Lignocellulose Saccharification via Cellulose Solvent Based Fractionation Followed by Enzymatic Hydrolysis: the Last Obstacle to Integrated Biorefineries

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ABSTRACT

The production of biofuels and biobased products from low-cost abundant renewable non-food lignocellulosic biomass will be vital to sustainable development because it will bring benefits to the environment, the economy, and the national security. The largest technical and economic challenge for emerging biorefineries is cost-effective release of fermentable sugars from recalcitrant structure of lignocellulosic biomass.

Cellulose- and organic-solvent-based lignocelluloses fractionation (COSLIF) technology was employed to overcome biomass recalcitrance. Surface response methodology (SRM) showed that optimal COSLIF pretreatment conditions were 85% (w/v) H_3PO_4 and ~50 ^oC, regardless of moisture contents in biomass from 5-15% (w/w) for common reed. Under these conditions, the pretreated biomass was hydrolyzed fast with high glucan digestibilities at low enzyme loadings (i.e., one FPU of cellulase per gram of glucan). Crystallinity index (CrI) measurements by X-ray diffraction (XRD) and cross polarization/magic angle spinning (CP/MAS) ^{13}C nuclear magnetic resonance (NMR), and cellulose accessibility to cellulase (CAC) determinations of COSLIF-pretreated biomass confirmed that highly ordered hydrogen-bonding networks in cellulose fibers of biomass were disrupted through cellulose dissolution in a cellulose solvent. This disruption of hydrogen bonding networks among cellulose chains resulted in a drastic increase in CAC values. Fourier transform infrared (FTIR) analyses on COSLIF-

pretreated biomass revealed conformational changes in specific hydrogen bonding among cellulose chains due to COSLIF.

While CrI is believed to be a key substrate characteristic that impacts enzymatic cellulose hydrolysis, studies in this thesis showed CrI values varied greatly depending on measurement techniques, calculation approaches, and sample preparation conditions. A correlation between CAC values and glucan digestibility of pretreated biomass showed that substrate accessibility is a key substrate characteristic impacting enzymatic cellulose hydrolysis.

In summary, COSLIF can effectively overcome biomass recalcitrance. The resulting pretreated biomass has high CAC values, resulting in fast hydrolysis rates and high enzymatic glucan digestibilities of COSLIF-pretreated biomass at low enzyme usage.

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LIST OF PUBLICATIONS

This thesis is based on the work presented in the following publications. They are referred to in the text by their Arabic numerals.

1. Sathitsuksanoh N., Zhu Z., Templeton N., Rollin J., Harvey S., and Zhang YHP.
   Saccharification of a potential bioenergy crop, *Phragmites australis* (common reed), by lignocellulose fractionation followed by enzymatic hydrolysis at decreased cellulase loadings
   Ind. Eng. Chem. Res. 2009, 48: 6441-6447.....21

2. Sathitsuksanoh N., Zhu Z., Ho T., Bai M., and Zhang YHP.
   Bamboo saccharification through cellulose solvent-based biomass pretreatment followed by enzymatic hydrolysis at ultra-low cellulase loadings
   Bioresour. Technol. 2010, 101:4926-4929.....29

3. Sathitsuksanoh N., Zhu Z., Wi S., Zhang YHP.
   Cellulose solvent-based biomass pretreatment breaks highly ordered hydrogen bonds in cellulose fibers of switchgrass
   Biotechnol. Bioeng. 2011, 108(3): 521-529.....34

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PREFACE

My favorite quote states, “Genuine passion cannot simply be taught or given, it must be experienced.” To me, this adage rings remarkably true. In looking back on my personal interests and professional goals, one common element has been passion. This thesis describes not only the most groundbreaking results achieved during my Ph.D. study in Biological Systems Engineering at Virginia Tech, but also my passion.

Working in this exciting high-impact field has been a rewarding experience. I would like to thank my advisor, Dr. Percival Zhang. His insight and knowledge have helped guide my research and support my work. His comments, suggestions and constructive criticisms have stimulated my way of thinking and progress. His trust in me and freedom to pursue my weird and bizarre ideas has made my research enjoyable. Without his guidance, this thesis would not have developed into the work that it has become.

Many of my friends have selected their committee members for the sake of fulfilling the graduate school’s requirement. In my case, all my committee members have been a part of my research progress and lend me their invaluable time and expertise. I would like to thank all three of my dissertation committee members, Drs. Justin Barone, Scott Renneckar, William Reynolds. The knowledge I have gained from them is more than coursework could ever teach.

The open, friendly, and crazy atmosphere in the Biofuels laboratory has made me keen and excited to come to work everyday. I would like to thank all my colleagues and fellow students for three very good years. In particular, Zhiguang Zhu, and Drs. Xu Bin, and Hong-Ge Chen, Chun You, and Xiaozhou Zhang–working on numerous projects with you has been fun.

Finally, I would like to thank my parents for their endless love, support, and encouragement. Talking to them over the phone always makes me absolutely distraught. Special thank to my mom who has been describing what I do in graduate school to our neighbors as "he is making booze from grass!"

Novozymes North America has been a great contributor for the enzymes used in all my study. In particular, I would like to thank Mr. Kurt Creamer for fresh batches of enzymes every year-some of which are referred in Biofuels lab as those greenish stuff!

My study could not be fruitful without lignocellulosic substrates generously procured from various institutions, National Renewable Energy Laboratory (NREL, Golden, CO) for corn stover, Oak Ridge National Laboratory (Oak Ridge, TN) for switchgrass, ITRD (Taiwan) for sugarcane bagasse, rice straw, and bamboo. I would like to also thank Dr. Stephen Harvey at US Army EBCB for common reed, Dr. Jack Saddler at University of British Columbia for providing me with douglas fir, Dr. Chip Frazer at Virginia Tech for yellow pine, and Drs. Steven Long and Frank Dohlman at UIUC and Monsanto for Miscanthus.

None of these projects in my thesis could have even formulated without financial supports from various agencies, ICTAS scholar program, DOE BioEnergy Science Center (BESC), USDA Bioprocessing and Biodesign Center, and Air Force Office of Scientific Research (AFOSR).

Passion, much like any emotion, is a trait that can be perceived negatively if taken to the extreme. However, in tempering my enthusiasm with passion in science for discovery of breakthroughs in bio-based economy, I still have been able to maintain excitement everyday. I hope this thesis presents my passion to others.

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INTRODUCTION

Concerns about accumulation of atmospheric greenhouse gases and depletion of fossil fuel reserve are driving us to seek for sustainable energy sources, such as solar power, nuclear power, wind power, hydropower, tidal energy, and so on. Transportation ability usually reflects the levels of civilization. The basic requirements for transportation fuels are high energy density (MJ/kg or MJ/L) and high power density (W/kg) in a compact space. Currently, the combination of liquid fuels (gasoline/bioethanol and diesel/biodiesel) and internal combustion engines are meeting with affordable transportation needs. In the future, automobiles may enter the age of hydrogen/electricity based on fuel cells (Olah et al. 2006). However, this transition might cause a big change in infrastructure of automotive industries.

Liquid ethanol is a near-term alternative transportation fuel. Currently, most ethanol is produced through yeast fermentation of soluble sugars that come from sugarcane and corn. However, the use of corn as the first-generation energy crop drives the food prices to increase. A rapid economic growth in developed and developing countries has led to higher food demand while supply is split into both food and fuel productions resulting in higher feedstock price, which constitutes into higher ethanol price. As a result, widely available lignocellulosic biomass (cellulosic biomass) comes into play. Lignocellulosic biomass includes:

- Agricultural residues (e.g., straws, corn stovers (cobs and stalks), bagasse, cotton gin trash, and palm oil and potato wastes)

- Energy crops grown specifically (e.g., sweet sorghum, switchgrass and common reeds)
- Paper sludge (e.g., recycled newspaper, pulp/paper mill sludge, and municipal solid wastes)
- Wood wastes (e.g., pruning's, wood chips, and sawdust)
- Brewery wastes (e.g., beverage wastes, such as beer)

Cellulosic biomass can be converted into sugar in an economically viable manner providing inexpensive materials for second-generation energy crops. Unlike starch and sugar, cellulosic biomass is made up of complex structure rendering it more difficult to unlock sugars. Overcoming lignocellulosic biomass recalcitrance followed by enzymatic hydrolysis of structural polymeric carbohydrates (i.e., cost-efficient liberation of fermentable sugars) is perhaps the most challenging technical and economic barrier to success of biorefineries (Fortman et al. 2008; Lynd et al. 2008; Zhang 2008). Lignocellulosic biomass is a natural composite having three main biopolymers (i.e., cellulose, hemicellulose, and lignin) intertwined chemically and physically. Pretreatment is among the most costly steps in biochemical conversion of biomass (Eggeman and Elander 2005; Wyman et al. 2005b), accounting for up to 40% of the total processing cost (Lynd 1996). Also, it affects the costs of other operations including size reduction prior to pretreatment and enzymatic hydrolysis and fermentation after pretreatment. Pretreatment can also strongly influence downstream costs involving detoxification if inhibitors were generated, enzymatic hydrolysis rates and enzyme loadings, mixing power, product concentration, product purification, power generation, waste treatment demands, and other process variables (Wyman et al. 2005b).

Lignocellulosic biomass

In most crop plants (e.g., corn, sugar cane, and wheat), carbohydrates are stored as starch in the plant cells. Although ethanol currently produced in commercial ethanol plants is from sugar cane in Brazil and from corn kernels in the United States, lignocellulosic biomass, such as agricultural and forest residues, municipal solid wastes, and herbaceous and woody plants can also be utilized as they are not competitive with food sources and shows great potential as a viable feedstock for transportation fuels (International Energy Agency. 2004; Worldwatch Institute. 2007). In the future, forest debris such as brush and small trees that are below pulping grade may be utilized in cellulosic ethanol production as well as switchgrass (Graham 2003). The cellulosic ethanol production may soon be economically viable on a commercial scale offering the potential to produce large quantities of ethanol. With transportation costs minimized, cellulosic ethanol demand should increase. They are abundant and can be converted into cellulosic ethanol and other co-products leading to an increase in revenue making it possible to commercialization. Cellulosic ethanol production is a relatively new process and theoretically more efficient then starch-based ethanol production systems (Tembo et al. 2003). If fermentable sugars from lignocellulose can be produced at a price competitive with those made from corn kernels, then profitable biomass biorefineries will be established solidly. But a narrow margin between sugar values extracted from biomass ($0.18/kg = $~110 sugar/ton biomass) and purchasing cost of delivered lignocellulosic biomass ($~50/ton dry biomass) raises a serious challenge – how to produce the low-cost sugars including costs associated with processing, capital depreciation, labor, or even transport (Hess et al. 2007; Zhang 2008; Zhang et al. 2006b). Therefore, it is clear that

co-utilization of lignocellulose components (acetic acid and lignin) will be vital to economic success of biomass biorefineries (Zhang et al. 2007). That is to say in addition to ethanol production from carbohydrate fractionation of biomass, more revenues from extra biofuels production from lignin and relatively high value acetic acid separation before fermentation are more valuable for the whole integrated biorefineries. Similar cases have happened to wet milling corn ethanol biorefineries.

Key challenges

Since cellulosic biomass is competitive in price with oil, a key challenge to commercializing production of fuels and chemicals from cellulosic materials is to reduce processing costs enough to achieve attractive investment returns. Biological conversion of biomass holds a great promise to achieve such low cost. With technological advancement in ethanol production from cellulosic materials, overall costs of ethanol production has been dropped to be competitive with grain ethanol. However, the risk of financing first-of-a-kind technology stalls commercial use (Wyman et al. 2005b).

Recalcitrant cellulosic biomass must be pretreated to achieve reactive forms of cellulose susceptible to enzymatic and microbial interactions to obtain high sugar yields, which are crucial to commercial success in biological conversion. Pretreatment is among the most costly steps in ethanol production accounting for up to 40% of the total processing cost depending on pretreatment technologies (Eggeman and Elander 2005; Lynd 1996; Wyman et al. 2005b). Pretreatment is a key step to unlock sugars from low-cost cellulose

materials. In order to understand the issues impacting cellulosic biomass pretreatment, it is helpful to first understand the chemical structure of cellulosic biomass and their potential applications.

Plant cell walls consists of cellulose, hemicellulose, and lignin, which together form a complex and rigid structure. The complex structure of biomass makes it difficult to chemical and biological degradation. There are many factors affecting biomass recalcitrance, such as substrate accessibility to cellulase, degree of polymerization, cellulose crystallinity, and lignin and hemicelluloses contents; however, two main factors are (1) low cellulose accessibility to cellulase (CAC) hindering cellulases from working efficiently and (2) lignin and hemicelluloses on the surface of cellulose blocking cellulases from accessing cellulose efficiently. The challenge is to break down the cellulose and hemicellulose chains made up of two-thirds to three-quarters of the biomass into fermentable sugars prior to subsequent fermentation. Figure 1 shows a schematic illustration of 2 main approaches to cellulosic ethanol production from lignocellulosic biomass. Many researchers have focused on the enzymatic strategy on the cellulosome enzyme system to unlock sugars rendering less pretreatment steps or avoiding it completely. However, this is a long term goal and many researchers have been actively investigating cellulosome systems (Bayer et al. 1994; Beguin and Lemaire 1996; Doi and Kosugi 2004; Schwarz 2001; Shoham et al. 1999). As a result, the near-term solution should focus on substrate strategy via effective pretreatment to release cost-competitive fermentable sugars from recalcitrant biomass via depolymerization and solubilization of one of the three major components of biomass (Lynd et al. 2008). Effectively releasing

soluble fermentable sugars from low-cost lignocellulosic biomass remain among the greatest challenges involved in the commercialization of second-generation biofuel biorefineries. For cellulosic ethanol to become a reality, biotechnological solutions should focus on optimizing the conversion of biomass to sugars (Lynd et al. 2008). A key to energizing a new biofuel industry based on conversion of cellulosic biomass to ethanol is to understand plant cell-wall chemical and physical structures---how they are synthesized and how they can be destructed.

Cellulose

Cellulose is a main component of lignocellulosic biomass made up around 38-50% of dry biomass located predominantly in the secondary cell wall. Cellulose chains are formed into microfibrils, which constitute into the basic framework of the cell giving rigidity and strength to cell walls. Cellulose is a homopolysaccharide of anhydroglucopyranose linked by β-1,4-glycosidic linkages. Bundles of cellulose molecules are aggregated together forming microfibrils ---diameter of individual microfibril is around 2-5 nm (Zhang et al. 2007)--- via orderly hydrogen bonds and Van der Waal's forces leading to highly ordered (crystalline) regions resulting in low accessibility to enzyme.

Certain structural features of cellulose contributing to low cellulose susceptibility to enzymatic hydrolysis are cellulose surface area and degree of cellulose polymerization. The cellulase enzyme mixture is mainly consisting of endo-, exo-glucanases, and b-glucosidase. Endoglucanase creates reactive ends by cleaving cellulose bonds, which are susceptible to exoglucanase. As a result, the more reactive cellulose, the higher cellulase efficiency will be. Although there are many factors affecting cellulose

enzymatic hydrolysis, the functionally-based enzymatic cellulose hydrolysis model clearly suggested that limited cellulose accessibility to cellulase (CAC) is the most crucial cellulose characteristics that affect enzymatic hydrolysis rates (Zhang and Lynd 2006). Although there are many means to determine surface area of cellulose, such as Brunauer-Emmett-Teller (BET), size exclusion, small angle x-ray scattering (SAXS), and nitrogen factor (NF) (Kumar and Wyman 2008; Zhang and Lynd 2004), the view on CAC is a controversial subject since it is difficult to measure CAC. Hong et al (Hong et al. 2008a; Hong et al. 2008b; Hong et al. 2007) established a new quantitative determination of CAC via means of a non-hydrolytic fusion protein containing a green fluorescence protein and cellulose-binding module. This method is believed to be more accurate in representing substrate cellulose-hydrolysis-related properties.

Hemicellulose

Hemicellulose, the second most common polysaccharides in nature, is a polymer containing primarily pentoses (xylose and arabinose) with some glucose and mannose dispersed throughout forming a short-chain polymer that interacts with cellulose and lignin forming a matrix in the plant cell walls (Wilkie 1979). In recent years, conversion of hemicellulose has been of interest since hemicelluloses represent 20-35% of lignocellulosic biomass. An efficient hemicellulose conversion processes can be utilized to increase ethanol yield and produce other coporducts. Enzymes that can degrade or help to degrade hemicelluloses hold great promise in saccharification of lignocelluloses biomass to fermentable sugars leading in an increase in product and coproduct revenues.

Such an enzyme, for example, cellulase-free xylanase can facilitate lignin removal from biomass (Saha 2003).

Hemicellulose hydrolyzates from pretreatment steps can be utilized to produce value-added products. Hemicelluloses has been utilized as plant gum for thickeners, adhesives, protective colloids, emulsifiers, and stabilizers as well as a biodegradable oxygen barrier films (Hartman et al. 2006; Kamm and Kamm 2004). Oligosaccharides may provide a source of higher value-added products, such as animal feed additives (Davis et al. 2002; Fernandez et al. 2002). Monomeric sugar, xylose, can be fermented to sugar alcohol, xylitol (Buhner and Agblevor 2004; Mussatto et al. 2005; Walther et al. 2001). Fufural, a xylose degradation product, can be utilized for production of lubricants, coatings, adhesives, and furan resins. Effective fermentation of xylose to ethanol or organic acids can increase the market size of xylose and xylose derivatives.

Lignin

Lignin is the most abundant phenolic polymer in nature, but its applications are limited. Most low-quality industrial lignin isolated from paper pulping industries are burned for energy generation and chemical recycling. Lignin research and development remains a stage of chicken and egg game. Without a sufficient amount of high-quality lignin supplies, there is no strong motivation to develop its new applications. Without broad lignin markets, there is no desire to isolate a large amount of high-quality lignin. High-quality lignin has been demonstrated to work as a substitute for polymeric materials, such as phenolic powder resins, polyurethane, and polyisocyanuratre foams as well as epoxy resins on a laboratory scale. Also, lignin as a raw material can be used as a precursor for

DMSO, vanilla, phenol, and ethylene (Eckert et al. 2007; Lora and Glasser 2002; Reddy and Yang 2005). Large amount of high-quality lignin isolated from biorefinery will be further processed to fuel additives (e.g.,), or even to synthetic diesel (Zhang 2008). Nearly pure lignin can be processed much easily and efficiently by chemical catalysis (e.g., pyrolysis or gasification) than lignocellulosic biomass. Researchers have been actively seeking new uses for lignin; the ones that are more applicable and profitable (Klausner 1984).

Cellulose solvent based lignocellulose pretreatment

A number of pretreatment technologies have been developed over the years. The key idea of pretreatment is to unlock sugars from its protective shells of lignin and increase enzymatic susceptibility of cellulose. Current pretreatment technologies are not efficient and cost effective due to specific feedstock dependency (i.e. corn and sugarcane). The solution to this energy challenge will not only be different in each country but also in different regions of a country, depending on available biomass resources. As a result, generic pretreatment (independency of feedstock) is required to accommodate different biomass properties and diverse biomass sources. Many researchers have focused on hemicellulose-targeted, cellulose-targeted, and lignin-targeted pretreatments (Dale et al. 1984). A good commercial pretreatment process should achieve the following objectives: (1) it must be green and economical; (2) it must promote an effective release of fermentable sugars from the recalcitrant matrix such that sugar yield is maximized while degradation products are avoided; (3) it must be conducted at low enzyme loadings such that nonspecific binding of enzymes to lignin and other components of pretreated

biomass can be avoided; (4) efficient chemical recovery and reconcentration; (5) all co-products are usable and marketable; (6) generic such that it can be applied with different feedstocks such that ethanol production plants can be sited in any geographic area. The capability to utilize low-cost cellulosic biomass to produce second-generation ethanol might not be enough to make biorefinery possible. The production of co-products that sell into high efficient markets gives a viable attractive business solution for investors. Fermentation of various sugars to produce beverage-alcohol and fuel-ethanol as well as chemicals is not something new. However, the high price of sugars and the low cost of competing petroleum based fuel have kept the production of chemicals confined to producing ethanol from corn---until the 1973 oil crisis and the 1979 energy crisis. However, from the mid-1980s to the mid-2003, the inflation adjusted price of crude oil on NYMEX was under $25/barrel. A series of events led the price to exceed $75/barrel in the mid-2006 and reached $110.20/barrel on March 12, 2008. Although ethanol production from cellulosic materials shows a great potential to solve energy challenge, currently there is no pretreatment technology that can achieve these criteria. The integration of pretreatment is needed in order to utilize advantages of individual treatment for environmentally sound, cost-effective, and low waste generating process.

Cellulose solvent only lignocellulose pretreatment

The use of appropriate solvents makes it possible to selectively remove lignin or hemicellulose from the native matrix. Solvents not only function to isolate cellulose from its lignin shell but also decrystallize cellulose structure by dissolution and subsequent regeneration to a highly active amorphous cellulose form. Most solvent pretreatment

technologies employ a large amount of solvents contributing to the cost of cellulose saccharification process. However, if the solvent utilized can be recovered, the economics of the process can be improved.

Cadoxen, an alkali solution of CdO in aqueous ethylenediamine was utilized as a cellulose solvent (Ladisch et al. 1978; Tsao 1978). However, cadoxen is very aggressive and time-consuming to prepare. In addition, toxic components in it interfere with subsequent enzyme hydrolysis and fermentation steps, and also prohibit use of the hydrolyzates recovered as an animal feed. Other solvents include HCl, H_2SO_4, H_3PO_4, and alkali.

Cellulose- and organic-solvent based lignocelluloses fractionation (COSLIF)

The current leading lignocellulose pretreatments (e.g., steam explosion, dilute acid, ammonia based pretreatment) cannot efficiently disrupt orderly hydrogen bonds among glucan chains in crystalline cellulose. They partially resulted in slow hydrolysis rates and low cellulose digestibility (i.e., modest sugar yields) (Wyman et al. 2005a; Wyman et al. 2005b). Moreover, many pretreatment methods are feedstock-specific. For example, ammonia fiber explosion (AFEX) has been found to be relatively ineffective for pretreating woody biomass (Dale et al. 1984; Sun and Cheng 2002). In order to effectively deal with two root causes of the recalcitrance of lignocellulose – breaking up orderly hydrogen bonds in crystalline cellulose and removing lignin and hemicellulose from the surface of cellulose, we have designed a novel chemical process, cellulose solvent and organic solvent lignocelluloses fractionation (COSLIF). COSLIF utilizes the

concept of “mix and match” to increase efficiency of the overall process. COSLIF not only fractionates lignocellulose components based on the significantly different solubility of cellulose, hemicellulose, and lignin in the cellulose solvent, organic solvent, and water, but also easily recycles the solvents due to a large difference in volatility between the solvents (Zhang et al. 2007).

The COSLIF has several-fold benefits, which are (1) de-crystallization of cellulose fibers (i.e., more cellulose accessibility so that cellulase can work on the substrate more efficiently) (Zhang et al. 2006a; Zhang and Lynd 2006), (2) removal of partial lignin and hemicelluloses from cellulose (i.e., fewer substrate obstacles to enzyme so that cellulase can access the substrate more efficiently) (Pan et al. 2006; Pan et al. 2005; Wyman 2007; Zhang 2008), and (3) modest reaction conditions (i.e., a decrease in sugar degradation, less inhibitor formation, lower utility consumption, and less capital investment) (McMillan 1994).

This thesis is based on the work presented in the following publications. They are referred to in the text by their arabic numerals.

1. Chapter 1 employed statistical analysis tool to obtain an optimal condition of COSLIF pretreatment. The central composite design consisted of 3-factor 2-level pattern with 20 experiments was utilized. The goal was to evaluate the effect of pretreatment time, pretreatment temperature, and biomass moisture content on pretreatment efficiency and enzymatic glucan digestibility. Common reed was utilized as a model feedstock in this study. Our results showed the relationships between these independent

variables and glucan retention of the solids after COSLIF, as well as enzymatic glucan digestibility of COSLIF-pretreated biomass.

2. Chapter 2 adopted the optimal condition from the previous study with some modification. The goal was to evaluate the COSLIF pretreatment efficiency on bamboo as a model feedstock. Different enzyme loadings were employed to investigate how COSLIF-pretreated substrate performed at an ultra-low enzyme usage.
3. My previous studies showed that COSLIF-pretreated biomass yielded fast hydrolysis rate and high enzymatic glucan release at low enzyme loadings. Our hypothesis was COSLIF mechanisms enhanced substrate accessibility greatly, and resulting solids were more accessible to hydrolytic cellulase enzymes. Surface accessibility measurement using TGC fusion protein, combined with other spectroscopic analyses (i.e., wide-angle x-ray diffraction (XRD), Fourier-transform Infrared spectroscopy (FTIR), and cross polarization/magic angle spinning (CP/MAS) ^{13}C nuclear magnetic resonance (NMR) was employed to investigate changes in substrate properties by COSLIF. Our results showed fast hydrolysis rate and high glucan digestibility for COSLIF-pretreated switchgrass were mainly attributed to an increase in cellulose accessibility to cellulase, because highly ordered hydrogen bonding networks of crystalline cellulose within switchgrass were disrupted through cellulose dissolution in cellulose solvent, as evidence by CP/MAS ^{13}C NMR and FTIR.
4. Chapter 4 entailed the study employing different cellulose solvents in pretreatment of biomass. Published results in literatures showed that ionic liquids and concentrated phosphoric acid appeared to be promising cellulose solvents for pretreatment.

Consequently, a direct side-by-side comparison between these two cellulose solvents was conducted to elucidate the influence from dissolution mechanisms, cellulose solvents remaining, and presence of lignin in biomass.

5. Integrated biorefineries with existing forest and agricultural biomass-based industries will allow a significant economic growth through new value-added products and higher resource utilization, a better alternative to landfilling and incineration is to incorporate cellulosic biomass in a biorefinery. With US diverse feedstocks in different regions of the country, the use of "generic" pretreatment technology that can accommodate different type of biomass would be one of the scenarios, which helps in feedstock logistical issue. In this chapter, COSLIF was applied to biomass mixture of Miscanthus and poplar wood at different mass ratios.
6. The presence of lignin in biomass is believed to be the major factor that causes biomass recalcitrant to enzymatic saccharification. One of the promising approaches was to employ a genetic modification tool to reduce lignin levels and/or chemical structure of biomass. We down-regulated 4CL expression in lignin biosynthesis pathway of switchgrass. Dilute sulfuric acid (DA) and COSLIF pretreatments were applied to transgenic plants. Enzymatic glucan digestibility for the solids resulting from the two pretreatments at a low enzyme loading was investigated.

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Chapter 1

Saccharification of a potential bioenergy crop, *Phragmites australis* (common reed), by lignocellulose fractionation followed by enzymatic hydrolysis at decreased cellulase loadings

Saccharification of a Potential Bioenergy Crop, *Phragmites australis* (Common Reed), by Lignocellulose Fractionation Followed by Enzymatic Hydrolysis at Decreased Cellulase Loadings

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Cost-effective biological saccharification of nonfood lignocellulosic biomass is vital to the establishment of a carbohydrate economy. *Phragmites australis* (common reed) is regarded as an invasive perennial weed with a productivity of up to 18−28 tons of dry weight per acre per year. We applied the cellulose solvent- and organic solvent-based lignocellulose fractionation (COSLIF) to the stems and leaves of *Phragmites* and optimized the pretreatment conditions (e.g., temperature, reaction time, and biomass moisture content) through response surface methodology (RSM). The optimal pretreatment conditions were 85% (w/v) H_3PO_4, 50 °C, and 60 min, regardless of the biomass moisture contents from 5−15% (w/w). Glucan digestibility of the COSLIF-pretreated common reed was 90% at hour 24 at a low cellulase loading (5 filter paper units and 10 β-glucosidase units per gram of glucan). Under these conditions, the overall sugar yields were 88% for glucose and 71% for xylose, respectively. Cellulose accessibility to cellulase (CAC) was increased 93.6-fold from 0.14 ± 0.035 to 13.1 ± 1.1 m^2 per gram of biomass with the COSLIF pretreatment. Results showed that cellulase concentrations could be reduced by 3-fold with only a slight reduction in sugar yield. This study suggested that *Phragmites* could be used as a carbon-neutral bioenergy feedstock, while its harvesting could help control its invasive growth and decrease nutrient pollution in adjacent waterways.

