzates by physical, chemical and biological methods have been studied. The goal of detoxification methods is to reduce the concentrations of one or more inhibitors and increase ethanol fermentation yields from the resulting detoxified hydrolyzate in comparison to the hydorlyzate prior to detoxification. Additionally, fermenting microorganisms have demonstrated their ability to acclimatize to high concentrations of inhibitors and overcome severe inhibition, thus eliminating the need for hydrolyzate detoxification prior to fermentation.

2.2 Introduction

The world currently relies heavily on petroleum-derived fuels to meet its growing energy needs. Moreover, some analysts believe that global oil production may have already hit its peak and political tensions around the world may cause a decrease in the

production of crude oil (Kerr 2005, Aleklett, Hook et al. 2010). In order to meet this ever increasing gap between the production and demand of liquid fuels, biomass-derived fuels, particularly bioethanol, have been proposed as sustainable alternative fuels (Council 1999). Currently, bioethanol is produced from sugars in sugarcane and starch in corn (Solomon, Barnes et al. 2007). However, starch-derived ethanol is dependent on feedstock that is considered surplus to human and livestock food. Additionally, corn ethanol technologies require high amounts of water and can have unfavorable environmental impacts upon scale up (Giampietro, Ulgiati et al. 1997). Alternatively, lignocellulosic biomass provides a unique and promising feedstock for the long term sustainable generation of fuels and chemicals with greater than 1 billion tons of dry lignocellulosic matter predicted to be available by 2030 (M. H. Langholtz 2016). Additionally, the US Environmental Protection Agency (EPA) has issued proposed volume requirements in Renewable Fuel Standard (2017) of 238 million gallons of cellulosic biofuels for 2019.

Lignocellulosic feedstocks consist of two types of materials: agricultural and forest residues, and dedicated energy crops. Agricultural and forest residues including materials such as corn stover and sugar cane bagasse, are available in abundance throughout the world as much of this material is left unused or burned (Kim and Dale 2004). Energy crops, such as switchgrass, poplar, and other woody feedstocks are of particular interest due to their comparatively high biomass yields and perennial nature (Na, Sollenberger et al. 2014). Lignocellulosic biomass is comprised of polysaccharides, lignin, protein, and inorganics. Cellulose and hemicellulose are structural polysaccharides

present in lignocellulosic biomass. Cellulose is a polymer of the six carbon sugar glucose. Hemicellulose is a polymer mostly consisting of five carbon sugars, such as xylose and arabinose, and is more structurally variable than cellulose. Pectins are also plant polysaccharides that are restricted to the primary cell wall and provide assistance during cell wall growth (Willats, McCartney et al. 2001). Due to their relatively high abundance in plant matter, both cellulose and hemicellulose are targets for hydrolysis to monomeric five and six carbon sugars that can be further fermented to ethanol. During plant cell wall synthesis however, cell wall polysaccharides are locked in a complex matrix that limits their access for conversion to monomeric units (Mcmillan 1994, Cosgrove 2005, Wyman, Dale et al. 2005). Lignin, formed by the polymerization of monolignol precursors, is deposited in plant cell walls to structurally reinforce cellulose microfibrils in both primary and secondary cell wall (Iiyama, Lam et al. 1994). The resulting cell wall, composed of lignin and polysaccharides is generally called the lignin-carbohydrate complex (LCC) (Whitmore 1978) and the highly hydrophobic and cross-linked nature of lignin is a major factor responsible for the resistance of plant cell walls to breakdown, termed biomass recalcitrance (Grabber 2005). Numerous contributing factors have been identified towards recalcitrance, namely crosslinking due to lignin and hemicellulose, cellulose crystallinity, and degree of depolymerization amongst others (DeMartini, Pattathil et al. 2013, Silveira, Stoyanov et al. 2013).

To remedy this, biomass pretreatment technologies have been developed to disrupt the cell wall matrix and increase access to cellulose for further downstream operations (Mosier, Wyman et al. 2005). Pretreatment techniques have been touted as

having great potential to increase efficiency of a biomass refinery and reduce the overall cost of biomass processing (Kohlmann, Sarikaya et al. 1995, Ladisch, Mosier et al. 2003). The goal of an effective pretreatment is to maximize total sugar release during pretreatment and subsequent downstream processing at an affordable cost (Mosier, Wyman et al. 2005). Numerous pretreatment technologies have been developed aimed at tackling recalcitrance with diverse focuses. Broadly, pretreatments can be classified into physical and chemical methods. Physical pretreatment methods were first developed to involve mechanical size reduction to improve process handling and increase enzymatic access to polysaccharides (Millett, Effland et al. 1979, Himmel, Tucker et al. 1986, Sidiras and Koukios 1989). The energy requirements for most physical pretreatment methods, however, are considered uneconomical (Kumar, Barrett et al. 2009, Lin, Huang et al. 2010, Barakat, de Vries et al. 2013). Owing to the added energy input requirements associated with mechanical size reduction (Tassinari, Macy et al. 1980, Mani, Tabil et al. 2004), physical pretreatment methods have recently been investigated in tandem with chemical methods (Hu and Wen 2008, Harun, Radiah et al. 2011). Further, microwave irradiation of lignocellulosic materials has been shown to improve enzymatic accessibility and digestibility (Azuma, Tanaka et al. 1984, Ma, Liu et al. 2009, Binod, Satyanagalakshmi et al. 2012). Recently, co-treatment by combining ball milling with biological breakdown using *Clostridium thermocellum* has been shown to achieve nearly 90% digestion of senescent switchgrass (Balch, Holwerda et al. 2017). Chemical pretreatment methods, also referred to as thermochemical pretreatment, typically utilize elevated temperatures, sometimes with acid or base catalysts, to promote hydrolysis of

cellulose by aiming to chemically remove hemicellulose and lignin from the cell wall. Steam explosion and liquid hot water pretreatments were developed to take advantage of water's acidic nature at high temperatures to solubilize hemicellulose and increase enzymatic hydrolysis yields of cellulose (Bobleter, Niesner et al. 1976, Brownell and Saddler 1987, Bobleter 1994, Kubikova, Zemann et al. 1996, VanWalsum, Allen et al. 1996, Weil, Sarikaya et al. 1997). Dilute acid pretreatment was developed with the goal of solubilizing hemicellulose to monomeric sugars and disrupting the lignin-cellulose matrix for greater enzymatic activity (Bienkowski, Ladisch et al. 1984, Lee, Iyer et al. 1999). Alkali pretreatments are able to heavily delignify plant cell walls, thus leaving behind cellulose and hemicellulose for enzymes to hydrolyze (Sun, Lawther et al. 1995). Ammonia fiber explosion (AFEX) combines steam explosion and liquid ammonia to achieve nearly complete glucose yield after pretreatment (Teymouri, Laureano-Perez et al. 2004, Alizadeh, Teymouri et al. 2005). Biological pretreatments, many utilizing white rot fungi, have been shown to be promising when implemented as additions to existing chemical pretreatment technologies (Muller and Trosch 1986, Itoh, Wada et al. 2003, Hakala, Lundell et al. 2005).

Aqueous chemical pretreatments may offer the advantages of being environmentally friendly, reducing capital and operational costs, and reducing separation complexity (Garrote, Dominguez et al. 1999, Wyman, Dale et al. 2005). Moreover, advanced pretreatments have been shown to reduce the amount of expensive enzyme required to achieve high sugar yields (Li, Knierim et al. 2010, Nguyen, Cai et al. 2015). These advances have been able to improve sugar yields from cellulose. However,

following chemical pretreatment, compounds can be produced and become part of pretreated solids that are potentially inhibitory to cellulolytic enzymes (Selig, Viamajala et al. 2007, Hu, Jung et al. 2012, Kumar, Hu et al. 2013). Further, hemicellulose sugars are typically solubilized into the liquid fraction along with numerous potential inhibitory compounds (Palmqvist, HahnHagerdal et al. 1996, Klinke, Ahring et al. 2002, Klinke, Olsson et al. 2003).

2.3 Biomass Chemical Pretreatment Technologies

The nature of inhibitory products formed during pretreatment are dependent on the pretreatment type employed. Therefore, this review first briefly describes major pretreatment types.

2.3.1 Hydrothermal Pretreatment

Hydrothermal pretreatment utilizes liquid or vapor phase water during pretreatment to solubilize hemicellulose and swell cell wall structure for greater cellulose accessibility to enzymes (Bobleter, Niesner et al. 1976, Mok and Antal 1992, Walch, Zemann et al. 1992, Bobleter 1994, Allen, Kam et al. 1996, VanWalsum, Allen et al. 1996). Autoionization in water at pretreatment temperatures releases H^+ ions that catalyze hemicellulose solubilization. In addition to hemicellulose oligomers, acetic and uronic acids are also introduced into the pretreatment hydrolyzate during hydrothermal pretreatment. While acetic acid is itself inhibitory to microorganisms (Vanzyl, Prior et al. 1991, Narendranath, Thomas et al. 2001), the presence of acids lowers the pH of pretreatment hydrolyzate and can drive the acid-catalyzed dehydration of solubilized

sugars to sugar degradation products, such as furfural and 5-hydroxymethylfurfural (HMF) at high pretreatment severities (Moller, Nilges et al. 2011).

2.3.2 Acid Pretreatment

Acid-based pretreatments have been the research and commercial benchmark for biomass pretreatment. The introduction of small amounts of sulfuric acid during pretreatment has been demonstrated to be highly effective at converting hemicellulose to monomeric sugars and releasing cellulose for the lignin matrix for improved enzymatic digestibility (Lloyd and Wyman 2005). However, in addition to acetic acid and sugar dehydration products that are released during hydrothermal pretreatment, dilute sulfuric acid releases highly inhibitory phenolic groups into solution as a result of the interaction with inter-unit lignin linkages (Mcmillan 1994, Parajo, Dominguez et al. 1998, Villa, Felipe et al. 1998). Under acidic conditions, HMF can also further degrade to levulinic and formic acid (Hall 1984). Furfural also degrades in the presence of acid, and particularly quickly in the presence of sugars (Danon, van der Aa et al. 2013). Further, because of the severity of dilute acid pretreatments, corrosion of reactor vessels over time can release metal ions into the hydrolyzate. Heavy metal ions have been shown to inhibit growth and sugar metabolism in microorganisms (Watson, Prior et al. 1984). Recent pretreatment technologies, such as sulfite pretreatment (SPORL) have been able to reduce sugar degradation during pretreatment (Zhu, Pan et al. 2009).

2.3.3 Alkali Pretreatment

Alkali pretreatments involve using bases, such as sodium hydroxide and potassium hydroxide, to delignify biomass and improve enzymatic yield (Mosier, Wyman

et al. 2005). Alkali pretreatments are commonly performed at temperatures lower than acid pretreatments, and solubilize less hemicellulose and inhibitors. Other alkaline compounds, such as calcium hydroxide and ammonia, have been used to perform lime pretreatments and ammonia fiber expansion (AFEX) (Watson, Prior et al. 1984). As alkali methods leave behind the majority of polysaccharides in the pretreated solids, the hydrolyzate is usually not fermented.

2.3.4 Solvent Pretreatments

Pretreatments that employ solvents, such as organic solvents (Pan, Xie et al. 2008) or ionic liquids (Karatzos, Edye et al. 2012) aim to remove lignin from biomass to ensure limited interference with enzymatic digestion. Depending on the nature of solvent used during pretreatment, residual solvent amounts can remain in pretreatment hydrolyzate and may be themselves inhibitory to fermentation microorganisms (Yang and Wyman 2008). Organosolv pretreatment utilizes high severities to solubilize lignin and hemicellulose into pretreatment liquid (Holtzapple and Humphrey 1981, Pan, Arato et al. 2005, Pan, Gilkes et al. 2006, Pan, Xie et al. 2008). At typical organosolv reaction conditions, soluble sugars are converted to dehydration products and low fermentation yields are observed using organosolv hydrolyzate (Zhao, Cheng et al. 2009, Amiri, Karimi et al. 2014).

2.4 Pretreatment Products and By-Products Found in Hydrolyzate

2.4.1 Sugars

During the course of acid-catalyzed pretreatment, polysaccharides are solubilized to form either oligomeric or monomeric sugars. Based on the feedstock type being

utilized, these sugars can vary. However, in general, sugars solubilized during pretreatment include glucose, xylose, arabinose, mannose, galactose, rhamnose, and their oligomers. The amount of sugars solubilized during pretreatment depends on the crystallinity of the polysaccharides and severity of pretreatment. These sugars are the target substrates of downstream fermentation processes.

2.4.2 Sugar-derived By-products

As sugars are solubilized, they can further react to form other products. Hemicellulose-derived pentose sugars dehydrate to furfural, while glucose dehydrates to HMF (Mosier, Wyman et al. 2005, Saha, Iten et al. 2005). Furfural can further degrade to formic acid and can also condense to form resins or further degrade to formic acid (Danon, van der Aa et al. 2013). HMF is more stable than furfural but also degrades at high severities to levulinic and formic acids (Hall 1984). Under alkaline conditions, while the majority of polysaccharides are left in the solids, some degradation occurs to saccharinic acid, dihydroxy and dicarboxylic acids (Hall 1984). Acetic acid from hemicellulose side groups, while not directly derived from sugars, is also solubilized in both acid and alkaline pretreatments.

2.4.3 Lignin-derived By-products

Lignin contains many inter-unit linkages, including β-O-4 aryl ether and other acid-sensitive linkages. The hydrolysis of these bonds releases phenolic compounds into acid pretreatment hydrolyzate. The molecular weight of these phenolic compounds can vary greatly based on the feedstock and pretreatment severity. The most common phenolic compounds found in acid pretreatment hydrolyzate are p-coumaric acid, ferulic

acid, vanillin, 4-hydroxybenzoic acid, syringyl aldehyde and coniferyl aldehyde (Larsson, Reimann et al. 1999, Palmqvist and Hahn-Hagerdal 2000).

2.4.4 Extractives and Heavy Metals

Different biomass feedstocks contain varying amounts of extractives (which include acidic resins, sterols and terpenes) (Thammasouk, Tandjo et al. 1997) that are solubilized during some pretreatment types. These compounds can be extracted from feedstocks by water or solvent-washing (Sluiter, Ruiz et al. 2008, Passos, Freire et al. 2014). Heavy metals can also be solubilized into pretreatment hydrolyzate, either during feedstock harvesting and handling, or during corrosion of pretreatment reactors.

2.5 Fermentation Microorganisms and Methods

Numerous microorganisms have been investigated as potential candidates for fermentation of sugars to ethanol. *Saccharomyces cerevisiae*, however, remains the organism of choice for commercial fermentation processes. In recent years, however, *Zymamonas mobilis* has been proposed as a potential replacement to *S. cerevisiae* as some of its traits can be advantageous to fermentation processes.

2.5.1 *Saccharomyces cerevisiae*

Saccharomyces cerevisiae has been the outstanding candidate for commercial fermentation processes due to its high ethanol tolerance, ability to grow under strict anaerobic conditions and tolerance of low pH (Van Maris, Winkler et al. 2007). The main metabolic pathway for sugar fermentation in *S. cerevisiae* is the Embden-Meyerhof-Parnas (EMP) pathway shown in Figure 2.1 during which one molecule of glucose is converted to two molecules of pyruvate, which is further converted to ethanol under

anaerobic conditions (Zaldivar, Borges et al. 2002). *S. cerevisiae* has been engineered to ferment xylose and other pentose sugars that it does not natively uptake (Kotter and Ciriacy 1993). *S. cerevisiae* is able to slowly metabolize the pentose sugar xylulose, which is a keto-isomer of xylose. Therefore, strategies to incorporate xylose fermentation into *S. cerevisiae* involve strategies to convert xylose to xylulose (Wang and Schneider 1980, Matsushika, Inoue et al. 2009). Further, recent research has focused on improved xylose uptake into the cell to facilitate higher ethanol yields (Jin, Alper et al. 2005, Apel, Ouellet et al. 2016). Theoretical mass yield of ethanol from sugars is 0.511 (Bai, Anderson et al. 2008) and yields of 90-93% of theoretical maximum can be achieved. Additionally, CO_2 and other by-products, including glycerol, and organic acids are produced during fermentation.

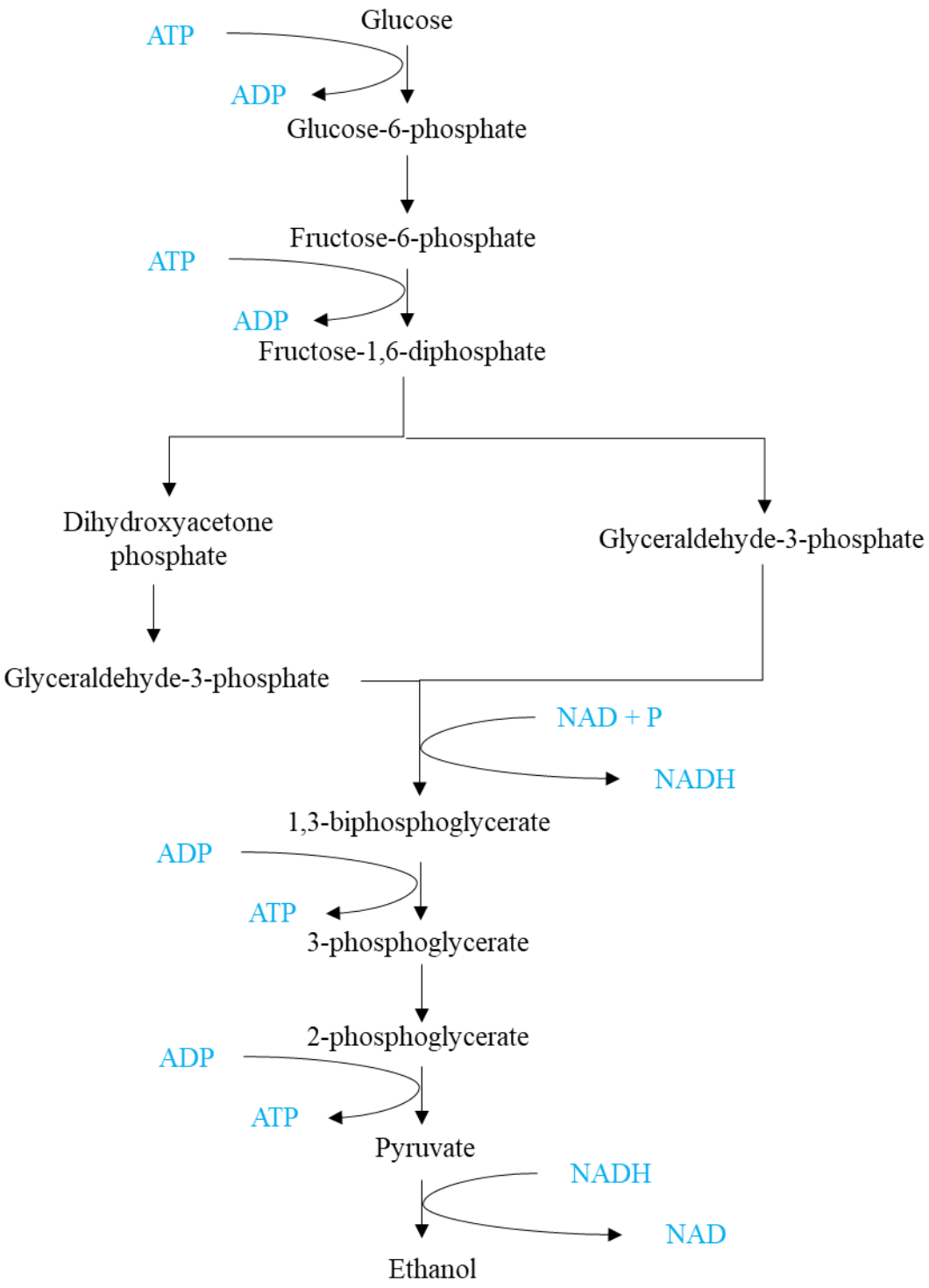


Figure 2.1. Simplified metabolic pathway of ethanol fermentation in *S. cerevisiae* (Bai, Anderson et al. 2008).

2.5.2 *Zymomonas mobilis*

Zymomonas mobilis is a gram-negative bacterium that converts glucose to ethanol and has significant advantages over *S. cerevisiae*. The metabolism for ethanol production is the Entner-Doudoroff (ED) pathway shown in Figure 2.2 (Conway 1992). During the ED pathway, less sugar is utilized in biomass production, and therefore, greater ethanol yields (as high as 97%) can be achieved (Sprenger 1996). Additionally, *Z. mobilis* maintains higher glucose metabolic flux, and can achieve ethanol productivity 3-5 times higher than of *S. cerevisiae* (Sprenger 1996). *Z. mobilis* has also been engineered to convert xylose and other pentose sugars by introducing pentose phosphase pathways (Zhang, Eddy et al. 1995). However, *Z. mobilis* has its disadvantages that have prevented it from being adopted at commercial scales. In particular, *Z. mobilis* biomass is not commonly acceptable to be used as animal feed, which generates the problem of its biomass disposal if adopted at industrial scales (Bai, Anderson et al. 2008). Further, continuous ethanol fermentation with *Z. mobilis* tends to be oscillatory, which may reduce overall ethanol yields (Daugulis, McLellan et al. 1997).

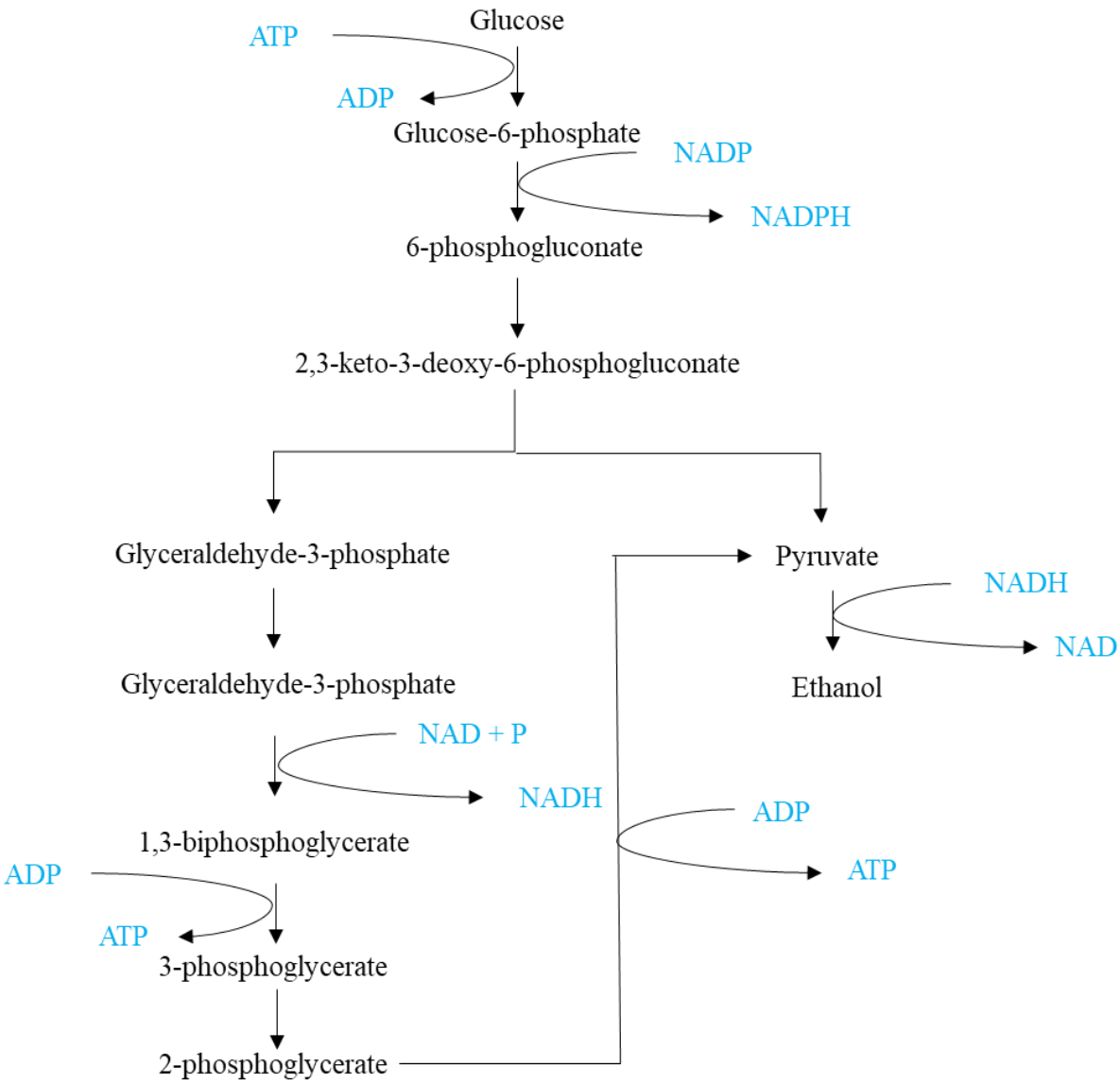


Figure 2.2. Simplified metabolic pathway of ethanol fermentation in *Z. mobilis* (Yang, Fei et al. 2016).

2.5.3 Fermentation Methods

Fermentation processes are performed in batch, fed-batch or continuous configuration. Each has its advantages and drawbacks, and the choice of fermentation

method is based on the nature of the process and potential inhibitors that may be introduced or arise during fermentation.

2.5.3.1 Batch and Fed-Batch Fermentation

Batch fermentation refers to a process in a vessel with an initial volume of medium containing substrates, nutrients and cells that is not altered by further addition or removal. This type of fermentation is simple and is widely used both in the laboratory and industrially (Shuler and Kargi 2002). The seed culture for batch fermentation is either grown in a separate fermenter or more economically, the cells are recycled from previous runs after completion (Palmqvist and Hahn-Hagerdal 2000). A drawback of batch pretreatments is that the internal conditions of the reactor cannot be controlled during operation. Therefore, the accumulation of various types of inhibitors or changes in reactor pH and dissolved oxygen concentration can impact fermentation process yields. An improvement on this is fed-batch processes, which involve the addition of substrate at a low rate to reduce the effect of high inhibitor concentrations that may be introduced with the substrate (Taherzadeh, Niklasson et al. 1999). While inhibitor amounts gradually increase, it provides sufficient time for the fermenting microorganisms to either bioconvert or acclimatize to inhibiting compounds.

2.5.3.2 Continuous Fermentation

The environment within a batch reactor is constantly changing as a result of substrate and nutrient consumption and product accumulation. In continuous fermentation, fresh substrate and nutrients streams are constantly being supplied into a well-stirred reactor while products and cells are simultaneously withdrawn (Shuler and

Kargi 2002). While continuous fermenters were thought to be incapable of maintaining high fermentation rates (Cysewski and Wilke 1978), modern continuous fermentation processes have been optimized to achieve high productivities, high process flexibility and are considered to be less expensive for ethanol production than batch processes (Zanin, Santana et al. 2000, Brethauer and Wyman 2010). Further, as the outlet concentrations are measured in real time, the internal reactor conditions can be adjusted to optimize fermentation conditions by means of pH adjustment, nutrient addition, etc.

2.6 Effect of Products and By-Products on Fermentation Microorganisms

Many of the products and by-products described in section 2.4 can have an effect on microorganisms during fermentation processes. The configuration of the fermentation process can have an impact on the accumulation of products and by-products that can impact fermentation performance. This section outlines the effect of various pretreatment products and by-products, as well as fermentation products, on fermenting microorganisms.

2.6.1 Effect of Sugars

Sugars are the primary carbon source for aerobic growth and the primary substrate during anaerobic fermentation by microorganisms. However, high sugar fermentations, while desirable for industrial applications, can cause a decrease in growth and cell viability likely due to increased osmotic stress on the cells (Xu, Thomas et al. 1996, Bafrncova, Smogrovicova et al. 1999, Ivorra, Perez-Ortin et al. 1999). It is believed that sugar concentrations and nitrogen limitation during fermentation regulate the synthesis of glucolytic enzymes (Thomas, Hynes et al. 1996). Further, inhibition of sugar transport by

limited nitrogen availability is a major factor limiting fermentation metabolism of high sugar concentrations (Salmon, Vincent et al. 1993, Salmon and Barre 1998).

2.6.2 Effect of Sugar and Carbohydrate By-Products

By-products of pretreatment arising from sugars and carbohydrates include weak acids, sugar dehydration products and sugar oligomers. Weak acids, including acetic, formic, and levulinic acids, can diffuse into the cell membrane as undissociated acids and dissociate within the cell to reduce inter-cellular pH (Pampulha and Loureirodias 1989, Palmqvist and Hahn-Hagerdal 2000). The mechanism of inhibition after diffusion into the cell has been hypothesized to be one of two possibilities: uncoupling and intracellular anion accumulation (Russell 1992). The former hypothesizes that a lower intracellular pH is neutralized by ATPase, which pumps protons out of the cell, and in turn, reduces ATP hydrolysis rates (Palmqvist and Hahn-Hagerdal 2000). At high acid concentrations, this results in a depletion of ATP and reduction of fermentation yields. The latter hypothesis of intercellular anion accumulation is suggested from the fact that the anionic form of weak acids do not traverse the cell membrane (Casal, Cardoso et al. 1996). Accordingly, the anionic form is captured within the cell and the undissociated form diffuses into the cell until equilibrium is achieved, resulting in high concentrations of the anionic form at low extracellular pH (Palmqvist and Hahn-Hagerdal 2000). The activity of glycolytic enzymes in the presence of weak acids, such as acetic acid, has been shown to decrease, thus reducing fermentation performance (Pampulha and Loureirodias 1990). At low concentrations, however, weak acids have been demonstrated to be stimulating to ethanol production by *S. cerevisiae* (Pampulha and Loureirodias 1989).

Furfural and HMF are the other major sugar-derived by-products that are released during pretreatment. Both furfural and HMF are metabolized by *S. cerevisiae* (Taherzadeh, Gustafsson et al. 1999, Taherzadeh, Gustafsson et al. 2000). Furfural is reduced to furfuryl alcohol during fermentation (Taherzadeh, Gustafsson et al. 1999). Furfural has been shown to reduce aerobic and anaerobic performance in microorganisms (Palmqvist and Hahn-Hagerdal 2000). Furfural inhibition is a function of furfural concentration, cell density, culture conditions, and aeration (Palmqvist, Almeida et al. 1999). Furfural was found to inhibit the enzyme alcohol dehydrogenase which is responsible for providing a constant supply of NAD^+, thus causing reduced ethanol yields during fermentation by *S. cerevisiae* (Banerjee, Bhatnagar et al. 1981, Modig, Liden et al. 2002). Further, furfural has been reported to affect glycolytic and TCA fluxes, which are involved in energy metabolism (Horvath, Franzen et al. 2003). HMF is converted to 5-hydroxymethyl furfuryl alcohol, suggesting that a similar mechanism of HMF inhibition occurs (Palmqvist and Hahn-Hagerdal 2000). In general, furans, such as furfural and HMF, inhibit ethanol fermentation by re-directing cellular energy towards addressing damage caused by furans, and by reduced intracellular levels of ATP or NADH, by inhibiting enzymatic activity (Almeida, Modig et al. 2007).

2.6.3 Effect of Lignin-Derived By-Products

Phenolic compounds that are released as a result of lignin depolymerization can penetrate the cell membrane and cause a loss of integrity (Palmqvist and Hahn-Hagerdal 2000). Low molecular weight phenolics have been reported to be more inhibitory to *S. cerevisiae* than high molecular weight phenolics (Klinke, Thomsen et al. 2004). In

general, aldehydes and ketones are more inhibitory than acids, which are more inhibitory than alcohols (Almeida, Modig et al. 2007). Mechanisms of inhibition from phenolics have yet to be studied in detail and have not yet been clearly elucidated, largely due to their heterogeneity and poor analytical techniques (Almeida, Modig et al. 2007). However, phenolics are believed to disrupt biological membranes, affecting their ability to serve as selective barriers and enzyme matrices (Heipieper, Weber et al. 1994). Further, weakly acidic phenolics may transport protons back across the mitochondrial membranes and destroy the electrochemical gradient (Almeida, Modig et al. 2007).

2.6.4 Effect of Extractives and Heavy Metals

Heavy metals have been reported to be toxic to microorganisms, being used as fungicides (Ross 1975, Gadd and Griffiths 1978). Nickel inhibits yeast fermentation following its transport into a non-exchangeable compartment of the cell and inhibiting alcohol dehydrogenase (Fuhrmann and Rothstein 1968). Further, copper, cadmium, silver, silver and mercury have all been found to be toxic to yeasts (White and Munns 1951).

2.6.5 Effect of Fermentation Products

In addition to pretreatment by-products present in fermentation broths, fermentation products can cause inhibition at high concentrations. Ethanol inhibition typically occurs to *Saccharomyces cerevisiae* at concentrations > 150 g/L (Bai, Anderson et al. 2008), however, ethanol tolerance varies for different strains. High ethanol concentrations affect the integrity of the cell membrane, damage permeability to ionic species, acidify intracellular conditions, and perturb key enzyme conformation preventing

effective sugar conversion (Ma and Liu 2010). Further, ethanol may also affect the activity of membrane ATPase (Casey and Ingledew 1986).

2.6.6 Combined Effects

Acetic acid and furfural have been shown to interact and cause greater inhibition than expected from the accumulation of individual effects (Palmqvist and Hahn-Hagerdal 2000). Ethanol inhibition has been observed to be exacerbated by the presence of fermentation by-products such as aldehydes and ketones, and other stresses such as high temperatures (Jones 1989). Ethanol has also been shown to increase the effect of acetic acid inhibition on fermenting yeasts (Pampulha and Loureirodias 1989).

2.7 Methods of Overcoming Inhibition

The inhibition to fermentation from several compounds as described in section 2.6 can be overcome by two major methods: detoxification of hydrolyzate and microorganism acclimatization.

2.7.1 Detoxification of Hydrolyzate

Detoxification involves the use of physical, chemical or biological means of reducing the effect of inhibitory compounds in pretreatment hydrolyzates. This is done either by lowering the concentration of inhibitory compounds, or by converting them to less toxic compounds.

2.7.1.1 Physical Detoxification Methods

The physical properties of inhibitors in hydrolyzate, such as volatility and solubility, can be utilized to design detoxification methods for their removal. Hydrolyzate from *Aspen* has been shown to be detoxified by evaporation to near dryness and

subsequent resuspension in fermentation medium, due to the reduction in concentrations of volatile compounds such as acetic acid, furfural and vanillin (Wilson, Deschatelets et al. 1989, Converti, Dominguez et al. 2000). Solvent extraction using organic solvents, such as diethyl ether and ethyl acetate, has been shown to be effective at reducing the concentrations of furfural, HMF and phenolics by preferentially solvating those compounds in the organic solvent, thus increasing fermentation yield from pretreatment hydrolyzate (Clark and Mackie 1984, Wilson, Deschatelets et al. 1989). Activated charcoal is also used as an adsorbent in solid-phase extraction for the removal of lignin-derived phenolics from hydrolyzate, after which the activated charcoal can be recovered and recycled (Converti, Dominguez et al. 2000). Ion exchange resins are also effective for the removal of acetic acid from hydrolyzate. Over 80% of acetic acid has been reported to be removed using an anion exchange resin to increase hydrolyzate fermentation yields (Vanzyl, Prior et al. 1991). Waste fly ash has recently been investigated as an alternative to expensive activated charcoal as an adsorbent (Yucel and Aksu 2015).

2.7.1.2 Chemical Detoxification Methods

Overliming of pretreatment hydrolyzate has been the overwhelming choice of detoxification. Increasing the pH of hydrolyzate to 9-10 with the addition of $Ca(OH)_2$ during overliming and readjustment to 5.5 with sulfuric acid has been reported to improve fermentability of hydrolyzates (Vanzyl, Prior et al. 1988, Palmqvist and Hahn-Hagerdal 2000). Overliming is believed to precipitate inhibitory compounds when a solid precipitate is formed at pH 10, while also providing an unstable environment for some

inhibitors at high pH, thus facilitating their degradation (Palmqvist and Hahn-Hagerdal 2000). This has been demonstrated by the fact that overliming of hydrolyzate to pH 10 and readjustment to pH 5.5 resulted in higher fermentation yields than direct neutralization to pH 5.5 (Palmqvist and Hahn-Hagerdal 2000). Overliming in an effective method of hydrolyzate detoxification, however, with the drawback of excessive gypsum production (Aden, Ruth et al. 2002). A major drawback of overliming is that some sugar loss occurs due to base deconstruction at pH 10 (Martinez, Rodriguez et al. 2000). Recently, bioenergy residues have been pyrolyzed to form pyrochars that were effective as bio-adsorbents for the removal of >90% of furans from pretreatment hydrolyzate (Monlau, Sambusiti et al. 2015). Electrochemical oxidation has been studied with regard to wastewater treatment and detoxification of lignocellulosic hydrolyzates by completely mineralizing most phenolics and reduction in concentrations of lignin-derived phenolics, such as ferulic acid, p-coumaric acid, and vanillin (Canizares, Lobato et al. 2005, Lee, Min et al. 2015).

2.7.1.3 Biological Detoxification Methods

During biological detoxification of hydrolyzates, lignin-degrading enzymes, such as laccases and peroxidases, are utilized to selectively remove phenolics and increase ethanol productivity (Jonsson, Palmqvist et al. 1998). The mechanism of detoxification is believed to be oxidative polymerization of low molecular weight phenolics (Palmqvist and Hahn-Hagerdal 2000). The soft-rot fungus *Trichoderma reesei* has also been reported to degrade fermentation inhibitors by the removal of furfural, acetic acid and phenolics (Palmqvist, HahnHagerdal et al. 1997).

2.7.2 Microorganism Acclimatization

Each of the detoxification methods mentioned above incur added capital and operating cost to a biomass refinery. Microorganism acclimatization is a means by which the fermenting microorganisms are able to adapt and develop a resistance to the inhibitors present in hydrolyzates without prior detoxification. Yeast cells have long been observed to be capable of acclimatization to external factors, leading to eventual increased tolerance (Gray 1946, Johnson and Harris 1948). Further, by continuous adaptation selection, particular yeast strains with higher tolerance of inhibitors have been identified (Chen and Gong 1985). In particular, adaptation of *S. cerevisiae* to furfural has been observed (Chung and Lee 1985, Villa, Bartroli et al. 1992). Such adaptation may occur due to the synthesis of new enzymes for the reduction of furfural as the alcohol dehydrogenase activity has been reported to increase after 48 hours of fermentation at 2 g/L furfural concentrations (Palmqvist and Hahn-Hagerdal 2000). In response to increased ethanol exposure, *S. cerevisiae* has been observed to increase its production of cellular oleic acid that is hypothesized to be incorporated into the cell membrane effecting a compensatory decrease in membrane fluidity to counteract the membrane fluidizing effects of ethanol (You, Rosenfield et al. 2003). *S. cerevisiae* also possesses the ability to metabolize some phenolic compounds (Larsson, Quintana-Sainz et al. 2000, Klinke, Olsson et al. 2003), likely due to the presence of phenylacrylic acid decarboxylase, which is able to convert cinnamic, p-coumaric and ferulic acids (Almeida, Modig et al. 2007). Developing improved strains of fermenting microorganisms via directed evolution is also becoming an increasingly utilized strategy for improvement of

inhibitor and ethanol tolerance (Smolke 2010). Lignin-degrading laccase from fungi have been expressed in *S. cerevisiae* with the goal of incorporating phenolic degradation and reduce inhibition (Bulter, Alcalde et al. 2003). Increased initial inoculum has also been observed to reduce the effect of inhibitors (Leonard and Hajny 1945, Chung and Lee 1985, Yucel and Aksu 2015). High initial cell densities are likely to increase the depletion rate of bioconversible inhibitors. Additionally, growth is more affected by inhibitors than volumetric ethanol productivity due to uncoupling by the presence of weak acids (Palmqvist, Grage et al. 1999), or due to low glycerol production in the presence of furfural (Palmqvist, Almeida et al. 1999). Fed-batch and continuous fermenters aim to achieve cell acclimatization by introducing inhibitors at a low rate and retaining cells in the fermenter to increase cell density (Palmqvist and Hahn-Hagerdal 2000).

2.8 Conclusions

The major inhibitors of fermentation microorganisms arising from chemical pretreatment of lignocellulosic and methods to overcome this inhibition were discussed. The major inhibitors of fermenting microorganisms are furans and phenolics arising during pretreatment. These can be selectively removed from pretreatment hydrolyzates by physical, chemical or biological detoxification methods. However, detoxification requires the incorporation of an additional processing step, thus potentially increasing capital and operating costs. Additionally, some detoxification methods, such as overliming, can cause sugar degradation in hydrolyzate due to the harsh chemical conditions utilized. Inhibition can also be caused by ethanol that is a primary product of

fermentation. High ethanol concentrations can lower cell viability and in turn, reduce overall fermentation efficiency. Fermentation reactors can be configured as fed-batch or continuous to alleviate some of the inhibition caused by hydrolyzate inhibitors and ethanol. Promisingly, microorganisms are able to acclimatize to external conditions and develop resistances to inhibition by various mechanisms, thus allowing for increased ethanol tolerance and the potential of eliminating detoxification steps altogether. Modern genetic engineering techniques have also been studied to incorporate detoxification abilities within the fermenting microorganism.

2.9 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 Lab., Golden, CO.(US).

Aleklett, K., M. Hook, K. Jakobsson, M. Lardelli, S. Snowden and B. Soderbergh (2010). "The peak of the oil age - analyzing the world oil production reference scenario in world energy outlook 2008." Energy Policy **38**(3): 1398-1414.

Alizadeh, H., F. Teymouri, T. I. Gilbert and B. E. Dale (2005). "Pretreatment of switchgrass by ammonia fiber explosion (AFEX)." Applied Biochemistry and Biotechnology **121**: 1133-1141.

Allen, S. G., L. C. Kam, A. J. Zemann and M. J. Antal (1996). "Fractionation of sugar cane with hot, compressed, liquid water." Industrial & Engineering Chemistry Research **35**(8): 2709-2715.

Almeida, J. R. M., T. Modig, A. Petersson, B. Hahn-Hagerdal, G. Liden and M. F. Gorwa-Grauslund (2007). "Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*." Journal of Chemical Technology and Biotechnology **82**(4): 340-349.

Amiri, H., K. Karimi and H. Zilouei (2014). "Organosolv pretreatment of rice straw for efficient acetone, butanol, and ethanol production." Bioresource Technology **152**: 450-456.

Apel, A. R., M. Ouellet, H. Szmidt-Middleton, J. D. Keasling and A. Mukhopadhyay (2016). "Evolved hexose transporter enhances xylose uptake and glucose/xylose co-utilization in *Saccharomyces cerevisiae*." Scientific Reports **6**.

Azuma, J. I., F. Tanaka and T. Koshijima (1984). "Enhancement of enzymatic susceptibility of lignocellulosic wastes by microwave irradiation." Journal of Fermentation Technology **62**(4): 377-384.

Bafrncova, P., D. Smogrovicova, I. Slavikova, J. Patkova and Z. Domeny (1999). "Improvement of very high gravity ethanol fermentation by media supplementation using *Saccharomyces cerevisiae*." Biotechnology Letters **21**(4): 337-341.

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.

Balch, M. L., E. K. Holwerda, M. F. Davis, R. W. Sykes, R. M. Happs, R. Kumar, C. E. Wyman and L. R. Lynd (2017). "Lignocellulose fermentation and residual solids characterization for senescent switchgrass fermentation by *Clostridium thermocellum* in the presence and absence of continuous in situ ball-milling." Energy & Environmental Science **10**(5): 1252-1261.

Banerjee, N., R. Bhatnagar and L. Viswanathan (1981). "Inhibition of glycolysis by furfural in *Saccharomyces cerevisiae*." European Journal of Applied Microbiology and Biotechnology **11**(4): 226-228.

Barakat, A., H. de Vries and X. Rouau (2013). "Dry fractionation process as an important step in current and future lignocellulose biorefineries: a review." Bioresource Technology **134**: 362-373.

Bienkowski, P. R., M. R. Ladisch, M. Voloch and G. T. Tsao (1984). "Acid-hydrolysis of pretreated lignocellulose from corn residue." Biotechnology and Bioengineering: 511-524.

Binod, P., K. Satyanagalakshmi, R. Sindhu, K. U. Janu, R. K. Sukumaran and A. Pandey (2012). "Short duration microwave assisted pretreatment enhances the enzymatic saccharification and fermentable sugar yield from sugarcane bagasse." Renewable Energy **37**(1): 109-116.

Bobleter, O. (1994). "Hydrothermal degradation of polymers derived from plants." Progress in Polymer Science **19**(5): 797-841.

Bobleter, O., R. Niesner and M. Rohr (1976). "Hydrothermal degradation of cellulosic matter to sugars and their fermentative conversion to protein." Journal of Applied Polymer Science **20**(8): 2083-2093.

Brethauer, S. and C. E. Wyman (2010). "Review: continuous hydrolysis and fermentation for cellulosic ethanol production." Bioresource Technology **101**(13): 4862-4874.

Brownell, H. H. and J. N. Saddler (1987). "Steam pretreatment of lignocellulosic material for enhanced enzymatic hydrolysis." Biotechnology and Bioengineering **29**(2): 228-235.

Bulter, T., M. Alcalde, V. Sieber, P. Meinhold, C. Schlachtbauer and F. H. Arnold (2003). "Functional expression of a fungal laccase in *Saccharomyces cerevisiae* by directed evolution." Applied and Environmental Microbiology **69**(2): 987-995.

Canizares, P., J. Lobato, R. Paz, M. A. Rodrigo and C. Saez (2005). "Electrochemical oxidation of phenolic wastes with boron-doped diamond anodes." Water Research **39**(12): 2687-2703.

Casal, M., H. Cardoso and C. Leao (1996). "Mechanisms regulating the transport of acetic acid in *Saccharomyces cerevisiae*." Microbiology-Uk **142**: 1385-1390.

Casey, G. P. and W. M. M. Ingledew (1986). "Ethanol tolerance in yeasts." Crc Critical Reviews in Microbiology **13**(3): 219-280.

Chen, L. F. and C. S. Gong (1985). "Fermentation of sugarcane bagasse hemicellulose hydrolysate to xylitol by a hydrolysate-acclimatized yeast." Journal of Food Science **50**(1): 226-228.

Chung, I. S. and Y. Y. Lee (1985). "Ethanol fermentation of crude acid hydrolyzate of cellulose using high-level yeast inocula." Biotechnology and Bioengineering **27**(3): 308-315.

Clark, T. A. and K. L. Mackie (1984). "Fermentation inhibitors in wood hydrolysates derived from the softwood *Pinus Radiata*." Journal of Chemical Technology and Biotechnology B-Biotechnology **34**(2): 101-110.

Converti, A., J. M. Dominguez, P. Perego, S. S. da Silva and M. Zilli (2000). "Wood hydrolysis and hydrolysate detoxification for subsequent xylitol production." Chemical Engineering & Technology **23**(11): 1013-1020.

Conway, T. (1992). "The Entner-Doudoroff pathway - history, physiology and molecular-biology." Fems Microbiology Letters **103**(1): 1-28.

Cosgrove, D. J. (2005). "Growth of the plant cell wall." Nature Reviews Molecular Cell Biology **6**(11): 850-861.

Council, N. R. (1999). Review of the research strategy for biomass-derived transportation fuels, National Academies Press.

Cysewski, G. R. and C. R. Wilke (1978). "Process design and economic studies of alternative fermentation methods for production of ethanol." Biotechnology and Bioengineering **20**(9): 1421-1444.

Danon, B., L. van der Aa and W. de Jong (2013). "Furfural degradation in a dilute acidic and saline solution in the presence of glucose." Carbohydrate Research **375**: 145-152.

Daugulis, A. J., P. J. McLellan and J. H. Li (1997). "Experimental investigation and modeling of oscillatory behavior in the continuous culture of *Zymomonas mobilis*." Biotechnology and Bioengineering **56**(1): 99-105.

DeMartini, J. D., S. Pattathil, J. S. Miller, H. J. Li, M. G. Hahn and C. E. Wyman (2013). "Investigating plant cell wall components that affect biomass recalcitrance in poplar and switchgrass." Energy & Environmental Science **6**(3): 898-909.

Fuhrmann, G. F. and A. Rothstein (1968). "Mechanism of partial inhibition of fermentation in yeast by nickel ions." Biochimica Et Biophysica Acta **163**(3): 331-+.

Gadd, G. M. and A. J. Griffiths (1978). "Microorganisms and heavy-metal toxicity." Microbial Ecology **4**(4): 303-317.

Garrote, G., H. Dominguez and J. C. Parajo (1999). "Mild autohydrolysis: an environmentally friendly technology for xylooligosaccharide production from wood." Journal of Chemical Technology and Biotechnology **74**(11): 1101-1109.

Giampietro, M., S. Ulgiati and D. Pimentel (1997). "Feasibility of large-scale biofuel production - Does an enlargement of scale change the picture?" Bioscience **47**(9): 587-600.

Grabber, J. H. (2005). "How do lignin composition, structure, and cross-linking affect degradability? A review of cell wall model studies." Crop Science **45**(3): 820-831.

Gray, W. D. (1946). "The acclimatization of yeast to high concentrations of glucose - the subsequent effect upon alcohol tolerance." Journal of Bacteriology **52**(6): 703-709.

Hakala, T. K., T. Lundell, S. Galkin, P. Maijala, N. Kalkkinen and A. Hatakka (2005). "Manganese peroxidases, laccases and oxalic acid from the selective white-rot fungus *Physisporinus rivulosus* grown on spruce wood chips." Enzyme and Microbial Technology **36**(4): 461-468.

Hall, G. S. (1984). "Wood - chemistry, ultrastructure, reactions - Fengel,D, Wegener,G." Nature **310**(5977): 521-521.

Harun, M. Y., A. B. D. Radiah, Z. Z. Abidin and R. Yunus (2011). "Effect of physical pretreatment on dilute acid hydrolysis of water hyacinth (*Eichhornia crassipes*)." Bioresource Technology **102**(8): 5193-5199.

Heipieper, H. J., F. J. Weber, J. Sikkema, H. Keweloh and J. A. M. Debont (1994). "Mechanisms of resistance of whole cells to toxic organic-solvents." Trends in Biotechnology **12**(10): 409-415.

Himmel, M., M. Tucker, J. Baker, C. Rivard, K. Oh and K. Grohmann (1986). Comminution of biomass: hammer and knife mills. New York, John Wiley**:** 39-58.

Holtzapple, M. and A. E. Humphrey (1981). "The effect of organosolv pretreatment on the enzymatic-hydrolysis of Poplar." Abstracts of Papers of the American Chemical Society **182**(Aug): 61-Micr.

Horvath, I. S., C. J. Franzen, M. J. Taherzadeh, C. Niklasson and G. Liden (2003). "Effects of furfural on the respiratory metabolism of *Saccharomyces cerevisiae* in glucose-limited chemostats." Applied and Environmental Microbiology **69**(7): 4076-4086.

Hu, F., S. Jung and A. Ragauskas (2012). "Pseudo-lignin formation and its impact on enzymatic hydrolysis." Bioresource Technology **117**: 7-12.

Hu, Z. H. and Z. Y. Wen (2008). "Enhancing enzymatic digestibility of switchgrass by microwave-assisted alkali pretreatment." Biochemical Engineering Journal **38**(3): 369-378.

Iiyama, K., T. B. T. Lam and B. A. Stone (1994). "Covalent cross-links in the cell-wall." Plant Physiology **104**(2): 315-320.

Itoh, H., M. Wada, Y. Honda, M. Kuwahara and T. Watanabe (2003). "Bioorganosolve pretreatments for simultaneous saccharification and fermentation of beech wood by ethanolysis and white rot fungi." Journal of Biotechnology **103**(3): 273-280.

Ivorra, C., J. E. Perez-Ortin and M. L. del Olmo (1999). "An inverse correlation between stress resistance and stuck fermentations in wine yeasts. A molecular study." Biotechnology and Bioengineering **64**(6): 698-708.

Jin, Y. S., H. Alper, Y. T. Yang and G. Stephanopoulos (2005). "Improvement of xylose uptake and ethanol production in recombinant *Saccharomyces cerevisiae* through an inverse metabolic engineering approach." Applied and Environmental Microbiology **71**(12): 8249-8256.

Johnson, M. C. and E. E. Harris (1948). "Acclimatization of various yeasts to wood sugar." Journal of the American Chemical Society **70**(9): 2961-2963.

Jones, R. P. (1989). "Biological principles for the effects of ethanol." Enzyme and Microbial Technology **11**(3): 130-153.

Jonsson, L. J., E. Palmqvist, N. O. Nilvebrant and B. Hahn-Hagerdal (1998). "Detoxification of wood hydrolysates with laccase and peroxidase from the white-rot fungus *Trametes versicolor*." Applied Microbiology and Biotechnology **49**(6): 691-697.

Karatzos, S. K., L. A. Edye and W. O. S. Doherty (2012). "Sugarcane bagasse pretreatment using three imidazolium-based ionic liquids; mass balances and enzyme kinetics." Biotechnology for Biofuels **5**.

Kerr, R. A. (2005). "Bumpy road ahead for world's oil." Science **310**(5751): 1106-1108.

Kim, S. and B. E. Dale (2004). "Global potential bioethanol production from wasted crops and crop residues." Biomass & Bioenergy **26**(4): 361-375.

Klinke, H. B., B. K. Ahring, A. S. Schmidt and A. B. Thomsen (2002). "Characterization of degradation products from alkaline wet oxidation of wheat straw." Bioresource Technology **82**(1): 15-26.

Klinke, H. B., L. Olsson, A. B. Thomsen and B. K. Ahring (2003). "Potential inhibitors from wet oxidation of wheat straw and their effect on ethanol production of *Saccharomyces cerevisiae*: wet oxidation and fermentation by yeast." Biotechnology and Bioengineering **81**(6): 738-747.

Klinke, H. B., A. B. Thomsen and B. K. Ahring (2004). "Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass." Applied Microbiology and Biotechnology **66**(1): 10-26.

Kohlmann, K. L., A. Sarikaya, P. J. Westgate, J. Weil, A. Velayudhan, R. Hendrickson and M. R. Ladisch (1995). "Enhanced enzyme activities on hydrated lignocellulosic substrates." Enzymatic Degradation of Insoluble Carbohydrates **618**: 237-255.

Kotter, P. and M. Ciriacy (1993). "Xylose fermentation by *Saccharomyces cerevisiae*." Applied Microbiology and Biotechnology **38**(6): 776-783.

Kubikova, J., A. Zemann, P. Krkoska and O. Bobleter (1996). "Hydrothermal pretreatment of wheat straw for the production of pulp and paper." Tappi Journal **79**(7): 163-169.

Kumar, P., D. M. Barrett, M. J. Delwiche and P. Stroeve (2009). "Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production." Industrial & Engineering Chemistry Research **48**(8): 3713-3729.

Kumar, R., F. Hu, P. Sannigrahi, S. Jung, A. J. Ragauskas and C. E. Wyman (2013). "Carbohydrate derived-pseudo-lignin can retard cellulose biological conversion." Biotechnology and Bioengineering **110**(3): 737-753.

Ladisch, M. R., N. Mosier, R. Hendrickson, R. Dreschel, G. Welch, B. S. Dien and R. Bothast (2003). "Principles and economics of pretreating cellulose in water for ethanol production." Abstracts of Papers of the American Chemical Society **225**: U85-U85.

Larsson, S., A. Quintana-Sainz, A. Reimann, N. O. Nilvebrant and L. J. Jonsson (2000). "Influence of lignocellulose-derived aromatic compounds on oxygen-limited growth and ethanolic fermentation by *Saccharomyces cerevisiae*." Applied Biochemistry and Biotechnology **84-6**: 617-632.

Larsson, S., A. Reimann, N. O. Nilvebrant and L. J. Jonsson (1999). "Comparison of different methods for the detoxification of lignocellulose hydrolyzates of spruce." Applied Biochemistry and Biotechnology **77-9**: 91-103.

Lee, K. M., K. Min, O. Choi, K. Y. Kim, H. M. Woo, Y. Kim, S. O. Han and Y. Um (2015). "Electrochemical detoxification of phenolic compounds in lignocellulosic hydrolysate for *Clostridium* fermentation." Bioresource Technology **187**: 228-234.

