ABSTRACT

WANG, ZIYU. Alkaline Pretreatment of Genetically-Engineered Switchgrass for Improved Carbohydrates Conversion Efficiency. (Under the direction of Jay J. Cheng).

Switchgrass (*Panicum virgatum L.*) is considered as a promising cellulosic energy crop due to its high biomass yield, low agricultural inputs, and positive environmental benefits. The recalcitrance of lignocellulosic biomass to biochemical conversion is a major hurdle for cost-effective production of cellulosic sugars that can be further processed into fuels and valuable chemicals. Pretreatment is required to disrupt the recalcitrant structure for better accessibility of inner carbohydrates to hydrolytic enzymes. Using genetic technology to manipulate lignin synthesis in plants is another effective means to reduce lignin content and/or alter the structure of lignin-carbohydrate complex, and consequently facilitate improved sugar release from the biomass. The overall goal of this research was to gain new insight into the impact of alkaline pretreatment on hydrolysis efficiency of carbohydrates in transgenic switchgrass.

In this study, switchgrass (cv. Alamo) was genetically transformed to suppress the expression of 4-coumarate-CoA ligase (4CL). The transgenic plants were determined to have lignin content reductions of up to 8.5%, while the ratios of acid soluble lignin (ASL) to acid insoluble lignin (AIL) and syringyl/guaiacyl (S/G) were remarkably higher in the transgenic plants than those in the conventional biomass. In the investigation of the effects of lignin down-regulation on pretreatment efficiency, both conventional and transgenic plants were pretreated with 0.5, 1, and 2% (w/v) NaOH for 15, 30, and 60 min at 121 ℃, followed by enzymatic hydrolysis. At the optimal conditions of 1% NaOH and 30 min, the glucan and xylan conversion efficiency in the best transgenic plants were 16 and 18% higher than the

conventional plant, respectively. The results suggest that higher ASL/AIL and S/G ratios may alleviate the negative influence of high lignin content on biomass saccharification.

The best transgenic switchgrass and its conventional counterpart were further pretreated using two groups of conditions: lime at 50 ℃ and the combined alkali (lime and NaOH) at ambient temperature (21 ℃). At the recommended conditions (0.1 g/g raw biomass and 12 h) for lime pretreatment at 50 ℃, the glucan and xylan conversions of transgenic switchgrass were improved by 12 and 10%, respectively, in comparison to those of conventional plant. These increases were reduced to 7 and 8% for glucan and xylan conversions, respectively, when the best conditions (0.025 g lime/g raw biomass, 0.1 g/g raw biomass, and 6 h) for combined alkali pretreatment at ambient temperature were employed. A comparison of all three alkali pretreatments indicates that the advantage of transgenics over conventional plant in sugar production could be maximized if proper pretreatment conditions were used.

The yields of glucose and xylose after lime pretreatment and enzymatic hydrolysis of switchgrass were predicted using two different modeling approaches: multiple linear regression (MLR) and a modified SIR (S: susceptible; I: infectious; R: recovered) (mSIR) model. The amount of alkali loading and residence time applied in the pretreatment were used as predictors in both models. For the MLR model, the values of correlation coefficient R^2 (ranging from 0.90 to 0.97) for both glucose and xylose yields were comparable between the training and testing data sets, while the corresponding root mean square error (RMSE) values for the testing data were higher than those obtained with the training data. The differences between the predicted and experimental values for glucose and xylose yields were within 3-6% and 3-10%, respectively. The mSIR model yielded comparable R^2 and

RMSE values for glucose yield with the MLR model. However, the predictions for xylose yield by the mSIR model were more accurate than those obtained with the MLR model. Further validation of the mSIR model for two other alkali pretreatments (NaOH, combined alkali) shows that the mSIR model had the best performance for combined alkali pretreatment.

Alkaline Pretreatment of Genetically-Engineered Switchgrass for Improved Carbohydrates Conversion Efficiency

by
Ziyu Wang

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APPROVED BY:

_____	_____
Dr. Jay J. Cheng Chair of Advisory Committee	Dr. Ratna R. Sharma-Shivappa
_____	_____
Dr. Larry F. Stikeleather	Dr. Zhilin Li

DEDICATION

This dissertation is dedicated to my beloved wife who has supported me through the past two years with her constant love.

BIOGRAPHY

Ziyu Wang was born and raised in Fuzhou, Jiangxi, P.R. China. As the only child of his parents, he had a wonderful but unusual childhood with their love and support. Ziyu moved to Humen in Guangdong with his parents when he was 8 years old. In the following two years, he went to a Kungfu school to get trained both mentally and physically. The two years' special experience taught him the importance of perseverance, independence, team work, and self-confidence. After finishing the study in the Kungfu school, Ziyu went back to Fuzhou to continue his education until he was matriculated to the undergraduate program in Bioengineering at Beijing University of Chemical Technology in September 2002. The year of 2006, in which Ziyu obtained his B.S. degree and came to the United States for graduate study, is one of the most important and meaningful years to him. Ziyu was initially admitted to the Ph.D. program in the Chemistry department at NC State in fall 2006. After one month study there, he started thinking about his future career plan and made a decision to switch from che7mistry to bioprocess engineering. Ziyu completed his M.S. program in the Department of Biological and Agricultural Engineering at NC State in December 2008. To go after his interest in developing renewable energy sources for resolving environmental and social sustainability issues, Ziyu continued his graduate study to pursue his Ph.D. degree in the same department under the direction of Dr. Jay J. Cheng. After completing his Ph.D., Ziyu is interested in starting his career in the biofuels industry.

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understanding, and support always accompany me no matter where I am. Lastly, I must thank my dear wife for her constant love, because without her support I would not accomplish this dissertation timely.

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CHAPTER 1: INTRODUCTION

1. BACKGROUND

The whole world has relied heavily on petroleum since the inception of its exploitation, as it can be used as raw material for energy sources and a wide variety of derivative products. However, the unsustainability of petroleum in its long-term supply and impact to environment has been a critical issue to healthy development of worldwide economy that needs to be addressed seriously. In addition, global energy demand, especially in the emerging economies of China and India, is expected to continue to increase rapidly (IEA, 2009). Therefore, there is considerable interest in developing renewable alternatives to petroleum.

Although there is a debate on the timing of peak crude oil production (Campell and Laherrere, 1998; Jackson, 2007; USGS, 2000), the reserves of crude oil are projected to be unavoidably depleted (IEA, 2009). The declining trend of oil reserves, together with other factors such as increasing energy demand and unstable production of oil, results in markedly higher costs of petroleum-based energy supply than those in the past. This in turn adversely affects the global economic development (Sanchez and Cardona, 2008).