Introduction

The production of biofuels and biobased products from renewable lignocellulosic biomass will promote rural economy, decrease greenhouse gas emissions, and enhance energy security.[1−3] Biomass saccharification usually involves two sequential steps: lignocellulose pretreatment/fractionation and enzymatic hydrolysis of cellulose. The largest technological and economical challenge for biomass biorefineries is the efficient release of fermentable soluble sugars from low-cost lignocellulosic biomass at competitive costs.[4−7] Currently, the production of second generation biofuels, that is, cellulosic ethanol, cannot compete with that made from corn grain and sugar cane, because of its high processing costs (ca. $1−3 per gallon of cellulosic ethanol), huge capital investment ($2−10 per annual gallon ethanol capacity), and relatively low revenues from ethanol ($2−3 per gallon of cellulosic ethanol).[4,7,8]

Recently, a new technology called cellulose solvent- and organic solvent-based lignocellulose fractionation (COSLIF) has been developed to separate lignocellulose components (cellulose, hemicellulose, lignin, and acetic acid) by using a cellulose solvent, an organic solvent, and water.[4] Different from other lignocellulose pretreatments, this technology can be conducted at modest reaction conditions (e.g., 50 °C and atmospheric pressure) for minimizing sugar degradation. The key ideas of COSLIF are (1) partial removal of lignin and hemicellulose from cellulose, allowing more cellulose exposure to cellulase, (2) decrystallization of cellulose fibers (allowing cellusase to work more efficiently), and (3) modest reaction conditions (i.e., a decrease in sugar degradation, less inhibitor formation, lower energy requirement, and less capital investment). Higher glucan digestibility of the COSLIF-pretreated biomass was attributed to greater cellulose accessibility and more lignin removal, as compared to the dilute acid pretreatment.[9] In addition, COSLIF can separate lignocellulose components on the basis of their different solubilities in solvents and exhibit coutilization of lignocellulose components such as lignin.[4,7,10]

The DOE cellulosic ethanol workshop has summarized three distinct goals associated with potential bioenergy feedstocks: (1) maximizing the total amount of biomass produced per acre per year, (2) producing sustainable biomass with minimal inputs (e.g., pesticides, fertilizers, seeds, and harvesting), and (3) maximizing the amount of biofuels that can be produced per unit of biomass.[11] A yield of 20 dry tons per acre per year may be considered as a reasonable target in an area with adequate rainfall and good soil.[11]

Pharagmites australis (common reed) is a widespread perennial grass that grows in wetlands or near inland waterways throughout the world. Although it is harvested for thatched roofs, ropes, baskets, pulping feedstock, etc., in some areas of the world, common reed is typically regarded as an invasive weed, due to its vigorous growth and difficulty of eradication. *Phragmitez*, a C4 photosynthesis plant, can grow as high as 18 feet, with enormously high productivities of 18−28 tons of dry biomass per acre per year.[12] This productivity is approximately

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Table 1. The Features of *Phragmites* and Its Potential Advantages as a Bioenergy Crop

features	advantages
perennial grass	harvested yearly long canopy duration
C4 photosynthesis	high photosynthesis efficiency, e.g., 18−28 tons of dry biomass per acre per year
having seeds	easy large-scale planting at the beginning
growing from rhizomes	no yearly replanting
removing pollutant nutrients	no fertilizers waste water treatment
growing in wetland	no irrigation
few pests	low pesticide needed
invasive	harvesting = weed control
winter standing	long harvesting time (several months) low moisture content feedstock for winter harvesting
having rhizomes	recycling nutrients to rhizomes in winter
temperate and tropical regions	worldwide
marginal lands	no competition for arable land, preferring neutral and even alkaline wetlands

three to five times higher than a dedicated bioenergy crop—switchgrass. Since it produces seeds in addition to its growth from rhizomes, large-scale planting would be easier as compared to another potential bioenergy plant *Miscanthus*. Judging from annual inputs, the use of common reed as a bioenergy plant would have several advantages: growth from rhizomes after initial establishment from seeds or rhizomes, no or low fertilizer requirement, no irrigation (growing in wetlands), and low pesticides needed. Since common reed is regarded as an invasive weed by the U.S. Environmental Protection Agency (EPA), annual harvesting of common reed as a bioenergy feedstock can be regarded as weed control. In addition, existing strands of *Pragmites* are huge in the USA, and its further planting as a bioenergy crop seems promising. In fact, growth features of common reed are very good for biomass harvesting. Its winter standing allows a much longer harvesting time, as compared to corn stover. Also, harvesting of standing naturally dried strands with decreased moisture contents of ∼5−15% would save drying costs and biomass transportation costs. Before winter, it can recycle its nutrients to rhizomes for the growth in next year. Common reed usually grows in neutral pH or alkaline tropical and temperate water lands or wetlands, which are not suitable for most crops. Because it can take up nutrients efficiently, harvesting of existing strands will effectively remove phosphorus and nitrogen from inland waterways, and prevent algal blooms and other microbial pollution.[13−16] The features and associated advantages of common reed are presented in Table 1.

In this study, we investigated the feasibility of applying the COSLIF technology to common reed. We also sought to further improve the COSLIF technology by replacing the organic solvent (acetone) with ethanol for reductions in processing costs and capital investment for recycling of organic solvent. We optimized key pretreatment conditions by using response surface methodology (RSM), studied the release of soluble sugars from this potential bioenergy plant at decreased enzyme loadings, and analyzed potential economic benefits associated with low use of costly enzyme.

Materials and Methods

Chemicals and Materials. All chemicals were reagent grade and purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise noted. Phosphoric acid (85%) and 95% ethanol were purchased from Fisher Scientific (Houston, TX). The *Trichoderma* cellulase (Novozyme 50013) and β-glucosidase (Novozyme 50010) were gifts from Novozymes North American (Franklinton, NC). They had activities of 84 filter paper units (FPUs) per mL and 270 β-glucosidase units per mL, respectively.

Common reed was obtained from the U.S. Army Edgewood Chemical Biological Center (Aberdeen, MD) in the early winter of 2007. The naturally dried common reed was milled into small particles by using the Pallmann counter-rotating knife ring flaker (Clifton, NJ). The resulting particulates were screened to the sizes of less than 40 mesh (i.e., smaller than 0.420 mm) and greater than 60 mesh (i.e, larger than 0.250 mm). The milled materials were slowly dried to a moisture content of ∼5% at room temperature, whose moisture contents were determined by complete drying in a convection oven, at 105 ± 3 °C for 4 h or longer, until a constant weight was achieved. The different moisture contents of biomass samples were prepared by mixing ∼5% moisture content biomass with water, and then equilibrating in a closed container at room temperature overnight.

COSLIF Procedure. The COSLIF pretreatment for common reed was conducted as described elsewhere,[4,10] with some modifications. Acetone was replaced with 95% (v/v) ethanol. One gram of dry common reed with a moisture content, varying from 5%, 10% to 15%, was mixed with 8 mL of 85% phosphoric acid at different temperatures (40, 50, and 60 °C) for different lengths of time (30, 60, and 90 min) in 50-mL plastic centrifuge tubes. The biomass dissolution and weak hydrolysis reactions were stopped by adding 20 mL ethanol. After mixing well, solid/liquid separation was conducted in a swinging bucket centrifuge at 4500 rpm at room temperature for 15 min. After the supernatant was decanted, an additional 40 mL of ethanol was mixed with the slurry containing cellulose and hemicellulose. The solid/liquid separation was again conducted by centrifugation. After the supernatant was decanted, the pellets were resuspended and washed twice with 40 mL of water. The residual amorphous solid pellet was neutralized to pH 5−7 with a small amount of 2 M sodium carbonate.

Carbohydrate and Lignin Assays. The structural carbohydrate composition of the biomass was determined with a modified quantitative saccharification (QS) procedure.[17] In the modified QS, the secondary hydrolysis was conducted in the presence of 1% (w/w) sulfuric acid, rather than 4% sulfuric acid at 121 °C, for 1 h for more accurate determination of acid-labile carbohydrates (e.g., xylan and arabinan).[17] Monomeric sugars were measured by a Shimadzu HPLC, with a Bio-Rad Aminex HPX-87P column (Richmond, CA), at 65 °C with a distilled water as a mobile phase at a rate of 0.6 mL per min.[17] Lignin and ash were measured according to the standard NREL biomass protocol.[18] The concentrations of glucose and xylose in the enzymatic hydrolysate were measured by a Shimadzu HPLC with a Bio-Rad Aminex HPX-87H chromatography column by using 0.1% (v/v) sulfuric acid as a mobile phase at a flow rate of 0.6 mL per minute and a column temperature of 65 °C.[4]

Enzymatic Hydrolysis. The pretreated common reed samples were diluted to 10 g glucan per liter in a 50 mM sodium citrate buffer (pH 4.8) with supplementary addition of 0.1% (w/v) NaN_3, which prevented the growth of microorganisms.[4] All hydrolysis experiments were carried out in a rotary shaker at 250 rpm and 50 °C. Four enzyme loadings were tested: (1) 5 FPUs cellulase and 30 units of β-glucosidase per gram of glucan; (2) 10 FPUs cellulase and 30 units of β-glucosidase per gram of glucan; (3) 15 FPUs cellulase and 30 units of β-glucosidase per gram of glucan; (4) 5 FPUs cellulase and 10 units of β-glucosidase per gram of glucan. Eight hundred microliters of well-mixed hydrolysate were removed, followed by immediate centrifugation at 13 000 rpm for 5 min. Then exactly 500 μL

of the supernatant was transferred to another microcentrifuge tube and incubated at room temperature for 30 min, enabling the conversion of (nearly) all cellobiose to glucose, by β-glucosidase in the supernatant. The supernatant was acidified by adding 50 μL of 10% (w/w) sulfuric acid, and then was frozen overnight. The thawed liquid samples were mixed well and then centrifuged at 13 000 rpm for 5 min, to remove any solid sediment. The clear supernatants were used for determination of the released glucose by HPLC. After 72-h hydrolysis, the remaining hydrolysate was transferred to a 50 mL centrifuge tube, and centrifuged at 4500 rpm for 15 min. After decanting, the pellet was resuspended in 20 mL of water and centrifuged to remove soluble sugars. Following centrifugation, the remaining sugars and lignin in the lyophilized pellets were measured by QS. The soluble glucose and xylose (including galactose and mannose) in the enzymatic hydrolysate were measured by HPLC using a Bio-Rad HPX-87H column, as described above.

The enzymatic glucan digestibility (X)[19] can be calculated in percent as

$$X = \frac{G_f}{(180/162)G_i}100 \quad (1)$$

where G_f, is the amount of soluble glucose plus cellodextrins in the liquid phase after hydrolysis (g glucose equivalent, GE) and G_i, is the initially added glucan in solid cellulosic samples before hydrolysis (g).

For biomass pretreatment and subsequent enzymatic hydrolysis, the biomass input (stream 1) generated two streams (pretreatment hydrolysate—stream 2 and pretreated biomass—stream 3) and then enzymatic hydrolysis (stream 3) produced the solid residue (stream 4) and the enzymatic hydrolysate (stream 5).[20] The overall glucose yield (Y_{Glu}), during the COSLIF pretreatment and enzymatic cellulose hydrolysis, is calculated in percent as

$$Y_{Glu} = \frac{Glu_2 + Glu_5}{(180/162)Glu_1}100 \quad (2)$$

where Glu_2 and Glu_5 are mass amounts of glucose equivalent in streams 2 (the pretreatment liquid hydrolysate) and 5 (the enzymatic hydrolysate), respectively. Glu_1 is the initial glucan content before pretreatment. It is worth noting that commercial cellulase and β-glucosidase solutions contain very high concentrations of sugars (~20−100 g glucose per liter of enzyme solution).[21] Therefore the glucose concentration in stream 5 needs to be reduced by the amount of sugars already present in the enzyme solutions.[21]

Since a significant amount of xylooligosaccharides that cannot be measured in the presence of cellodextrins, by the regular HPLC columns, could exist in stream 5, the overall xylose yield Y_{Xyl} can be calculated in an alternative way as

$$Y_{Xyl} = \frac{Xyl_2 + (150/132)(Xly_3 - Xly_4)}{(150/132)Xyl_1}100 \quad (3)$$

where Xyl_3 and Xyl_4 are mass amounts of xylan in steams 3 and 4, respectively. Xylan contents in streams 1, 3, and 4 were measured by the HPLC HPX-87P column after quantitative saccharification.

Scanning Electron Microscopy (SEM). The biomass materials were imaged with a Zeiss-DSM940 (Carl Zeiss, Okerkochen, Germany). All samples were sputter-coated with gold and imaged by SEM, as described elsewhere.[22]

Substrate Accessibility Assays. The total substrate accessibility to cellulase (TSAC) was determined on the basis of the maximum adsorption capacity of the TGC protein.[9] The TGC protein is a nonhydrolytic fusion protein, containing a green fluorescence protein and cellulose-binding module.[23] The recombinant TGC fusion protein was produced in *Escherichia coli* BL21 (pNT02),[23] and purified by affinity adsorption on regenerated amorphous cellulose,[22] followed by modest desorption using ethylene glycol (EG).[24] EG was removed by membrane dialysis in a 50 mM sodium citrate buffer (pH 6.0). The TGC protein solution was reconcentrated using a 10000 Da molecular weight cutoff centrifugal ultrafilter column (Millipore, Billerica, MA). Mass concentration of the nonadsorbed TGC protein was measured on the basis of a fluorescent reading using a BioTek multidetection microplate reader, as described elsewhere.[23] Cellulose accessibility to cellulase (CAC, m^2/g biomass) can be measured on the basis of the maximum TGC adsorption capacity after the blocking by a large amount of BSA (e.g., 5 g/L). Noncellulose accessibility to cellulase (NCAC, m^2/g biomass) was calculated as NCAC = TSAC − CAC.[9]

Results

The common reed sample was harvested at the Aberdeen Proving Ground of Maryland. After complete drying, it contains 32.7 ± 2.5% glucan, 18.1 ± 2.2% xylan, 1.2 ± 0.2% galactan, 2.5 ± 0.3% arabinan, 22 ± 2.0% lignin, as well as 20 ± 3.6% mass weight for extractives, ashes, proteins, and so on.

Modified COSLIF Technology. The original version of COSLIF used a highly volatile organic solvent (acetone) between a cellulose solvent (concentrated phosphoric acid) and water. The functions of this organic solvent are (1) to partially remove lignin by dissolving it, (2) to decrease cellulose solvent recycling costs, and (3) to separate water-soluble depolymerized hemicellulose fragments and water-insoluble amorphous cellulose.[4,10] Low boiling-point acetone can be recycled easily by simple flashing, but it must be recycled with very high yields (e.g., > 99.99%). Any loss in acetone would negatively impact the economics of COSLIF implementation.

Here we replaced acetone by using ethanol for the modified COSLIF. This modification brought several benefits such that (1) a much lower recycling efficiency of ethanol is acceptable because the remaining ethanol in the hydrolysate and cellulose phase can be recycled after ethanol fermentation, (2) ethanol is more chemically stable than acetone, and (3) ethanol is less corrosive to the following membrane-based separations. Furthermore, we decreased organic solvent use nearly 2-fold, from 100 volumes to 60 volumes.

Optimization of COSLIF Pretreatment Conditions. The yield of fermentable sugars from the lignocellulosic biomass is a critical factor for evaluating the overall performance of the saccharification process, because sugar yields correlate closely with revenue.[10,19,20] Biomass saccharification usually involves two sequential steps: pretreatment and enzymatic hydrolysis. The COSLIF pretreatment conditions (temperature, time, and biomass moisture content) were optimized by using RSM.[25] The pretreatment temperature (T, 40, 50, and 60 °C), reaction time (t, 30, 60, and 90 min), and biomass moisture content (MC, 5, 10, and 15%) were chosen as independent variables (Table 2). The experimental design consisted of a 3-factor 2-level pattern with 20 experiments—14 combinations with 6 replications of the central point. The statistical software Design-Expert 6.0 (Stat-Ease Inc., Minneapolis, MN) was used to analyze the experimental results. The glucan retention after the COSLIF pretreatment, glucan digestibility, and glucan yield are presented in Table 2. The quadratic equation was obtained for the

Table 2. Pretreatment Conditions and Experimental Results for Glucan Retention after the COSLIF Pretreatment, Glucan Digestibility after Enzymatic Hydrolysis, And Glucan Yield That Equals Glucan Retention × Glucan Digestibility[a]

run	T	MC	t	glucan retention (%)	glucan digestibility (%)	glucan yield (%)
1	33.2	10	60	98.0	82.5	80.9
2	40	5	30	93.9	87.8	82.4
3	40	5	90	95.1	92.6	88.1
4	40	15	30	95.1	87.3	83.0
5	40	15	90	91.1	91.9	83.8
6	50	1.6	60	88.6	93.1	82.5
7	50	10	9.6	92.4	73.1	67.5
8	50	10	60	92.7	93.8	86.9
9	50	10	60	91.9	93.9	86.3
10	50	10	60	94.3	92.3	87.0
11	50	10	60	94.5	93.6	88.4
12	50	10	60	91.6	93.5	85.6
13	50	10	60	91.4	93.8	85.8
14	50	10	110.5	85.9	93.2	80.1
15	50	18	60	92.8	88.9	82.5
16	60	5	30	84.4	93.2	78.6
17	60	5	90	68.7	89.9	61.8
18	60	15	30	79.3	92.4	73.3
19	60	15	90	59.0	89.9	53.0
20	66.8	10	60	47.5	85.3	40.5

[a] All hydrolysis experiments were carried out at the same enzyme loading of 15 FPUs of cellulase and 30 units of β-glucosidase per gram of glucan for 24 h.

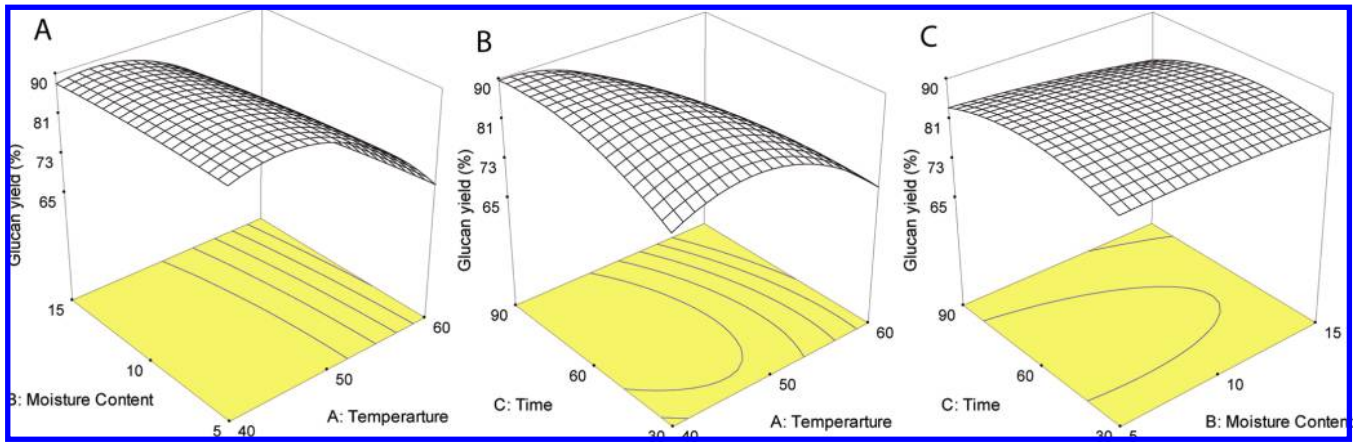

Figure 1. Response surface for the glucose yield from common reed pretreated by COSLIF at (a) temperature and moisture content, (b) temperature and reaction time, (c) moisture content and reaction time, followed by enzymatic cellulose hydrolysis (15 FPUs of cellulase per gram of glucan).

maximum glucose release, from pretreatment and enzymatic hydrolysis as

$$\text{glucan yield (\%)} = -155.68 + 8.66T + 1.94\text{MC} + 1.44t - 0.083T^2 - 0.025(\text{MC})^2 - 4.09 \times 10^{-3}t^2 - 0.026T(\text{MC}) - 0.018Tt - 6.69 \times 10^{-3}(\text{MC})t \quad (4)$$

Figure 1A shows the effects of reaction temperature and biomass moisture content on glucan yield that equals glucan retention multiplied by glucan digestibility. Regardless of temperature, the moisture content alone, between 5 and 15% had little effect on glucan yield. Reaction temperatures, between 40 and 50 °C, did not differentially impact glucose yields, while the higher reaction temperature (60 °C) resulted in a much lower sugar yield. As shown in Figure 1B, at a long reaction time (90 min), increasing reaction temperature significantly decreased the sugar yield, mainly due to overhydrolysis of polysaccharides. There was a maximum glucan yield at approximately 50 °C for a short reaction time (30 min). Low reaction temperatures prevented overhydrolysis of the glucan, resulting in high glucan retention. However, when pretreatment conditions were not sufficient, the enzymatic digestibilities were much lower than those of well-pretreated samples (90%) (Table 2). Therefore, a trade-off between pretreatment and hydrolysis was identified (i.e., maximum glucan yield) at two points: 40 °C for 90 min and 50 °C for 60 min. Figure 1C suggests that the maximum sugar yields were obtained when the reaction time was approximately 60 min, regardless of biomass moisture contents. All data suggested that biomass with a moisture content ranging from 5−15% did not affect pretreatment efficiency. The optimal pretreatment conditions for common reed were found to be 50 °C and 60 min, regardless of moisture content between 5 and 15%. After COSLIF treatment, ~93% of the glucan was retained, while 65% of the xylan and 28% of the lignin were removed.

Effect of Enzyme Loading and Mass Balance. Since cellulase is still a relatively costly biocatalyst accounting for a significant fraction of the processing costs for cellulosic ethanol production (approximately 30−100 cents per gallon of ethanol),[2] we studied the effects of an enzyme decrease from 15 to 5 FPUs per gram on glucan digestibility. Figure 2 shows the glucan digestibility profiles of the common reed pretreated under the optimum condition (50 °C, 1 atm, and 60 min) at different enzyme loadings. Since enzymatic cellulose hydrolysis involves a rate-limiting primary cellulose hydrolysis (soluble cellodextrin

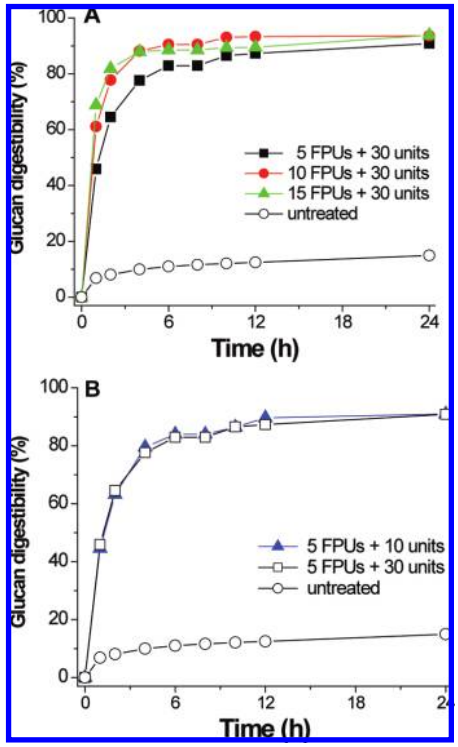

Figure 2. Enzymatic cellulose hydrolysis profiles for the COSLIF-pretreated common reed at different enzyme loadings (A, various cellulase and 30 units of beta-glucosidase; and B, 5 FPUs of cellulase and 10 or 30 units of beta-glucosidase).

release from solid cellulose), and a fast secondary cellulose hydrolysis (glucose generation from cellodextrins mainly mediated by β-glucosidase),[26] we first tested the effects of decreased cellulase loadings from 15 to 10 to 5 FPUs per gram of glucan, with a fixed high β-glucosidase loading (30 units per gram of glucan). High β-glucosidase loading can prevent any possible cellobiose inhibition. At a high enzyme loading (15 FPUs per gram of glucan), glucan digestibility reached 94% at hour 24 before leveling off (Figure 2A). When cellulase loading was decreased, glucan digestibility decreased slightly. At a low cellulase loading (5 FPUs per gram of glucan), glucan digestibilities were 87% at hour 12, 90% at hour 24, and nearly leveled off after 24 h.

Furthermore, we investigated the effect of 3-fold reduction in β-glucosidase from 30 to 10 units of per gram of glucan on glucan digestibility at 5 FPUs per gram of glucan. As shown in Figure 2C, a 3-fold reduction in total cellulase loading resulted in only 1−2% decrease in final glucan digestibility, and decreased hydrolysis rate only during the first 12 h. There was no significant difference in hydrolysis rates and final glucan digestibilities.

Figure 3 presents the mass balance of common reed pretreated by the COSLIF process and hydrolysis with 5 FPUs of cellulase as well as 10 units of β-glucosidase per gram of glucan. The enzymatic digestibilities at a low enzyme loading were 90% for glucan and 46% for xylan, respectively. The overall glucose and xylose yields, including enzymatic hydrolysis and pretreatment (water stream), were 88% and 71%, respectively.

Surface Morphology and Substrate Accessibility. Figure 4 shows the surface morphology changes in intact and COSLIF-treated common reed samples. The intact plant cell wall structures of common reed presents its plant cell vascular bundles and its fibril structure (Figure 4A). Concentrated H_3PO_4 can overcome biomass recalcitrance by dissolving crystalline cellulose fibers, accompanied with increasing cellulose accessibility.[4,9,22] The sequential washing by the organic solvent can partially remove lignin.[9] A well-treated lignocellulose sample (85% H_3PO_4, 50 °C and 60 min) shows no fibrous structure (Figure 4C), suggesting that all fibrous structures of the lignocellulose were completely disrupted. However, this disruption required sufficient reaction time at the set temperature.[9] Figure 4B shows that 20 min reaction time at 50 °C looks to break large fibrils of common reed but is not as efficient as that in Figure 4C. We further measured the substrate accessibility before and after the COSLIF pretreatment. This measurement was based on adsorption of a nonhydrolytic fusion protein TGC containing green fluorescent protein and a cellulose-binding module.[23] Through the COSLIF pretreatment, the total substrate accessibility to cellulase (TSAC) increased from 0.35 ± 0.056 to 16.1 ± 1.3 m^2 per gram of biomass (Table 3). To eliminate interference from the remaining lignin and other noncellulose components, cellulose accessibility to cellulase (CAC) was measured on the basis of the adsorption of TGC after blocking with BSA. The CAC values of the intact common reed and pretreated common reed were 0.14 ± 0.035 and 13.1 ± 1.1 m^2 per gram of biomass, respectively. This result suggested that COSLIF can increase substrate accessibility 93.6-fold and yield a cellulosic product with high substrate digestibility mediated by cellulase and fast enzymatic hydrolysis rate even at a low enzyme loading. A 14.4-fold increase in noncellulose accessibility (NCAC), from 0.21 to 3.03 m^2 per gram of biomass, was much lower than a 93.6-fold increase in CAC, suggesting the importance of increasing cellulose accessibility through biomass pretreatment.