Lee, Y., P. Iyer and R. W. Torget (1999). Dilute-acid hydrolysis of lignocellulosic biomass. Recent progress in bioconversion of lignocellulosics, Springer**:** 93-115.

Leonard, R. H. and G. J. Hajny (1945). "Fermentation of wood sugars to ethyl alcohol." Industrial and Engineering Chemistry **37**(4): 390-395.

Li, C. L., B. Knierim, C. Manisseri, R. Arora, H. V. Scheller, M. Auer, K. P. Vogel, B. A. Simmons and S. Singh (2010). "Comparison of dilute acid and ionic liquid pretreatment of switchgrass: biomass recalcitrance, delignification and enzymatic saccharification." Bioresource Technology **101**(13): 4900-4906.

Lin, Z. X., H. Huang, H. M. Zhang, L. Zhang, L. S. Yan and J. W. Chen (2010). "Ball milling pretreatment of corn stover for enhancing the efficiency of enzymatic hydrolysis." Applied Biochemistry and Biotechnology **162**(7): 1872-1880.

Lloyd, T. A. and C. E. Wyman (2005). "Combined sugar yields for dilute sulfuric acid pretreatment of corn stover followed by enzymatic hydrolysis of the remaining solids." Bioresource Technology **96**(18): 1967-1977.

M. H. Langholtz, B. J. S., and L. M. Eaton (Leads) (2016). "2016 billion-ton report: advancing domestic resources for a thriving bioeconomy, volume 1: economic availability of feedstocks." U.S. Department of Energy **ORNL/TM-2016/160.**(Oak Ridge National Laboratory, Oak Ridge, TN): 448.

Ma, H., W. W. Liu, X. Chen, Y. J. Wu and Z. L. Yu (2009). "Enhanced enzymatic saccharification of rice straw by microwave pretreatment." Bioresource Technology **100**(3): 1279-1284.

Ma, M. G. and Z. L. Liu (2010). "Mechanisms of ethanol tolerance in *Saccharomyces cerevisiae*." Applied Microbiology and Biotechnology **87**(3): 829-845.

Mani, S., L. G. Tabil and S. Sokhansanj (2004). "Grinding performance and physical properties of wheat and barley straws, corn stover and switchgrass." Biomass & Bioenergy **27**(4): 339-352.

Martinez, A., M. E. Rodriguez, S. W. York, J. F. Preston and L. O. Ingram (2000). "Effects of Ca(OH)(2) treatments ("overliming") on the composition and toxicity of bagasse hemicellulose hydrolysates." Biotechnology and Bioengineering **69**(5): 526-536.

Matsushika, A., H. Inoue, T. Kodaki and S. Sawayama (2009). "Ethanol production from xylose in engineered *Saccharomyces cerevisiae* strains: current state and perspectives." Applied Microbiology and Biotechnology **84**(1): 37-53.

Mcmillan, J. D. (1994). "Pretreatment of lignocellulosic biomass." Enzymatic Conversion of Biomass for Fuels Production **566**: 292-324.

Millett, M. A., M. J. Effland and D. F. Caulfield (1979). Influence of Fine Grinding on the Hydrolysis of Cellulosic Materials - Acid Vs. Enzymatic, Hydrolysis of Cellulose: Mechanisms of Enzymatic and Acid Catalysis: 71-89

Modig, T., G. Liden and M. J. Taherzadeh (2002). "Inhibition effects of furfural on alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase." Biochemical Journal **363**: 769-776.

Mok, W. S. L. and M. J. Antal (1992). "Uncatalyzed solvolysis of whole biomass hemicellulose by hot compressed liquid water." Industrial & Engineering Chemistry Research **31**(4): 1157-1161.

Moller, M., P. Nilges, F. Harnisch and U. Schroder (2011). "Subcritical water as reaction environment: fundamentals of hydrothermal biomass transformation." Chemsuschem **4**(5): 566-579.

Monlau, F., C. Sambusiti, N. Antoniou, A. Zabaniotou, A. Solhy and A. Barakat (2015). "Pyrochars from bioenergy residue as novel bio-adsorbents for lignocellulosic hydrolysate detoxification." Bioresource Technology **187**: 379-386.

Mosier, N., C. Wyman, B. Dale, R. Elander, Y. Y. Lee, M. Holtzapple and M. Ladisch (2005). "Features of promising technologies for pretreatment of lignocellulosic biomass." Bioresource Technology **96**(6): 673-686.

Muller, H. W. and W. Trosch (1986). "Screening of white-rot fungi for biological pretreatment of wheat straw for biogas production." Applied Microbiology and Biotechnology **24**(2): 180-185.

Na, C. I., L. E. Sollenberger, J. E. Erickson, K. R. Woodard, M. O. Wallau and N. C. Krueger (2014). "Biomass yield and composition of perennial bioenergy grasses at harvests following a freeze event." Agronomy Journal **106**(6): 2255-2262.

Narendranath, N. V., K. C. Thomas and W. M. Ingledew (2001). "Acetic acid and lactic acid inhibition of growth of *Saccharomyces cerevisiae* by different mechanisms." Journal of the American Society of Brewing Chemists **59**(4): 187-194.

Nguyen, T. Y., C. M. Cai, R. Kumar and C. E. Wyman (2015). "Co-solvent pretreatment reduces costly enzyme requirements for high sugar and ethanol yields from lignocellulosic biomass." ChemSusChem **8**(10): 1716-1725.

Palmqvist, E., J. S. Almeida and B. Hahn-Hagerdal (1999). "Influence of furfural on anaerobic glycolytic kinetics of *Saccharomyces cerevisiae* in batch culture." Biotechnology and Bioengineering **62**(4): 447-454.

Palmqvist, E., H. Grage, N. Q. Meinander and B. Hahn-Hagerdal (1999). "Main and interaction effects of acetic acid, furfural, and p-hydroxybenzoic acid on growth and ethanol productivity of yeasts." Biotechnology and Bioengineering **63**(1): 46-55.

Palmqvist, E. and B. Hahn-Hagerdal (2000). "Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification." Bioresource Technology **74**(1): 17-24.

Palmqvist, E. and B. Hahn-Hagerdal (2000). "Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition." Bioresource Technology **74**(1): 25-33.

Palmqvist, E., B. HahnHagerdal, M. Galbe and G. Zacchi (1996). "The effect of water-soluble inhibitors from steam-pretreated willow on enzymatic hydrolysis and ethanol fermentation." Enzyme and Microbial Technology **19**(6): 470-476.

Palmqvist, E., B. HahnHagerdal, Z. Szengyel, G. Zacchi and K. Reczey (1997). "Simultaneous detoxification and enzyme production of hemicellulose hydrolysates obtained after steam pretreatment." Enzyme and Microbial Technology **20**(4): 286-293.

Pampulha, M. E. and M. C. Loureirodias (1989). "Combined effect of acetic-acid, pH and ethanol on intracellular pH of fermenting yeast." Applied Microbiology and Biotechnology **31**(5-6): 547-550.

Pampulha, M. E. and M. C. Loureirodias (1990). "Activity of glycolytic-enzymes of *Saccharomyces cerevisiae* in the presence of acetic-acid." Applied Microbiology and Biotechnology **34**(3): 375-380.

Pan, X., N. Gilkes, J. Kadla, K. Pye, S. Saka, D. Gregg, K. Ehara, D. Xie, D. Lam and J. Saddler (2006). "Bioconversion of hybrid poplar to ethanol and co-products using an organosolv fractionation process: optimization of process yields." Biotechnol Bioeng **94**(5): 851-861.

Pan, X. J., C. Arato, N. Gilkes, D. Gregg, W. Mabee, K. Pye, Z. Z. Xiao, X. Zhang and J. Saddler (2005). "Biorefining of softwoods using ethanol organosolv pulping: preliminary evaluation of process streams for manufacture of fuel-grade ethanol and co-products." Biotechnology and Bioengineering **90**(4): 473-481.

Pan, X. J., D. Xie, R. W. Yu and J. N. Saddler (2008). "The bioconversion of mountain pine beetle-killed lodgepole pine to fuel ethanol using the organosolv process." Biotechnology and Bioengineering **101**(1): 39-48.

Parajo, J. C., H. Dominguez and J. M. Dominguez (1998). "Biotechnological production of xylitol. Part 3: Operation in culture media made from lignocellulose hydrolysates." Bioresource Technology **66**(1): 25-40.

Passos, H., M. G. Freire and J. A. Coutinho (2014). "Ionic liquid solutions as extractive solvents for value-added compounds from biomass." Green Chemistry **16**(12): 4786-4815.

Ross, I. S. (1975). "Some effects of heavy-metals on fungal cells." Transactions of the British Mycological Society **64**(Apr): 175-193.

Russell, J. B. (1992). "Another explanation for the toxicity of fermentation acids at low pH - anion accumulation versus uncoupling." Journal of Applied Bacteriology **73**(5): 363-370.

Saha, B. C., L. B. Iten, M. A. Cotta and Y. V. Wu (2005). "Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol." Process Biochemistry **40**(12): 3693-3700.

Salmon, J. M. and P. Barre (1998). "Improvement of nitrogen assimilation and fermentation kinetics under enological conditions by derepression of alternative nitrogen-assimilatory pathways in an industrial *Saccharomyces cerevisiae* strain." Applied and Environmental Microbiology **64**(10): 3831-3837.

Salmon, J. M., O. Vincent, J. C. Mauricio, M. Bely and P. Barre (1993). "Sugar-transport inhibition and apparent loss of activity in *Saccharomyces cerevisiae* as a major limiting factor of enological fermentations." American Journal of Enology and Viticulture **44**(1): 56-64.

Selig, M. J., S. Viamajala, S. R. Decker, M. P. Tucker, M. E. Himmel and T. B. Vinzant (2007). "Deposition of lignin droplets produced during dilute acid pretreatment of maize stems retards enzymatic hydrolysis of cellulose." Biotechnology Progress **23**(6): 1333-1339.

Shuler, M. L. and F. Kargi (2002). Bioprocess engineering: basic concepts. Upper Saddle River, NJ, Prentice Hall.

Sidiras, D. K. and E. G. Koukios (1989). "Acid saccharification of ball-milled straw." Biomass **19**(4): 289-306.

Silveira, R. L., S. R. Stoyanov, S. Gusarov, M. S. Skaf and A. Kovalenko (2013). "Plant biomass recalcitrance: effect of hemicellulose composition on nanoscale forces that control cell wall strength." Journal of the American Chemical Society **135**(51): 19048-19051.

Sluiter, A., R. Ruiz, C. Scarlata, J. Sluiter and D. Templeton (2008). "Determination of extractives in biomass." National Renewable Energy Laboratory.

Smolke, C. D. (2010). The metabolic pathway engineering handbook : tools and applications. Boca Raton, CRC Press.

Solomon, B. D., J. R. Barnes and K. E. Halvorsen (2007). "Grain and cellulosic ethanol: History, economics, and energy policy." Biomass & Bioenergy **31**(6): 416-425.

Sprenger, G. A. (1996). "Carbohydrate metabolism in *Zymomonas mobilis*: A catabolic highway with some scenic routes." Fems Microbiology Letters **145**(3): 301-307.

Sun, R. C., J. M. Lawther and W. B. Banks (1995). "Influence of alkaline pretreatments on the cell-wall components of wheat-straw." Industrial Crops and Products **4**(2): 127-145.

Taherzadeh, M. J., L. Gustafsson, C. Niklasson and G. Liden (1999). "Conversion of furfural in aerobic and anaerobic batch fermentation of glucose by *Saccharomyces cerevisiae*." Journal of Bioscience and Bioengineering **87**(2): 169-174.

Taherzadeh, M. J., L. Gustafsson, C. Niklasson and G. Liden (2000). "Physiological effects of 5-hydroxymethylfurfural on *Saccharomyces cerevisiae*." Applied Microbiology and Biotechnology **53**(6): 701-708.

Taherzadeh, M. J., C. Niklasson and G. Liden (1999). "Conversion of dilute-acid hydrolyzates of spruce and birch to ethanol by fed-batch fermentation." Bioresource Technology **69**(1): 59-66.

Tassinari, T., C. Macy, L. Spano and D. D. Y. Ryu (1980). "Energy-requirements and process design considerations in compression-milling pretreatment of cellulosic wastes for enzymatic-hydrolysis." Biotechnology and Bioengineering **22**(8): 1689-1705.

Teymouri, F., L. Laureano-Perez, H. Alizadeh and B. E. Dale (2004). "Ammonia fiber explosion treatment of corn stover." Applied Biochemistry and Biotechnology **113**: 951-963.

Thammasouk, K., D. Tandjo and M. H. Penner (1997). "Influence of extractives on the analysis of herbaceous biomass." Journal of Agricultural and Food Chemistry **45**(2): 437-443.

Thomas, K., S. Hynes and W. Ingledew (1996). "Effect of nitrogen limitaton on synthesis of enzymes in *Saccharomyces cerevisiae* during fermentation of high concentration of carbohydrates." Biotechnology letters **18**(10): 1165-1168.

Van Maris, A. J., A. A. Winkler, M. Kuyper, W. T. De Laat, J. P. Van Dijken and J. T. Pronk (2007). Development of efficient xylose fermentation in *Saccharomyces cerevisiae*: xylose isomerase as a key component. Biofuels, Springer**:** 179-204.

VanWalsum, G. P., S. G. Allen, M. J. Spencer, M. S. Laser, M. J. Antal and L. R. Lynd (1996). "Conversion of lignocellulosics pretreated with liquid hot water to ethanol." Applied Biochemistry and Biotechnology **57-8**: 157-170.

Vanzyl, C., B. A. Prior and J. C. Dupreez (1988). "Production of ethanol from sugar-cane bagasse hemicellulose hydrolyzate by *Pichia stipitis*." Applied Biochemistry and Biotechnology **17**: 357-369.

Vanzyl, C., B. A. Prior and J. C. Dupreez (1991). "Acetic-acid inhibition of D-xylose fermentation by *Pichia stipitis*." Enzyme and Microbial Technology **13**(1): 82-86.

Villa, G. P., R. Bartroli, R. Lopez, M. Guerra, M. Enrique, M. Penas, E. Rodriquez, D. Redondo, I. Iglesias and M. Diaz (1992). "Microbial transformation of furfural to furfuryl alcohol by *Saccharomyces cerevisiae*." Engineering in Life Sciences **12**(6): 509-512.

Villa, P., M. Felipe, R. Rodriguez, M. Vitolo, E. Luis dos Reis, S. Silva, A. Napoles and I. Mancilha (1998). Influence of phenolic compounds on the bioprocess of xylitol production by *Candida guilliermondii*. Esbes-2 European Symposium on Biochemical Engineering Science, 2.

Walch, E., A. Zemann, F. Schinner, G. Bonn and O. Bobleter (1992). "Enzymatic saccharification of hemicellulose obtained from hydrothermally pretreated sugar-cane bagasse and beech Bark." Bioresource Technology **39**(2): 173-177.

Wang, P. Y. and H. Schneider (1980). "Growth of yeasts on D-xylulose." Canadian Journal of Microbiology **26**(9): 1165-1168.

Watson, N. E., B. A. Prior, P. M. Lategan and M. Lussi (1984). "Factors in acid-treated bagasse inhibiting ethanol-production from D-xylose by *Pachysolen tannophilus*." Enzyme and Microbial Technology **6**(10): 451-456.

Weil, J., A. Sarikaya, S. L. Rau, J. Goetz, C. M. Ladisch, M. Brewer, R. Hendrickson and M. R. Ladisch (1997). "Pretreatment of yellow poplar sawdust by pressure cooking in water." Applied Biochemistry and Biotechnology **68**(1-2): 21-40.

White, J. and D. Munns (1951). "Inhibitory effect of common elements towards yeast growth." Journal of the Institute of Brewing **57**(3): 175-179.

Whitmore, F. W. (1978). "Lignin-carbohydrate complex formed in isolated cell-walls of *Callus*." Phytochemistry **17**(3): 421-425.

Willats, W. G. T., L. McCartney, W. Mackie and J. P. Knox (2001). "Pectin: cell biology and prospects for functional analysis." Plant Molecular Biology **47**(1-2): 9-27.

Wilson, J. J., L. Deschatelets and N. K. Nishikawa (1989). "Comparative fermentability of enzymatic and acid hydrolysates of steam-pretreated Aspen wood hemicellulose by *Pichia stipitis* Cbs-5776." Applied Microbiology and Biotechnology **31**(5-6): 592-596.

Wyman, C. E., B. E. Dale, R. T. Elander, M. Holtzapple, M. R. Ladisch and Y. Y. Lee (2005). "Coordinated development of leading biomass pretreatment technologies." Bioresource Technology **96**(18): 1959-1966.

Xu, P., A. Thomas and C. D. Gilson (1996). "Combined use of three methods for high concentration ethanol production by *Saccharomyces cerevisiae*." Biotechnology Letters **18**(12): 1439-1440.

Yang, B. and C. E. Wyman (2008). "Pretreatment: the key to unlocking low-cost cellulosic ethanol." Biofuels Bioproducts & Biorefining-Biofpr **2**(1): 26-40.

Yang, S. H., Q. Fei, Y. P. Zhang, L. M. Contreras, S. M. Utturkar, S. D. Brown, M. E. Himmel and M. Zhang (2016). "*Zymomonas mobilis* as a model system for production of biofuels and biochemicals." Microbial Biotechnology **9**(6): 699-717.

You, K. M., C. L. Rosenfield and D. C. Knipple (2003). "Ethanol tolerance in the yeast *Saccharomyces cerevisiae* is dependent on cellular oleic acid content." Applied and Environmental Microbiology **69**(3): 1499-1503.

Yucel, H. G. and Z. Aksu (2015). "Ethanol fermentation characteristics of *Pichia stipitis* yeast from sugar beet pulp hydrolysate: Use of new detoxification methods." Fuel **158**: 793-799.

Zaldivar, J., A. Borges, B. Johansson, H. P. Smits, S. G. Villas-Boas, J. Nielsen and L. Olsson (2002). "Fermentation performance and intracellular metabolite patterns in laboratory and industrial xylose-fermenting *Saccharomyces cerevisiae*." Applied Microbiology and Biotechnology **59**(4-5): 436-442.

Zanin, G. M., C. C. Santana, E. P. S. Bon, R. L. C. Giordano, F. F. de Moraes, S. R. Andrietta, C. C. D. Neto, I. C. Macedo, D. L. Fo, L. P. Ramos and J. D. Fontana (2000). "Brazilian bioethanol program." Applied Biochemistry and Biotechnology **84-6**: 1147-1161.

Zhang, M., C. Eddy, K. Deanda, M. Finkestein and S. Picataggio (1995). "Metabolic engineering of a pentose metabolism pathway in ethanologenic *Zymomonas mobilis*." Science **267**(5195): 240-243.

Zhao, X., K. Cheng and D. Liu (2009). "Organosolv pretreatment of lignocellulosic biomass for enzymatic hydrolysis." Appl Microbiol Biotechnol **82**(5): 815-827.

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): 2411-2418.

Chapter 3

Miscible THF-Water Mixture Removes Lignin from the Lignin-Carbohydrate Complex and Prevents Redeposition from Interfering with Enzymes*

*This chapter was submitted under the following citation:
Patri, A.S., Mohan, R., Cai, C. M., Kumar, R., Wyman, C.E., 2018. Miscible THF-water mixture removes lignin from the lignin-carbohydrate complex and prevents redeposition from interfering with enzymes.

3.1 Abstract

Dilute sulfuric acid (DSA) pretreatment of lignocellulosic biomass facilitates hemicellulose solubilization and enhances enzymatic hydrolysis of cellulose to glucose. However, most of the lignin either redeposits on the surface of DSA pretreated solids or remains intact in the cell wall. This lignin blocks the surface and deactivates enzymes and results in the need for uneconomically high enzyme loadings to achieve high yields. In this study, miscible mixtures of tetrahydrofuran (THF) with water and dilute acid via Co-solvent Enhanced Lignocellulosic Fractionation (CELF) removed nearly 80% of native lignin and most of the hemicellulose from Alamo switchgrass to produce solids that were highly enriched in glucan. Furthermore, enzyme dosages as low as 2 mg protein/g glucan sustained glucan hydrolysis for over 5 weeks that resulted in 90% glucose yields from CELF pretreated switchgrass solids. A modified Ninhydrin-based protein assay revealed that the free-enzyme concentration in the liquid during enzymatic hydrolysis of CELF solids remained unchanged over long culture times. By contrast, a 40% drop in free enzymes in solution was observed for hydrolysis of DSA solids. Furthermore, measuring enzyme adsorption per gram of lignin showed that CELF appeared to prevent redeposition of lignin onto the biomass surface, and the low amount of lignin left on the solids was mostly integral to the original lignin-carbohydrate complex (LCC). Scanning electron micrographs further supported this mechanism. The prolonged activity of cellulase on CELF pretreated solids could be attributed to low lignin in the LCC making more enzymes available for hydrolysis of the readily accessible glucan.

3.2 Introduction

Lignocellulosic biomass is a uniquely abundant resource for the sustainable production of non-petroleum derived fuels and chemicals (Wyman, Dale et al. 2005). Switchgrass, in particular, is being developed as a bioenergy feedstock for the United States, due to its adaptability to varying climate conditions that would allow it to be grown on land not used for production of primary food or cash crops (McLaughlin and Kszos 2005, David and Ragauskas 2010, Shen, Poovaiah et al. 2013). Pretreatment is a key step required to alter the structure of biomass to make the polysaccharides more accessible to enzymes that deconstruct these polymeric carbohydrates to dissolved sugars suitable for fermentation (Mosier, Wyman et al. 2005). During pretreatment, sufficient severity is required to expose cellulose fibers embedded in the complex lignocellulosic matrix. However, at elevated temperatures, sugar degradation to dehydration products lowers the overall total sugar yield from biomass (Kumar, Hu et al. 2013). Further, sugar dehydration products can be severely inhibitory to fermenting microorganisms (Palmqvist and Hahn-Hagerdal 2000). Several pretreatment technologies have been developed to disrupt the lignocellulose matrix and allow for greater accessibility to enzymes, thereby enhancing yields (Mosier, Wyman et al. 2005). However, uneconomically high enzyme loadings are required to achieve high sugar yields during enzymatic hydrolysis from traditionally pretreated biomass mainly due to the presence of lignin which typically remains attached to the solid fraction after pretreatment (Kumar, Tabatabaei et al. 2016, Wyman, Cai et al. 2017). Lignin has been shown to competitively bind enzymes during enzymatic hydrolysis (Yang and Wyman 2006, Vermaas, Petridis et

al. 2015), thus reducing the availability and activity of enzymes during hydrolysis and further affecting potential recovery and recycle of expensive enzymes (Ramos, Breuil et al. 1993). Therefore, an effective pretreatment should minimize sugar degradation during pretreatment (Stage 1) and allow for high sugar yields by subsequent enzymatic hydrolysis (Stage 2) at affordable enzyme loadings.

Dilute sulfuric acid (DSA) pretreatment of lignocellulosic biomass has been shown to be effective at solubilizing the hemicellulose fraction while disrupting the lignocellulose matrix to allow for increased enzymatic access to carbohydrates (Torget, Walter et al. 1991, Lloyd and Wyman 2005). However, during DSA, lignin condenses and relocates on the cellulose surface, thus acting as a physical barrier to enzymatic access of cellulose (Donohoe, Decker et al. 2008, Li, Pu et al. 2014). Co-solvent Enhanced Lignocellulosic Fractionation (CELF) has recently been developed as a pretreatment technology capable of removing the majority of lignin from biomass, while realizing high sugar yields at low enzyme loadings (Nguyen, Cai et al. 2015). The miscible mixture of tetrahydrofuran (THF) with water and dilute acid used for CELF has been demonstrated to preferentially solvate lignin, thus allowing for its facile removal from cellulose and preventing lignin self-aggregation (Smith, Mostofian et al. 2016). In this study, overall release of glucan and xylan was compared for application of DSA and CELF pretreatments to Alamo switchgrass at varying temperatures and times, followed by release of these sugars by enzymatic digestion of DSA and CELF pretreated solids over a range of enzyme loadings. Additionally, the amount of enzyme adsorbed on the residual solids after solubilization of carbohydrates in DSA and CELF pretreated

switchgrass was measured to understand factors affecting sustained enzyme activity and sugar yields. Finally, scanning electron microscope images were employed to picture the extent of surface morphology modifications of switchgrass samples by DSA and CELF pretreatments.

3.3 Experimental

3.3.1 Materials

Senescent Alamo switchgrass provided by Genera Energy Inc. (Vonore, TN) was knife milled to ~ 1 mm particle size using a Wiley Mill (Model 4, Arthur H. Thomas Company, Philadelphia, PA) with a 1 mm particle size interior sieve. A fungal cellulolytic enzyme cocktail, Accellerase® 1500, was provided by DuPont Industrial Biosciences (Palo Alto, CA). The protein concentration was measured, by applying the standard BCA method with bovine serum albumin as a standard, to be 82 mg/ml (Smith, Krohn et al. 1985).

3.3.2 Pretreatment

Pretreatments were performed in a 1 L Hastelloy Parr® autoclave reactor (236HC Series, Parr Instruments Co., Moline, IL) equipped with a double stacked pitch blade impeller rotated at 200 rpm. For DSA reactions, solutions were loaded with 0.5 wt% (based on liquid mass) sulfuric acid (Ricca Chemical Company, Arlington, TX), while in CELF reactions, THF (>99% purity, Fisher Scientific, Pittsburgh, PA) was added to a 0.5 wt% sulfuric acid solution in water at a 0.889:1 THF to acidic water mass ratio. Temperatures for CELF reactions were 140°C and 150°C, while for DSA reactions were run at 150 and 160 °C. Prior to each pretreatment, milled switchgrass (7.5 wt%) was added to the solution and soaked overnight at 4°C. All reactions were maintained at reaction

temperature (± 1°C) by convective heating with a 4 kW fluidized sand bath (Model SBL-2D, Techne, Princeton, NJ). The reaction temperature was directly measured by using an in-line K-type thermocouple (Omega Engineering Inc., Stamford, Connecticut). Following pretreatment, solids were separated from the liquid by vacuum filtration at room temperature through glass fiber filter paper (Fisher Scientific, Pittsburgh, PA) and washed with room temperature deionized water until the filtrate pH reached neutral. The solids were carefully transferred to a Ziplock bag and weighed. The moisture content of the solids was determined by a halogen moisture analyzer (Model HB43, Mettler Toledo, Columbus, OH). Lignin-deposited Avicel (LDA) was prepared as per Li et. al. (Li, Pu et al. 2014).

3.3.3 Enzymatic Hydrolysis

Enzymatic hydrolysis was performed as per the NREL protocol (Selig, Weiss et al. 2008) in triplicate in 125 mL Erlenmeyer flasks with a 50 g total working mass made up of 50 mM sodium citrate buffer (pH 4.9) to maintain the hydrolysis pH and 0.02% sodium azide to prevent microbial contamination together with enough pretreated solids to result in approximately 1 wt% glucan. Accellerase® 1500 cellulase loading was varied from 2 - 65 mg protein/g glucan in unpretreated biomass (Gao, Kumar et al. 2014). Bovine serum albumin (BSA) (Sigma-Aldrich Corp., St. Louis, MO) was added to select enzymatic hydrolysis flasks at a loading of 0.1 g/g glucan roughly 2 hours prior to the addition of cellulase. Enzymatic hydrolysis flasks were placed in a Multitron orbital shaker (Infors HT, Laurel, MD) set at 150 rpm and 50°C and allowed to equilibrate for 1 hour before enzyme addition. Homogenous samples of approximately 500 μL were

collected at 4 hours, 24 hours, and every 24 hours and subsequently loaded into 2 mL centrifuge tubes (Fisher Scientific, Pittsburg, PA) and then centrifuged at 15000 rpm for 10 minutes before analysis of the supernatant by HPLC.

3.3.4 Analytical Procedures

All chemical analyses were performed based on Laboratory Analytical Procedures (LAPs) documented by the National Renewable Energy Laboratory (NREL, Golden, CO). Compositional analysis of unpretreated and pretreated switchgrass was performed according to the NREL procedure (version 8-03-2012) in triplicates (Sluiter, Hames et al. 2008). Liquid samples along with appropriate calibration standards were analyzed on HPLC (Waters Alliance e2695) equipped with a Bio-Rad Aminex® HPX-87H column and RI detector (Waters 2414) with an eluent (5 mM sulfuric acid) flow rate at 0.6 mL/min. The chromatograms were integrated using an Empower® 2 software package (Waters Co., Milford, MA).

3.3.5 Quantification of Free Protein Content in Enzymatic Hydrolysis Liquid

A $NaBH_4$-based modified Ninhydrin assay was used to quantify the protein in enzymatic hydrolysis liquor with reduced interferences from solubilized sugars (Mok, Arantes et al. 2015). In brief, one hundred microliters of sample or standard was incubated at room temperature for 60 minutes with 50 µL of 6.7 g/L $NaBH_4$ in a 1.5 mL microcentrifuge tube. Bovine serum albumin (BSA) in the range of 0 – 2000 mg/L was used as the protein standard. This was followed by the addition of 300 µL of 9 M HCl and subsequent heated in a dry oven at 130°C for 2 hours. After cooling to room temperature, 100 µL of the sample was transferred into a fresh 1.5 mL microcentrifuge tube and neutralized with 100

µL of 5 M NaOH. Upon neutralization, 200 µL of 2% ninhydrin reagent (Sigma-Aldrich Corp., St. Louis, MO) was added to the tubes, which were then heated at 100°C for 10 minutes in a dry oven. After cooling to room temperature, 500 µL of 50% (v/v) ethanol was added to each tube. Finally, 200 µL of colored solution was transferred to a 96-well microplate, and absorbance was read at 560 nm using a SpectraMax M2e Microplate Reader (Molecular Devices, Sunnyvale, CA). All samples were analyzed in triplicate.

3.3.6 Scanning Electron Microscopy

Unpretreated and pretreated switchgrass 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 with Pt/Pd (Cressington 108 Auto) for 90 seconds to form a conductive coating (~ 10-15 nanometer thickness), and subsequently examined with a Tescan MIRA3 GMU scanning electron microscope at an accelerating voltage of 5 kV and a working distance of 5 mm.

3.3.7 Calculations

Following HPLC quantification, the mass of each sugar was converted to the mass of the corresponding anhydrous form by multiplying cellobiose values by 0.95, glucose values by 0.90, and xylose values by 0.88 to compensate for the mass of water added during hydrolysis.

Mass of sugar released in pretreatment hydrolysate = Sugar concentration from HPLC* Volume of pretreatment hydrolysate

Volume of pretreatment hydrolysate = (Total reaction mass – (Mass of wet pretreated solids * Moisture content))/ Hydrolysate density

Stage 1 and Stage 2 yields, referring to sugar yields during pretreatment and enzymatic hydrolysis, respectively, were calculated as follows:

Stage 1 sugar (glucan or xylan) yield = 100 * (Concentration of monomeric sugar measured by HPLC * anhydrous correction factor * volume of pretreatment hydrolyzate)/ Absolute sugar (glucan or xylan) in unpretreated biomass

Enzyme loading = mg of protein per gram of glucan in enzymatic hydrolysis flask/ glucan yield fraction after pretreatment

Glucose Yield % = (Concentration of monomeric sugar measured by HPLC * anhydrous correction factor * total reaction volume of enzymatic hydrolysis flask)/ Mass of glucan in enzymatic hydrolysis flask

Stage 2 sugar yield = (Enzymatic glucose or xylose yield % * mass of glucan or xylan fed to stage 2)/ Mass of polymeric sugar in solid before pretreatment

Stage 1 + 2 (glucan + xylan) yield = (Stage 1 glucan + xylan yield %) + (Stage 2 glucan + xylan yield %)

Following free protein quantification using a spectrophotometer, the free protein mass in solution was calculated as follows:

Free protein measured in solution, mg = Free protein concentration * Volume of enzymatic hydrolysis liquid

Protein adsorbed per gram of residual solids = (Free protein measured before hydrolysis – Free protein measured after complete glucan hydrolysis)/ Mass of residual solids after hydrolysis

% Protein adsorbed after complete glucan hydrolysis = 100 * (Free protein measured before hydrolysis – Free protein measured after complete glucan hydrolysis)/ Free protein measured before hydrolysis

3.4 Results and Discussion

3.4.1 Maximizing Overall Glucose and Xylose Sugar Yields from Switchgrass by DSA and CELF Pretreatments Followed by Enzymatic Hydrolysis

Switchgrass was treated with DSA and CELF pretreatments to identify conditions that maximized glucan and xylan yields from each pretreatment coupled with subsequent enzymatic hydrolysis of the pretreated solids. DSA pretreatments were performed at 150 and 160°C and CELF pretreatments were performed at 140 and 150 °C as these temperature ranges have previously been shown to be optimum for switchgrass (Shi, Ebrik et al. 2011) and corn stover (Nguyen, Cai et al. 2015), respectively. The reaction sets at each temperature were carried out over a range of reaction times to be sure that differences in biomass sources did not alter the time to achieve the highest yields. The liquid hydrolysates from both pretreatments were analyzed for total glucose and xylose including gluco- and xylo-oligomers. The sugar yields from either pretreatment step alone were termed Stage 1 release. The pretreated solids were then subjected to

enzymatic hydrolysis at a high cellulase loading of 65 mg protein/g glucan in unpretreated switchgrass to determine the maximum possible sugar release at each pretreatment condition. The yields from enzymatic hydrolysis of the washed pretreated solids were termed Stage 2 release. Figures 3.1 and 3.2 illustrate the trends in glucan and xylan released in Stages 1 and 2 alone, as well as the combined glucan and xylan yields from Stage 1+2 together.

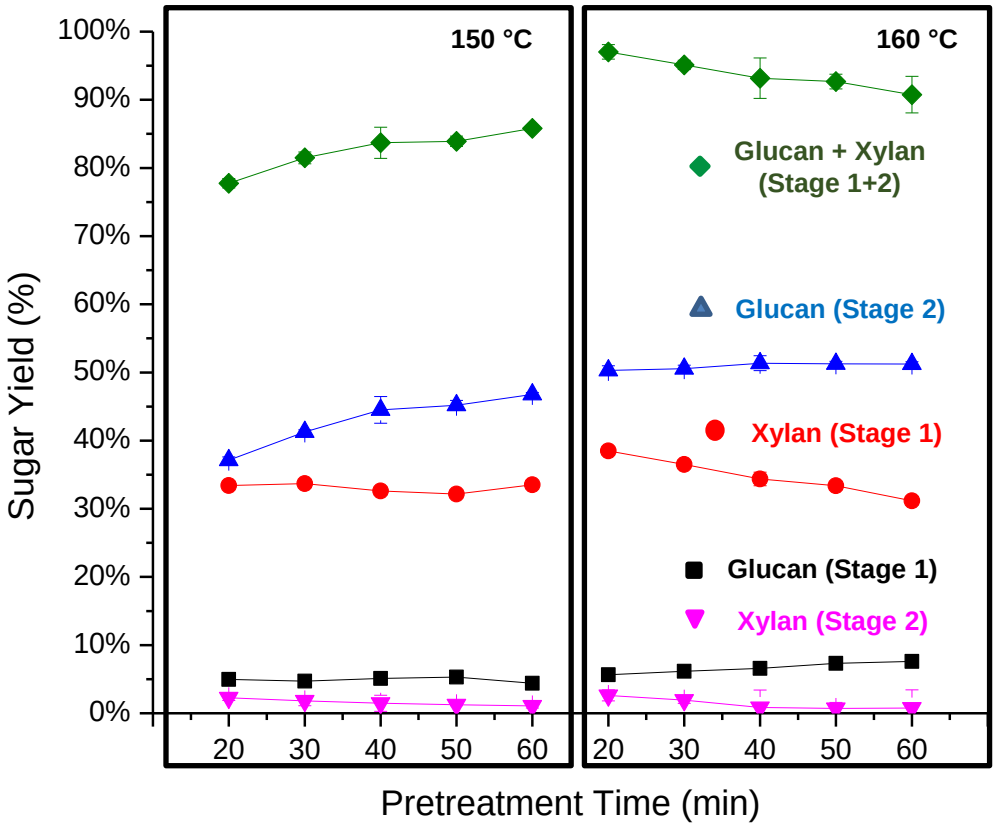


Figure 3.1. Effect of pretreatment time at 150°C and 160°C on glucan, xylan, and total glucan plus xylan yields from dilute sulfuric acid (DSA) pretreatment (Stage 1) of switchgrass, enzymatic hydrolysis of the pretreated solids (Stage 2), and the two stages combined. Stage 1 reaction conditions: solids loading- 7.5 wt%; acid loading- 0.5 wt%. Stage 2 enzymatic hydrolysis was performed on pretreated solids at a 10 g/L glucan loading by 65 mg of Accellerase 1500® protein/g glucan in unpretreated switchgrass.

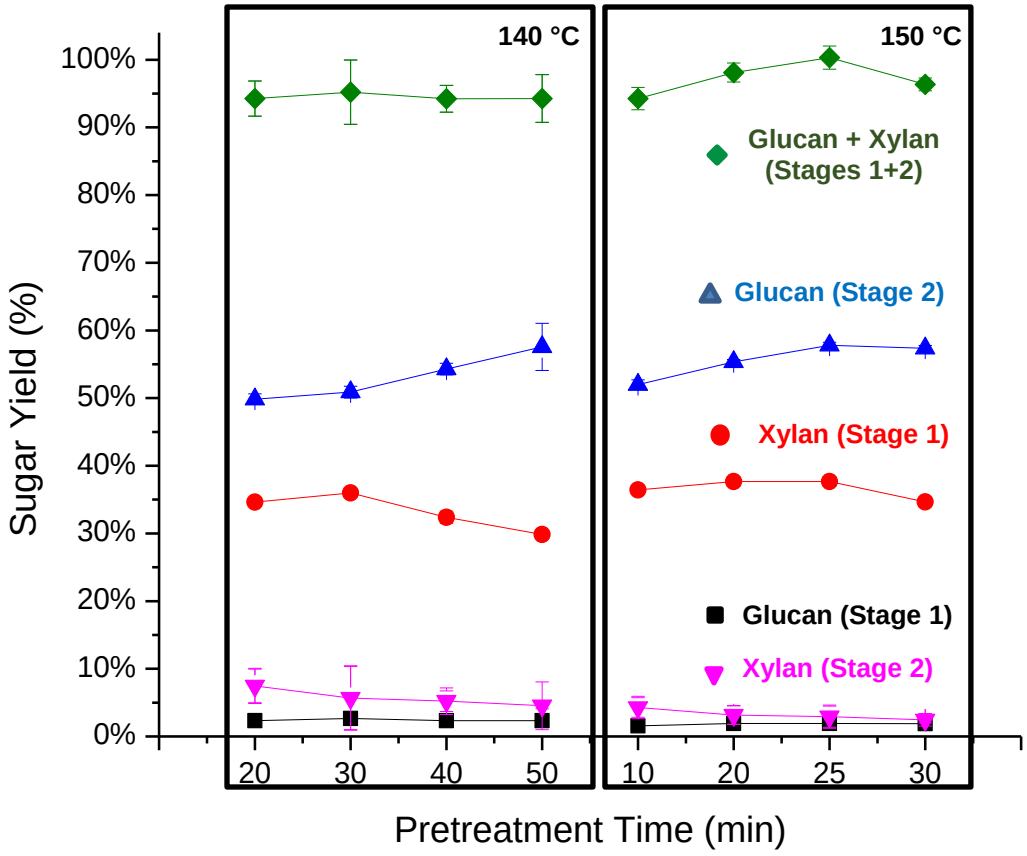


Figure 3.2. Effect of pretreatment time at 140°C and 150°C on glucan, xylan, and total glucan plus xylan yields from CELF pretreatment (Stage 1) of switchgrass, enzymatic hydrolysis of the pretreated solids (Stage 2), and the two stages combined. Stage 1 reaction conditions: solids loading- 7.5 wt%, acid loading- 0.5 wt% based on liquid weight, THF: water mass ratio-0.889:1. Stage 2 enzymatic hydrolysis was performed on pretreated solids at a 10 g/L glucan loading by 65 mg protein of Accellerase 1500® cellulase/g glucan in unpretreated switchgrass.

As expected, increasing time during both pretreatments initially increased Stage 1 xylan release as more xylan from the hemicellulose fraction was solubilized by the acid catalyst. However, at pretreatment times >30 minutes, significant amounts of xylose were dehydrated to furfural, which reduced the maximum possible xylose yield. Furfural at

higher severities was measured to close mass balances for both pretreatments. Glucan, on the other hand, was largely conserved in the solid in Stage 1 for both pretreatments, as the pretreatment conditions were not severe enough to solubilize significant amounts of crystalline cellulose. The small amount of glucan released during pretreatment, termed Stage 1 glucan, was likely mostly from hemicellulose and the amorphous portion of cellulose and was robust enough to suffer little degradation at the pretreatment conditions applied (Samuel, Pu et al. 2010). Increasing pretreatment time made biomass more susceptible to enzymatic breakdown by hydrolysis of pretreated solids, as illustrated by the increase in Stage 2 glucan release with increasing pretreatment time. Since the Accellerase® 1500 cellulase cocktail contains some hemicellulases and auxiliary enzymes as well (Chundawat, Lipton et al. 2011), residual xylan in pretreated solids was also solubilized during enzymatic hydrolysis and reported as Stage 2 xylan. The conditions that maximized sugar release for DSA and CELF pretreatments were 160°C, 20 minutes and 150 °C, 25 minutes, respectively, demonstrating that CELF reduced the temperature needed to achieve maximum sugar yields by 10°C from that needed for DSA (Figures 3.1 and 3.2).

The compositions of pretreated solids prepared at all pretreatment conditions were analyzed to determine the fate of components in the solids left by pretreatment. The mass of components in solids produced by application of the maximum sugar recovery pretreatment conditions for both DSA and CELF pretreatments were then adjusted to a basis of 100 g of unpretreated switchgrass (Figure 3.10 in Additional Information). For both pretreatments at maximum sugar recovery conditions, most of the hemicellulose

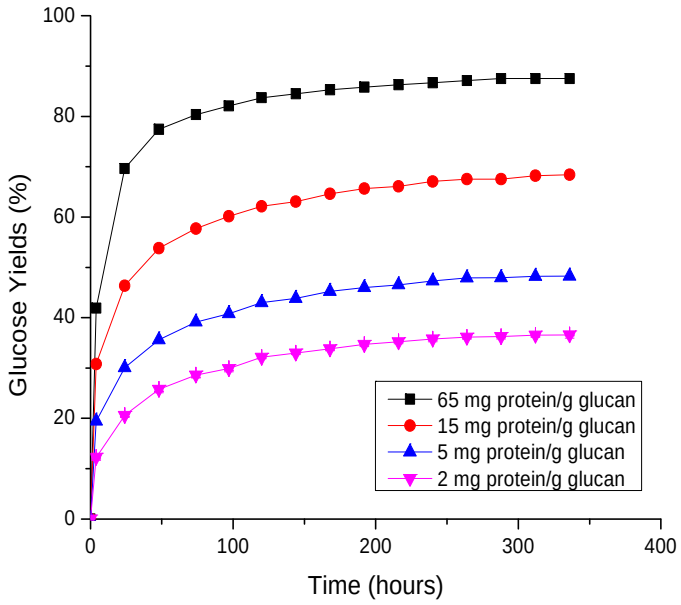
sugars (mostly xylan) were solubilized during pretreatment, in agreement with previous results for both of these pretreatments (*20*). Glucan was largely conserved in both pretreatments as expected. The major difference between DSA and CELF pretreated solids was the amount of lignin left in pretreated solids. While DSA removed roughly 14 wt% of Klason lignin (K-lignin), CELF removed 77 wt% of K-lignin at optimized conditions. This greater degree of delignification is encouraging as lignin has been shown to be a major contributor to biomass recalcitrance (Zeng, Zhao et al. 2014). At conditions optimized for maximum sugar recovery, the solids produced by DSA pretreatment contained 65% glucan, 4% xylan, and 32% K-lignin. CELF pretreated solids, on the other hand, contained 86% glucan, 4% xylan, and 11% K-lignin at optimized conditions, consistent with enhanced lignin removal by CELF (Figure 3.10 in Additional Information). Compositional analyses on solids resulting from more severe CELF pretreatments revealed that more lignin was removed at higher severities. However, a drawback was that more xylan was lost to dehydration products.

3.4.2 Enzymatic Hydrolysis Glucose Yields of DSA and CELF Pretreated Switchgrass at Lower Enzyme Loadings

The digestibility of solids prepared by DSA and CELF pretreatments of switchgrass was determined for enzymatic hydrolysis at Accellerase® 1500 cellulase loadings ranging from 2 to 65 mg protein/g glucan in unpretreated switchgrass. Enzyme loadings were based on unpretreated switchgrass so as not to penalize a pretreatment if it released more glucose in Stage 1. Ten days of hydrolysis at 65 mg protein/g glucan enzyme loading achieved a maximum glucose yield of 88% from DSA switchgrass

(Figure 3.3 (i)). On the other hand, CELF pretreated switchgrass reached 100% glucose yields in less than 2 days for both 65 and 15 mg/g enzyme loadings, and in 14 days at a 5 mg/g enzyme loading (Figure 3.3 (ii)). Further, at a considerably lower enzyme loading of just 2 mg/g, CELF pretreated switchgrass continued to be enzymatically hydrolyzed for as long as 5 weeks while enzymatic hydrolysis of DSA pretreated switchgrass virtually stopped after 2 weeks. This prolonged activity of cellulase enzymes on CELF pretreated switchgrass could be attributed to the low lignin content of CELF pretreated solids compared to DSA pretreated solids in that lignin has been shown to unproductively bind cellulase as well as block the surface of cellulose substrate (Zhang and Lynd 2004, Li, Pu et al. 2014, Whitehead, Bandi et al. 2017). Thus, the data here strongly suggest that lignin removal from solid substrates is critical to achieving high glucose yields by hydrolysis at low enzyme loadings as well as prolonging cellulase activity.

i



ii

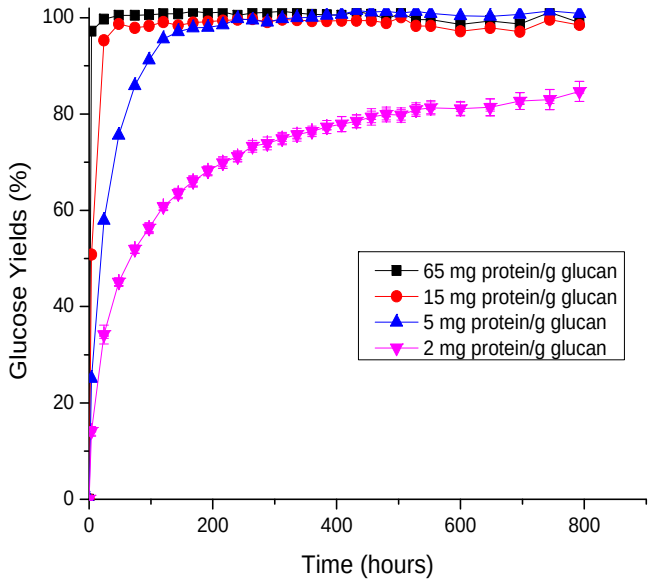


Figure 3.3. Comparison of glucose yields from enzymatic hydrolysis of solids prepared by (i) DSA and (ii) CELF pretreatments of switchgrass at cellulase loadings of 2-65 mg protein/g glucan in unpretreated switchgrass. Pretreatment reaction conditions were those that gave the highest total combined sugar yields at a loading of 65 mg protein/g enzyme, i.e., for DSA: 160°C, 20 minutes, and 0.5 wt% sulfuric acid; for CELF: 150°C, 25 minutes, and 0.5 wt% sulfuric acid at a 0.889:1 THF: water mass ratio.

3.4.3 Enzyme-Lignin Binding During Enzymatic Hydrolysis of DSA and CELF Switchgrass

Since CELF resulted in highly digestible solids and prolonged enzymatic activity, it was sought to understand mechanisms that could account for such enhanced enzyme performance. It has previously been shown that the cellulose in CELF pretreated solids is more or less equally accessible to cellulase as the solids produced by DSA pretreatment, as evidenced by the similar amounts of cellulase adsorbed by both CELF and DSA pretreated solids (Thomas, Donohoe et al. 2017). However, it appears that enzyme effectiveness (i.e., unit sugar produced/unit amount of bound enzyme) for CELF pretreated solids may be much higher than for DSA treated solids, owing to the lower lignin content of CELF solids. To assess this possibility, the free protein concentration in the liquid was measured before (at 0 h) and after complete enzymatic hydrolysis of the glucan in solids produced by DSA and CELF pretreatments of switchgrass at 10 g/L glucan loadings, with the results shown in Figure 3.4. An extremely high enzyme loading of 100 mg protein/g glucan was needed to achieve complete solubilization of glucan in DSA pretreated switchgrass. On the other hand, for solids from CELF pretreatment of switchgrass, complete glucan solubilization was achieved at enzyme loadings of 65, 15, and 5 mg protein/g glucan. The amount of initial and final protein in solution for DSA pretreated solids revealed a 40% drop in free protein after enzymatic hydrolysis, whereas no significant drop in free protein amount was measured after hydrolysis of CELF pretreated solids. These results suggested that the high level of delignification by CELF resulted in negligible binding of enzyme to residual solids and prolonged enzymatic

activity during enzymatic hydrolysis. However, as also shown in Figure 3.4, this result could stem from the very low amounts of residual solids left after complete removal of glucan by enzymatic hydrolysis of CELF solids compared to DSA solids.

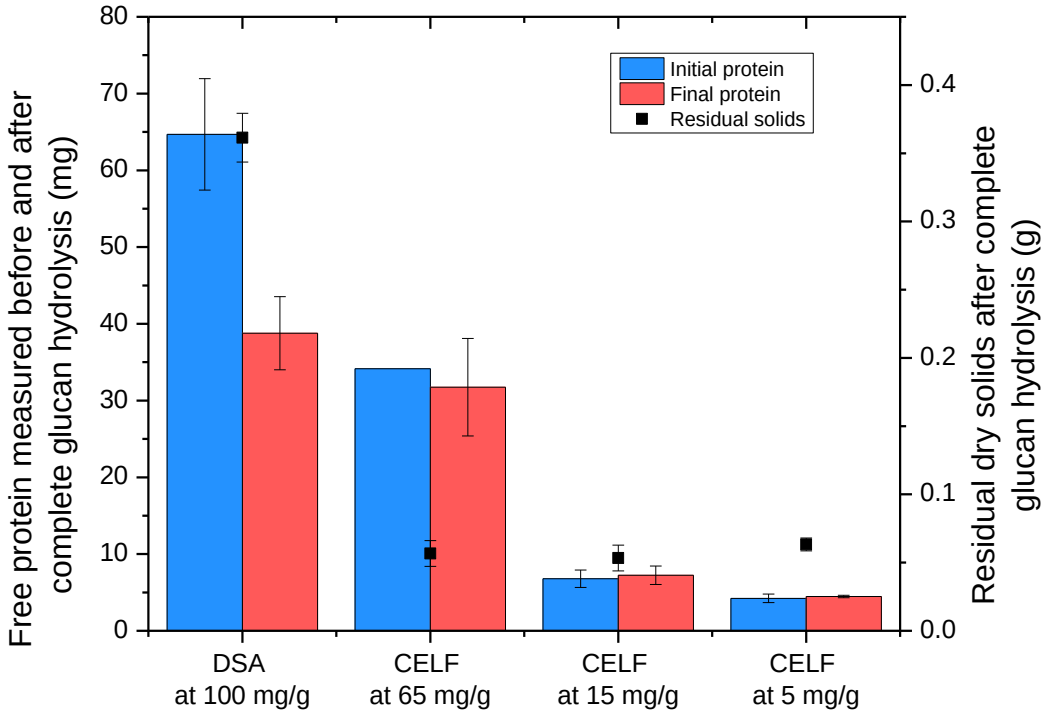


Figure 3.4. Initial and final protein measured in solution before (at 0 h) and after complete glucan removal by enzymatic hydrolysis at a 10 g/L glucan loading and cellulase loading of 100 mg protein/g glucan in unpretreated switchgrass for DSA pretreated solids and 65, 15, and 5 mg protein/ g glucan for CELF pretreated switchgrass solids. Residual dry solids after complete glucan hydrolysis for DSA and CELF pretreated switchgrass are shown on the right axis.

Since Figure 3.4 shows that CELF residual solids composed mostly of lignin adsorbed negligible amounts of cellulase enzyme at equal glucan loadings, the question arises as to whether differences in DSA and CELF lignin could be responsible for this result. Therefore, enzyme adsorption by equivalent amounts of residual lignin was

investigated to understand the enzyme binding behavior of lignin on solids prepared by DSA and CELF. The glucan loading for CELF solids was increased to 40 g/L to match the amount of lignin present in a 10 g/L glucan loading of DSA pretreated switchgrass. The initial amount of enzyme was kept at 1.72 g protein/L for both cases to give an equivalent loading of 350 mg protein/g lignin. This approach was applied to ensure complete solubilization of both substrates and allow a direct comparison to the total amount of free protein after complete glucan solubilization. Figure 3.5 shows that after complete glucan saccharification, equal amounts of residual solids were left. However, because the final amount of free protein for CELF pretreated solids was 65% less than the initial amount, CELF residual solids mostly containing K-lignin actually adsorbed >50% more enzyme on a per gram of lignin basis than residual DSA lignin.

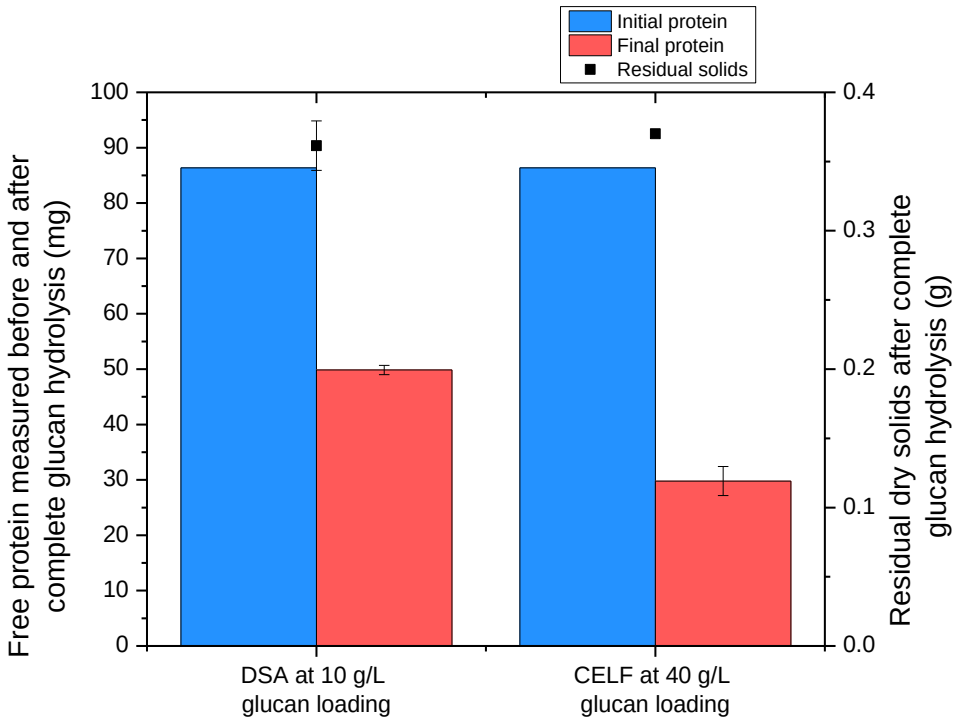


Figure 3.5. Initial and final free protein content (on the left axis) in enzymatic hydrolysis solutions measured before (at 0 h) and after complete glucan hydrolysis for DSA pretreated switchgrass at 10 g/L glucan loading and CELF pretreated switchgrass at 40 g/L glucan loading. Initial protein added in both cases was 1.72 g/L. Residual dry solids after complete glucan hydrolysis for DSA pretreated switchgrass and CELF pretreated switchgrass are shown on the right y-axis.