According to the International Energy Agency (IEA), the total world energy consumption is projected to see an overall increase of 40% from 2007 to almost 17 billion tonnes of oil equivalent (toe) in 2030 (IEA, 2009). Fossil fuels account for nearly 77% of this overall increase in energy demand over the projection period. The majority of this increase comes from the countries outside the Organization for Economic Cooperation and Development (non-OECD nations) such as China, India, and Middle East because of their

stronger long-term economic growth (EIA, 2011a,b). In contrast, the increase in petroleum demand in the United States (US) is marginal. Nevertheless, a large portion of petroleum consumed in the US is imported, which leads to balance of trade deficits and national energy security concerns (Um et al., 2003). The US transportation sector represents about two thirds of its total petroleum consumption and is almost exclusively fueled by oil (Wyman et al., 2005). Demand for liquid transportation fuels is projected to increase by 4.7 quadrillion Btu from 2009 to 2035 (EIA, 2011a).

Greenhouse gases such as carbon dioxide emitted mainly from the combustion of fossil fuels are detrimental to the environment, which are commonly believed to contribute to global climate change and air pollution. According to the International Energy Agency, energy consumption accounts for approximately 65% of the world's greenhouse gas emissions (IEA, 2009). In 2008, annual worldwide energy-related carbon dioxide emissions were 30.2 billion metric tons and are projected to be over 43 billion metric tons in 2035 (EIA, 2011b). In particular, the growth of liquid fuels consumption from 2008 to 2035 corresponds to an absolute increase of 3.3 billion metric tons of carbon dioxide emissions during the projection period (EIA, 2011b).

As a result of the aforementioned problems associated with the use of petroleum-based fuels, seeking renewable energy sources is imperative. In fact, the petroleum share of the increases in liquid fuels consumption is declining as the use of biofuels climbs (EIA, 2011a). Liquid biofuels mainly include bioethanol and biodiesel which can be used to greatly reduce the world's reliance on gasoline and diesel, respectively. Since gasoline is the main

energy source in the US transportation sector, developing bioethanol capacity is of particular interest in the nation. Burning fuel ethanol produces less carbon monoxide, nitric oxide, nitrogen oxide, and photochemical pollutants in comparison with gasoline (Wheals et al., 1999). Current commercial-scale bioethanol is produced from sugary food crops such as sugarcane and corn. The US primarily uses corn as feedstock for ethanol production. However, this approach is not considered viable in the long term due to the use of arable land and the competition with food and feed production (Elobied et al., 2007; Sun and Cheng, 2002). Lignocellulosic materials such as herbaceous crops, agricultural residues, and woody biomass are promising alternatives to corn for ethanol production because they are abundant across the globe, non-edible, and renewable (Lynd et al., 1999; Wyman 2007).

2. RECALCITRANCE OF LIGNOCELLULOSIC BIOMASS

Unlike starchy feedstock, lignocellulosic biomass is structurally complex, consisting of three major components including cellulose, hemicelluloses, and lignin. Cellulose, a linear polymer of anhydro D-glucose units linked by β-1-4 glycosidic bonds, is an important sugar source in plants. The formation of cellulose chains due to the nature of β-1-4 glycosidic bond, along with the interactions among the chains via inter-molecular and intra-molecular hydrogen bonds, results in highly ordered microfibrils known as crystalline cellulose. A small portion of cellulose structure can be amorphous. The crystallinity of cellulose is one of the major hurdles for effective sugar release from the biomass. Hemicelluloses are heteropolysaccharides that are mainly composed of hexoses (D-glucose, D-mannose, and D-galactose), pentoses (D-xylose, L-arabinose, and D-arabinose), and deoxyhexoses (L-

rhamnose or 6-dexoy-L-mannose and rare L-fucose or 6-deoxy-L-galactose) with small amounts of uronic acids present (Brown, 2003). The chemical composition and structure of hemicelluloses can vary for different types of cellulosic biomass. In general, arabinoxylan (major hemicellulose) and glucomannan (minor hemicellulose) are present in herbaceous biomass. The chemical and thermal stability of hemicelluloses is lower than that of cellulose, most likely because of its lack of crystallinity and lower degree of polymerization.

Lignin, the largest non-carbohydrate fraction in lignocelluloses, is a phenylpropane-based polymer that cannot be depolymerized to its original monomers. In contrast to cellulose and hemicellulose, the structure of lignin is much more complex. There are three types of monolignols including *p*-coumaryl alcohol, coniferyl alcohol, and synapyl lignin present in lignin. Various types of carbon-carbon and ether bonds between individual monolignols contribute to the complicated structure of lignin. The intrinsic nature of lignin structure, together with cellulose crystallinity and the sheath formed by lignin and hemicellulose, forms the recalcitrance of lignocellulosic biomass that needs to be overcome for easy access of hydrolytic enzymes to the carbohydrates fraction (Himmel et al., 2007).

3. APPROACHES TO UNLOCK LOW-COST CELLULOSIC SUGAR

Although lignocellulosic materials are competitive in price with oil, the complex structure of lignocellulosic biomass, especially the lignin barrier, makes the inner carbohydrates more difficult to be hydrolyzed by enzymes compared to corn starch. The major challenge to commercializing cellulosic ethanol production is to develop low-cost technology for conquering the biomass recalcitrance (Lynd et al., 2008; Wyman, 2003,

2007). The conversion of lignocellulosic biomass to ethanol involves three main steps: pretreatment, hydrolysis of carbohydrates present in pretreated biomass to fermentable sugars, and fermentation of the sugars to ethanol. Regardless of advances made in the past two decades, the cost of cellulosic ethanol production remains high, mainly caused by the high costs associated with releasing fermentable sugars from biomass through pretreatment and enzymatic hydrolysis (O'Dwyer et al., 2007; Yang and Wyman, 2008). Hence, developing effective approaches to achieve inexpensive cellulosic sugar production is necessary.

3.1 Genetic modification of lignin

Modifying lignin content and lignin-carbohydrate structure using genetic techniques is a potent strategy for overcoming the inherent recalcitrance of cellulosic biomass (Hisano et al., 2009). Genetic transformation of biomass is normally achieved by down-regulating central genes that govern lignin biosynthesis, with attempts to reduce lignin content and/or alter the structure of lignin-carbohydrate complex. A variety of plant materials such as switchgrass (Fu et al., 2011; Li and Qu, 2011), alfalfa (Chen and Dixon, 2007), and poplar (Stewart et al., 2009) has been genetically manipulated with reduced lignin content. Additionally, these studies have shown different lignin composition in transgenic plants from the non-transgenic biomass. The altered lignin content and composition can be favorable for pretreatment efficiency, leading to improved sugar release after enzymatic hydrolysis (Chen and Dixon, 2007; Fu et al., 2011). This also presents a potential to lower pretreatment severity and/or reduce enzyme loadings for cost-effective sugar production.