Discussion

This study showed that very high overall yields (88% for glucose and 71% for xylose) were achieved for the COSLIF-pretreated common reed at a low cellulase loading (5 FPUs of cellulase and 10 units of β-glucosidase per gram of glucan) within 24 h hydrolysis. The optimal pretreatment conditions through surface response methodology were 50 °C, 1 atm, and 60 min in the presence of 85% H_3PO_4, regardless of the moisture contents of the feedstock, from 5 to 15% (w/w). Glucan digestibility (94%) of the pretreated common reed at a high enzyme loading was slightly lower than the previous results (i.e., 96−97%) for corn stover, switchgrass, poplar, and hemp hurds. This small difference was attributed to less efficient lignin removal (28% of overall lignin) in the modified COSLIF as compared to those achieved (40−50% of overall lignin)[4,10] by decreasing the use of organic solvent.

Water in ∼5−15% moisture content biomass did not dilute concentrated phosphoric acid significantly below the critical values (e.g., 80−83%) as a cellulose solvent.[10,22] The moisture contents of harvested biomass range widely from ∼5 to 40% w/w, depending on the harvesting season and biomass type.[27] Winter harvesting of the standing bioenergy plants after natural drying to 5−15% moisture contents, such as common reed, would save feedstock transportation costs as compared to that of freshly cut wet biomass feedstock. This study suggested the technological feasibility of efficient sugar release from a perennial grass, the common reed.

Cost analysis associated with enzyme costs and sugar-to-ethanol revenues suggests that decreasing cellulase use would compensate for the slight revenue loss, resulting from a slightly low overall sugar yields at a decreased enzyme loading (Figure 5). It was estimated that approximately 79.4 and 75.4 gallons of cellulosic ethanol per ton of common reed could be produced at high (15 FPUs per gram of glucan) and low (5 FPUs per

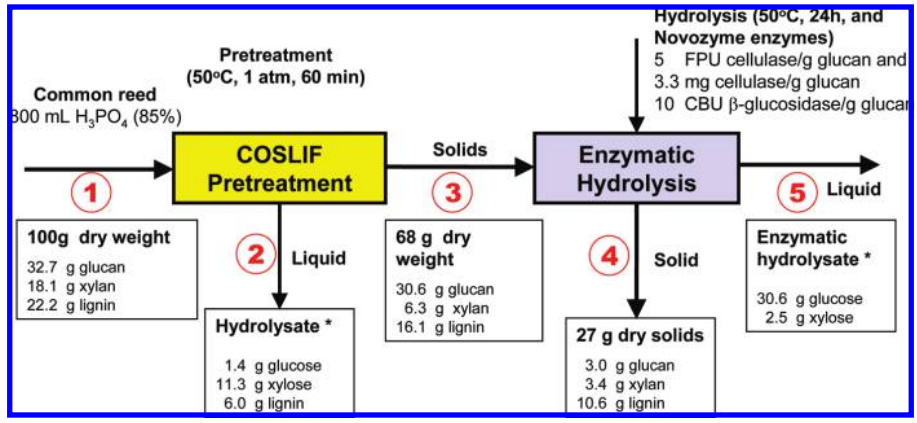

Figure 3. Mass balance for common reed pretreated by COSLIF followed by enzymatic hydrolysis at a low enzyme loading (5 FPUs of cellulase and 10 units of β-glucosidase per gram of glucan).

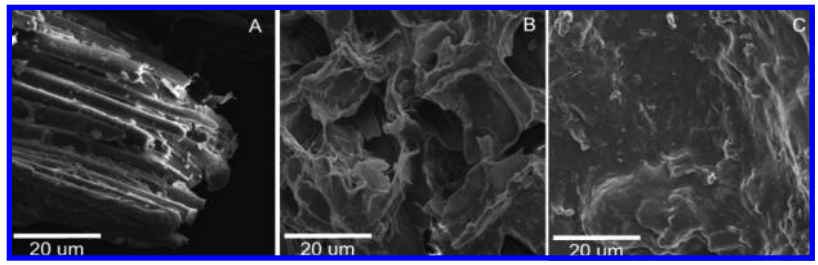

Figure 4. SEM images for the common reed samples before (A) and after the COSLIF pretreatment (B, 20-min dissolution; and C, 40-min dissolution).

Table 3. The Substrate Accessibilities (TSAC, Total Substrate Accessibility to Cellulase; CAC, Cellulose Accessibility to Cellulase; and NCAC, Noncellulose Accessibility to Cellulase) for Intact and COSLIF-Pretreated Biomass

sample	TSAC (m^2/g biomass)	CAC (m^2/g biomass)	NCAC (m^2/g biomass)
intact	0.35 ± 0.056	0.14 ± 0.035	0.21 ± 0.066
pretreated	16.1 ± 1.3	13.1 ± 1.1	3.0 ± 1.7

gram of glucan) cellulase loadings, respectively. If cellulase costs are \$0.30 per gallon based on an enzyme use of 15 FPUs per gram of glucan, a 3-fold reduction in cellulase use can save \$0.20 per gallon and decrease the ethanol revenues of \$0.126 per gallon, resulting in a net savings of 7.4 cents per gallon of ethanol, \$5.55 per ton of common reed, or a \$3.7 million of annual cost savings for a biorefinery processing 2000 tons of biomass per day. The cost saving would increase drastically to \$0.27 and \$0.53 per gallon of ethanol, if the cellulase costs were

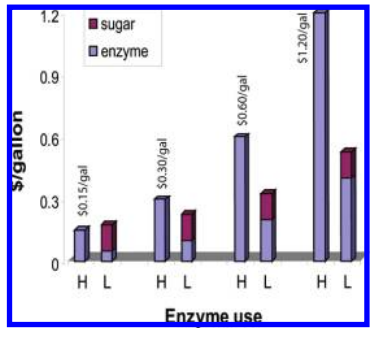

Figure 5. Enzyme cost analysis under the low (L, 5 FPUs of cellulase per gram of glucan) and high (H, 15 FPUs of cellulase per gram of glucan) cellulase loadings. Given an assumption of ethanol fermentation yield = 95% of theoretical yield for glucose and xylose, the overall ethanol yields were 79.4 and 75.4 gallons per ton of dry common reed, at the high and low enzyme loadings, respectively. The selling price of cellulosic ethanol was assumed to be \$2.50 per gallon.

\$0.60 and \$1.20 per gallon, respectively. On the other hand, if cellulase costs were decreased to \$0.15 per gallon of ethanol, a saving in enzyme cost could not be enough to compensate for the sugar loss.

The COSLIF technology may be regarded as a nearly generic pretreatment. It has previously been shown to efficiently increase the glucan digestibility of a relatively broad range of feedstocks, including corn stover, switchgrass, hemp hurds, and poplar.[4,10] This study extended the range of feedstocks to the common reed and also made improvements in the COSLIF process. Different from widely studied dilute acid pretreatment,[28−31] which substantially removes hemicelluloses thereby disrupting the linkages among cellulose, hemicellulose, and lignin, the COSLIF pretreatment not only partially removes lignin and hemicelluloses, but also substantially disrupts the fibrillar structure of biomass. The resulting fast hydrolysis rates and high glucan enzymatic digestibilities of the COSLIF-pretreated common reed are attributed to (i) more efficient biomass structure destruction, qualitatively shown by SEM images (Figure 4), and (ii) higher substrate accessibility to cellulase (Table 3).

Conclusion

The pretreatment conditions were optimized for the common reed through surface response methodology for the maximal release of soluble sugars. At a low enzyme loading (5 FPUs of cellulase and 10 units of β-glucosidase), the overall glucose and xylose yields were 88% and 71%, respectively. Low use of costly cellulase would significantly improve the overall economics of cellulosic ethanol production. Since the COSLIF technology is still at a relatively early stage of development, more detailed economic analyses, based on rigorous Aspen-plus models are needed to understand its potential for practical applications. This study clearly suggests that currently growing *Phragmitez*, an invasive weed, can be used for bioenergy

feedstock and would be planted as a bioenergy crop at marginal wetlands in the future.

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Note Added after ASAP Publication: The version of this paper that was published on the ASAP website May 28, 2009 had errors in the presentation of data for run 20 in Table 2. The corrected version of this paper was reposted to the Web June 2, 2009.

Nomenclature

BSA = bovine serum albumin
CAC = cellulose accessibility to cellulase
EG = ethylene glycol
GE = glucose equivalent
NCAC = noncellulose accessibility to cellulase
MC = moisture content (%)
QS = quantitative saccharification
RSM = response surface methodology
TGC = a nonhydrolytic fusion protein, containing a green fluorescence protein and a cellulose-binding module
TSAC = total substrate accessibility to cellulase

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Chapter 2

Bamboo saccharification through cellulose solvent-based biomass pretreatment followed by enzymatic hydrolysis at ultra-low cellulase loadings

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Bamboo saccharification through cellulose solvent-based biomass pretreatment followed by enzymatic hydrolysis at ultra-low cellulase loadings

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ABSTRACT

The modified cellulose solvent- (concentrated phosphoric acid) and organic solvent- (95% ethanol) based lignocellulose fractionation (COSLIF) was applied to a naturally-dry moso bamboo sample. The biomass dissolution conditions were 50 °C, 1 atm for 60 min. Glucan digestibility was 88.2% at an ultra-low cellulase loading of one filter paper unit per gram of glucan. The overall glucose and xylose yields were 86.0% and 82.6%, respectively. COSLIF efficiently destructed bamboo's fibril structure, resulting in a ~33-fold increase in cellulose accessibility to cellulase (CAC) from 0.27 to 9.14 m^2 per gram of biomass. Cost analysis indicated that a 15-fold decrease in use of costly cellulase would be of importance to decrease overall costs of biomass saccharification when cellulase costs are higher than $0.15 per gallon of cellulosic ethanol.

1. Introduction

The production of second generation biofuels (e.g., cellulosic ethanol) or third generation biofuels (e.g., hydrogen) from renewable lignocellulosic biomass will result in a new industrial revolution from a fossil fuel-based economy to a sustainable carbohydrate economy (Lynd et al., 2008; Zhang, 2008, 2009). Cost-effective production of fermentable sugars from recalcitrant biomass remains the largest obstacle to emerging cellulosic ethanol biorefineries (Lynd et al., 2008; Wyman, 2007; Zhang, 2008). Significant advances in reduction of 20- to 30-fold of enzyme costs have been made through enzyme production process optimization and cellulase engineering (Himmel et al., 2007; Zhang et al., 2006b), but cellulase, whose costs may range from ~30 cents to more than 100 cents per gallon of cellulosic ethanol at a typical cellulase loading of 15 filter paper units per gram of glucan, is still far more expensive than that of starch-hydrolyzing enzymes for corn kernel-based ethanol biorefineries (e.g., ~5 cents per gallon of starch ethanol).

Bamboos are giant woody, tree-like, perennial evergreen C_4 grasses with more than 70 genera and about 1000 species. Bamboos grow naturally in tropical, subtropical, and temperate regions around the world (Gratani et al., 2008). Bamboos are of economic and high cultural significance in East Asia and South East Asia. Since they are both lightweight and exceptionally durable, the treated bamboos are used extensively as building materials for houses, construction scaffolding, flooring, bridges, etc. Also, they are extensively used to make furniture, chopsticks, food steamers, paper pulp, etc., and are grown as ornamental plants. *Phyllostachys pubescens* (moso bamboo), one of the most popular bamboos, can grow to heights of over 20 m with a diameter of nearly 18 cm. Moso bamboo flourishes in moist, well drained, and fertilized soils with pH from 4.5 to 7.0 and annual precipitation between 800 and 1800 mm. Marginal lands, such as mountain valley, foot of mountain, and gentle slope, are suitable for moso bamboo growth.

Cellulose solvent- and organic solvent-based lignocellulose fractionation (COSLIF) has been developed to separate lignocellulose components using a cellulose solvent (concentrated phosphoric acid), an organic solvent (acetone), and water (Moxley et al.,

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2008; Zhang et al., 2007). High glucan enzymatic digestibility of the COSLIF-pretreated biomass is mainly attributed to large cellulose accessibility to enzymes (Zhu et al., 2009).

In this study, we investigated the feasibility of bamboo saccharification by the modified COSLIF followed by enzymatic hydrolysis at ultra-low cellulase loadings.

2. Methods

2.1. Chemicals and materials

All chemicals were reagent grade and purchased from Sigma–Aldrich (St. Louis, MO), unless otherwise noted. Phosphoric acid (85%) and ethanol (95%) were purchased from Fisher Scientific (Houston, TX). The *Trichoderma* cellulase (Novozyme® 50013) and beta-glucosidase (Novozyme® 50010) were gifts from Novozymes North American (Franklinton, NC). They had activities of 84 filter paper units (FPUs) per mL and 270 beta-glucosidase units per mL, respectively. The bamboo used in this study was moso bamboo grown in Taiwan. The full-size culm with around a half- to one-year age was harvested in April 2008, and then dried naturally from April to August 2008. The naturally-dried material was milled to small particles by a Pallmann counter rotating knife ring flaker (Clifton, NJ) to the nominal sizes of <40 and >60 mesh.

2.2. Modified COSLIF procedure

The modified COSLIF pretreatment for bamboo was conducted using 95% (v/v) ethanol as an organic solvent, as described elsewhere (Sathitsuksanoh et al., 2009).

2.3. Carbohydrate and lignin assays

The structural carbohydrate composition of the completely dry biomass was determined using a modified quantitative saccharification (QS) (Moxley and Zhang, 2007). Monomeric sugars were measured by a Shimadzu HPLC with a Bio-Rad Aminex HPX-87P column (Moxley et al., 2008). Lignin and ash were measured according to the standard NREL biomass protocol (Sluiter et al., 2006).

2.4. Enzymatic hydrolysis

The pretreated bamboo samples were diluted to 10 g of glucan per liter in a 50 mM sodium citrate buffer (pH 4.8), as described elsewhere (Moxley et al., 2008; Sathitsuksanoh et al., 2009; Zhu et al., 2009). The enzymatic glucan digestibility, overall glucose and xylose yields during the modified COSLIF pretreatment and enzymatic cellulose hydrolysis were calculated, as described elsewhere (Sathitsuksanoh et al., 2009; Zhang et al., 2009).

2.5. Substrate accessibility assays

The total substrate accessibility to cellulase (TSAC) was determined based on the maximum adsorption capacity of the TGC protein containing a green fluorescence protein and a cellulose-binding module (Hong et al., 2007; Zhu et al., 2009). The recombinant TGC fusion protein was produced in *Escherichia coli* BL21 (pNT02) (Hong et al., 2007) and purified by affinity adsorption on regenerated amorphous cellulose (Zhang et al., 2006a,b) followed by modest desorption using ethylene glycol (Hong et al., 2008). Cellulose accessibility to cellulase (CAC, m^2/g biomass) was measured based on the maximum TGC adsorption capacity after BSA blocking (Zhu et al., 2009).

3. Results and discussion

The functionally-based model for enzymatic cellulose hydrolysis suggests that increasing substrate accessibility is more important than decreasing the degree of polymerization of cellulose for biomass pretreatment (Zhang and Lynd, 2006). The most efficient way for increasing cellulose accessibility is to dissolve cellulose fibers and regenerate them as amorphous form (Kuo and Lee, 2009; Zhang et al., 2006a,b). The COSLIF V1.0 utilizes a highly-volatile organic solvent (acetone) between a cellulose solvent (concentrated phosphoric acid) and water (Moxley et al., 2008; Zhang et al., 2007). Low boiling-point acetone can be recycled easily by simple flashing, but it must be recycled with very high yields (e.g., >99.99%). Any loss in acetone would negatively impact the economics of COSLIF implementation and cause environmental pollution. Since it was found that removal of lignin from the COSLIF-pretreated biomass was not as important as increasing cellulose accessibility to cellulase (Zhu et al., 2009), here we used ethanol as the organic solvent for the modified COSLIF V2.0. The use of ethanol as an organic solvent would bring several benefits: (1) lower recycling efficiencies of ethanol during the pretreatment (e.g., 98–99%) compared to those of acetone, because the remaining ethanol in the hydrolysate and cellulose phase can be recycled after the subsequent ethanol fermentation, (2) ethanol is more chemically stable than acetone for sequential solid/liquid separation, and (3) ethanol is less corrosive to membrane-based separation. Furthermore, the replacement of acetone by ethanol allows a decrease in organic solvent utilization from 100 to 60 volumes. Since bamboo is a wood-like grass, similar to common reed and poplar, the modified COSLIF pretreatment conditions were determined to be 8 mL of 85% phosphoric acid for 1.06 g of bamboo with a moisture content of ~6% at 50 °C and 1 atm for 60 min.

The COSLIF-pretreated bamboo samples were hydrolyzed by a commercial *Trichoderma* cellulase at the enzyme loadings of 1, 2, 5, and 15 filter paper units and 10 units of beta-glucosidase at 50 °C (Fig. 1A). At 15 FPUs per gram of glucan, the sample was hydrolyzed fast for the first 12 h; the glucan digestibility was 89.9% at hour 12 and rose slightly to 94.9% at hour 72. Decreasing cellulase loadings from 5 to 2 to 1 FPU per gram of glucan significantly decreased initial hydrolysis rates but slightly decreased final glucan digestibilities from 93.3% to 89.8% to 88.2%, respectively. Glucan digestibilities at different enzyme loadings were much greater for 12-h or 24-h hydrolysis than for 72-h hydrolysis (Fig. 1B). The results clearly suggested efficient enzymatic hydrolysis at an ultra-low cellulase loading for 72 h.

Fig. 2 presents the mass balance for modified COSLIF pretreatment and enzymatic hydrolysis based on 100 g of dry biomass. After modified COSLIF pretreatment, 2.1 g of soluble glucose equivalent and 10.9 g of soluble xylose equivalent were removed before enzymatic hydrolysis. The reactive cellulosic material was hydrolyzed at one FPU per gram of glucan, releasing 39.1 g of soluble glucose and 3.0 g of xylose equivalent. The overall glucose and xylose yields were 86.0% and 82.6%, respectively. By contrast, the overall glucose and xylose yields were 92.8% and 85.0%, respectively, when 15 filter paper units per gram of glucan were used.

The high glucan digestibility was obtained for the COSLIF-pretreated bamboo sample even when cellulase loading was decreased by 15-fold from a typical 15 FPUs per gram of glucan. This result was mainly attributed to drastic changes in surface morphology of intact and COSLIF-treated bamboo samples. The intact plant cell wall structure of bamboo had its microfibril structure; while a COSLIF-treated lignocellulose sample showed no obvious fibrous structure (data not shown), as shown elsewhere for other biomass samples (Moxley et al., 2008; Zhu et al., 2009). In addition to qualitative images, we further measured the substrate accessibility before and after the modified pretreatment.

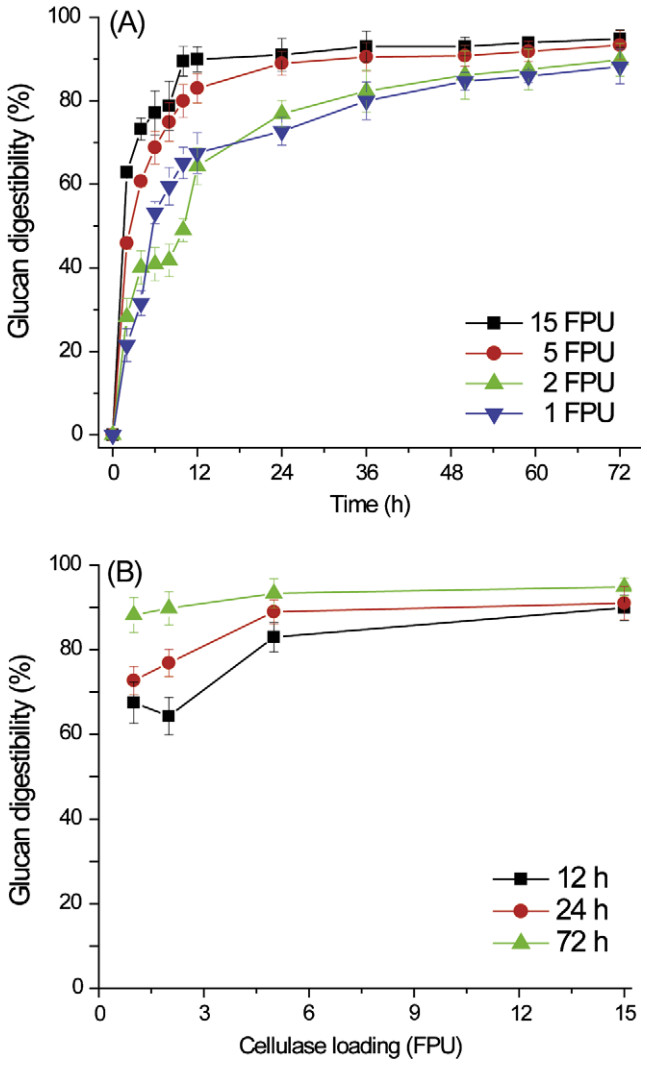

Fig. 1. Enzymatic cellulose hydrolysis profiles for the COSLIF-pretreated bamboo at different enzyme loadings (A, 1, 2, 5, and 15 FPUs of cellulase as well as 10 units of beta-glucosidase) and glucan digestibilities in terms of cellulase loadings for different hydrolysis time lengths (B).

The total substrate accessibility to cellulase (TSAC) increased from 0.45 ± 0.04 to 9.68 ± 0.76 m^2 per gram of COSLIF-pretreated biomass. In order to eliminate interference from the remaining lignin and other non-cellulose components, cellulose accessibility to cellulase (CAC) was measured based on the adsorption of TGC after blocking with BSA. The CAC values of the intact bamboo and pretreated bamboo were 0.27 ± 0.03 and 9.14 ± 0.64 m^2 per gram of biomass, respectively. This result suggested that COSLIF can increase substrate accessibility by 33-fold and yield a cellulosic product with high substrate digestibility mediated by cellulase and a fast enzymatic hydrolysis rate even at a low enzyme loading.

Bamboos, giant woody tree-like perennial evergreen grasses, are one of the fastest growing woody plants in the world. It is estimated that approximately 26,650 culms are produced per hectare in a bamboo plantation during a 10-year growing cycle (Shanmughavel and Francis, 2001). The average yearly biomass productivity in a 10-year growth cycle is approximately 95 tons of dry biomass per year per hectare (i.e., ~39 tons/acre/year), in which culms account for ~80% of total biomass (Shanmughavel and Francis, 2001). The above results indicate that bamboo are among the highest biomass producers as compared to other bioenergy plants in terms of tons of dry weight per acre per year, such as switchgrass (~4–8), dedicated forests including poplar (~8), Miscanthus (~10–25), *Phragmites australis* (common reed, ~18–28) (Sathitsuksanoh et al., 2009), and sugarcane (~25–30) (Zhang, 2008). In addition, existing systems for bamboo plantation, harvesting, and transportation would provide advantageous opportunities to build bamboo-based refineries as compared to other potential bioenergy plants, such as switchgrass and Miscanthus.

Current fungal cellulase cost per gallon of cellulosic ethanol may range from ~30 cents to more than 100 cents, based on a typical cellulase loading (15 filter paper units per gram of diluted acid-pretreated glucan) (Himmel et al., 2007; Zhang et al., 2006b). Obviously, ethanol production costs would reduce greatly as specific activity of cellulase increases (Fig. 3). When a typical cellulase loading of 15 FPUs/g glucan was decreased to 1 FPU/g glucan, the ethanol yield decreased from 88.1 to 83.6 gallons per ton of bamboo. Trade-off points between enzyme saving and ethanol revenue loss ($2 and $3 per gallon of ethanol) are shown in Fig. 3. If the selling price of ethanol was $3 per gallon and cellulase selling prices were greater than ~$0.16 of cellulase per gallon, a 15-fold reduction in cellulase loading would generate a more positive revenue increase than a loss of ethanol revenue. The enzyme-saving advantage (e.g., 84 cents per gallon) was far greater than the ethanol loss (e.g., 0.15 cents per gallon) when the cellulase cost was 90 cents per gallon. The above economy analysis remained relatively simple because a lot of factors, such as, large-size reactors needed for longer hydrolysis time, more utilities for mixing and cooling/heating, and waste treatment are not accounted. In general, cellulase is

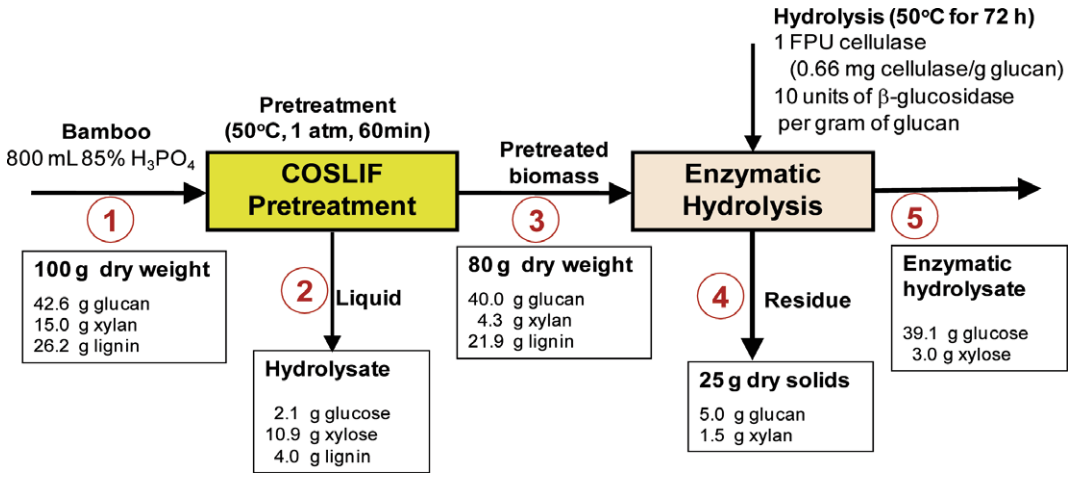

Fig. 2. Mass balance for bamboo pretreated by COSLIF followed by enzymatic hydrolysis by 1 FPU of cellulase and 10 units of beta-glucosidase per gram of glucan.

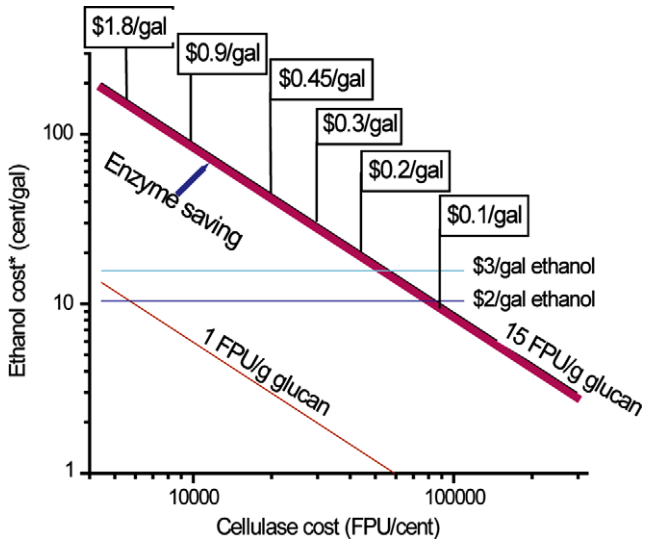

Fig. 3. Cost analysis of ethanol production* (involving only cellulase costs and ethanol yield loss due to a decreased cellulase loading) in terms of cellulase production costs. It was estimated that 29,665 FPUs were needed for one gallon of cellulosic ethanol produced based on an assumption that 15 FPUs per gram of glucan could cost ranging from 3 to 200 cents. The bamboo-to-ethanol yields were 88.1 and 83.6 gallons per ton of bamboo at 15 and 1 FPU per gram of glucan, respectively, based on 90% ethanol fermentation yields.

still one of the dominant cost fractions of the whole process (Himmel et al., 2007; Zhang, 2008; Zhang et al., 2006b).