A potential hypothesis to explain these differences is that the K-lignin in pretreated solids prepared by aqueous pretreatments, such as DSA, has large amounts of lignin globules deposited on the cellulose surface (Selig, Viamajala et al. 2007, Donohoe, Decker et al. 2008) and that lignin may not adsorb as much enzyme as lignin within the lignin-carbohydrate complex (LCC). On the other hand, CELF maintained lignin solubility, preventing redeposition onto cellulose (Smith, Mostofian et al. 2016). Therefore, the lignin remaining in CELF pretreated solids was mostly locked within the lignin-carbohydrate complex (LCC) that could bind more cellulase. On the other hand,

because DSA pretreated solids contained a very large amount of lignin redeposited on the cellulose in addition to LCC lignin, DSA lignin bound a lower mass of cellulose per gram of lignin.

It has been suggested that redeposited lignin globules do not strongly bind enzymes but merely provide a physical obstacle between cellulose and enzymes during hydrolysis (Li, Pu et al. 2014). To test this hypothesis, DSA pretreated solids were washed once at room temperature with 500 mL THF to remove lignin deposited on the cellulose surface. Figure 3.11 in Additional Information shows that THF washing removed surface deposited lignin without removing major carbohydrates or lignin from the LCC.

Bulk level compositional analysis of the THF-washed DSA switchgrass showed that 33% of the K-lignin was removed by the THF wash (Figure 3.10 in Additional Information). Conversely, THF washing of CELF pretreated solids resulted in a negligible reduction in K-lignin content (Figure 3.10 in Additional Information). Following complete glucan solubilization of the THF-washed DSA solids, the amount of protein adsorbed per gram of residual solids was very similar to that of CELF residual solids (Figure 3.6), suggesting that LCC lignin binds more cellulase than surface deposited lignin, possibly due to different structural and/or compositional differences.

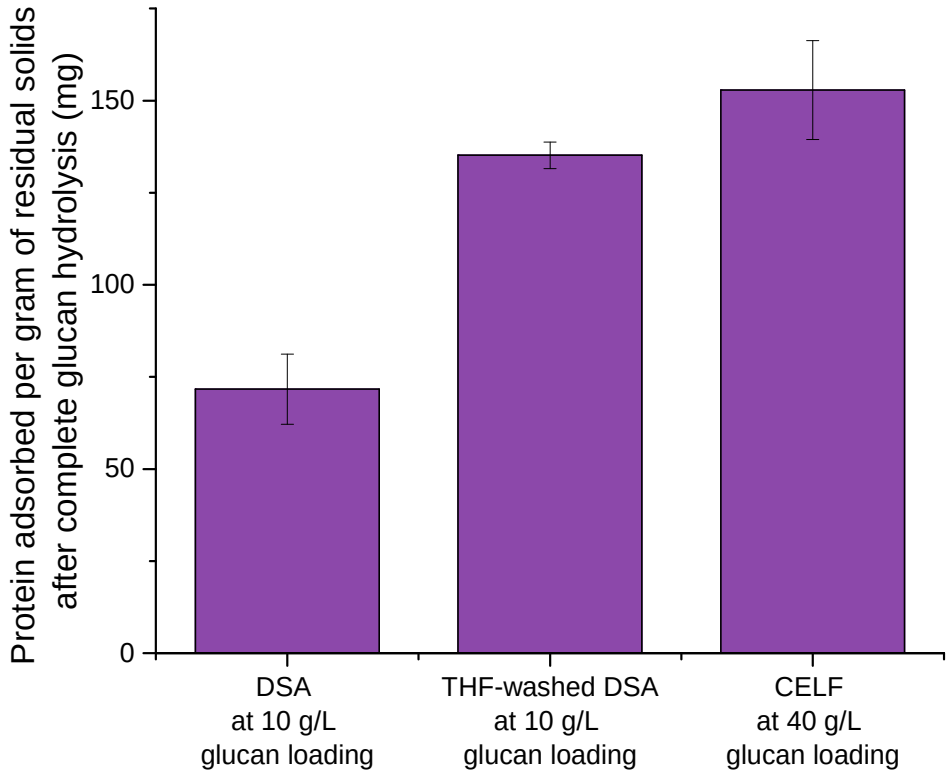


Figure 3.6. Protein adsorbed per gram of residual dry solids containing mostly K-lignin resulting from complete enzymatic hydrolysis of DSA and THF-washed DSA pretreated switchgrass at 10 g/L glucan loading, and CELF pretreated switchgrass at 40 g/L glucan loading. Initial protein added in all cases was 1.72 g/L.

3.4.4 Scanning Electron Microscopy of DSA and CELF Switchgrass

Scanning electron micrographs were used to visualize differences in lignin deposited on the surface by DSA and CELF pretreatments of switchgrass. Additionally, THF washed DSA samples were imaged to examine the removal of surface deposited lignin by THF. After DSA pretreatment, a high concentration of lignin globules was observed on the surface of the pretreated biomass (Figure 3.7 (ii)), while washing with THF dramatically dropped the amount of lignin globules observable and the average

diameter of individual droplets (Figure 3.7 (iii)). As expected, CELF pretreated switchgrass appeared to have a low concentration of redeposited lignin globules (Figure 3.7 (iv)). These images confirmed the limited amount of surface deposited lignin on CELF pretreated switchgrass and suggested that most of the K-lignin detected was part of the LCC.

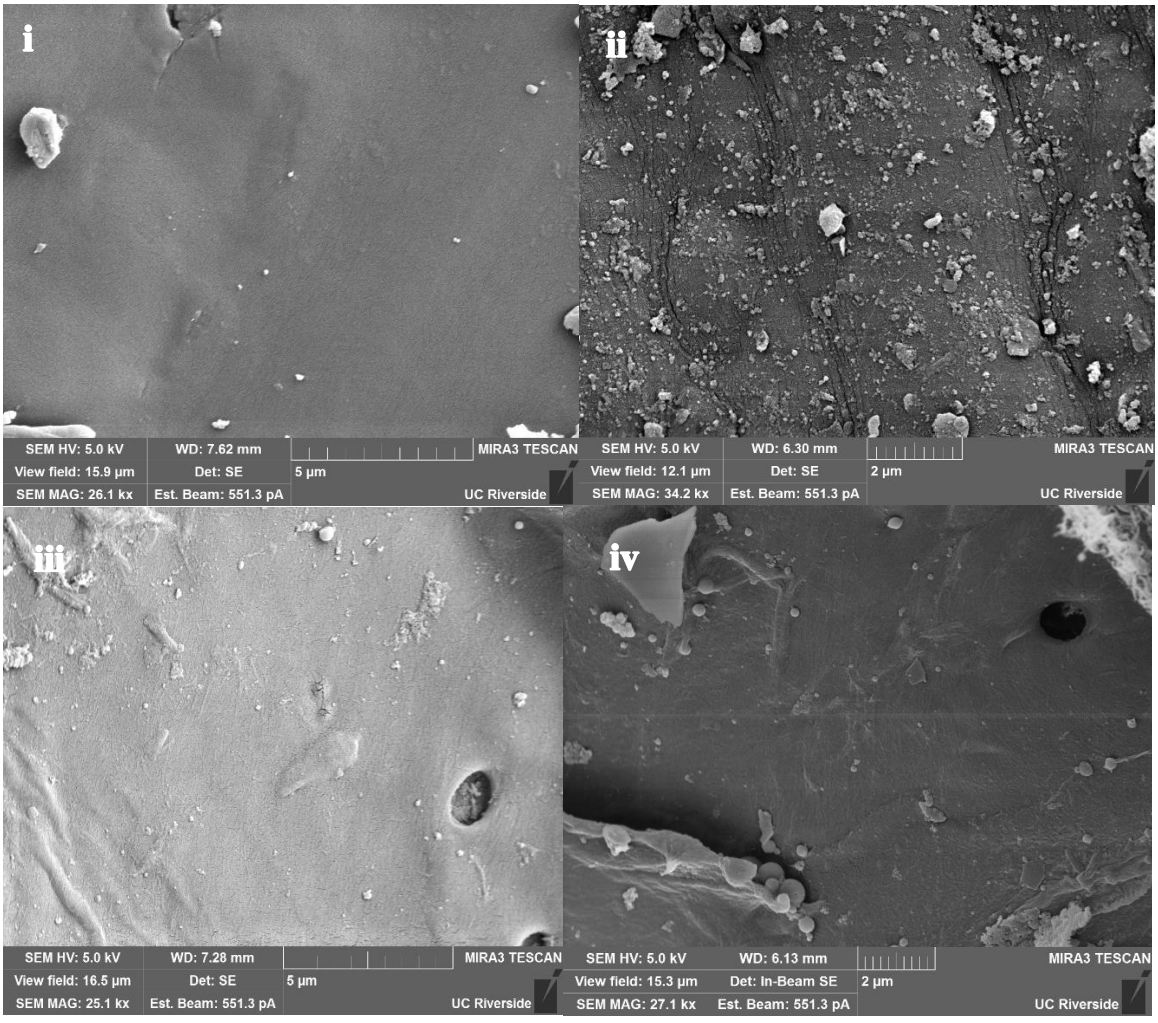


Figure 3.7. SEM images of (i) unpretreated, (ii) DSA pretreated, (iii) THF-washed DSA pretreated, and (iv) CELF pretreated pretreated switchgrass.

CELF pretreatment has been shown to delignify Alamo switchgrass to a very high extent, producing a glucan-rich solid that was highly digestible and resulted in prolonged hydrolytic enzymatic activity for at least 5 weeks. The low amount of lignin in CELF pretreated solids resulted in negligible amounts of enzyme being unproductively bound, thus preserving enzymatic activity. DSA solids, on the other hand, adsorbed roughly 40% of the enzyme after complete glucan hydrolysis and glucose yields plateaued after 10 days. The observation that THF washing removed a large fraction of surface redeposited lignin from DSA pretreated solids while leaving most of the lignin on CELF solids suggested that CELF solids contain very little lignin on the surface, as confirmed by SEM images, and that most of the measured K-lignin is part of the LCC. This result implies that most lignin is removed directly from the LCC during CELF, thereby reducing the amount of enzyme that could unproductively adsorb during enzymatic hydrolysis. Additionally, the prevention of lignin redeposition on the surface of pretreated biomass resulted in faster initial enzymatic rates because barriers to enzyme action on the surface of the glucan rich solids were significantly reduced.

It must be noted, however, that removal of surface redeposited lignin alone did not enhance enzymatic yields (Lai, Tu et al. 2014, Lai, Tu et al. 2015), particularly when significant lignin remained in the LCC. As shown in Figure 3.8, enzymatic hydrolysis yields from THF-washed DSA switchgrass were only enhanced at the high enzyme loading of 65 mg protein/g glucan, while at lower enzyme loadings, glucose yields plateaued at lower values for THF-washed DSA solids compared to unwashed DSA solids. It is hypothesized that this difference was due to redeposited lignin shielding

lignin in addition to cellulose in the LCC from enzymes. Thus, THF washing of redeposited lignin from the surface exposed considerably more of the remaining LCC lignin to enzymes that were in turn unproductively bound earlier in the hydrolysis process. When high enzyme loadings were applied to DSA solids, enough enzyme could be left in solution despite some binding to lignin in the LCC that cellulose could still be hydrolyzed to glucose with high yields. However, at lower enzyme loadings, significant amounts of enzyme may be bound to the exposed lignin in the LCC earlier, thus resulting in lower glucose yields.

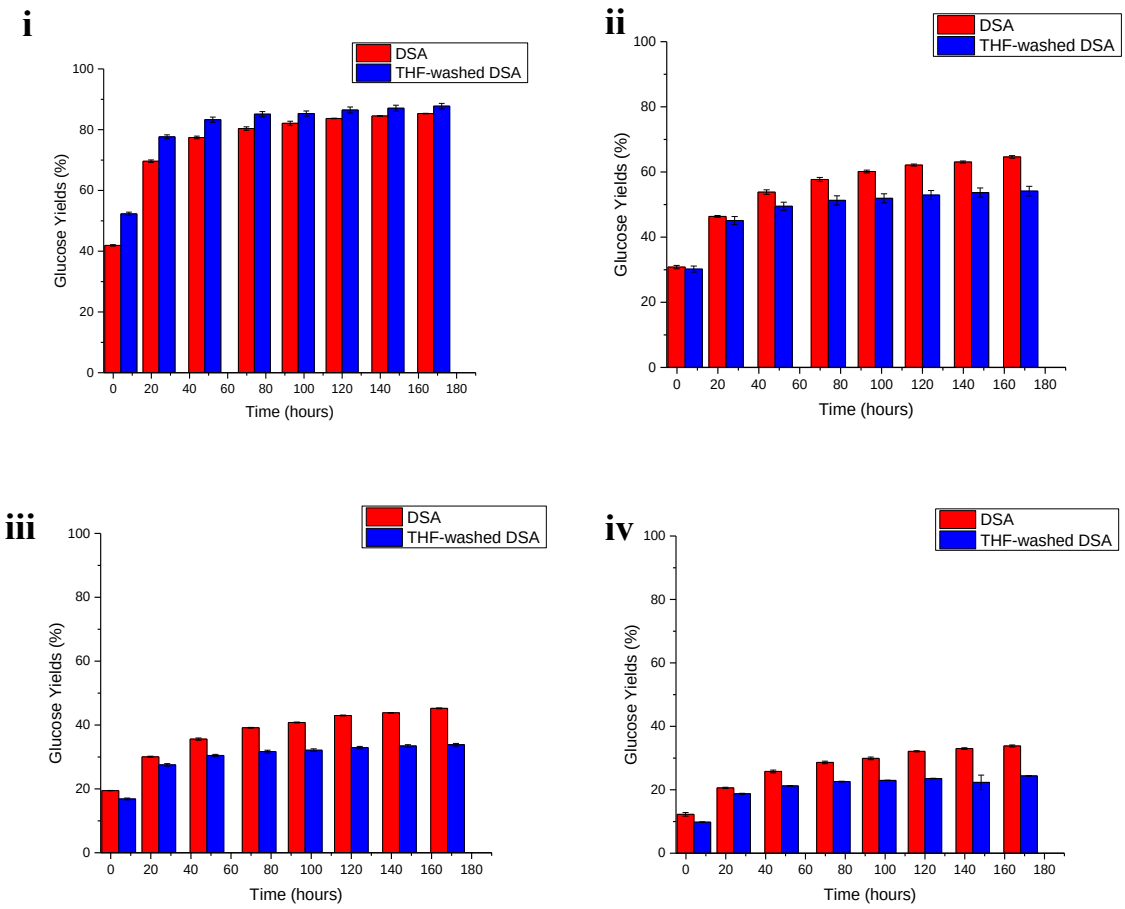


Figure 3.8. Comparison of glucose yields from enzymatic hydrolysis DSA and THF-washed DSA switchgrass at cellulase loadings of (i) 65 mg protein/g glucan, (ii) 15 mg protein/g glucan, (iii) 5 mg protein/g glucan, and (iv) 2 mg protein/g glucan in unpretreated switchgrass. Pretreatment reaction conditions for DSA: 160°C, 20 minutes, 0.5 wt% sulfuric acid; THF wash performed with 500 mL of THF at room temperature.

When bovine serum albumin (BSA) was added to bind with lignin in the LCC (Yang and Wyman 2006) prior to hydrolysis at the lower enzyme loadings, enhancement of glucose yields for THF-washed DSA switchgrass was greater than from DSA switchgrass (Figure 3.9). This result suggested that once lignin in the LCC was bound by BSA, the more exposed cellulose in THF-washed DSA switchgrass was more easily

hydrolyzed than cellulose in DSA switchgrass. Faster hydrolysis rates were only observed with THF-washed DSA switchgrass once BSA was attached to lignin in the LCC to prevent it binding with cellulase. The lower hydrolysis rates and final yields observed without the addition of BSA supports the hypothesis that redeposited lignin in DSA switchgrass not only shields enzymes from cellulose but also from lignin in the LCC.

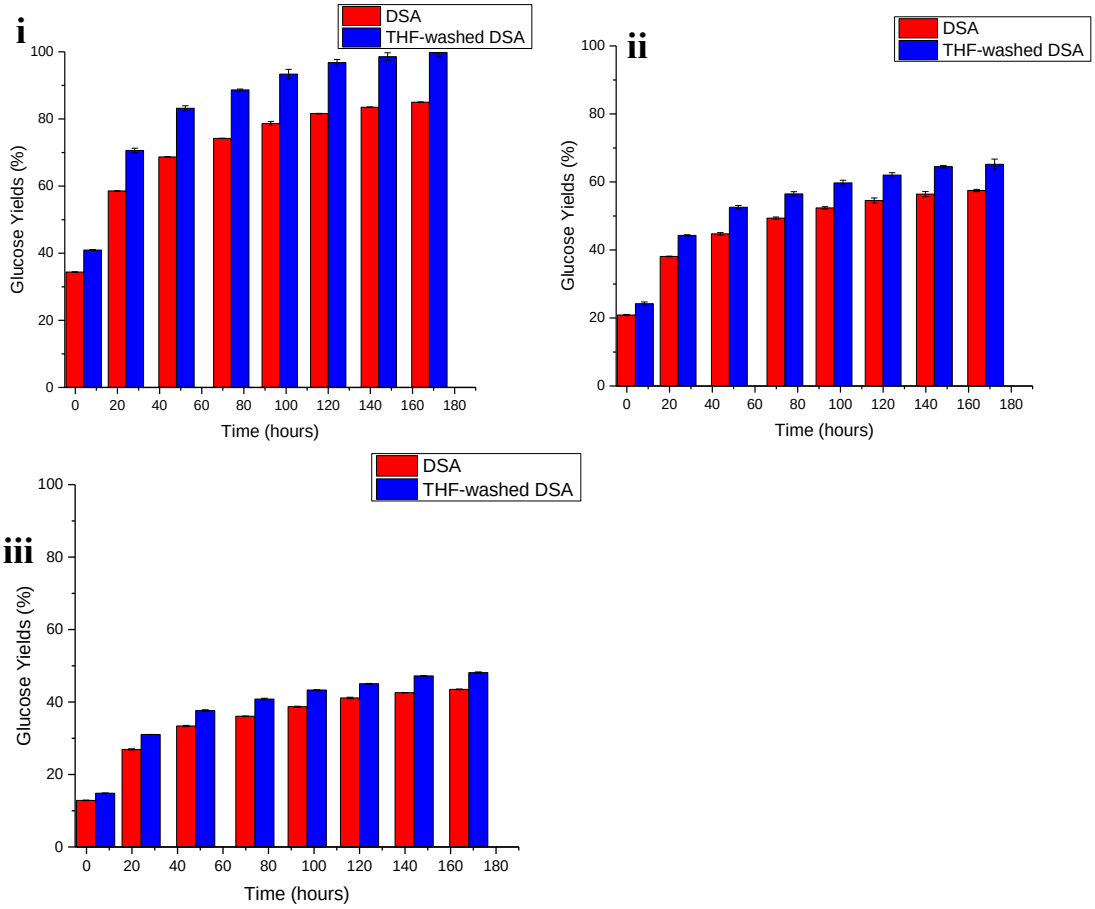


Figure 3.9. Comparison of glucose yields from enzymatic hydrolysis DSA and THF-washed DSA switchgrass with the addition of bovine serum albumin (BSA) at cellulase loadings of (i) 15 mg protein/g glucan, (ii) 5 mg protein/g glucan, and (iii) 2 mg protein/g glucan in unpretreated switchgrass. Pretreatment reaction conditions for DSA: 160°C, 20 minutes, 0.5 wt% sulfuric acid; THF wash performed with 500 mL of THF at room temperature. BSA loading was 0.1 g BSA/g glucan.

CELF appeared to remove most of the lignin from the LCC, thus leaving the bulk of the pretreated solid rich in cellulose. Even for application of low amounts of cellulolytic enzymes, the limited lignin redeposited on CELF solids implied that more of the cellulose in the CELF LCC was exposed to free enzymes, thus enhancing enzyme-cellulose binding, as evidenced by the rapid initial rate of hydrolysis for CELF pretreated switchgrass (Figure 3.3). The low amount of lignin in the CELF LCC suggested that less enzyme could unproductively bind to lignin, prolonging enzyme activity. From these results, it can also be hypothesized that although much less lignin is available to unproductively tie up enzymes, LCC lignin has a higher binding affinity for cellulolytic enzymes than surface redeposited lignin; however, further in-depth work is required to understand the differences between these two types of lignin.

3.5 Conclusions

THF as a co-solvent with water and dilute acid (CELF) can be an attractive pretreatment for biofuels production in that the highly digestible glucan rich solids produced by CELF pretreatment can achieve nearly theoretical glucose yields at enzyme loadings as low as 5 mg protein/g glucan. CELF significantly enhanced lignin removal from switchgrass (up to 77% lignin removal) and substantially lowered lignin redeposition onto the cellulose surface compared to dilute acid alone. Additionally, the preservation of cellulase activity for much longer periods of time during hydrolysis of CELF pretreated solids compared to DSA highlights the importance of delignification of the plant cell walls prior to biological deconstruction. Further, the low lignin content of CELF pretreated switchgrass was shown to result in much less cellulase being bound to

lignin and thereby unavailable for further cellulose hydrolysis. This outcome contrasts with the 40% loss in cellulase to unproductive binding to lignin in solids produced by DSA, the current pretreatment benchmark (Humbird, Davis et al. 2011). The latter results in less cellulase available for hydrolysis in addition to reduced accessiblity of cellulose to enzymes. On the other hand, although lignin left in CELF solids was mostly part of the LCC, CELF enhanced cellulase availability by dramatically reducing the total amount of K-lignin, thus preserving enzymatic activity for prolonged hydrolysis times.

3.6 Acknowledgments

We acknowledge support from the Office of Biological and Environmental Research in the US Department of Energy (DOE) Office of Science through the BioEnergy Science Center (BESC) and the Center for Bioenergy Innovation (CBI), both at Oak Ridge National Laboratory. The award of a fellowship to the lead author by the National Center for Sustainable Transportation facilitated his participation. We also acknowledge the Center for Environmental Research and Technology (CE-CERT) of the Bourns College of Engineering for providing the facilities and the Ford Motor Company for funding the Chair in Environmental Engineering that facilitates projects such as this one. D.K. acknowledges support by the Air Force Office of Scientific Research, Multi-University Research Initiative (AFOSR-FA9550-15-1-0009). D.K. also acknowledges financial support by the Army Research Office DURIP Grant W911NF-16-1-0208 for the MIRA SEM. R.M. and support by the National Institute of Justice Graduate Research (STEM) Fellowship Award #2016-R2-CX-0015.

3.7 References

Chen, S. F., R. A. Mowery, R. S. Sevcik, C. J. Scarlata and C. K. Chambliss (2010). "Compositional analysis of water-soluble materials in switchgrass." Journal of Agricultural and Food Chemistry **58**(6): 3251-3258.

Chundawat, S. P. S., M. S. Lipton, S. O. Purvine, N. Uppugundla, D. H. Gao, V. Balan and B. E. Dale (2011). "Proteomics-based compositional analysis of complex cellulase-hemicellulase mixtures." Journal of Proteome Research **10**(10): 4365-4372.

David, K. and A. J. Ragauskas (2010). "Switchgrass as an energy crop for biofuel production: A review of its ligno-cellulosic chemical properties." Energy & Environmental Science **3**(9): 1182-1190.

Donohoe, B. S., S. R. Decker, M. P. Tucker, M. E. Himmel and T. B. Vinzant (2008). "Visualizing lignin coalescence and migration through maize cell walls following thermochemical pretreatment." Biotechnology and Bioengineering **101**(5): 913-925.

Gao, X. D., R. Kumar, S. Singh, B. A. Simmons, V. Balan, B. E. Dale and C. E. Wyman (2014). "Comparison of enzymatic reactivity of corn stover solids prepared by dilute acid, AFEX (TM), and ionic liquid pretreatments." Biotechnology for Biofuels **7**.

Humbird, D., R. Davis, L. Tao, C. Kinchin, D. Hsu, A. Aden, P. Schoen, J. Lukas, B. Olthof and M. Worley (2011). Process design and economics for biochemical conversion of lignocellulosic biomass to ethanol: dilute-acid pretreatment and enzymatic hydrolysis of corn stover, National Renewable Energy Laboratory (NREL), Golden, CO.

Kumar, R., F. Hu, P. Sannigrahi, S. Jung, A. J. Ragauskas and C. E. Wyman (2013). "Carbohydrate derived-pseudo-lignin can retard cellulose biological conversion." Biotechnology and Bioengineering **110**(3): 737-753.

Kumar, R., M. Tabatabaei, K. Karimi and I. S. Horvath (2016). "Recent updates on lignocellulosic biomass derived ethanol - A review." Biofuel Research Journal **3**(1): 347-356.

Lai, C. H., M. B. Tu, M. Li and S. Y. Yu (2014). "Remarkable solvent and extractable lignin effects on enzymatic digestibility of organosolv pretreated hardwood." Bioresource Technology **156**: 92-99.

Lai, C. H., M. B. Tu, Q. Yong and S. Y. Yu (2015). "Disparate roles of solvent extractable lignin and residual bulk lignin in enzymatic hydrolysis of pretreated sweetgum." Rsc Advances **5**(119): 97966-97974.

Li, H. J., Y. Q. Pu, R. Kumar, A. J. Ragauskas and C. E. Wyman (2014). "Investigation of lignin deposition on cellulose during hydrothermal pretreatment, its effect on cellulose

hydrolysis, and underlying Mechanisms." Biotechnology and Bioengineering **111**(3): 485-492.

Lloyd, T. A. and C. E. Wyman (2005). "Combined sugar yields for dilute sulfuric acid pretreatment of corn stover followed by enzymatic hydrolysis of the remaining solids." Bioresource Technology **96**(18): 1967-1977.

McLaughlin, S. B. and L. A. Kszos (2005). "Development of switchgrass (*Panicum virgatum*) as a bioenergy feedstock in the United States." Biomass & Bioenergy **28**(6): 515-535.

Mok, Y. K., V. Arantes and J. N. Saddler (2015). "A NaBH4 coupled Ninhydrin-based assay for the quantification of protein/enzymes during the enzymatic hydrolysis of pretreated lignocellulosic biomass." Applied Biochemistry and Biotechnology **176**(6): 1564-1580.

Mosier, N., C. Wyman, B. Dale, R. Elander, Y. Y. Lee, M. Holtzapple and M. Ladisch (2005). "Features of promising technologies for pretreatment of lignocellulosic biomass." Bioresource Technology **96**(6): 673-686.

Nguyen, T., C. Cai, R. Kumar and C. Wyman (2015). "Co-solvent pretreatment reduces costly enzyme requirements for high sugar and ethanol yields from lignocellulosic biomass." ChemSusChem **8**(10): 1716-1725.

Palmqvist, E. and B. Hahn-Hagerdal (2000). "Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition." Bioresource Technology **74**(1): 25-33.

Ramos, L. P., C. Breuil and J. N. Saddler (1993). "The use of enzyme recycling and the influence of sugar accumulation on cellulose hydrolysis by *Trichoderma* cellulases." Enzyme and Microbial Technology **15**(1): 19-25.

Samuel, R., Y. Pu, M. Foston and A. J. Ragauskas (2010). "Solid-state NMR characterization of switchgrass cellulose after dilute acid pretreatment." Biofuels **1**(1): 85-90 %@ 1759-7269.

Selig, M., N. Weiss and Y. Ji (2008). Enzymatic Saccharification of Lignocellulosic Biomass: Laboratory Analytical Procedure (LAP): Issue Date, 3/21/2008, National Renewable Energy Laboratory.

Selig, M. J., S. Viamajala, S. R. Decker, M. P. Tucker, M. E. Himmel and T. B. Vinzant (2007). "Deposition of lignin droplets produced during dilute acid pretreatment of maize stems retards enzymatic hydrolysis of cellulose." Biotechnology Progress **23**(6): 1333-1339.

Shen, H., C. R. Poovaiah, A. Ziebell, T. J. Tschaplinski, S. Pattathil, E. Gjersing, N. L. Engle, R. Katahira, Y. Q. Pu, R. Sykes, F. Chen, A. J. Ragauskas, J. R. Mielenz, M. G. Hahn, M. Davis, C. N. Stewart and R. A. Dixon (2013). "Enhanced characteristics of genetically modified switchgrass (*Panicum virgatum* L.) for high biofuel production." Biotechnology for Biofuels **6**.

Shi, J., M. A. Ebrik and C. E. Wyman (2011). "Sugar yields from dilute sulfuric acid and sulfur dioxide pretreatments and subsequent enzymatic hydrolysis of switchgrass." Bioresource Technology **102**(19): 8930-8938.

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 **1617**: 1-16.

Smith, M. D., B. Mostofian, X. L. Cheng, L. Petridis, C. M. Cai, C. E. Wyman and J. C. Smith (2016). "Cosolvent pretreatment in cellulosic biofuel production: effect of tetrahydrofuran-water on lignin structure and dynamics." Green Chemistry **18**(5): 1268-1277.

Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson and D. C. Klenk (1985). "Measurement of protein using bicinchoninic acid." Analytical Biochemistry **150**(1): 76-85.

Thomas, V. A., B. S. Donohoe, M. Li, Y. Pu, A. J. Ragauskas, R. Kumar, T. Y. Nguyen, C. M. Cai and C. 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**.

Torget, R., P. Walter, M. Himmel and K. Grohmann (1991). "Dilute-acid pretreatment of corn residues and short-rotation woody crops." Applied Biochemistry and Biotechnology **28-9**: 75-86.

Vermaas, J., L. Petridis, X. Qi, R. Schulz, B. Lindner and J. Smith (2015). "Mechanism of lignin inhibition of enzymatic biomass deconstruction." Biotechnology for Biofuels **8**(1): 1-16.

Whitehead, T. A., C. K. Bandi, M. Berger, J. Park and S. P. S. Chundawat (2017). "Negatively supercharging cellulases render them lignin-resistant." ACS Sustainable Chemistry & Engineering **5**(7): 6247-6252.

Wyman, C. E., C. M. Cai and R. Kumar (2017). "Bioethanol from lignocellulosic biomass." Encyclopedia of Sustainability Science and Technology: 1-27.

Wyman, C. E., B. E. Dale, R. T. Elander, M. Holtzapple, M. R. Ladisch and Y. Y. Lee (2005). "Coordinated development of leading biomass pretreatment technologies." Bioresource Technology **96**(18): 1959-1966.

Yang, B. and C. E. Wyman (2006). "BSA treatment to enhance enzymatic hydrolysis of cellulose in lignin containing substrates." Biotechnology and Bioengineering **94**(4): 611-617.

Zeng, Y. N., S. Zhao, S. H. Yang and S. Y. Ding (2014). "Lignin plays a negative role in the biochemical process for producing lignocellulosic biofuels." Current Opinion in Biotechnology **27**: 38-45.

Zhang, Y. H. P. and L. R. Lynd (2004). "Toward an aggregated understanding of enzymatic hydrolysis of cellulose: Noncomplexed cellulase systems." Biotechnology and Bioengineering **88**(7): 797-824.

3.8 Additional Information

To study the effect of enzyme binding between lignin still linked to the LCC and lignin redeposited on the cellulose surface after dilute acid pretreatment, DSA switchgrass was washed with 500 mL of room temperature THF. The effect of the THF wash on removing redeposited lignin is shown in Figure 3.10. From bulk compositional analysis, roughly 33% of K-lignin was removed by the THF wash, indicating that one-third of K-lignin was that redeposited onto cellulose during DSA. THF washing of CELF switchgrass, on the other hand, removed a very small amount of lignin. The small amount of lignin that was removed was presumed to be lignin that had precipitated as some THF evaporated at the top of the Buchner funnel during solid-liquid separation of the CELF mixture.

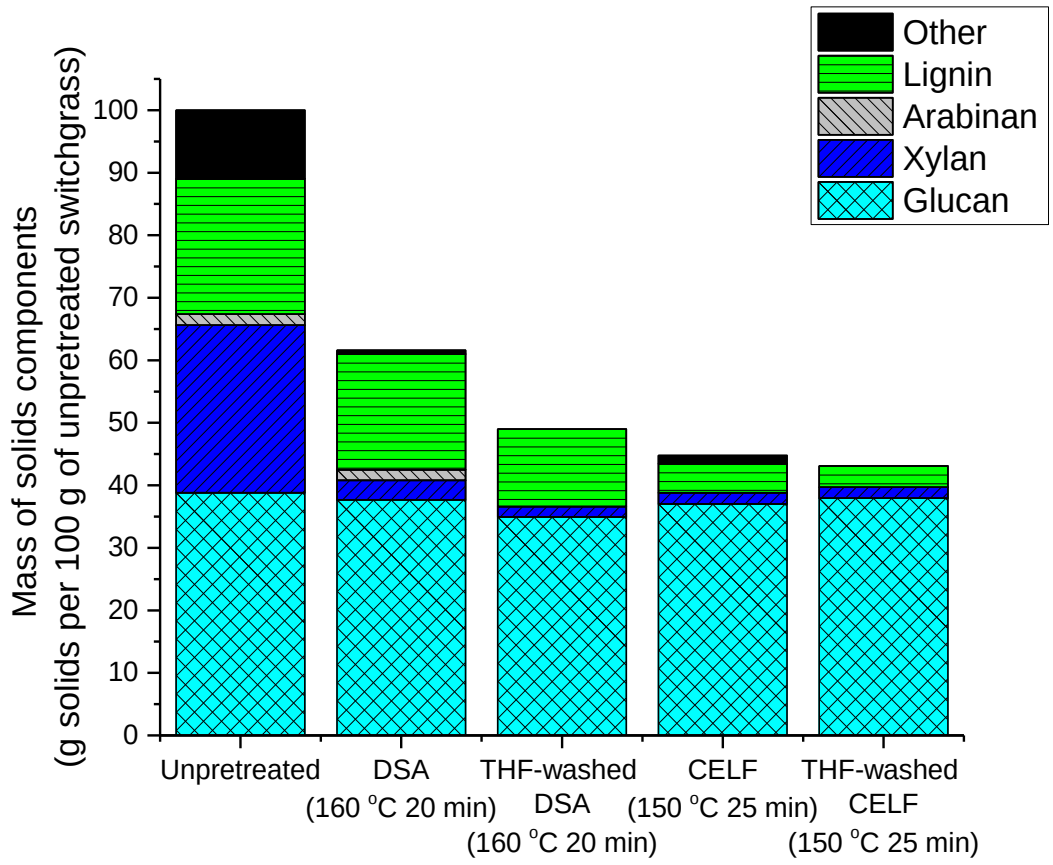


Figure 3.10. Tracking mass of glucan, xylan, and lignin left in the solids produced by DSA and CELF pretreatments at conditions optimized for recovery of highest overall glucan and xylan yields and for THF-washed DSA pretreated solids. The values shown are based on the content of each component in 100 g of switchgrass before pretreatment. Reaction conditions: DSA: 160°C, 20 minutes, 0.5 wt% sulfuric acid. CELF: 150°C, 25 minutes, 0.5 wt% sulfuric acid, 0.889:1 THF/water mass ratio.

THF washing of biomass samples at room temperature was utilized to solubilize surface deposited lignin and remove it from the solid samples. To ensure that this process in fact only removed surface deposited lignin and not lignin or carbohydrates in the LCC, unpretreated switchgrass and lignin-deposited Avicel (LDA) were washed with THF. As shown in Figure 3.11, upon washing with THF, no change in major carbohydrates or

lignin in unpretreated switchgrass was observed. The mass lost was attributed to the extractives present in switchgrass that are typically solubilized during pretreatment (Chen, Mowery et al. 2010). Additionally, upon washing LDA with THF, the deposited lignin was removed from the solid fraction, leaving behind cellulose-rich Avicel.

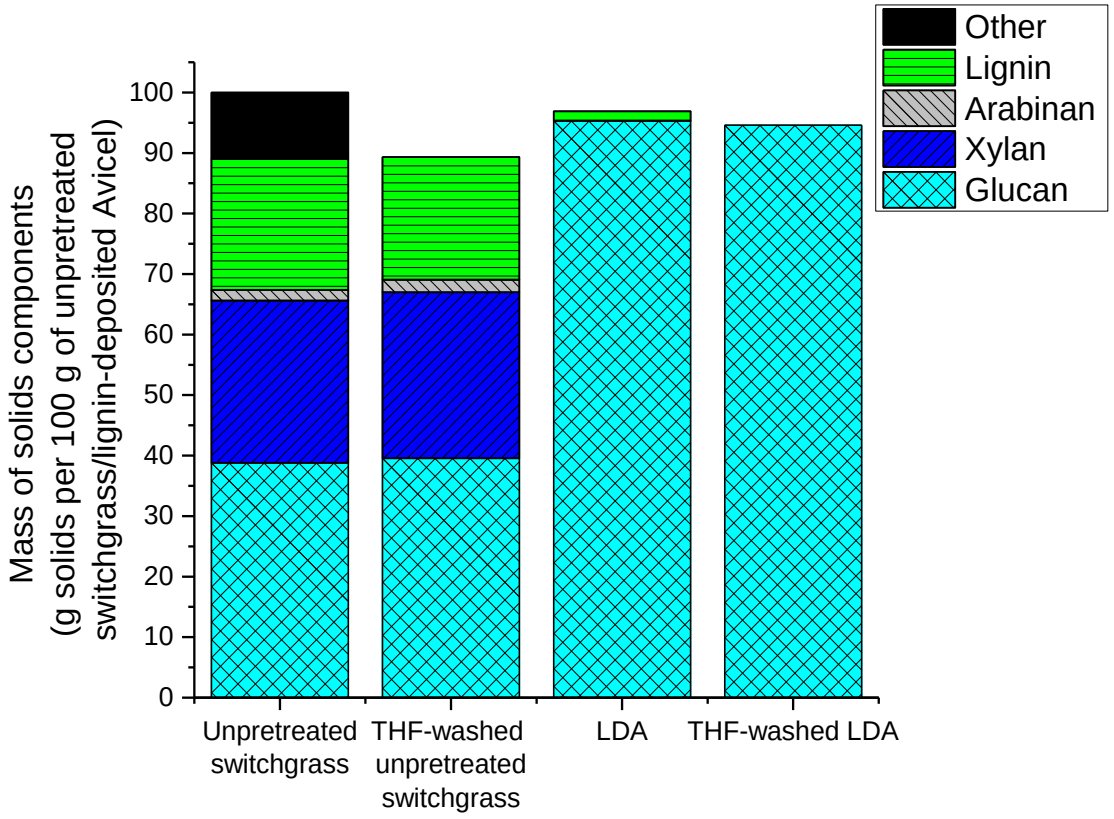


Figure 3.11. Tracking mass of glucan, xylan, and lignin in unpretreated and THF-washed unpretreated switchgrass, and lignin-deposited Avicel (LDA) and THF-washed LDA. THF wash performed with 500 mL of THF at room temperature.

Chapter 4

A Multifunctional Co-Solvent Pair Reveals Molecular Principles of Biomass Deconstruction*

*This chapter was conducted in collaboration with researchers at Oak Ridge National Laboratory, University of Central Florida, and University of Tennessee Knoxville. It submitted under the following citation:
Patri, A.S., Mostofian, B., Pu, Y., Ciaffone, N., Soliman, M., Tetard, L., Smith, M.D., Cheng, X., Kumar, R., Ragauskas, A., Wyman, C.E., Smith, J.C., Petridis, L., Cai, C.M., 2018. A multifunctional co-solvent pair reveals molecular principles of biomass deconstruction.

4.1 Abstract

Plants have evolved complex structural and chemical mechanisms that resist breakdown, challenging the deconstruction of inexpensive lignocellulosic biomass into valuable chemical building blocks. Here, experimental and computational evidence reveal synergistic mechanisms during treatment of biomass within a miscible co-solvent pair, tetrahydrofuran and water that functionally overcome biomass recalcitrance to deconstruction by simultaneously improving sugar accessibility and promoting lignin release for valorization. Molecular simulations reveal how lignin globules expand in the co-solvent, dissociating from themselves and cellulose, while nanoscale infrared sensors track the migration of lignin molecules outwards of the plant cell wall. This expansion of the lignin molecules exposes inter-unit linkages, rendering them susceptible to acid-catalyzed hydrolysis, leading to extensive depolymerization by cleaving aryl-ether bonds while the co-solvent pair prevents unwanted re-condensation reactions. The resulting simultaneous liberation of lignin and hemicellulose from cellulose allows unfettered access of cellulolytic enzymes, leading to sustained high rates of hydrolysis without further manipulation, portending a paradigm shift towards a synergistic approach to biomass deconstruction.

4.2 Introduction

One of the greatest scientific and engineering challenges of our time is to provide pathways for modern chemical and fuel industries to transition to a fully sustainable practice of utilizing renewable resources (van Renssen 2011, Trancik 2014, Elliott 2016, Jeffries 2016). Lignocellulosic biomass is the most abundant source of organic carbon on

Earth and is the only suitable feedstock capable of supporting renewable energy production in a developing bioeconomy at scales relevant to positively impacting global warming (Lynd, Cushman et al. 1991, Rostrup-Nielsen 2005, Ragauskas, Williams et al. 2006, Dodds and Gross 2007, Himmel, Ding et al. 2007, Simmons, Loque et al. 2008). However, plant biomass has evolved to form complex structural and chemical mechanisms that resist its breakdown to valuable precursors amenable for conversion. Lignin in biomass serves as both a shield to block chemical and biological access to sugars as well as an inhibitor to enzymes (Scheiding, Thoma et al. 1984, Converse, Matsuno et al. 1988, Holtzapple, Cognata et al. 1990, Eriksson, Karlsson et al. 2002, Vermaas, Petridis et al. 2015). In addition, cellulose is tightly packed in microfibrils that exhibit high crystallinity and degree of polymerization contributing to its recalcitrance (Zhao, Zhang et al. 2012). Additionally, xylan oligomers and other polysaccharides from hemicellulose cause severe cellulase inhibition in cellulose conversion to sugars (Kumar and Wyman 2009, Kumar and Wyman 2014).

Methods for biomass deconstruction have traditionally focused on improving recovery of monomeric sugars from biomass. Aqueous technologies, such as hydrothermal and dilute acid pretreatments have been developed to reduce limiting factors, such as cellulose degree of polymerization and enzyme inhibition by hemicellulose sugars, thus achieving modest improvements in sugar yields upon enzymatic digestion while largely ignoring lignin (Lloyd and Wyman 2005, Kumar, Mago et al. 2009, Pu, Hu et al. 2013). Lignin removal from the cell wall and subsequent processing is challenging due to its chemical structure and resistance to chemical and

biological manipulation in aqueous environments. Lignin is also more chemically diverse and energy dense than cellulose and its potential value as a primary feedstock for conversion to renewable chemicals, materials, and fuels has long been recognized (Chum, Parker et al. 1985, Linger, Vardon et al. 2014, Ragauskas, Beckham et al. 2014). However, limited understanding of lignin-solvent-cellulose interactions has prevented effective utilization of alternate solvent and co-solvents to augment treatment of biomass towards promoting sugar recovery and lignin utilization.

Furthermore, recent focus has emphasized disparate approaches to either achieving high yields from polysaccharide hydrolysis or targeted extraction and depolymerization of residual lignin towards value-added products (Lloyd and Wyman 2005, Zhao, Cheng et al. 2009, Galkin and Samec 2016, Rodriguez, Salvachúa et al. 2017). Ammonia fiber expansion (AFEX) (Alizadeh, Teymouri et al. 2005) and organosolv (Lora and Aziz 1985, Johansson, Aaltonen et al. 1987, Pan, Arato et al. 2005, Pan, Gilkes et al. 2006, Huijgen, Smit et al. 2012) pretreatments are capable of delignifying biomass to achieve high cellulose digestibility, however, with drawbacks such as costly process economics (Bals, Wedding et al. 2011) and reduced overall sugar yields due to severe degradation of hemicellulose sugars. Additionally, sulfite and ionic liquid pretreatments have made strides towards increasing enzymatic sugar yields and improving ethanol titers achieved from biomass feedstocks, albeit still requiring significant enzyme loadings (Wang, Pan et al. 2009, Zhu, Pan et al. 2009, Shuai, Yang et al. 2010, Xu, Sun et al. 2016). Recently, significant progress has been made with co-solvent pretreatment termed Co-solvent Enhanced Lignocellulosic Fractionation (CELF)

of biomass to demonstrate its performance, yield and cost advantages over traditional technologies. In particular, previous research has reported 90% recovery of fermentable sugars while lowering enzyme loadings by an order of magnitude and achieving >80 g/L ethanol titers from high-solids fermentations of pretreated biomass after selective lignin removal by CELF (Cai, Zhang et al. 2013, Cai 2014, Cai, Nagane et al. 2014, Nguyen, Cai et al. 2015, Nguyen, Cai et al. 2017). Further, CELF has been successfully integrated with promising advanced fermentation technologies (Thomas, Donohoe et al. 2017). Moving forward, it is critical for efficient and green techniques to be developed whereby high yields of biomass polymers suitable for subsequent valorization is paramount (Ragauskas, Williams et al. 2006, Dodds and Gross 2007). Key to this is maximizing total utilization of carbon by simultaneously enhancing both the efficient hydrolysis of polysaccharides and the extraction and depolymerization of lignin (Ragauskas, Williams et al. 2006). However, the molecular mechanisms necessary for rational and integrative biomass deconstruction methods remain largely unexplored.

We characterize here, at multiple scales, the physical and chemical changes that occur to the primary cell wall components, lignin, cellulose, and hemicellulose during co-solvent treatment in equivolume mixtures of tetrahydrofuran (THF) and water at elevated temperatures. This unique co-solvent pair also promotes lignin release from cellulose and their subsequent depolymerization to enhance biomass deconstruction. Cellulose that is liberated from lignin and hemicellulose in this co-solvent environment is shown to allow unfettered access to cellulolytic enzymes, exhibiting negligible loss in the rate of hydrolysis over conversion, even at low enzyme loadings. A highly pure form of lignin

powder is also extracted that is most suitable for further catalytic or biological conversion. Thus, elucidation of the molecular principles governing the synergistic interactions of this co-solvent pair with lignin and cellulose can reveal the associated mechanisms for enhanced biomass deconstruction over other aqueous methods.

4.3 Experimental

4.3.1 Pretreatment

Acer reactions were carried out using air-dried *Acer* chips obtained in New York State by Mascoma Corporation (now of Lallemand Inc., Lebanon, NH). *Populus* reactions were carried out using air-dried BESC (Bioenergy Science Center) standard *Populus* chips provided by the National Renewable Energy Laboratory (NREL). Chips were knife milled to below 1 mm particle size in a Model 4 Wiley Mill Thomas Scientific (Swedesboro, NJ) at the University of California, Riverside. Co-solvent pretreatment solutions were made up of a 1:1 volumetric THF mixture (>99% purity, Fisher Scientific, Pittsburgh, PA) and deionized water. Concentrated sulfuric acid (72 wt%, Ricca Chemical Company) was diluted in solution to obtain acid reaction concentrations of 0.5 wt%. The pretreatment reactions were performed at 10 wt% solids loading in a 1 L Hastelloy Parr autoclave reactor (236HC Series, Parr Instruments Co., Des Moines, IL) equipped with a double-stacked pitch blade impeller operating at 200 rpm at reaction temperature of 160 °C for 25 minutes. The reactor temperature was measured directly using an in-line thermocouple (Omega, K-type). Pretreatment reaction temperatures were maintained by convective heating using a 4 kW fluidized sand bath (Techne, Princeton, NJ). At the conclusion of pretreatment time, the reactor was cooled by quickly immersing

it into a large water bath at room temperature. Following pretreatment, pretreated solids were vacuum filtered and washed with deionized water until they reached a pH of 5. The wet solids were then weighed and moisture content was determined to calculate total dry solids yield during pretreatment. Post-pretreatment liquid was collected and neutralized with 30% ammonium hydroxide until pH of 7 was achieved. The liquid was then boiled in a water bath set to 70 °C to remove the THF co-solvent and precipitate extracted lignin. The recovered lignin samples were washed with deionized water and diethyl ether before being dried at 30 °C for 12 hours.

4.3.2 Sodium Chlorite Delignification (Lignin Removal)

Chlorite delignification was carried out in a fume hood at 70 °C for 8 hours in a water bath (StableTemp, Cole-Parmer, Vernon Hills, IL) by mixing 5 g of dry biomass in triplicate with 160 mL of deionized water, followed by 3 g of sodium chlorite and 3 mL of acetic acid in a 500 mL Erlenmeyer flask (Fischer Scientific, Pittsburg, PA). The contents were thoroughly mixed by shaking the flasks, and a 50 mL Erlenmeyer flask was inverted in the mouth of the reaction flask to contain the reaction contents. Fresh additions of sodium chlorite and acetic acid were added every 2 hours. The contents were allowed to incubate in the water bath placed inside a fume hood for 8 hours. Following completion of the reaction, the delignified solids were vacuum filtered and washed with deionized water until they reached a pH of 5. Similar to pretreated solids, total dry solids yield was determined during pretreatment.

4.3.3 Enzymatic Hydrolysis

As per the NREL protocol for Enzymatic Saccharification of Lignocellulosic Biomass, enzymatic hydrolysis of pretreated solids, and sodium chlorite bleached solids was performed in triplicate using 125 mL Erlenmeyer flasks with a 50 g total working mass containing 50 mM citrate buffer (pH 4.9) to maintain hydrolysis pH, 0.02% sodium azide to prevent microbial contamination, and roughly 1 wt% glucan from pretreated solids. Cellulase enzyme cocktail Accellerase 1500® (BCA Protein Content ~ 85 mg/mL, DuPont Industrial Biosciences, Palo Alto, CA) loadings were varied from 2 - 15 mg protein/g glucan in unpretreated biomass. Enzymatic hydrolysis flasks were placed in a Multitron orbital shaker (Infors HT, Laurel, MD) set at 150 rpm and 50 °C and allowed to stabilize for 1 hour before the addition of enzyme. Samples of approximately 500 µL were periodically taken into 2 mL centrifuge tubes (Fisher Scientific, Pittsburg, PA). Samples were centrifuged at 15000 rpm for 10 minutes before being analyzed by HPLC.

4.3.4. Quantification of Free Protein Content in Enzymatic Hydrolysis Liquid

A $NaBH_4$-based modified Ninhydrin assay was used to quantify total protein in enzymatic hydrolysis liquor with reduced interference from solubilized sugars (Mok, Arantes et al. 2015). One hundred microliters of sample or standard was incubated with 50 µL of 6.7 g/L $NaBH_4$ in a 1.5 mL microcentrifuge tube. Bovine serum albumin (BSA) in the range of 0 – 2000 mg/L was utilized as a protein standard. This was followed by the addition of 300 µL of 9 M HCl and subsequent heating in a dry oven at 130 °C for 2 hours. After cooling to room temperature, 100 µL of the sample was transferred to a fresh 1.5 mL microcentrifuge tube and neutralized with 100 µL of 5M NaOH. Upon

neutralization, 200 µL of 2% Ninhydrin reagent (Sigma-Aldrich Corp., St. Louis, MO) was added and heated at 100 °C for 10 minutes in a dry oven. After cooling to room temperature, 500 µL of 50% (v/v) ethanol was added. Finally, 200 µL of colored solution was transferred to a 96-well microplate and absorbance was read at 560 nm using a SpectraMax M2e Microplate Reader (Molecular Devices, Sunnyvale, CA). All samples were performed in triplicate.

4.3.5 Fractal Modeling of Enzymatic Hydrolysis Kinetics

Fractal kinetic models were based on first-order cellulose saccharification kinetics to form glucose with rate coefficient k_t, and fractal exponent h (equation (1)) (Wang and Feng 2010):

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

Non-linear regression using MATLAB 7.0 was used to fit experimental data from enzymatic hydrolysis to the model described in equation (2) in which X [%] is conversion and t [hours] is time:

$$X = 100 * \left\{1 - exp\left[-k\left(1 + \frac{t^{1-h}-1}{1-h}\right)\right]\right\} \quad (2)$$

4.3.6 Raman, AFM and nanoIR Imaging and Material Preparation

Cross sections of untreated *Populus* (obtained from ORNL green house) were prepared using a custom microtome. The sections were solvated in a 1:1 THF:water mixture. The reactor was heated at T = 160 °C for 15 min, 30 min and 60 min. Before and after treatment, the cross sections were characterized using Raman confocal spectroscopy (WITec Alpha300RA) and nanoscale infrared imaging (nanoIR2, Anasys Instruments).

Raman spectra were acquired using a 20x objective with 532 nm laser excitation and a 600 g/mm grating. Laser power and integration time were optimized to maintain the plant cell wall intact. AFM images were acquired with an Au-coated cantilever (PR-EX-nIR2 k ~ 0.07-0.4 N/m) for contact mode imaging and nanoIR mode. NanoIR measurements were carried out as described in (Dazzi and Prater 2016). The laser pulse was tuned to match with one of the cantilever resonances. At a fixed point, the wavelength of the laser was swept from 1530 cm^{-1} to 1800 cm^{-1} with a 2 cm^{-1} step. At each wavelength the position and intensity of the cantilever contact resonance was recorded to form an 'absorption vs. wavenumber' spectrum. When the laser wavelength coincides with an absorption band of the material, the amplitude measured is large.

4.3.7 Molecular Dynamics (MD) Simulations

A cellulose fiber bound by 10 lignin molecules (G-lignin 60mers) was solvated in a THF:water (1:1 v/v) mixture and in pure water, respectively. Both the cellulose:lignin weight ratio (~2:1) and the solid loading (~5 wt%) roughly correspond to the values from the experiments. The total number of atoms in a simulation system was greater than 1.5 million. After energy minimization and equilibration in the NPT ensemble at T = 445 K and ambient pressure, MD simulations with the CHARMM (Vorobyov, Anisimov et al. 2007, Guvench, Hatcher et al. 2009, Petridis and Smith 2009, Guvench, Mallajosyula et al. 2011) force field parameters and the TIP3P (Jorgensen, Chandrasekhar et al. 1983) water model were run with a time step of 2 fs for a total simulation time of >1.5 μs. Three simulations of lignocellulose in the co-solvent mixture were run for up to 750 ns and only one simulation in water-only was run for 50 ns. All simulations were performed and

analyzed with the GROMACS software version 5.0.1 (Pronk, Páll et al. 2013) on the TITAN supercomputer located at the Oak Ridge Leadership Computing Facility (OLCF). Visualization and rendering of molecular images was performed using the VMD software (Humphrey, Dalke et al. 1996).

4.3.8 Analytical Methods on Raw Biomass

Compositional analysis of raw and pretreated *Acer* and *Populus* was conducted according to the established NREL procedure (version 8-30-2010) in triplicate. All chemical analyses performed were based on Laboratory Analytical Procedures (LAPs) documented by NREL (Golden, CO). Liquid samples and appropriate calibration standards were analyzed using High Precision Liquid Chromatography (Agilent 1200 system equipped with a Bio-Rad Aminex HPX-87H column and refractive index (RI) detector) with a 5 mM sulfuric acid eluent at a flowrate of 0.6 mL/min. HPLC chromatograms were integrated using Agilent Chemstation software package.

4.3.9 Lignin HSQC NMR Analysis

Two-dimensional ^{13}C-^{1}H HSQC NMR experiments of control and recovered lignin samples were carried out in a Bruker Avance 400-MHz spectrometer operating at a frequency of 100.59 MHz for ^{13}C(Pu, Chen et al. 2009, Hallac and Ragauskas 2011). A standard Bruker heteronuclear single quantum coherence pulse sequence (hsqcetgpspsi2) was used on a BBFO probe. The lignin samples were dissolved in deuterated dimethyl sulfoxide (DMSO-d_6) solvent. The spectra were acquired with the following conditions: 13 ppm spectra width in F2 (^{1}H) dimension (1024 data points) and 210 ppm spectra width in F1 (^{13}C) dimension (256 data points), a 1.5 s pulse delay, a 90^{o} pulse, and a $^{1}J_{C\text{-}H}$ of

145 Hz. The DMSO solvent peak (δ_C 39.5 ppm; δ_H 2.5 ppm) was used for chemical shifts calibration. Relative lignin interunit linkage abundance and monomer compositions were semi-quantitatively calculated by using volume integration of contours in HSQC spectra.(Ragauskas, Pu et al. 2014) NMR data and spectra processing was performed using TopSpin 2.1 software (Bruker BioSpin) and Adobe Illustrator CC (Adobe Inc.).