3.2 Pretreatment of cellulosic biomass

Another approach to unlock low-cost cellulosic sugar is effectively pretreating biomass with chemicals in cost-effective manner for easy release of fermentable sugars. These sugars can either be converted into liquid biofuels or other value-added products such as xylitol, lactic acid, and vanillin. Pretreatment facilitates the efficiency of enzymatic hydrolysis by disrupting the structure of biomass and increasing accessibility of cellulolytic enzymes to the substrates, as described in Figure 1. The goal of pretreatment is to increase

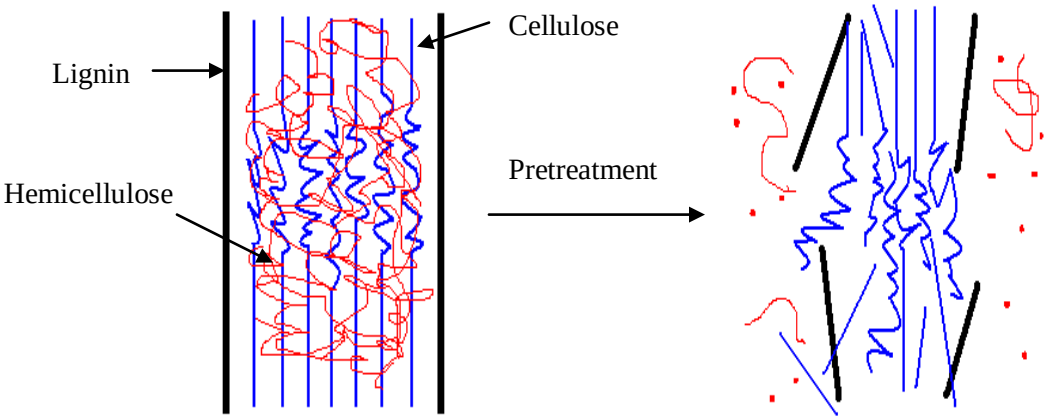

Figure 1. Effects of pretreatment on structure and components of lignocellulosic biomass (adapted from Mosier et al., 2005).

the accessible surface area of cellulose by breaking up the lignin seal, disrupting cellulose crystallinity, solubilizing hemicellulose, and/or increasing pore size of biomass (Mosier et al., 2005). Pretreatment is one of the most costly processes and has a major impact on the cost of prior and subsequent operations (Thorsell et al., 2004; Wooley et al., 1999; Yang and Wyman, 2008). For instance, more efficient pretreatment contributes to less loading of

expensive enzymes employed in the subsequent hydrolysis step, while less recalcitrant structure of biomass resulting from genetic modification of lignin mitigates the severity of pretreatment required.

Pretreatment technologies for cellulosic biomass conversion are categorized into physical, physico-chemical, chemical, and biological processes. The advantages and disadvantages of various pretreatment methods have been extensively reviewed in previously published work (Mosier et al., 2005; Sun and Cheng, 2002; Yang and Wyman, 2008). Of the promising pretreatment technologies, alkali pretreatment has attracted much attention because of its potential low cost and effectiveness in removing lignin from biomass.

Apart from delignification effect, alkali pretreatment also removes acetyl and different kinds of uronic acid substitutions on hemicellulose, thus increases the efficiency of enzymatic hydrolysis of carbohydrates (Chang and Holtzapple, 2000). Sodium hydroxide, lime, and ammonia are the common alkali reagents applied in pretreatment of cellulosic biomass. Sodium hydroxide is the strongest base among the three, giving the highest degree of lignin removal (Wang et al., 2010; Xu et al., 2010). However, it is much more expensive than the other two chemicals. In contrast to sodium hydroxide, lime and ammonia are relatively weak in terms of the ability of yielding hydroxide ions in solution. This typically results in longer residence time required for comparable biomass pretreatment efficiency. Compared with sodium hydroxide pretreatment that is normally carried out at temperatures above 100 ℃, lime pretreatment can be employed at moderate temperatures (eg. 50 ℃) because lime is more soluble in water at lower temperatures (Wang and Cheng, 2011).

4. SWITCHGRASS AS ENERGY CROP

Switchgrass, a warm-season perennial grass native to North America, has been identified as an energy crop that could reduce our dependence on the imported oil for transportation fuel use (McLaughlin, 1992). It can be cultivated widely across the US due to its high biomass yield and low agricultural inputs (Keshwani and Cheng, 2009). The environmental benefits associated with switchgrass include the potential for significant carbon sequestration, nutrient recovery from run-off, soil remediation and provision of habitats for grassland birds (Keshwani and Cheng, 2009). According to a study by Schmer et al. (2008), an estimated reduction of greenhouse gas emissions of 94% can be achieved from burning switchgrass-derived ethanol in comparison with gasoline.

Previous studies have shown the potential of switchgrass as raw material for ethanol production through proper pretreatment, hydrolysis, and fermentation. The low efficiency of ethanol production from switchgrass, however, is mainly due to its high lignin content and high cellulose crystallinity. In addition, low cellulose content in the biomass represents another intrinsic barrier for economically viable conversion of switchgrass to ethanol. In general, wild-type switchgrass consists of 28-37% cellulose, 25-30% hemicellulose and 15-21% lignin. With the help of genetic technology, conventional switchgrass can be modified to reduce its lignin content and/or increase its carbohydrates content for effortless conversion of biomass to fermentable sugars. Consequently, the improved carbohydrates conversion efficiency could lead to lower cost of cellulosic ethanol production.

5. RESEARCH OBJECTIVES

The overall goal of the study presented in this dissertation is to gain new insight into the impact of alkaline pretreatment on hydrolysis efficiency of carbohydrates in genetically-engineered switchgrass. Specific research objectives are as follows:

Objective 1: Review published research on modeling various pretreatment processes and enzymatic hydrolysis of lignocellulosic biomass for sugar production to enlighten potential improvements for process design and optimization as well as economic assessment of the processes. This objective is addressed in Chapter 2.

Objective 2: Examine the impact of lignin down-regulation on biomass composition and the effectiveness of high temperature sodium hydroxide pretreatment of switchgrass. This objective is addressed in Chapter 3.

Objective 3: Investigate the effects of chemical loading and residence time on the efficiency of low temperature alkaline pretreatment for both conventional and transgenic switchgrass plants. This objective is addressed in Chapter 4.

Objective 4: Develop models to predict sugar production from switchgrass after alkaline pretreatment and enzymatic hydrolysis. This objective is addressed in Chapter 5.