4. Conclusions

The modified COSLIF pretreatment effectively disrupt recalcitrance of bamboo, generating highly reactive cellulosic materials as shown in a high glucan digestibility of 88.2% with 1 filter paper unit per gram of glucan at hour 72. A 15-fold reduction in cellulase usage will be of great importance in profitable production of cellulosic ethanol.

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Chapter 3

Cellulose solvent-based biomass pretreatment breaks highly ordered hydrogen bonds in cellulose fibers of switchgrass

ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Cellulose Solvent-Based Biomass Pretreatment Breaks Highly Ordered Hydrogen Bonds in Cellulose Fibers of Switchgrass

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ABSTRACT: The switchgrass (SG) samples pretreated by cellulose solvent- and organic solvent-based lignocellulose fractionation were characterized by enzymatic hydrolysis, substrate accessibility assay, scanning electron microscopy, X-ray diffraction (XRD), cross polarization/magic angle spinning (CP/MAS) ^{13}C nuclear magnetic resonance (NMR), and Fourier transform infrared spectroscopy (FTIR). Glucan digestibility of the pretreated SG was 89% at hour 36 at one filter paper unit of cellulase per gram of glucan. Crystallinity index (CrI) of pure cellulosic materials and SG before and after cellulose solvent-based pretreatment were determined by XRD and NMR. CrI values varied greatly depending on measurement techniques, calculation approaches, and sample drying conditions, suggesting that the effects of CrI data obtained from dried samples on enzymatic hydrolysis of hydrated cellulosic materials should be interpreted with caution. Fast hydrolysis rates and high glucan digestibilities for pretreated SG were mainly attributed to a 16.3-fold increase in cellulose accessibility to cellulase from 0.49 to 8.0 m^2/g biomass, because the highly ordered hydrogen-bonding networks in cellulose fibers of biomass were broken through cellulose dissolution in a cellulose solvent, as evidenced by CP/MAS ^{13}C-NMR and FTIR.

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KEYWORDS: biomass; biofuels; cellulose accessibility; cellulose solvent-based pretreatment; crystallinity index; drying of cellulose; switchgrass

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Introduction

The production of biofuels and biobased products from renewable cellulosic biomass would promote rural economy, decrease greenhouse gas emissions, and enhance national energy security (Lynd et al., 2002; Zhang, 2009). The current production of second generation biofuels, cellulosic ethanol, cannot compete with that made from corn and sugarcane, because of high processing costs, the requirement for huge capital investment, and relatively low revenues from low-value ethanol (Zhang, 2008). Biomass saccharification usually involves two sequential steps: lignocellulose pretreatment/fractionation and enzymatic hydrolysis. The root causes of biomass recalcitrance have been attributed to several factors: low substrate accessibility to enzymes, high crystallinity of cellulose, presence of hemicellulose, lignin, and other components, and high degree polymerization of cellulose chains (Zhang and Lynd, 2004; Himmel et al., 2007; Rollin et al., 2010). Highly ordered hydrogen bonds and *van der Waals* forces among sugar chains in crystalline fibers result in high crystallinity index (CrI) values and very low substrate accessibility (Lynd et al., 2002; Hong et al., 2007).

Switchgrass (SG) (*Panicum virgatum* L.) is a native, warm-seasoned, perennial, C_4 grass in North America and is distributed in more than half of the United States. It has several valuable features, such as modest/high productivities, adaptation to many types of soil and climate,

efficient water use, and low input of fertilizers and herbicides (i.e., high ratio of energy output to energy input). Therefore, SG has been regarded as a promising bioenergy crop.

Cellulose solvent- and organic solvent-based lignocellulose fractionation (COSLIF) has been developed to fractionate lignocellulose by using a combination of a concentrated phosphoric acid as a cellulose solvent and an organic solvent (e.g., acetone or ethanol) under modest reaction conditions (Zhang et al., 2007; Sathitsuksanoh et al., 2009). Concentrated phosphoric acid beyond a critical concentration disrupts the linkage among cellulose, hemicellulose, and lignin through biomass dissolution and increases cellulose accessibility greatly (Moxley et al., 2008). Our earlier work has shown that COSLIF-pretreated biomass can be hydrolyzed quickly by cellulase with very high glucan digestibilities (Zhang et al., 2007; Sathitsuksanoh et al., 2009; Zhu et al., 2009).

Crystallinity index (CrI) of cellulose is widely regarded as a key substrate characteristic that affects enzymatic cellulose hydrolysis (Chang and Holtzapple, 2000; Laureano-Perez et al., 2005), because amorphous cellulose can be hydrolyzed much faster than crystalline cellulose (Zhang et al., 2006). Some studies showed that crystallinity of cellulosic materials increased over the course of enzymatic cellulose hydrolysis (Betrabet and Paralikar, 1977; Ooshima et al., 1983), while others represent contradictory results (Ohmine et al., 1983; Puls and Wood, 1991). Many techniques, such as X-ray diffraction (XRD) (Teeäär et al., 1987), cross polarization/magic angle spinning (CP/MAS) ^{13}C nuclear magnetic resonance (CP/MAS ^{13}C-NMR) (Newman, 1999; Park et al., 2009), and Fourier-transform infrared spectroscopy (FTIR) (Oh et al., 2005) have been employed to determine CrI values. Park et al. (2009) have presented significantly different crystallinity values for the same materials by XRD and CP/MAS ^{13}C-NMR approaches (Park et al., 2009). Therefore, CrI roles in biomass pretreatment and enzymatic hydrolysis remain in debate.

Drying conditions of cellulosic samples greatly influence their characteristics and rates of enzymatic hydrolysis (Fan et al., 1980; Laivins and Scallan, 1996; Zhang and Lynd, 2004). Saddler and co-workers (Esteghlalian et al., 2001) show that different drying methods greatly affect substrate accessibility measured by Simon's stain. Oven drying results in the collapse or closure of the large pores in the samples, accompanied with significant reductions in enzymatic hydrolysis rates. Therefore, it is recommended to measure cellulose accessibility to cellulase (CAC) under hydrated conditions without drying (Hong et al., 2007).

In this study, we applied COSLIF pretreatment to SG. In order to understand the extent of CrI on enzymatic hydrolysis, many characteristics of SG and pure cellulosic materials before and after the pretreatment were investigated via scanning electron microscopy (SEM), XRD, CP/MAS ^{13}C-NMR, and FTIR. Moreover, effects of drying conditions (i.e., air drying and freeze drying) on enzymatic hydrolysis and substrate characteristics were studied.

Materials and Methods

Chemicals and Materials

All chemicals were reagent grade and purchased from Sigma–Aldrich (St. Louis, MO), unless otherwise noted. Phosphoric acid (85%) and ethanol (95%) were purchased from Fisher Scientific (Houston, TX). The *Trichoderma* cellulase (Novozyme® 50013) and β-glucosidase (Novozyme® 50010) were donated by Novozymes North America (Franklinton, NC). They had activities of 84 filter paper units (FPU)/mL and 270 cellobiase units/mL, respectively. Microcrystalline cellulose (Avicel PH105) was purchased from FMC (Philadelphia, PA). Regenerated amorphous cellulose (RAC) was prepared through Avicel dissolution in concentrated phosphoric acid, followed by regeneration in water as described elsewhere (Zhang et al., 2006). Birchwood xylan was purchased from Sigma–Aldrich. Lignin was isolated from sugar cane bagasse through Kraft pulping and NaOH treatment at 170°C. SG was procured from the National Renewable Energy Laboratory (Boulder, CO). The naturally dried SG was milled into small particles by a Pallmann counter-rotating knife ring flaker (Clifton, NJ). The resulting particulates were screened to the nominal sizes of 40-60 mesh. Bovine serum albumin was purchased from Alfa Aesar (Ward Hill, MA).

COSLIF Procedure

The COSLIF pretreatment was conducted as described elsewhere (Zhang et al., 2007), with some modifications. The COSLIF was conducted using 85% H_3PO_4 at 50°C, 1 atm, and 45 min. Acetone was replaced with 95% (v/v) ethanol (Sathitsuksanoh et al., 2009; Sathitsuksanoh et al., 2010). The residual amorphous solid pellets were neutralized to pH 5–7 with a small amount of 2 M sodium carbonate. The air-drying COSLIF-pretreated switchgrass (PSG) samples were prepared by direct drying in a Precision oven (Thermo Fisher Scientific Inc., Waltham, MA) at 37°C for 24 h. The freeze-drying samples were frozen at −70°C for 60 min and then was lyophilized in a VirTis freeze-drying apparatus (Gardiner, NY).

Carbohydrate and Lignin Assays

The structural carbohydrate composition of SG was determined by a modified quantitative saccharification (QS) procedure (Moxley and Zhang, 2007). In the modified QS, the secondary hydrolysis was conducted in the presence of 1% (w/w) sulfuric acid, rather than 4% (w/w) sulfuric acid at 121°C, for 1 h, creating a more accurate determination of acid-labile carbohydrates (e.g., xylan and arabinan). Monomeric sugars were measured by a Shimadzu HPLC equipped with a Bio-Rad Aminex HPX-87P column (Richmond, CA). Lignin and ash were measured according to the standard NREL biomass protocol (Sluiter et al., 2006; Moxley and Zhang, 2007). The concentrations of glucose

and xylose in the enzymatic hydrolysate were measured by a Shimadzu HPLC (Columbia, MD) with a Bio-Rad Aminex HPX-87H chromatography column equipped with refractive index detector. Galactose and mannose were co-eluted with xylose. The column was operated with 5 mM H_2SO_4 as a mobile phase at 60°C and at a flow rate of 0.6 mL/min.

Enzymatic Hydrolysis

The COSLIF-PSG samples were diluted to 10 g glucan/L in a 50 mM sodium citrate buffer (pH 4.8) with supplementary addition of 0.1% (w/v) NaN_3. All hydrolysis experiments were carried out in a rotary shaker at 250 rpm and 50°C. Four enzyme loadings were tested: 15, 10, 5, and 1 FPU of cellulase, supplemented with 10 units of β-glucosidase per gram of glucan. After enzymatic hydrolysis, enzymatic hydrolysis digestibility for glucan was calculated as previously described (Zhang et al., 2009).

Crystallinity Index Assays and Calculations

The CrI value can be determined by XRD and CP/MAS ^{13}C-NMR, along with different calculation approaches. The X-ray diffractograms of all samples were measured by the PANalytical X'Pert Pro X-ray diffractometer (Westborough, MA) with CoKα radiation ($\lambda = 1.78901$ Å) with the scanning rate of 4°/min ranging from 10° to 60°. With and without amorphous halo correction, deconvolution of the XRD spectra was conducted using PeakFit® 4.12 software (Systat Software Inc., Chicago, IL) assuming Gaussian distribution function as the shape of the resolved peaks. With regard to the peak height method, the CrI value was calculated based on the height of the peak corresponding to (002) lattice plane (I_{002}) and the minimum between 110 and 002 lattice planes (I_{am}) as below (Segal et al., 1959; Zhang and Lynd, 2004).

$$CrI\ (\%)\ = \frac{I_{002} - I_{am}}{I_{002}}\ \times\ 100$$

Based on the peak-deconvolution method, five crystalline peaks corresponding to (101), (10$\bar{1}$), (021), (002), and (040) lattice planes. These crystalline scattering is superimposed on an amorphous scattering. The spectra were deconvolved using Gaussian line shape function. The CrI value was calculated from the ratio of the crystalline area over the total area (Park et al., 2009). Based on the amorphous subtraction method, the separation of peaks from amorphous and crystalline contributions were conducted by subtracting the scattering of an amorphous standard (Ruland, 1961), where xylan was chosen as an amorphous standard. The CrI value was calculated from the ratio of the crystalline area over the total area after all spectra were deconvolved by using Gaussian line shape function.

The cross-polarization magic-angle spinning (CP/MAS) ^{13}C-NMR spectra of all samples were obtained on a Bruker II Avance-300 spectrometer operating at the resonance frequencies of 300.12 MHz for 1H, and 75.47 MHz for ^{13}C, using a Bruker 4.0 mm MAS NMR probe spinning at 6 kHz. Cross-polarization for 1 ms mixing time was achieved at 50 kHz rf-field at the 1H channel and linearly ramping the ^{13}C rf-field over a 25% range centered at 38 kHz. Total accumulation time was 8 min (2048 transient signals) by using 63 kHz of two-pulse phase modulated proton decoupling technique (Bennett et al., 1995). All spectra were collected at room temperature with polyethylene as an internal standard. According to the C_4 peak-deconvolution method, the CrI value was calculated from the ratio of the crystalline area over the total area, where separation of crystalline (86–92 ppm) and amorphous (79–86 ppm) fractions were based on Guassian line shape function. According to the NMR amorphous subtraction method, amorphous contribution was separated from the original spectrum prior to deconvolution of signals in the C_4 resonance region, where xylan was an amorphous standard.

Other Assays

The total substrate accessibility to cellulase (TSAC), CAC, and non-cellulose accessibility to cellulase (NCAC) were determined based on the maximum adsorption capacity of the TGC protein containing a green fluorescence protein and a cellulose-binding module (Zhu et al., 2009; Rollin et al., 2010). The recombinant TGC fusion protein was produced in *Escherichia coli* BL21 (pNT02) (Hong et al., 2007) and purified by affinity adsorption (Hong et al., 2008). The SEM images of the biomass materials were taken with a Zeiss-DSM940 (Carl Zeiss, Okerkochen, Germany), as described elsewhere (Moxley et al., 2008). All samples were sputter-coated with gold and imaged by SEM. All FTIR spectra were subjected to Saviszky-Golay smoothing. The absorbance of the bands obtained were resolved using Voigt distribution function by PeakFit® 4.12 software. The FTIR spectroscopy was conducted using a Thermo Nicolet 6700 ATR/FT-IR spectrometer (Thermo Fisher Scientific Inc.). Two hundred and fifty-six scans at a resolution of 6/cm were averaged for each sample.

Results

Enzymatic Hydrolysis of COSLIF-Pretreated Switchgrass

Switchgrass samples were pretreated by the modified COSLIF procedure, where ethanol was used as an organic solvent (Sathitsuksanoh et al., 2009). The completely dry SG contained 34.6% glucan, 18.6% xylan, 1.9% galactan, 3.0% arabinan, 21.0% lignin, as well as 20.9% extractives, ashes, and proteins. After COSLIF pretreatment, PSG contained 43.4% glucan, 8.2% xylan, 1.0% galactan, 1.0% arabinan, and 20.3% lignin.

The glucan hydrolysis profiles of the COSLIF-PSG were examined at different enzyme loadings from 1 to

15 FPU/g of glucan (Fig. 1). At a high enzyme loading of 15 FPU/g of glucan (~9.9 mg cellulase/g of glucan), glucan digestibility reached 94.6% at hour 24. When cellulase loading was reduced, glucan digestibility decreased slightly. At a low cellulase loading of 1 FPU/g of glucan (~0.67 mg cellulase/g of glucan), glucan digestibilities were 68% at hour 12, 83% at hour 24, and 92% at hour 72.

At hour 12, low cellulase loadings resulted in low glucan digestibilities. However, the glucan digestibility still reached 92% at hour 72 when enzyme loading was only 1 FPU of cellulase/g of glucan. A 15-fold reduction in cellulase loading for hydrolysis while maintaining high glucan digestibility would greatly reduce the amount of costly cellulase required for soluble sugar release.

Supramolecular Structure and Substrate Accessibility

The drastic changes in the surface morphology of SG before and after COSLIF were observed by using SEM (Fig. 2). The intact SG shows fiber cells (Fig. 2A). By contrast, PSG had no obvious fibrous structure (Fig. 2B and C), suggesting that all fibrous structures of the lignocellulose were completely disrupted by cellulose solvent-based pretreatment.

Substrate accessibilities of SG were measured based on adsorption of a non-hydrolytic fusion protein TGC (Zhu et al., 2009). Through COSLIF pretreatment, TSAC and CAC increased from 1.3 ± 0.1 to $9.6 \pm 0.6\ m^2/g$ of biomass and from 0.49 ± 0.05 to $8.0 \pm 1.1\ m^2/g$, respectively (Table I). A drastic increase in CAC by 16.3-fold led to high enzymatic digestibilities and fast hydrolysis rates even at low enzyme loadings. The two-fold increase in NCAC, from 0.78 to 1.62 m^2/g of biomass, was lower than the 16.3-fold increase in CAC, suggesting the accessibility of the cellulose fraction is preferably increased by COSLIF, compared to other cellulase-adsorptive surfaces in the pretreated biomass.

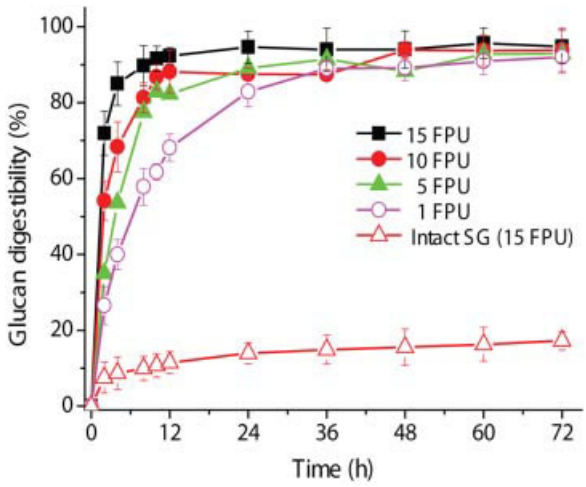

Figure 1. Enzymatic cellulose hydrolysis profiles for the COSLIF-PSG at different enzyme loading: 1, 5, 10, and 15 FPU of cellulase, supplemented by 10 units of β-glucosidase per gram of glucan.

X-Ray Diffraction Analysis

The XRD spectra of Avicel, RAC, xylan, and lignin are shown in Figure 3A. The XRD spectrum of Avicel showed five peaks, corresponding to (101), (10$\bar{1}$), (021), (002), and (040) lattice planes of crystalline cellulose I polymorph. The XRD spectrum of freeze-dried RAC, which was made from dissolution of Avicel and precipitation in water, showed a significant reduction in intensities of these five peaks. The intensities of peaks corresponding to (101), (10$\bar{1}$), and (040) lattice planes were greatly reduced to an undetectable level. The remaining two peaks corresponding to (021) and (002) lattice planes were broader and weaker than those of Avicel, indicating a significant reduction in crystallinity after cellulose dissolution and regeneration. A similar spectrum was observed for the air-dried RAC. However, the intensities of peaks of air-dried RAC corresponding to (021) and (002) lattice planes were higher than those of freeze-dried RAC, implying an increase in crystallinity due to air drying. Xylan and lignin did not exhibit any significant peaks, indicating that they are amorphous (Fig. 3A).

The XRD spectra of intact SG, air-dried PSG, and freeze-dried PSG are shown in Figure 3B. The spectrum of intact SG showed two significant peaks corresponding to (101) and (002) lattice planes. The peaks corresponding to (10$\bar{1}$), (021), and (040) lattice planes were not observed, which might be due to interference from other components in SG (e.g., hemicellulose and lignin), which may affect the peak broadening while part of a composite with cellulose. The spectrum of freeze-dried PSG showed a drastic reduction of intensity of the 101 peak to an undetectable level. The 002 peak in freeze-dried PSG was greatly reduced compared to that of intact SG, indicating a significant reduction in crystalline fraction of SG via COSLIF. Similar to the spectrum of freeze-dried PSG, the spectrum of air-dried PSG showed a broad 002 peak. Comparison of intensities of the peaks between freeze- and air-dried PSG spectra showed slightly different spectra; based on CrI calculation method, different conclusions may be drawn about the relative crystallinity of these different drying conditions.

Effect of Drying on Enzymatic Cellulose Hydrolysis

Previous work suggested that drying might greatly change the characteristics of hydrated cellulosic materials (Fan et al., 1981; Weimer et al., 1990; Zhang and Lynd, 2004). Since enzymatic cellulose hydrolysis must be conducted in hydrated conditions, we investigated the effects of air drying and freeze drying on enzymatic hydrolysis of PSG (Fig. 4A) and pure cellulose (Fig. 4B). The wet PSG was hydrolyzed fast at an enzyme loading of 15 FPU/g of glucan, reaching more than 90% digestibility within 12 h (Fig. 4A).

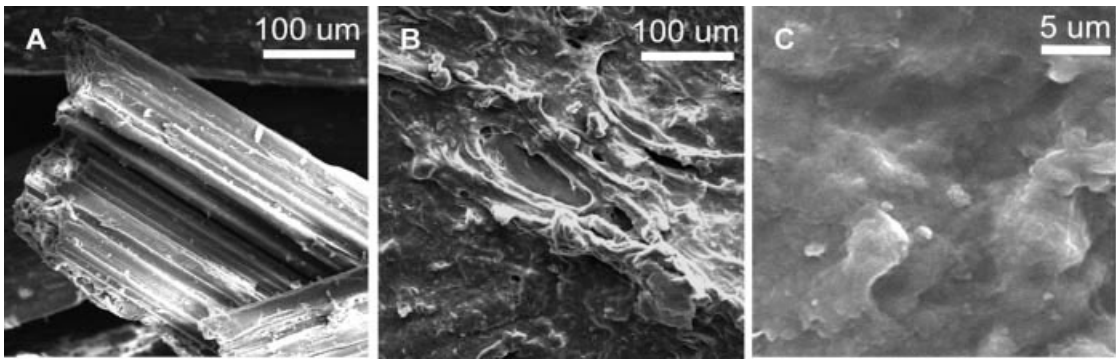

Figure 2. SEM micrographs of SG samples before (**A**) and after the COSLIF pretreatment (**B** and **C**).

Air-dried PSG that was hydrated before enzymatic hydrolysis had a poor digestibility of 63% at hour 72, suggesting that air drying caused a great reduction in substrate reactivity possibly due to a collapse in its supramolecular structures. The freeze-dried sample had a similar hydrolysis profile to hydrated COSLIF-PSG, suggesting that freeze drying can better preserve the substrate structure and reactivity compared to air drying, but still decrease the substrate accessibility relative to samples that were never dried (Table I). For both PSG (Fig. 4A) and pretreated pure cellulose (Fig. 4B), air drying resulted in a great reduction of enzymatic substrate reactivity which cannot be recovered by re-hydration, while freeze drying largely preserved the substrate reactivity.

Solid State ^{13}C-NMR Spectroscopy

Solid-state ^{13}C-NMR spectrum of Avicel (Fig. 5A) exhibited six singlets corresponding to ^{13}C chemical shifts of cellulose carbons, C_1 (105 ppm), C_4 (79–92 ppm), $C_2/C_3/C_5$ (70–80 ppm), and C_6 (60–69 ppm) of the anhydroglucose units of cellulose (Dudley et al., 1983). The C_4 resonance region is commonly used for determining cellulose crystallinity (Isogai et al., 1989). Moreover, the changes in the C_6 resonance region may indicate changes of specific hydrogen bonding with other hydroxyl groups in the adjacent cellulose chains. Broad shoulders in the C_4 region at 83 ppm and in the C_6 region at 63 ppm indicate the presence of amorphous cellulose (Atalla et al., 1980). Our NMR spectrum of Avicel showed strong signals at 89 and 65 ppm and broad signals at 83 and 63 ppm, indicating that Avicel contains both crystalline and amorphous fractions. In comparison with Avicel, the NMR spectrum of freeze-dried RAC showed a reduction in signals at 89 and 65 ppm, suggesting a decrease in crystalline fractions and possible disruption of orderly hydrogen-bonding networks in cellulose dissolution and regeneration. Similar results were observed in the air-dried pretreated sample.

Table I. Total substrate accessibility to cellulase (TSAC), cellulose accessibility to cellulase (CAC), and non-cellulose accessibility to cellulase (NCAC).

Materials	TSAC (m^2/g biomass)	CAC (m^2/g biomass)	NCAC (m^2/g biomass)
Intact switchgrass	1.3 ± 0.1	0.49 ± 0.05	0.78 ± 0.09
COSLIF-pretreated switchgrass[a]	9.6 ± 0.6	8.0 ± 1.1	1.6 ± 1.3
Freeze-dried COSLIF-pretreated switchgrass	2.7 ± 0.4	2.1 ± 0.4	0.59 ± 0.40
Avicel		2.3 ± 0.1	
RAC[a]		51.9 ± 0.9	
Freeze-dried RAC		19.1 ± 1.3	

[a]Never-dried samples.

The CP/MAS ^{13}C-NMR spectra of intact SG, air-dried PSG, and freeze-dried PSG are shown in Figure 5B. The spectrum of intact SG showed a broad C_4 region (79–92 ppm) and a peak at 65 ppm with a shoulder (63 ppm) in the C_6 region. PSG regardless of drying means did not show significant differences in the C_4 region. The broader C_4 regions for SG samples (Fig. 5B) than that of Avicel (Fig. 5A) may be attributed to the presence of hemicellulose and lignin in SG. After COSLIF pretreatment, the peak in the C_6 region (65 ppm) appeared to be weaker relative to that of intact SG, indicating that highly ordered hydrogen-bonding networks in SG was disrupted by using COSLIF.

The spectrum of isolated lignin (Fig. 5A) shows a strong signal at 56 ppm, corresponding to aromatic methoxyl (–OCH_3) resonance (Bartuska et al., 1980; Maciel et al., 1981). The NMR spectrum of intact SG exhibits a weak peak signal at 56 ppm, indicating a presence of lignin (Fig. 5B). After COSLIF pretreatment, air-dried PSG shows a very weak peak at 56 ppm while there is no significant peak at 56 ppm in freeze-dried PSG sample, implying that lignin characteristics may be changed by different drying methods.

Fourier Transform Infrared Spectroscopy Analysis

Comparison of FTIR spectra of Avicel and RAC shows differences in band intensities at 898 (ν_{as}(ring), anomeric

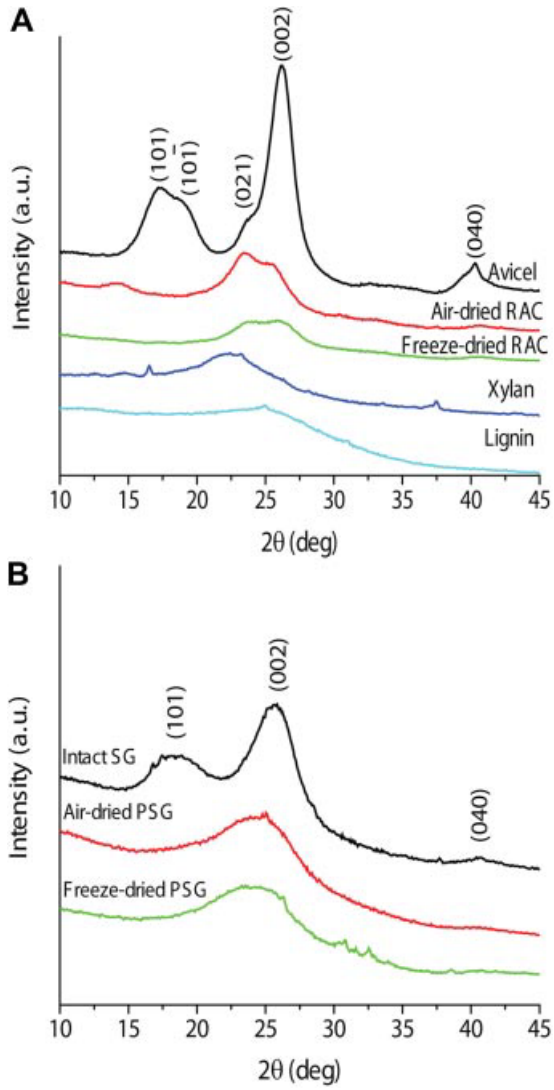

Figure 3. XRD spectra of Avicel, air-dried RAC, freeze-dried RAC, xylan, and lignin (**A**), as well as intact SG, PSG samples followed by air drying or freeze drying, and wet PSG that was never dried (**B**).