4.3.10 Lignin ^{31}P NMR Analysis

Quantitative ^{31}P NMR experiments were conducted on a Bruker Avance 400-MHz spectrometer. Lignin samples (~ 15 mg) were dissolved in a solvent mixture of pyridine and deuterated chloroform (1.6/1.0, v/v, 0.50 mL). The mixture was then further derivatized with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (TMDP)(Pu, Cao et al. 2011). Chromium acetylacetonate and endo-*N*-hydroxy-5-norbornene-2,3-dicarboximide (NHND) were also added into the solution as relaxation agent and an internal standard, respectively. The spectrum was acquired using an inverse-gated decoupling pulse sequence (Waltz-16), 90° pulse, 25-s pulse delay, and 128-256 scans. All the NMR data were processed using the TopSpin 2.1 software (Bruker BioSpin) and MestreNova (Mestre Laboratories) software packages.

4.3.11 Lignin Molecular Weight Analysis

The lignin molecular weight analysis was performed with gel permeation chromatography (GPC) after acetylation (Hallac and Ragauskas 2011). The dry lignin samples were dissolved a mixture of acetic anhydride/ pyridine (1:1, v/v) and stirred at room temperature for 24 h. The solvents were removed by roto-evaporation at 45 ºC with ethanol. The addition and removal of ethanol was repeated until trace of acetic acid was

removed from the samples. The acetylated lignin samples were dried under vacuum at 45 ºC overnight prior to GPC analysis. The molecular weight distributions of the acetylated lignin samples were analyzed on a PSS-Polymer Standards Service (Warwick, RI, USA) GPC SECurity 1200 system featuring Agilent HPLC 1200 components equipped with four Waters Styragel columns (HR1, HR2, HR4 and HR6) and an UV detector (270 nm). Tetrahydrofuran was used as the mobile phase with the flow rate of 1.0 mL/min. Polystyrene narrow standards were used for establishing the calibration curve. Data collection and processing were performed using Polymer Standards Service WinGPC Unity software (Build 6807) and molecular weights were calculated by the software relative to the polystyrene calibration curve.

4.4 Results and Discussion

4.4. 1 Molecular Dynamics Simulations Predict Lignin Dissociation from Cellulose in a Co-Solvent Environment

Atomic-detail molecular dynamics (MD) simulations were applied to a lignocellulose aggregate with lignin molecules based on guaicyl and bound to the surface of a linear cellulose fiber, as shown in Figure 4.1. The model was solvated first in pure water (aqueous) and subsequently in an equivolume THF-water (co-solvent) environment at 445K (which is similar to standard pretreatment conditions). Simulation snapshots of lignocellulose in aqueous and co-solvent media (Figure 4.1 (i)) reveal two striking differences in the physical behavior of the solutes in the two environments. First, the lignin molecules dissociated from the cellulose and from each other, as indicated by the substantial decrease in the lignin-cellulose and lignin-lignin atomic contacts (Figure 4.1

(ii), left, mid-left). Second, the solvated lignin molecules changed from compact to extended conformations as shown by the increase in their radii of gyration (Figure 4.1 (ii), mid-right), suggesting that the cleavage of ether linkages (ß-O-4) may be enhanced due to greater solvent accessible surface area (SASA; Figure 4.1 (ii), right) in the extended macromolecular configurations. Thus, the presence of a co-solvent disaggregated and dissolved lignin molecules, allowing them to separate from the solid cellulose fraction and from each other while exposing lignin linkages to the solvent environment. The co-solvent induced separation of lignin molecules found in the simulations (Figure 4.5 in Additional Information) suggests that condensation (re-polymerization) reactions of lignin polymers are unlikely to occur in the presence of the co-solvent.

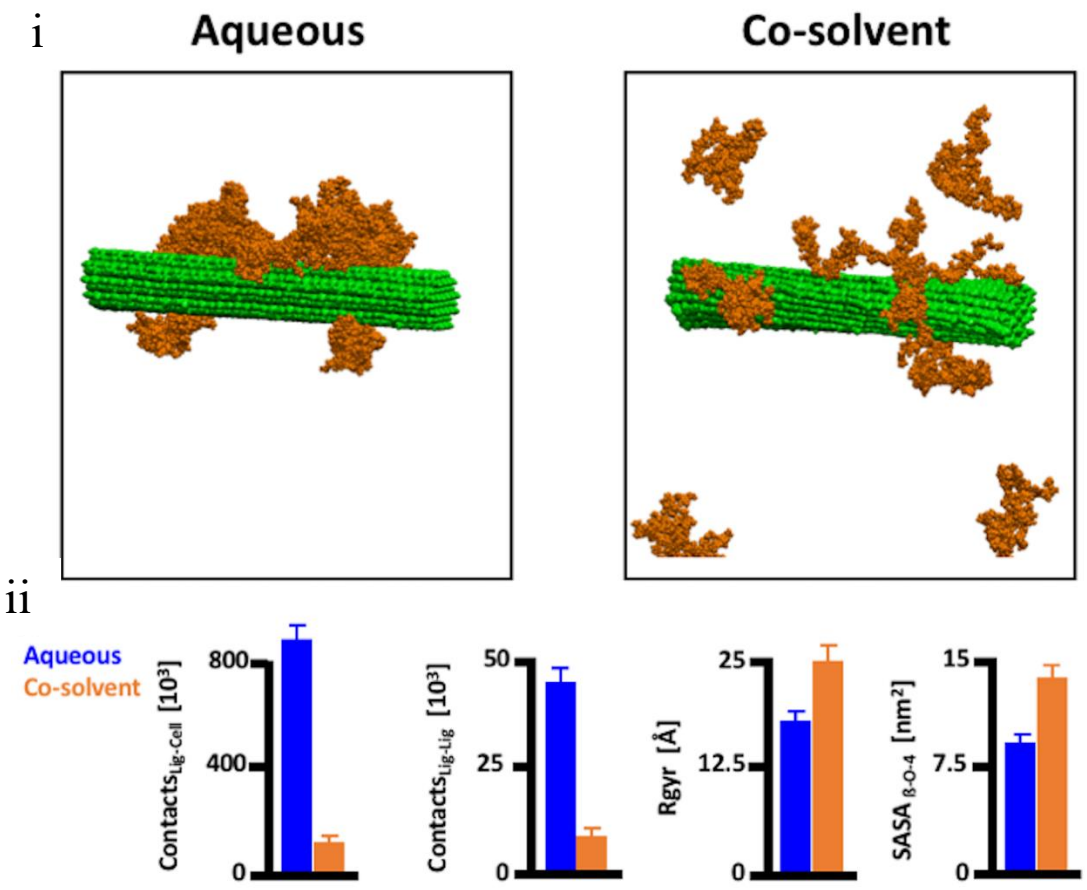


Figure 4.1. (i) MD simulation of lignocellulose in aqueous solution (left) and co-solvent mixture (right) after ~50 ns. The cellulose fiber is shown in green and the lignin molecules in brown. In the co-solvent environment, almost all lignin molecules have dissociated from the cellulose fiber and from each other, changing their structure from compact (globular) to extended (coil) states. **(ii)** Average lignin-cellulose (left) and lignin-lignin contact numbers (mid-left), lignin radius of gyration (mid-right), and solvent accessible surface area of all ß-O-4 lignin linkage atoms (right) in the aqueous (blue) and in the co-solvent mixture (orange).

4.4.2 Raman and NanoIR Spectra Reveal Lignin Undergoes Molecular Changes in a Co-Solvent Environment

Both the rearrangement of the lignin structure and enhanced chemical accessibility of the ß-O-4 lignin linkages unveiled by MD simulations were then

confirmed experimentally by co-solvent reactions of thin cross sections of hardwood *Populus*, exposing its cell wall. First, Raman spectra collected in multiple regions of the solids remaining after the co-solvent reaction (Figure 4.2 (i)) show that the cellulose remained mostly unchanged while the lignin underwent significant changes over time. After 15 min of reaction at 160 °C in the co-solvent (without acid), the intensities of the ring conjugated C=C stretching of lignin coniferyl alcohol (1660 cm^{-1}) and coniferaldehyde (1620 cm^{-1}) bands decreased. Variations of the lignin bands in the 1200-1700 cm^{-1} range and around 2940 cm^{-1} (asymmetric CH stretching in lignin O-CH_3 groups) as well as an increase in the fluorescence background also suggested that lignin underwent molecular changes during the reaction. The pronounced reductions in lignin peak intensities observed in these spectra clearly demonstrated the ability of the co-solvent mixture to delignify biomass. In contrast, cellulose bands around 1350 cm^{-1} and 1150 cm^{-1} and a carbohydrate band around 2897 cm^{-1} remained.

To better understand where the local chemical changes occur in the cell wall, nanoscale functional imaging (nanoIR) was applied to biomass cross sections at sub-100 nm lateral resolution. In the untreated sample, the nanoIR spectra obtained at different locations show similar signatures with slight differences in lignin bands around 1650 cm^{-1} (Figure 4.2 (iv)) in the lamella and the secondary cell wall. Clear structural (Figure 4.2 (ii) and 4.2 (iii)) and chemical (Figure 4.2 (v)) differences can be noted after the co-solvent reaction at different locations of the deconstructed cell walls. The IR spectra obtained on the co-solvent reacted tissues reveal the presence of a very strong aromatic band at 1595 cm^{-1}, corresponding to symmetric aryl ring stretching. The absorption from

1720-1610 cm^{-1} significantly decreased after the reaction, while the absorption peak centered at 1730 cm^{-1} broadened. The bands related to ring conjugated C=C coniferyl/sinapyl alcohol and to ring conjugated C=O stretch of coniferaldehyde/sinapaldehyde, present in untreated cell walls, were not present in the sample after reaction, suggesting a rearrangement of the polymers. The Raman and nanoIR data are indicative of a rearrangement of lignin, which is in line with the MD simulations.

When dilute sulfuric acid was supplemented to the co-solvent environment (equivalent to CELF pretreatment methods), the hardwood slices after 160 °C reaction contained only bands consistent with cellulose, as shown in Figure 4.6 (Additional Information). We then performed bulk scale reactions of 1 mm sized wood chips of hardwood *Acer* in 1 L pressure vessels to perform chemical composition, molar mass, and NMR analysis (Figure 4.8 and Table 4.2 in Additional Information). First, compositional analysis revealed that, in the absence of acid, the majority of the carbohydrates remained in the solid fraction after reaction in co-solvent mixture and the amount of lignin was only slightly reduced (Table 4.2 in Additional Information). The addition of as little as 0.5 wt% dilute sulfuric acid in the co-solvent mixture removed as much as 83% of the lignin and 95% of the hemicellulose from the raw wood chips after only 25 minutes reaction at 160 °C.

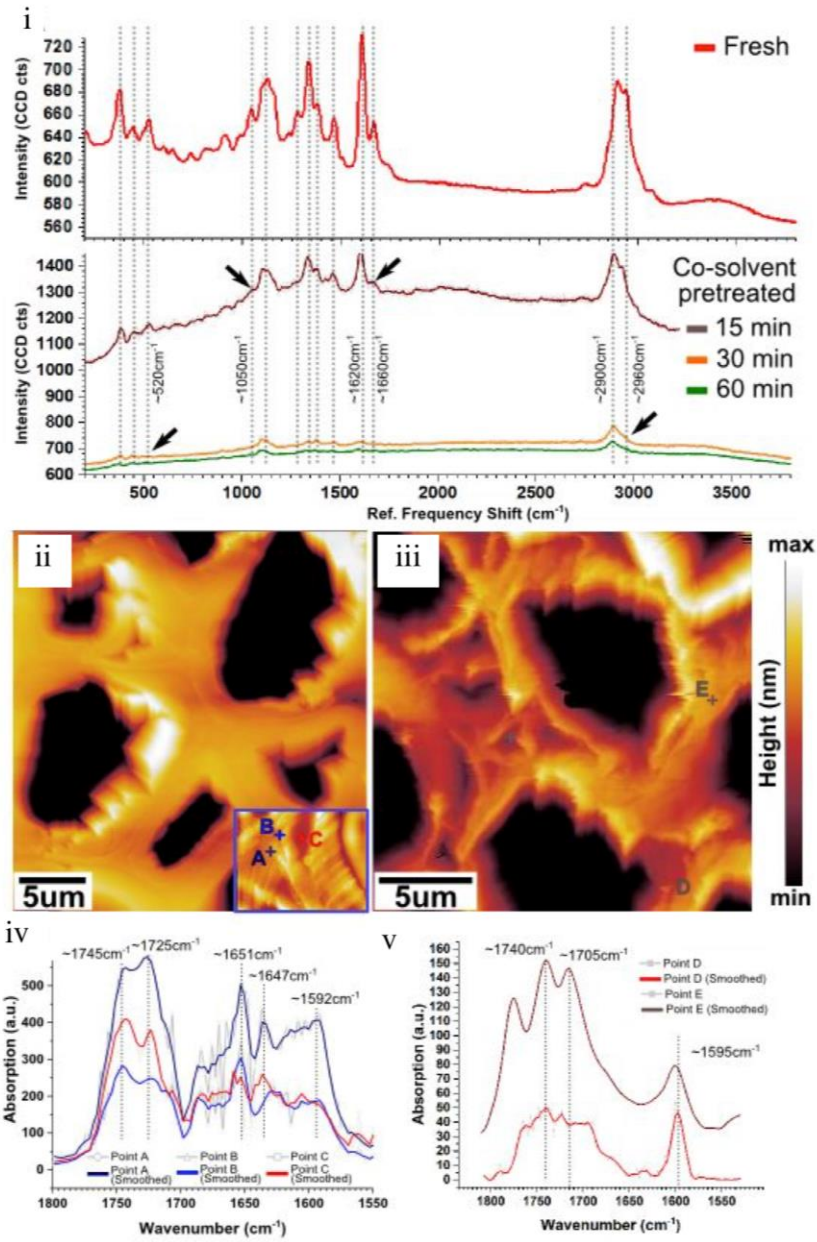


Figure 4.2. Micro-and nanoscale chemical analysis of **(i)** Raman spectra of the cell walls of unpretreated sample (red), sample reacted in co-solvent environment for 15min (brown), 30min (orange) and sample reacted in co-solvent environment until bands representative of lignin disappear (green). **(ii, iii)** Topography images of the unpretreated *Populus* cross section (ii) and the co-solvent reacted cross section (iii). **(iv)** Local IR spectra obtained at selected regions across the cell wall including the middle lamella (points A, B) and secondary wall (point C). **(v)** Spectra (from point D and E) of the sample treated in co-solvent environment exhibiting significant differences, in accordance with the changes in cell wall structure (iv).

4.4.3 Extensive Delignification by Co-Solvent Pretreatment Allows for Sustained Enzymatic Activity during Cellulose Hydrolysis

A significant result of the efficient solubilization of hemicellulose and lignin in the co-solvent mixture is the unrestricted accessibility of the cellulose substrate to enzymes arising from the minimal interference from lignin (Tatsumoto, Baker et al. 1988, Yang and Wyman 2006), suggesting that high glucose yields at affordable enzyme loadings may be achievable (Klein-Marcuschamer, Oleskowicz-Popiel et al. 2012). To investigate the effect of selective removal of hemicellulose and lignin on recalcitrance, dilute sulfuric acid pretreatment (DSA) and sodium chlorite delignification were chosen as a conventional benchmark pretreatment and a laboratory-scale delignification method respectively. DSA generated hemicellulose-free (H-) *Acer*, and sodium chlorite delignification generated lignin-free (L-) *Acer*. Further, delignification of H- biomass produced hemicellulose and lignin-free (H- L-) biomass. L-, and H-L- biomass, as well as acidic co-solvent pretreated biomass from CELF, show that delignification corresponded with an increase in enzymatic digestibility, while the additional removal of hemicellulose contributed to very high sugar yields (Table 4.1 and Figure 4.7 in Additional Information). However, with near complete removal of hemicellulose and lignin, as with H-L- *Acer*, the cellulose microfiber structure is believed to collapse thus limiting cellulase action (Ishizawa, Jeoh et al. 2009). Co-solvent pretreatment, however, was capable of delignifying biomass sufficiently while preserving essential structural lignin and thus allowing near complete cellulose digestibility (Table 4.1).

The kinetics of enzymatic cellulose hydrolysis (Figure 4.3) further indicates that the removal of lignin is key to increased hydrolytic activity due to greater substrate accessibility. The transient rate parameter based on a fractal kinetic model k_t was calculated as a measure of enzyme-cellulose binding (Wang and Feng 2010). When lignin was significantly removed from the biomass substrates, k_t appears to decrease at a far slower rate with increasing conversion than for the case where hemicellulose alone is removed. The softer slope obtained for the CELF, L- and H- L- materials with increasing conversion indicates that the removal of lignin was key to increasing cellulase enzyme accessibility to substrates over the course of hydrolysis. These results are further supported by free protein content before and after complete cellulose hydrolysis by enzymes (Table 4.4 in Additional Information). Following complete cellulose solubilization in H- *Acer*, the final free protein concentration was roughly 40% less than that before the start of hydrolysis, whereas there was no significant change in free protein concentration after complete solubilization of cellulose from CELF *Acer*. The conservation of free protein after hydrolysis of CELF solids indicates that the high degree of lignin removal during co-solvent reactions was critical to eliminating loss of enzyme activity due to unproductive binding to lignin and achieving prolonged hydrolysis yields as evidenced by the more constant transient rate parameter.

Table 4.1. Glucan, xylan, and lignin compositions of unpretreated, H-, L-, H-L-, and CELF treated *Acer* and their enzymatic digestibility to glucose.

Substrate	**Glucan** **%**	**Xylan** **%**	**Lignin** **%**	**Glucose yield*** **%**
Unpretreated *Acer*	45.7	20.3	24.4	2.9
Hemicellulose-free (H-) *Acer*	63.3	2.1	32.6	20.4
Lignin-free (L-) *Acer*	55.2	22.1	1.7	32.0
Hemicellulose-free and lignin-free (H-L-) *Acer*	96.4	3.1	0	79.5
CELF pretreated *Acer*	89.1	2.3	8.1	98.6

*After 5 days of incubation with Accellerase® 1500 at an enzyme loading of 5 mg-protein g-glucan-in-raw^{-1}

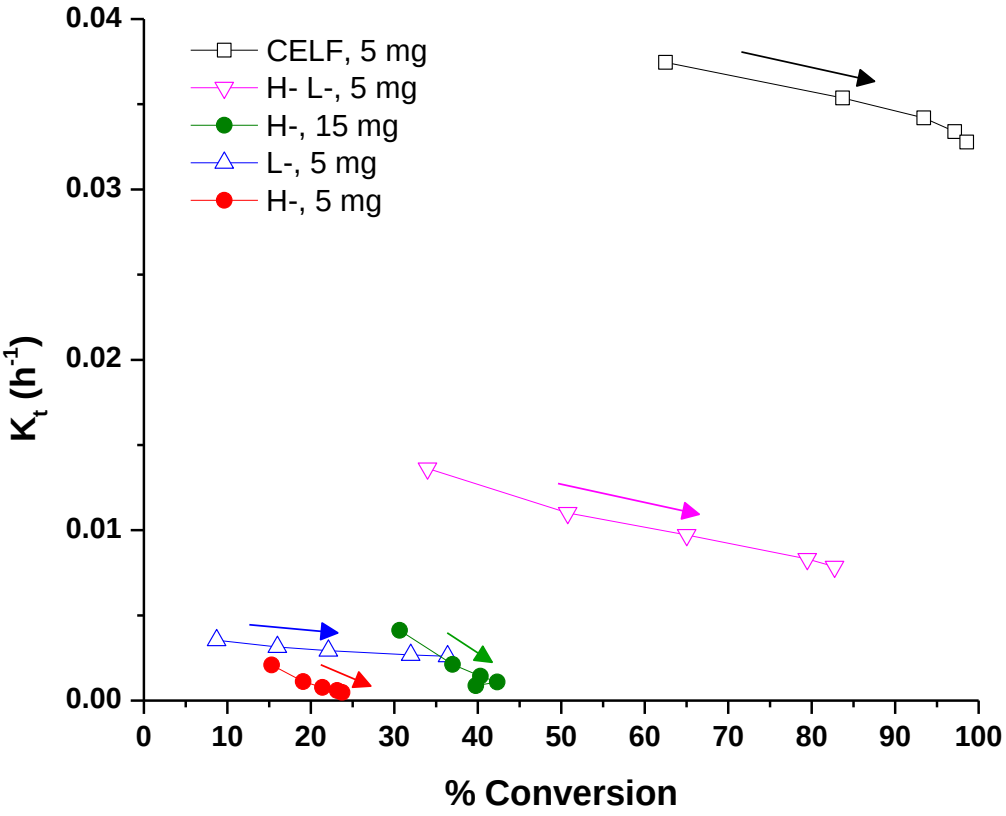


Figure 4.3. Comparison of the change in fractal kinetic rate parameter, k_t, with respect to percent conversion for hemicellulose-free *Acer* (H-) at enzyme loadings of 5 and 15 mg protein/g glucan, lignin-free (L-), hemicellulose and lignin-free (H- L-), and CELF *Acer* at enzyme loadings of 5 mg protein/g glucan. Data points for samples where lignin is removed are shown in unfilled markers. Data points for acidic aqueous reactions are shown in solid circles. Data points for acidic co-solvent reactions are shown in unfilled squares.

4.4.4 Co-Solvent Pretreatment Encourages Depolymerization of Lignin while Limiting its Recondensation

The question then arises as to whether the physical modifications in lignin structure and bulk level delignification described above are accompanied by chemical modifications as a result of the interactions with the co-solvent environment. To evaluate this possibility, we investigated the structure of lignin solubilized and extracted from

Acer by a co-solvent reaction with dilute sulfuric acid by GPC and NMR. The solubilized lignin was isolated by low temperature distillation of THF from the post-pretreatment liquid. The GPC analysis reveals that the molecular weight of solubilized lignin was reduced by ~80% compared to that from the unpretreated control (Figure 4.4), indicating that solubilized lignin was highly degraded during the acidic co-solvent reaction. Moreover, HSQC, ^{13}C and ^{31}P NMR were applied to determine the chemical structures of lignin in the unpretreated biomass (Figure 4.8 in Additional Information) showing a decrease of about ~70% of total β-*O*-4 linkage content in the solubilized lignin compared to the untreated polymer. A peak at around 152 ppm in ^{13}C NMR (Figure 4.8 (ii) in Additional Information), attributed to the C3/5 in the etherified syringyl unit, was significantly reduced for the solubilized lignin, suggesting that etherified syringyl units (i.e., syringyl β-*O*-4 linkage) were very reactive and largely cleaved upon acidic co-solvent reaction. Furthermore, the cleavage of the β-O-4 aryl ether bonds was supported by ^{31}P NMR data (Figure 4.8 (iii) in Additional Information), which resulted in a dramatic increase in phenolic OH groups, both from guaiacyl (~60% increase) and syringyl units (>10-fold increase), in the liquid fraction. This was supported by the nanoIR data above.

Under acidic conditions at elevated temperature, the predominant reactions in lignin are competitive fragmentation by acidolysis of aryl-ether (primarily β-O-4) linkages and repolymerization by acid catalyzed condensation (Li, Henriksson et al. 2007, Pu, Hu et al. 2013). While the former reaction results in the formation of new phenolic end groups and decreased molecular weights of lignin, the latter gives rise to a

new carbon-carbon linkage between two lignin units resulting in an undesirable increase in molecular size negatively impacting its functionality. The GPC results in Figure 4.4 demonstrate that the co-solvent reaction significantly favored acid-catalyzed depolymerization of native lignin while limiting its condensation, leading to low molecular weight products. Evidence of preventing repolymerization was consistent with the MD simulations showing lignin molecules became individually solvated in the co-solvent environment, whereas they aggregated in water (Figures 4.1 and 4.5).

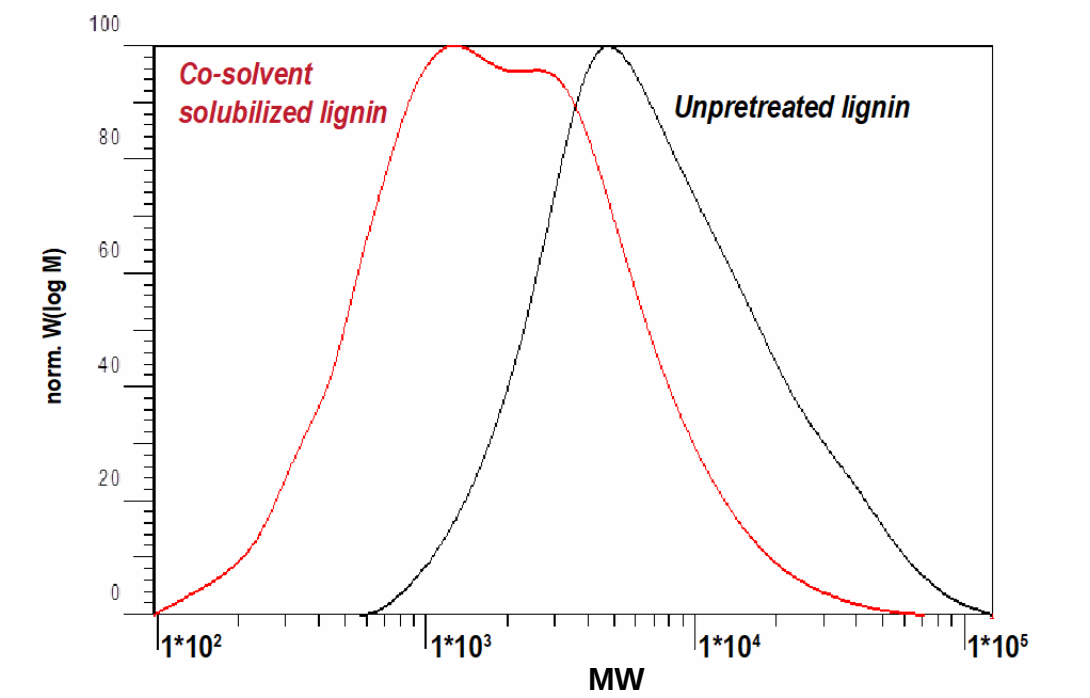


Figure 4.4. Molecular weights distribution of lignin from unpretreated *Acer* (control) and acidic co-solvent solubilized lignin from CELF. Lignin molecular weight decreases by more than 80% after CELF.

Our study presents a set of molecular principles supported by multi-scale evidence to elucidate the fundamental principles supporting efficient breakdown of lignocellulosic matter for deployment at production scale. Recent advances have been

made in understanding acid-catalyzed lignin depolymerization (Sturgeon, Kim et al. 2014) with the aim of achieving lignin breakdown and gaining access to carbohydrates in lignocellulose. However, realizing high degrees of lignin depolymerization is challenging due to lignin condensation reactions that occur during typical acidic pretreatment conditions (Kobayashi, Kohn et al. 2011). Previous studies have suggested that the addition of a protecting agent can stabilize lignin during pretreatment for subsequent depolymerization (Shuai, Amiri et al. 2016). Nevertheless, the amalgamation of effective lignin depolymerization with biofuels production at a commercial scale has yet to be demonstrated. Our work illustrates how a co-solvent pair can effectively coordinate with and expose native inter lignin bonds to facilitate their cleavage during pretreatment (Figure 4.5 in Additional Information). This co-solvent pretreatment has previously been reported to achieve industrially relevant bioethanol titers during high solids fermentations using affordable enzyme loadings (Nguyen, Cai et al. 2015, Nguyen, Cai et al. 2017), thus demonstrating that a co-solvent based technology is an ideal candidate for the study of molecular principles that determine effective biomass deconstruction. Together, nanoscale functional imaging, NMR and MD simulations reveal that lignin dissociates from cellulose in a co-solvent environment, with aryl ring residues remaining in the plant cell walls, whereas the presence of acid in the reactor completely removes lignin and solubilizes hemicellulose. Further, the decrease in the number of aryl-ether inter-lignin linkages in the overall molecular weight of recovered lignin indicates that lignin undergoes significant depolymerization by acid-catalyzed hydrolysis, likely facilitated by lignin adopting extended macromolecular configurations in which linkages are

significantly exposed to the solvent. Observations made at various time points with Raman and nanoIR measurements suggest that the nature and quantity of lignin removed could possibly be tuned by selecting collection times. After acid-catalyzed depolymerization has taken place, lignin fragments are individually solvated in the co-solvent, thus hindering the formation of C-C linkages that lead to repolymerization. Figures 4.4 and 4.8C show evidence of lignin depolymerization in reduced molecular weight distribution and increased phenolic group content in syringyl and guaiacyl units, indicating extensive cleavage of aryl ether interunit linkages. Further, GPC data indicates that lignin repolymerization was minimized and Figure 4.2 further demonstrated how extracted lignin does not recondense onto the cell wall surface. Finally, with the majority of lignin extracted from the bulk of the biomass, facile enzymatic digestion of cellulose is achievable, without any significant loss of enzyme activity (Figures 4.3 and 4.7), suggesting that economically feasible enzyme loadings can be employed without compromising on overall sugar yield. Thus, simultaneous improvements to releasing both sugar and lignin from biomass without the need to employ complex multi-component processes is attainable using a co-solvent mediated process.

4.5 Conclusion

The use of a co-solvent during pretreatment ushers a new-age of technologies for the sustainable single-step selective removal of lignin for valorization and hydrolysis of cellulose. The multi-scale approach applied here reveals the molecular processes responsible for the removal of lignin and its impact on subsequent enzymatic hydrolysis. The coupling of physical (changes in lignin conformations) and chemical (catalysis of

bond cleavage) processes emerges as a key principle behind optimal pretreatment design. These results portend a paradigm shift in the field of biomass conversion away from solely addressing cellulose recalcitrance, hemicellulose inhibition or lignin extraction/refining and towards employing a rationally selected solvent marriage capable of synergistically deconstructing whole biomass systems, so as to yield unfettered access both to sugars and lignin for amenable valorization.

4.6 Acknowledgments

We acknowledge support from the Office of Biological and Environmental Research in the Department of Energy (DOE) Office of Science through the BioEnergy Science Center (BESC) at Oak Ridge National Laboratory (Contract DE-PS02-06ER64304). The award of a fellowship to the lead author by the National Center for Sustainable Transportation made his participation in this project possible. We also acknowledge to support from the Genomic Science Program, Office of Biological and Environmental Research, U. S. Department of Energy (Contract FWP ERKP752). This research used resources of the Oak Ridge Leadership Computing Facility under an INCITE award. We also acknowledge the Center for Environmental Research and Technology (CE-CERT) of the Bourns College of Engineering for providing the facilities and the Ford Motor Company for funding the Chair in Environmental Engineering that facilitates projects such as this one.

4.7 References

Alizadeh, H., F. Teymouri, T. I. Gilbert and B. E. Dale (2005). "Pretreatment of switchgrass by ammonia fiber explosion (AFEX)." Applied Biochemistry and Biotechnology **121**: 1133-1141.

Bals, B., C. Wedding, V. Balan, E. Sendich and B. Dale (2011). "Evaluating the impact of ammonia fiber expansion (AFEX) pretreatment conditions on the cost of ethanol production." Bioresource Technology **102**(2): 1277-1283.

Cai, C. M. Z. (2014). Co-Solvent Enhanced Production of Platform Fuel Precursors From Lignocellulosic Biomass, Doctoral Dissertation, University of California, Riverside.

Cai, C. M., N. Nagane, R. Kumar and C. E. Wyman (2014). "Coupling metal halides with a co-solvent to produce furfural and 5-HMF at high yields directly from lignocellulosic biomass as an integrated biofuels strategy." Green Chemistry **16**(8): 3819-3829.

Cai, C. M., T. Y. Zhang, R. Kumar and C. E. Wyman (2013). "THF co-solvent enhances hydrocarbon fuel precursor yields from lignocellulosic biomass." Green Chemistry **15**(11): 3140-3145.

Chang, V. S. and M. T. Holtzapple (2000). "Fundamental factors affecting biomass enzymatic reactivity." Applied Biochemistry and Biotechnology **84-6**: 5-37.

Chum, H. L., S. K. Parker, D. A. Feinberg, J. D. Wright, P. A. Rice, S. A. Sinclair and W. G. Glasser (1985). Economic contribution of lignins to ethanol production from biomass, Solar Energy Research Inst., Golden, CO (USA)**:** Medium: X; Size: Pages: 102.

Converse, A. O., R. Matsuno, M. Tanaka and M. Taniguchi (1988). "A model of enzyme adsorption and hydrolysis of microcrystalline cellulose with slow deactivation of the adsorbed enzyme." Biotechnology and Bioengineering **32**(1): 38-45.

Dazzi, A. and C. B. Prater (2016). "AFM-IR: Technology and applications in nanoscale infrared spectroscopy and chemical imaging." Chemical Reviews.

Dodds, D. and R. Gross (2007). "Chemicals from biomass." Science **318**(5854): 1250-1251.

Elliott, D. (2016). "A balancing act for renewables." Nature Energy **1**: 15003.

Eriksson, T., J. Karlsson and F. Tjerneld (2002). "A model explaining declining rate in hydrolysis of lignocellulose substrates with cellobiohydrolase I (Cel7A) and endoglucanase I (Cel7B) of *Trichoderma reesei*." Applied Biochemistry and Biotechnology **101**(1): 41-60.

Galkin, M. V. and J. S. M. Samec (2016). "Lignin valorization through catalytic lignocellulose fractionation: a fundamental platform for the future biorefinery." ChemSusChem **9**(13): 1544-1558.

Guvench, O., E. Hatcher, R. Venable, R. Pastor and A. MacKerell (2009). "CHARMM additive all-atom force field for glycosidic linkages between hexopyranoses." Journal of Chemical Theory and Computation **5**(9): 2353-2370.

Guvench, O., S. Mallajosyula, P. Raman, E. Hatcher, K. Vanommeslaeghe, T. Foster, F. Jamison and A. MacKerell (2011). "CHARMM additive all-atom force field for carbohydrate derivatives and its utility in polysaccharide and carbohydrate–protein modeling." Journal of Chemical Theory and Computation **7**(10): 3162-3180.

Hallac, B. B. and A. J. Ragauskas (2011). "Analyzing cellulose degree of polymerization and its relevancy to cellulosic ethanol." Biofuels Bioproducts & Biorefining **5**(2): 215-225.

Himmel, M. E., S. Y. Ding, D. K. Johnson, W. S. Adney, M. R. Nimlos, J. W. Brady and T. D. Foust (2007). "Biomass recalcitrance: engineering plants and enzymes for biofuels production." Science **315**(5813): 804-807.

Holtzapple, M., M. Cognata, Y. Shu and C. Hendrickson (1990). "Inhibition of *Trichoderma reesei* cellulase by sugars and solvents." Biotechnology and Bioengineering **36**(3): 275-287.

Huijgen, W. J. J., A. T. Smit, P. J. de Wild and H. den Uil (2012). "Fractionation of wheat straw by prehydrolysis, organosolv delignification and enzymatic hydrolysis for production of sugars and lignin." Bioresource Technology **114**: 389-398.

Humphrey, W., A. Dalke and K. Schulten (1996). "VMD: Visual molecular dynamics." Journal of Molecular Graphics & Modelling **14**(1): 33-38.

Ishizawa, C. I., T. Jeoh, W. S. Adney, M. E. Himmel, D. K. Johnson and M. F. Davis (2009). "Can delignification decrease cellulose digestibility in acid pretreated corn stover?" Cellulose **16**(4): 677-686.

Jeffries, E. (2016). "News feature: Freeing fossil fuels." Nature Climate Change **6**(2): 125-126.

Johansson, A., O. Aaltonen and P. Ylinen (1987). "Organosolv pulping - methods and pulp properties." Biomass **13**(1): 45-65.

Jorgensen, W., J. Chandrasekhar, J. Madura, R. Impey and M. Klein (1983). "Comparison of simple potential functions for simulating liquid water." The Journal of Chemical Physics **79**(2): 926-935.

Klein-Marcuschamer, D., P. Oleskowicz-Popiel, B. A. Simmons and H. W. Blanch (2012). "The challenge of enzyme cost in the production of lignocellulosic biofuels." Biotechnology and Bioengineering **109**(4): 1083-1087.

Kobayashi, T., B. Kohn, L. Holmes, R. Faulkner, M. Davis and G. E. Maciel (2011). "Molecular-level consequences of biomass pretreatment by dilute sulfuric acid at various temperatures." Energy & Fuels **25**(4): 1790-1797.

Kumar, R., G. Mago, V. Balan and C. E. Wyman (2009). "Physical and chemical characterizations of corn stover and poplar solids resulting from leading pretreatment technologies." Bioresource Technology **100**(17): 3948-3962.

Kumar, R. and C. E. Wyman (2009). "Effect of xylanase supplementation of cellulase on digestion of corn stover solids prepared by leading pretreatment technologies." Bioresource Technology **100**(18): 4203-4213.

Kumar, R. and C. E. Wyman (2014). "Strong cellulase inhibition by mannan polysaccharides in cellulose conversion to sugars." Biotechnology and Bioengineering **111**(7): 1341-1353.

Li, J. B., G. Henriksson and G. Gellerstedt (2007). "Lignin depolymerization/repolymerization and its critical role for delignification of aspen wood by steam explosion." Bioresource Technology **98**(16): 3061-3068.

Linger, J. G., D. R. Vardon, M. T. Guarnieri, E. M. Karp, G. B. Hunsinger, M. A. Franden, C. W. Johnson, G. Chupka, T. J. Strathmann, P. T. Pienkos and G. T. Beckham (2014). "Lignin valorization through integrated biological funneling and chemical catalysis." Proceedings of the National Academy of Sciences of the United States of America **111**(33): 12013-12018.

Lloyd, T. A. and C. E. Wyman (2005). "Combined sugar yields for dilute sulfuric acid pretreatment of corn stover followed by enzymatic hydrolysis of the remaining solids." Bioresource Technology **96**(18): 1967-1977.

Lora, J. H. and S. Aziz (1985). "Organosolv pulping - a versatile approach to wood refining." Tappi; (United States) **68**(8): 94-97.

Lynd, L. R., J. H. Cushman, R. J. Nichols and C. E. Wyman (1991). "Fuel ethanol from cellulosic biomass." Science (New York, N.Y.) **251**(4999): 1318-1323.

Mok, Y. K., V. Arantes and J. N. Saddler (2015). "A NaBH4 coupled Ninhydrin-based assay for the quantification of protein/enzymes during the enzymatic hydrolysis of pretreated lignocellulosic biomass." Applied Biochemistry and Biotechnology **176**(6): 1564-1580.

Nguyen, T. Y., C. M. Cai, R. Kumar and C. E. Wyman (2015). "Co-solvent pretreatment reduces costly enzyme requirements for high sugar and ethanol yields from lignocellulosic biomass." ChemSusChem **8**(10): 1716-1725.

Nguyen, T. Y., C. M. Cai, R. Kumar and C. 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): 11673-11678.

Pan, X. J., C. Arato, N. Gilkes, D. Gregg, W. Mabee, K. Pye, Z. Z. Xiao, X. Zhang and J. Saddler (2005). "Biorefining of softwoods using ethanol organosolv pulping: Preliminary evaluation of process streams for manufacture of fuel-grade ethanol and co-products." Biotechnology and Bioengineering **90**(4): 473-481.

Pan, X. J., N. Gilkes, J. Kadla, K. Pye, S. Saka, D. Gregg, K. Ehara, D. Xie, D. Lam and J. Saddler (2006). "Bioconversion of hybrid poplar to ethanol and co-products using an organosolv fractionation process: Optimization of process yields." Biotechnology and Bioengineering **94**(5): 851-861.

Petridis, L. and J. C. Smith (2009). "A molecular mechanics force field for lignin." Journal of Computational Chemistry **30**(3): 457-467.

Pronk, S., S. Páll, R. Schulz, P. Larsson, P. Bjelkmar, R. Apostolov, M. Shirts, J. Smith, P. Kasson, D. van der Spoel, B. Hess and E. Lindahl (2013). "GROMACS 4.5: a high-throughput and highly parallel open source molecular simulation toolkit." Bioinformatics **29**(7): 845-854.

Pu, Y., F. Hu, F. Huang, B. H. Davison and A. J. Ragauskas (2013). "Assessing the molecular structure basis for biomass recalcitrance during dilute acid and hydrothermal pretreatments." Biotechnology for Biofuels **6**(1): 15.

Pu, Y. Q., S. L. Cao and A. J. Ragauskas (2011). "Application of quantitative P-31 NMR in biomass lignin and biofuel precursors characterization." Energy & Environmental Science **4**(9): 3154-3166.

Pu, Y. Q., F. Chen, A. Ziebell, B. H. Davison and A. J. Ragauskas (2009). "NMR characterization of C3H and HCT down-regulated Alfalfa lignin." Bioenergy Research **2**(4): 198-208.

Pu, Y. Q., F. Hu, F. Huang, B. H. Davison and A. J. Ragauskas (2013). "Assessing the molecular structure basis for biomass recalcitrance during dilute acid and hydrothermal pretreatments." Biotechnology for Biofuels **6**.

Ragauskas, A., Y. Pu, R. Samuel, N. Jiang, C. Fu and Z.-Y. Wang (2014). "Structural characterization of lignin in wild-type versus COMT down-regulated switchgrass." Frontiers in Energy Research **1**(14).

Ragauskas, A., C. Williams, B. Davison, G. Britovsek, J. Cairney, C. Eckert, W. Frederick, J. Hallett, D. Leak, C. Liotta, J. Mielenz, R. Murphy, R. Templer and T. Tschaplinski (2006). "The path forward for biofuels and biomaterials." Science (New York, N.Y.) **311**(5760): 484-489.

Ragauskas, A. J., G. T. Beckham, M. J. Biddy, R. Chandra, F. Chen, M. F. Davis, B. H. Davison, R. A. Dixon, P. Gilna, M. Keller, P. Langan, A. K. Naskar, J. N. Saddler, T. J. Tschaplinski, G. A. Tuskan and C. E. Wyman (2014). "Lignin valorization: Improving lignin processing in the biorefinery." Science **344**(6185): 1246843.

Ragauskas, A. J., C. K. Williams, B. H. Davison, G. Britovsek, J. Cairney, C. A. Eckert, W. J. Frederick, J. P. Hallett, D. J. Leak, C. L. Liotta, J. R. Mielenz, R. Murphy, R. Templer and T. Tschaplinski (2006). "The path forward for biofuels and biomaterials." Science **311**(5760): 484-489.

Rodriguez, A., D. Salvachúa, R. Katahira, B. A. Black, N. S. Cleveland, M. L. Reed, H. Smith, E. E. Baidoo, J. D. Keasling and B. A. Simmons (2017). "Base-catalyzed depolymerization of solid lignin-rich streams enables microbial conversion." ACS Sustainable Chemistry & Engineering **5**(9): 8171-8180.

Rostrup-Nielsen, J. (2005). "Making Fuels from Biomass." Science **308**(5727): 1421-1422.

Scheiding, W., M. Thoma, A. Ross and K. Schugerl (1984). "Modeling of the enzymatic-hydrolysis of cellobiose and cellulose by a complex enzyme mixture of *Trichoderma reesei* Qm 9414." Applied Microbiology and Biotechnology **20**(3): 176-182.

Shuai, L., M. T. Amiri, Y. M. Questell-Santiago, F. Heroguel, Y. D. Li, H. Kim, R. Meilan, C. Chapple, J. Ralph and J. S. Luterbacher (2016). "Formaldehyde stabilization facilitates lignin monomer production during biomass depolymerization." Science **354**(6310): 329-333.

Shuai, L., Q. Yang, J. Y. Zhu, F. C. Lu, P. J. Weimer, J. Ralph and X. J. Pan (2010). "Comparative study of SPORL and dilute-acid pretreatments of spruce for cellulosic ethanol production." Bioresource Technology **101**(9): 3106-3114.

Simmons, B., D. Loque and H. Blanch (2008). "Next-generation biomass feedstocks for biofuel production." Genome biology **9**(12): 242.

Sturgeon, M. R., S. Kim, K. Lawrence, R. S. Paton, S. C. Chmely, M. Nimlos, T. D. Foust and G. T. Beckham (2014). "A mechanistic investigation of acid-catalyzed

cleavage of aryl-ether linkages: Implications for lignin depolymerization in acidic environments." Acs Sustainable Chemistry & Engineering **2**(3): 472-485.

Tatsumoto, K., J. O. Baker, M. P. Tucker, K. K. Oh, A. Mohagheghi, K. Grohmann and M. E. Himmel (1988). "Digestion of pretreated Aspen substrates - hydrolysis rates and adsorptive loss of cellulase enzymes." Applied Biochemistry and Biotechnology **18**: 159-174.

Thomas, V. A., B. S. Donohoe, M. Li, Y. Pu, A. J. Ragauskas, R. Kumar, T. Y. Nguyen, C. M. Cai and C. 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**: 252.

Trancik, J. (2014). "Renewable energy: Back the renewables boom." Nature **507**: 300-302.

van Renssen, S. (2011). "An energy revolution." Nature Climate Change **2**(1): 19-20.

Vermaas, J. V., L. Petridis, X. H. Qi, R. Schulz, B. Lindner and J. C. Smith (2015). "Mechanism of lignin inhibition of enzymatic biomass deconstruction." Biotechnology for Biofuels **8**: 217.

Vorobyov, I., V. Anisimov, S. Greene, R. Venable, A. Moser, R. Pastor and A. MacKerell (2007). "Additive and classical drude polarizable force fields for linear and cyclic ethers." Journal of Chemical Theory and Computation **3**(3): 1120-1133.

Wang, G. S., X. J. Pan, J. Y. Zhu, R. Gleisner and D. Rockwood (2009). "Sulfite pretreatment to overcome recalcitrance of lignocellulose (SPORL) for robust enzymatic saccharification of hardwoods." Biotechnology Progress **25**(4): 1086-1093.

Wang, Z. and H. Feng (2010). "Fractal kinetic analysis of the enzymatic saccharification of cellulose under different conditions." Bioresource Technology **101**(20): 7995-8000.

Xu, F., J. Sun, N. V. S. N. M. Konda, J. Shi, T. Dutta, C. D. Scown, B. A. Simmons and S. Singh (2016). "Transforming biomass conversion with ionic liquids: process intensification and the development of a high-gravity, one-pot process for the production of cellulosic ethanol." Energy & Environmental Science **9**(3): 1042-1049.

Yang, B. and C. E. Wyman (2006). "BSA treatment to enhance enzymatic hydrolysis of cellulose in lignin containing substrates." Biotechnology and Bioengineering **94**(4): 611-617.

Zhao, X., K. Cheng and D. Liu (2009). "Organosolv pretreatment of lignocellulosic biomass for enzymatic hydrolysis." Applied Microbioloigy and Biotechnology **82**(5): 815-827.

Zhao, X., L. Zhang and D. Liu (2012). "Biomass recalcitrance. Part II: Fundamentals of different pre-treatments to increase the enzymatic digestibility of lignocellulose." Biofuels, Bioproducts and Biorefining **6**(5): 561-579.

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): 2411-2418.

Zhu, L., J. P. O'Dwyer, V. S. Chang, C. B. Granda and M. T. Holtzapple (2008). "Structural features affecting biomass enzymatic digestibility." Bioresource Technology **99**(9): 3817-3828.

4.8 Additional Information

Figure 4.5 examines in more detail the simulation of the system in Figure 4.1 and compares the time evolution of lignin-lignin intermolecular contacts (Figure 4.5 (i)) and the average distance between any two lignin molecules (Figure 4.5 (ii)) in co-solvent mixture with those in aqueous simulations. It is evident that the co-solvent counteracts the effects of water, resulting in lignin dissolution (small number of inter-lignin contacts and larger intermolecular distances) instead of aggregation (larger number of inter-lignin contacts and smaller intermolecular distances). The co-solvent reduces the possibility of lignin association, thus rendering repolymerization after hydrolysis less likely.

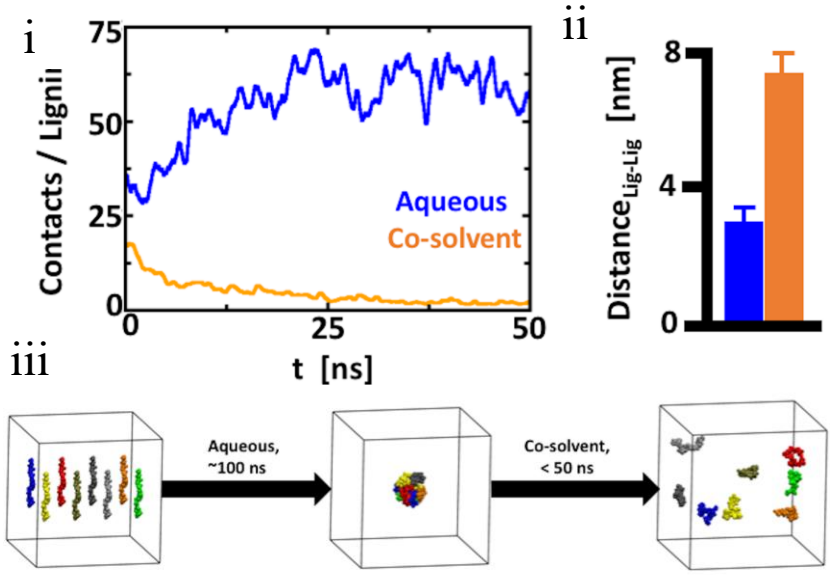


Figure 4.5 (i) Normalized contact numbers between any two lignin molecules over time for simulations in aqeuous (blue) and in THF:water co-solvent (orange) environments. Although the same cellulose-lignin system is used as the starting structure, the numbers at t = 0 ns differ slightly because they are reported after 10 ns of equilibration simulations during which lignin-lignin contacts are increased in water and reduced in co-solvent, respectively. **(ii)** Average lignin-lignin distance (i.e. minimum separation) in the simulations in aqeuous (blue) and in co-solvent (orange) environments. **(iii) (Left)** Starting structure of lignin simulation in water with different lignin decamers shown in different colors. **(Middle)** Within ~100 ns, all lignin polymers have collapsed to a spherical aggregate. **(Right)** The aggregate dissolves to individual lignin molecules in co-solvent mixture in less than 50 ns All simulations were performed at 375 K, the water boiling temperature, and the co-solvent concentration corresponds to that from experiments (1:1 v/v).

In order to further assess the effect of the co-solvent on lignin aggregation, we performed additional simulations of smaller lignin polymers in the two solvent environments. Eight fully-elongated lignin decamers were placed separated from each other in a water medium (see Figure 4.5 (iii)). Within few ns of simulation, the lignin molecules collapse, reducing their average end-to-end distance d_e from 6.2 nm to 2.2 nm

and bind non-covalently to each other, suggesting that contacts to the solvent are not favorable. After ~100 ns, all lignin molecules precipitate to a single aggregate for the first time and this formation remains stable for longer simulation times. Lignin aggregation in pure water facilitates re-polymerization reactions. The addition of the co-solvent changes the behavior of the lignin molecules, which become more solvent-exposed by increasing their size (d_e = 3.4 nm) and disaggregate to become individually solvated. All lignin molecules are fully dissolved in co-solvent mixture in less than 50 ns.

These findings show that the THF:water co-solvent system has the same effect on smaller lignin molecules as on larger aggregates, shown in the main text, suggesting that condensation (re-polymerization) reactions of lignin polymers are unlikely to occur because of their separation in the presence of the co-solvent. Altogether, the simulations shed light on the molecular principles behind the enhanced fractionation of lignin and cellulose in CELF pretreatment, accentuating the merits of this co-solvent medium in allowing enzymes to access cellulose and in processing lignin for its recovery.

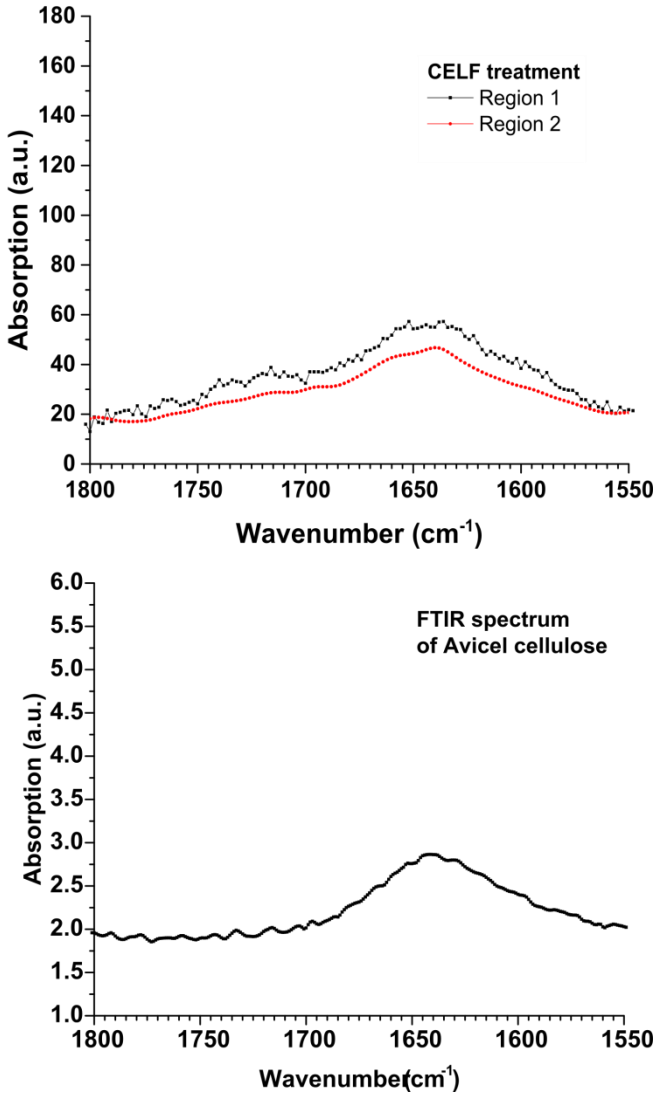


Figure 4.6. NanoIR spectra of CELF pretreated solids (top) and FTIR spectrum of pure cellulose (bottom) indicating that only cellulose remains in the solid after acid-catalyzed THF:water treatment.

Table 4.2. Solids yields and components mass of unpretreated, non-catalyzed co-solvent and aqueous pretreated, and acid catalyzed aqueous and co-solvent pretreated *Acer*.

Substrate	**Solids Yield %**	**Glucan (g)**	**Xylan (g)**	**Lignin (g)**	**Other (g)**
Unpretreated *Acer*	-	45.7	20.3	24.4	2.9
Non-acid co-solvent pretreated *Acer*	92.0	42.1	18.7	19.7	8.4
Non-acid aqueous pretreated *Acer*	85.0	45.5	14.3	21.1	4.2
Acidic aqueous pretreated *Acer*	64.2	40.6	1.4	20.9	1.3
Acidic co-solvent pretreated *Acer*	41.8	37.2	1.0	3.4	0.2

All pretreatments are performed at 160 °C and 25 minutes. Co-solvent pretreatments were performed in a 1:1 THF:water (v/v) co-solvent mixture. Acid catalyzed reactions were performed with 0.5 wt% sulfuric acid. Solids components are based on a starting material of 100 g of untreated *Acer*. Mass loss is attributed to solids removed and solubilized into liquid fraction during pretreatment.