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CHAPTER 2: MODELING BIOCHEMICAL CONVERSION OF LIGNOCELLULOSIC MATERIALS FOR SUGAR PRODUCTION: A REVIEW

Reprint of the paper "Wang, Z., Xu, J., Cheng, J.J., 2011. Modeling biochemical conversion of lignocellulosic materials for sugar production: a review. BioResources. 6(4), 5282-5306."

ABSTRACT

To deeply understand the factors that affect the conversion of lignocellulosic biomass to fermentable sugars, experimental results should be bridged with process simulations. The objective of this paper is to review published research on modeling of the pretreatment process using leading technologies such as dilute acid, alkaline, and steam explosion pretreatment, as well as the enzymatic hydrolysis process for converting lignocellulose to sugars. The most commonly developed models for the pretreatment are kinetic models with assumptions of a first-order dependence of reaction rate on biomass components and an Arrhenius-type correlation between rate constant and temperature. In view of the heterogeneous nature of the reactions involved in the pretreatment, the uses of severity factor, artificial neural network, and fuzzy inference systems present alternative approaches for predicting the behavior of the systems. Kinetics of the enzymatic hydrolysis of cellulosic biomass has been simulated using various modeling approaches, among which the models developed based on Langmuir-type adsorption mechanism and the modified Michaelis-Menten models that incorporate appropriate rate-limiting factors have the most potential. Factors including substrate reactivity, enzyme activity and accessibility, irreversible binding of enzymes to lignin, and enzyme deactivation at high conversion levels, need to be considered in modeling the hydrolysis process. Future prospects for research should focus on thorough understanding of the interactions between biomass reactants and chemicals/enzymes — the key to developing sophisticated models for the entire conversion process.

Keywords: Lignocellulose; Modeling; Pretreatment; Enzymatic Hydrolysis; Kinetics

1. INTRODUCTION

Ethanol, a renewable energy source obtained through fermentation of simple sugars, is one of the sustainable alternatives to gasoline that can be used to enhance energy security and mitigate greenhouse gas emissions (Lynd et al. 2008; Sun and Cheng 2002; Wyman 2007). Current fuel ethanol production in the world is mainly based on the fermentation of glucose derived from food based crops. This approach, however, is limited by the arable land and the food and feed applications for the crops such as corn and sugarcane (Elobied et al. 2007; Somerville et al. 2010; Sun and Cheng 2002). Lignocellulosic materials such as agricultural residues, perennial grasses, and forestry biomass are major promising energy sources that have the potential to substitute a large portion of fossil fuels use across the globe, particularly being able to replace 30% of current petroleum consumption in the US market by 2030 (Perlack et al. 2005; Wyman 2007).

Ethanol production from lignocellulosic biomass features a biological conversion that involves the hydrolysis of cellulose and hemicellulose to fermentable reducing sugars and the fermentation of the sugars to ethanol (Lynd et al. 2008; Sun and Cheng 2002). Unlike the relatively simple structure of sugar or starch-based feedstocks, biomass recalcitrance is one of the major challenges in realizing the full cellulosic ethanol production potential (Himmel et al. 2007; Zhang et al. 2007). This recalcitrance is due to the complex structure of lignocellulosic biomass and the way specific components (cellulose, hemicellulose, and lignin) interact with each other (Kumar et al. 2009; Balan et al. 2009).

In light of the natural resistance of cellulosic biomass to attack by hydrolytic enzymes, a pretreatment step is required to alter the structure of biomass and increase the digestibility of cellulose and hemicellulose by the enzymes (Mosier et al. 2005; Sun and Cheng 2002; Yang and Wyman 2008). The current costly cellulosic ethanol production is due to the high costs associated with releasing fermentable sugars from biomass through pretreatment and enzymatic hydrolysis (Lynd et al. 2008; O'Dwyer et al. 2007; Yang and Wyman 2008).

Improving conversion efficiency from lignocellulosic biomass to sugars will require new biotechnological approaches developed through a thorough understanding of what factors impede the sugar production (Lynd et al. 2008; O'Dwyer et al. 2007). This deep perception can be acquired via bridging computer simulations and experimental results together to identify common problematic features that hinder the progress of cellulosic ethanol industry. Given the areas being advanced for lignocellulose-to-ethanol conversion, improving the existing models or developing new models should focus on the two technologically and economically critical steps, including pretreatment and enzymatic hydrolysis.

Kinetic modeling is the most widely investigated method for simulating the two processes, as it is developed based on inherent reaction mechanisms (Conner et al. 1985; Dang and Nguyen 2006, 2007; Fuentes et al. 2011; Jacobsen and Wyman 2000; Kadam et al. 2004; Kim and Holtzapple 2006; Keshwani and Cheng 2010; Ladisch et al. 1983; South et al. 1995), while some non-kinetic or fuzzy inference models are of particular interest when

considering complex systems such as conversion of cellulosic biomass to ethanol (O'Dwyer et al. 2008; Redding 2009; Keshwani and Cheng 2010).

Although much work has been carried out to model a single or hybrid process for the biochemical conversion of lignocellulosic materials to sugars, there has been a need for a comprehensive review on the studies of modeling the entire process. Therefore, it is of great interest to summarize these valuable works to enlighten potential improvements for process design and optimization as well as economic assessment of the processes. The objective of this paper is to review published research on modeling different pretreatment processes and enzymatic hydrolysis of lignocellulosic biomass for sugar production.

2. PRETREATMENT PROCESS MODELING

2.1 Process overview

Pretreatment offers a key to unlock lignocellulosic biomass to achieve high sugar yields after enzymatic hydrolysis, despite the fact that it accounts for only 20% of the total cost for cellulosic ethanol production (Yang and Wyman 2008). Pretreatment processes typically reduce cellulose crystallinity, increase biomass porosity, and remove lignin and/or hemicellulose (McMillan 1994; Xu et al. 2011). A variety of pretreatments can be achieved through physical, chemical, physico-chemical, thermo-chemical, or biological processes, with different approaches having distinct modes of action (Kumar et al. 2008; Sun and Cheng 2002). Careful selection of appropriate pretreatment technologies for process design is essential, as pretreatment has pervasive effects on all other operations in the overall conversion process, and these affected units incorporate the choice of feedstock, size

reduction, enzymatic hydrolysis, sugar fermentation, product recovery, residue usage, and waste treatment (Wyman 2007; Yang and Wyman 2008).