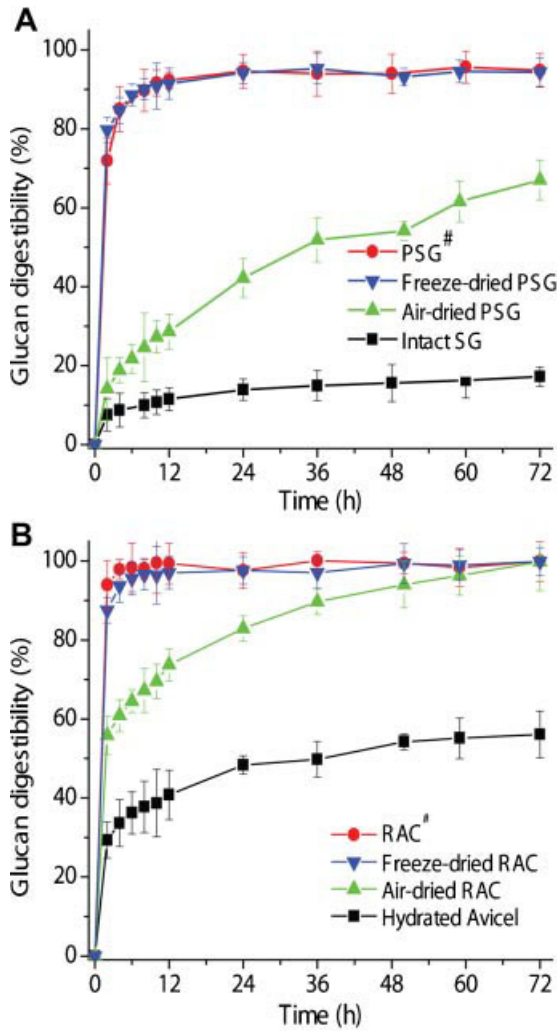

Figure 4. Enzymatic cellulose hydrolysis profiles of intact SG, air-dried PSG, and freeze-dried PSG (**A**), and Avicel, RAC, air-dried RAC, and freeze-dried RAC (**B**). #Never-dried samples.

vibration at β-glycosidic linkage), 1055 (ν(CO), CO stretching), 1105 (ν_{as}(ring), antisymmetric in-phase ring stretching), 1161 (ν_{as}(COC), COC antisymmetric stretching), 1281 (δ(CH), CH bending), 1315 (ω(CH_2), CH_2 wagging), 1335 (δ(OH), OH rocking), 1368 (δ(CH), CH bending), 1428 (δ_s(CH_2), CH_2 symmetric bending), and 2897 (ν(CH), CH stretching) cm^{-1} whose assignments are given in parentheses (Nelson and O'Connor, 1964; Hulleman et al., 1994; Dumitriu, 1998). Figure 6A shows that these band intensities decrease from Avicel to RAC, implying that highly ordered hydrogen bonds in Avicel were disrupted through cellulose dissolution and regeneration. The air-dried RAC showed stronger band intensities at 2897 and 898 cm^{-1} than freeze-dried RAC, suggesting that some RAC may re-crystallize during air drying, as partially supported by an increase in the peak intensity corresponding to the (002) lattice plane in the XRD spectra (Fig. 3A).

The FTIR spectra of intact SG and freeze-dried PSG are shown in Figure 6B. The freeze-dried PSG sample showed more significant decreases in the band intensities at 2919, 1422, 1368, 1337, 1319, 1158, and 898 cm^{-1} than intact SG, suggesting that highly ordered hydrogen bonds in crystalline cellulose of SG were disrupted after the pretreatment.

Discussion

Crystallinty is believed to be a key substrate characteristic affecting enzymatic hydrolysis. However, these results presented here suggest that the different conclusion can be drawn. Table II shows the CrI values of pure cellulosic materials and pretreated biomass by XRD employing three

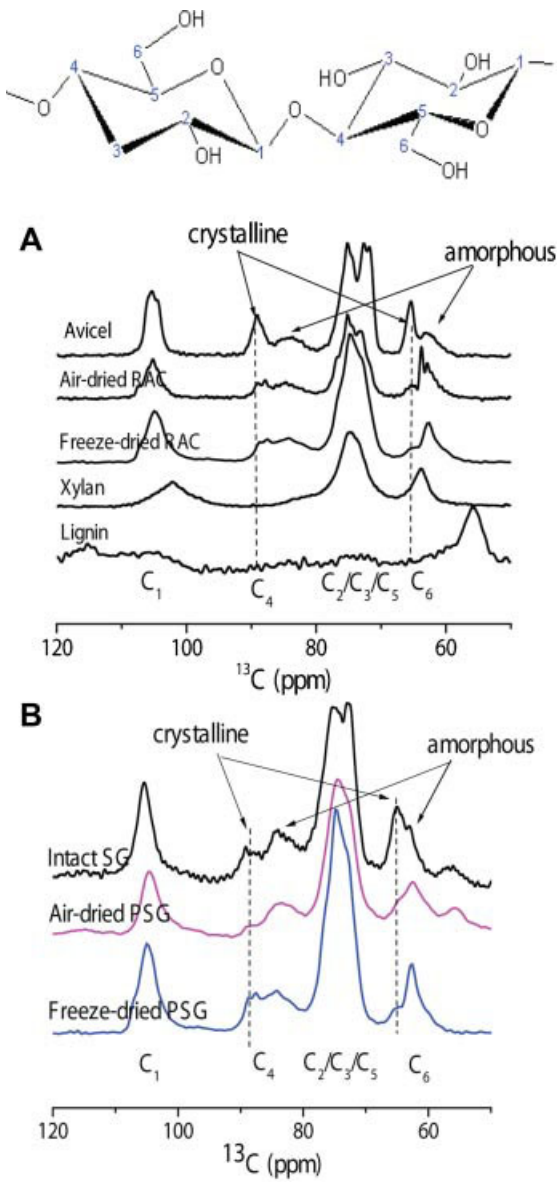

Figure 5. CP/MAS ^{13}C-NMR spectra of intact SG, air-dried PSG, and freeze-dried PSG.

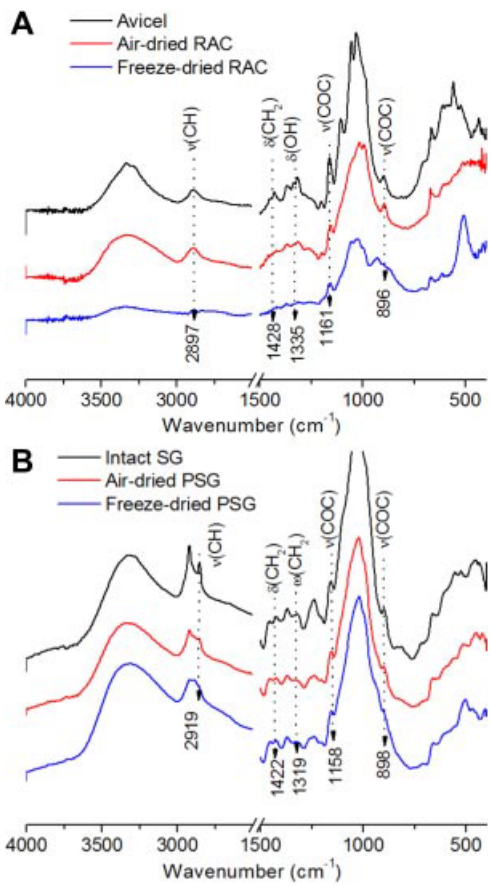

Figure 6. FTIR spectra of Avicel, air-dried RAC, and freeze-dried RAC (**A**), as well as intact SG, air-dried PSG, and freeze-dried PSG (**B**).

different calculation approaches based on peak height (Segal et al., 1959; Zhang and Lynd, 2004), peak deconvolution (Teeäär et al., 1987), and amorphous subtraction (Ruland, 1961), as well as CP/MAS ^{13}C-NMR using two different calculation approaches based on C_4 peak separation (Newman, 1999), and NMR amorphous subtraction (Park et al., 2009). CrI values of Avicel ranged from 52.8% to 91.8%, depending on the measurement techniques and calculation approaches. CrI values of Avicel from NMR were ~53–58%, suggesting that Avicel is a mixture of crystalline and amorphous cellulose, while the CrI value from the peak height method via XRD was 91.8%, suggesting that Avicel was highly crystalline. CrI values were 70.0% and 73.1% for Avicel based on peak-deconvolution and amorphous substration via XRD, respectively. These large variations in dry Avicel suggested that CrI determination were influenced greatly by the techniques and calculation means. RAC samples have no detectable CrI values (Table II). Amorphous subtraction-based NMR methods showed that air-dried RAC had a much higher CrI value than freeze-dried RAC, indicating that air drying may re-crystallize some fractions of amorphous cellulose.

Measurement of the CrI value of lignocellulose is more complicated than that of pure cellulose because the presence of hemicellulose, lignin and other components interferes with the fingerprint regions of cellulose. The CrI values of intact SG ranged from 33.6% to 67.0%, depending on its measurement techniques and calculation methods (Table II). After pretreatment, freeze-dried PSG had much lower CrI values (between 3.2% and 32.0%). Clearly, COSLIF greatly reduced crytallinity of biomass.

The relationship among CrI changes, pretreatment efficacies and subsequent enzymatic hydrolysis is very complicated. Some biomass pretreatments, such as COSLIF and ionic liquid-based pretreatment, decrease CrI values

Table II. The CrI values of pure and pretreated cellulosic materials by different measurement techniques and calculation approaches.

Materials	CrI (%)				
	XRD			NMR	
	Peak height	Peak de-convolution	Amorphous subtraction[a]	C_4 peak separation	Amorphous subtraction[a]
Dry Avicel	91.8	70.0	73.1	53.2	58.2
Air-dried RAC	ND	ND	ND	ND	33.5
Freeze-dried RAC	ND	ND	ND	ND	5.7
Intact switchgrass	67.0	59.4	60.9	38.9	33.6
Air-dried COSLIF-pretreated switchgrass	12.5	10.9	ND	21.8	15.6
Freeze-dried COSLIF-pretreated switchgrass	3.2	14.0	ND	17.6	19.1

ND, not detectable.
[a]Xylan was chosen as a standard for amorphous subtraction calculations via XRD and CP/MAS ^{13}C-NMR.

greatly by disrupting highly ordered hydrogen bonds in crystalline cellulose fibers (Gollapalli et al., 2002; Lee et al., 2009; Zhao et al., 2009). By contrast, other biomass pretreatments, such as dilute acid and steam explosion, increased composite CrI values by removing amorphous fractions (i.e., hemicellulose) (Deschamps et al., 1996; Kim and Lee, 2005). Considering the multiple facts that: (i) the CrI values of lignocellulose are mainly correlated with crystalline cellulose fraction, (ii) the composite CrI values of lignocellulose are also influenced by other lignocellulose components, such as hemicellulose and lignin, (iii) CrI determination is greatly influenced by measurement techniques, calculation methodologies, and drying conditions during sample preparation (Table II), and (iv) enzymatic hydrolysis is heavily influenced by substrate characteristics (e.g., cellulose accessibility), remaining hemicellulose and lignin, as well as properties of remaining lignin. We believe that the determination of CrI values of biomass after pretreatment lose some significance relating to glucan digestibility and pretreatment efficacy.

Conclusions

COSLIF-PSG was hydrolyzed fast with high digestibility at low enzyme loadings because the cellulose solvent (concentrated phosphoric acid) disrupted the linkage among cellulose, hemicelluloses, and lignin, as well as dissolved cellulose fibers, resulting in disruption of highly ordered hydrogen bonds in crystalline cellulose in SG, as evidenced by CP/MAS ^{13}C-NMR and FTIR. The CrI values for the pure cellulosic materials and SG varied largely, depending on its measurement techniques, calculation means, and drying conditions for the sample preparation. These results suggested that CrI was not a key substrate characteristic impacting enzymatic cellulose hydrolysis and relating to pretreatment efficacy.

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Cellulose Solvent-Based Pretreatment for Corn Stover and Avicel: Concentrated Phosphoric Acid *versus* Ionic Liquid [BMIM]Cl

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Running title: COSLIF vs ionic liquid

Abstract

Since cellulose accessibility has become more recognized as the major substrate characteristic limiting hydrolysis rates and glucan digestibilities, cellulose solvent-based lignocellulose pretreatments have gained attention. In this study, we investigated two cellulose solvent-based lignocellulose pretreatments: cellulose solvent- and organic solvent-based lignocellulose fractionation (COSLIF) using concentrated phosphoric acid (~85% (w/w) H_3PO_4) and ionic liquid pretreatment (IL) using [BMIM]Cl. Enzymatic glucan digestibilities of COSLIF- and IL-pretreated corn stover (CS) were 96% and 55% after 72 h at five filter paper units of cellulase per gram of glucan, respectively. Regenerated amorphous cellulose by COSLIF and IL had digestibilities of 100% and 92%, respectively. Our results suggested that differences in enzymatic glucan digestibilities of COSLIF- and IL-pretreated CS were attributed to combinatory factors. These results provide insights into mechanisms of cellulose solvent-based pretreatment and effects of residual cellulose solvents and lignin on enzymatic cellulose hydrolysis.

Keywords: biofuels, biomass pretreatment, cellulase inhibition, cellulose accessibility to cellulase, cellulose solvent and organic solvent-based lignocellulose fractionation (COSLIF), enzymatic cellulose hydrolysis, ionic liquid

Introduction

The issues of climate change associated with processing of fossil fuels and concerns pertaining to depleting fossil fuels have driven the search for sustainable and economically viable renewable energy options. Most current liquid transportation fuels are derived from crude oil. The production of cellulosic ethanol and advanced biofuels from the most abundant, low-cost, and non-food lignocellulosic biomass is anticipated to replace a significant fraction of liquid fossil fuel consumption in the future. But the largest obstacle to commercial biorefineries is cost-effective release of fermentable soluble sugars from lignocellulosic biomass, such as agricultural wastes, bioenergy crops, and woody biomass (Zhang 2008; Lynd et al. 2008). Lignocellulosic biomass is mainly composed of cellulose, hemicellulose, and lignin intertwined together, forming a recalcitrant matrix. Biological saccharification of biomass usually involves two steps: pretreatment and enzymatic hydrolysis. Pretreatment converts recalcitrant biomass to reactive (solid) cellulosic intermediate, and cellulases (mainly) hydrolyze pretreated biomass to soluble sugars.

Maize or corn (*Zea mays* L. spp.) is one of the most important agricultural crops in the world. In 2009, nearly one third of corn kernels in the USA were used to produce ~10.6 billion gallons of ethanol, which was blended with gasoline as a transportation fuel. Since corn kernels are mainly used as food and animal feed, production of biofuels from corn kernels negatively impacts food price and supply. Corn stover, agricultural wastes after harvesting corn kernels, is a potential biofuels feedstock and is not fully utilized. Currently only a small fraction of corn stover is used for animal feeding, barn bedding, and heating fuel. Therefore, employing corn stover would greatly increase biofuels production potential in the future without affecting food markets.

To extract soluble sugars from corn stover, it is necessary to overcome its recalcitrance to biological saccharification. Recalcitrance of lignocellulosic biomass is attributed to numerous factors: low substrate accessibility, high degree of polymerization (DP) of cellulose, presence of lignin and hemicellulose, high crystallinity, particle size, and porosity (Himmel et al. 2007). Most of these factors are correlated with substrate accessibility, which is suggested to be the most important substrate parameter impacting hydrolysis rate. Ball milling and cellulose dissolution in cellulose solvents followed by regeneration in anti-solvents can greatly increase cellulose accessibility before enzymatic hydrolysis. Although ball milling may be among the most efficient biomass pretreatment, it consumes so much energy that employing ball milling is not pragmatic for biorefineries (Ryu and Lee 1982; Chang et al. 1981). Alternatively, dissolution of cellulose in cellulose solvents followed by regeneration in anti-solvents can increase cellulose accessibility by disrupting highly-ordered hydrogen bonds in crystalline cellulose fibers (Ladisch et al. 1978; Swatloski et al. 2002). The resulting amorphous cellulose has much larger surface accessibility than that before pretreatment (Zhu et al. 2009).

Cellulose solvent-based biomass pretreatment was first developed by Ladisch et al. (Ladisch et al. 1978). They utilized Cadoxen, an alkali solution of CdO in aqueous ethylenediamine, to dissolve cellulose and biomass. The resulting regenerated amorphous cellulose (RAC) from pure cellulose is hydrolyzed quickly by cellulase with 100% cellulose digestibility, while enzymatic glucan digestibilities of pretreated biomass vary from modest to high, depending on biomass type. But toxicity and corrosiveness of cadoxen along with costly recycling technology prevents its commercialization. As such, many green cellulose solvent systems have been developed to pretreat cellulose and biomass, such as *N*-Methylmorpholine-*N*-oxide (NMMO) (Kuo and Lee

2009b), NaOH/urea (Zhao et al. 2008), concentrated phosphoric acid (Zhang et al. 2007), and ionic liquids (Dadi et al. 2006).

Zhang and his coworkers developed a cellulose solvent- and organic solvent-based lignocellulose fractionation (COSLIF) by using a concentrated phosphoric acid as a cellulose solvent followed by an organic solvent (e.g., acetone or ethanol) under modest reaction conditions (e.g., ~50 ^{o}C) (Zhang et al. 2007). COSLIF has been applied to a number of feedstocks, such as corn stover, switchgrass, poplar, and douglas fir (Zhang et al. 2007; Zhu et al. 2009). Although COSLIF-pretreated materials yield high sugar yields in short enzymatic saccharification times, a large amount of concentrated phosphoric acid usage needs to be optimized.

Similarly, another type of cellulose solvents, ionic liquids (ILs), can dissolve cellulose (Swatloski et al. 2002). A number of ionic liquids have been synthesized and employed to pretreat pure cellulose (Dadi et al. 2006; Zhao et al. 2009b; Kuo and Lee 2009a; Yang et al. 2010) and lignocellulosic biomass (Liu and Chen 2006; Singh et al. 2009; Fu et al. 2010; Nguyen et al. 2010; Lee et al. 2009; Zhao et al. 2009a). ILs, such as, 1-ally-3-methylimidazolium chloride (AMIM[Cl]), 1-ethyl-3-methylimidazolium acetate (EMIM[OAc]), 1-butyl-3-methylimidazolium chloride (BMIM[Cl]), and 1-ethyl-3-methylimidazolium diethyl-phosphate (EMIM[DEP]) have gained increasing interest as cellulose solvents in the pretreatment step (Zavrel et al. 2009; Li et al. 2009). The dissolved cellulose in ILs can be regenerated with an addition of anti-solvents, such as water, methanol, and ethanol. The IL-pretreated biomass exhibit reduced crystallinity, which enhances enzymatic glucan digestibility. Enzymatic hydrolysis of IL-pretreated biomass appears to depend on many factors, such as type of

lignocellulose, type of IL, size of lignocellulose particles, lignocellulose loading, water contents in lignocellulose and ionic liquid, as well as dissolution conditions (Lee et al. 2009; Sun et al. 2009).

The use of cellulose solvents, such as concentrated H_3PO_4 and ILs, for lignocellulose pretreatment has shown great promise. By exploiting different solubilities of different lignocellulosic components in different organic solvents, lignocellulose can be fractionated for production of not only biofuels but also value-added by-products. But development of cellulose solvent-based pretreatment is still in the early stage; very little is known about how cellulose solvents interact with enzymes. Understanding dissolution mechanisms of lignocellulose by cellulose solvents would greatly enhance potential usage of cellulose solvents, optimize overall conversion process parameters, and lower usage amount of cellulose solvents and costly enzymes. In this study, we employed two cellulose solvent-based pretreatment technologies–COSLIF and IL ([BMIM]Cl)–to pretreat corn stover and microcrystalline cellulose (Avicel). Pretreated materials were hydrolyzed by cellulase enzymes under a low enzyme loading. The influences of dissolution mechanisms, cellulose solvents remaining, and presence of lignin on enzymatic saccharification were investigated.

Materials and Methods

Chemicals and materials. All chemicals were reagent grade and purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise noted. Phosphoric acid (85% w/w) and ethanol (95% v/v) were purchased from Fisher Scientific (Houston, TX). 1-n-Butyl-3-methylimidazolium chloride ([BMIM]Cl, 96%) was purchased from Alfa Aesar (Ward Hill, MA). The *Trichoderma reesei*

cellulase (Novozyme® 50013) and β-glucosidase (Novozyme® 50010) were gifted by Novozymes North America (Franklinton, NC). They had activities of 84 filter paper units (FPUs) of cellulase per mL and 270 units of β-glucosidase per mL. Microcrystalline cellulose (Avicel PH105) was purchased from FMC (Philadelphia, PA). Lignin was isolated from sugar cane bagasse through Kraft pulping and NaOH treatment at 170˚C. Corn stover was procured from the National Renewable Energy Laboratory (Boulder, CO). The naturally-dried corn stover was milled into small particles by a Pallmann counter-rotating knife ring flaker (Clifton, NJ) to the nominal sizes of 40-60 mesh (250-400 μm). Both Avicel and milled corn stover were stored in a sealed container at 4°C. All feedstocks were dried in the convection oven at 105°C over night prior to pretreatment to eliminate the effect of moisture content.

COSLIF procedure. Regenerated amorphous cellulose via COSLIF (RAC_{COSLIF}) was prepared from Avicel through cellulose dissolution in 85% (w/w) phosphoric acid at 2% (w/v) solid loading, followed by regeneration in water as described elsewhere (Zhang et al. 2006a). The COSLIF-pretreated corn stover (CS_{COSLIF}) was prepared as described previously (Zhu et al. 2009) with some modification. In short, one gram of corn stover was mixed with 8 mL of 85% (w/w) H_3PO_4 at 50°C, 1 atm for 30 min. The corn stover/phosphoric acid slurry was stopped by adding 20 mL of 95% (v/v) ethanol and then mixed well. Solid-liquid separation was conducted in a swing bucket centrifuge at 4,500 rpm at room temperature for 10 min. After the supernatant was discarded, the pellets were suspended in 40 mL of 95% (v/v) ethanol. After centrifugation, the solid pellets were washed by 80 mL of deionized water. After centrifugation, the remaining solid pellet was neutralized with 2 M sodium carbonate.

IL pretreatment. Regenerated amorphous cellulose made by using IL (RAC_{IL}) was prepared through Avicel dissolution in [BMIM]Cl. The dissolution condition was adopted from Zhao et al.

(Zhao et al. 2009a). In short, one gram of Avicel was mixed with 20 g of [BMIM]Cl at a solid loading of ~5% (w/w) on a block heater at 105-110 ^{o}C for 15 min. The hydrogel-like solution was allowed to cool, and 20 mL of water was added to precipitate the solid. After centrifugation, the supernatant was discarded. An additional 400 mL of water was then used to wash residual [BMIM]Cl from the solid pellets. The IL-pretreated corn stover (CS_{IL}) was conducted by mixing one gram of corn stover with 20 g of [BMIM]Cl at 105-110 ^{o}C for 30 min. The mixture was allowed to cool, and 20 mL of 95% (v/v) ethanol was added to precipitate the solid. After centrifugation, the supernatant was discarded. The solid pellets were suspended by additional 160 mL of 95% (v/v) ethanol. After centrifugation, the pellets were washed by 320 mL of deionized water to remove residual [BMIM]Cl from the solid pellets. The resulting solid pellets, denoted as CS_{IL}, were used in the enzymatic hydrolysis experiments. It should be noted that Avicel and corn stover were added step-wise to lessen the over-heating and aggregation during dissolution.

Carbohydrate and lignin assays. The structural carbohydrate composition of corn stover was determined by the standard NREL biomass protocol (Sluiter et al. 2006). Monomeric sugars were measured by a Shimadzu HPLC with a Bio-Rad Aminex HPX-87H column (Richmond, CA) equipped with refractive index (RI) detector. The concentrations of glucose and xylose were measured in enzymatic hydrolysate, whereby galactose and mannose were co-eluted with xylose. The column was operated with five mM H_2SO_4 as a mobile phase at 60^{o}C and a flow rate of 0.6 mL/min. Intact CS contained approximately 37.1 ± 1.8 wt.% glucan, 18.1 ± 0.6 % xylan, 1.9 ± 0.1 wt.% galactan, 3.4 ± 0.4 wt.% arabinan, and 19.0 ± 0.1 wt.% lignin.

Enzymatic hydrolysis. The COSLIF- and IL-pretreated samples were diluted to 10 g of glucan per liter in a 50 mM sodium citrate buffer (pH 4.8) supplemented with 0.1% (w/v) NaN_3.

COSLIF- and IL-pretreated samples were completely suspended in the reaction media to ensure that the cellulose particle surface was accessible to the cellulases, thereby optimizing cellulase adsorption and activity. All hydrolysis experiments were carried out in a rotary shaker at 250 rpm at 50 °C. The enzyme loadings were five FPU per gram of glucan and 10 units of β-glucosidase per gram of glucan, otherwise noted.

Other assays. Total substrate accessibility to cellulase (TSAC), cellulose accessibility to cellulase (CAC), and noncellulose accessibility to cellulase (NCAC) were determined based on the maximum adsorption capacity of the TGC protein containing a green fluorescence protein and a cellulose-binding module (Zhu et al. 2009). The recombinant TGC fusion protein was produced in *Escherichia coli* BL21 (pNT02) and purified by affinity adsorption followed by ethylene glycol washing. The scanning electron microscopic (SEM) images of the pure cellulosic and lignocellulosic materials were procured from a Zeiss-DSM940 (Carl Zeiss, Okerkochen, Germany). All samples were sputter-coated with gold prior to imaging.

Results and discussion

Corn stover was pretreated by two cellulose solvent-based pretreatments: COSLIF and IL--[BMIM]Cl. Intact corn stover showed plant fibril structure under SEM (Fig.1B). After two cellulose solvent pretreatments, pretreated corn stover samples did not have any fibril structure (Fig. 1C&D). COSLIF- and IL-pretreated corn stover samples were hydrolyzed by 5 FPU of cellulase and 10 units of β-glucosidase per gram of glucan (Fig. 1A). Corn stover pretreated by COSLIF (CS_{COSLIF}) was hydrolyzed fast and glucan digestibilities were 83% after 24 and 93% after 72 h. In contrast, the hydrolysis rates of corn stover pretreated by IL (CS_{IL}) were slower and only 55% glucan digestibility was reached at after 72 h. Incomplete hydrolysis of pretreated

corn stover might be due to combination of factors, such as residual cellulose solvents and the presence of lignin.