In order to test the enzymatic digestibility of *Acer* after lignin removal, we measured the hydrolysis yields of pretreated solids at different pretreatment conditions with and without hemicellulose and lignin removal (Figures 4.7 (i) and (ii) for enzyme loadings of 2 mg and 5 mg cellulase/g glucan, respectively). After hemicellulose removal (H-), the enzymes lose their hydrolytic activity after ~3 days and the glucose yield plateaus at 10-20%, depending on the enzyme loading, in good agreement with standard processes for biomass conversion. Sequentially removing hemicellulose followed by

lignin (H- L-) from the solid biomass fraction allows enzymes to remain active for as long as 13 days. At low enzyme loading (Figure 4.7 (i)), a comparable enzymatic activity is achieved with the CELF pretreatment method (up to 70% of glucose yield within 12 days). Interestingly, at higher enzyme loading (Figure 4.7 (ii)), the entire glucan is converted to glucose within only 5 days on CELF pretreated biomass while the solid conversion after sequential hemicellulose and lignin removal remains below 85% even after 9 days. This extremely high glucose yield during CELF pretreatment is remarkable because it is achieved in a single step process, allowing both hemicellulose and lignin to be removed for subsequent valorization, which is a significant advantage over traditional pretreatment methods.

We studied the kinetics of enzymatic cellulose hydrolysis based on a first-order fractal model (Wang and Feng 2010). Here, the transient rate coefficient k_t with a fractal exponent h replaces the rate constant k by the following relationship: $k_t = kt^{-h}$. The value of k_t over hydrolysis time is shown in Figure 4.7 (iii) for different pretreatment conditions and enzyme loadings. For hemicellulose-free (H-) *Acer*, k_t = ~0 h^{-1} after ~150 hours of hydrolysis but it remains at constantly higher values beyond 200 hours of hydrolysis for all other substrates, in particular for CELF ($k_t > 0.03$ h^{-1}). k_t is shown as a function of the amount of enzymatic cellulose conversion in Figure 4.3. The softer slope of k_t with increasing conversion for CELF pretreatment and for those methods involving lignin removal indicates that the removal of lignin is key to increased hydrolytic activity due to greater substrate accessibility. In particular, the CELF pretreatment shows near-complete digestion of lignocellulosic biomass at a much higher rate than observed for pure

cellulose (Avicel) and at a lower enzyme loading than H- *Acer*, demonstrating the effectiveness of CELF and its advantage over other methods.

The enzymatic digestibility of *Populus* and *Acer* after CELF pretreatment was compared. Figure 4.7 (iv) shows the glucose yields from enzymatic hydrolysis of CELF pretreated *Populus* and *Acer* at an enzyme loading of 5 mg protein/g glucan. Both substrates show near-identical digestibility after CELF pretreatment, thus making them equivalent as hardwood substrates for the purposes of this study.

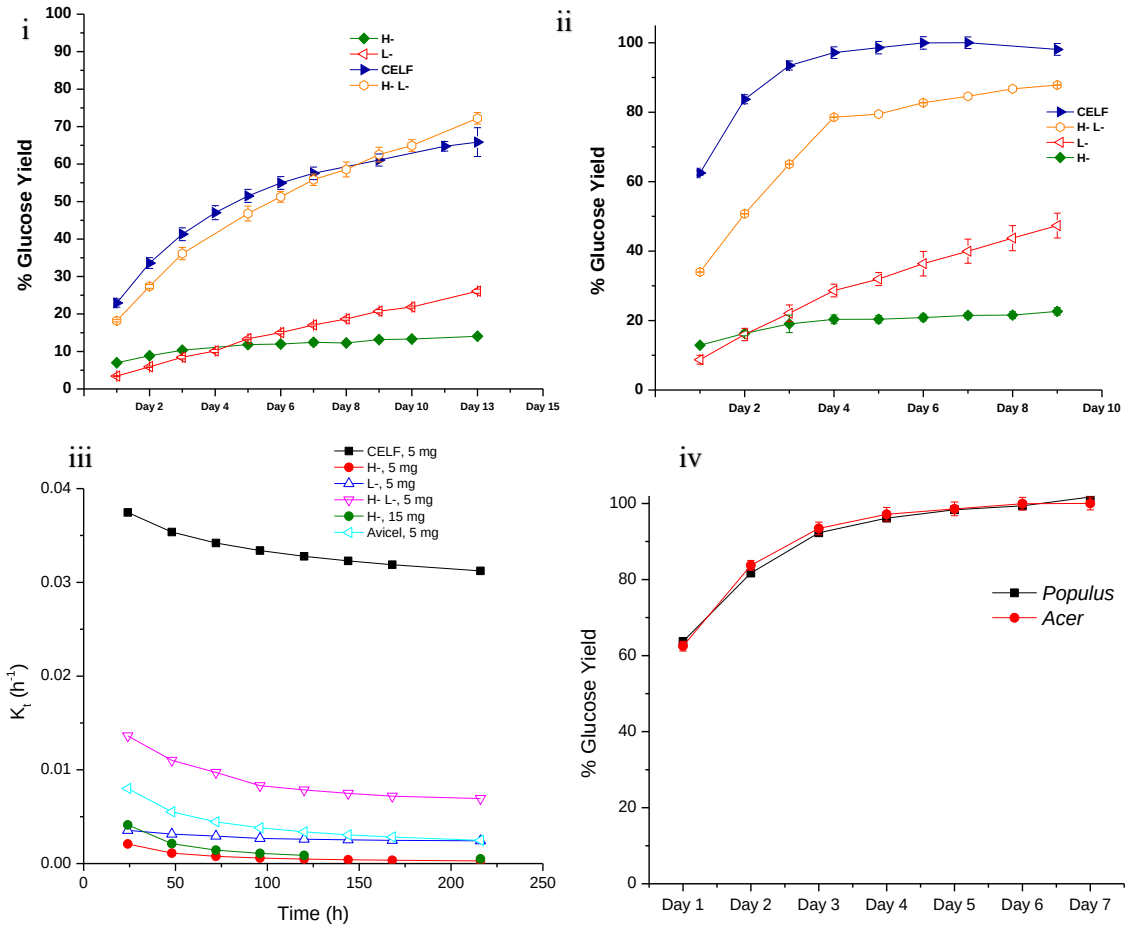


Figure 4.7. Glucose yields from enzymatic hydrolysis of pretreated solids of hemicellulose-free (H-), lignin-free (L-), hemicellulose and lignin-free (H- L-), and CELF pretreated *Acer* at enzyme loading of **(i)** 2 mg protein/g glucan based on *Acer* before pretreatment and **(ii)** 5 mg protein/g glucan. **(iii)** Comparison of the change in fractal kinetic rate coefficient with respect to enzymatic hydrolysis time for H- *Acer* at enzyme loadings of 5 and 15 mg protein/g glucan (denoted as 5 mg and 15 mg), L-, H- L-, Avicel and CELF pretreatments of *Acer* at 5 mg protein/g glucan. **(iv)** Glucose yields from enzymatic hydrolysis of pretreated solids CELF pretreated *Populus* and *Acer* at enzyme loading of 5 mg protein/g glucan based on biomass before pretreatment.

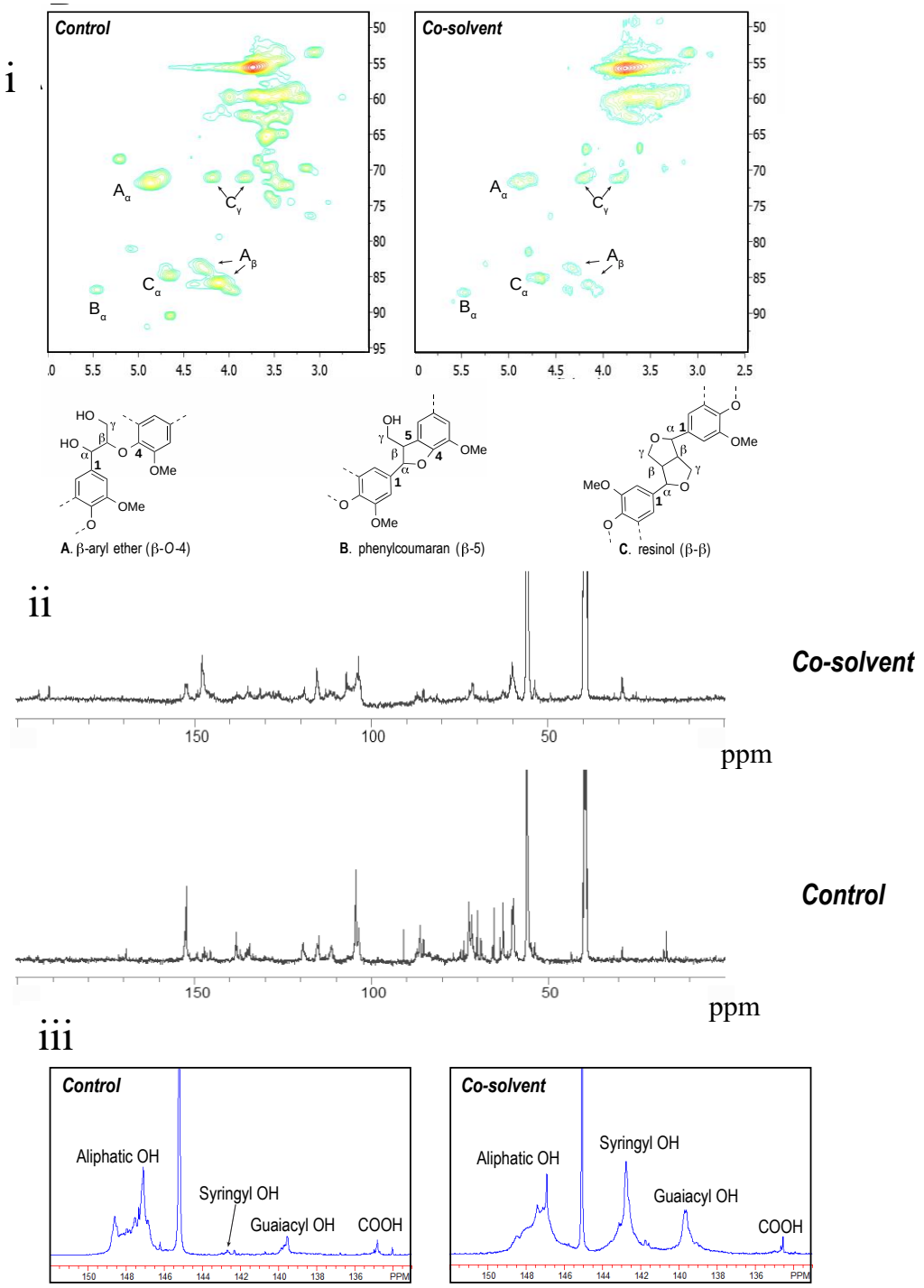


Figure 4.8. (i) $^{13}C/^{1}H$ HSQC spectra (aliphatic regions) of lignin isolated from unpretreated *Acer* (control) and CELF lignin. A: β-O-4 ether; B: β-5/α-O-4 phenylcoumaran; C: resinol. **(ii)** ^{13}C NMR spectra of lignin isolated from untreated *Acer* (control) and co-solvent lignin. **(iii)** ^{31}P NMR spectra of lignin isolated from untreated *Acer* (control) and CELF lignin.

Following pretreatment, lignin presents in two forms – lignin extracted from the cell wall and solubilized in the pretreatment liquid, and lignin present in biomass after pretreatment. The lignin present in the various pretreated *Acer* solid samples along with the extracted lignin from co-solvent reactions were analyzed for relative abundance of major lignin interunit linkages by HSQC NMR spectroscopy and compared to that in lignin present in raw (unpretreated) *Acer* (Table 4.3). Following acidic aqueous pretreatment, the percentage of β-O-4 linkages in lignin in pretreated *Acer* was reduced, indicating that the acid-catalyzed hydrolysis of β-aryl ether bonds occurred during the reaction. Following non-acid catalyzed co-solvent pretreatment, the lignin in pretreated *Acer* contains a much higher percentage of β-O-4 linkages than lignin from raw *Acer*, likely due to the reaction solubilizing a very small amount of low molecular weight lignin end chains, also evidenced in the minimal reduction in bulk lignin (Table 4.2). The percentage of β-O-4 linkages in lignin in pretreated *Acer* is greatly decreased following acidic co-solvent pretreatment illustrating greater lignin depolymerization during acid catalyzed co-solvent reactions. Lignin extracted during acid-catalyzed co-solvent reaction was shown to be highly depolymerized with very significant reduction in β-O-4 linkages.

Table 4.3: Relative abundance (%) of major interunit linkages and monolignol units syringyl to guaiacyl ratios analyzed with HSQC NMR spectroscopy in residual and extracted lignin from variously treated *Acer*.

	Raw *Acer* lignin	Acidic aqueous[a] Lignin in pretreated *Acer*	No acid co-solvent*		Acidic co-solvent[a]*	
			Lignin in pretreated *Acer*	Extracted lignin	Lignin in pretreated *Acer*	Extracted lignin
β-O-4	74.9	63.1	90.3	74.5	60.2	64.8
α-O-4/β-5	14.6	21.3	3.4	11.9	11.2	16.7
β-β	10.3	15.6	6.3	13.6	28.6	14.5
S/G ratio	1.1	5.5	2.6	1.0	4.3	1.6

[a]Acid catalyzed reactions were performed with 0.5 wt% sulfuric acid.
*Co-solvent pretreatments were performed in a 1:1 THF:water (v/v) co-solvent mixture
Note: The relative abundance of interunit linkages were expressed as the percentage of the total interunit linkages. β-O-4: β-aryl ether; α-O-4/β-5: phenylcoumaran; β-β: resinol; S: syringyl units; G: guaiacyl units.

To determine enzyme adsorption to residual solids following complete hydrolysis of cellulose from samples with and without the removal of lignin, a modified Ninhydrin assay was performed on post-enzymatic hydrolysis liquid samples from enzymatic hydrolysis of H- and CELF pretreated biomass at enzyme loadings of 100 mg protein/ g glucan and 5 mg protein/g glucan based on biomass before pretreatment respectively. Table 4.4 shows the protein concentration in the enzymatic hydrolysis liquid before and after complete hydrolysis of the cellulose fraction left in solids from H- and CELF pretreated *Acer*, as well as the mass of residual solids. These data show that no significant

change in the free enzyme concentration during enzymatic hydrolysis of CELF pretreated *Acer*. However, for the case of H- *Acer*, the mass of solids left after enzymatic hydrolysis is roughly 3 times that left after hydrolysis of CELF pretreated solids and only about 60% of the protein is left in solution. These results indicate that roughly 40% of protein is irreversibly adsorbed on these H- solids. Thus, the much lower lignin content of CELF material appears responsible for more enzyme remaining in solution and could explain why CELF pretreated solids continue to be digestible after multiple weeks of hydrolysis by cellulolytic enzymes. These results also suggest that hemicellulose contributes to biomass recalcitrance because of the significantly higher value of k_t for H- L- *Acer* as compared to L- *Acer*, in which hemicellulose is not removed.

Table 4.4. Protein measured before and after enzymatic hydrolysis and residual solids after complete cellulose hydrolysis by H- *Acer* and CELF *Acer* at 100 and 5 mg protein/g glucan enzyme loadings respectively.

Substrate	**Protein Before (mg)**	**Protein After (mg)**	**Residual Solids (g)**
Hemicellulose-free (H-) *Acer*	85	52	0.36
CELF *Acer*	3	3	0.11

Structural features of cellulose, such as crystallinity and degree of polymerization have been observed to be factors that affect enzymatic digestibility of unpretreated biomass (Chang and Holtzapple 2000, Zhu, O'Dwyer et al. 2008). To consider the possibility of structural features affecting digestibility of pretreated biomass, the degree of polymerization and crystallinity index of hemicellulose-free (H-) and CELF pretreated

maple were measured (Table 4.5). The DP_w of cellulose inunpretreated *Acer* was 2762, and after both pretreatment methods was reduced by > 65% (DP_w = 892 and 672, for H- and CELF respectively) thus indicating that both pretreatments were able to depolymerize cellulose significantly to increase enzymatic digestibility. However, the DP_w of H- and CELF pretreated *Acer* were not significantly different when compared to the reduction from unpretreated *Acer*. Additionally, the cellulose crystallinity index % was found to increase after pretreatment to nearly identical values for both methods, possibly due to solubilization of the amorphous cellulose fraction. Thus, as the DP_w and crystallinity index measurements of the H- and CELF pretreated *Acer* are comparable, the increase in digestibility of CELF *Acer* in comparison to H- *Acer* (as shown in Table 4.1) is not attributed to changes in cellulose crystallinity and degree of polymerization, and is attributed to the lower lignin content in CELF *Acer*.

Table 4.5. Cellulose crystallinity index, degrees of polymerization (DP_w and DP_n) and polydispersity index (PI) for unpretreated, hemicellulose-free (H-), and CELF pretreated *Acer*.

Substrate	**Crystallinity Index (%)**	**DP_w**	**DP_n**	**PI**
Unpretreated *Acer*	52.1	2762	382	7.2
Hemicellulose-free (H-) *Acer*	60.2	892	148	6.0
CELF *Acer*	60.3	672	126	5.3

Chapter 5

Adding Tetrahydrofuran to Dilute Sulfuric Acid Pretreatment Reduces Presoaking and Milling Requirements of Alamo Switchgrass*

*This chapter will be submitted under the following citation:
Patri, A.S., McAlister, L., Cai, C.M., Kumar, R., Wyman, C.E., 2018. Adding tetrahydrofuran to dilute sulfuric acid pretreatment reduces presoaking and milling requirements of Alamo switchgrass.

5.1 Abstract

Pretreatment is effective in reducing the natural recalcitrance of plant biomass so cell walls can be accessed for conversion to sugars. However, biomass is typically reduced in size to increase the pretreatment effectiveness and realize high sugar yields. Nonetheless, biomass size reduction is a very energy intensive operation and contributes significantly to the overall capital cost. In this study, the effect particle size reduction on the deconstruction of Alamo switchgrass was examined prior to pretreatment by dilute sulfuric acid (DSA) and Co-solvent Enhanced Lignocellulosic Fractionation (CELF). In addition, the impact of biomass presoaking was also evaluated. Pretreated solids were analyzed for compositional differences, and sugar yields by enzymatic hydrolysis were measured over a range of enzyme loadings. In general, DSA successfully solubilized hemicellulose while CELF removed nearly 80% of lignin from switchgrass in addition to majority of hemicellulose. Presoaking and particle size reduction did not have a significant impact on biomass compositions after pretreatment. However, presoaking for 4 hours increased sugar yields by enzymatic hydrolysis of DSA pretreated switchgrass compared to unsoaked samples, whereas sugar yields from enzymatic hydrolysis of CELF solids continued to increase for up to 18 hours of presoaking time. DSA required particle size reduction by knife milling to be < 2 mm in order to achieve adequate sugar yields during enzymatic hydrolysis. CELF solids, on the other hand, realized nearly identical sugar yields from unmilled and milled switchgrass even at very low enzyme loadings. These results imply that CELF may be capable of eliminating particle size reduction prior to pretreatment and reduce overall costs of biomass processing to fuels.

5.2 Introduction

Biofuels derived from lignocellulosic biomass have the potential to substantially reduce greenhouse emissions and dependence on vulnerable and depletable fossil fuel resources (Lynd, Cushman et al. 1991). Switchgrass (*Panicum virgatum*) is a leading candidate as an effective bioenergy feedstock due to its perennial nature, high productivity, and soil restoration properties (Samson and Omielan 1994, Brown, Rosenberg et al. 2000, Fike, Parrish et al. 2006). Switchgrass is mostly composed of carbohydrates and lesser amounts of lignin, with minor contributions from ash, extractives, and protein (Dien, Jung et al. 2006). Cellulose and hemicellulose are the carbohydrates of primary interest for biological production of biofuels, as they can be broken down into five and six carbon sugars that microorganisms can ferment to ethanol with high yields. However, due to the complex nature of plant cell walls, pretreatment is typically required prior to enzymatic and biological conversion to expose carbohydrates from the lignin shield (Yang and Wyman 2008). Various pretreatments that can be broadly categorized as mechanical, thermal, chemical, or their combination methods have been developed over the years to overcome this recalcitrance to sugar release (Wyman, Dale et al. 2005). Mechanical methods typically involve particle size reduction by milling to increase enzyme access to cell wall carbohydrates (Bridgeman, Darvell et al. 2007, Zhu, O'Dwyer et al. 2008). Thermochemical pretreatments utilize chemical reagents, such as acids, bases or solvents, at elevated temperatures to disrupt the cell wall structure and achieve greater access to carbohydrates (Mosier, Wyman et al. 2005). Dilute sulfuric acid (DSA) pretreatment is a research and commercial benchmark that solubilizes

hemicellulose to sugars with high yields and increases digestibility of pretreated biomass, although high enzyme loadings are required to achieve satisfactory sugar yields (Lloyd and Wyman 2005). Co-solvent Enhanced Lignocellulosic Fractionation (CELF) is a recently developed advanced pretreatment that utilizes dilute acid in a miscible mixture of tetrahydrofuran (THF) and water to recover about 80-90% of the lignin and >95% hemicellulose sugars in solution and achieve nearly theoretical sugar yields from the glucan and hemicellulose left in the resulting carbohydrate-rich solids at low enzyme loadings (Nguyen, Cai et al. 2015).

Several challenges are yet to be addressed before biomass-derived fuels can be considered cost effective (Himmel, Ding et al. 2007). For one, because pretreatment is one of the most expensive single unit operation in a biomass processing plant (Wyman 2007), pretreatment cost reductions would be a significant step to achieve cost competitive cellulosic biofuels. Particle size reduction and presoaking prior to pretreatment are typically needed to increase biomass surface area and effectively distribute acid throughout the biomass solids, respectively, (Cadoche and Lopez 1989, Athmanathan and Trupia 2016) resulting in high sugar yields in pretreatment step as well enzymatic hydrolysis (Tillman, Lee et al. 1990). Presoaking of biomass with the reaction ingredients at ambient temperatures prior to thermochemical pretreatment has also been shown to increase biomass wetting and improve inter-particle diffusion of acid catalysts (Ghose, Pannirselvam et al. 1983, Kim and Lee 2002, Ewanick and Bura 2011). However, these additional processing steps increase overall capital and operating costs associated with biomass-derived fuels. Particle size reduction, in particular, can require

intensive energy inputs (Hinman, Schell et al. 1992, Schell and Harwood 1994, Jannasch, Quan et al. 2001, Mani, Tabil et al. 2004), and reducing milling or eliminating it altogether has been proposed to lower pretreatment costs. Thus, in this study, the impact of biomass presoaking and particle size reduction by knife milling were assessed for DSA and CELF pretreatment of Alamo switchgrass. Solids after both pretreatments were analyzed for compositional differences at varying presoaking times and particle sizes. Furthermore, sugar yields from enzymatic hydrolysis of pretreated solids were compared over a range of enzyme loadings to determine the impact of presoaking and knife milling of biomass sugar release following DSA and CELF pretreatments.

5.3 Experimental

5.3.1 Materials

All pretreatments were carried out with senescent Alamo switchgrass chopped (5 cm in length and 0.5 cm in diameter) provided by Genera Energy Inc. (Vonore, TN). DuPont Industrial Biosciences (Palo Alto, CA) provided the Accellerase® 1500 fungal cellulolytic enzyme cocktail used for enzymatic hydrolysis. The protein concentration was measured as 82 mg/ml by following the standard BCA method with bovine serum albumin as a standard (Smith, Krohn et al. 1985). Tetrahydrofuran (THF) was purchased from Fisher Scientific (Pittsburgh, PA).

5.3.2 Milling and Soaking

Knife milling was performed using a Wiley Mill (Model 4, Arthur H. Thomas Company, Philadelphia, PA) with a 1 mm or 2 mm particle size interior sieve. Prior to pretreatment, milled and unmilled switchgrass solids were soaked for times varying from 0 to 18 hours

in appropriate reaction ingredients (see Pretreatment section below) in the pretreatment reactor at 4 °C in a refrigerator to lower reaction kinetics during pre-soaking and minimize solvent evaporation.

5.3.3 Pretreatment

Pretreatments were performed in a 1 L Hastelloy Parr® autoclave reactor (236HC Series, Parr Instruments Co., Moline, IL) equipped with a double stacked pitch blade impeller rotated at 200 rpm. For DSA reactions, solutions were loaded with 0.5 wt% (based on liquid mass) sulfuric acid (Ricca Chemical Company, Arlington, TX), while in CELF reactions, THF (>99% purity, Fisher Scientific, Pittsburgh, PA) was added to a 0.5 wt% sulfuric acid solution in water at a 0.889:1 THF to acidic water mass ratio. CELF reactions were performed at 150 °C for 25 minutes, while DSA reactions were run at 160 °C for 20 minutes as these conditions were determined to be optimum for maximum sugar release in a previous study (see Chapter 3). All reactions were maintained at reaction temperature (± 1°C) by convective heating with a 4 kW fluidized sand bath (Model SBL-2D, Techne, Princeton, NJ). The reaction temperature was directly measured by an in-line K-type thermocouple (Omega Engineering Inc., Stamford, Connecticut). Following pretreatment, solids were separated from the liquid by vacuum filtration at room temperature through glass fiber filter paper (Fisher Scientific, Pittsburgh, PA) and washed with room temperature deionized water until the filtrate pH reached neutral. The solids were carefully transferred to Ziplock bags and weighed. Moisture content of the solids was determined by a halogen moisture analyzer (Model HB43, Mettler Toledo, Columbus, OH).

5.3.4 Enzymatic Hydrolysis

Enzymatic hydrolysis was performed as per the NREL protocol (Selig, Weiss et al. 2008) in triplicate in 125 mL Erlenmeyer flasks with a 50 g total working mass made up of 50 mM sodium citrate buffer (pH 4.9) to maintain the hydrolysis pH and 0.02% sodium azide to prevent microbial contamination together with enough pretreated solids to result in approximately 1 wt% glucan. Accellerase® 1500 cellulase loadings for enzymatic hydrolysis were varied from 2 - 65 mg protein/g glucan in unpretreated biomass (Gao, Kumar et al. 2014). Enzyme loadings were based on unpretreated switchgrass so as not to penalize a pretreatment if it released more glucose in the pretreatment step. Enzymatic hydrolysis flasks were placed in a Multitron orbital shaker (Infors HT, Laurel, MD) set at 150 rpm and 50 °C and allowed to equilibrate for 1 hour before enzyme addition. Homogenous samples of approximately 500 µL were collected at 4 hours, 24 hours, and then every subsequent 24 hours, loaded into 2 mL centrifuge tubes (Fisher Scientific, Pittsburg, PA), and then centrifuged at 15000 rpm for 10 minutes before analysis of the supernatant by HPLC.

5.3.5 Analytical Procedures

All chemical analyses followed Laboratory Analytical Procedures (LAPs) documented by the National Renewable Energy Laboratory (NREL, Golden, CO). Compositional analyses of unpretreated and pretreated switchgrass were performed according to the NREL protocol in triplicates (Sluiter, Hames et al. 2008). Liquid samples along with appropriate calibration standards were analyzed on an HPLC (Waters Alliance e2695) equipped with a Bio-Rad Aminex® HPX-87H column and RI detector (Waters 2414)

with an eluent (5 mM sulfuric acid) flow rate at 0.6 mL/min. The chromatograms were integrated using an Empower® 2 software package (Waters Co., Milford, MA).

5.3.6 Calculations

Following HPLC quantification, the following formulae were applied to calculate mass, volumes, enzyme loadings, and yields:

Mass of sugar released in pretreatment hydrolysate = Sugar concentration from HPLC* Volume of pretreatment hydrolysate

Volume of pretreatment hydrolysate = (Total reaction mass – (Mass of wet pretreated solids * Moisture content))/ Hydrolysate density

Glucan yield fraction after pretreatment = (Mass of wet pretreated solids*(100 – Moisture content)*% of glucan in pretreated solids)/ (Mass of unpretreated solids*% of glucan in unpretreated solids)

Enzyme loading = mg of protein per gram of glucan in enzymatic hydrolysis flask/ glucan yield fraction after pretreatment

The mass of anhydrous sugar in enzymatic hydrolysis substrates was converted to the mass of the corresponding hydrous form by dividing cellobiose values by 0.95, glucose values by 0.90, and xylose values by 0.88 to compensate for the mass of water added during hydrolysis.

Glucose Yield, % = 100*(Concentration of monomeric sugar measured by HPLC * total reaction volume of enzymatic hydrolysis flask)/ (Mass of glucan in enzymatic hydrolysis flask/anhydrous correction factor)

5.4 Results and Discussion

CELF and DSA pretreatments were performed on Alamo switchgrass at conditions previously determined to maximize overall sugar release. To study the effect of presoaking on DSA and CELF, unpretreated switchgrass that was knife milled to < 1 mm was soaked for 4 and 18 hours at 4 °C prior to pretreatment. These solids were compared to samples that were not soaked prior to pretreatment. The effect of particle size reduction on switchgrass was studied by presoaking unmilled and knife milled biomass for 18 hours at 4 °C before pretreatment. Figure 5.1 shows images of unmilled switchgrass and switchgrass knife milled through sieve sizes of 2 mm and 1 mm.

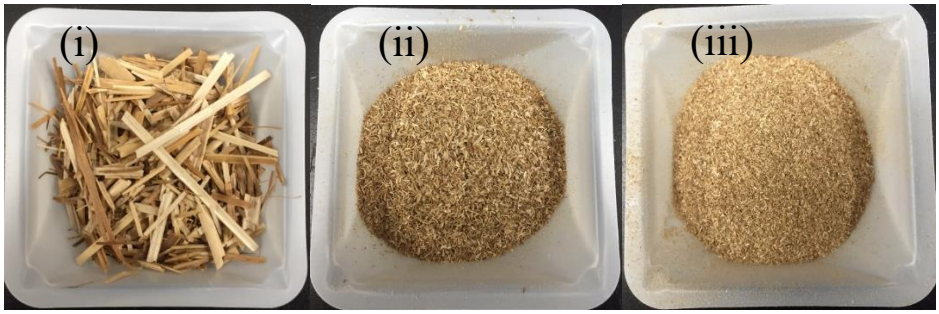


Figure 5.1. Alamo switchgrass (i) before knife milling and after milling to (ii) < 2 mm and (iii) < 1 mm.

5.4.1 Effects of presoaking and particle size reduction on compositions of Alamo switchgrass solids pretreated by DSA and CELF

The masses of major components of Alamo switchgrass solids after DSA and CELF pretreatments at varying presoaking times and particle sizes are listed in Table 5.1. As expected, both pretreatments removed a large fraction of the xylan in the hemicellulose fraction of switchgrass from the biomass. Additionally, CELF pretreatment removed the majority of lignin from the biomass to leave highly glucan-rich solids.

Minimal differences were observed in major component masses as a function of presoaking times for both pretreatments. However, unsoaked DSA pretreated switchgrass contained slightly more glucan in the solids compared to soaked samples, implying that pretreatment acid was not able to fully reach amorphous cellulose without soaking thus resulting in less glucan removal. On the other hand, CELF pretreated switchgrass that was soaked for 4 and 18 hours prior to pretreatment was slightly more delignified than samples that were not soaked prior to CELF. No major compositional differences were observed in solids produced by pretreatment of the range of particle sizes. As expected, both pretreatments removed hemicellulose from the solids, and CELF removed the majority of lignin from solid biomass. These results suggest that CELF removed nearly 80% of the lignin from switchgrass even without presoaking or particle size reduction prior to pretreatment.

Table 5.1. Masses of glucan, xylan and lignin in solids for unpretreated switchgrass and following DSA and CELF pretreatments of switchgrass for varying presoaking times and particle sizes starting with 100 g of unpretreated switchgrass.

Pretreatment	**Presoaking time***	**Particle size****	**Glucan (g)**	**Xylan (g)**	**Lignin (g)**
Unpretreated	-	-	38.8	26.8	21.7
DSA	0 hours	< 1 mm	37.5	2.8	17.7
DSA	4 hours	< 1 mm	36.3	2.7	17.8
DSA	18 hours	< 1 mm	35.8	2.9	17.6
CELF	0 hours	< 1 mm	37.4	2.7	4.6
CELF	4 hours	< 1 mm	36.8	2.3	4.0
CELF	18 hours	< 1 mm	37.0	1.7	3.8
DSA	18 hours	Unmilled	36.0	2.7	18.6
DSA	18 hours	< 2 mm	35.9	2.7	17.6
DSA	18 hours	< 1 mm	35.8	2.9	17.6
CELF	18 hours	Unmilled	37.2	1.6	4.1
CELF	18 hours	< 2 mm	36.8	1.7	4.0
CELF	18 hours	< 1 mm	37.0	1.7	3.8

*Dilute sulfuric acid (DSA) samples were presoaked in 0.5 wt% sulfuric acid and Co-solvent Enhanced Lignocellulosic Fractionation (CELF) samples were presoaked in 0.5 wt% sulfuric acid at a 0.889:1 THF: water mass ratio, both at 4 °C.
**Particle size reduction achieved by knife milling.

5.4.2 Effect of presoaking on enzymatic hydrolysis of DSA and CELF pretreated Alamo switchgrass solids

Enzymatic hydrolysis of solids pretreated by DSA and CELF after presoaking for 4 and 18 hours at 4 °C and without soaking was assessed with varying Accellerase® 1500 cellulase loadings. Figure 5.2 shows sugar released from DSA pretreated switchgrass that was hydrolyzed with enzyme loadings of 65, 15, 5, and 2 mg protein/g glucan in unpretreated solids. At the highest enzyme loading of 65 mg protein/g glucan, Figure 5.2 (i) points out that presoaking for 4 hours increased glucose yields slightly (3%) at the end of 7 days of enzymatic hydrolysis compared to solids that were not presoaked. However, increasing presoaking further to 18 hours did not affect glucose yields. Similar trends were observed at the lower enzyme loadings, with the minor differences in yields between presoaking times of 4 and 18 hours indicating that 4 hours of presoaking prior to DSA pretreatment was sufficient to realize virtually maximum glucan release.

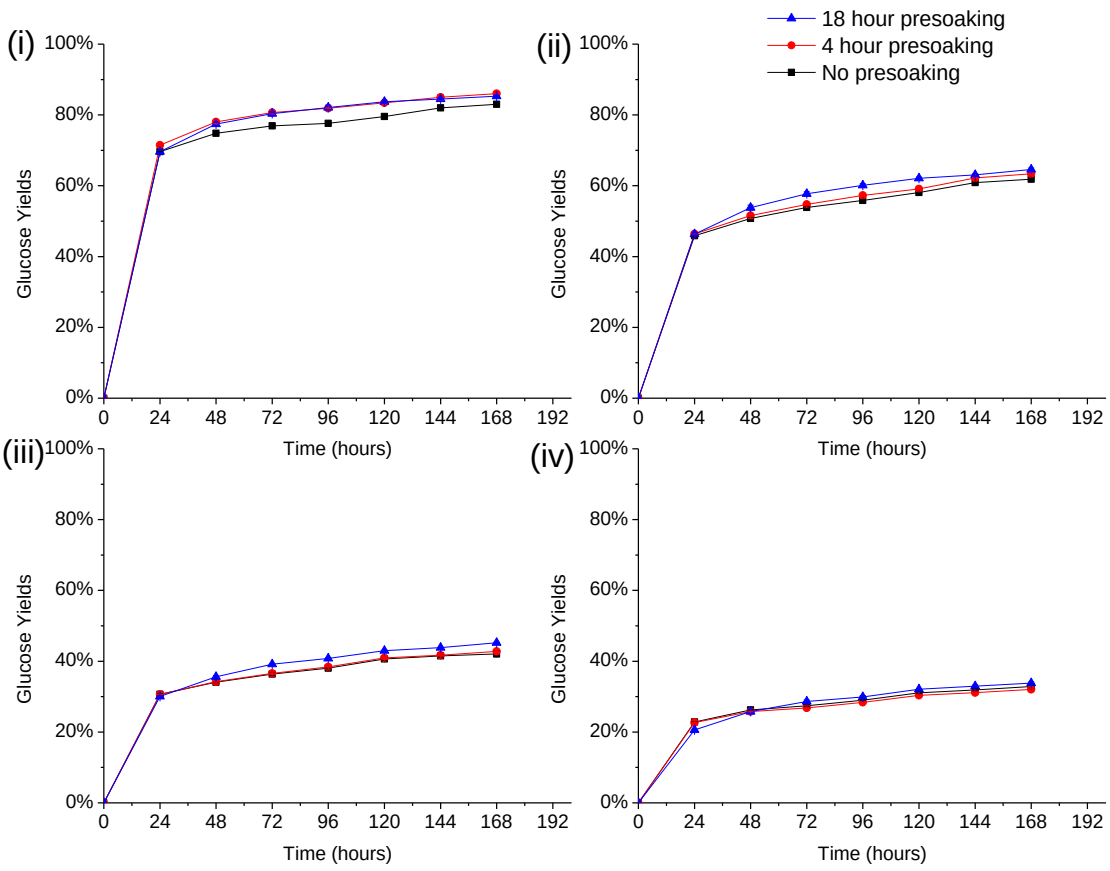


Figure 5.2. Effect of presoaking time on enzymatic glucose yields for pretreated solids prepared by DSA at Accellerase 1500 cellulase loadings of (i) 65 mg (ii) 15 mg (iii) 5 mg, and (iv) 2 mg cellulase protein/g glucan in unpretreated switchgrass. All DSA pretreatments were performed on switchgrass knife milled to < 1 mm at 7.5 wt% solid loading at 160 °C, 20 minutes, and 0.5 wt% sulfuric acid.

As previously shown, CELF produces highly digestible solids that are virtually completely hydrolyzed to glucose in 48 hours at enzyme loadings of 65 and 15 mg protein/g glucan. Thus, enzyme loadings of 5 and 2 mg protein/g glucan were applied here to more clearly show the effect of presoaking times on enzymatic hydrolysis of CELF switchgrass. Figure 5.3 (i) shows that presoaking of switchgrass for 4 hours prior to CELF increased glucose yields by 3% from 2 weeks of hydrolysis at an enzyme

loading of 5 mg protein/g glucan compared to unsoaked switchgrass. Furthermore, presoaking switchgrass for 18 hours increased glucose yields an additional 4% to reach 100% in 2 weeks, as also shown in Figure 5.3 (i). For the enzyme loading of 2 mg protein/g glucan in Figure 5.3 (ii), 18 hours of presoaking prior to CELF increased glucose yields from 2 weeks of enzymatic hydrolysis by 14% compared to unsoaked switchgrass. However, presoaking for more than 18 hours resulted in no change in glucose yields (data not shown).

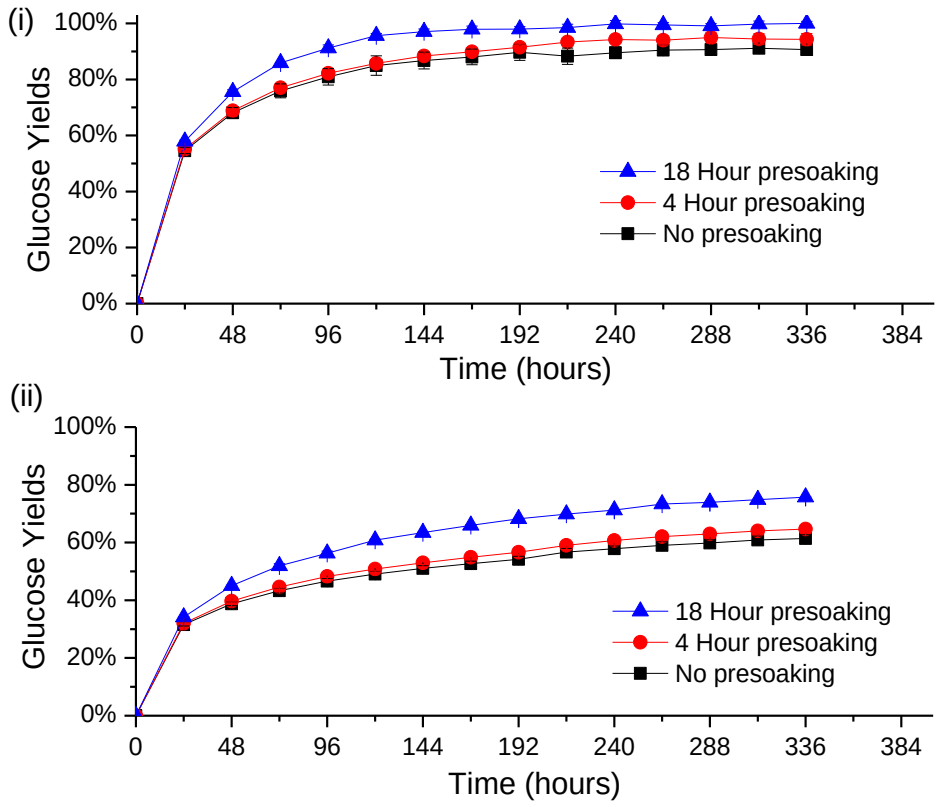


Figure 5.3. Effect of presoaking time on enzymatic glucose yields for pretreated solids prepared by CELF at Accellerase 1500 cellulase loadings of (i) 5 mg protein/g glucan in unpretreated switchgrass and (ii) 2 mg protein/g glucan in unpretreated switchgrass. All CELF pretreatments were performed on switchgrass knife milled to < 1 mm at 7.5 wt% solid loading at 150 °C, 25 minutes, and 0.5 wt% sulfuric acid at a 0.889:1 THF: water mass ratio.

5.4.3 Effect of particle size prior to DSA and CELF pretreatment on enzymatic hydrolysis of Alamo switchgrass

Following CELF and DSA pretreatment of switchgrass presoaked for 18 hours with and without prior milling, the resulting solids were hydrolyzed by Accellerase® 1500 over a range of enzyme loadings. Figure 5.4 shows that milling significantly improved sugar yields from enzymatic hydrolysis of DSA pretreated switchgrass solids. For example, glucose yields from DSA pretreatment of unmilled switchgrass were 14% lower than those from milled switchgrass even at a very high enzyme loading of 65 mg protein/g glucan (Figure 5.4 (i)). Furthermore, yields from enzymatic hydrolysis of solids produced by DSA pretreatment of unmilled switchgrass were lower at enzyme loadings of 15, 5, and 2 mg protein/g glucan compared to those from DSA on milled switchgrass. The sieve size used during milling, however, only had a slight effect on glucose yields from DSA pretreated switchgrass.

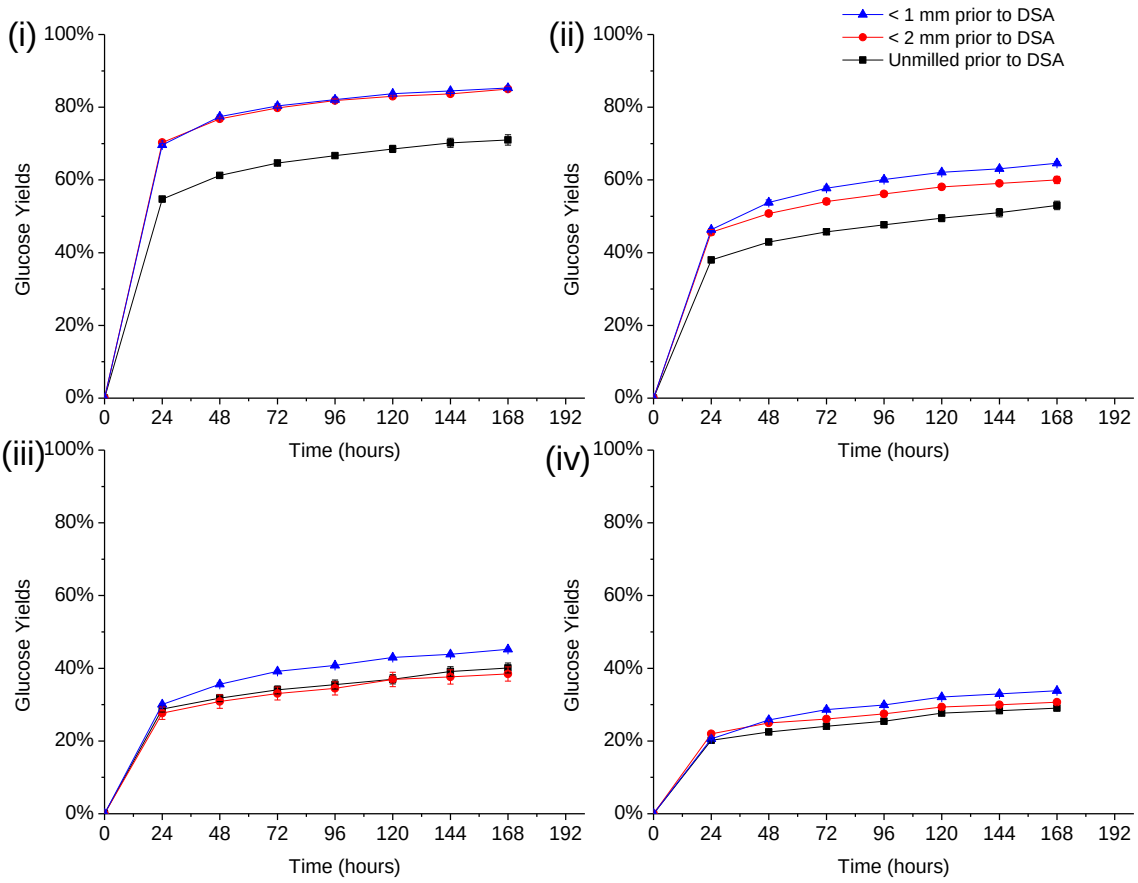


Figure 5.4. Effect of milling size on enzymatic glucose yields for pretreated solids prepared by DSA at Accellerase 1500 cellulase loadings of (i) 65 mg (ii) 15 mg (iii) 5 mg, and (iv) 2 mg cellulase protein/g glucan in unpretreated switchgrass. All DSA pretreatments were performed on switchgrass (presoaked for 18 hours at 4 °C) at 7.5 wt% solid loading at 160 °C, 20 minutes, and 0.5 wt% sulfuric acid.

Because CELF pretreatment achieves nearly theoretical yields from enzymatic hydrolysis at high enzyme loadings, only low loadings of 5 and 2 mg protein/g glucan were applied so the effects of enzyme loading could be distinguished. As shown in Figure 5.5, all samples were highly digestible after 8 days of hydrolysis even at these very low enzyme loadings. Furthermore, glucose yields from enzymatic hydrolysis of CELF pretreated switchgrass were virtually the same regardless of whether the switchgrass was

milled or not and the particle size achieved after milling did not affect glucose yields. These results suggest that CELF is capable of achieving high sugar yields from switchgrass even without prior particle size reduction.

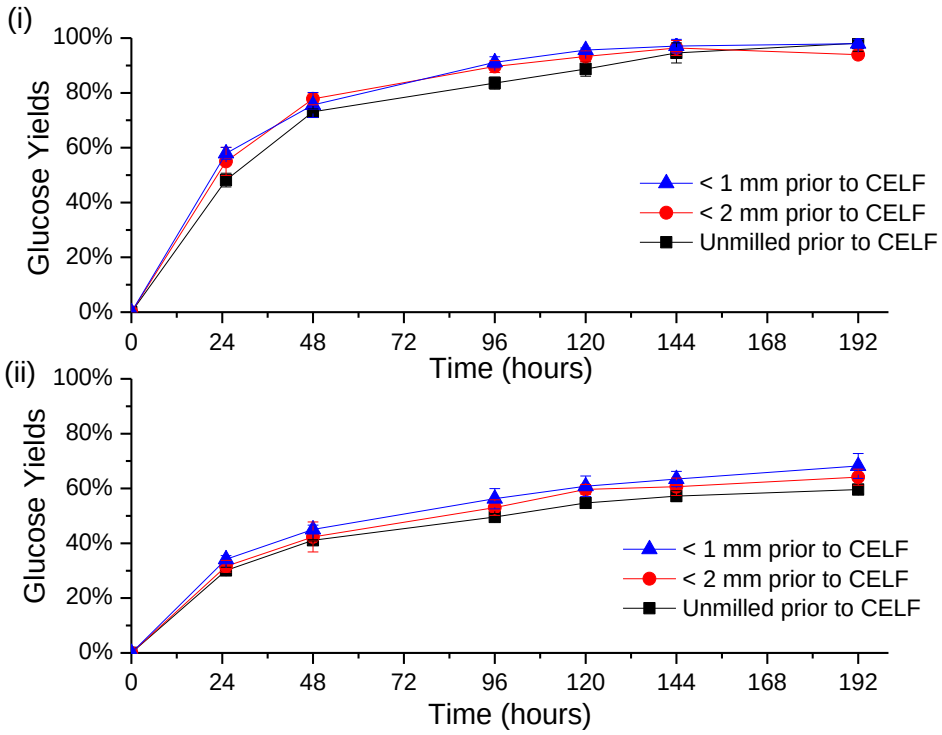


Figure 5.5. Effect of milling size on enzymatic glucose yields for pretreated solids prepared by CELF at Accellerase 1500 cellulase loadings of (i) 5 mg, and (ii) 2 mg cellulase protein/g glucan in unpretreated switchgrass. All CELF pretreatments were performed on switchgrass (presoaked for 18 hours at 4 °C) at 7.5 wt% solid loading at 150 °C, 25 minutes, and 0.5 wt% sulfuric acid at a 0.889:1 THF: water mass ratio.

These results demonstrate that CELF pretreatment can remove a large portion of the lignin and hemicellulose without particle size reduction by knife milling. They also show that milling has very little effect on glucose yields from enzymatic hydrolysis of CELF pretreated solids. These results are in stark contrast to those from DSA pretreatment for which particle size reduction to at least < 2 mm was required to achieve

comparable sugar yields to CELF albeit at much greater loadings of expensive enzymes. Milling prior to pretreatment had a minor effect on the composition of solids produced by DSA pretreatment of switchgrass, implying that the increase in enzymatic hydrolysis yields with milling of DSA switchgrass resulted from enhanced micro-accessibility of cellulose (Kumar and Wyman 2013). Micro-accessibility of cellulose can be improved by reducing its crystallinity or degree of polymerization (Pu, Hu et al. 2013). Because DSA solubilizes hemicellulose and increases cellulose accessibility without physically removing much of the lignin from biomass, it is likely that acid for DSA pretreatment does not effectively diffuse through the entire particle to contact all of the cellulose microfibrils and make them more micro-accessible to cellulolytic enzymes. During CELF pretreatment, on the other hand, the THF:water co-solvent solubilizes a large fraction of the lignin as well as hemicellulose to thus increase the glucan content in the pretreated solids. As lignin in plants coats cell wall polysaccharides to thereby impair their access to water and impart structural strength to the cell wall (Iiyama, Lam et al. 1994), removal of most of the lignin in addition to hemicellulose by CELF pretreatment of unmilled switchgrass disintegrates the cell wall structure and allows acid catalyst to freely contact cellulose fibers.

CELF pretreatment has previously been demonstrated to reduce the amount of enzyme required to achieve high glucose yields and high ethanol titers from corn stover (Nguyen, Cai et al. 2015, Nguyen, Cai et al. 2017). This study show that those findings apply to CELF pretreatment of switchgrass with 100% glucose yields achieved at enzyme loadings as low as 5 mg protein/g glucan. Because enzymes are a major contributor to the

cost of cellulosic fuel production (Klein-Marcuschamer, Oleskowicz-Popiel et al. 2012), reduction of the amount of enzyme required to realize high sugar yields can have a major impact on process economics. In addition, the results presented here suggest that energy intensive milling can be eliminated as well for application of CELF pretreatment instead of DSA to switchgrass. Although elimination of particle size reduction could have significant commercial implications, the effect at higher solids loadings in pretreatment and enzymatic hydrolysis must still be ascertained.

5.5 Conclusions

Most biological operations for biomass conversion require particle size reduction prior to pretreatment to realize high sugar yields by enzymatic hydrolysis. Biomass is also presoaked prior to most pretreatments to provide adequate reactant contact. Both particle size reduction and presoaking are performed to increase reactant diffusion into the biomass particle. However, the high milling energy required to reduce particle size sufficiently to realize high yields are a significant contributor to processing costs, and extended presoaking increases processing times. In this study, the effect of presoaking and knife milling on glucose release from Alamo switchgrass prior to DSA and CELF pretreatment. The results presented here indicate that CELF pretreatment is capable of achieving high glucose yields from subsequent enzymatic hydrolysis even at low enzyme loadings without particle size reduction, in definite contrast to DSA.

5.6 Acknowledgments

Support by the Office of Biological and Environmental Research in the US Department of Energy (DOE) Office of Science through the BioEnergy Science Center (BESC) and

the Center for Bioenergy Innovation (CBI), both managed by Oak Ridge National Laboratory, made this research possible. The award of a fellowship to the lead author by the National Center for Sustainable Transportation facilitated his participation. We also acknowledge the Ford Motor Company for funding the Chair in Environmental Engineering that facilitates projects such as this one and the Center for Environmental Research and Technology (CE-CERT) of the Bourns College of Engineering for providing facilities.

5.7 References

Athmanathan, A. and S. Trupia (2016). "Examining the role of particle size on ammonia-based bioprocessing of maize stover." Biotechnology Progress **32**(1): 134-140.

Bridgeman, T. G., L. I. Darvell, J. M. Jones, P. T. Williams, R. Fahmi, A. V. Bridgwater, T. Barraclough, I. Shield, N. Yates, S. C. Thain and I. S. Donnison (2007). "Influence of particle size on the analytical and chemical properties of two energy crops." Fuel **86**(1-2): 60-72.

Brown, R. A., N. J. Rosenberg, C. J. Hays, W. E. Easterling and L. O. Mearns (2000). "Potential production and environmental effects of switchgrass and traditional crops under current and greenhouse-altered climate in the central United States: a simulation study." Agriculture Ecosystems & Environment **78**(1): 31-47.

Cadoche, L. and G. D. Lopez (1989). "Assessment of size-reduction as a preliminary step in the production of ethanol from lignocellulosic wastes." Biological Wastes **30**(2): 153-157.

Dien, B. S., H. J. G. Jung, K. P. Vogel, M. D. Casler, J. F. S. Lamb, L. Iten, R. B. Mitchell and G. Sarath (2006). "Chemical composition and response to dilute-acid pretreatment and enzymatic saccharification of alfalfa, reed canarygrass, and switchgrass." Biomass & Bioenergy **30**(10): 880-891.

Ewanick, S. and R. Bura (2011). "The effect of biomass moisture content on bioethanol yields from steam pretreated switchgrass and sugarcane bagasse." Bioresource Technology **102**(3): 2651-2658.

Fike, J. H., D. J. Parrish, D. D. Wolf, J. A. Balasko, J. T. Green, M. Rasnake and J. H. Reynolds (2006). "Long-term yield potential of switchgrass-for-biofuel systems." Biomass & Bioenergy **30**(3): 198-206.

Gao, X. D., R. Kumar, S. Singh, B. A. Simmons, V. Balan, B. E. Dale and C. E. Wyman (2014). "Comparison of enzymatic reactivity of corn stover solids prepared by dilute acid, AFEX (TM), and ionic liquid pretreatments." Biotechnology for Biofuels **7**(1): 71.

Ghose, T. K., P. V. Pannirselvam and P. Ghosh (1983). "Catalytic solvent delignification of agricultural residues - organic catalysts." Biotechnology and Bioengineering **25**(11): 2577-2590.

Himmel, M. E., S. Y. Ding, D. K. Johnson, W. S. Adney, M. R. Nimlos, J. W. Brady and T. D. Foust (2007). "Biomass recalcitrance: engineering plants and enzymes for biofuels production." Science **315**(5813): 804-807.

Hinman, N. D., D. J. Schell, C. J. Riley, P. W. Bergeron and P. J. Walter (1992). "Preliminary estimate of the cost of ethanol-production for SSF technology." Applied Biochemistry and Biotechnology **34-5**: 639-649.

Iiyama, K., T. B. T. Lam and B. A. Stone (1994). "Covalent cross-links in the cell-wall." Plant Physiology **104**(2): 315-320.

Jannasch, R., Y. Quan and R. Samson (2001). "A process and energy analysis of pelletizing switchgrass." Prepared by REAP-Canada (www. reap-canada. com) for Natural Resources Canada.

Kim, S. B. and Y. Y. Lee (2002). "Diffusion of sulfuric acid within lignocellulosic biomass particles and its impact on dilute-acid pretreatment." Bioresource Technology **83**(2): 165-171.

Klein-Marcuschamer, D., P. Oleskowicz-Popiel, B. A. Simmons and H. W. Blanch (2012). "The challenge of enzyme cost in the production of lignocellulosic biofuels." Biotechnology and Bioengineering **109**(4): 1083-1087.