Successfully modeling compositional changes of biomass during pretreatment is conducive to process design and optimization for better conversion (Jacobsen and Wyman 2000). Previous reviews state that efficient pretreatments need to employ chemicals for high product yields and low costs (Xu et al. 2011; Yang and Wyman 2008). In this respect, kinetic models would shed light on understanding the physical and chemical changes of biomass components and their correlations with chemicals implemented in pretreatments. Apart from the application of kinetic models on pretreatment, a fuzzy-logic-based inference system has been applied to simulate the process (Keshwani and Cheng 2010). Process simulation for acid pretreatment in the context of cellulosic ethanol production has drawn much attention for the last two decades. As is the case with acid pretreatment, modeling compositional changes of lignocellulosic biomass during alkaline pretreatment is also widely investigated, with the focus on delignification kinetics inspired from modeling the alkaline pulping process. A unified model, from which the entire conversion efficiency will benefit, is highly desired for predicting the performance of different pretreatment technologies.

2.2 Acid pretreatment simulation

Dilute acid pretreatment of lignocellulosic biomass for ethanol production has attracted extensive attention as to technology improvement and process simulation. A critical review on acid-based pretreatment/hydrolysis processes for the conversion of lignocellulosic materials to ethanol was carried out by Taherzadeh and Karimi (2007a), highlighting the

dependence of acid-based hydrolysis efficiency on properties of the substrate, acidity, and rate of decomposition of the biomass components during the process. In general, the acid reagent used in this pretreatment technology is sulfuric acid, of which the effectiveness on sugar production after enzymatic hydrolysis has been the focus of research efforts (Canilha et al. 2011; George et al. 2010; Guo et al. 2008; Redding et al. 2011; Saha et al. 2005; Schell et al. 2003; Sun and Cheng 2005; Yang et al. 2009; Yat et al. 2008). The major impact of acid pretreatment is to solubilize hemicellulose to form xylose, with little or high lignin removal for batch or flow-through reactors, respectively (Moiser et al. 2005; Yang and Wyman 2004). With this commonly-agreed mechanism, predicting the change of hemicellulose content in lignocellulosic biomass becomes the priority for model development. Although dilute acid pretreatment has insignificant influence on the dissolution of cellulose (Redding et al. 2011; Taherzadeh and Karimi 2007a), inclusion of equations relating cellulose saccharification with pretreatment conditions and biomass physical or chemical properties in model development favors the process design of subsequent enzymatic hydrolysis.

2.2.1 Kinetic model approach

Substantial knowledge of the kinetics of cellulose and hemicellulose fractionation is extremely beneficial to the design, development, and modeling of acid pretreatment processes. Figure 1 illustrates the generic kinetic processes representing xylan removal and cellulose saccharification during acid pretreatment (Conner et al. 1985; Jacobsen and Wyman 2000; Mehlberg and Tsao 1979; Saeman 1945). The initial kinetic model developed for predicting the changes of cellulose content and its associated products was reported by

Saeman (1945). This model consists of two consecutive reactions, of which the first one has a rate constant more sensitive to the changes of temperature and acid concentration than the second one (Saeman 1945). Instead of studying the kinetic conversion of cellulose to glucose, an approach that simulates cellobiose hydrolysis was used to simplify the process (Mosier et al. 2002). Based on Saeman's study, Jacobsen and Wyman (2000) proposed a simple kinetic model for xylan removal during dilute acid pretreatment. In their model, xylan is considered as a chemically unified component that is hydrolyzed to xylose, from which degradation products are formed. Several studies have applied this simple kinetic model to predicting xylan solubilization for acid pretreatment of corn stover (Lu et al 2008), a variety of timbers and switchgrass (Yat et al. 2008), and sugarcane bagasse (Bustos et al. 2003).

A biphasic reaction kinetic model is also applicable to predict cellulose and xylan hydrolysis during dilute acid pretreatment (Bustos et al. 2003; Carrasco and Roy 1992; Conner et al. 1985; Esteghlalian et al. 1997; Mehlberg and Tsao 1979). The first attempt to model the kinetics of hemicellulose hydrolysis during acid pretreatment was made by Mehlberg and Tsao (1979), who suggested two parallel paths for xylan reacting with hydrochloric acid. According to Conner (1984), the slow-reacting rate of xylan solubilization may be due to the entanglement of a portion of xylan with lignin via intermolecular bonds. The inclusion of oligosaccharides in the parallel reaction model takes into account scenarios where breaking down oligomers to monomers is not necessarily much faster than their formation. A relatively complex model for cellulose saccharification was developed by Conner et al. (1985) who took into consideration the presence of amorphous cellulose, and

the reversion reactions of glucose. At high acid concentrations and low temperatures, a remarkable reversible formation of disaccharides from glucose is commonly observed (Pilath et al. 2010).

All the aforementioned kinetic models, as shown in Fig. 1, involve the use of a first-order dependence of reaction rate on the biomass component. The reaction rate equation for the biomass constituent, *Rx*, is expressed as follows,

$$Rx = -\mathrm{d}X/\mathrm{d}t = kX \quad (1)$$

where X is the fraction of original biomass component remaining in the solid residue and k is the rate constant. The commonly used reaction rate constants are assumed to be dependent on temperature and acid concentration and can be calculated from a modified Arrhenius equation (Carrasco and Roy 1992; Jacobsen and Wyman 2000),

$$k = A \cdot C^{m_i} \cdot \exp(-Ea/RT) \quad (2)$$

where A is the Arrhenius constant or the pre-exponential factor (1/time depending on the unit of k); C is the concentration of acid (wt%); m_i is a constant; Ea is the activation energy (J/mol); R is the ideal gas constant, 8.314 J/(mol K); and T is the absolute temperature (K).

The effect of the neutralizing capacity of the biomass was also evaluated and incorporated in the kinetic models due to the presence of ash-forming constituents (Conner et al. 1985; Esteghlalian et al. 1997). In light of this attention, the acid concentration C can be substituted with the molar hydrogen-ion concentration [H+] (Conner et al. 1985). These

kinetic models are applicable not only to single-stage pretreatment but also to multi-stage pretreatment. Baugh et al. (1988) predicted the saccharification of both cellulose and hemicellulose during a multistage acid pretreatment using a typical first-order model indicated in Fig. 1.