Due to the factors associated with the lignin in corn stover, it is beneficial to first investigate the isolated interaction of pure cellulose, cellulose solvents, and cellulase. Avicel (microcrystalline cellulose), a pure cellulosic material containing crystalline and amorphous cellulose, can be dissolved completely in concentrated phosphoric acids and [BMIM]Cl (Fig. 2B&C). After cellulose dissolution, cellulose solutions appeared transparent, indicating that both cellulose solvents completely dissolved Avicel. Regenerated amorphous cellulose samples by COSLIF and IL (RAC_{COSLIF} and RAC_{IL}) were hydrolyzed by 5 FPU of cellulase and 10 units of β-glucosidase per gram of glucan (Fig. 2A). Initial hydrolysis rates of RAC_{COSLIF} and RAC_{IL} (from 0 - 6 h) were almost the same, but the overall hydrolysis rates of RAC_{IL} were slower than those of RAC_{COSLIF} and had a glucan digestibility of ~ 90% after 72 h. Complete hydrolysis was achieved for RAC_{COSLIF} after 6 h. Higher glucan digestibilities and faster hydrolysis rates were obtained on RAC_{COSLIF} compared to that of RAC_{IL}, in agreement with the previous report (Kuo and Lee 2009a). The slower overall hydrolysis rates of RAC_{IL} might be due to a decreased stability of cellulases in the presence of residual [BMIM]Cl. Moreover, it was noted that cellulose was dissolved in concentrated phosphoric acid at ~ 0 ^{o}C. At this temperature, spontaneous hydrolysis of cellulose was minimized so that RAC_{COSLIF} had the same degree of polymerization as that of Avicel (Zhang and Lynd 2005). At evaluated temperatures, however, cellulose dissolution was accompanied with DP decrease (data not shown).

One major drawback of cellulose solvent-based pretreatment is removal of residual cellulose solvents. It would be impractical to completely wash residual cellulose solvents out, as it requires a large amount of water. Measuring residual cellulose solvents in the pretreated solids is difficult. RAC_{COSLIF} was hydrolyzed completely in a short time, suggesting a slight or no negative effect of residual H_3PO_4 on cellulase activities. With regards to IL-pretreated materials, ~10-15% (v/v) of residual ionic liquid always remains in the reaction media (Engel et al. 2010). Consequently, IL effects on cellulase activities from 1-10 g [BMIM]Cl/L were investigated on pure cellulose of different structures: microcrystalline cellulose (Avicel) and RAC_{COSLIF} (Fig. 3). The presence of [BMIM]Cl concentrations from 1-10 g/L slightly decreased hydrolysis rates of RAC_{COSLIF}, but such a decrease was not statistically significant (Fig. 3A). In contrast, the presence of [BMIM]Cl had significant negative effects on enzymatic Avicel hydrolysis (Fig. 3B). Without [BMIM]Cl, enzymatic hydrolysis of Avicel had glucan digestibility of 68% after 72 h. In the presence of 10 g/L [BMIM]Cl, the glucan digestibility was decreased to 40%. The normalized instantaneous hydrolysis rates of RAC_{COSLIF} and Avicel were shown in Fig. 3C and D, respectively. These decreases of instantaneous hydrolysis rates of RAC and Avicel over time might be due to decreased substrate reactivity, product inhibition, and enzyme deactivation. For RAC_{COSLIF}, different levels of IL did not cause significant decreases in hydrolysis rates (Fig. 3C), while high levels of IL resulted in significant decreases in hydrolysis rates of Avicel (Fig. 3D). Such large differences pertaining to the effect of residual [BMIM]Cl on cellulase activities on Avicel and RAC_{COSLIF} can be explained by two major factors: (i) RAC_{COSLIF} is a homogeneous large surface substrate while Avicel is a heterogeneous low surface substrate (Zhang et al. 2006b); and (ii) different modes of action of cellulase components (endoglucanse and exoglucanase) had different sensitivity to [BMIM]Cl (Engel et al. 2010).

We hypothesized that lignin might play a synergistically negative role in lowered substrate reactivities. Negative effects of residual lignin on enzymatic cellulose hydrolysis may be attributed to (i) cellulase adsorption by lignin (Ooshima et al. 1986; Zhu et al. 2009) and (ii) blockage of lignin on the surface of cellulose so that cellulase cannot access cellulose (Zhang et al. 2007; Kumar and Wyman 2009; Pan 2008). Therefore, we investigated the effects of lignin addition on enzymatic hydrolysis of pretreated cellulosic materials. When isolated lignin was mixed with Avicel, RAC_{COSLIF}, and RAC_{IL} in a similar ratio present in untreated corn stover, the enzymatic hydrolysis profiles were examined (Fig. 4). It should be noted that currently there is no available lignin isolation method that does not modify lignin, so isolated lignin is commonly used to observe its influence on enzymatic hydrolysis (Pan 2008; Nakagame et al. 2010b; Nakagame et al. 2010a). Direct addition of lignin clearly decreased glucan digestibility regardless of cellulosic substrates: Avicel, RAC_{COSLIF} and RAC_{IL} (Fig. 4). Another possibility pertaining to lignin's negative effects may be due to changes of lignin property after the pretreatment. But this possibility was eliminated by the control experiments of added COSLIF-pretreated lignin ($lignin_{COSLIF}$) and IL-pretreated lignin ($lignin_{IL}$). The lignin pretreated by cellulose solvents had slightly weaker negative impacts on enzymatic digestibility than those cases of direct lignin addition (Fig. 4).

It was hypothesized that dissolved lignin during pretreatment can redeposit and redistribute on the surface of cellulose, which can block cellulose accessibility to cellulase (Selig et al. 2007; Donohoe et al. 2008; Singh et al. 2009). To test this hypothesis, we mixed isolated lignin and Avicel, followed by cellulose solvent pretreatments, obtaining two substrates of (Avicel +

lignin)$_{COSLIF}$ and (Avicel + lignin)$_{IL}$. Such pretreated cellulosic materials in the presence and absence of lignin were hydrolyzed by cellulases (Fig. 5). There were no significant differences in hydrolysis rates and glucan digestibilities between (Avicel + lignin)$_{COSLIF}$ and (Avicel + lignin)$_{IL}$ as compared to respective RAC_{COSLIF} + $lignin_{COSLIF}$ and RAC_{IL} + $lignin_{IL}$, respectively. These results suggested that there was no significant effect due to lignin redistribution. One possible reason was both COSLIF and IL pretreatments enhanced cellulose accessibility to cellulase, which played a greater role in enzymatic hydrolysis performance than any specific change in the adsorption of the lignin to the enzymes (Nakagame et al. 2010a; Nakagame et al. 2010b).

Both cellulose solvents can dissolve cellulose and biomass well (Fig. 2) by disrupting highly ordered hydrogen bonds in cellulose fibers. Consequently, regenerated cellulosic materials had very low crystallinities (Kuo and Lee 2009a). Intact lignocellulosic biomass had a low cellulose accessibility to cellulase (CAC) of 0.42 ± 0.01 m^2/g cellulose, based on the adsorption of a fusion protein, thioredoxin-GFP-cellulose binding module (TGC). After pretreatments, CS_{COSLIF} had the highest CAC value of 11.6 ± 0.8 m^2/g biomass, as compared to those pretreated by IL and dilute acid pretreatment (DA) (Table 1). At the same enzyme loading of 5 FPU/g glucan, enzymatic glucan digestibilities in a decreasing order were 96% (COSLIF), 60% (DA) (Zhu et al. 2009), and 55% (IL). This decreasing digestibility trend complied with the CAC values (Table 1), suggesting that CAC values were highly likely related to enzymatic glucan digestibilities.

The hydrolysis rates and final digestibilities for pretreated cellulose and lignocellulosic biomass by using cellulose solvents varied greatly in the literature, depending on selection of cellulose

solvent, type of biomass, and pretreatment conditions. The strategic illustration of utilization of cellulose solvents for pretreatment and saccharification is shown in Figure 6. Solid black lines represent the preferred biomass saccharification pathway–biomass pretreatment followed by enzymatic hydrolysis. Zhang et al. (Zhang et al. 2007) suggested that COSLIF operating at higher temperatures (> 50 ^{o}C) resulted in a loss in solid weight of regenerated cellulose due to strong acid hydrolysis and/or thermal degradation of sugars. Fu et al. (Fu et al. 2010) found that when the cellulose dissolution temperature in IL increased from 90 ^{o}C to 150 ^{o}C, the solid weight of regenerated cellulose decreased greatly. This finding suggested that a significant fraction of cellulose was hydrolyzed at the elevated temperatures during washing step with anti-solvents. Although biomass pretreated by IL had much better enzymatic digestibility at high temperatures, net glucose yields from enzymatic hydrolysis at high temperature were lower than those at low temperatures (as shown in the present study). Consequently, development of ionic liquids that can dissolve cellulose at low temperatures (< 100 ^{o}C) would be of technical and economic importance. The elevated temperature operation would present great technical and economic challenges in separation of a large amount of soluble sugars from cellulose solvents and efficient recycle of cellulose solvents (Mora Pale et al. 2011; Zhang 2008). Since it was vital to retain most cellulose as a solid substrate for the following enzymatic or acid hydrolysis, it was not recommended to dissolve biomass in cellulose solvents at high temperatures. This was to avoid auto-hydrolysis, high energy input from high temperature biomass pretreatment, high overall loss of sugars via thermal degradation, and high cost associated with separation of soluble sugars and cellulose solvents, making biorefinering processes more expensive.

The use of cellulose solvents, specifically concentrated phosphoric acid and ionic liquids, for pretreatment of lignocellulose in the future industrial processes in biorefineries holds much promise, including environmental friendly, high yield of sugars over short saccharification times, and possible integrated processes for production of value-added products (Li et al. 2010; Zhang 2008; Volynets and Dahman 2011). However, the large amount of cellulose solvents usage and recycle of the cellulose solvents are major technical and economic challenges for the rapid commercialization in biorefineries (Chundawat et al. 2011). In the case of concentrated phosphoric acid, phosphoric acid is highly water soluble, and it can be easily washed from pretreated materials. Moreover, residual phosphoric acid from pretreatment and enzymatic hydrolysis in the solid material may be used as a medium nutrient for the following microbial fermentation. Furthermore, it has been proposed that the fermentation broth containing unrecovered phosphate after fermentation and distillation may be used as a fertilizer for nearby dedicated bioenergy crop plantations. Ionic liquids with hydrophilic anions–being non volatile, non-flammable, recyclable and designer friendly–have been increasingly recognized as green cellulose solvents for dissolution and pretreatment of cellulose and lignocelulose. The choice of ILs in biological saccharification of lignocellulose imposes a trade-off between biomass dissolution and enzymatic activity (Murugesan and Linhardt 2005). Some authors have reported decreased enzyme activity in the presence of ILs (Docherty and Kulpa 2005; Turner et al. 2003; Turner et al. 2004). Datta et al. (Datta et al. 2010) reported that a commercial endoglucanase from *Tricoderma viridie* lost its activity in the presence of small concentration of EMIM[OAc]. Complete removal of ILs is not pragmatic, as it requires a large amount of water and complex recycling systems (Engel et al. 2010). Consequently, Datta et al suggested leaving residual ILs

in the reaction media and developed IL-tolerant hydrophillic cellulases. Other authors employ acid-catalyzed hydrolysis of IL-pretreated cellulose (Kim et al. 2010; Dee and Bell 2011).

Currently there are no perfect cellulose solvents for pretreatment; however, cellulose solvent-based lignocellulose pretreatments offer many strategic advantages to the development of integrated biorefineries–from high sugar yields in short saccharification times to fractionation of biomass components for possible integrated co-products utilization processes. Based on previously published results and our present study, the ideal cellulose solvent for pretreatment in biorefineries should have numerous features: (i) dissolving cellulose at low temperatures, (ii) dissolving wet cellulose (i.e., no biomass drying step required), (iii) less costly or high recyclable, (iv) less volatile for easy recycling, (v) thermostable and chemostable, (vi) nontoxic to the subsequent enzymatic hydrolysis and microbial fermentation, (vii) high cellulose dissolution capacity (> 10% wt. cellulose/vol), and (viii) fast diffusion rate in solid lignocellulose composite (Zhang 2008; Engel et al. 2010; Klein-Marcuschamer et al. 2011; Wu et al. 2011).

Conclusions

Corn stover samples pretreated by COSLIF and IL exhibited glucan digestibilities of 96% and 55% after 72 h at five FPU of cellulase per gram of glucan, respectively. Our results suggested that an incomplete hydrolysis was attributed to combinatorial factors: (i) a tradeoff between disrupting crystalline cellulose in biomass and maintaining reactive cellulose in the solid phase; (ii) residual cellulose solvent in the pretreated biomass decreased cellulase activity; and (iii) residual lignin on the cellulosic materials after pretreatments decreased cellulase hydrolysis performance. Although cellulose solvent-based biomass pretreatments are receiving more

interest, many obstacles require attentions, such as validating cellulose solvent recycling technologies on large scales, decreasing cellulose solvent volume use, and conducting detailed techno-economic analysis.

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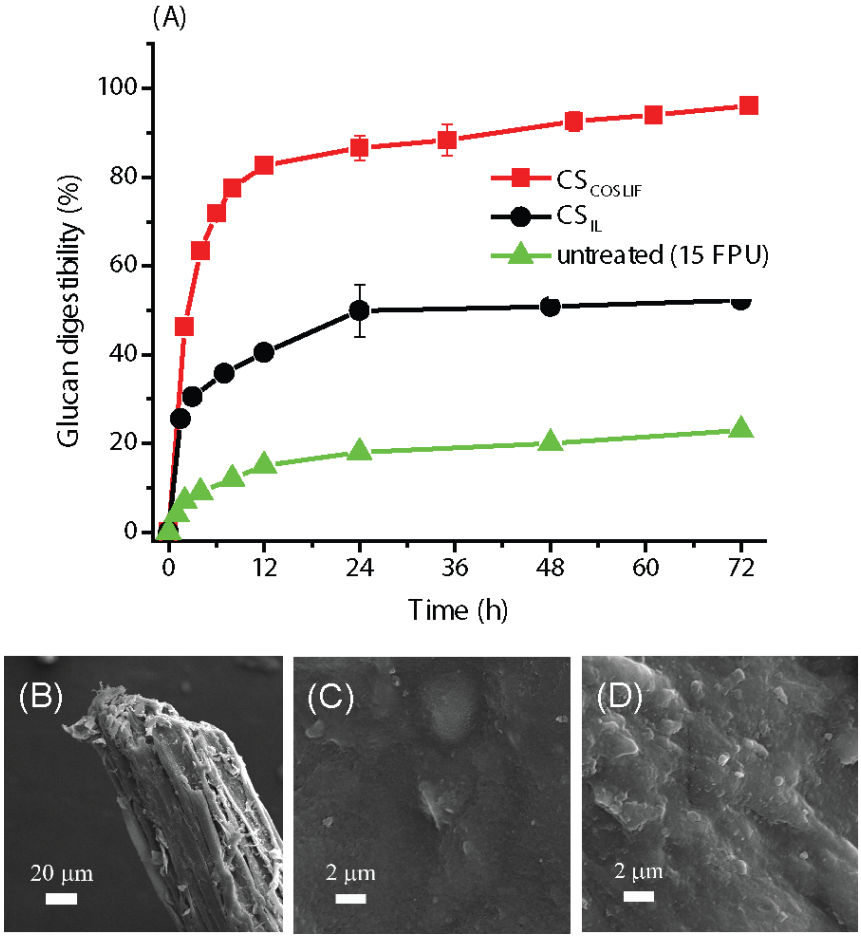

Figure 1. Enzymatic hydrolysis profiles of intact corn stover and corn stover samples pretreated by COSLIF and IL at an enzyme loading of 5 FPU of cellulase per gram of glucan (A); SEM micrographs of intact corn stover (B), after COSLIF (C), and after IL (D).

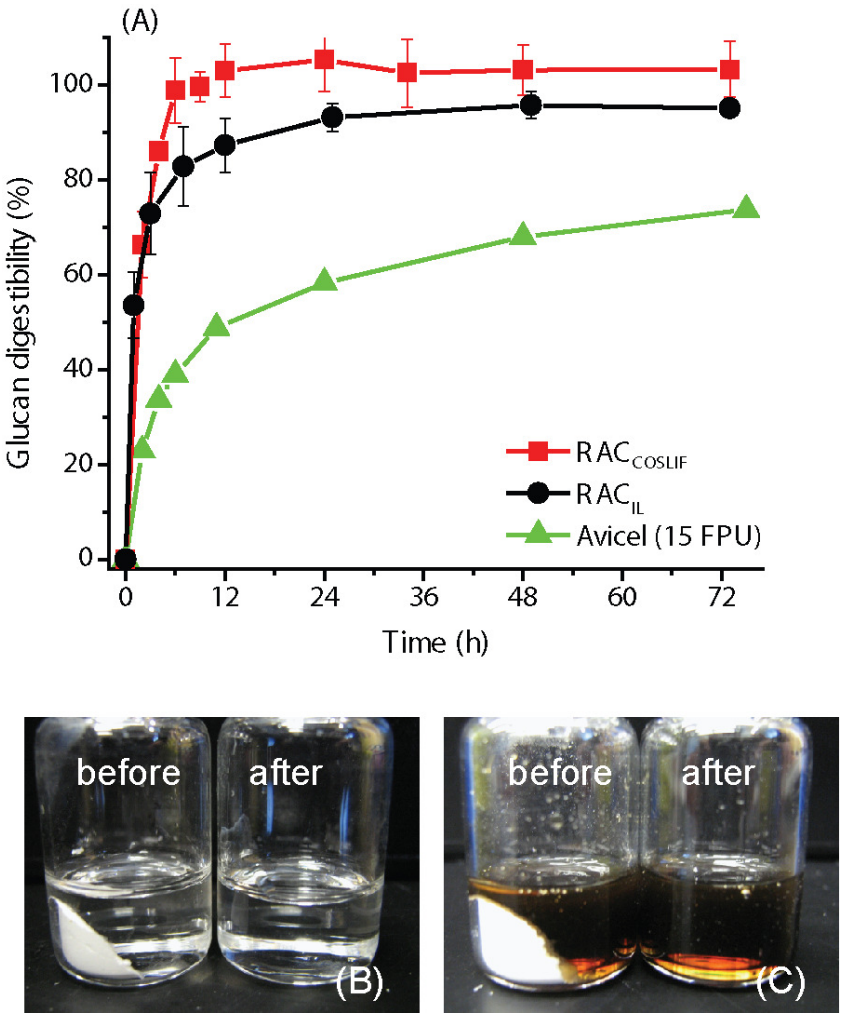

Figure 2. Enzymatic cellulose hydrolysis profiles of Avicel, RAC_{COSLIF}, and RAC_{IL} (A); illustrations of Avicel dissolution in 85% (w/w) H_3PO_4 (B) and [BMIM]Cl (C).

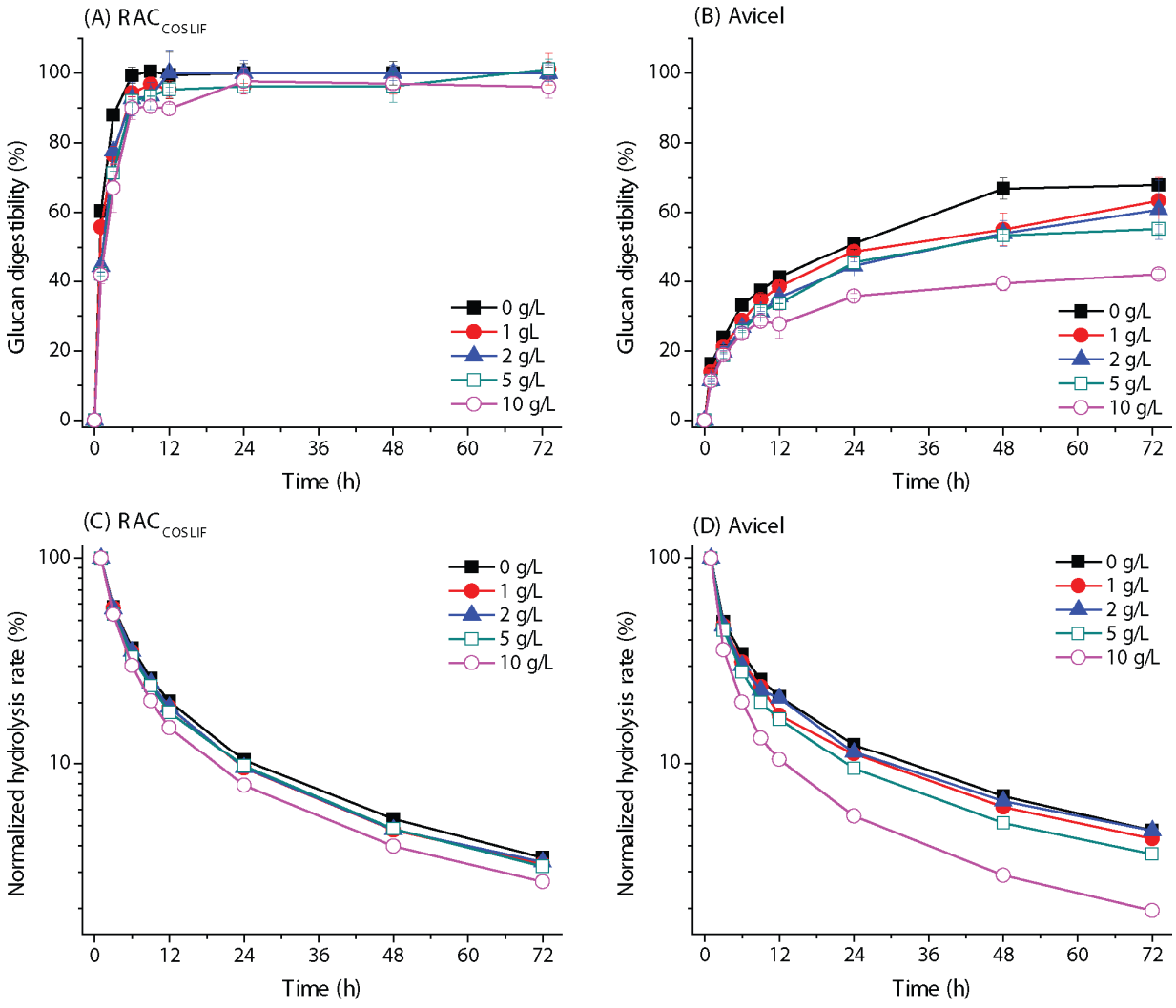

Figure 3. Enzymatic hydrolysis profiles of RAC_{COSLIF} (A) and Avicel (B) in the presence of increasing [BMIM]Cl as well as their instantaneous hydrolysis rates on RAC_{COSLIF} (C) and Avicel (D).

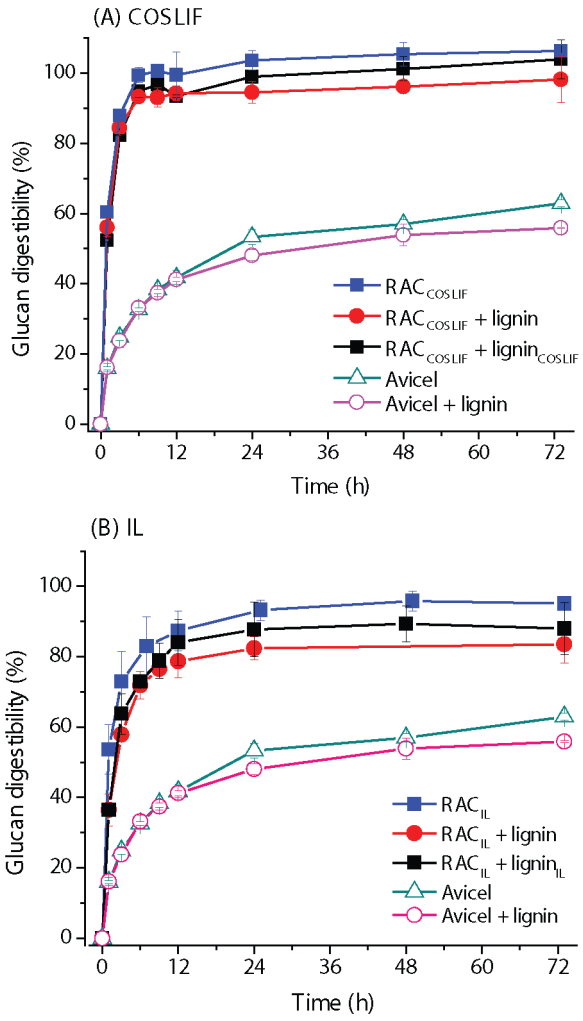

Figure 4. Effects of isolated lignin and pretreated isolated lignins on enzymatic hydrolysis of RAC_{COSLIF} (A) and RAC_{IL} (B).

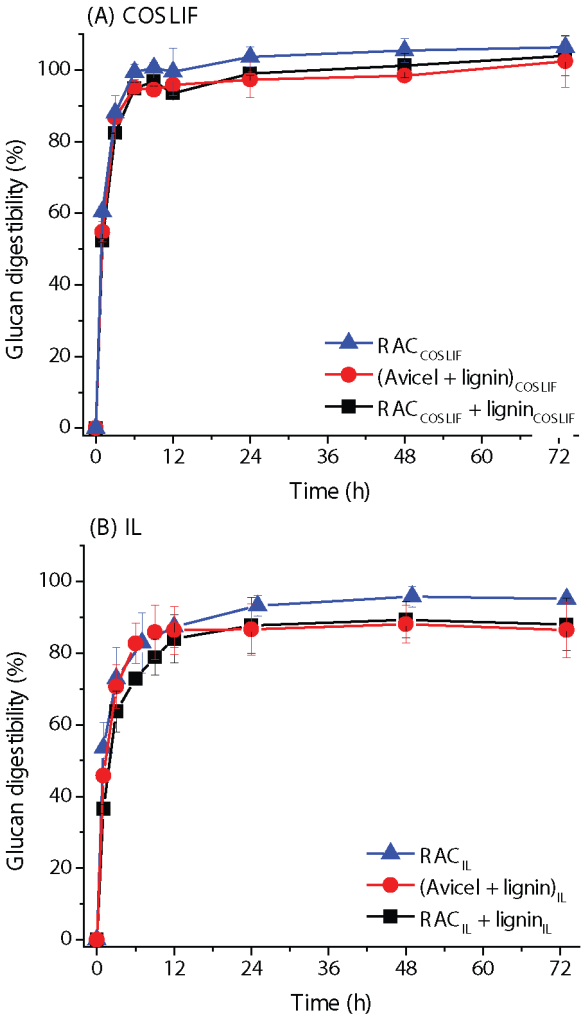

Figure 5. Effects of lignin redistribution on the surface of RAC pretreated by COSLIF (A) and IL (B).

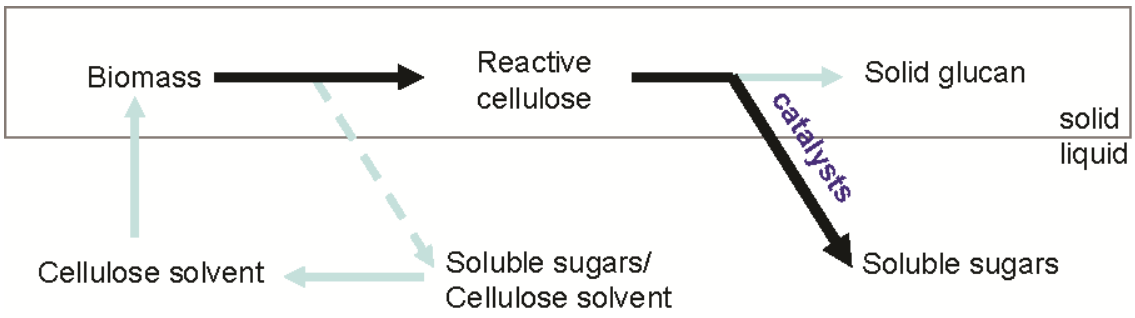

Figure 6. Biomass saccharification strategies based on cellulose solvent followed by hydrolysis by cellulase involving the separation and recycling of cellulose solvent. The solid black line represents the preferred way of biomass saccharification so to avoid excessive separation of soluble sugars/cellulose solvent.

Table 1. Total surface accessibility to celulase (TSAC), cellulose accessibility to cellulase (CAC) non-cellulose accessibility of cellulase (NCAC), and glucan digestibility after 72 h under 5 FPU of cellulase and 10 units of β-glucosidase per gram of glucan of corn stover (CS), and CS pretreated by COSLIF, IL, and dilute acid (DA).