Kumar, R. and C. E. Wyman (2013). "Physical and chemical features of pretreated biomass that influence macro-/micro-accessibility and biological processing." Aqueous Pretreatment of Plant Biomass for Biological and Chemical Conversion to Fuels and Chemicals: 281-310.

Lloyd, T. A. and C. E. Wyman (2005). "Combined sugar yields for dilute sulfuric acid pretreatment of corn stover followed by enzymatic hydrolysis of the remaining solids." Bioresource Technology **96**(18): 1967-1977.

Lynd, L. R., J. H. Cushman, R. J. Nichols and C. E. Wyman (1991). "Fuel ethanol from cellulosic biomass." Science **251**(4999): 1318-1323.

Mani, S., L. G. Tabil and S. Sokhansanj (2004). "Grinding performance and physical properties of wheat and barley straws, corn stover and switchgrass." Biomass & Bioenergy **27**(4): 339-352.

Mosier, N., C. Wyman, B. Dale, R. Elander, Y. Y. Lee, M. Holtzapple and M. Ladisch (2005). "Features of promising technologies for pretreatment of lignocellulosic biomass." Bioresource Technology **96**(6): 673-686.

Nguyen, T. Y., C. M. Cai, R. Kumar and C. E. Wyman (2015). "Co-solvent pretreatment reduces costly enzyme requirements for high sugar and ethanol yields from lignocellulosic biomass." ChemSusChem **8**(10): 1716-1725.

Nguyen, T. Y., C. M. Cai, R. Kumar and C. 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): 11673-11678.

Pu, Y. Q., F. Hu, F. Huang, B. H. Davison and A. J. Ragauskas (2013). "Assessing the molecular structure basis for biomass recalcitrance during dilute acid and hydrothermal pretreatments." Biotechnology for Biofuels **6**(1): 15.

Samson, R. A. and J. A. Omielan (1994). "Switchgrass - a potential biomass energy crop for ethanol production." Proceedings of the Thirteenth North American Prairie Conference: Spirit of the Land, Our Prairie Legacy: 253-258.

Schell, D. J. and C. Harwood (1994). "Milling of lignocellulosic biomass - Results of pilot-scale testing." Applied Biochemistry and Biotechnology **45-6**: 159-168.

Selig, M., N. Weiss and Y. Ji (2008). Enzymatic Saccharification of Lignocellulosic Biomass: Laboratory Analytical Procedure (LAP): Issue Date, 3/21/2008, National Renewable Energy Laboratory.

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 **1617**: 1-16.

Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson and D. C. Klenk (1985). "Measurement of protein using bicinchoninic acid." Analytical Biochemistry **150**(1): 76-85.

Tillman, L. M., Y. Y. Lee and R. Torget (1990). "Effect of transient acid diffusion on pretreatment hydrolysis of hardwood hemicellulose." Applied Biochemistry and Biotechnology **24-5**: 103-113.

Wyman, C. E. (2007). "What is (and is not) vital to advancing cellulosic ethanol." Trends in Biotechnology **25**(4): 153-157.

Wyman, C. E., B. E. Dale, R. T. Elander, M. Holtzapple, M. R. Ladisch and Y. Y. Lee (2005). "Coordinated development of leading biomass pretreatment technologies." Bioresource Technology **96**(18): 1959-1966.

Yang, B. and C. E. Wyman (2008). "Pretreatment: the key to unlocking low-cost cellulosic ethanol." Biofuels Bioproducts & Biorefining **2**(1): 26-40.

Zhu, L., J. P. O'Dwyer, V. S. Chang, C. B. Granda and M. T. Holtzapple (2008). "Structural features affecting biomass enzymatic digestibility." Bioresource Technology **99**(9): 3817-3828.

Chapter 6

Liquid-Liquid Extraction of Lignin-Derived Phenolics in CELF Hydrolyzate to Reduce Inhibition of *Saccharomyces cerevisiae* Fermentations

*This chapter will be submitted under the following citation:
Patri, A.S., Cai, C.M., Kumar, R., Wyman, C.E., 2018. Liquid-liquid extraction of lignin-derived phenolics in CELF hydrolyzate to reduce inhibition of *Saccharomyces cerevisiae* fermentations

6.1 Abstract

Co-solvent Enhanced Lignocellulosic Fractionation (CELF) is an advanced pretreatment technology that employs a miscible solution of tetrahydrofuran with water containing dilute acid to solubilize the majority of hemicellulose and lignin into the pretreatment hydrolyzate and leave behind solids greatly enriched in glucan that are highly digestible by enzymes. Furthermore, CELF realizes high yields of dissolved hemicellulose sugars, and microbial conversion of these sugars into ethanol is essential to realize competitive ethanol production costs. This study identified tetrahydrofuran (THF) and lignin-derived phenolics produced during CELF lignin depolymerization as the major inhibitors to *Saccharomyces cerevisiae* fermentations of CELF hydrolyzate sugars. However, because of its high volatility compared to water, THF was easily evaporated from the hydrolyzate to concentrations < 5 g/L that did not inhibit fermentations. Lignin-derived phenolics were effectively extracted out of solution using methyl isobutyl ketone (MIBK) and toluene as organic solvents during liquid-liquid extraction (LLE) of CELF hydrolyzate. The concentrations of lignin-derived phenolics after LLE were low enough to not severely inhibit genetically engineered and wild type strains of *Saccharomyces cerevisiae*. The combination of THF removal by boiling and LLE extraction of lignin-derived phenolics produced an aqueous phase that yielded 90% of theoretical ethanol yields from hemicellulose sugars solubilized by CELF.

6.2 Introduction

Biofuels provide an attractive substitute for conventional fossil fuels due to the use of renewable feedstocks that reduce greenhouse gas emissions and foreign resource dependence (Lynd, Cushman et al. 1991, Wyman 2007). Pretreatments are typically required prior to biological conversions to deconstruct plant cell wall structures to sugars with high yields (Mosier, Wyman et al. 2005, Yang and Wyman 2008). Acid-based thermochemical pretreatments have been historically studied to solubilize hemicellulose and disrupt the cell wall structure to increase access to cellulose (Grohmann, Torget et al. 1987, Torget, Himmel et al. 1991, Lloyd and Wyman 2005, Saha, Iten et al. 2005). The liquid hydrolyzate after dilute sulfuric acid (DSA) pretreatment, a current research and commercial benchmark, typically contains a large portion of the hemicellulose sugars, often composed mostly of xylose, and fermentation organisms have been genetically engineered to ferment these sugars to ethanol with high yields (Gong, Ladisch et al. 1981, Schneider, Wang et al. 1981, Vanzyl, Prior et al. 1988, Moniruzzaman and Ingram 1998, Sreenath and Jeffries 2000). However, most of the lignin is left in the solids after DSA where it competitively binds with enzymes, reducing their effectiveness at deconstructing cellulose to free glucose (Zeng, Zhao et al. 2014, Vermaas, Petridis et al. 2015). Co-solvent Enhanced Lignocellulosic Fractionation (CELF) is an advanced pretreatment technology that utilizes a tetrahydrofuran (THF):water co-solvent mixture during acid pretreatment to solubilize the majority of lignin from biomass in addition to hemicellulose and leave behind highly digestible glucan-rich solids (Nguyen, Cai et al. 2015, Nguyen, Cai et al. 2017, Thomas, Donohoe et al. 2017). As with DSA hydrolyzate,

the liquid hydrolyzate after CELF contains the majority of hemicellulose sugars solubilized during pretreatment.

The conversion of these hemicellulose sugars to ethanol is critical to maximizing sugar conversion and making biorefineries competitive. During acid pretreatments, however, numerous chemical inhibitors are either solubilized or generated that prevent fermentation microorganisms from achieving high ethanol yields from hydrolyzate sugars (Palmqvist, HahnHagerdal et al. 1996, Palmqvist and Hahn-Hagerdal 2000). The fermentability of CELF hydrolyzate and identification and removal of potential inhibitors it may produce have yet to be determined. In this study, an engineered xylose fermenting strain of *Saccharomyces cerevisiae* was employed to ferment glucose and xylose in CELF hydrolyzate, and its results were compared to those for fermentation of a sugar control with identical glucose and xylose concentrations. The concentrations of THF, lignin-derived compounds, and hemicellulose-derived compounds were measured by high precision liquid chromatography (HPLC), and the effect of adding each of these individually on fermentation of control solutions glucose and xylose was quantified. Finally, organic solvents were employed to reduce inhibitor concentrations in CELF hydrolyzate by liquid-liquid extraction to increase fermentation yields.

6.3 Experimental

6.3.1 Materials

Alamo switchgrass was provided by Genera Energy Inc. (Vonore, TN). The switchgrass was knife milled using a Thomas Wiley Laboratory Mill Model 4 (Arthur H. Thomas Company, Philadelphia, PA) with a 1 mm particle size interior sieve. Mascoma LLC (a

Lallemand company) provided M11205, a xylose fermenting engineered strain of *Saccharomyces cerevisiae* (Lebanon, NH). The glucose and xylose used in sugar standards and stock solutions were purchased from Sigma Aldrich (St. Louis, MO). Tetrahydrofuran (THF) was purchased from Fisher Scientific (Pittsburgh, PA).

6.3.2 Pretreatment

Pretreatments were performed in a 1 L Hastelloy Parr® autoclave reactor (236HC Series, Parr Instruments Co., Moline, IL) equipped with a double stacked pitch blade impeller rotated at 200 rpm. CELF reaction solutions contained THF (>99% purity, Fisher Scientific, Pittsburgh, PA) and water at a volume ratio of 1:1 (or mass ratio of 0.889:1) and 0.5 wt% (based on liquid mass) sulfuric acid (Ricca Chemical Company, Arlington, TX) as a catalyst. Prior to each reaction, milled switchgrass (7.5 wt%) was added to the solution and soaked overnight at 4°C. CELF reactions were performed at 150 °C for 25 minutes, CELF conditions that were previously determined to maximize sugar recovery from switchgrass by the combined operations of pretreatment and enzymatic hydrolysis (see Chapter 3). Reactions were maintained at temperature (± 1 °C) by convective heating with a 4 kW fluidized sand bath (Model SBL-2D, Techne, Princeotn, NJ). Reaction temperature was directly measured using an in-line K-type thermocouple (Omega Engineering Inc., Stamford, Connecticut). Following reaction, liquid hydrolyzate was separated from the solid fraction by vacuum filtration at room temperature through glass fiber filter paper (Fisher Scientific, Pittsburgh, PA. The pH of the liquid hydrolyzate was measured using an Orion^{TM} Model 91-72 Sure-Flow pH Electrode (ThermoFisher Scientific, Waltham, MA).

6.3.3 Liquid Hydrolyzate Treatment

Ammonium hydroxide solution (30%, Sigma Aldrich, St. Louis, MO) was slowly added to the liquid hydrolyzate obtained from CELF pretreatment until a pH of 6 was achieved. The hydrolyzate was then poured into 500 mL flasks and placed in a water bath (Model 14575-12, Cole Palmer, Vernon Hills, IL) set at 75°C in a fume hood, and the hydrolzate solution was boiled for 8 hours to remove THF. Hydrolyzate was then filtered through a 0.22 µm sterile filter (Stericup, Millipore Sigma, St Louis, MO) to separate solid lignin precipitate from sterile filtrate.

6.3.4 Liquid-Liquid Extraction

CELF hydrolyzate was mixed with an equal mass of cyclohexane, methyl isobutyl ketone, or toluene purchased from Sigma Aldrich (St. Louis, MO) or 2-methyl tetrahydrofuran from Fisher Scientific (Hampton, NH). All chemicals were used without purification. The extraction was performed using a 1 L separatory funnel (Fisher Scientific, Pittsburgh, PA). The mixture was allowed to settle in the separatory funnel until two distinct phases were visible. Both phases were collected and stored separately. The aqueous layer was then neutralized, boiled to remove THF and filtered as described in section 6.3.3.

6.3.5 Cell Cultivation and Hydrolyzate Fermentations

1 mL of the M11205 yeast strain (Mascoma Corporation, Lebanon, NH) that had been frozen at -80 °C was combined with 5 mL of glucose (Sigma Aldrich, St. Louis, MO) at 500 g/L, 5 mL yeast extract and peptone (100 g/L and 200 g/L, respectively, Becton, Dickinson and Company, Redlands, CA) and 39 mL of deionized (DI) water in a 500 mL

Erlenmeyer baffled flask equipped with a vent cap (Fisher Scientific, Hampton, NH). After 24 hours of incubation, the optical density at 600 nm (OD_{600}) was measured to determine cell density. The amount of cells to be transferred to anaerobic flasks was calculated by the following equation:

$$Volume\ from\ seed\ flask = \frac{Anaerobic\ flask\ volume * 0.5}{Seed\ flask\ OD} * (Number\ of\ anaerobic\ flasks + 1)$$

The appropriate volume from the seed flask was centrifuged at 2400 rpm for 15 minutes in a benchtop centrifuge (Allegra X15-R, Beckman Coulter, Brea, CA). The supernatant was decanted, and the cells resuspended in sterile deionized (DI) water before being centrifuged again. Finally, the cells were resuspended in water volume in mL equivalent to the number of anaerobic flasks + 1. Anaerobic hydrolyzate fermentations were performed in triplicate in 125 mL flasks with a 50 g working mass that contained CELF hydrolyzate after THF removal, sodium citrate buffer (50 mM, pH 4.8), yeast extract and peptone (10 g/L and 20 /L, respectively, Becton, Dickinson and Company, Redlands, CA), tetracycline (40 mg/L, Sigma Aldrich, St. Louis, MO) as an antimicrobial agent, and the inoculum of M11205 yeast strain (Mascoma Corporation, Lebanon, NH) from the seed culture. Empty flasks with bubble traps attached were autoclaved at 121 °C for 35 minutes. Flasks were then cooled and moved into a laminar flow hood (Baker and Baker Ruskinn, Sanford, ME) for aseptic addition of yeast extract and peptone, citrate buffer, tetracycline, and cell inoculum. 500 µL samples of fermentation liquid were taken at time zero and subsequently every 24 hours. Samples were centrifuged and the supernatant

diluted four times in a glass 2 mL screw top vial (Agilent Technologies, Santa Clara, CA) for analysis by HPLC.

6.3.6 Analytical Procedures

All chemical analyses were performed based on Laboratory Analytical Procedures (LAPs) documented by the National Renewable Energy Laboratory (NREL, Golden, CO). 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. The chromatograms were integrated using the Empower® 2 software package (Water Co., Milford, MA).

6.3.7 Quantification of Total Dissolved Phenols

The Folin-Ciocalteau Reagent (FCR) colorimetric assay was utilized to qualitatively determine the concentration of total dissolved phenols in CELF hydrolyzate (Singleton and Rossi 1965, Singleton, Orthofer et al. 1999) with gallic acid (10 g/L, Sigma Aldrich, St. Louis, MO) as a phenol standard. Hydrolyzate samples were diluted 1:4 (v/v) with deionized water before performing the assay. 500 µL of sample, blank, or standard was added to 30 mL of deionized water in a 50 mL volumetric flask, followed by the addition of 2.5 mL of FCR (Sigma Aldrich, St. Louis, MO). After 5 minutes, 15 mL of sodium carbonate solution (20%, Sigma Aldrich, St. Louis, MO) was added to the flask, and the final volume was adjusted to 50 mL. After 2 hours, the 760 nm absorbance was read at 23 °C for the solution in a 1-cm cuvette using a SpectraMax M2e Microplate Reader (Molecular Devices, Sunnyvale, CA). All samples were analyzed in duplicate.

6.3.8 Calculations

Following HPLC quantification, the percent ethanol yield was calculated as follows:

Ethanol yield as percent of theoretical maximum = 100*(Mass of ethanol/(0.51* Initial mass of glucose and xylose))

Mass of ethanol = Concentration of ethanol as determined by HPLC * Working volume of fermentation flask

6.4 Results and Discussion

6.4.1 Quantification of CELF Hydrolyzate Components and Fermentability of CELF Hydrolyzate Sugars

CELF pretreatment was performed on switchgrass at conditions previously determined to maximize sugar release from pretreatment combined with subsequent enzymatic hydrolysis of pretreated solids (see Chapter 3). Following pretreatment, the liquid CELF hydrolyzate was separated from the pretreated solids and neutralized with ammonium hydroxide to a pH of 6 before THF was boiled out of solution. The hydrolyzate was then collected and filtered to remove precipitated lignin. The concentrations of major components in CELF hydrolyzate after neutralization and boiling were determined by HPLC analysis. As displayed in Table 6.1, glucose, xylose, and arabinose were detected in the CELF hydrolyzate. The concentration of oligomers as measured according to the NREL LAP for determination of sugars in liquid sample was less than 2% of the total sugars (Sluiter, Hames et al. 2008). As the THF concentration was negligible (Table 6.1), the hydrolyzate after neutralization and THF removal was termed “THF-free CELF hydrolyzate.”

Table 6.1: Concentrations of soluble compounds in CELF hydrolyzate after neutralization and boiling as detected by HPLC.

Compound	Concentration (g/L)
Glucose	6.5
Xylose	42.7
Arabinose	5.0
Acetic Acid	3.8
Xylo-oligomers	0.8
Tetrahydrofuran (THF)	< 1
1,4-butanediol (BDO)	4.2

*THF concentration was below detection limit of column used.

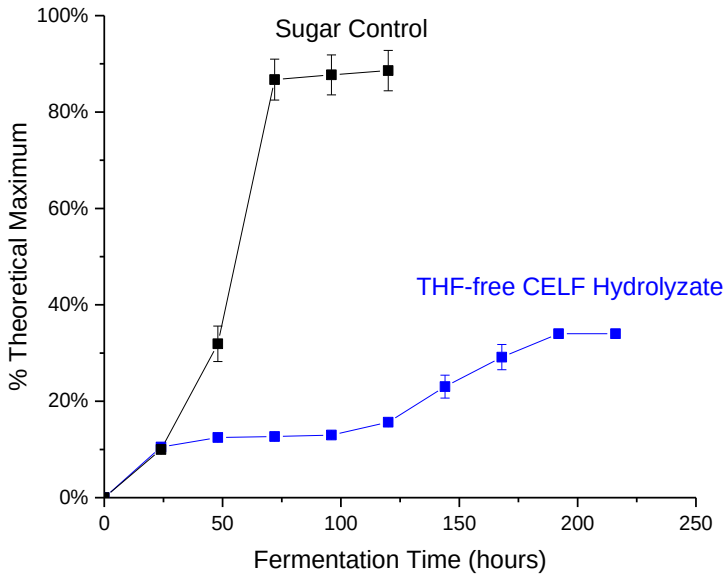


Figure 6.1. Fermentation ethanol yields by *Saccharomyces* cerevisiae M11205 as percent of the theoretical maximum for fermentation of THF-free CELF hydrolyzate and a pure sugar control containing the same amount of initial sugars as the THF-free CELF hydrolyzate.

Figure 6.1 compares the ethanol yields from fermentation of THF-free CELF hydrolyzate and a sugar control by *Saccharomyces* cerevisiae M11205 with identical glucose and xylose concentrations. As shown, sugars in the control were almost completely fermented to ethanol in three days, while the maximum ethanol yield from fermenting sugars in the THF-free CELF hydrolyzate plateaued at roughly 35% of the theoretical maximum. These results indicated that something in the THF-free CELF hydrolyzate inhibited M11205 and limited fermentation yields of hemicellulose sugars solubilized during CELF.

6.4.2 Effect of THF on M11205 Fermentation Yields

To determine which components in the THF-free hydrolyzate were responsible for limiting M11205 fermentations, each of the components that were identified by the analysis reported in Table 6.1 was added to pure sugar control solutions. Although acetic acid has previously been shown to inhibit yeast fermentations (Narendranath, Thomas et al. 2001), the concentrations in THF-free hydrolyzate were below previously reported inhibitory levels for *Saccharomyces cerevisiae* (Taherzadeh, Niklasson et al. 1997, Palmqvist, Grage et al. 1999). By adding acetic acid to pure sugars, we confirmed that concentrations below 6 g/L did not inhibit M11205 (Table 6.4 in Additional Information). 1,4-butanediol (BDO), the THF hydrolysis product, was also detected in THF-free CELF hydrolyzate, but BDO at concentrations detected in the THF-free CELF hydrolyzate did not have an observable effect on pure sugar fermentation yields (Table 6.5 in Additional Information). THF has also been shown to be inhibitory to microorganisms (Wang and

Peng 2003, Yao, Lv et al. 2009, Yao, Guan et al. 2010) but the concentrations in the hydrolyzate were found to be minute (Table 6.1). Furthermore, pure glucose and xylose fermentations by M11205 were not affected by THF concentrations below 5 g/L, as shown in Figure 6.2. Thus, it can be concluded that THF was not responsible for slowing fermentations of THF-free hydrolyzate.

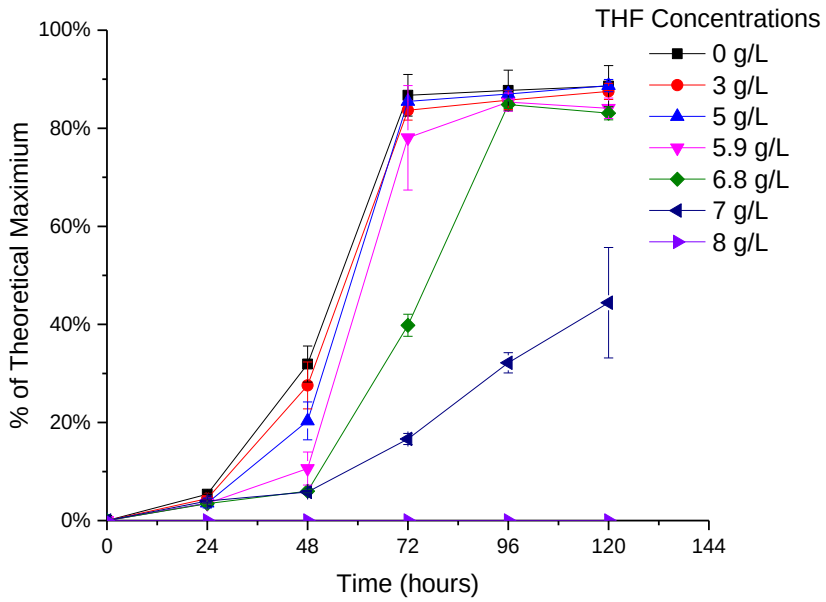


Figure 6.2. Ethanol yields as a percent of theoretical maximum from *Saccharomyces* cerevisiae M11205 fermentations of sugar controls containing a range of THF concentrations.

6.4.3 Effect of Lignin-Derived Phenolics on M11205 Fermentation Yields

Because the concentrations of compounds shown in Table 6.1 were lower than those experimentally determined to inhibit M11205, it was apparent that compounds that were not detected by HPLC were responsible for the inhibition shown in Figure 6.1. CELF solubilized nearly 80% of the lignin into the hydrolyzate (Chapter 3) and

significantly depolymerized lignin, with some remaining in solution (Chapter 4). Although some of that lignin precipitated out of solution upon THF removal by boiling, the THF-free CELF hydrolyzate still contained lignin-derived compounds. Thus, the total concentration of dissolved lignin-derived phenolics was quantified using the Folin-Ciocalteau Reagent (FCR) assay (Singleton and Rossi 1965) that has been shown to be more accurate at quantifying phenolics than alternate methods, such as the Prussian Blue assay (Persson, Larsson et al. 2002). Applying FCR revealed the total dissolved phenols concentration in THF-free CELF hydrolyzate to be 3.30 g gallic acid equivalents (GAE)/L, the phenol standard for this assay. Thus, it was determined that phenolics could cause inhibition.

Unfortunately, because the actual concentration of each phenolic species could not be directly quantified, it could not be conclusively determined if they are solely responsible for inhibited fermentations or their effect was compounded by other components in solution. To assess the effect of lignin-derived phenolics and possible other compounds on fermentation yields, THF-free CELF hydrolyzate was diluted with a sugar control solution of identical glucose and xylose concentrations to vary the proportion of THF-free hydrolyzate while keeping total sugar concentrations constant. Figure 6.3 shows that diluting THF-free CELF hydrolyzate reduced inhibition. Furthermore, at very low concentrations of THF-free CELF hydrolyzate, the fermentation rates appeared to be greater than those for the sugar control (The cause of this phenomenon is further investigated in Chapter 7). These results suggest that reduction of

concentrations of lignin-derived phenolics and possibly other compounds in the CELF hydrolyzate can overcome inhibition of M11205.

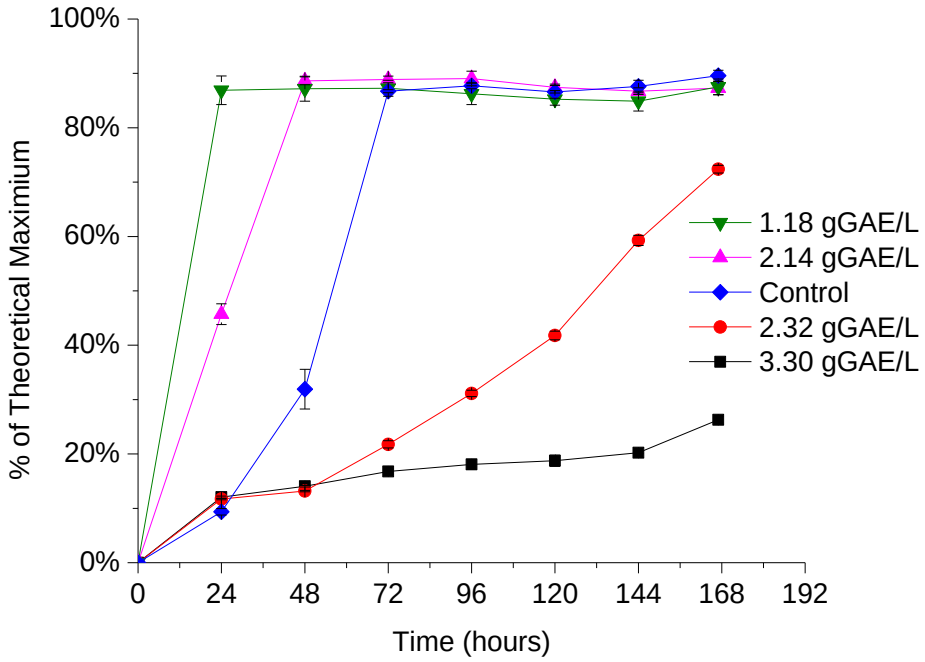


Figure 6.3. Ethanol yields as a percent of the theoretical maximum for *Saccharomyces* cerevisiae M11205 fermentations of dilutions of THF-free CELF hydrolyzate with sugar solutions at the same glucose and xylose concentrations as THF-free CELF hydrolyzate.

6.4.4 Liquid-Liquid Extraction of CELF Hydrolyzate to Remove Lignin-Derived Phenolics

Liquid-liquid extraction (LLE) is a separation technique based on transfer of a solute from one solvent to another. Typically, LLE uses an organic solvent that is immiscible in water to extract specific solutes from one liquid phase to the other based on favorable partitioning of the solute into one phase compared to the other (Müller, Berger et al. 1985). Solvent extraction has been applied to remove fermentation inhibitors from

hydrolyzates (Luo, Brink et al. 2002, Zautsen, Maugeri et al. 2009). Here, organic solvent extraction was evaluated for extraction of lignin-derived phenolics and possibly other compounds from CELF hydrolyzate using equivalent masses of CELF hydrolyzate and an organic solvent. For this approach, solvents were selected for low solubility in water to ensure two distinct phases after extraction and minimize solvent losses to water and high boiling point to facilitate separation from THF. Table 6.2 reports boiling points and water solubility data details the four organic solvents selected based on these criteria.

Table 6.2. Boiling points and water solubility of organic solvents selected for liquid-liquid extraction of phenolics and possible other compounds that inhibited fermentations of CELF hydrolyzate (Gerhartz, Thomas et al. 1989).

Solvent	**Boiling point (°C) (at standard pressure)**	**Solubility in water (g of solvent per 100 g water) (at 20 °C)**
Methyl isobutyl ketone (MIBK)	117.5	1.9
Toluene	110.6	5.2
Cyclohexane	80.7	0
2-methyl tetrahydrofuran (MTHF)	80.2	4

Liquid-liquid extraction with the four solvents in Table 6.2 were applied to CELF hydrolyzate prior to neutralization and boiling. The aqueous phases from all four extractions were then collected and analyzed by HPLC followed by boiling in a water bath to remove residual THF. Table 6.3 reports THF and phenolic concentrations in the aqueous phases from all four extractions before and after boiling to remove THF. MIBK and MTHF removed the bulk of phenolics during extraction while all solvents significantly reduced aqueous phase THF concentrations from the starting concentration

of roughly 430 g/L. Following boiling of the aqueous phases to reduce THF concentrations to < 5 g/L, the liquids from cyclohexane and MTHF extractions still contained high concentrations of THF. In these cases, HPLC analysis revealed that significant amounts of cyclohexane and MTHF migrated into the aqueous phase and were solubilized in the residual THF. It is hypothesized that adding a third solvent to the mixture altered the boiling points of the solvents, thus making THF removal more difficult. As the THF concentrations in cyclohexane and MTHF liquids were well above the established upper limit of acceptable THF from Figure 6.2, no attempt was made to ferment these by M11205. Because MIBK and toluene, on the other hand, reduced phenolic concentrations and dropped THF concentrations below 5 g/L, the post LLE hydrolyzates were good candidates for fermentations. Figure 6.4 displays ethanol yields for M11205 fermentation of these streams in comparison to a sugar control. Promisingly, the MIBK and toluene-extracted aqueous phases showed very little inhibition, with the result that M11205 fermented all hemicellulose sugars and achieved a nearly 90% theoretical ethanol yield.

Table 6.3. Concentrations of THF and dissolved phenolics (expressed as gGAE/L) in aqueous phases following LLE by four solvents measured before and after removal of residual THF by boiling*.

Solvent	Before boiling		After boiling	
	THF concentration (g/L)	**Phenolics concentration (gGAE/L)**	**THF concentration (g/L)**	**Phenolics concentration (gGAE/L)**
MIBK	78.9	0.55	1	1.35
Toluene	68.2	1.00	4	2.06
Cyclohexane	95.4	1.40	11	1.88
MTHF	86.5	0.39	30	1.00

*LLE was performed with equal masses of selected solvent and CELF hydrolyzate

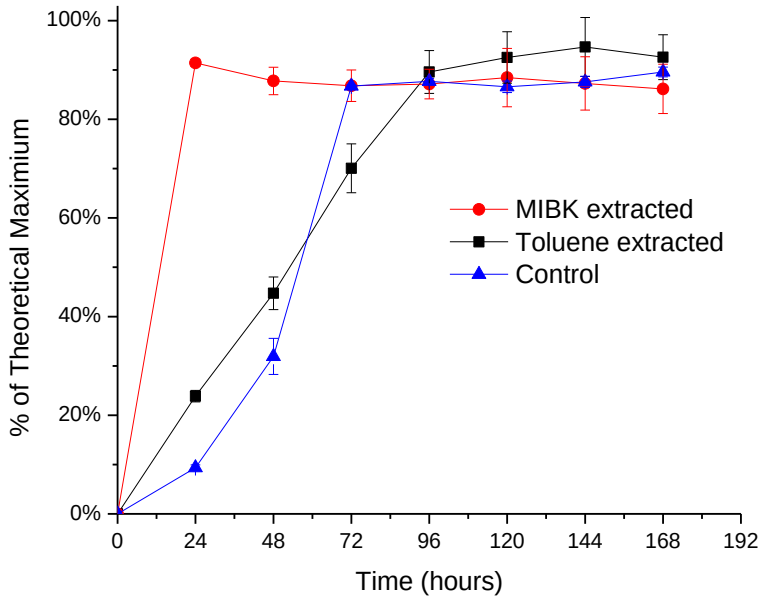


Figure 6.4. Fermentation ethanol yields as percent of theoretical maximum for *Saccharomyces* cerevisiae M11205 fermentations of hydrolyzates after MIBK or toluene extractions o followed by THF removal by boiling. Control solution was prepared with glucose and xylose (in concentrations identical to those in hydrolyzates) in deionized water.

CELF hydrolyzate contained monomeric hemicellulose sugars that should be amenable to fermentation to ethanol, along with acetic acid, tetrahydrofuran, 1,4-butanediol, and lignin-derived phenolics. Of these, tetrahydrofuran, lignin-derived phenolics, and possibly other undetectable compounds were shown to inhibit *Saccharomyces cerevisiae* fermentations of the sugar monomers in CELF hydrolyzate. The maximum THF tolerance of other microorganisms has been reported to range from 5 to 15 g/L (Kohlweyer, Thiemer et al. 2000, Yao, Lv et al. 2009). While those species were also observed to metabolize THF as a primary carbon source, the THF concentration did not change during fermentation by M11205. Although our results established THF to inhibit pure sugar fermentations at concentrations above 5 g/L, the low boiling point of THF allowed straightforward THF removal by boiling the hydrolyzate.

Phenolics have been shown to inhibit fermenting microorganisms (Palmqvist and Hahn-Hagerdal 2000, Palmqvist and Hahn-Hagerdal 2000, Kato, Saito et al. 2002, Thomas, Lawson et al. 2003, Jeong and Jeong 2010, Wu, Lin et al. 2011). These compounds penetrate into the membranes of microorganisms and cause a loss of integrity, thus affecting the cell's ability to effectively transport substrates and products (Heipieper, Weber et al. 1994). Our study showed that lignin-derived phenolics, arising from the depolymerization of lignin during CELF, measured in CELF hydrolyzate and possibly other undetectable compounds were major inhibitors of fermentation microorganisms that could be overcome by reducing their concentration through dilution.

Inhibitors from pretreatment hydrolyzates have been removed by adsorptive materials, biodegradation, and solvent extraction (Glancer and Ban 1989, Jonsson, Palmqvist et al. 1998, Ranjan, Thust et al. 2009, Zautsen, Maugeri et al. 2009, Lee, Venditti et al. 2011, Sainio, Turku et al. 2011). Two organic solvents with low solubility in water, toluene and MIBK, were able to remove a large enough fraction of lignin-derived phenolics and possibly other inhibitors from CELF hydrolyzate to overcome inhibition and achieve nearly complete conversion of hemicellulose sugars to ethanol. However, liquid-liquid extraction was only effective when applied prior to neutralization and THF removal by boiling. When performed after neutralization and boiling, the phenolics concentration did not drop significantly even when greater amounts of organic solvent were applied for LLE (see Table 6.6 in Additional Information). This outcome suggests that phenolics either undergo chemical changes during boiling that make them more difficult to extract with an organic solvent or the THF migration into the organic phase during LLE shown in Table 6.3 is critical to the extraction of phenolics. Further research is required to answer these questions. A fascinating result is that the fermentation rates are far greater after significant reduction of phenolics concentrations by MIBK extraction (Figure 6.4) and hydrolyzate dilution (Figure 6.3) than for the sugar control at the same sugar concentrations. The cause of this intriguing behavior is further explored in Chapter 7.

6.5 Conclusions

This study showed that THF was not inhibitory to engineered *Saccharomyces cerevisiae* M11205 at concentrations below 5 g/L. Thus, although the THF concentration

in CELF hydrolyzate was greater than 400 g/L, its low boiling point made it easily possible to reduce its concentration to well below the inhibition limit. Lignin-derived phenolics and possibly other undetectable compounds present in the CELF hydrolyzate severely inhibited hemicellulose sugar fermentations by M11205. However, dilution of CELF hydrolyzate with solutions containing the same concentration of sugars as in the hydrolyzate or application of liquid-liquid extraction with methyl isobutyl ketone or toluene were effective in reducing inhibitor concentrations to fermentable levels. Of particular note, fermentation rates of CELF hydrolyzates following LLE were greater than fermentations rates of solutions containing the same sugar concentrations as the CELF hydrolyzate, an outcome that deserves more study to identify what caused this acceleration.

6.6 Acknowledgements

Support by the Office of Biological and Environmental Research in the US Department of Energy (DOE) Office of Science through the BioEnergy Science Center (BESC) and the Center for Bioenergy Innovation (CBI), both managed by Oak Ridge National Laboratory, made this research possible. The award of a fellowship to the lead author by the National Center for Sustainable Transportation facilitated his participation. We also acknowledge the Ford Motor Company for funding the Chair in Environmental Engineering that facilitates projects such as this one and the Center for Environmental Research and Technology (CE-CERT) of the Bourns College of Engineering for providing facilities. We are also grateful to Mascoma LLC (a Lallemand company) for

providing the M11205 xylose fermenting engineered strain of *Saccharomyces cerevisiae* used in these experiments.

6.7 References

Chadwick, S. S. (1988). “Ullmann's encyclopedia of industrial chemistry.” Reference Services Review **16**(4): 31-34.

Glancer, M. and S. N. Ban (1989). "Biodegradation of lignin from the acid hydrolysate of cornstover by selected mixed culture of yeasts." Process Biochemistry **24**(3): 109-113.

Gong, C. S., M. R. Ladisch and G. T. Tsao (1981). "Production of ethanol from wood hemicellulose hydrolyzates by a xylose-fermenting yeast mutant, *Candida*-Sp Xf-217." Biotechnology Letters **3**(11): 657-662.

Grohmann, K., R. W. Torget, M. E. Himmel and C. E. Wyman (1987). "Dilute acid pretreatment of biomass." Abstracts of Papers of the American Chemical Society **193**: 39-Cell.

Heipieper, H. J., F. J. Weber, J. Sikkema, H. Keweloh and J. A. M. Debont (1994). "Mechanisms of resistance of whole cells to toxic organic-solvents." Trends in Biotechnology **12**(10): 409-415.

Jeong, J. B. and H. J. Jeong (2010). "2-Methoxy-4-vinylphenol can induce cell cycle arrest by blocking the hyper-phosphorylation of retinoblastoma protein in benzo[a]pyrene-treated NIH3T3 cells." Biochemical and Biophysical Research Communications **400**(4): 752-757.

Jonsson, L. J., E. Palmqvist, N. O. Nilvebrant and B. Hahn-Hagerdal (1998). "Detoxification of wood hydrolysates with laccase and peroxidase from the white-rot fungus *Trametes versicolor*." Applied Microbiology and Biotechnology **49**(6): 691-697.

Kato, T., N. Saito, K. Kashimura, M. Shinohara, T. Kurahashi and K. Taniguchi (2002). "Germination and growth inhibitors from wheat (*Triticum aestivium* L.) husks." Journal of Agricultural and Food Chemistry **50**(22): 6307-6312.

Kohlweyer, U., B. Thiemer, T. Schrader and J. R. Andreesen (2000). "Tetrahydrofuran degradation by a newly isolated culture of *Pseudonocardia* sp strain K1." Fems Microbiology Letters **186**(2): 301-306.

Lee, J. M., R. A. Venditti, H. Jameel and W. R. Kenealy (2011). "Detoxification of woody hydrolyzates with activated carbon for bioconversion to ethanol by the thermophilic anaerobic bacterium *Thermoanaerobacterium saccharolyticum*." Biomass & Bioenergy **35**(1): 626-636.

Lloyd, T. A. and C. E. Wyman (2005). "Combined sugar yields for dilute sulfuric acid pretreatment of corn stover followed by enzymatic hydrolysis of the remaining solids." Bioresource Technology **96**(18): 1967-1977.

Luo, C. D., D. L. Brink and H. W. Blanch (2002). "Identification of potential fermentation inhibitors in conversion of hybrid poplar hydrolyzate to ethanol." Biomass & Bioenergy **22**(2): 125-138.

Lynd, L. R., J. H. Cushman, R. J. Nichols and C. E. Wyman (1991). "Fuel ethanol from cellulosic biomass." Science (New York, N.Y.) **251**(4999): 1318-1323.

Moniruzzaman, M. and L. O. Ingram (1998). "Ethanol production from dilute acid hydrolysate of rice hulls using genetically engineered *Escherichia coli*." Biotechnology Letters **20**(10): 943-947.

Mosier, N., C. Wyman, B. Dale, R. Elander, Y. Y. Lee, M. Holtzapple and M. Ladisch (2005). "Features of promising technologies for pretreatment of lignocellulosic biomass." Bioresource Technology **96**(6): 673-686.

Müller, E., R. Berger, E. Blass and D. Sluyts (1985). "Liquid–liquid extraction." Ullmann's Encyclopedia of Industrial Chemistry.

Narendranath, N. V., K. C. Thomas and W. M. Ingledew (2001). "Effects of acetic acid and lactic acid on the growth of *Saccharomyces cerevisiae* in a minimal medium." Journal of Industrial Microbiology & Biotechnology **26**(3): 171-177.

Nguyen, T., C. Cai, R. Kumar and C. Wyman (2015). "Co-solvent pretreatment reduces costly enzyme requirements for high sugar and ethanol yields from lignocellulosic biomass." ChemSusChem **8**(10): 1716-1725.

Nguyen, T. Y., C. M. Cai, R. Kumar and C. 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): 11673-11678.

Palmqvist, E., H. Grage, N. Q. Meinander and B. Hahn-Hagerdal (1999). "Main and interaction effects of acetic acid, furfural, and p-hydroxybenzoic acid on growth and ethanol productivity of yeasts." Biotechnology and Bioengineering **63**(1): 46-55.

Palmqvist, E. and B. Hahn-Hagerdal (2000). "Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification." Bioresource Technology **74**(1): 17-24.

Palmqvist, E. and B. Hahn-Hagerdal (2000). "Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition." Bioresource Technology **74**(1): 25-33.

Palmqvist, E., B. HahnHagerdal, M. Galbe and G. Zacchi (1996). "The effect of water-soluble inhibitors from steam-pretreated willow on enzymatic hydrolysis and ethanol fermentation." Enzyme and Microbial Technology **19**(6): 470-476.

Persson, P., S. Larsson, L. J. Jonsson, N. O. Nilvebrant, B. Sivik, F. Munteanu, L. Thorneby and L. Gorton (2002). "Supercritical fluid extraction of a lignocellulosic hydrolysate of spruce for detoxification and to facilitate analysis of inhibitors." Biotechnology and Bioengineering **79**(6): 694-700.

Ranjan, R., S. Thust, C. E. Gounaris, M. Woo, C. A. Floudas, M. von Keitz, K. J. Valentas, J. Wei and M. Tsapatsis (2009). "Adsorption of fermentation inhibitors from lignocellulosic biomass hydrolyzates for improved ethanol yield and value-added product recovery." Microporous and Mesoporous Materials **122**(1-3): 143-148.

Saha, B. C., L. B. Iten, M. A. Cotta and Y. V. Wu (2005). "Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol." Process Biochemistry **40**(12): 3693-3700.

Sainio, T., I. Turku and J. Heinonen (2011). "Adsorptive removal of fermentation inhibitors from concentrated acid hydrolyzates of lignocellulosic biomass." Bioresource Technology **102**(10): 6048-6057.

Schneider, H., P. Y. Wang, Y. K. Chan and R. Maleszka (1981). "Conversion of D-Xylose into Ethanol by the Yeast *Pachysolen tannophilus*." Biotechnology Letters **3**(2): 89-92.

Singleton, V. L., R. Orthofer and R. M. Lamuela-Raventos (1999). "Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent." Oxidants and Antioxidants, Pt A **299**: 152-178.

Singleton, V. L. and J. A. Rossi (1965). "Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents." American journal of Enology and Viticulture **16**(3): 144-158.

Sluiter, A., B. Hames, R. Ruiz, C. Scarlata, J. Sluiter and D. Templeton (2008). "Determination of sugars, byproducts, and degradation products in liquid fraction process samples." Golden, National Renewable Energy Laboratory.

Sreenath, H. K. and T. W. Jeffries (2000). "Production of ethanol from wood hydrolyzate by yeasts." Bioresource Technology **72**(3): 253-260.

Taherzadeh, M. J., C. Niklasson and G. Liden (1997). "Acetic acid - friend or foe in anaerobic batch conversion of glucose to ethanol by *Saccharomyces cerevisiae*?" Chemical Engineering Science **52**(15): 2653-2659.

Thomas, M. G., C. Lawson, N. M. Allanson, B. W. Leslie, J. R. Bottomley, A. McBride and O. A. Olusanya (2003). "A series of 2(Z)-2-benzylidene-6,7-dihydroxybenzofuran-3[2H]-ones as inhibitors of chorismate synthase." Bioorganic & Medicinal Chemistry Letters **13**(3): 423-426.

Thomas, V. A., B. S. Donohoe, M. Li, Y. Pu, A. J. Ragauskas, R. Kumar, T. Y. Nguyen, C. M. Cai and C. 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.

Torget, R., M. E. Himmel and K. Grohmann (1991). "Dilute sulfuric-acid pretreatment of hardwood bark." Bioresource Technology **35**(3): 239-246.

Vanzyl, C., B. A. Prior and J. C. Dupreez (1988). "Production of ethanol from sugar-cane bagasse hemicellulose hydrolyzate by *Pichia stipitis*." Applied Biochemistry and Biotechnology **17**: 357-369.

Vermaas, J. V., L. Petridis, X. H. Qi, R. Schulz, B. Lindner and J. C. Smith (2015). "Mechanism of lignin inhibition of enzymatic biomass deconstruction." Biotechnology for Biofuels **8**.

Wang, Y. and D.-c. Peng (2003). "Biodegradability of wastewater containing tetrahydrofuran." Environmental Science and Technology: S2.

Wu, C. S., Z. M. Lin, L. N. Wang, D. X. Guo, S. Q. Wang, Y. Q. Liu, H. Q. Yuan and H. X. Lou (2011). "Phenolic compounds with NF-kappa B inhibitory effects from the fungus *Phellinus baumii*." Bioorganic & Medicinal Chemistry Letters **21**(11): 3261-3267.

Wyman, C. E. (2007). "What is (and is not) vital to advancing cellulosic ethanol." Trends in Biotechnology **25**(4): 153-157.

Yang, B. and C. E. Wyman (2008). "Pretreatment: the key to unlocking low-cost cellulosic ethanol." Biofuels, Bioproducts & Biorefining **2**(1): 26-40.

Yao, Y. L., J. Guan, P. Tang, H. P. Jiao, C. Lin, J. J. Wang, Z. M. Lu, H. Min and H. C. Gao (2010). "Assessment of toxicity of tetrahydrofuran on the microbial community in activated sludge." Bioresource Technology **101**(14): 5213-5221.

Yao, Y. L., Z. M. Lv, H. Min, Z. H. Lv and H. P. Jiao (2009). "Isolation, identification and characterization of a novel *Rhodococcus* sp strain in biodegradation of tetrahydrofuran and its medium optimization using sequential statistics-based experimental designs." Bioresource Technology **100**(11): 2762-2769.

Zautsen, R. R. M., F. Maugeri, C. E. Vaz-Rossell, A. J. J. Straathof, L. A. M. van der Wielen and J. A. M. de Bont (2009). "Liquid-liquid extraction of fermentation inhibiting compounds in lignocellulose hydrolysate." Biotechnology and Bioengineering **102**(5): 1354-1360.

Zeng, Y. N., S. Zhao, S. H. Yang and S. Y. Ding (2014). "Lignin plays a negative role in the biochemical process for producing lignocellulosic biofuels." Current Opinion in Biotechnology **27**: 38-45.

6.8 Additional Information

Table 6.4 Day 5 fermentation ethanol yields (expressed as % of theoretical maximum) for fermentations of model sugar solutions with incremental amounts of acetic acid introduced[a].

Acetic acid concentration (g/L)	Day 5 % Ethanol yield of theoretical maximum, %
0	88.7
1.1	89.0
2.5	89.1
3.8	88.8
5.2	89.1
6.0	86.8

[a] Model sugar solutions were made up of glucose and xylose in concentrations similar to those found in THF-free CELF hydrolyzate

Table 6.5 Day 5 fermentation ethanol yields (expressed as % of theoretical maximum) for fermentations of model sugar solutions with incremental amounts of 1,4-butanediol (BDO) introduced[a].

BDO concentration (g/L)	Day 5 % Ethanol yield of theoretical maximum, %
0	88.7
0.5	88.8
1.8	89.0
4.3	88.9
5.6	89.0
7.5	89.0

[a] Model sugar solutions were made up of glucose and xylose in concentrations similar to those found in THF-free CELF hydrolyzate

Table 6.6 Concentrations of dissolved phenolics (expressed as gGAE/L) in THF-free CELF hydrolyzate before and after liquid-liquid extraction (LLE) with varying amounts of MIBK.

Sample	Phenolics concentration (gGAE/L)
THF-free CELF hydrolyzate	2.32
Aqueous phase after 1:1 (m/m) MIBK:THF-free CELF hydrolyzate LLE	2.30
Aqueous phase after 2:1 (m/m) MIBK:THF-free CELF hydrolyzate LLE	2.28

Chapter 7

Surfactant Made from Sugars Dramatically Enhances Aerobic and Anaerobic Performance of the Yeast *Saccharomyces cerevisiae*

*This chapter will be submitted under the following citation:
Patri, A.S., Cai, C.M., Kumar, R., Wyman, C.E., 2018. Surfactant made from sugars dramatically enhances aerobic and anaerobic performance of the yeast *Saccharomyces cerevisiae*

7.1 Abstract

Saccharomyces cerevisiae is one of the leading microorganisms used for commercial fermentations. It has been engineered to convert the pentose sugars xylose and arabinose into ethanol in addition to hexose sugars it uses naturally. However, xylose uptake lags glucose consumption in engineered strains due to the diauxic effect, slowing fermentations and often hurting ethanol yields. We demonstrate that a highly potent yeast stimulant can be produced from sugars that greatly enhances xylose uptake rates and ethanol yields from anaerobic fermentations by engineered *S. cerevisiae*. Furthermore, glucose uptake rates and aerobic cell growth were also enhanced, thus accelerating anaerobic sugar fermentations to ethanol. These important results are attributed to production of 4-hydroxybutyl glucopyranoside, an alkyl glycoside, by the Fischer glycosidation of glucose and 1,4-butanediol in the presence of an acid catalyst. Addition of dilute concentrations of pretreatment hydrolyzate generated by Co-solvent Enhanced Lignocellulosic Fractionation (CELF) of cellulosic biomass and model cellulose substrates also enhanced *S. cerevisiae* performance. This stimulant has potentially significant benefits for yeast fermentations of sugar to a variety of products.

7.2 Introduction

Biologically mediated processes have the potential to significantly advance the production of sustainable fuels and chemicals from cellulosic biomass (Lynd, van Zyl et al. 2005, Lynd, Laser et al. 2008). Metabolic engineering of microbes has improved existing product production pathways as well as made it possible to make new products (Bailey 1991, Nielsen, Larsson et al. 2013). The yeast *Saccharomyces cerevisiae* has

been utilized for centuries for anaerobic fermentation of sugars to ethanol (Legras, Merdinoglu et al. 2007) with high yields and relatively high concentrations and remains the primary organism for commercial ethanol production. Its high ethanol tolerance, ability to grow under strictly anaerobic conditions, and low pH tolerance contribute to it being ideal for commercial fermentations (Van Maris, Winkler et al. 2007). Because the *S. cerevisiae* genome has been completely sequenced, selective modification of the organism's genes is relatively straightforward (Goffeau, Barrell et al. 1996). However, although *S. cerevisiae* rapidly ferments hexose sugars, such as glucose, fructose, and mannose, it is unable to anaerobically metabolize pentose sugars, such as xylose and arabinose, with its native genome (Harper 1984, van Maris, Abbott et al. 2006). This limitation is of particular significance as the majority of sugars solubilized by acid and some other pretreatments of lignocellulosic biomass to make it accessible for enzymatic deconstruction are pentose sugars whose conversion to ethanol is crucial to cost effective processing of biomass to fuels (Lee 1997, van Maris, Abbott et al. 2006). Fortunately, *S. cerevisiae* strains have been engineered to ferment pentose sugars to ethanol (Van Maris, Winkler et al. 2007). However, xylose consumption lags glucose metabolism due to the diauxic effect that slows fermentations and can result in lower yields from pentose sugars (Hamacher, Becker et al. 2002, Sedlak and Ho 2004). Therefore, novel methods are needed increase xylose fermentation rates and enhance xylose conversions.

The addition of non-ionic surfactants has been reported to enhance fermentation of sugars to ethanol (Lee, Lee et al. 1996, Alkasrawi, Eriksson et al. 2003, Tu, Zhang et al. 2009, Wei, Shrestha et al. 2011). Further, surfactants have been demonstrated to

stimulate microorganisms and product formation (Reese and Maguire 1969). Alkyl glycosides are non-ionic surfactants that are applied industrially (von Rybinski and Hill 1998) and can be produced by Fischer glycosidation of glucose with an alcohol in the presence of an acid catalyst (von Rybinski and Hill 1998). Alkyl glycosides have been reported to stimulate anaerobic fermentations (Zhao, Yang et al. 2015). Additionally, the production of alkyl glycosides from glucose released by cellulose hydrolysis has been investigated (Villandier and Corma 2010).

Recent results have shown that *Saccharomyces cerevisiae* fermentations of xylose and glucose mixtures released by Co-solvent Enhanced Lignocellulosic Fractionation (CELF) pretreatment of switchgrass proceed at faster rates and achieve higher yields than fermentations of mixtures of pure sugars at the same concentrations. We hypothesize that 1,4-butanediol (BDO) formed from the tetrahydrofuran (THF) employed for CELF pretreatments reacts with glucose to produce an alkyl glycoside that is responsible for this improvement in aerobic and anaerobic fermentation performance. In this paper, alkyl glycoside production by acid catalyzed reaction of BDO with glucose, maple wood, and switchgrass is demonstrated, and its enhancement of xylose and glucose uptake rates and fermentation yields is described for two strains of *Saccharomyces cerevisiae*.

7.3 Experimental

7.3.1 Materials

Alamo switchgrass was provided by Genera Energy Inc. (Vonore, TN). Maple wood chips were obtained in New York State by Mascoma LLC (a Lallemand Company, Lebanon, NH). Both of these lignocellulosic biomass materials were knife milled using a

Thomas Wiley Laboratory Mill Model 4 (Arthur H. Thomas Company, Philadelphia, PA) with a 1 mm particle size interior sieve. Mascoma LLC provided M11205, a xylose fermenting engineered strain of *Saccharomyces cerevisiae* (Lebanon, NH). NREL provided D_5A, a non-xylose fermenting strain of *Saccharomyces* cerevisiae. Xylan from beechwood, α-cellulose, Avicel® PH-101, cellulose from cotton linters, glucose, and xylose were all purchased from Sigma Aldrich (St. Louis, MO). Tetrahydrofuran (THF) and 1,4-butanediol (BDO) were purchased from Fisher Scientific (Pittsburgh, PA). Yeast extract and peptone were obtained from Becton Dickinson Company, Redlands, CA), and tetracycline from Sigma Aldrich (St. Louis, MO).

7.3.2 Reactions

All reactions, including pretreatment of biomass, were performed in a 1 L Hastelloy Parr® autoclave reactor (236HC Series, Parr Instruments Co., Moline, IL) equipped with a double stacked pitch blade impeller rotated at 200 rpm. Glucose reaction solutions contained 5 g of glucose and 2 g of BDO in a 0.5 wt% sulfuric acid (Ricca Chemical Company, Arlington, TX) solution. CELF reactions with switchgrass, maple wood, xylan from beechwood, α-cellulose, Avicel® PH-101, and cellulose from cotton linters were performed at 150 °C for 25 minutes. Dilute sulfuric acid (DSA) reactions with switchgrass and maple wood were performed at 160 °C for 30 minutes. CELF reaction solutions contained THF (>99% purity, Fisher Scientific, Pittsburgh, PA) and water at 1:1volume ratio (0.889:1 mass ratio) plus 0.5 wt% (based on liquid mass) sulfuric acid as a catalyst. Prior to each reaction,7.5 wt% of milled biomass was added to the solution and soaked overnight at 4°C. Reactions were maintained at temperature (± 1 °C) by

convective heating with a 4 kW fluidized sand bath (Model SBL-2D, Techne, Princeotn, NJ). Reaction temperature was directly measured by an in-line K-type thermocouple (Omega Engineering Inc., Stamford, Connecticut). Following reaction, if necessary, liquid hydrolyzate was separated from the solid fraction by vacuum filtration at room temperature through glass fiber filter paper (Fisher Scientific, Pittsburgh, PA). The pH of the liquid hydrolyzate was measured by an Orion^{TM} Model 91-72 Sure-Flow pH Electrode (ThermoFisher Scientific, Waltham, MA).