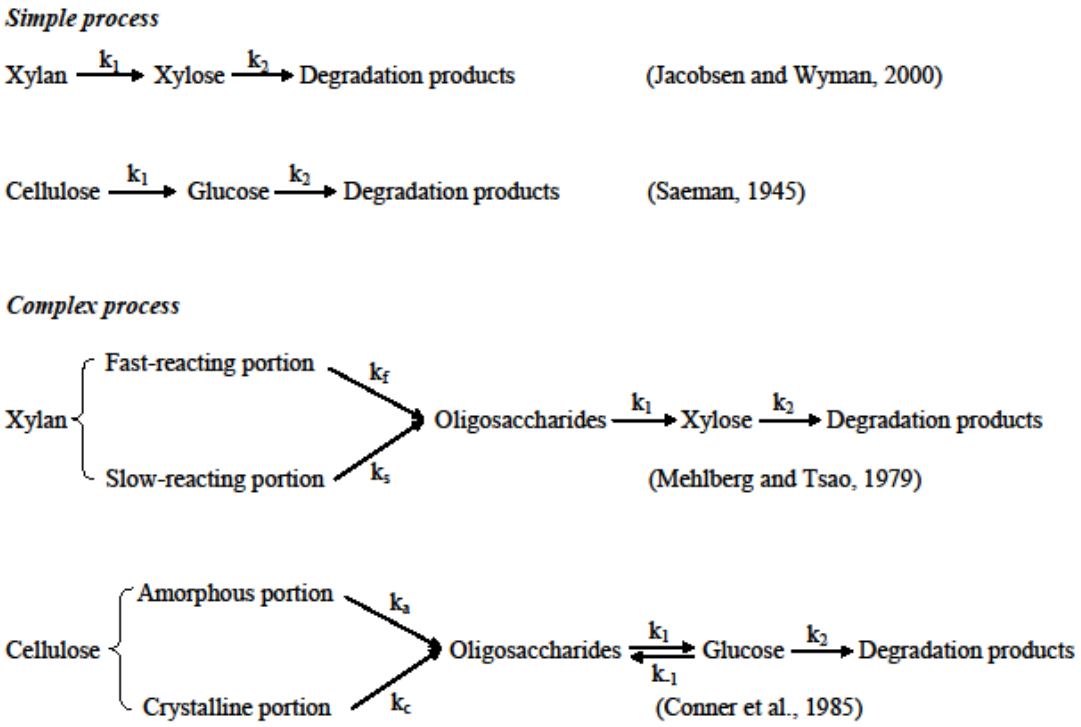

Figure 1. Generic kinetic processes of xylan removal and cellulose saccharification during dilute acid pretreatment.

The validity of a first-order kinetic model for pretreatment of a mixture of lignocellulosic feedstocks is essential, because in reality a cellulosic ethanol plant may use a mixture of feedstocks for the purpose of cost savings. Jensen et al. (2008) proved the applicability of the kinetics approach in dilute sulfuric acid pretreatment of a mixture of switchgrass and woody biomass, and stated that the impact of the interactions among these

biomass varieties on the kinetics of hemicellulose saccharification is negligible. To accurately determine the kinetic parameters, preheating time needs to be as short as possible to fulfill an isothermal condition. In a case of non-isothermal condition, heat transfer limitations resulting from reactor configurations need to be considered when developing a kinetic model for biomass saccharification during pretreatment (Jacobsen and Wyman 2001). The existing kinetic models provide a useful tool for predicting the changes of cellulose and hemicellulose during dilute acid pretreatment under various conditions. However, more progress is needed to make these models more reliable and effective by taking into account the effects of the interactions among lignin and the two carbohydrates.

2.2.2 Severity factor approach

The concept of combining the effects of reaction temperature and residence time into one single factor in empirical models to predict the solubilization of biomass components during acid pretreatments has roots in the pulp and paper industry. Overend and Chornet (1987) first examined the use of a severity parameter that incorporated temperature and time for steam explosion pretreatment. The inclusion of acid concentration into the severity factor was later realized in acid pretreatment of aspen (Chum et al. 1990). This modified severity factor (M) is determined as,

$$M = t \cdot C^{n} \cdot \exp[(T_r - 100)/14.75] \quad (3)$$

where t is the residence time (min); C is the acid concentration (wt%); T_r is the reaction temperature (℃); and n is an arbitrary constant. For non-isothermal conditions, T_r is not an

equilibrium temperature but a temperature profile varying with time. A similar combined severity factor replacing the acid concentration with pH value was derived to effectively model the dilute acid pretreatment of softwoods (Nguyen et al. 2000). The performance of this severity factor approach has been evaluated in different studies (Bower et al. 2008; Jacobsen and Wyman 2000; Silverstein et al. 2007). In particular, Silverstein et al. (2007) found that this approach enables good predictive ability of severity factor-incorporated models in xylan solubilization.

2.2.3 Artificial neural network approach

Artificial neural network (ANN) modeling has apparent advantages over traditional kinetic modeling approaches in predicting the behavior of complex systems, mainly because it avoids the derivation of mathematical equations but behaves analogously to a biological neural structure (O'Dwyer et al. 2008). An ANN is normally composed of three layers including input, hidden, and output, from which a typical feed-forward back-propagation ANN can be built. Figure 2 shows an example of a typical scheme of three-layer neural network. In this network, there are an input layer with three inputs, a hidden layer consisting of three neurons, scalar weights, biases, and transfer functions (normally sigmoid), and an output layer including one neuron, scalar weights, a bias, and a transfer function (normally linear). The mechanism of feed-forward back-propagation ANN is to feed the weighted inputs to the neuron in the hidden layer and make a summation that will be passed to the neuron in the output layer through a sigmoid transfer function, and then further to the output using a linear transfer function.

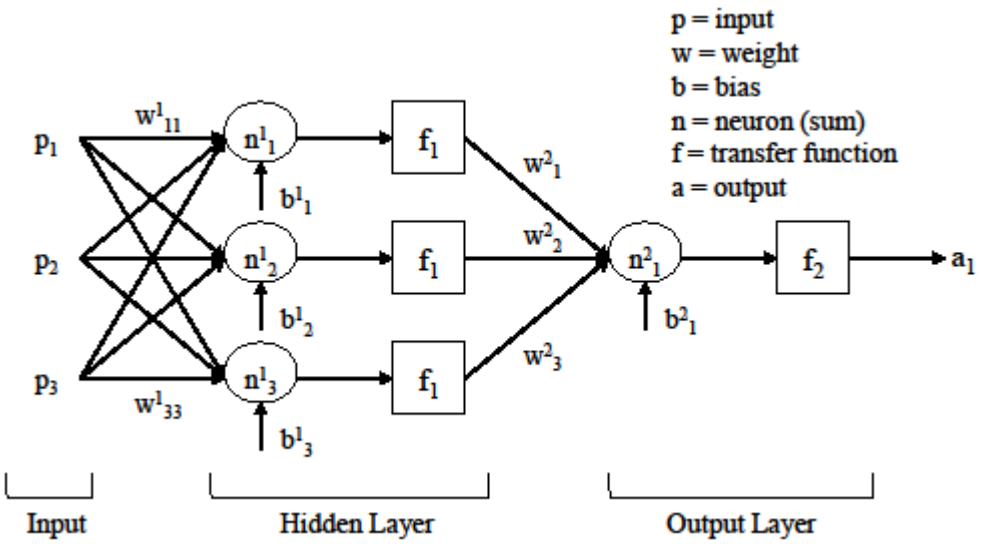

Figure 2. Typical scheme of three-layer feed-forward back-propagation artificial neural network.