Materials	TSAC m^2/g biomass	CAC m^2/g biomass	NCAC m^2/g biomass	Glucan digestibility (%)	References
Corn stover (CS)	1.13 ± 0.01	0.42 ± 0.01	0.71 ± 0.01	23%	This study
CS_{COSLIF}	14.4 ± 1.1	11.6 ± 0.8	2.9 ± 0.2	96%	This study
CS_{IL}	5.8 ±0.3	5.0 ± 0.2	0.73 ± 0.09	55%	This study
CS_{DA}	7.7 ± 0.6	5.9 ± 0.3	1.8 ± 0.6	60%	(Zhu et al. 2009)

Chapter 5

Cellulose solvent- and organic solvent-based lignocellulose fractionation (COSLIF) enables to pretreat various feedstocks: Miscanthus, poplar, Miscanthus-poplar mixtures, bagasse, wheat straw, and rice straw

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Abstract

Concerns pertaining to depleting crude oil, accumulating greenhouse gases, and national energy security are motivating to seek for the sustainable production of alternative biofuels from non-food lignocellulosic biomass. To efficiently release soluble sugars from biomass, cellulose solvent- and organic solvent-based lignocellulose fractionation (COSLIF) followed by enzymatic hydrolysis was applied to various feedstocks, including Miscanthus, poplar, their mixtures at different ratios, rice straw, wheat straw, and bagasse. COSLIF increased cellulose accessibility to cellulase by disrupting highly order hydrogen bonding networks of cellulose fibers in biomass. Pretreated Miscanthus, poplar, and their mixtures regardless of their ratios showed similar hydrolysis profiles, and their glucan digestibilities were ~93% at 5 filter paper units per gram of glucan after 72 h. The overall glucose and xylose yields were 93% and 85%, respectively. Fast hydrolysis rate and high glucan digestibility were attributed to the ~125-fold increase in cellulose accessibility to cellulase from 0.12 to 15.0 m^2/g of biomass mixture (Miscanthus: poplar =1:2). These results suggested that COSLIF could be regarded as a feedstock-independent pretreatment suitable for processing diverse feedstocks.

Keywords: biofuels, biomass pretreatment, cellulose accessibility to cellulase, cellulose solvent and organic solvent-based lignocellulose fractionation (COSLIF), enzymatic cellulose hydrolysis

Introduction

The sustainable production of alternative biofuels from non-food lignocellulosic biomass would bring benefits to the environment, economy and nation security. Non-food lignocellulosic biomass is the most abundant bioresource and is less costly compared to crude oil based on energy content. The largest obstacle to cellulosic biorefineries is cost-effective release of fermentable sugars from lignocellulosic biomass (Lynd et al. 2008). Lignocellulosic biomass is composed of three major components: cellulose, hemicellulose, and lignin. Their interwoven linkage results in a recalcitrant natural composite (Himmel et al. 2007). Biological conversion of lignocellulose usually involves two sequential steps: (1) pretreatment, which increases substrate reactivity for hydrolytic enzymes, and (2) enzymatic hydrolysis, which releases soluble sugars by using hydrolytic enzymes.

A billion ton study, jointly conducted by the US department of Agriculture (USDA) and the US department of Energy (DOE), has estimated that more than one billion tons of plant biomass will be available to meet the Advance Energy Initiative (AEI) renewable targets (DOE and USDA 2011). This amount of biomass could be obtained without interfering food production. The forest and agricultural sectors were projected to produce 368 and 998 million of dry tons, respectively (Heaton et al. 2008). Among the various sources of biomass, bioenergy crops, such as *Miscanthus x giganteus* (known as Miscanthus) and *Populus nigra x Populus maximowiczii* (hybrid poplar) are of special interest due to their high productivities. Miscanthus is a perennial C_4 grass, featuring a long production lifetime (e.g., 10-15 years) (Wang et al. 2010). Extensive trials in Europe projected an average productivity of Miscanthus over 30 dry metric tons per hectare per year with minimal agricultural inputs, much higher than an average yield of 10-15

tons per hectare per year of switchgrass (Heaton et al. 2004; Khanna et al. 2008; Miguez et al. 2009; Somerville et al. 2010). Therefore, Miscanthus is regarded as a promising bioenergy grass crop. Poplar and their hybrids are fast-growing and short-rotation woody crops and can be grown in marginal lands with a mean above-ground biomass productivity of ~14 dry metric tons per hectare per year (Sannigrahi et al. 2010). Since hybrid poplar has a wide spatial distribution in North America and Canada, it can be grown close to the processing facilities. Moreover, woody biomass has several advantages compared with most agricultural residues and bioenergy grass crops, such as higher mass density, rendering lower transportation cost, and higher polysaccharide content (i.e. 40-50% glucan and 20-30% xylan) (Balan et al. 2009). With consideration of diverse feedstocks in different regions of the United States and the heterogeneity of the same feedstock due to different growth conditions, harvesting seasons and storage conditions, developing feedstock-independent pretreatment without significant changes in pretreatment conditions is of importance for large-scale implementation of second generation biorefineries. Also, this operation decreases feedstock logistical hurdles and maintains biodiversity.

Many pretreatment technologies, such as dilute acid, steam explosion, ammonia fiber explosion (AFEX), aqueous ammonia recycle percolation (ARP), and lime, have shown to be effective to pretreat herbaceous biomass (i.e., corn stover and switchgrass) (Mosier et al. 2005; Rollin et al. 2011; Wyman et al. 2005; Wyman et al. 2009). However, many of these pretreatments are ineffective for woody biomass. For example, enzymatic glucan disgestibilities of dilute acid-, AFEX-, ARP-pretreated poplar were 47%, 39%, and 36%, respectively, at 15 filter paper units (FPU) of cellulase per gram of glucan (Balan et al. 2009; Wyman et al. 2009). These low

enzymatic conversions were due to a more recalcitrance of woody poplar compared to herbaceous biomass. Cellulose solvent- and organic solvent-based lignocellulose fractionation (COSLIF) has been developed to fractionate lignocellulose by using a combination of a concentrated phosphoric acid as a cellulose solvent and an organic solvent (e.g., acetone or ethanol) under modest reaction conditions (Rollin et al. 2011). Previous work has shown that COSLIF effectively work on different feedstocks, such as corn stove, switchgrass, hemp hurd, bamboo, and common reed and the cellulose solvent can effectively disrupt highly ordered hydrogen bonding network of crystalline cellulose, increasing cellulose accessibility to hydrolytic enzymes (Sathitsuksanoh et al. 2011). Consequently, COSLIF-pretreated biomass can be hydrolyzed rapidly by cellulase, and very high glucan digestibilities were obtained even at low cellulase loadings (Sathitsuksanoh et al. 2010).

Here, we examined efficiency of COSLIF on two potential bioenergy crops – Miscanthus, hybrid poplar and their mixtures at various mass ratios. The biomass mixtures were pretreated by COSLIF, followed by enzymatic hydrolysis. In addition, we studied pretreatment feasibility of COSLIF on bagasse, wheat straw, and rice straw.

Materials and Methods

Chemicals and materials. All chemicals were reagent grade and purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise noted. Phosphoric acid (85% w/w) and ethanol (95% v/v) were purchased from Fisher Scientific (Houston, TX). The *Trichoderma reesei* cellulase (Novozyme® 50013) and β-glucosidase (Novozyme® 50010) were gifted by Novozymes North America (Franklinton, NC). They had activities of 84 filter paper units (FPU) of cellulase per

mL and 270 units of β-glucosidase per mL, measured as described elsewhere. Hybrid poplar and Miscanthus samples were procured from the National Renewable Energy Laboratory (Boulder, CO) and University of Illinois (Urbana, IL), respectively. The naturally-dried biomass was milled into small particles by a Pallmann counter-rotating knife ring flaker (Clifton, NJ). The resulting particulates with nominal sizes of 40-60 mesh (250-400 μm) were used for pretreatment experiments.

COSLIF procedure The COSLIF-pretreated biomass was prepared as described previously (Sathitsuksanoh et al. 2009). In short, one gram of biomass was mixed with 8 mL of 85% (w/w) H_3PO_4 at 50°C, 1 atm for 60 min. The reaction between biomass and concentrated phosphoric acid was ceased by adding 20 mL of 95% (v/v) ethanol and then was mixed well. Solid-liquid separation was conducted in a swing bucket centrifuge at 4,500 rpm at room temperature for 10 min. After the supernatant was discarded, the pellets were suspended in 40 mL of 95% (v/v) ethanol. After centrifugation, the solid pellets were washed by 80 mL of deionized water. After centrifugation, the remaining solid pellet was neutralized by 2 M sodium carbonate.

Carbohydrate and lignin assays. The structural carbohydrate composition of intact poplar and Miscanthus was determined with a modified quantitative saccharification (QS) procedure (Moxley and Zhang 2007). Monomeric sugars were measured by a Shimadzu HPLC with a Bio-Rad Aminex HPX-87H column (Richmond, CA) equipped with refractive index (RI) detector. The concentrations of glucose and xylose were measured in enzymatic hydrolysate, whereby galactose and mannose were co-eluted with xylose. The column was operated with 5 mM H_2SO_4 as a mobile phase at 60°C and a flow rate of 0.6 mL/min. Lignin and ash were measured according to the standard NREL biomass protocol (Sluiter et al. 2006).

Enzymatic hydrolysis. The COSLIF-pretreated samples were diluted to 10 g glucan per liter in a 50 mM sodium citrate buffer (pH 4.8) supplemented with 0.1% (w/v) NaN_3. COSLIF-pretreated samples were completely suspended in the reaction media to ensure that the cellulose particle surface was accessible to the cellulases, thereby optimizing cellulase adsorption and activity. All hydrolysis experiments were carried out in a rotary shaker at 250 rpm at 50 °C. The enzyme loadings were 5 FPU per gram of glucan and 10 units of β-glucosidase per gram of glucan, otherwise noted.

Other assays. The total substrate accessibility to cellulase (TSAC), cellulose accessibility to cellulase (CAC), and noncellulose accessibility to cellulase (NCAC) were determined based on the maximum adsorption capacity of the TGC protein containing a green fluorescence protein and a cellulose-binding module (Rollin et al. 2011; Zhu et al. 2009).

Results and discussion

The carbohydrate and lignin compositions of Miscanthus and poplar samples are shown in Table 1. Two feedstocks have comparative overall carbohydrate contents but differ in sugar compositions (Table 1). Poplar contained a higher lignin content of 28 wt.% compared to 23 wt.% of Miscanthus. Miscanthus and poplar samples were mixed at different ratios, i.e., 1:0, 1:2, 2:1, and 0:1. Non-pretreated biomass samples regardless of their ratios showed similar hydrolysis profile with a glucan digestibility of 8% after 72 h. COSLIF-pretreated biomass mixture samples were hydrolyzed at the enzyme loading of 5 FPU of cellulase and 10 units of β-glucosidase per gram of glucan (Fig. 1). All four COSLIF-pretreated biomass mixtures had similar hydrolysis profiles. The pretreated biomass mixtures were hydrolyzed fast, and 50% of substrate was hydrolyzed after 3 h. The glucan digestibilities were ~90% after 24 and ~93%

after 72 h. These results suggested efficient enzymatic hydrolysis of COSLIF-pretreated bioenergy crops regardless of their ratios at a low enzyme loading. Different lignin contents in Mascanthus and poplar did not cause significantly different digestibilities of both pretreated feedstocks, partially suggesting that decreasing lignin content in feedstock may be not important when cellulose accessibility to cellulase of pretreated biomass was enhanced to very high levels by using the cellulose solvent (Rollin et al. 2011).

Mass balance on the basis of 100 g dry biomass (Miscanthus: poplar = 1:2) was shown to include COSLIF pretreatment followed by enzymatic hydrolysis under 5 FPU of cellulase per gram of glucan (Fig. 2). After COSLIF, 6.0 g of soluble glucose equivalent and 10.7 g of soluble xylose equivalent were removed. The reactive cellulose material was hydrolyzed by cellulase, releasing 36.4 g of soluble glucose and 2.5 g of soluble xylose equivalent. The overall glucose and xylose yields were 92.8% and 84.7%, respectively.

The high glucan digestibility of COSLIF-pretreated biomass was attributed to drastic changes in macromolecular structure of biomass by using COSLIF. Via COSLIF, highly ordered hydrogen bonding network of crystalline cellulose was disrupted, resulting in a drastic increase in substrate accessibility to cellulase (Sathitsuksanoh et al. 2011). Total substrate accessibility to cellulase (TSAC) increased from 0.21 (i.e., 0.18*1/3 + 0.23 *2/3) to 16.8 m^2 per gram of Miscanthus: poplar = 1:2 (Table 2). The CAC values of intact Miscanthus and poplar were 0.09 and 0.14 m^2 per gram of biomass, respectively. After COSLIF, the CAC value of pretreated biomass (Miscanthus: poplar = 1:2) was 14.99 m^2 per gram of biomass. This result suggested that COSLIF can enhance cellulose accessibility to cellulase by ~125-fold, resulting in highly

reactive cellulosic materials suitable for enzymatic cellulose hydrolysis even at a low enzyme loading.

COSLIF was also tested to pretreat three other feedstocks, i.e., bagasse, wheat straw, and rice straw. These intact foodstocks exhibited different enzymatic hydrolysis profiles, indicating their different degrees of recalcitrance. Intact rice straw and wheat straw yielded low glucan digestibilities (< 10%) after 72 h at 15 FPU of cellulase per gram of glucan, whereas intact bagasse yielded a very high glucan digestibility of ~47% (Fig. 3C). The tested bagasse was pre-processed to extract soluble sugars by mechanical forces; this operation might cause the biomass structure to be disrupted more than simple milling. Regardless of large differences in their initial glucan digestibilities before pretreatment, all COSLIF-pretreated biomass samples showed similar hydrolysis profiles and comparatively high glucan digestibilities (> 80-90%) after 24 h (Fig. 3), suggesting that COSLIF has an ability of converting different recalcitrant degrees of biomass feedstocks to the same reactivity solids suitable for enzymatic hydrolysis.

In summary, three different categories of feedstocks: agricultural wastes, bioenergy crops, and woody biomass were pretreated by COSLIF (Fig. 4). The standard COSLIF pretreatment conditions were 50 ^{o}C and atmospheric pressure with different pretreatment times from 30 to 105 min – depending on the type of feedstocks. Generally speaking, concentrated phosphoric acid at 50^{o}C can efficiently dissolve biomass so to open its recalcitrant structure while avoiding harsh hydrolysis of polymeric carbohydrates. Under these conditions, concentrated phosphoric acid works as a cellulose solvent rather than a strong acid (e.g., H_2SO_4 or HCl) can hydrolyze cellulose and hemicellulose to monomeric sugars. Intact biomass of different species showed a

large variation of their glucan digestibilities under 15 FPU of cellulase per gram of glucan. Agricultural wastes showed different recalcitrance to enzymatic hydrolysis in a decreasing order: bagasse (47%) > corn stover (23%) > hurd of industrial hemp (14%) > wheat straw (11%) > rice straw (10%). After COSLIF, pretreated agricultural waste samples had high glucan digestibilities (i.e., 86-94%) at a 3-fold reduction in enzyme loading (i.e., 5 FPU of cellulase per gram of glucan). As compared to agricultural wastes, intact bioenergy crops had lower enzymatic glucan digestibilities in a descending order of common reed (19%) > switchgrass (17%) > Miscanthus (8%) > bamboo (3%). However, all COSLIF-pretreated bioenergy crops had similarly high glucan digestibilities at 5 FPU of cellulase per gram of glucan. In general, woody biomass materials are densely packed and contain higher lignin contents compared to agricultural wastes and bioenergy crops, while COSLIF-pretreated hybrid poplar yielded a high glucan digestibility of 93.7% at 5 FPU of cellulase per gram of glucan. Clearly, when a cellulose solvent enables to dissolve cellulose fibers so to effectively disrupt highly ordered hydrogen bonding networks within crystalline cellulose, regenerated cellulosic materials regardless of feedstock types with different degrees of recalcitrance exhibited similar substrate reactivities to cellulase. Such high-reactivity pretreated biomass samples feature fast enzymatic hydrolysis rate and high glucan digestibility.

The results in this study indicated that COSLIF was a “nearly” feedstock-independent pretreatment technology. COSLIF effectively works on a wide range of feedstock and the pretreated biomass feedstocks yield high glucan digestibilities, which were attributed to very high substrate accessibility to cellulase.

Conclusions

COSLIF-pretreated Miscanthus, poplar, and their mixtures had fast hydrolysis rates and high glucan digestibilities of 93% at 5 FPU of cellulase per g glucan after 72 h. The overall glucose and xylose yields were 93% and 85%, respectively. CAC values of pretreated biomass mixtures suggested that COSLIF increased substrate accessibility by ~125-fold. COSLIF was shown to be "nearly" generic pretreatment, effectively dealing with herbaceous biomass, bioenergy crop, and woody biomass with high glucan digestibilities (> 80-90%) at low cellulase loading (5 FPU per gram glucan). These results show that COSLIF could be a viable process to deal with diverse feedstock so that it might be possible to build biorefineries even in some areas without a constraint of a sole biomass source and an impairment of natural biodiversity.

Acknowledgments. This work was supported partially by the DOE BioEnergy Science Center (BESC) and USDA Bioprocessing and Biodesign Center. NS was partially supported by the ICTAS scholar program. The authors would like to thank Dr. Frank Dohleman and Dr. Steve Long from the University of Illinois for providing the Miscanthus sample utilized in this study.

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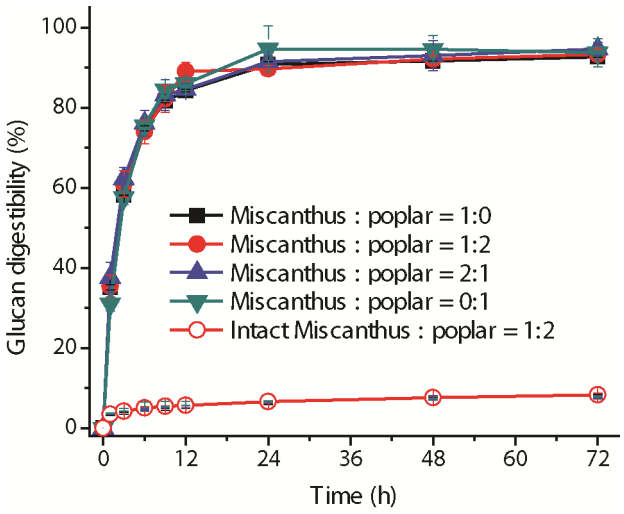

Figure 1. Enzymatic hydrolysis profiles of COSLIF-pretreated biomass mixtures at the enzyme loading of 5 FPU of cellulase and 10 units of β-glucosidase per gram of glucan at 50°C. Enzymatic hydrolysis of intact biomass mixtures at 15 FPU of cellulase per gram of glucan exhibited similar hydrolysis profiles. For simplification, the hydrolysis profile of an untreated Miscanthus: poplar ratio of 1:2 was shown only.

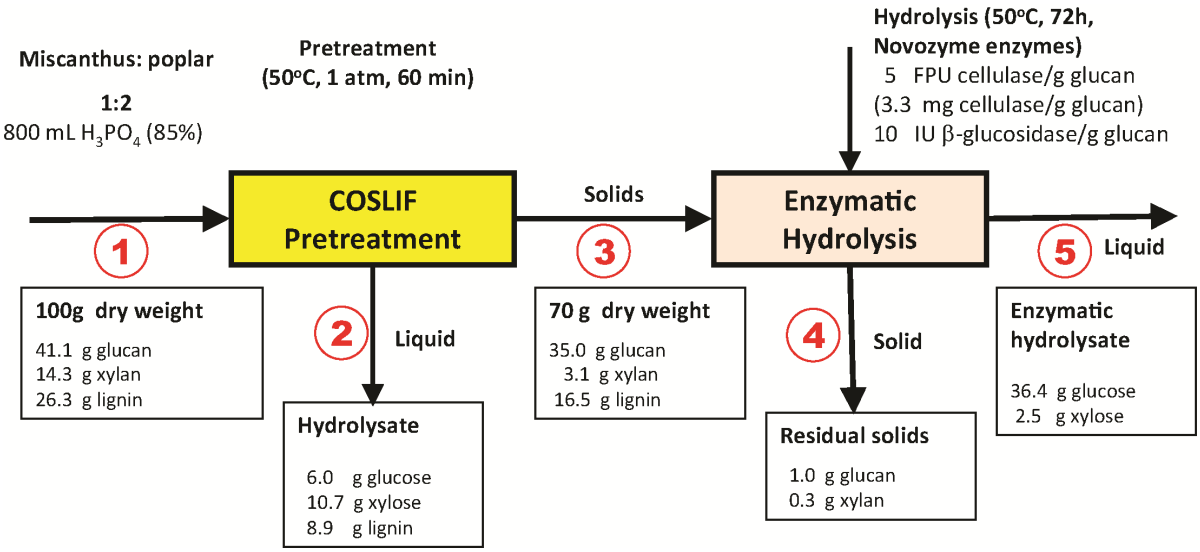

Figure 2. Mass balance of biomass mixture (Miscanthus: poplar = 1:2) pretreated by COSLIF followed by enzymatic hydrolysis by 5 FPU of cellulase per gram of glucan

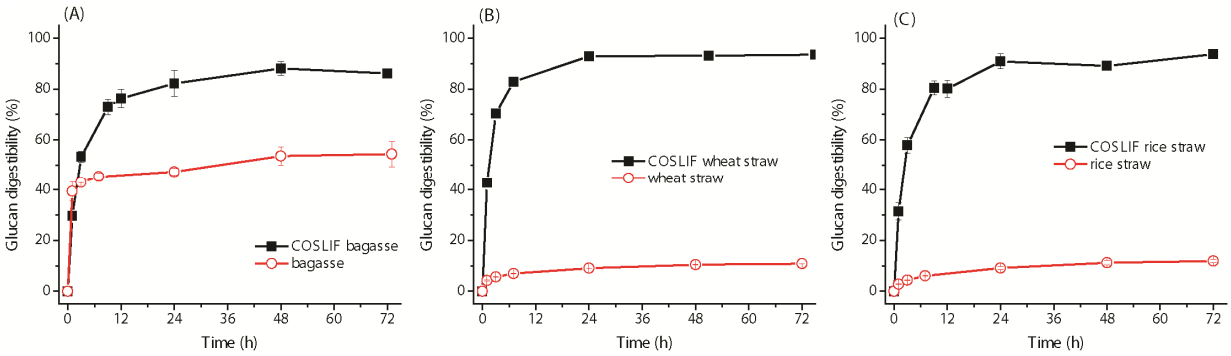

Figure 3. Enzymatic hydrolysis profiles of COSLIF-pretreated bagasse (A), wheat straw (B), and rice straw (C). COSLIF pretreatment conditions were 50 °C, atmospheric pressure, and pretreatment temperature of 45-min for wheat straw as well as 30-min for bagasse and rice straw.

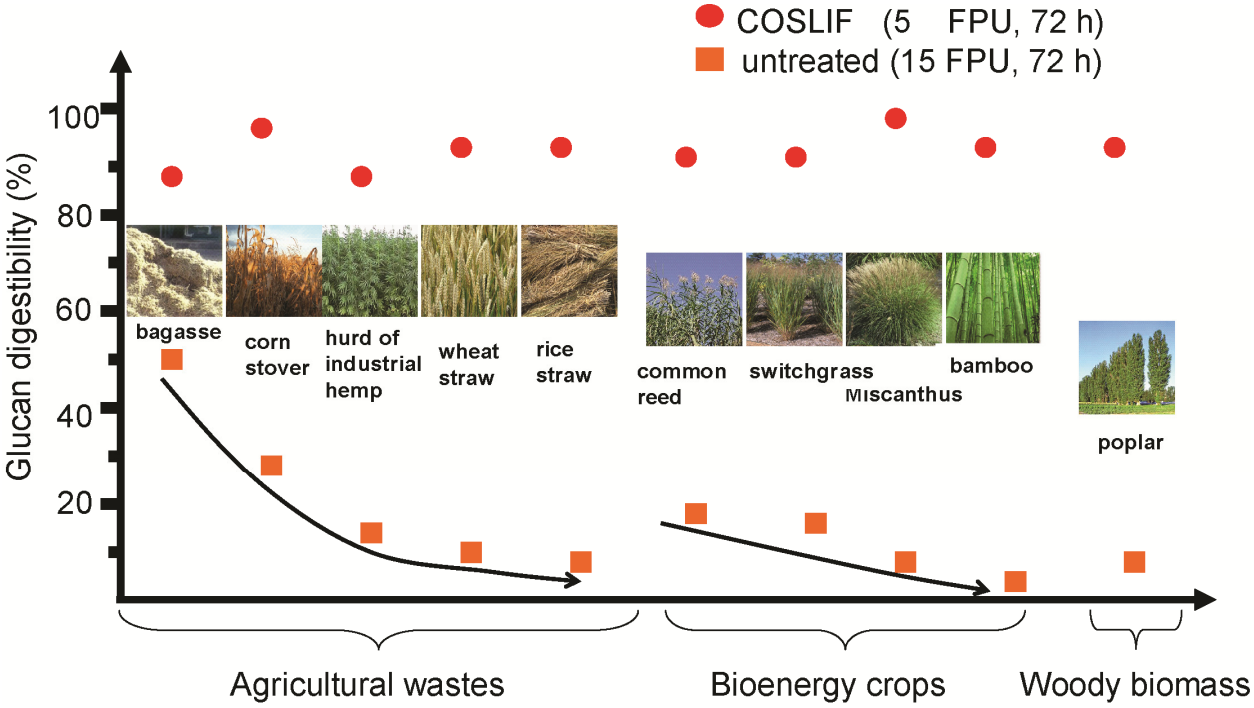

Figure 4. COSLIF appears to be a feedstock-independent technology. All biomass feedstocks were pretreated by COSLIF at 50 °C and atmospheric pressure with different reaction times of 30 min (bagasse, corn stover, and rice straw), 45 min (wheat straw, switchgrass, and hurd of industrial hemp), 60 min (common reed, Miscanthus, bamboo, and poplar).

Table 1. Compositional analysis of Miscanthus and hybrid poplar

Compositions (wt.%)	Miscanthus	Hybrid poplar
Glucan	41.00 ± 0.09	40.13 ± 0.43
Xylan	18.42 ± 0.05	11.95 ± 0.13
Galactan	ND	1.26 ± 0.01
Arabinan	2.08 ± 0.01	1.12 ± 0.20
Mannan	ND	3.33 ± 0.01
Lignin	23.10 ± 0.20	28.1 ± 2.11

ND indicates not detected

Table 2. Total surface accessibility to celulase (TSAC), cellulose accessibility to cellulase (CAC), and glucan digestibility after 72 h under 5 FPU of cellulase and 10 units of β-glucosidase per gram of glucan.