7.3.3 Liquid Hydrolyzate Treatment

Ammonium hydroxide solution (30%, Sigma Aldrich, St. Louis, MO) was slowly added to the liquid hydrolyzate produced by pretreatment until a pH of 6 was achieved. For samples containing THF, the hydrolyzate was poured into 500 mL flasks and placed in a water bath (Model 14575-12, Cole Palmer, Vernon Hills, IL) set at 75°C in a fume hood for 8 hours to allow the THF to boil out of solution. All liquid samples were then filtered through a 0.22 µm sterile filter (Stericup, Millipore Sigma, St Louis, MO) to separate solid lignin precipitate from sterile filtrate.

7.3.4 Cell Cultivation and Hydrolyzate Fermentation

1 mL of M11205 or D_5A yeast stock that was frozen at -80 °C was added to 500 mL Erlenmeyer baffled flasks equipped with vent caps (Fisher Scientific, Hampton, NH) along with 5 mL of 500 g/L glucose, 5 mL of yeast extract, and peptone (100 g/L and 200 g/L) and 39 mL of deionized (DI) water. After 24 hours of incubation for M11205 and 12 hours of for D_5A, the optical density at 600 nm (OD_{600}) was measured to determine cell density. Growth times were set to achieve OD_{600} in the range of 6-8 for both strains. The

amount of cells to be transferred to anaerobic flasks was determined by the following calculation:

$$Volume\ from\ seed\ flask = \frac{Anaerobic\ flask\ volume * 0.5}{Seed\ flask\ OD} * (Number\ of\ anaerobic\ flasks + 1)$$

The appropriate volume from the seed flask was centrifuged at 2400 rpm for 15 minutes in a benchtop centrifuge (Allegra X15-R, Beckman Coulter, Brea, CA). The supernatant was decanted and the cells resuspended in sterile deionized (DI) water before being centrifuged again. Finally, the cells were resuspended in a volumetric amount of water measured in mL equivalent to the number of anaerobic flasks + 1. Anaerobic hydrolyzate fermentations were performed in triplicate in 125 mL flasks with a 50 g working mass that contained THF-free CELF hydrolyzate, sodium citrate buffer (50 mM, pH 4.8), yeast extract and peptone (10 g/L and 20 /L, Becton, respectively, Dickinson and Company, Redlands, CA), tetracycline (40 mg/L, Sigma Aldrich, St. Louis, MO) as an antimicrobial agent, and yeast inoculum from the seed culture. Empty flasks with bubble traps attached were autoclaved at 121 °C for 35 minutes. Flasks were then cooled and moved into a laminar flow hood (Baker and Baker Ruskinn, Sanford, ME) for aseptic addition of yeast extract, peptone, citrate buffer, tetracycline, and cell inoculum. 500 µL samples of fermentation liquid were taken at time zero and every 24 hours thereafter. Samples were centrifuged, and the supernatant diluted four times in a glass 2 mL screw top vial (Agilent Technologies, Santa Clara, CA) for analysis by HPLC.

7.3.5 Analytical Procedures

All chemical analyses were performed according to Laboratory Analytical Procedures (LAPs) documented by the National Renewable Energy Laboratory (NREL, Golden, CO). 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 (5mM sulfuric acid) flow rate of 0.6 mL/min. The chromatograms were integrated via the Empower® 2 software package (Water Co., Milford, MA).

7.3.6 Calculations

Following HPLC quantification, the percent ethanol yield was calculated as follows:

% Ethanol yield of theoretical maximum = 100*(Mass of ethanol/(0.51* Initial mass of glucose and xylose))

Mass of ethanol = Concentration of ethanol as determined by HPLC * Working volume of fermentation flask

7.4 Results and Discussion

7.4.1 Stimulation of *Saccharomyces cerevisiae* Fermentations by Low Concentrations of CELF Hydrolyzate

CELF hydrolyzate was previously shown to consist mostly of hemicellulose-derived monomeric sugars, THF, and lignin-derived phenolics, the latter two inhibiting *S. cerevisiae* fermentations (see Chapter 6). Following facile THF evaporation by boiling to concentrations < 5 g/L that had little inhibitory effect, lignin-derived phenolics were the major inhibiting compounds left in this hydrolyzate termed “THF-free CELF

hydrolyzate" (TFCH). Table 7.1 shows optical densities (OD_{600}) when M11205 and D_5A yeast strains were grown aerobically with the addition of 1 mL of switchgrass TFCH to 50 g/L of glucose in seed flasks compared to results for controls without TFCH addition at cell harvest times. These results show that addition of as little as 1 mL of TFCH to a 50 mL seed flask increased OD_{600} significantly for both strains. Based on these positive results, increasing proportions of TFCH over the range of 0 – 33% by volume were added to anaerobic M11205 fermentations of sugar solutions for which glucose and xylose concentrations in each flask were identical to those in TFCH, i.e., 6.5 g/L glucose, 43 g/L xylose. The results reported in Figure 7.1 show that a TFCH concentration as low as 0.3% increased fermentation rates compared to a sugar control without any TFCH added (labeled 0% TFCH). For TFCH concentrations in the range of 2 – 33% TFCH, the sugars were completely converted to ethanol within 1 day of anaerobic fermentation. However, at TFCH concentrations > 33%, inhibition by lignin-derived phenolics and possibly other compounds in the TFCH was greater than stimulation (see Chapter 6). TFCH addition also improved anaerobic fermentations by the non-xylose fermenting strain of *S. cerevisiae* D_5A (Figure 7.5 in Additional Information). These results suggest that TFCH contains a stimulant that enhanced both aerobic and anaerobic fermentations by *Saccharomyces cerevisiae*.

Table 7.1: Effect of TFCH addition on growth of M11205 and D_5A yeast in 50 g/L glucose for seed flasks as measured by OD_{600} compared to results for controls grown on just 50 g/L glucose.

Strain	Flask Contents	OD_{600} at cell harvest*
M11205	Glucose	7.68
	Glucose + 1 mL TFCH	9.43
D_5A	Glucose	6.60
	Glucose + 1 mL TFCH	8.08

*M11205 cells harvested after 24 hours of aerobic growth. D_5A cells harvested after 12 hours of aerobic growth.

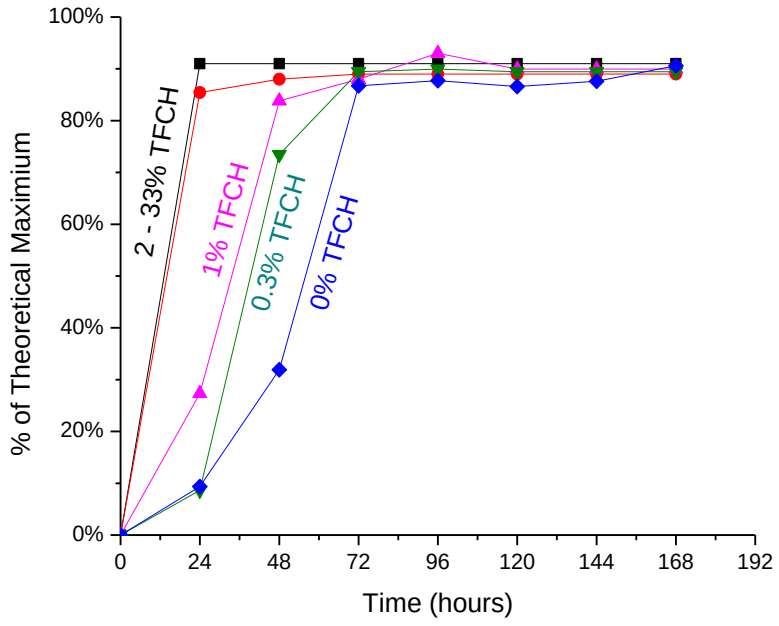


Figure 7.1. M11205 fermentation ethanol yields as percent of theoretical maximum for fermentation of sugar control containing the same amount of initial sugars as TFCH with incremental addition of TFCH over the range of 0-33% by volume. Sugar concentrations in all flasks were identical.

The fermentation broths employed to develop the results in Figure 7.1 contained a mixture of glucose and xylose. Typically, *Saccharomyces cerevisiae* preferentially consumes glucose before xylose (Hamacher, Becker et al. 2002, Sedlak and Ho 2004). To assess whether this diauxic effect was sustained with addition of low concentrations of TFCH, samples were taken every 2 hours over the first 16 hours of M11205 fermentations of a 10% concentration of TFCH. Figure 7.2 shows that while ethanol yields increased almost immediately, glucose was consumed first, while xylose concentrations remained constant during this period. Thus, M11205 preferred glucose to xylose as a primary carbon source, and the enhancement of fermentation rates was likely due to low concentrations of TFCH increasing glucose and xylose consumption rates. Furthermore, recycle of the cells that benefited from TFCH addition at identical optical density after consumption of glucose and xylose to a flask containing just pure glucose and xylose in the same concentrations as a control (0% TFCH) did not accelerate subsequent runs without TFCH addition (see Figure 7.6 in Additional Information).

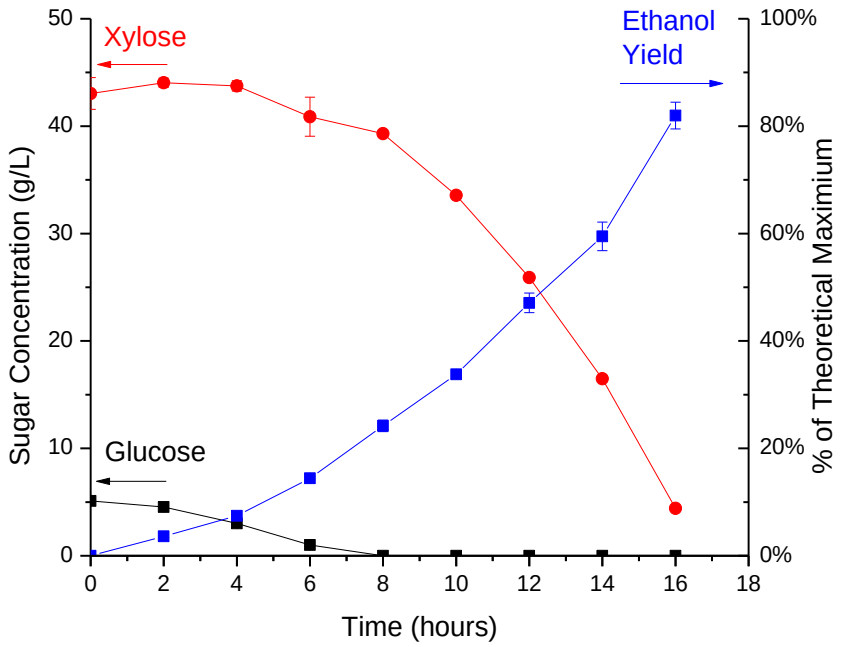


Figure 7.2. Glucose and xylose concentrations (plotted on the left y-axis) and M11205 fermentation ethanol yields as percent of theoretical maximum (plotted on the right y-axis) for fermentation of sugar solution (containing glucose and xylose at concentrations identical to TFCH) with a 10% concentration of TFCH.

7.4.2 Production of Yeast Stimulant from Cellulose and Glucose

The results in Figures 7.1 and 7.2 indicate that TFCH contains a compound that stimulates yeast growth and ethanol production. Since TFCH results from neutralizing CELF hydrolyzate and boiling it to remove most of the THF, a control containing glucose, xylose, and sulfuric acid concentrations identical to those in CELF hydrolyzate was subjected to the same routine of neutralization and boiling to determine whether the stimulant was generated by these steps. Figure 7.7 in Additional Information shows that the fermentation yields and rates did not change for solutions prepared in this way, indicating that the stimulant was produced during the CELF reaction of biomass.

To further identify the component that stimulated fermentations, switchgrass and maple wood were subjected to CELF and dilute sulfuric acid (DSA) pretreatments. Additionally, α-cellulose, cellulose from cotton linters, Avicel® PH-101, and beechwood xylan were pretreated at CELF conditions. Finally, a control reaction was also run for just THF mixed with water and dilute sulfuric acid. The solutions produced by all these reactions were then neutralized, boiled to remove THF, and supplemented with glucose and xylose so the final concentrations were identical to those for TFCH. Table 7.2 reports the major components measured in the resulting solutions compositions as well as whether the liquids enhanced M11205 anaerobic fermentation yields.

Table 7.2: Compositions of liquids produced by submitting various substrates to CELF pretreatment conditions and the effect of these hydrolyzates on M11205 fermentation yields.

Substrate	Glucan[a]	Xylan[a]	K-lignin[a]	Reaction	Fermentation Enhancement (Yes/No)[b]
Switchgrass	39%	27%	22%	CELF	Yes
				DSA	No
Maple wood	46%	20%	24%	CELF	Yes
				DSA	No
α-cellulose	91%	6%	-	CELF	Yes
Cellulose from cotton linters	98%	-	-	CELF	Yes
Avicel® PH-101	97%	-	-	CELF	Yes
Xylan from beechwood	2%	70%	-	CELF	No
Control[c]	-	-	-	CELF	No

[a]Glucan, xylan and lignin compositions determined by NREL LAP.
[b]Yes indicates that all sugars were converted to ethanol by 24 hours of fermentation; no indicates 72 hours were requires for complete sugar conversion
[c]Control was performed by reacting 1:1 (v/v) THF:water and 0.5 wt% sulfuric acid.

These results show that fermentation rates were enhanced by hydrolyzate produced by application of CELF pretreatment conditions to all substrates containing glucan. Furthermore, hydrolyzates resulting from DSA pretreatment of switchgrass and maple wood or application of CELF pretreatment conditions to beechwood xylan or the CELF control had no stimulating effects. These results suggest that reaction of THF with glucan catalyzed by dilute acid produced the yeast stimulant. CELF pretreatment solubilizes some glucose and hydrolyzes some THF to BDO. The combination of BDO,

glucose, and acid likely react to form an alkyl glycoside, 4-hydroxybutyl glucopyranoside, by Fischer glycosylation (Figure 7.3). To verify this hypothesis, 6.25 g/L of glucose was subjected to CELF pretreatment and a mixture containing 6.25 g/L glucose, 2.5 g/L BDO, and 0.5 wt% sulfuric acid was also reacted at 150 °C for 25 minutes. The resulting hydrolyzates were supplemented with glucose and xylose to reach concentrations identical to those in TFCH. The yeast M11205 completely then completely converted the sugars in these hydrolyzate samples to ethanol in just 24 hours. This outcome is consistent with the stimulating effect shown in Figure 7.1 and supports the hypothesis that 4-hydroxybutyl glucopyranoside produced at CELF pretreatment conditions was responsible for enhancing *Saccharomyces cervisiae* performance.

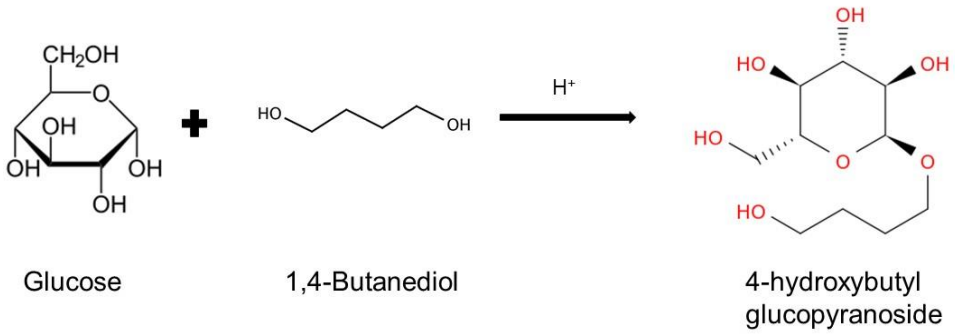


Figure 7.3. Proposed mechanism for 4-hydroxybutyl glucopyranoside production from glucose and 1,4-butanediol (BDO) at CELF reaction conditions. Glucose results from cellulose hydrolysis while BDO is produced by THF hydrolysis at CELF reaction conditions.

Non-ionic surfactants have previously been reported to increase cell viability and aid in high gravity ethanol fermentations (Zhang and Liu 2016). Further, their ability to increase fermentation rates has also been reported (Lee, Lee et al. 1996). The trends observed with the addition of CELF hydrolyzate suggest the presence of this surfactant

was responsible for the resulting increased substrate uptake even at concentrations as low as 2% TFCH (Figure 7.1). For comparison, addition of Tween 20, a commercially available surfactant, at a concentration of 5 g/L did not improve fermentation yields as dramatically as TFCH (see Figure 7.8 in Additional Information). Surfactants have previously been reported to improve cellular mechanisms by enhancing permeation of molecules through the cell membrane (Volkering, Breure et al. 1995, Koley and Bard 2010). To verify that something in TFCH enhanced membrane transport, M11205 yields and sugar concentrations were compared for fermentation of two solutions containing 100 g/L xylose, one of which also contained 10% TFCH by volume (Figure 7.4). The results demonstrated that the sample containing TFCH consumed xylose more rapidly, possibly due to the surfactant facilitating transport of xylose into the cells that in turn increased ethanol production. Characterization and optimization of the production of the surfactant as well as the exact mechanism behind the improvement in sugar uptake and yield requires further study.

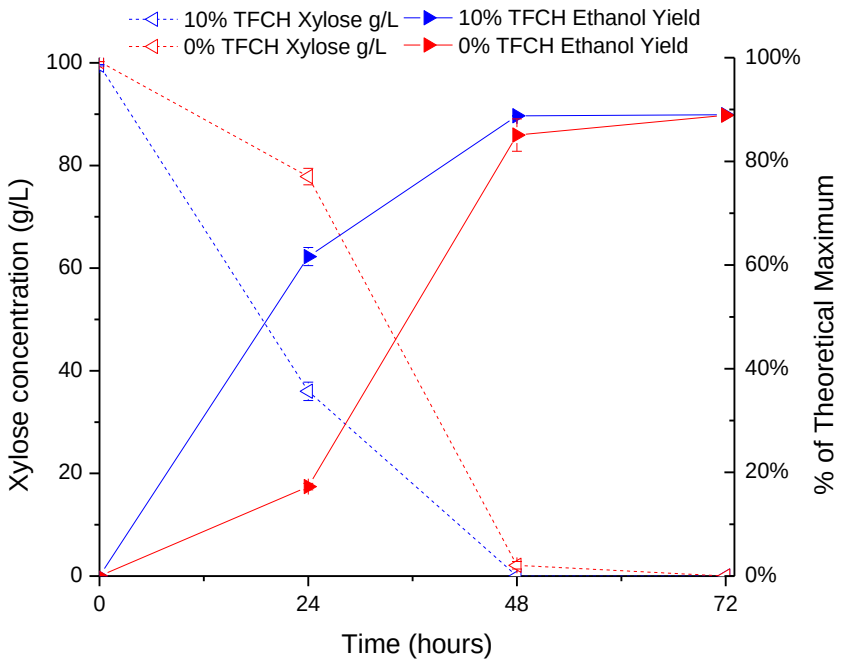


Figure 7.4. Xylose concentrations (left axis) and M11205 fermentation ethanol yields as percent of theoretical maximum (right axis) resulting from fermentations of a 100 g/L xylose control and 100 g/L xylose to which had been added a 10% concentration of TFCH.

A number of approaches have been proposed to improve xylose uptake by *Saccharomyces cerevisiae* (Madhavan, Tamalampudi et al. 2009, Matsushika, Inoue et al. 2009, Wisselink, Toirkens et al. 2009, Ma, Liu et al. 2012, Hou, Qiu et al. 2017). Here, addition of liquid produced by acid catalyzed reaction of THF with glucan or glucose with BDO accelerated xylose uptake into yeast cells, likely through formation of the alkyl glycoside, 4-hydroxybutyl glucopyranoside. Production of this stimulant by reaction of glucose with small amounts of BDO could have major commercial implications for accelerating a number of industrial fermentation processes that use glucose and future cellulosic biorefineries that must use pentose and hexose sugars.

7.5 Conclusions

CELF hydrolyzate from pretreatment of switchgrass, maple wood, and model cellulose substrates enhanced aerobic and anaerobic performance of two strains of *Saccharomyces cerevisiae*. Pure sugar fermentations were also enhanced by addition of a solution that resulted from reaction of glucose with 1,4-butanediol in the presence of an acid catalyst, implying that the stimulating compound was 4-hydroxybutyl glucopyranoside, an alkyl glycoside produced by Fischer glycosidation. The compound contained in these solutions likely accelerated sugar uptake by yeast cells so they could produce ethanol at a faster rate. This stimulant could have significant commercial implications for a number of commercial fermentation processes.

7.6 Acknowledgements

Support by the Office of Biological and Environmental Research in the US Department of Energy (DOE) Office of Science through the BioEnergy Science Center (BESC) and the Center for Bioenergy Innovation (CBI), both managed by Oak Ridge National Laboratory, made this research possible. The award of a fellowship to the lead author by the National Center for Sustainable Transportation facilitated his participation. We also acknowledge the Ford Motor Company for funding the Chair in Environmental Engineering that facilitates projects such as this one and the Center for Environmental Research and Technology (CE-CERT) of the Bourns College of Engineering for providing facilities. We are also grateful to Mascoma LLC (a Lallemand company) for providing the M11205 xylose fermenting engineered strain of *Saccharomyces cerevisiae* used in these experiments.

7.7 References

Alkasrawi, M., T. Eriksson, J. Borjesson, A. Wingren, M. Galbe, F. Tjerneld and G. Zacchi (2003). "The effect of Tween-20 on simultaneous saccharification and fermentation of softwood to ethanol." Enzyme and Microbial Technology **33**(1): 71-78.

Bailey, J. E. (1991). "Toward a science of metabolic engineering." Science **252**(5013): 1668-1675.

Goffeau, A., B. G. Barrell, H. Bussey, R. W. Davis, B. Dujon, H. Feldmann, F. Galibert, J. D. Hoheisel, C. Jacq, M. Johnston, E. J. Louis, H. W. Mewes, Y. Murakami, P. Philippsen, H. Tettelin and S. G. Oliver (1996). "Life with 6000 genes." Science **274**(5287): 546-&.

Hamacher, T., J. Becker, M. Gardonyi, B. Hahn-Hagerdal and E. Boles (2002). "Characterization of the xylose-transporting properties of yeast hexose transporters and their influence on xylose utilization." Microbiology **148**: 2783-2788.

Harper, D. R. (1984). "Yeasts - Characteristics and identification - Barnett,Ja, Payne,Rw, Yarrow,D." Nature **307**(5949): 394-394.

Hou, J., C. X. Qiu, Y. Shen, H. X. Li and X. M. Bao (2017). "Engineering of *Saccharomyces cerevisiae* for the efficient co-utilization of glucose and xylose." FEMS Yeast Research **17**(4).

Koley, D. and A. J. Bard (2010). "Triton X-100 concentration effects on membrane permeability of a single HeLa cell by scanning electrochemical microscopy (SECM)." Proceedings of the National Academy of Sciences of the United States of America **107**(39): 16783-16787.

Lee, J. (1997). "Biological conversion of lignocellulosic biomass to ethanol." Journal of Biotechnology **56**(1): 1-24.

Lee, W. G., J. S. Lee, J. P. Lee, C. S. Shin, M. S. Kim and S. C. Park (1996). "Effect of surfactants on ethanol fermentation using glucose and cellulosic hydrolyzates." Biotechnology Letters **18**(3): 299-304.

Legras, J. L., D. Merdinoglu, J. M. Cornuet and F. Karst (2007). "Bread, beer and wine: *Saccharomyces cerevisiae* diversity reflects human history." Molecular Ecology **16**(10): 2091-2102.

Lynd, L., M. Laser, D. Bransby, B. Dale, B. Davison, R. Hamilton, M. Himmel, M. Keller, J. McMillan, J. Sheehan and C. Wyman (2008). "How biotech can transform biofuels." Nature Biotechnology **26**(2): 169-172.

Lynd, L. R., W. H. van Zyl, J. E. McBride and M. Laser (2005). "Consolidated bioprocessing of cellulosic biomass: an update." Current Opinion in Biotechnology **16**(5): 577-583.

Ma, M., Z. L. Liu and J. Moon (2012). "Genetic engineering of inhibitor-tolerant *Saccharomyces cerevisiae* for improved xylose utilization in ethanol production." Bioenergy Research **5**(2): 459-469.

Madhavan, A., S. Tamalampudi, A. Srivastava, H. Fukuda, V. S. Bisaria and A. Kondo (2009). "Alcoholic fermentation of xylose and mixed sugars using recombinant *Saccharomyces cerevisiae* engineered for xylose utilization." Applied Microbiology and Biotechnology **82**(6): 1037-1047.

Matsushika, A., H. Inoue, T. Kodaki and S. Sawayama (2009). "Ethanol production from xylose in engineered *Saccharomyces cerevisiae* strains: current state and perspectives." Applied Microbiology and Biotechnology **84**(1): 37-53.

Nielsen, J., C. Larsson, A. van Maris and J. Pronk (2013). "Metabolic engineering of yeast for production of fuels and chemicals." Current Opinion in Biotechnology **24**(3): 398-404.

Reese, E. and A. Maguire (1969). "Surfactants as stimulants of enzyme production by microorganisms." Applied Microbiology **17**(2): 242-245.

Sedlak, M. and N. W. Y. Ho (2004). "Characterization of the effectiveness of hexose transporters for transporting xylose during glucose and xylose co-fermentation by a recombinant *Saccharomyces* yeast." Yeast **21**(8): 671-684.

Tu, M. B., X. Zhang, M. Paice, P. McFarlane and J. N. Saddler (2009). "Effect of surfactants on separate hydrolysis fermentation and simultaneous saccharification fermentation of pretreated lodgepole Pine." Biotechnology Progress **25**(4): 1122-1129.

Van Maris, A. J., A. A. Winkler, M. Kuyper, W. T. De Laat, J. P. Van Dijken and J. T. Pronk (2007). Development of efficient xylose fermentation in *Saccharomyces cerevisiae*: xylose isomerase as a key component. Biofuels, Springer**:** 179-204.

van Maris, A. J. A., D. A. Abbott, E. Bellissimi, J. van den Brink, M. Kuyper, M. A. H. Luttik, H. W. Wisselink, W. A. Scheffers, J. P. van Dijken and J. T. Pronk (2006). "Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: current status." Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology **90**(4): 391-418.

Villandier, N. and A. Corma (2010). "One pot catalytic conversion of cellulose into biodegradable surfactants." Chemical Communications **46**(24): 4408-4410.

Volkering, F., A. M. Breure, J. G. Vanandel and W. H. Rulkens (1995). "Influence of nonionic surfactants on bioavailability and biodegradation of polycyclic aromatic-hydrocarbons." Applied and Environmental Microbiology **61**(5): 1699-1705.

von Rybinski, W. and K. Hill (1998). "Alkyl glycosides - Properties and applications of a new class of surfactants." Angewandte Chemie-International Edition **37**(10): 1328-1345.

Wei, L., A. Shrestha, M. Tu and S. Adhikari (2011). "Effects of surfactant on biochemical and hydrothermal conversion of softwood hemicellulose to ethanol and furan derivatives." Process Biochemistry **46**(9): 1785-1792.

Wisselink, H. W., M. J. Toirkens, Q. Wu, J. T. Pronk and A. J. A. van Maris (2009). "Novel evolutionary engineering approach for accelerated utilization of glucose, xylose, and arabinose mixtures by engineered *Saccharomyces cerevisiae* strains." Applied and Environmental Microbiology **75**(4): 907-914.

Zhang, Z. and X. Liu (2015). Ethanol fermentation method with surfactant improvement, Patent application. No. WO2015035734A1.

Zhao, J. W., Q. Yang, X. M. Li, D. B. Wang, K. Luo, Y. Zhong, Q. X. Xu and G. M. Zeng (2015). "Enhanced production of short-chain fatty acid from food waste stimulated by alkyl glycosides and its mechanism." Waste Management **46**: 133-139.

7.8 Additional Information

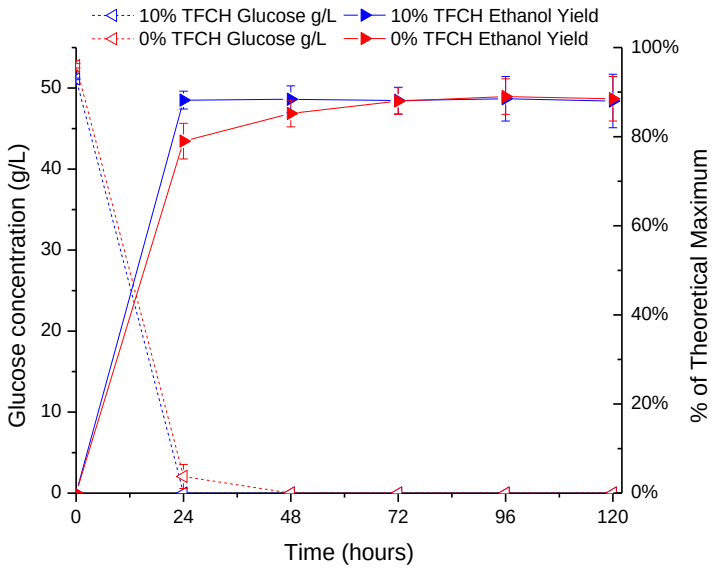


Figure 7.5. Glucose concentrations (left axis) and D_5A fermentation ethanol yields as percent of theoretical maximum (right axis) from fermentation of a 50 g/L glucose control solution (0% TFCH) and a 50 g/L glucose solution with 10% TFCH.

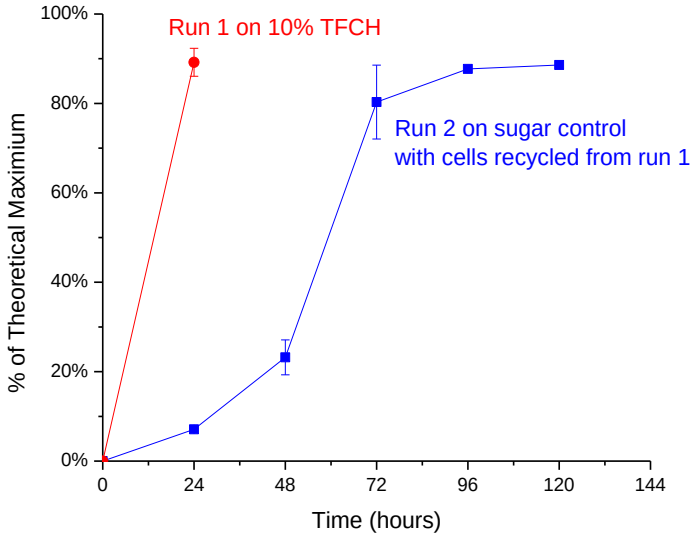


Figure 7.6. M11205 fermentation ethanol yields as percent of theoretical maximum from two fermentation runs. Run 1 employed cells to ferment a sugar solution containing 10% TFCH. Run 2 used the cells from run 1 to ferment a sugar control solution (0% TFCH). Sugar concentrations in both runs were identical.

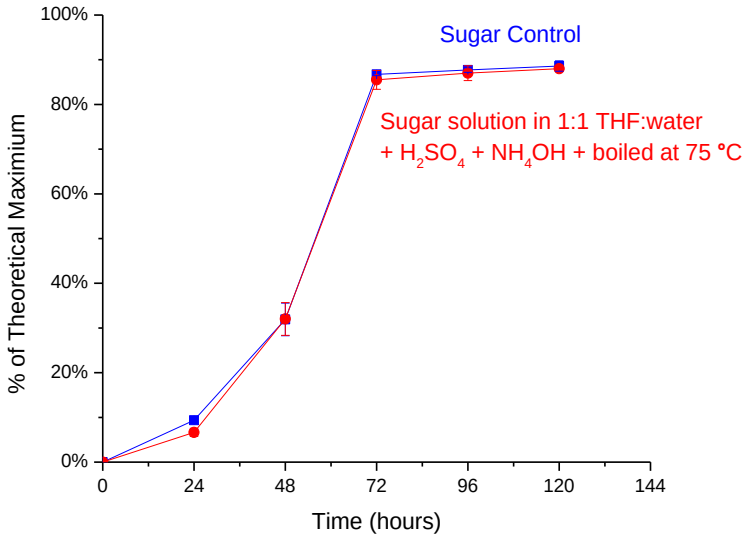


Figure 7.7. Comparison of M11205 fermentation ethanol yields as percent of theoretical maximum from fermentation of a sugar control solution with glucose and xylose concentrations identical to those in TFCH, and another sugar solution prepared in 1:1 THF:water and 0.5 wt% H_2SO_4, which was neutralized with ammonia and boiled at 75 °C to remove THF. Sugar concentrations in both runs were identical.

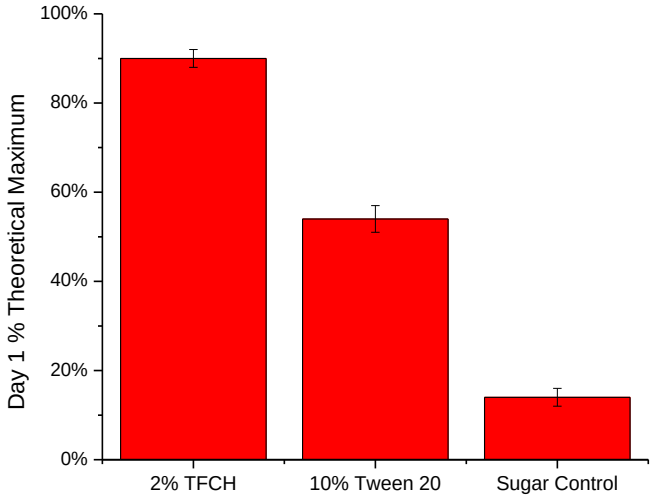


Figure 7.8. Day 1 M11205 fermentation ethanol yields as percent of theoretical maximum from fermentation of a sugar solution containing the same amount of initial sugars as TFCH with additions of 2% TFCH and 10% Tween 20 in comparison to sugar control. Sugar concentrations in all flasks were identical.

Chapter 8

Acclimatization of *Saccharomyces cerevisiae* to Ferment Sugars in Hydrolyzate Released by Co-Solvent Pretreatment of Alamo Switchgrass

*This chapter will be submitted under the following citation:
Patri, A.S., Katahira, R., Beckham G.T., Cai, C.M., Kumar, R., Wyman, C.E., 2018. Acclimatization of *Saccharomyces cerevisiae* to ferment sugars in hydrolyzate released by co-solvent pretreatment of Alamo switchgrass

8.1 Abstract

Co-solvent Enhanced Lignocellulosic Fractionation (CELF) is a recently developed advanced pretreatment that dissolves the majority of lignin and hemicellulose sugars from biomass in the liquid to leave highly digestible glucan-rich solids. Furthermore, CELF achieves high yields of hemicellulose sugars in solution. However, microbial conversion of these hemicellulose sugars in CELF hydrolyzate to ethanol is required to achieve economic viability. Following removal of the CELF pretreatment co-solvent, tetrahydrofuran (THF), the hydrolyzate contained large concentrations of lignin-derived inhibitors. As a result, fermentation of this hydrolyzate by an engineered *Saccharomyces cerevisiae* strain was only about to achieve a yield of 35% of the theoretical maximum. Two strategies were explored to overcome this inhibition and improve fermentation yields. Strategy A recycled whole cells to increase the cell density in subsequent fermentations that resulted in complete sugar conversion by the third fermentation cycle. Strategy B slowly acclimatized yeast cells at a constant cell density by increasing the ratio of CELF hydrolyzate to pure sugar solutions. After 4 cycles of acclimatization, the cells were able to achieve complete sugar conversion in 5 day fermentations. Combining strategies A and B significantly improved fermentation rates with two runs of whole cell recycle with acclimatized cells completely converting sugars in just 24 hours. Finally, characterization of post-fermentation hydrolyzate broth revealed that the lignin-derived phenols left in solution could be potential candidates for further bioconversion into fuels and chemicals. Combining slow acclimatization and whole cell recycle of yeast successfully overcame fermentation inhibition and dramatically improved fermentation

yields. These results suggest that a continuous fermentation strategy could completely convert hemicellulose sugars in CELF hydrolyzate to ethanol. Further, these strategies could potentially be applied to overcome higher inhibitor concentrations that would result from pretreatments at higher solids loadings.

8.2 Introduction

Fuel ethanol from lignocellulosic biomass has long been identified as providing a promising path with powerful economic, environmental, and strategic advantages to traditional fossil fuels (Lynd, Cushman et al. 1991, Wyman 2007). Numerous mechanical, chemical, and biological pretreatments have been developed to overcome the natural recalcitrance of plant biomass to biologically promoted release of sugars from the structural carbohydrates in plant cell walls (Mosier, Wyman et al. 2005, Yang and Wyman 2008). In particular, dilute sulfuric acid (DSA) has been used as a pretreatment catalyst to disrupt the cell wall matrix and solubilize hemicellulose with high sugar yields (Torget, Himmel et al. 1991, Lloyd and Wyman 2005, Saha, Iten et al. 2005). The liquid hydrolyzate after dilute sulfuric acid pretreatment typically contains solubilized hemicellulose sugars, often composed mostly of xylose, and fermentation organisms have been genetically engineered to ferment these sugars to ethanol with high yields (Gong, Ladisch et al. 1981, Schneider, Wang et al. 1981, Vanzyl, Prior et al. 1988, Sreenath and Jeffries 2000). However, a limitation of DSA pretreatment is the limited removal of lignin that nonproductively binds enzymes, with the result that uneconomically high enzyme loadings are needed for hydrolysis of pretreated solids with reasonably high sugar yields (Lloyd and Wyman 2005). Thus, a recent advances in pretreatment

technologies focused on solubilizing lignin during pretreatment to improve digestibility of pretreated solids (Connors, Johanson et al. 1980, Chang, Burr et al. 1997). Tetrahydrofuran (THF) in particular has recently been shown to be an effective co-solvent with aqueous dilute sulfuric acid solutions in a pretreatment called Co-solvent Enhanced Lignocellulosic Fractionation (CELF) that solubilizes the majority of hemicellulose and lignin from biomass (Nguyen, Cai et al. 2015) to produce a glucan-rich solid that is highly digestible at low enzyme loadings (Nguyen, Cai et al. 2015).

In addition to producing a solid that can be readily digested to recover glucose for fermentation, CELF produces a liquid hydrolyzate containing the majority of hemicellulose sugars and lignin removed from the solids. CELF can be tuned to maximize total sugar recovery, including pentose and hexose sugars in the hydrolyzate, without compromising digestibility of glucan in the pretreated solids (Nguyen, Cai et al. 2015, Nguyen, Cai et al. 2015). Fermentation of hemicellulose sugars to ethanol with high yields is critical to biorefinery competitiveness, but fermentation of CELF hydrolyzate sugars has yet to be demonstrated. In general, limitations in fermenting pretreatment hydrolyzates typically result from inhibitors generated during pretreatment, such as sugar-derived furans, lignin degradation compounds, and acetic acid (Larsson, Palmqvist et al. 1999, Luo, Brink et al. 2002, Islam, Elliott et al. 2017). Various methods of inhibitor removal have been investigated to condition hydrolyzate solutions so they are fermentable (Ranjan, Thust et al. 2009, Sainio, Turku et al. 2011, Zhang, Agrawal et al. 2011). Alternatively, yeast cells have been shown to adapt to inhibitors so they can realize higher fermentation yields (Johnson and Harris 1948, Fein, Tallim et al. 1984,

Chen and Gong 1985). Further, Brazilian fermentation processes recycle yeast cells from one fermentation run to the next to reach very high cell densities and shorter fermentation times (Basso, de Amorim et al. 2008).

In this study, an engineered xylose fermenting *S. cerevisiae* strain was applied to ferment the xylose and glucose in CELF pretreatment hydrolyzate. In addition, the broth left after fermentation was characterized by GC-MS to identify potential inhibitors. Two acclimatization strategies were then applied to overcome inhibition of the engineered *Saccharomyces cerevisiae* and ferment glucose and xylose present in CELF hydrolyzate to ethanol with high yields.

8.3 Experimental

8.3.1 Materials

Alamo switchgrass was provided by Genera Energy Inc. (Vonore, TN). The switchgrass was knife milled using a Thomas Wiley Laboratory Mill Model 4 (Arthur H. Thomas Company, Philadelphia, PA) with a 1 mm particle size interior sieve. Mascoma LLC (a Lallemand company) provided M11205, a xylose fermenting engineered strain of *Saccharomyces cerevisiae* (Lebanon, NH). The glucose and xylose used in sugar standards and stock solutions were purchased from Sigma Aldrich (St. Louis, MO). Tetrahydrofuran (THF) was purchased from Fisher Scientific (Pittsburgh, PA).

8.3.2 Pretreatment

Pretreatments were performed in a 1 L Hastelloy Parr® autoclave reactor (236HC Series, Parr Instruments Co., Moline, IL) equipped with a double stacked pitch blade impeller rotated at 200 rpm. CELF reaction solutions contained THF (>99% purity, Fisher

Scientific, Pittsburgh, PA) and water at volume ratio of 1:1 (or mass ratio of 0.889:1) and 0.5 wt% (based on liquid mass) sulfuric acid (Ricca Chemical Company, Arlington, TX) as a catalyst. Prior to each reaction, milled switchgrass (7.5 wt%) was added to the solution and soaked overnight at 4°C. CELF reactions were performed at 150 °C for 25 minutes, which were determined to be the optimum conditions for CELF for maximum sugar recovery for switchgrass (see Chapter 3). Reactions were maintained at temperature (± 1 °C) by convective heating with a 4 kW fluidized sand bath (Model SBL-2D, Techne, Princeotn, NJ). Reaction temperature was directly measured using an in-line K-type thermocouple (Omega Engineering Inc., Stamford, Connecticut). Following reaction, liquid hydrolyzate was separated from the solid fraction by vacuum filtration at room temperature through glass fiber filter paper (Fisher Scientific, Pittsburgh, PA. The pH of the liquid hydrolyzate was measured using an Orion^{TM} Model 91-72 Sure-Flow pH Electrode (ThermoFisher Scientific, Waltham, MA).

8.3.3 Liquid Hydrolyzate Treatment

Ammonium hydroxide solution (30%, Sigma Aldrich, St. Louis, MO) was slowly added to the liquid hydrolyzate obtained from pretreatment until a pH of 6 was achieved. The hydrolyzate was then poured into 500 mL flasks and placed in a water bath (Model 14575-12, Cole Palmer, Vernon Hills, IL) set at 75°C in a fume hood and THF was allowed to boil out of solution for 8 hours. Hydrolyzate samples were taken at the end of boiling time to ensure final THF concentrations below detection limit (<0.5 g/L). This hydrolyzate is hereby referred to as THF-free CELF hydrolyzate (TFCH). TFCH was

then filtered through a 0.22 µm sterile filter (Stericup, Millipore Sigma, St Louis, MO) to separate solid lignin precipitate from sterile filtrate.

8.3.4 Cell Cultivation and Hydrolyzate Fermentation

Yeast M11205 seed cultures were prepared in a 500 mL Erlenmeyer baffled flask with a vent cap (Fisher Scientific, Hampton, NH) with a 50 g working mass that contained 50 g/L glucose, yeast extract and peptone (10 g/L and 20 /L, respectively, Becton, Dickinson and Company, Redlands, CA) and yeast M11205 strain (Mascoma Corporation, Lebanon, NH) frozen stock culture. After 24 hours of incubation, the optical density at 600 nm (OD_{600}) was measured to determine cell density. The amount of cells to be transferred to anaerobic flasks was determine by the following calculation:

$$Volume\ from\ seed\ flask = \frac{Anaerobic\ flask\ volume * 0.5}{Seed\ flask\ OD} * (Number\ of\ anaerobic\ flasks + 1)$$

The volume was centrifuged at 2400 rpm for 15 minutes in a benchtop centrifuge (Allegra X15-R, Beckman Coulter, Brea, CA). The supernatant was decanted and the cells resuspended in sterile deionized (DI) water before being centrifuged again. Finally, the cells were resuspended in volume of water in mL equivalent to the number of anaerobic flasks + 1. Anaerobic hydrolyzate fermentations were performed in triplicate in 125 mL flasks with a 50 g working mass that contained THF-free CELF hydrolyzate, sodium citrate buffer (50 mM, pH 4.8), yeast extract and peptone (10 g/L and 20 /L, respectively, Becton, Dickinson and Company, Redlands, CA), tetracycline (40 mg/L, Sigma Aldrich, St. Louis, MO) as an antimicrobial agent, and yeast M11205 strain (Mascoma Corporation, Lebanon, NH) inoculum from the seed culture. Empty flasks

with bubble traps attached were autoclaved at 121 °C for 35 minutes. Flasks were then cooled and moved into a laminar flow hood (Baker and Baker Ruskinn, Sanford, ME) for aseptic addition of yeast extract and peptone, citrate buffer, tetracycline, and cell inoculum. 500 µL samples of fermentation liquid were taken at time zero and subsequently every 24 hours. Samples were centrifuged and the supernatant diluted four times in a glass 2 mL screw top vial (Agilent Technologies, Santa Clara, CA) for analysis by HPLC.

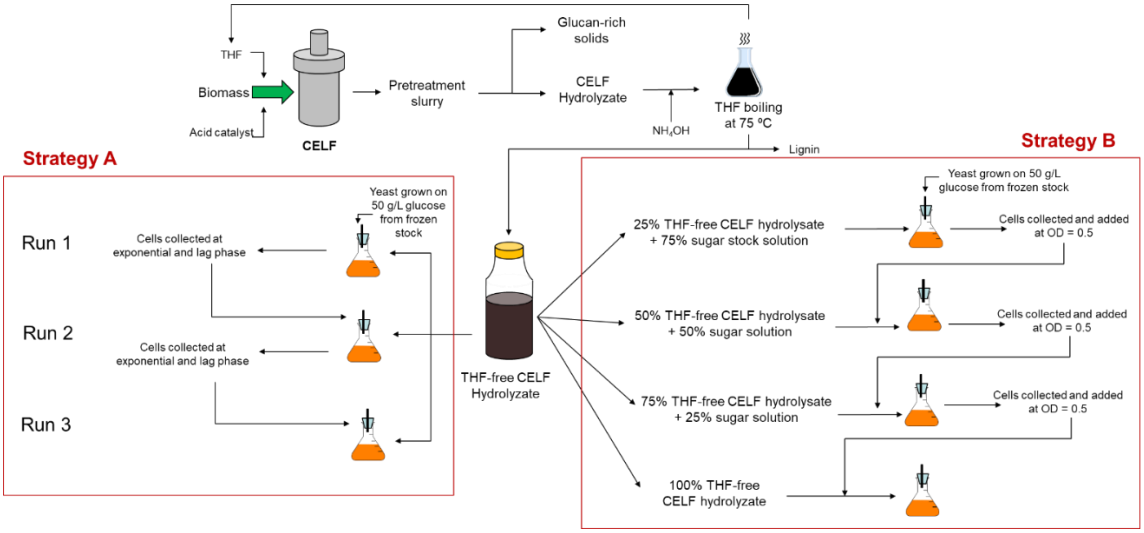


Figure 8.1. Experimental flow diagram for generation of THF-free CELF hydrolyzate from biomass and subsequent fermentation of solubilized hemicellulose sugars using two fermentation strategies. Strategy A involved exposing yeast cells to THF-free CELF hydrolyzate for three successive runs. Run 1 applied cells from seed culture, run 2 used cells from whole cell recycle of run 1, and run 3 used cells from whole cell recycle of run 2. Cells were collected at exponential and stationary phase for subsequent cycles. Strategy B involved acclimatizing cells with incremental concentrations of THF-free CELF hydrolyzate diluted in a sugar stock solution. Cells were collected from each run and added at constant OD_{600} to the next run.

Figure 8.1 illustrates the two strategies investigated for anaerobic fermentation of TFCH sugars using cells from the seed culture. In strategy A, cells from the seed culture were first added to an anaerobic fermentation flask with TFCH at OD_{600} = 0.5 in run 1. The ethanol yields were measured every 24 hours to determine what phase the cells were in. OD_{600} was measured at the time of cell harvest. Cells were collected from run 1 flasks when in exponential and stationary phase from separate fermentation flasks. Cells were centrifuged, washed, resuspended in 1 mL of sterile DI water and added to a run 2 anaerobic fermentation flask with TFCH. Similar to cell harvest from run 1 flasks, cells were collected from run 2 flasks when in exponential and stationary phase from separate fermentation flasks. OD_{600} was measured at the time of cell harvest. Cells were centrifuged, washed, resuspended in 1 mL of sterile DI water and added to a run 3 anaerobic fermentation flask with TFCH.

In strategy B, cells from seed culture were first added at OD_{600} = 0.5 to an anaerobic fermentation flask with TFCH diluted to 25% with a sugar stock solution prepared with glucose and xylose concentrations identical to that in TFCH, i.e., 25 mL TFCH + 75 mL sugar stock solution. Following complete conversion of sugars to ethanol, the OD_{600} was measured and the volume of inoculum required to transfer cells at an OD_{600} = 0.5 was calculated using the same equation as the seed culture. Cells were centrifuged, washed, resuspended in 1 mL of sterile DI water and added to an anaerobic fermentation flask with TFCH diluted to 50% with a sugar stock solution. Following complete conversion of sugars to ethanol, the OD_{600} was measured and volume and inoculum required was similarly calculated. Cells were added to an anaerobic fermentation flask with TFCH

diluted to 75% with a sugar stock solution, and cells were collected after conversion of sugars to ethanol. Finally, OD_{600} was measured, cells collected and added to an anaerobic fermentation flask with undiluted TFCH. All fermentation runs of TFCH were compared to anaerobic fermentation of a sugar stock solution with glucose and xylose concentrations identical to that in TFCH.

8.3.5 Analytical Procedures

All chemical analyses were performed based on Laboratory Analytical Procedures (LAPs) documented by the National Renewable Energy Laboratory (NREL, Golden, CO). 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 at 0.6 mL/min. The chromatograms were integrated using Empower® 2 software package (Water Co., Milford, MA).

8.3.6 Gel Permeation Chromatography (GPC) Analysis

Each lignin precipitate sample (20 mg) was acetylated in a mixture of pyridine (0.5 mL) and acetic anhydride (0.5 mL) at 40°C for 24h with stirring. The reaction was terminated by addition of methanol (0.2 mL). The acetylation solvents were then removed under a stream of nitrogen gas. The samples were further dried in a vacuum oven at 40°C overnight. The dried, acetylated lignin sample was dissolved in tetrahydrofuran (THF, Baker HPLC grade). The dissolved sample was filtered (0.45 μm, PTFE membrane syringe filters) before GPC analysis. GPC analysis was performed using an Agilent HPLC with 3 GPC columns (Polymer Laboratories, 300 x 7.5 mm) packed with

polystyrene-divinyl benzene copolymer gel (10 μm beads) having nominal pore diameters of 10^4, 10^3, and 50 Å. The eluent was THF and the flow rate of 1.0 mL/min. An injection volume of 25 μL was used. The HPLC was attached to a diode array detector measuring absorbance at 260 nm (band width 80 nm). Retention time was converted into molecular weight (Mw) by applying a calibration curve established using polystyrene standards of known molecular weight (1 x 10^6 to 580 Da) and toluene (92 Da).

8.3.7 Gas Chromatography Mass Spectrometry (GC-MS) Analysis

Analysis was performed on an Agilent 6890N GC equipped with a 5973N Mass Spectrometer Detector (MS) (Agilent Technologies, Palo Alto, CA). Sample compounds were separated using a 30m x 0.25mm x 0.25 μm HP-5MS column (122-5532, Agilent). HP MSD Chemstation G1701 software (Agilent) equipped with NIST11 database Rev. 2.0G (May 19, 2011 build) was used to identify analytes in each sample. Each sample was placed on an auto-sampler (Agilent) and injected at a volume of 1 uL into the GCMS (Agilent) in splitless mode. The analytes of interest were separated on a Restek Stabilwax-DA column (Restek Corporation, Bellefonte, PA, Cat # 11023). The GCMS method consisted of a front inlet temperature of 260º C, and an auxiliary transfer line temperature of 260 º C. A flow of 1 ml/min was held constant throughout the run. A starting temperature of 35º C was held for 3 minute and then ramped at 10º C/min to a temperature of 225º C and held for 1 minute, then ramped at 15º C/min to a temperature of 250º C and held for 10 minutes. The method resulted in a run time of 34.67 minutes

for each sample. The MS was set up with no solvent delay. Sample TIC's (Total Ion Count) were collected on the MS system from 35 m/z to 450 m/z.

8.3.8 Quantification of Total Dissolved Phenols

A colorimetric assay using the Folin-Ciocalteau Reagent (FCR) was utilized to qualitatively determine the concentration of total dissolved phenols in CELF hydrolyzate (Singleton and Rossi 1965, Singleton, Orthofer et al. 1999). Gallic acid (10 g/L, Sigma Aldrich, St. Louis, MO) was utilized as a phenol standard. Hydrolyzate samples were diluted 1:4 (v/v) with deionized water before performing the assay. 500 µL of sample, blank or standard was added to 30 mL of deionized water in a 50 mL volumetric flask, followed by the addition of 2.5 mL of FCR (Sigma Aldrich, St. Louis, MO). After 5 minutes, 15 mL of sodium carbonate solution (20%, Sigma Aldrich, St. Louis, MO) was added to the flask and the final volume was adjusted to 50 mL. After 2 hours, the absorbance of the solution was read at 23 °C at 760 nm in a 1-cm cuvette using a SpectraMax M2e Microplate Reader (Molecular Devices, Sunnyvale, CA). All samples were analyzed in duplicate.

8.3.9 Calculations

Following HPLC quantification, the % ethanol yield was calculated as follows:

% Ethanol yield of theoretical maximum = 100*(Mass of ethanol/(0.51* Initial mass of glucose and xylose))

Mass of ethanol = Concentration of ethanol as determined by HPLC * Working volume of fermentation flask

8.4 Results and Discussion

The major cause of the inhibition in THF-free CELF hydrolyzate to fermentation microorganisms was identified to result from lignin-derived products that CELF produced during lignin removal and depolymerization (see Chapter 6). Thus, the total concentration of dissolved phenolic compounds in THF-free CELF hydrolyzate was measured using the Folin-Ciocalteau Reagent (FCR) colorimetric assay that has been shown to more accurately quantify phenols than alternative approaches, such as the Prussian Blue assay (Persson, Larsson et al. 2002). Applying FCR revealed the concentration of total dissolved phenols in THF-free CELF hydrolyzate to be 3.30 g gallic acid equivalents (GAE)/L, the phenol standard for this assay.

8.4.1 Whole Yeast Cell Recycle in Successive Fermentations (Strategy A)

As outlined in Figure 8.1, Strategy A of whole cell recycle was one of two strategies applied to enhance yields from fermentation of glucose and xylose in THF-free CELF hydrolyzate by *Saccharomyces cerevisiae* strain M11205. Figure 8.2 reports ethanol yields for three successive fermentation runs using this strategy. Run 1 performed with cells from the seed culture achieved a maximum ethanol yield of only 37% in 8 days. This low yield can be attributed to severe inhibition by lignin-derived compounds and acetic acid. For run 2, the cells all were collected following the plateau in fermentation yield for run 1, i.e., the stationary phase, and introduced into fresh hydrolyzate that was identical to that used in run 1. After an initial lag phase of 3 days, these cells reached an ethanol yield of 55% at day 9. Finally, the cells from run 2 were similarly collected and introduced to ferment fresh hydrolyzate for run 3. Again, after a

lag phase of 3 days, the final ethanol yields from run 3 were 89% after day 6. To eliminate the lag phase seen in runs 2 and 3, cells were collected slightly earlier in the fermentation run when yields were trending upwards, i.e., during the exponential phase, before being introduced into fresh hydrolyzate. This approach resulted in similar increases in ethanol yields in successive runs as use of cells during the stationary phase but with faster fermentation rates for each successive run (solid lines).