The critical step in developing an effective ANN is to train the network with a series of inputs and associated outputs to minimize the error between the predicted and the actual output by adjusting the weights and biases (O'Dwyer et al. 2008; Redding 2009). Over-fitting, one of the most commonly encountered problems during the training process of neural networks, has to be mitigated by regularization to produce an ANN that performs well with any data within the range of training (O'Dwyer et al., 2008). The number of neurons in a hidden layer also needs to be controlled well so that the probability of over-fitting and under-fitting can be maintained at low levels. The successful development of an ANN would bring remarkable value to the design of cost-effective pretreatment and enzymatic hydrolysis processes.

There have been few studies so far applying this approach for predicting and optimizing cellulosic-to-ethanol process. The first if not the only application of ANN for

simulating dilute acid pretreatment of lignocellulosic biomass was examined by Redding (2009), who reported that the ANN predicted more accurately the xylose content of the prehydrolyzate than a multiple linear regression model. Additionally, he found that the optimum number of neurons in the hidden layer was six, which was double the number of inputs including acid concentration, temperature, and residence time. O'Dwyer et al. (2008) also successfully applied a feed-forward back-propagation ANN to correlate biomass structure properties such as lignin content, acetyl content, and cellulose crystallinity with glucan and xylan digestibility during enzymatic hydrolysis. Moreover, they showed the good flexibility of ANN in explaining the impact of glucan and xylan hydrolyses have on each other by increasing the dimensionality of the ANN input matrix.

2.3 Alkaline pretreatment simulation

Alkaline pretreatment includes the use of sodium hydroxide, lime, and ammonia water at dilute concentrations. Tarkow and Feist (1969) examined the mechanism of alkaline pretreatment and found that intermolecular ester bonds crosslinking hemicellulose and lignin are broken down, and the resulting structural disruptions increase biomass porosity. Delignification is considered to be the major impact of alkaline pretreatment to enhance the reactivity of the remaining carbohydrates (Iyer et al. 1996; Kim and Holtzapple 2005; Kim and Lee 2005; Wang et al. 2010; Wang and Cheng 2011; Xu et al. 2010a,b). In addition to separating lignin from biomass, alkaline pretreatment has the capability of effectively removing acetyl groups and uronic acid substitutions on hemicellulose, which also contributes to improvement in enzymatic hydrolysis (Chang and Holtzapple 2000; Selig et al.

2009). A novel study by Xu and Cheng (2011) found that combining the use of sodium hydroxide and lime can remarkably improve the cost-effectiveness of alkaline pretreatment of switchgrass at room temperature.

2.3.1 Delignification kinetics approach

Efforts aimed at modeling changes of biomass composition during alkaline pretreatment began with studies of delignification kinetics in wood pulping processes. Gustafson et al. (1983) briefly summarized the kinetic models developed during the late 1950s to the late 1970s, and pointed out that these kinetic models mostly lack considerations of mass transfer limitations. In response to this issue, Gustafson et al. (1983) developed a theoretical delignification model incorporating kinetics and diffusion and provided a good fit of the model to the pulping behavior of softwood. In their study, Fick's law for diffusion, which is applicable to homogeneous systems, was used to estimate the mass transfer rate of alkalis during the pulping process. However, the assumption of a homogeneous process for the alkaline pulping may not be necessarily appropriate due to the complexity of pulping reaction systems.

In light of the heterogeneous nature of the alkaline pulping, the delignification process can also be viewed as a superposition of three consecutive stages including initial, bulk, and residual phases. The three phases represent distinct fractions of lignin, and are described as first-order reaction sub-processes (Chiang et al. 1990). A mathematical formula that depicts the general delignification kinetics in kraft pulping is shown as,

$$W_L=a_i\cdot\exp(-k_i\cdot t)+a_b\cdot\exp(-k_b\cdot t)+a_r\cdot\exp(-k_r\cdot t) \quad (4)$$

where W_L is the fraction of the residual lignin (g lignin remaining/g lignin in raw biomass); a_i is the maximum fraction of lignin fragments released in the initial stage; a_b is the maximum fraction of lignin fragments released in the bulk stage; a_r is the maximum fraction of lignin fragments released in the residual stage; and k_i, k_b, and k_r are the reaction rate constants for the initial, bulk, and residual delignification stage, respectively (Chiang et al. 1990; Kim and Holtzapple 2006). Note that $a_i+a_b+a_r=1$ since $W_L=1$ at $t=0$. The well-known Arrhenius-type temperature dependence can be applied to relate the rate constants with reaction temperature.

Kim and Holtzapple (2006) and Fuentes et al. (2011) have examined the performance of the three-stage delignification kinetics on simulating lignin content change during lime pretreatment of corn stover, and sugarcane bagasse, respectively. The main difference between the two studies is that Kim and Holtzapple (2006) developed a modified kinetic model with the initial phase excluded for pretreatment time of weeks, while Fuentes et al. (2011) established a first-order reaction model without inclusion of the initial and residual phases for pretreatment time up to 108 h. Parameter estimation for constants (a_i, a_b, and a_r) and activation energy (Ea), and pretreatment conditions are summarized in Table 1. The results demonstrated the applicability of the first-order reaction corresponding to the initial, bulk, and residual phases of delignification to lime pretreatment under a wide range of pretreatment conditions. Depending on the variety of biomass feedstock and the pretreatment conditions, this delignification model may need to be modified in order to accurately predict compositional changes of biomass during alkaline pretreatment. Instead of the model based

on three parallel first-order reactions, a kinetic model based on time-dependent rate constant was developed by Montane et al. (1994) to reasonably simulate kraft pulping delignification. This approach described a distribution of activation energies using the Kohlraush distribution function by assuming a continuous distribution of parallel first-order processes in the pulping system.

Table 1. Pretreatment conditions and parameter estimation for delignification kinetic models of lime pretreatment

Pretreatment conditions & model parameters	Corn stover (Kim and Holtzapple 2006)		Sugarcane bagasse (Fuentes et al. 2011)
	Non-oxidative	Oxidative	
Lime loading (g $Ca(OH)_2$ /g dry biomass)	0.5		0.15, 0.25, 0.4, 0.55, 0.65
Temperature (℃)	25, 35, 45, 55		60, 70, 80, 90
Time	up to 16 weeks		up to 108 h
a_b	0.28	0.27	1
a_r	0.63	0.57	N/A
$E_{a(b)}$ (kJ/mol)	70.24	50.15	31.47
$E_{a(r)}$ (kJ/mol)	10.74	54.21	N/A

In a review of delignification kinetics, Bogren et al. (2007) described five successive events that occur during the delignification process by including the rates of mass transport of the involved species. In the delignification of a wood chip, as explained by Bogren et al. (2007), the chemicals initially transfer from the bulk liquor to the surface of the chip, and then transport to the reactive site for facilitating reactions and dissolution of wood components, after which the dissolved components move from the reactive site to the surface

and finally to the bulk liquor. The concentrations of hydrosulphide, hydroxide ion, sodium ion, dissolved wood components, and lignin-carbohydrate complex (LCC) that influence the performance of pulping system have to be considered in a model representing kraft delignification (Bogren et al. 2007).