Substrate	TSAC	CAC	Glucan digestibility
	m^2/g biomass	m^2/g biomass	(%)
Intact Miscanthus	0.18 ± 0.01	0.087 ± 0.001	7.9 ± 0.6
Intact poplar	0.23 ± 0.01	0.14 ± 0.01	7.8 ± 0.0
Miscanthus : poplar = 1:0	20.7 ± 1.2	18.9 ± 1.7	92.6 ± 0.0
Miscanthus : poplar = 1:2	16.8 ± 2.2	15.0 ± 1.2	93.3 ± 1.3
Miscanthus : poplar = 2:1	17.1 ± 1.3	15.7 ± 1.1	92.6 ± 1.7
Miscanthus : poplar = 0:1	18.2 ± 1.1	17.4 ± 0.9	93.7 ± 3.4

Table 3. Compositional analysis of bagasse, wheat straw, and rice straw

Compositions (wt.%)	Bagasse	Wheat straw	Rice straw
Glucan	31.81 ± 0.53	32.36 ± 0.13	27.93 ± 0.20
Xylan	13.28 ± 0.13	18.23 ± 0.09	13.86 ± 0.14
Galactan	0.218 ± 0.001	ND	1.99 ± 0.13
Arabinan	2.50 ± 0.10	2.676 ± 0.001	2.86 ± 0.02
Mannan	1.38 ± 0.09	ND	ND
Lignin	14.96 ± 0.09	17.72 ± 1.37	24.53 ± 0.10

ND indicates not detected

Chapter 6

Synergistic strategy to overcome biomass recalcitrance through genetic modification and cellulose solvent-based lignocellulose pretreatment

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Abstract

Decreasing lignin content of plant biomass by genetic engineering is believed to mitigate biomass recalcitrance and improve saccharification efficiency of transgenic biomass. Our results showed that lower lignin transgenic switchgrass samples had slightly higher enzymatic glucose releases compared to that of wild type. After dilute acid pretreatment, a strong negative correlation between lignin content of plant samples and overall glucose release was observed, wherein the highest overall enzymatic glucan digestibility was 70% for the sample containing 11 wt.% lignin. By using cellulose solvent and organic solvent-based lignocellulose fractionation (COSLIF), COSLIF-pretreated transgenic biomass feedstocks regardless of lignin content of the plant samples yielded high enzymatic glucan digestibilities (i.e., 80-90%). No obvious correlation between lignin content and sugar release was observed. These studies suggested whether decreasing lignin content in plant biomass is important to saccharification or not depended on pretreatment choice.

Keywords: biofuels, transgenic biomass, lignin, pretreatment, hydrolysis

Introduction

Biomass recalcitrance to saccharification is a major obstacle to cost-efficient production of biofuels and biochemical from lignocellulosic biomass. Biomass recalcitrance is often attributed to many factors, such as low substrate accessibility, high degree of polymerization (DP) of cellulose, presence of lignin and hemicellulose, high crystallinity, large particle size, and porosity (Himmel et al. 2007; Zhang 2011). Most of these factors (e.g., particle size, porosity, crystallinity, presence of lignin) are closely correlated with cellulose accessibility to cellulase, which has been recognized as the most important factor that limits efficient enzymatic hydrolysis of pretreated biomass (Arantes and Saddler 2011; Hong et al. 2007; Rollin et al. 2011).

Lignin in the plant cell walls is crucial for plant growth and development, and the presence of lignin is widely believed to block cellulose accessibility to cellulase and to bind cellulase so to decrease active cellulase working for cellulose hydrolysis (Guo et al. 2001). Some enzymes responsible for the synthesis of lignin have been identified and characterized in model plants (Li et al. 2008; Sticklen 2008). Down-regulation of the enzymes responsible for lignin biosynthesis, such as coumaroyl shikimate 3-hydroxylase (C3H) and hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase (HCT) in alfalfa, decreases lignin content, resulting in improved enzymatic saccharification performance for dilute sulfuric acid pretreated biomass (Chen and Dixon 2007). However, biomass yields are often decreased accompanied with lignin reduction (Chen and Dixon 2007). Switchgrass (*Panicum virgatum* L.) has been regarded as a promising bioenergy crop. To lower pretreatment costs for switchgrass, it may be important to

modulate its biomass recalcitrance by adjusting lignin content, while maintaining normal growth pattern, biomass productivity, and cellulose content (Fu et al. 2011).

Dilute acid (DA) pretreatment effectively depolymerize and solubilize the most labile biomass component -- hemicellulose so to leave cellulose- and lignin-rich solids, which are more easily hydrolyzed by cellulase for a better digestibility (Saha et al. 2005; Zhu et al. 2009). A high enzyme loading, however, is usually required to achieve high soluble sugar yields mainly due to non-specific binding of cellulase by lignin and not very high cellulose accessibility (Zhu et al. 2009). Cellulose solvent-based biomass pretreatments by employing concentrated H_3PO_4 (~85%) or ionic liquids have been recognized as an efficient mean to disrupt highly ordered hydrogen bonding in cellulose chains in biomass. The resulting solids are extremely reactive and highly accessible to cellulase, leading to very high sugar yields at a low cellulase loading within a short saccharification time (Rollin et al. 2011; Zhu et al. 2009).

We discovered that down-regulation of the 4-coumarate: CoA ligase (*4cl*) gene decreased lignin content in switchgrass while maintaining biomass productivity. Also we investigated the effects of different lignin content samples pretreated by two different pretreatments on sugar release efficiency.

Materials and Methods

Chemicals and materials. All chemicals were reagent grade and purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise noted. Phosphoric acid (85% w/w), sulfuric

acid (95% w/w), ethanol (95% v/v) were purchased from Fisher Scientific (Houston, TX). The *Trichoderma reesei* cellulase (Novozyme® 50013) and β-glucosidase (Novozyme® 50010) were gifted by Novozymes North America (Franklinton, NC). They had activities of 84 filter paper units (FPU) of cellulase per mL and 270 units of β-glucosidase per mL. The naturally-dried switchgrass samples were milled into small particles by a Pallmann counter-rotating knife ring flaker (Clifton, NJ). The resulting particulates with nominal sizes of 40-60 mesh (250-400 μm) were used for pretreatment experiments.

Down-regulation of the *4cl* gene in switchgrass. Mature seeds of switchgrass line HR8 selected from cv. Alamo were used for all tissue cultures and genetic transformations. A modified *Agrobacterium*-mediated transformation protocol was used to transform switchgrass with the RNAi binary vector (Somleva and Conger 2002). In short, somatic embryogenic calluses were suspended in *Agrobacterium* solution ($A_{600} = 0.6$) and vacuum infiltrated for 10 min with occasional shaking. After *Agrobacterium*-inoculation, the calluses were blotted on sterile paper towels and then transferred to the co-cultivation medium for 4 days at 23°C in the dark. After co-cultivation, the calluses were transferred onto callus and then regeneration media selected under 50 mg/l hygromycin B (Sigma Chemical Co., St. Louis, MO). The regenerated plants were verified by PCR, Southern blot, and GUS staining. The verified transgenic plants were grown in the Horticulture greenhouse at Virginia Tech, with temperature set at 22°C at night and 28°C during the day with a 12-14 hour light. The plants were grown in Miracle-Gro Potting Mix (Miracle-Gro Lawn Products, Inc., Marysville, OH) in 1.1×10^{-2} m^3 pots and watered twice a week. Wild type (WT) plants regenerated from non-transformed calluses were also grown in the same greenhouse under the same conditions. Each transgenic line (T_1-n) was multiplied

by splitting tillers and maintained in the greenhouse. All plant samples were harvested when 50% of the tillers had flowered.

Fluorescence microscopy and histological staining. The internodes of the WT and T_n plants were embedded in 2.5% agarose and cut with a Leica VT1200 vibrating blade microtome (Bannockburn, US) into 50 µm thick sections. Phloroglucinol and Mäule staining of the 50 µm thick stem sections were used to analyze the lignin deposition patterns by visualization under an Olympus SZXZ-RFL3 fluorescence microscope (Olympus America, Melville, NY) (Coleman et al. 2008).

COSLIF procedure. The COSLIF-pretreated switchgrass was prepared as described previously (Zhu et al. 2009). One gram of switchgrass was mixed with 8 mL of 85% (w/w) H_3PO_4 at 50^{o}C and 1 atm for 45 min. The switchgrass/phosphoric acid slurry was mixed with 20 mL of 95% (v/v) ethanol to stop the reaction. Solid-liquid separation was conducted in a swing bucket centrifuge at 4,500 rpm at room temperature for 10 min. After the supernatant was discarded, the pellets were suspended in 40 mL of 95% (v/v) ethanol. After centrifugation, the solid pellets were washed by 80 mL of deionized water. After centrifugation, the remaining solid pellet was neutralized to pH ~ 6 with 2 M sodium carbonate.

DA pretreatment. The DA pretreatment was conducted using 1.3% (w/w) H_2SO_4 at a solid loading of 10% (w/w) at 130 ^{o}C and 15 psi (autoclave) for 40 min (Chen and Dixon 2007). After DA, the hydrolysate was separated by centrifugation. The switchgrass residue was washed in water prior to enzymatic hydrolysis.

Carbohydrate and lignin assays. The structural carbohydrate composition of lignocellulose was determined by a modified quantitative saccharification procedure

(Moxley and Zhang 2007). Monomeric sugars were measured by a Shimadzu HPLC with a Bio-Rad Aminex HPX-87H column (Richmond, CA) equipped with refractive index detector. The concentrations of glucose and xylose were measured in enzymatic hydrolysate, whereby galactose and mannose were co-eluted with xylose. The column was operated with 5 mM H_2SO_4 as a mobile phase at 60 ^oC and a flow rate of 0.6 mL/min (Rollin et al. 2011; Zhu et al. 2009).

Enzymatic hydrolysis. The COSLIF- and DA-pretreated samples were diluted to 20 g glucan per liter in a 50 mM sodium citrate buffer (pH 4.8) supplemented with 0.1% (w/v) NaN_3. The enzyme loadings were 5 filter paper units (FPU) per gram of biomass and 10 units of β-glucosidase per gram of biomass, otherwise noted. Hydrolysis experiments were carried out in a rotary shaker at 250 rpm at 50 °C for 24 h and 72 h for COSLIF- and DA-pretreated switchgrass samples, respectively.

Results and discussion

Transgenic plants by down-regulating the 4CL expression in "Alamo" switchgrass yielded lower lignin contents (Fig. 1A), while maintaining biomass productivity and cellulose content (see Table S1 in the Supporting Information). Down-regulating 4CL expression in switchgrass had little to no influence on the biosynthesis of syringyl (S) monolignol but greatly decreased guaiacyl (G) monolignol. As a result, G:S ratios were decreased (see Table S1, in the Supporting Information). The lowest lignin content transgenic plant (T_0-4) had the same total carbohydrate level as wild-type (WT) (Table S1 in the Supporting Information).

Fluorescence microscopy and histological staining techniques were employed to identify changes in structure of plant cells and tissues. Although many compounds, such as chlorophyll, lignin, suberin, and cutin, can autofluoresce under UV excitation, lignin appears blue under fluorescence microscope. The blue fluorescence of transgenic plants (Fig. 1B') was weaker than that of WT (Fig. 1B), indicating that transgenic plants had a reduced lignin content. Because the cinnamaldehyde ends of lignin reacted with phloroglucinol-HCl to give a red-violet color, the transgenic specimen had a weaker red-violet color (Fig. 1C'), indicating a decreased lignin content. Since the S monolignol content can be stained with the Maule reagent, giving a brown color, there was no significant qualitative difference between WT and the transgenic plant, suggesting that down-regulating 4CL expression had no influence on S monolignol as reported previously (Xu et al. 2011).

Intact WT and transgenic plants were hydrolyzed by commercial cellulase supplemented with β-glucosidase. The enzymatic glucan releases of transgenic plants were higher than that of WT, indicating less recalcitrance in transgenic plants. However, no correlation between lignin level and enzymatic glucan release was observed (Fig. 2B). Since very low glucan digestibility was obtained, pretreatment was required. All samples were pretreated with DA and COSLIF pretreatments. After pretreatment, the solid (stream S1) and hydrolysate (stream L1) were separated (Fig. 2A). For DA-pretreated samples, the overall glucan release (stream L1 + stream L2) increased in proportion to a decrease in lignin level of plant samples (Fig. 2C). There was a strong negative correlation between lignin content and overall glucan release from DA ($R^2 = 0.767$). The highest value was

70% for the sample containing 11% lignin. By using COSLIF, the overall glucan release showed no correlation with lignin level (R^2 = 0.036) (Fig. 2C). The overall glucan release from COSLIF-pretreated solids was ~90% regardless of lignin content. A strong negative correlation between lignin level and enzymatic glucan release (stream L2) was observed in DA-pretreated solids, while enzymatic hydrolysis of COSLIF-solids had a very weak negative correlation with lignin levels (Fig. 2D).

Concentrated H_3PO_4 as a cellulose solvent in COSLIF disrupted highly ordered hydrogen bonding networks among cellulose chains. Biomass dissolution temperature for concentrated phosphoric acid greatly influenced hydrolysis degrees of polysaccharides (data not shown). Under a modest reaction temperature (e.g., 50 ^{o}C), acid hydrolysis of cellulose by concentrated H_3PO_4 was weak. Consequently, most cellulose after COSLIF was maintained as a reactive solid for the following enzymatic hydrolysis. Elevated biomass dissolution temperature resulted in excessive hydrolysis of cellulose and hemicellulose to soluble sugars, resulting in a separation challenge between soluble sugars from the cellulose solvent and costly recycling of the cellulose solvent, as occurred to concentrated sulfuric acid and hydrochloric acid. Therefore, relatively low COLSIF temperatures were preferred. Also, such low biomass dissolution temperature avoided hemicellulose and cellulose degradation (data not shown). The COSLIF-pretreated solids regardless of lignin content were so reactive, allowing a low enzyme usage, while maintaining high overall glucose yields (> 85%) in a short time (< 24h).

Conclusions

Our new findings were (1) whether lignin was a major factor limiting enzymatic saccharification of biomass was pretreatment based, and (2) down-regulating 4CL expression in switchgrass greatly decreased guaiacyl monolignol lignin content but not affected biomass productivity yield.

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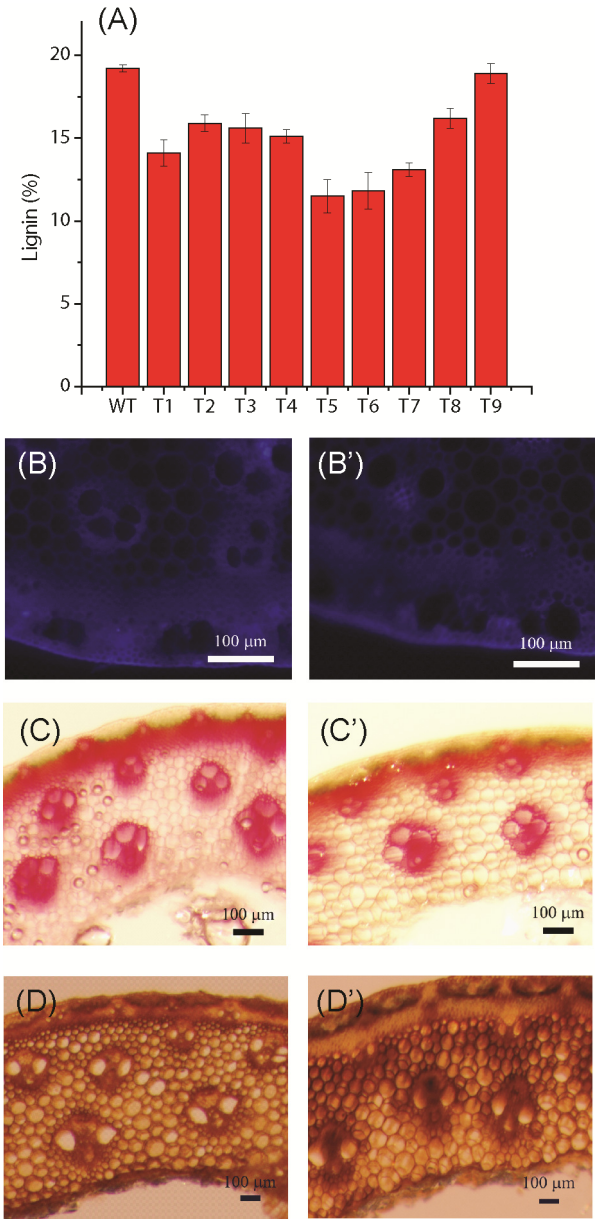

Figure 1. Analysis of wild-type and transgenic switchgrass lines. Lignin content of wild type and transgenic switchgrass lines (A). Lignin autofluorescence (B and B'), histological staining by phloroglucinol-HCl reagents (C and C') and mäule reagents (D and D') of cross-section of internode 3 of WT and transgenic T_0-4 line.

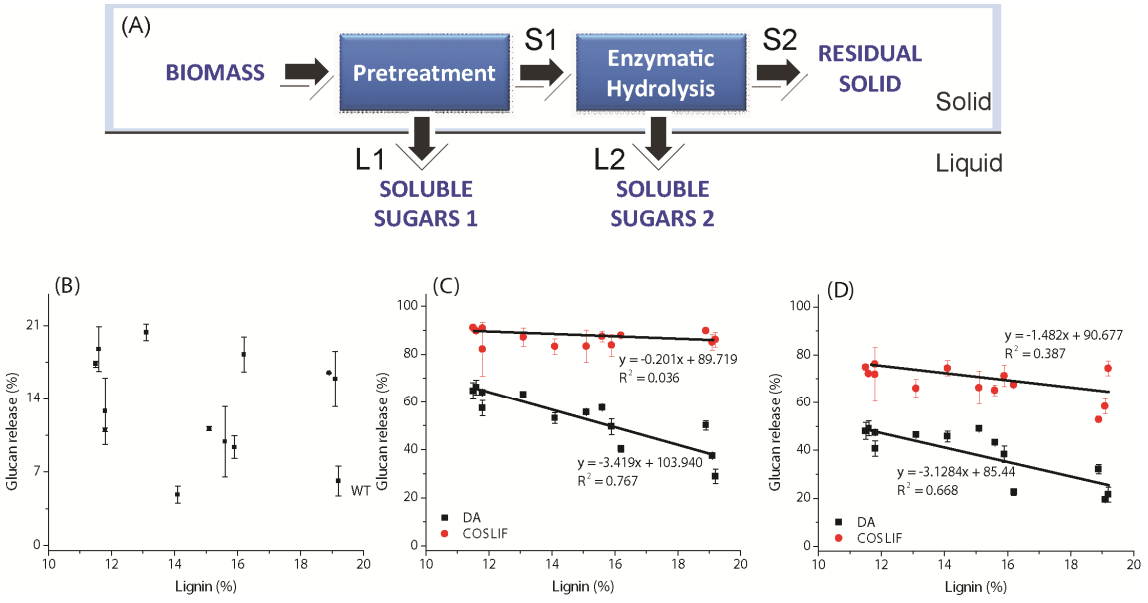

Figure 2. Schematic diagram of mass balance (A), as well as the relationship between glucan releases for untreated samples (B), overall glucan release by pretreatment and enzymatic hydrolysis (C), and enzymatic glucan release after pretreatments (D) and lignin content. Enzymatic hydrolysis was carried out at 20 g biomass/L. All pretreated solids were hydrolyzed at 5 FPU of cellulase and 10 units of β-glucosidase per gram of biomass. DA condition was 130°C and 20 psi for 40 min. COSLIF condition was 50 °C and 1 atm for 45 min. DA- and COSLIF-pretreated biomass samples were hydrolyzed by cellulases for 72 h and 24 h, respectively. Data are from triplicate.

Supporting Information

Figure S1 shows enzymatic total sugar and xylan releases from transgenic switchgrass samples. No correlations between lignin contents and enzymatic total sugar and xylan releases of untreated transgenic plants were observed (Fig. S1A and D). Similar to enzymatic glucan release from transgenic plants, a strong negative correlation between lignin content and overall total sugar release (Fig. S1B) was observed from DA-pretreated solids ($R^2 = 0.695$). However, a weak negative correlation between lignin content and overall total sugar release was observed for COSLIF-pretreated solids ($R^2 = 0.147$). The enzymatic total sugar release (Fig. S1C) of COSLIF-pretreated solids showed a weak negative correlation with lignin content ($R^2 = 0.304$), whereas a strong negative correlation was observed for DA-pretreated solids ($R^2 = 0.605$). DA-preteated solids appeared to have a weak negative correlation between overall and enzymatic xylan releases and lignin contents (Fig S1E and F). However, lignin content had little or no effect on overall and enzymatic xylan releases from COSLIF-pretreated solids.

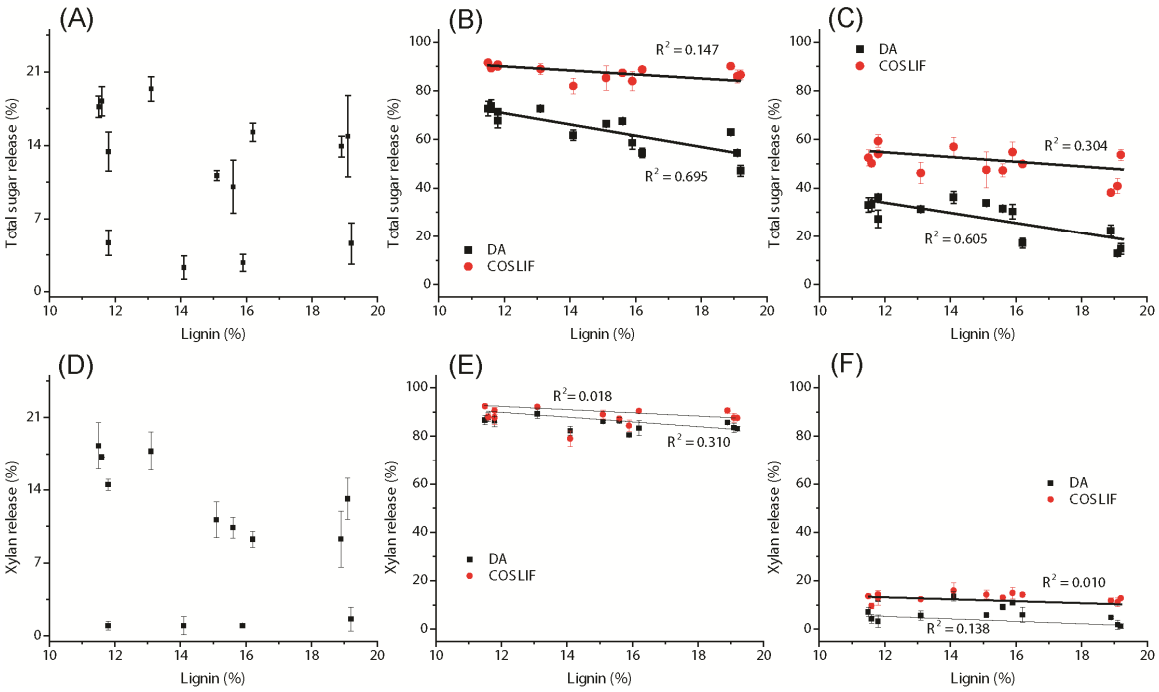

Figure S1. Relationship between total sugar and xylan releases from COSLIF- and DA-pretreated solids and lignin contents. Enzymatic hydrolysis was carried out under 20 g biomass/L. The glucan release for all pretreated solids was hydrolyzed by 5 FPU of cellulase and 10 units of β-glucosidase per gram of biomass. DA condition was 130^{o}C, 20 psi for 40 min. COSLIF condition was 50 ^{o}C, 1 atm for 45 min. DA- and COSLIF-pretreated biomass samples were hydrolyzed by cellulases for 72 h and 24 h, respectively. Data are from triplicate. Untreated samples (A and D), overall total sugar and xylan releases by pretreatment and enzymatic hydrolysis (B and E), enzymatic total sugar and xylan releases (C and F).

Table S1. Growth performance, carbohydrate content, and monolignol composition of transgenic and wild-type (WT) T_0 plants

Switchgrass T_0 Lines	Total dried weight (g)	Carbohydrates (%)		Lignin (%)			Monolignol composition (%)			G:S
		Glucan	Xylan	Acid-soluble	Acid-insoluble	Total Lignin	H	G	S	
Wild type	251.0 ± 27.3	34.8 ± 0.4	17.6 ± 0.3	18.5 ± 0.0	0.7 ± 0.2	19.2 ± 0.2	0.23 ± 0.09	11.60 ± 0.18	7.37 ± 0.27	1.57
T_0-1	224.9 ± 9.4	35.4 ± 1.2	14.6 ± 0.9	14.2 ± 0.4	1.7 ± 0.3	15.9 ± 0.5	0.30 ± 0.23	8.58 ± 1.04	7.01 ± 0.95	1.22
T_0-2	227.7 ± 47.6	36.9 ± 0.5	19.3 ± 0.0	14.2 ± 0.9	1.4 ± 0.2	15.6 ± 0.9	1.48 ± 0.23	7.20 ± 0.69	6.92 ± 0.92	1.04
T_0-3	226.0 ± 20.9	34.7 ± 0.8	18.8 ± 0.6	13.8 ± 0.2	1.3 ± 0.4	15.1 ± 0.4	1.41 ± 0.35	7.08 ± 0.08	6.61 ± 0.39	1.07
T_0-4	258.2 ± 11.3	39.2 ± 0.1	23.0 ± 0.3	12.2 ± 0.2	0.9 ± 0.3	13.1 ± 0.4	1.11 ± 0.19	3.60 ± 0.22	8.39 ± 0.03	0.43

Conclusions

Summary

Cellulosic biofuels are renewable alternatives to petroleum-based liquid fuels. By utilizing a low-cost, renewable, and locally available lignocellulosic feedstocks, cellulosic bioethanol can be produced and served as near-term and medium-term solutions to displace gasoline. However, the major technical and economic challenges to unlock soluble fermentable sugars from lignocellulose in a cost-effective manner must be overcome. This thesis focuses on (1) overcoming biomass recalcitrance to bioconversion by cellulose solvent- and organic solvent-based lignocellulose fractionation (COSLIF), (2) indentifying a key substrate characteristic that impacted effectiveness of enzymatic hydrolysis of biomass, and (3) investigating the relationship between lignin plant biomass contents and enzymatic saccharification efficiencies.

In the initial study, COSLIF pretreatment conditions were optimized for common reed by surface response methodology for maximal soluble sugar release. COSLIF was insensitive to moisture content in biomass [i.e., 5-15 % (w/w)]. The optimal pretreatment condition was ~50 ^{o}C in order to retain most cellulose in the solid phase and minimize sugar degradation products. The optimal COSLIF conditions were applied to pretreat bamboo, generating reactive cellulosic materials with a high glucan digestibility of 88.2% at one FPU cellulase per gram of glucan. This 15-fold reduction in cellulase usage would be of great economic importance for biorefinery development. Moreover, fast hydrolysis rates and high glucan digestibilities at low enzyme loadings of COSLIF-pretreated

biomass were attributed to the fact that highly ordered hydrogen bonding networks of cellulose within biomass were disrupted, as evidenced by CP/MAS ^{13}C NMR and FTIR. The CrI values calculated from XRD and NMR data suggested that CrI was not a key substrate characteristic impacting enzymatic cellulose hydrolysis and relating to pretreatment efficiency.

The correlation between CAC values and glucan digestibilities of pretreated biomass revealed that CAC was a key substrate characteristic influencing enzymatic cellulose hydrolysis of pretreated solids. By employing genetic modification, transgenic switchgrass had lower lignin contents than wild type. Decreasing lignin contents showed a strong negative correlation between over all glucan release of DA-pretreated switchgrass–with the highest glucan digestibility of ~70%. In contrast, COSLIF-pretreated switchgrass yielded high glucan diegestibilities (i.e., ~90%), regardless of lignin contents in biomass. The results in this thesis demonstrate that COSLIF would be a viable pretreatment process, which is nearly feedstock-independent and also effective regardless of lignin contents in plant biomass.

Perspectives

- COSLIF technology is still at an early stage of development; more detailed economic analyses based on Aspen-plus, and life cycle analysis models are needed to understand its potential applications in biorefineries.
- Hemicellulose hydrolysate and residual lignins from COSLIF process would be converted to a wide range of chemicals and polymeric materials. Chemical and/or

biological conversion routes of hemicellulose and lignin should be investigates, as co-product utilization for high-value applications allows an increase in revenues for biorefineries (Zhang 2011).

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