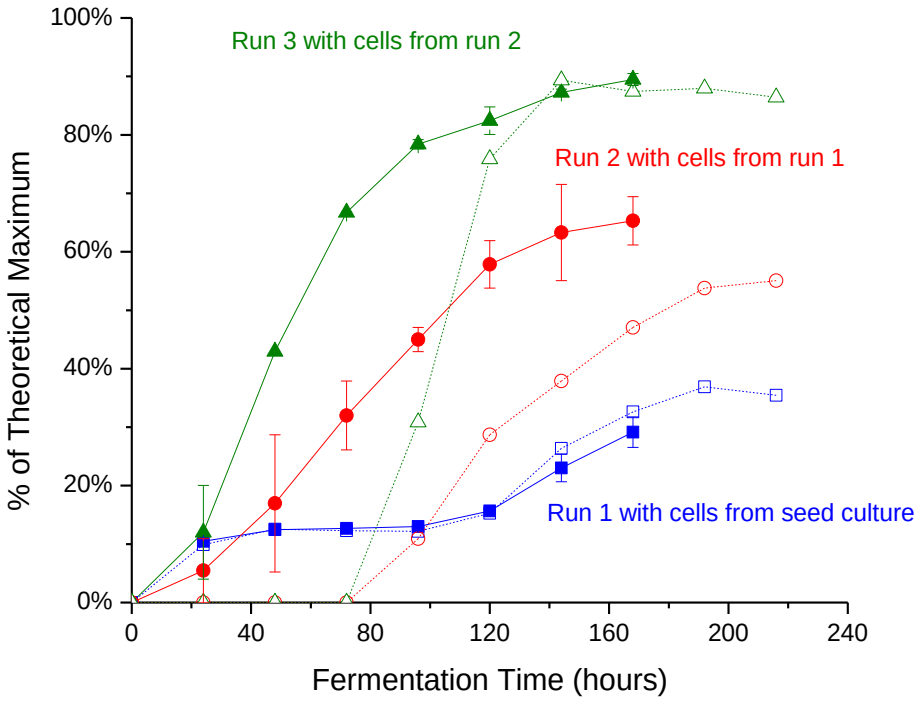


Figure 8.2. Ethanol yields as percent of theoretical maximum from application of whole cell recycle to fermentation of THF-free CELF hydrolyzate. Run 1 employed cells from the seed culture, run 2 used cells from run 1, and run 3 used cells from run 2. Dashed lines indicate runs that employed cells harvested in the stationary phase, while solid lines are for runs that used cells harvested during the exponential phase.

The successive improvement in ethanol yields upon whole cell recycle was encouraging as it illustrated that higher yeast cell densities can help overcome inhibition by compounds in CELF hydrolyzate. Table 8.1 reports the increase in cell densities measured at the start of each fermentation. Although harvesting cells for recycle during the exponential and stationary phases resulted in slight differences in cell densities, the advantages of high cell density cultures to improve productivity of biological reactions have been previously identified (Lee 1996).

Table 8.1: Yeast cell density (measured as OD_{600}) at the start of each run during strategy A.

Run*	**OD_{600} measured at the start of run**
1	0.50
2 – exponential	1.46
2 – stationary	1.92
3 – exponential	2.55
3 – stationary	2.88

*2-exponential and 3-exponential indicate runs where cells were harvested from previous runs at exponential phase; 2-stationary and 3-stationary indicate runs where cells were harvested from previous runs at stationary phase.

To determine whether the improvements in yield shown in Figure 8.2 were due to increases in cell density or the acclimatization of cells to CELF hydrolyzate, yeast cells from run 1 were collected during the exponential phase and added to fresh hydrolyzate in run 2 at a constant cell density of $OD_{600} = 0.5$ to maintain the same cell density between successive fermentation runs. All cells were assumed to be alive at harvest. Figure 8.3 shows the increase in ethanol yields in run 2 was roughly 10% of that in run 1, indicating that the much greater improvements in yield observed in Figure 8.2 were largely due to increased cell density (as shown in Table 8.1).

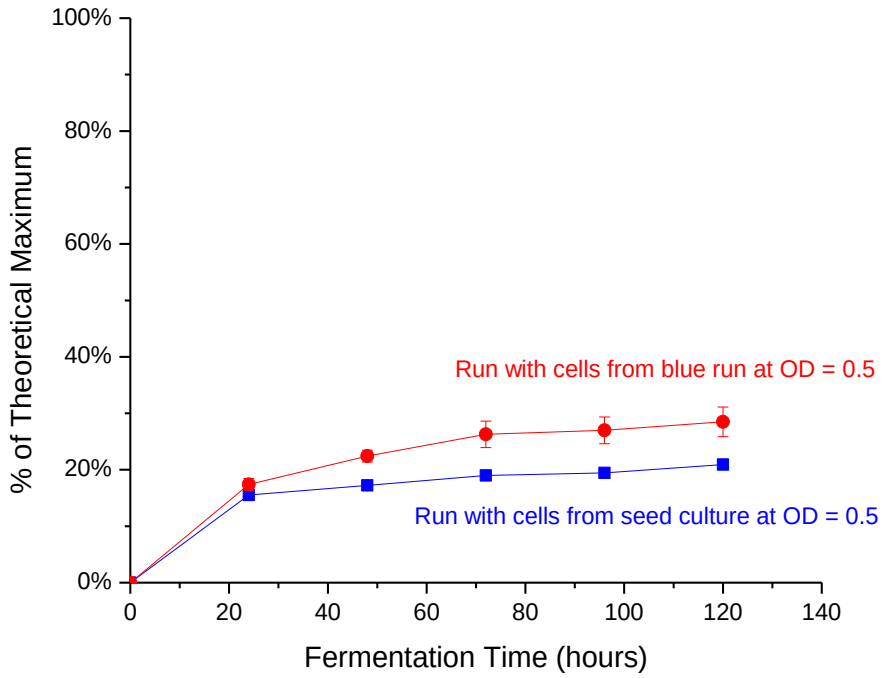


Figure 8.3. Fermentation ethanol yields as percent of theoretical maximum from fermentation of THF-free CELF hydrolyzate for cell recycle at constant optical density (OD). The first run (rectangle markers) applied cells from the seed culture at OD_{600} = 0.5, and the second run (circle markers) used cells from the first run at OD_{600} = 0.5.

8.4.2 Acclimatization of Yeast to Inhibitors (Strategy B)

The slight improvement in fermentation yields observed in Figure 8.3 suggested that yeast can acclimatize to inhibitors in THF-free CELF hydrolyzate, in line with other results that show yeast can acclimatize to unfavorable conditions (Gray 1946, Johnson and Harris 1948). However, as seen in Figure 8.3, exposing unacclimatized cells to high concentrations of inhibitors may only result in nominal improvements in yield. An alternative method of acclimatization is to recover cells for fermentation of successively higher proportions of hydrolyzate mixed with pure sugar solutions while maintaining

constant sugar concentrations (Chen and Gong 1985). Thus, unacclimatized cells were first applied to ferment a hydrolyzate stream diluted to one-fourth concentration with a pure sugar stock solution while maintaining the same ratio of glucose to xylose. Following complete conversion of these sugars, the cells were collected and introduced into higher concentrations of hydrolyzate at constant OD_{600} as outlined in Figure 8.1. After three acclimatization runs, fermentation yields were measured for cells fed just THF-free CELF hydrolyzate. Figure 8.4 compares yields resulting from application of cells following incremental hydrolyzate exposure to those for unacclimatized cells. The significant improvement in ethanol yield can be attributed to successive adaptation of yeast cells to inhibitors in the THF-free CELF hydrolyzate. This behavior was preserved in cells even after long-term cryogenic freezing (Figure 8.9 in Additional Information).

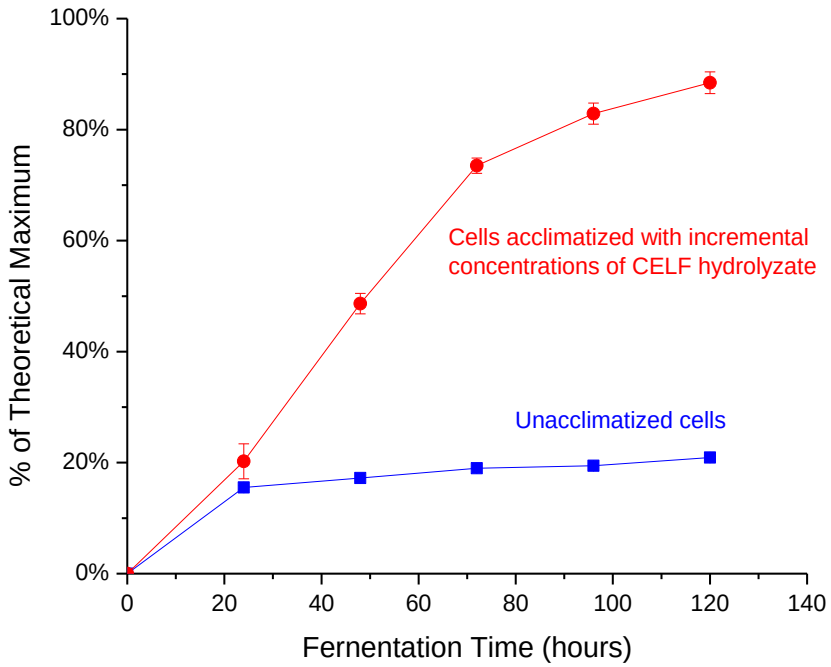


Figure 8.4: Ethanol yields as percent of theoretical maximum for fermentation of CELF hydrolyzate by cells at constant optical density (OD_{600}) that had been acclimatized to increasing concentrations of CELF hydrolyzate. The first run (rectangle marker) applied unacclimatized cells at OD_{600} = 0.5, while the second run (circle marker) employed cells acclimatized to increasing concentrations of CELF hydrolyzate at OD_{600} = 0.5.

8.4.3 Combining Strategies A and B to Overcome Inhibition

The FCR assay and lower amounts of acetic acid, one of the potent inhibitors of yeasts, suggested that lignin derived phenolic compounds are mainly responsible for strong inhibition of yeast by the THF-free CELF hydrolyzate. Both strategies A and B were individually effective in overcoming inhibition by lignin-derived compounds present in THF-free CELF hydrolyzate. Because the two strategies are independent and utilizing one does not perclude use of the other, the combined effect of both strategies A and B was investigated. In this case, following acclimatization by strategy B, the cells

that produced the results in Figure 8.4 were collected at the end of fermentation and all recycled to a fresh batch of THF-free CELF hydrolyzate. Additionally, all cells from this run were then recycled to a new fermentation flask of THF-free CELF hydrolyzate, thereby combining whole cell recycling (Strategy A) with cell acclimatization (Strategy B). Figure 8.5 shows ethanol yields for the two runs described (red and green lines) in comparison to a fermentation with unacclimatized cells (run 4). Both runs achieved dramatic improvements in fermentation rates, with the second run reaching a 90% ethanol yield in 24 hours. These results illustrate how combining strategies A and B completely overcame inhibition of yeast by lignin-derived compounds present in THF-free CELF hydrolyzate.

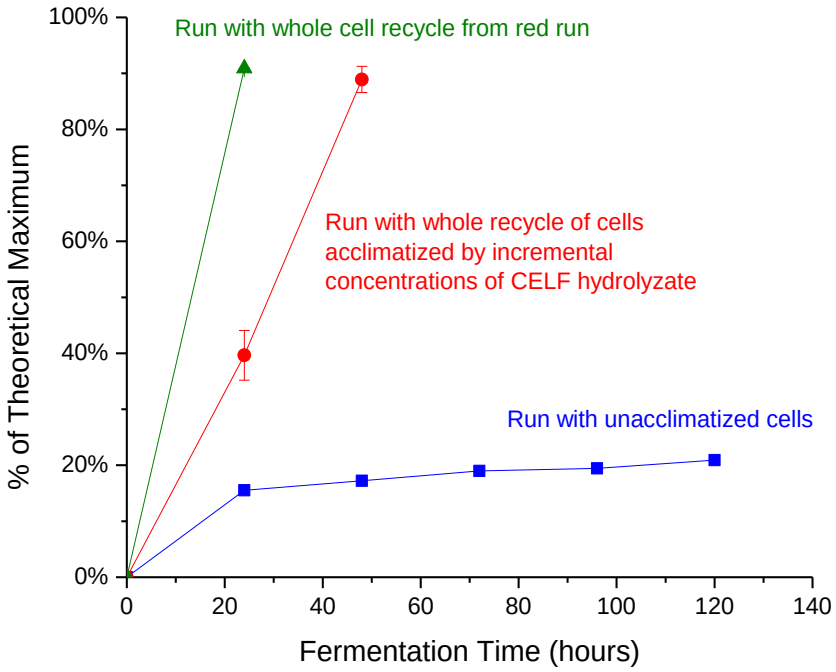


Figure 8.5. Ethanol yields as percent of theoretical maximum following fermentation of CELF hydrolyzate by cells acclimatizated to incremental increases in concentrations of CELF hydrolyzate coupled with whole cell recycle. The lowest yields resulted from unacclimatized cells at OD_{600} = 0.5. The first run with acclimatized cells (indicated with circle markers) and whole recycle of cells acclimatized using incremental concentrations of CELF hydrolyzate, and the second run using acclimatized cells (indicated with a triangle marker) used whole recycle cells from the first run.

8.4.4 GC-MS Analysis of Lignin-Derived Inhibitory Compounds

Following fermentation of sugars in THF-free CELF hydrolyzate to ethanol, GC-MS was utilized to identify lignin-derived compounds that CELF solubilized and likely inhibited fermentation microorganisms the most (Ando, Arai et al. 1986, Keweloh, Weyrauch et al. 1990, Larsson, Quintana-Sainz et al. 2000). Figure 8.6 shows the resulting GC-MS chromatograms of THF-free CELF hydrolyzate after sugar fermentation with Table 8.2 identifying the peaks labeled in Figure 8.6. Peaks 5, 6, and 7 were the only compounds that were not detected by HPLC. 2-methoxy-4-vinylphenol and

2,3-dihydroxybenzofuran are lignin-derivatives that have previously been shown to inhibit some microorganisms (Kato, Saito et al. 2002, Thomas, Lawson et al. 2003, Jeong and Jeong 2010, Wu, Lin et al. 2011) and likely caused inhibition observed for *Saccharomyces cerevisiae* during sugar fermentations. However, these compounds are also of particular interest in that engineered microorganisms have been shown to be capable of converting such lignin-derived compounds to valuable chemicals (Vardon, Franden et al. 2015), making phenols in post-fermentation CELF hydrolyzate potential candidates for bioconversion. However, further detailed characterization and investigation are required to truly asses the suitability of CELF hydrolyzate phenols for biological valorization.

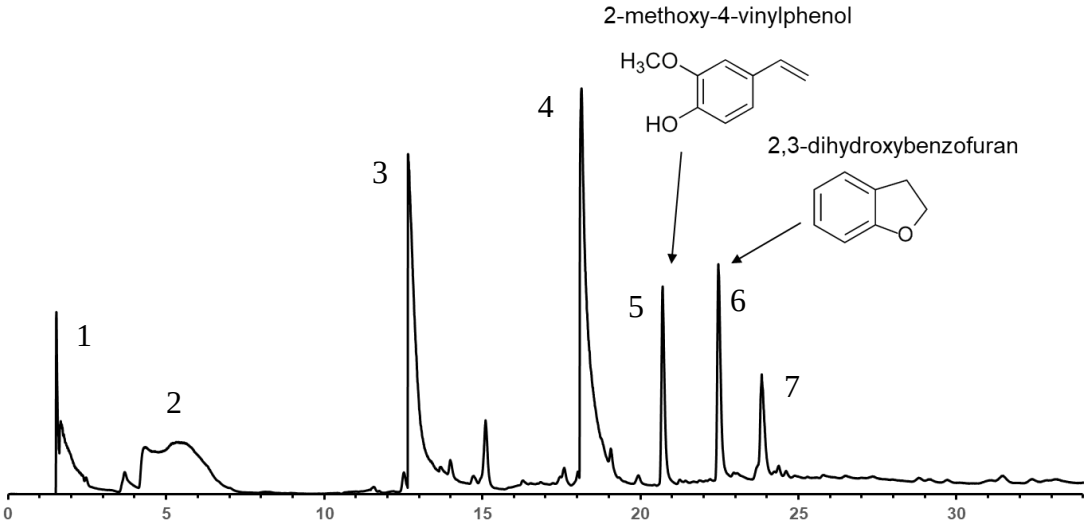


Figure 8.6. GC-MS chromatogram of THF-free CELF hydrolyzate after fermentation of glucose and xylose by *Saccharomyces cerevisiae* M11205. Peaks 5, 6 and 7 are 2-methoxy-4-vinylphenol, 2,3-dihydroxybenzofuran and texanol respectively.

Table 8.2: Compound name and retention time of peaks labeled in Figure 8.6.

Peak #	Compound	Retention Time
1	Carbon dioxide	1.6
2	Ethanol	4.33
3	Acetic acid	12.57
4	1,4-butanediol	18.41
5	2-methoxy-4-vinylphenol	20.73
6	2,3-dihydroxybenzofuran	22.5
7	Texanol	23.88

Hemicellulose in many types of lignocellulosic biomass is rich in pentose sugars, such as xylose, that can be released by acid pretreatments and must be fermented to achieve economically viable ethanol output. Increasing ethanol titers is crucial in the commercialization of a bioethanol process (Galbe, Sassner et al. 2007, Larsen, Petersen et al. 2008) and recent research has targeted higher titers during fermentation (Kang, Chung et al. 2015, Nguyen, Cai et al. 2015). CELF is capable of solubilizing the majority of hemicellulose sugars without significant acid-catalyzed dehydration that would lower yields. Thus, there is little furfural or other dehydration products in CELF hydrolyzate that inhibit fermenting microorganisms (Larsson, Palmqvist et al. 1999, Palmqvist, Grage et al. 1999). Although boiling THF from the CELF hydrolyzate is critical so that this solvent can be recycled to subsequent CELF reactions and keep solvent costs low, THF removal also increases sugar concentrations in the hydrolyzate, as roughly half the volume of CELF hydrolyzate is THF. As sugar concentrations increase, the theoretical ethanol titer achievable by fermenting hemicellulose sugars increases as well, providing an added benefit to commercial scale-up of CELF sugar fermentation.

Lignin has been shown to be highly inhibitory to cellulolytic enzymes and cause reduced sugar yields during enzymatic hydrolysis of solid biomass (Zeng, Zhao et al. 2014). Additionally, as shown in Chapter 6, lignin-derived phenols can cause severe inhibition to fermenting microorganisms when present in sugar hydrolyzates (Clark and Mackie 1984, Buchert, Puls et al. 1989, Jeong and Jeong 2010, Wu, Lin et al. 2011), likely due to their ability to penetrate the cell membrane of microorganisms (Heipieper, Weber et al. 1994). Numerous methods of detoxification of liquid hydrolyzates by biological methods have been employed to specifically target lignin-derived products (Glancer and Ban 1989, Palmqvist, Hahn-Hägerdal et al. 1997, Jonsson, Palmqvist et al. 1998). Further, solvent extractions have also been applied to remove inhibitors from hydrolyzates prior to fermentation (Ranjan, Thust et al. 2009, Lee, Venditti et al. 2011, Sainio, Turku et al. 2011). Such detoxification methods can reduce inhibition and thereby increase fermentation yields, however, with the drawback of more process steps that increase ethanol costs.

Acclimatization strategies have been applied to take advantage of the ability of microorganisms to adapt to their environments (Gray 1946, Johnson and Harris 1948, Chen and Gong 1985). In this work, we showed that a combination of increased cell density and cell acclimatization by slowly exposing yeast to increasing concentrations of inhibitors can be effective in overcoming inhibition of sugar-fermenting microorganisms by lignin-derived phenols. Of further significance, yield improvements in successive runs avoided the need for multiple runs in one particular environment to enhance performance. These results are encouraging as these strategies could be effective methods to achieve

significant ethanol yields in the presence of high concentrations of fermentation inhibitors that will result from pretreatment of higher solids loading. Additionally, the success of both strategies suggests that continuous fermentations would allow yeast to overcome severe inhibition through acclimatization and increased cell densities. If continuous fermentations are preferred commercially (Lee, Park et al. 2000, Kwon, Yoo et al. 2001), the acclimatization strategies outlined in our work could be potentially quite easily integrated with a continuous process. Finally, our characterization of lignin-derived phenols in post-fermentation THF-free CELF hydrolyzate suggested that further value might be realized from the dissolved lignin components after sugars fermentations. For example, lignin valorization is being explored as an avenue to improving the profitability of biorefineries (Ragauskas, Beckham et al. 2014, Salvachua, Karp et al. 2015, Sangchoom and Mokaya 2015, Abdelaziz, Brink et al. 2016, Beckham, Johnson et al. 2016), and the compounds identified here could have potential for conversion into valuable lignin-derived bioproducts.

Figure 8.7 outlines a proposed continuous process for conversion of lignocellulosic biomass that integrates CELF pretreatment with continuous fermentation of soluble sugars to ethanol followed by bio-conversion of lignin-derived molecules to valuable products. The major products from such a process would be ethanol from enzymatic hydrolysis of the highly susceptible glucan-rich solids (Nguyen, Cai et al. 2015), precipitated solid lignin, hemicellulose sugar-derived ethanol, and lignin fermentation products. Further, the much higher molecular weight of lignin precipitated by THF removal from CELF hydrolyzate compared to soluble lignin components (see

Figure 8.10 in Additional Information) could provide a potential precursor for synthesis of carbon fibers and other polymers (Ragauskas, Beckham et al. 2014).

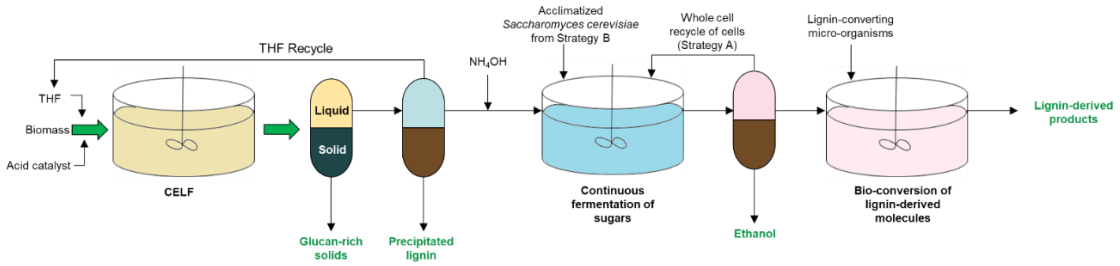


Figure 8.7. Process flow diagram of a proposed continuous biomass conversion process that integrates CELF pretreatment with ethanol production from hydrolyzate sugars using strategies A and B, and conversion of dissolved lignin-derived phenolics in hydrolyzate to products. Major products labeled in green.

8.5 Conclusions

In this study, we demonstrated that two strategies can overcome inhibition by lignin-derived phenols of fermentation of sugars released by CELF of Alamo switchgrass. The combination of whole yeast cell recycle and acclimatization resulted in ethanol yields from hemicellulose sugars that were 90% of the theoretical maximum without hydrolyzate detoxification or additional processing of the liquid produced by CELF pretreatment beyond THF recovery for recycle and lignin precipitation. Additionally, we identified lignin-derived compounds present in post-fermentation hydrolyzate that could potentially be converted into valuable products. Our acclimatization strategies hold promise to overcome inhibition by higher inhibitor concentrations that will result from pretreatments at higher solids concentrations needed to increase ethanol titers from fermentation.

8.6 Acknowledgments

Support by the Office of Biological and Environmental Research in the US Department of Energy (DOE) Office of Science through the BioEnergy Science Center (BESC) and the Center for Bioenergy Innovation (CBI), both managed by Oak Ridge National Laboratory, made this research possible. The award of a fellowship to the lead author by the National Center for Sustainable Transportation facilitated his participation. We also acknowledge the Ford Motor Company for funding the Chair in Environmental Engineering that facilitates projects such as this one and the Center for Environmental Research and Technology (CE-CERT) of the Bourns College of Engineering for providing facilities. We are also grateful to Mascoma LLC for providing the M11205 xylose fermenting engineered strain of *Saccharomyces cerevisiae* used in these experiments.

8.7 References

Abdelaziz, O. Y., D. P. Brink, J. Prothmann, K. Ravi, M. Z. Sun, J. Garcia-Hidalgo, M. Sandahl, C. P. Hulteberg, C. Turner, G. Liden and M. F. Gorwa-Grauslund (2016). "Biological valorization of low molecular weight lignin." Biotechnology Advances **34**(8): 1318-1346.

Ando, S., I. Arai, K. Kiyoto and S. Hanai (1986). "Identification of aromatic monomers in steam-exploded Poplar and their influences on ethanol fermentation by *Saccharomyces cerevisiae*." Journal of Fermentation Technology **64**(6): 567-570.

Basso, L. C., H. V. de Amorim, A. J. de Oliveira and M. L. Lopes (2008). "Yeast selection for fuel ethanol production in Brazil." FEMS Yeast Res **8**(7): 1155-1163.

Beckham, G. T., C. W. Johnson, E. M. Karp, D. Salvachua and D. R. Vardon (2016). "Opportunities and challenges in biological lignin valorization." Current Opinion in Biotechnology **42**: 40-53.

Buchert, J., J. Puls and K. Poutanen (1989). "The use of steamed hemicellulose as substrate in microbial conversions." Applied Biochemistry and Biotechnology **20-1**: 309-318.

Chang, V. S., B. Burr and M. T. Holtzapple (1997). "Lime pretreatment of switchgrass." Applied Biochemistry and Biotechnology **63-5**: 3-19.

Chen, L. F. and C. S. Gong (1985). "Fermentation of sugarcane bagasse hemicellulose hydrolysate to xylitol by a hydrolysate-acclimatized yeast." Journal of Food Science **50**(1): 226-228.

Clark, T. A. and K. L. Mackie (1984). "Fermentation inhibitors in wood hydrolysates derived from the softwood *Pinus radiata*." Journal of Chemical Technology and Biotechnology B-Biotechnology **34**(2): 101-110.

Connors, W. J., L. N. Johanson, K. V. Sarkanen and P. Winslow (1980). "Thermal-degradation of Kraft lignin in tetralin." Holzforschung **34**(1): 29-37.

Fein, J. E., S. R. Tallim and G. R. Lawford (1984). "Evaluation of D-xylose fermenting yeasts for utilization of a wood-derived hemicellulose hydrolysate." Canadian Journal of Microbiology **30**(5): 682-690.

Galbe, M., P. Sassner, A. Wingren and G. Zacchi (2007). "Process engineering economics of bioethanol production." Biofuels **108**: 303-327.

Glancer, M. and S. N. Ban (1989). "Biodegradation of lignin from the acid hydrolysate of cornstover by selected mixed culture of yeasts." Process Biochemistry **24**(3): 109-113.

Gong, C. S., M. R. Ladisch and G. T. Tsao (1981). "Production of ethanol from wood hemicellulose hydrolyzates by a xylose-fermenting yeast mutant, *Candida*-Sp Xf-217." Biotechnology Letters **3**(11): 657-662.

Gray, W. D. (1946). "The acclimatization of yeast to high concentrations of glucose - the subsequent effect upon alcohol tolerance." Journal of Bacteriology **52**(6): 703-709.

Heipieper, H. J., F. J. Weber, J. Sikkema, H. Keweloh and J. A. M. Debont (1994). "Mechanisms of resistance of whole cells to toxic organic-solvents." Trends in Biotechnology **12**(10): 409-415.

Islam, S. M., J. R. Elliott and L.-K. Ju (2017). "Minimization of fermentation inhibitor generation by carbon dioxide-water based pretreatment and enzyme hydrolysis of guayule biomass." Bioresource technology **251**: 84-92.

Jeong, J. B. and H. J. Jeong (2010). "2-Methoxy-4-vinylphenol can induce cell cycle arrest by blocking the hyper-phosphorylation of retinoblastoma protein in benzo[a]pyrene-treated NIH3T3 cells." Biochemical and Biophysical Research Communications **400**(4): 752-757.

Johnson, M. C. and E. E. Harris (1948). "Acclimatization of various yeasts to wood sugar." Journal of the American Chemical Society **70**(9): 2961-2963.

Jonsson, L. J., E. Palmqvist, N. O. Nilvebrant and B. Hahn-Hagerdal (1998). "Detoxification of wood hydrolysates with laccase and peroxidase from the white-rot fungus *Trametes versicolor*." Applied Microbiology and Biotechnology **49**(6): 691-697.

Kang, K. E., D. P. Chung, Y. Kim, B. W. Chung and G. W. Choi (2015). "High-titer ethanol production from simultaneous saccharification and fermentation using a continuous feeding system." Fuel **145**: 18-24.

Kato, T., N. Saito, K. Kashimura, M. Shinohara, T. Kurahashi and K. Taniguchi (2002). "Germination and growth inhibitors from wheat (*Triticum aestivium* L.) husks." Journal of Agricultural and Food Chemistry **50**(22): 6307-6312.

Keweloh, H., G. Weyrauch and H. J. Rehm (1990). "Phenol-induced membrane-changes in free and immobilized *Escherichia coli*." Applied Microbiology and Biotechnology **33**(1): 66-71.

Kwon, S., I. K. Yoo, W. G. Lee, H. N. Chang and Y. K. Chang (2001). "High-rate continuous production of lactic acid by *Lactobacillus rhamnosus* in a two-stage membrane cell-recycle bioreactor." Biotechnology and Bioengineering **73**(1): 25-34.

Larsen, J., M. O. Petersen, L. Thirup, H. W. Li and F. K. Iversen (2008). "The IBUS process - Lignocellulosic bioethanol close to a commercial reality." Chemical Engineering & Technology **31**(5): 765-772.

Larsson, S., E. Palmqvist, B. Hahn-Hagerdal, C. Tengborg, K. Stenberg, G. Zacchi and N. O. Nilvebrant (1999). "The generation of fermentation inhibitors during dilute acid hydrolysis of softwood." Enzyme and Microbial Technology **24**(3-4): 151-159.

Larsson, S., A. Quintana-Sainz, A. Reimann, N. O. Nilvebrant and L. J. Jonsson (2000). "Influence of lignocellulose-derived aromatic compounds on oxygen-limited growth and

ethanolic fermentation by *Saccharomyces cerevisiae*." Applied Biochemistry and Biotechnology **84-6**: 617-632.

Lee, J. M., R. A. Venditti, H. Jameel and W. R. Kenealy (2011). "Detoxification of woody hydrolyzates with activated carbon for bioconversion to ethanol by the thermophilic anaerobic bacterium *Thermoanaerobacterium saccharolyticum*." Biomass & Bioenergy **35**(1): 626-636.

Lee, S. Y. (1996). "High cell-density culture of *Escherichia coli*." Trends in Biotechnology **14**(3): 98-105.

Lee, W. G., B. G. Park, Y. K. Chang, H. N. Chang, J. S. Lee and S. C. Park (2000). "Continuous ethanol production from concentrated wood hydrolysates in an internal membrane-filtration bioreactor." Biotechnology Progress **16**(2): 302-304.

Lloyd, T. A. and C. E. Wyman (2005). "Combined sugar yields for dilute sulfuric acid pretreatment of corn stover followed by enzymatic hydrolysis of the remaining solids." Bioresource Technology **96**(18): 1967-1977.

Luo, C. D., D. L. Brink and H. W. Blanch (2002). "Identification of potential fermentation inhibitors in conversion of hybrid poplar hydrolyzate to ethanol." Biomass & Bioenergy **22**(2): 125-138.

Lynd, L. R., J. H. Cushman, R. J. Nichols and C. E. Wyman (1991). "Fuel ethanol from cellulosic biomass." Science **251**(4999): 1318-1323.

Mosier, N., C. Wyman, B. Dale, R. Elander, Y. Y. Lee, M. Holtzapple and M. Ladisch (2005). "Features of promising technologies for pretreatment of lignocellulosic biomass." Bioresource Technology **96**(6): 673-686.

Nguyen, T., C. Cai, R. Kumar and C. Wyman (2015). "Co-solvent pretreatment reduces costly enzyme requirements for high sugar and ethanol yields from lignocellulosic biomass." ChemSusChem **8**(10): 1716-1725.

Nguyen, T., C. Cai, O. Osman, R. Kumar and C. Wyman (2015). "CELF pretreatment of corn stover boosts ethanol titers and yields from high solids SSF with low enzyme loadings." Green Chem **18**(6): 1581-1589.

Palmqvist, E., H. Grage, N. Q. Meinander and B. Hahn-Hagerdal (1999). "Main and interaction effects of acetic acid, furfural, and p-hydroxybenzoic acid on growth and ethanol productivity of yeasts." Biotechnology and Bioengineering **63**(1): 46-55.

Palmqvist, E., B. Hahn-Hägerdal, Z. Szengyel, G. Zacchi and K. Rèczey (1997). "Simultaneous detoxification and enzyme production of hemicellulose hydrolysates obtained after steam pretreatment." Enzyme and Microbial Technology **20**(4): 286-293.

Persson, P., S. Larsson, L. J. Jonsson, N. O. Nilvebrant, B. Sivik, F. Munteanu, L. Thorneby and L. Gorton (2002). "Supercritical fluid extraction of a lignocellulosic

hydrolysate of spruce for detoxification and to facilitate analysis of inhibitors." Biotechnology and Bioengineering **79**(6): 694-700.

Ragauskas, A. J., G. T. Beckham, M. J. Biddy, R. Chandra, F. Chen, M. F. Davis, B. H. Davison, R. A. Dixon, P. Gilna, M. Keller, P. Langan, A. K. Naskar, J. N. Saddler, T. J. Tschaplinski, G. A. Tuskan and C. E. Wyman (2014). "Lignin valorization: Improving lignin processing in the biorefinery." Science **344**(6185): 709-+.

Ranjan, R., S. Thust, C. E. Gounaris, M. Woo, C. A. Floudas, M. von Keitz, K. J. Valentas, J. Wei and M. Tsapatsis (2009). "Adsorption of fermentation inhibitors from lignocellulosic biomass hydrolyzates for improved ethanol yield and value-added product recovery." Microporous and Mesoporous Materials **122**(1-3): 143-148.

Saha, B. C., L. B. Iten, M. A. Cotta and Y. V. Wu (2005). "Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol." Process Biochemistry **40**(12): 3693-3700.

Sainio, T., I. Turku and J. Heinonen (2011). "Adsorptive removal of fermentation inhibitors from concentrated acid hydrolyzates of lignocellulosic biomass." Bioresource Technology **102**(10): 6048-6057.

Salvachua, D., E. M. Karp, C. T. Nimlos, D. R. Vardon and G. T. Beckham (2015). "Towards lignin consolidated bioprocessing: simultaneous lignin depolymerization and product generation by bacteria." Green Chemistry **17**(11): 4951-4967.

Sangchoom, W. and R. Mokaya (2015). "Valorization of lignin waste: Carbons from hydrothermal carbonization of renewable lignin as superior sorbents for CO2 and hydrogen storage." ACS Sustainable Chemistry & Engineering **3**(7): 1658-1667.

Schneider, H., P. Y. Wang, Y. K. Chan and R. Maleszka (1981). "Conversion of D-xylose into ethanol by the yeast *Pachysolen tannophilus*." Biotechnology Letters **3**(2): 89-92.

Singleton, V. L., R. Orthofer and R. M. Lamuela-Raventos (1999). "Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent." Oxidants and Antioxidants, Pt A **299**: 152-178.

Singleton, V. L. and J. A. Rossi (1965). "Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents." American journal of Enology and Viticulture **16**(3): 144-158.

Sreenath, H. K. and T. W. Jeffries (2000). "Production of ethanol from wood hydrolyzate by yeasts." Bioresource Technology **72**(3): 253-260.

Thomas, M. G., C. Lawson, N. M. Allanson, B. W. Leslie, J. R. Bottomley, A. McBride and O. A. Olusanya (2003). "A series of 2(Z)-2-benzylidene-6,7-dihydroxybenzofuran-3[2H]-ones as inhibitors of chorismate synthase." Bioorganic & Medicinal Chemistry Letters **13**(3): 423-426.

Torget, R., M. E. Himmel and K. Grohmann (1991). "Dilute sulfuric-acid pretreatment of hardwood bark." Bioresource Technology **35**(3): 239-246.

Vanzyl, C., B. A. Prior and J. C. Dupreez (1988). "Production of ethanol from sugar-cane bagasse hemicellulose hydrolyzate by *Pichia stipitis*." Applied Biochemistry and Biotechnology **17**: 357-369.

Vardon, D. R., M. A. Franden, C. W. Johnson, E. M. Karp, M. T. Guarnieri, J. G. Linger, M. J. Salm, T. J. Strathmann and G. T. Beckham (2015). "Adipic acid production from lignin." Energy & Environmental Science **8**(2): 617-628.

Wu, C. S., Z. M. Lin, L. N. Wang, D. X. Guo, S. Q. Wang, Y. Q. Liu, H. Q. Yuan and H. X. Lou (2011). "Phenolic compounds with NF-kappa B inhibitory effects from the fungus *Phellinus baumii*." Bioorganic & Medicinal Chemistry Letters **21**(11): 3261-3267.

Wyman, C. E. (2007). "What is (and is not) vital to advancing cellulosic ethanol." Trends in Biotechnology **25**(4): 153-157.

Yang, B. and C. E. Wyman (2008). "Pretreatment: the key to unlocking low-cost cellulosic ethanol." Biofuels Bioproducts & Biorefining **2**(1): 26-40.

Zeng, Y. N., S. Zhao, S. H. Yang and S. Y. Ding (2014). "Lignin plays a negative role in the biochemical process for producing lignocellulosic biofuels." Current Opinion in Biotechnology **27**: 38-45.

Zhang, K., M. Agrawal, J. Harper, R. Chen and W. J. Koros (2011). "Removal of the fermentation inhibitor, furfural, using activated carbon in cellulosic-ethanol production." Industrial & Engineering Chemistry Research **50**(24): 14055-14060.

8.8 Additional Information

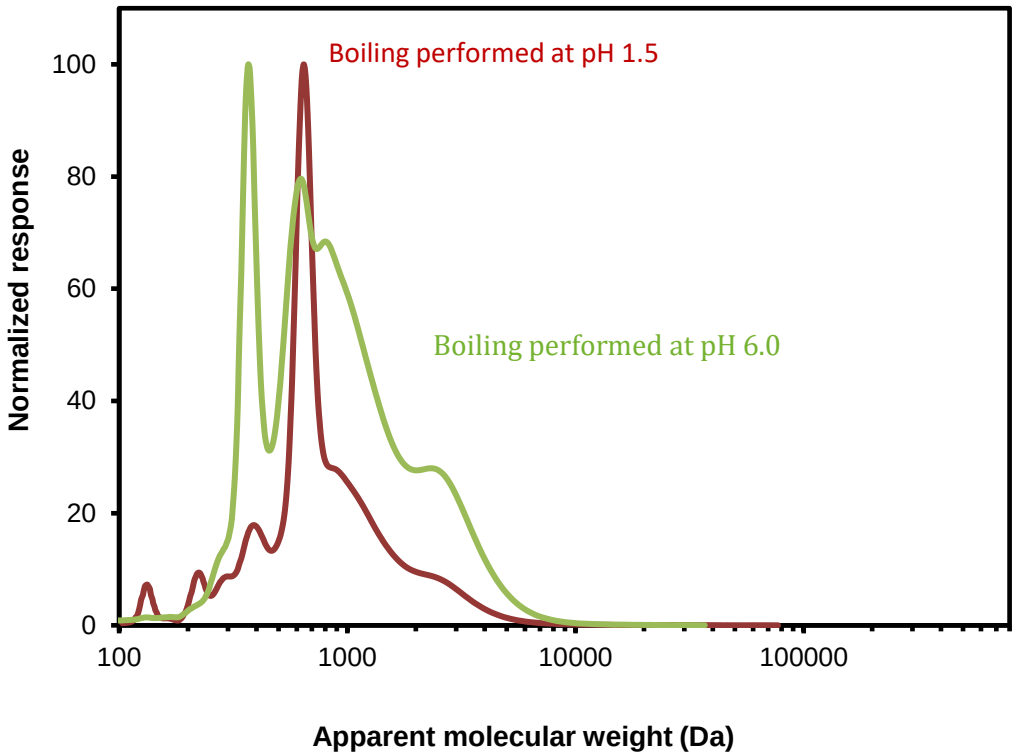


Figure 8.8. Gel permeation chromatogram of CELF hydrolyzate after boiling at pH 1.50 (red line) and CELF hydrolyzate after boiling at pH 6.0 (green line).

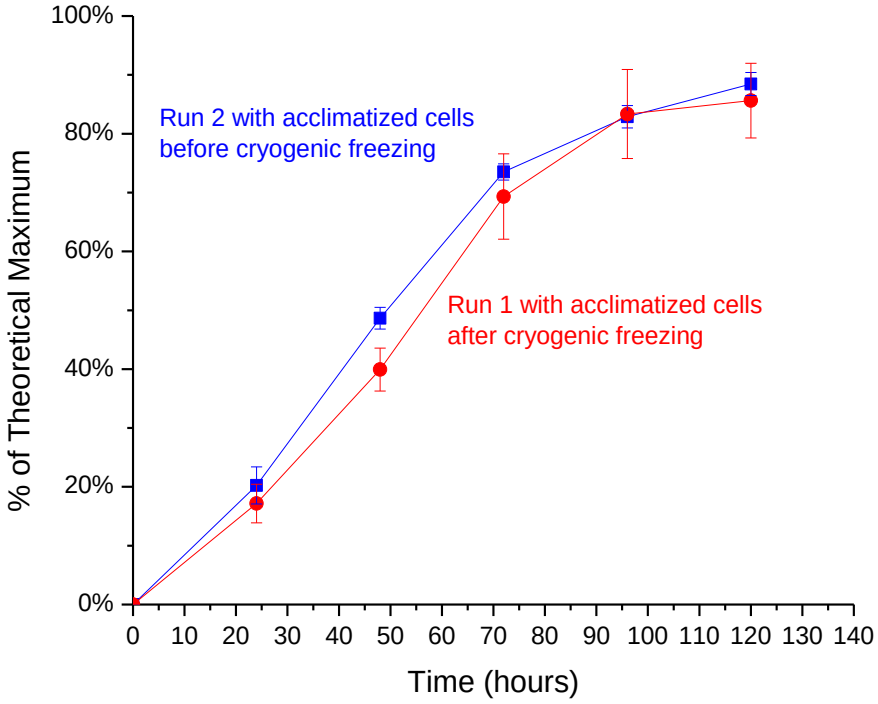


Figure 8.9: Ethanol yields (expressed as % of theoretical maximum) from fermentation of CELF hydrolyzate with acclimatized cells before and after cryogenic freezing. Run 1 applied acclimatized cells before cryogenic freezing at OD_{600} = 0.5, run 1 applied acclimatized cells after cryogenic freezing at OD_{600} = 0.5.

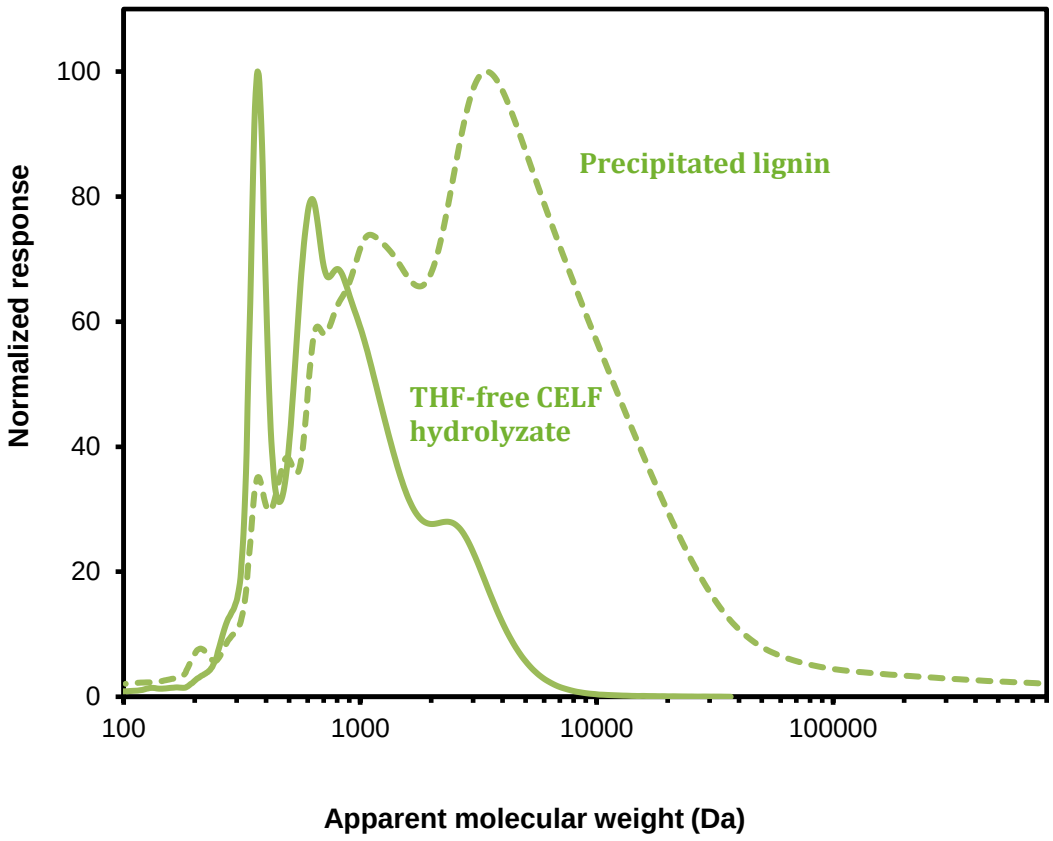


Figure 8.10. Gel permeation chromatogram of CELF hydrolyzate after boiling at pH 6.0 (solid line) and lignin precipitated during boiling (dashed line).

Chapter 9

Conclusions and Recommendations

9.1 Summary of Findings

Biomass pretreatment releases carbohydrates from the plant cell wall matrix and allows for greater access to cellulose. Co-solvent Enhanced Lignocellulosic Fractionation (CELF) was studied as an advanced pretreatment in comparison to established dilute sulfuric acid (DSA) pretreatment. Pretreatment conditions for maximum sugar release were established to be 160 °C and 20 minutes for DSA, and 150 °C and 25 minutes for CELF. At these conditions, CELF was found to delignify switchgrass up to 80% while preserving the major sugars completely. DSA, on the other hand, was found to solubilize only hemicellulose without any major change in lignin mass. Using molecular dynamics simulations, the high degree of delignification during CELF was determined to be caused by the unraveling of the lignin polymer in the tetrahydrofuran (THF)-water co-solvent environment, a behavior not seen in water-only environments. In its unraveled state, the inter-unit linkages in lignin are exposed to acid-catalyzed cleavage after which lignin fragments are individually solvated by THF, reducing the likelihood of recondensation to form new C-C bonds. The molecular weight of lignin extracted from CELF liquid hydrolyzate was found to be lower than that in unpretreated biomass, with an increase in syringyl and guaiacyl phenolic group content, suggesting extensive cleavage of aryl ether bonds interunit linkages.

The resulting pretreated solids after CELF were found to be comprised of nearly 90% glucan, which upon enzymatic hydrolysis, achieved theoretical glucose yields, even at enzyme loadings as low as 5 mg protein/g glucan. In comparison, solids from benchmark dilute sulfuric acid (DSA) pretreatment were only able to achieve 48%

glucose yields at the same enzyme loading. At very low enzyme loadings of 2 mg protein/g glucan, cellulolytic enzymes were found to be active for >5 weeks of hydrolysis of CELF solids, whereas enzymatic activity ceased after 2 weeks of hydrolysis on DSA solids. The free enzyme concentration before and after hydrolysis of cellulose in CELF solids was unchanged, implying that small amounts of residual lignin in CELF solids did not cause significant loss of enzymes to binding with lignin, whereas, residual lignin in DSA solids caused an approximately 40% loss of enzyme to lignin binding. This suggested that the reason for prolonged enzymatic activity was the preservation of free protein concentration in solution and mitigation of enzyme-lignin binding. Further, DSA solids were found to contain two forms of lignin – one that remains part of the lignin-carbohydrate complex (LCC) even after pretreatment, and the other that has recondensed onto the surface of the biomass during pretreatment. CELF lignin, on the other hand, was found to comprise mostly of lignin in the LCC, further supporting the earlier claim that the THF-water co-solvent prevents lignin aggregation and recondensation. The lignin in the LCC was determined to be responsible for the majority of protein binding, whereas the recondensed lignin did not bind much enzyme and merely provided a physical barrier between enzymes and the substrate. It was also hypothesized that the redeposited lignin does not only block access of enzymes to cellulose, but also blocks access of enzymes to lignin in the LCC. Therefore, removal of the recondensed lignin accelerated the unproductive enzyme-lignin binding, as evidenced by lower sugar yields from DSA solids where the recondensed lignin was washed away. CELF was also found to achieve identical sugar yields from unmilled and milled switchgrass, even at enzyme loadings of

2 mg protein/g glucan. This is significant as the elimination of the processing step of particle size reduction could have economic implications on the production of biofuels from switchgrass after CELF.

Pretreatment releases and generates inhibitors that can affect fermentation microorganisms, such as *Saccharomyces cerevisiae* and *Zymomonas mobilis*. The major inhibitors reported in literature are sugar dehydration products, such as furfural and 5-hydroxymethylfurfural, acetic acid, lignin-derived phenolics, high concentrations of ethanol, and heavy metals. Several methods of hydrolyzate detoxification have been previously reported, including solvent extraction, activated carbon extraction, and biological degradation of inhibitors. Additionally, fermentation microorganisms have long been known to have the ability to acclimatize to environmental conditions and overcome inhibition without the need for detoxification. CELF hydrolyzate contained high concentrations of THF (> 400 g/L), hemicellulose-derived sugars, acetic acid, 1,4-butanediol (BDO) and lignin-derived phenolics. THF has a low boiling point of 66 °C and was easily boiled out of hydrolyzate to concentrations below inhibitory levels to produce THF-free CELF hydrolyzate (TFCH). The major sugar present in TFCH was xylose, with small amounts of glucose also present. To ferment these sugars, an engineered strain of *S. cerevisiae* M11205 was employed during anaerobic fermentation. THF did not significantly inhibit fermentation of sugars by M11205 at concentrations < 5 g/L. Phenolics produced during the depolymerization of lignin during CELF were the major inhibitory compound present in TFCH, causing a maximum of 35% of theoretical maximum yield from sugars in TFCH. Methyl isobutyl ketone (MIBK) and toluene were

effective organic solvents that were used during liquid-liquid extraction to reduce phenolic concentrations in TFCH and increase fermentation yields to 90%.

CELF hydrolyzate was also found to contain a compound that drastically improved aerobic and anerobic performance of *S. cerevisiae* even at dilutions as low as 2% by volume. When phenolics concentrations were achieved below inhibitory levels in TFCH, either by liquid-liquid extraction or dilution, sugar uptake and fermentation rates by *S. cerevisiae* were observed to be significantly accelerated, with maximum ethanol yields being achieved 3 times as fast as those with a control solution. This behavior was reproducible with hydrolyzate from CELF on model cellulose substrates and on glucose, as well as the acid-catalyzed reaction of glucose with 1,4-butanediol. The proposed mechanism is the formation of an alkyl glycoside, 4-hydroxybutyl glucopyranoside, which acts as a non-ionic surfactant to increase sugar uptake rates into the cell and accelerate fermentation rates. The increased uptake rates were observed for both glucose and xylose, implying that the addition of small amounts of the proposed compound could have cause significant improvements in xylose uptake in *S. cerevisiae*, which has previously been reported to be slow. Thus, the proposed compound could potentially increase the overall productivity of ethanol fermentation plants, including current commercial corn starch and sucrose fermenting plants.

Finally, acclimatization strategies for *S. cerevisiae* to overcome inhibition by lignin-derived phenolics were explored. Whole recycle of cells from anaerobic flasks of TFCH fermentation was found to be effective at increasing ethanol yields. Cell harvest time played a role in the initial fermentation rates of subsequent runs. When cells were

harvested in stationary phase, the next fermentation run had a lag phase of 3 days. The lag phase was eliminated by collection of cells during exponential phase. This improvement in yield was the combined result of increase in cell density and cells developing a resistance to TFCH inhibitors, as cell recycle at identical cell density only showed a nominal improvement in fermentation yield. A second acclimatization strategy involved introduction of incremental concentrations of TFCH to fermentation flasks, while maintaining constant cell density, which yielded acclimatized cells after four runs. When these acclimatized cells were recycled at high cell densities, near-complete conversion of TFCH sugars was observed in 24 hours by the third run. The post-fermentation liquid was characterized by GC-MS to identify lignin-derived phenolics that may have caused inhibition. Two compounds, 2-methoxy-4-vinylphenol and 2,3-dihydroxybenzofuran were identified as lignin-derived compounds that could be potential candidates for downstream valorization to value-added products.

9.2 Novelty and Significance of this Dissertation

Prior research has shown that lignin removal during pretreatment of lignocellulosic biomass increases enzymatic digestibility of cellulose in the resulting solids. However, this is often done at the expense of hemicellulose sugar degradation due to severe pretreatment conditions. While some recent advances in pretreatment technology have been able to achieve greater overall sugar yields, they generally discard the lignin fraction as a waste stream. Further, the molecular principles behind effective biomass deconstruction have not been clearly elucidated. The research in this dissertation outlines, for the first time, synergistic mechanisms during co-solvent pretreatment that

simultaneously improves sugar accessibility and promotes lignin release for valorization. This work also proposes that lignin after typical aqueous pretreatments is present in two states with differing protein binding properties. This research proposes that during aqueous pretreatment lignin is redeposited not only on the cellulose surface, but also on the surface of lignin in the pretreated biomass structure. This inference is one that has previously not been reported and could usher a new understanding of lignin aggregation and redeposition during pretreatment.

Finally, the production of a novel yeast stimulating compound from glucose demonstrates improvements that have not been reported anywhere in literature, and cannot be matched by the addition of commercially available surfactants, such as Tween 20. Xylose utilization by fermenting microorganisms has been a topic of intense research for decades, and remains a bottleneck in commercial pentose sugar fermentations. The proposed fermentation stimulant in this dissertation is the first of its kind to demonstrate a dramatic increase in xylose sugar uptake and conversion in *S. cerevisiae* fermentations. While the majority of research on improving xylose uptake has involved genetically engineering microorganisms or converting xylose to isomers that are more bioavailable, the work in this dissertation proposes that the addition of minor amounts of the proposed stimulant in fermentation cultures can improve xylose uptake to degrees far beyond those previously reported in literature.

9.3 Recommendations for Future Research

The work described in this thesis brings up new questions that need further investigation to be answered. Evidence has been gathered to imply that surface

redeposited lignin does not bind much enzyme, and merely provides a barrier to enzymatic access. However, the rate of lignin solubilization and redeposition during DSA is yet to be understood. As the work in this thesis demonstrates a method of separation of redeposited lignin from LCC lignin, a time profile of lignin deposition on the biomass surface can be developed, which can be a useful metric during pretreatment design. This thesis describes the enzyme adsorbed to residual lignin in CELF and DSA solids. However, the mechanism behind the adsorption and whether these enzymes can be desorbed and released back into solution requires further research. CELF pretreatment could potentially eliminate particle size reduction of switchgrass, as identical sugar yields were observed with unmilled and milled switchgrass following CELF. Hardwoods are another primary feedstock for biofuel production, and previous research has shown that diffusion of acid catalyst into hardwood cell walls is limited. A similar study on the effect of particle size reduction on CELF pretreatment on hardwood feedstocks is required. This thesis identified the production of a novel yeast stimulant from glucose and 1,4-butanediol. However, the optimization of this reaction to produce the compound at high yields requires further detailed research. Further, understanding the mechanism behind improved sugar uptake and fermentation rates caused by addition of the stimulant will be of great value to the field. Finally, further process details, such as washing of solids after CELF and overall THF recovery following removal from hydrolyzate are needed before CELF can be considered a commercially viable pretreatment process.