2.3.2 Severity factor approach

In the history of development of kinetic models for the kraft pulping process, the H factor, which combines the reaction temperature and reaction time into one variable, as well as the effective alkali concentration, has been regarded as the core of process control schemes (Gustafson et al. 1983). The work conducted by Montane et al. (1994) indicated that the kinetic model based on a time-dependent rate constant is useless for non-isothermal processes. The reason is that the model does not incorporate the dependence of the reaction order regarding the SH^- concentration and the distribution of activation energies with the reaction temperature. This limitation prompted a formulation of a severity factor, combining reaction temperature, reaction time, and chemical (one or two active species) concentration (Montane et al. 1994). Satisfactory prediction in compositional changes and pulp yield using this severity factor has been demonstrated for sodium hydroxide, sodium bisulfate, and kraft pulping processes (Montane et al. 1994). The severity factor for acid pretreatment developed by Chum et al. (1990) has also given good performance in predicting the change of lignin content during alkaline pretreatment of cotton stalk (Silverstein et al. 2007). Based on the concept behind the severity factor, a simplified model, which is able to simulate and control the residual lignin content by simply determining the residual alkali concentration during the

entire pulping cycle, was derived by introducing a correlation between the alkali concentration of a cooking liquor and the time or the time-temperature variable of pulping characterized as H factor (Masura 1999). The model parameters have different values for the initial, bulk, and residual phases according to Masura's (1999) results.

2.3.3 Nuclei growth model approach

The aforementioned studies of modeling delignification during alkaline pretreatment lack an intensive investigation on simulating carbohydrate losses of lignocellulosic materials. With this concern, Dang and Nguyen (2006, 2007) developed a universal kinetic model, based on the power law of growth and nuclei growth concepts proposed by Avrami (1940), to predict the changes of both lignin and carbohydrate for a variety of alkaline pulping conditions. This model describes the heterogeneous characteristics of alkaline pulping kinetics, taking into account the heterogeneous mass transport phenomenon and the effects of active alkali concentration and reaction temperature, and can be expressed as the following rate equation,

$$-\mathrm{d}X/\mathrm{d}t = knt^{n-1} C^{b} X \qquad (5)$$

where X is the amount of the component (lignin, cellulose, or xylan) in the biomass during the course of the pretreatment; k and n are constants that are determined by the heterogeneous nature of the entire reaction system; and C is the chemical concentration (Dang and Nguyen 2006, 2007). Model (5) can be transformed to yield,

$$-\mathrm{d}X/\mathrm{d}t = a\cdot\exp[-E/(RT)]n\, t^{n-1} C^{b} X \qquad (6)$$

by assuming that the temperature effect follows the Arrhenius rule of thermodynamics (Dang and Nguyen 2006, 2007). The parameter *C* was then replaced with the dimension-less dielectric loss tangent of the alkali reagent for modeling compositional changes during microwave-based alkali pretreatment of switchgrass (Keshwani and Cheng 2010). Both kinetic models developed in these two studies show accurate predictions in delignification and xylan loss but not in cellulose loss. This is because cellulose has the most uniform interlinked structure among the three components, thus resulting in the least variability of the rate coefficient of cellulose loss with respect to time (Keshwani and Cheng 2010).

2.3.4 Fuzzy-logic-based model approach

Due to the inherent variability in lignocellulosic biomass characteristics and heterogeneity of pretreatment reactions, an alternative approach other than kinetic modeling that is able to deal with non-random uncertainty is highly desirable. Fuzzy-logic-based modeling is such a method that has been successfully utilized for predicting the behavior of complex systems in the real world. Factors that may contribute to the success of this approach include (1) it is based on natural language for qualitative description in a fast and efficient way; (2) it is able to model nonlinear functions of arbitrary complexity; (3) it is tolerant of imprecise data; and (4) it can build a model based on the experienced understanding on the system or process being modeled. The development of fuzzy-logic-based models can be fulfilled using the fuzzy logic toolbox embedded in MATLAB.

Of various types of fuzzy relational models, the Mamdani-type fuzzy inference system was applied for the first time by Keshwani and Cheng (2010) to model microwave-

based alkali pretreatment of switchgrass. A typical Mamdani-type fuzzy inference system comprises three components including input and output membership functions and the rule-base that maps the input space to the output space. Each rule in the Mamdani-type fuzzy inference system is developed based on an IF-THEN relationship between inputs and output. These components need to be determined based on expert opinion of the system being modeled and actual relationships between inputs and outputs observed from experimental data. In general, both input and output membership functions are represented by linguistic terms such as low, medium, and high which depict certain ranges in each dimension of data space. The fuzzy output set predicted by the rule-base has to be converted to crisp values by defuzzification routines for comparison with experimental values.

In Keshwani and Cheng's (2010) work, the levels of input factors were used to define the number of input membership functions and their location in the data space, while the number of output membership functions and their location were determined by clustering experimental output data with subtractive clustering followed by a fuzzy C-means algorithm. The rule-base was then established based on observations from the training data set. Their fuzzy-logic-based model has several improvements over the kinetic model using a time-dependent rate coefficient. They were able to predict multiple outputs using the same inference system, and reflect uncertainty and changes in the process by modifying membership functions and rules. The results obtained by Keshwani and Cheng (2010) have demonstrated the ability of the fuzzy inference system in predicting cellulose loss during the pretreatment with a high degree of accuracy. This good performance of the fuzzy-logic-based

modeling approach in predicting compositional changes during alkali pretreatment suggests its potential application in other types of pretreatment of lignocellulosic biomass.

2.4 Physico-chemical pretreatment simulation

Steam explosion (autohydrolysis), ammonia fiber explosion (AFEX), and CO_2 explosion are the three common physico-chemical pretreatment technologies used in the lignocellulose-to-ethanol process (Alizadeh et al. 2005; Balan et al. 2009; Kim and Hong 2001; Lee et al. 2010; Negro et al. 2003; Teymouri et al. 2005; Zheng et al. 1998). The most promising pretreatment options, among the above three, are steam explosion and AFEX (Mosier et al. 2005; Yang and Wyman 2008); therefore, investigations on developing models with good performance for these two pretreatments are valuable for process control and optimization.

2.4.1 Kinetic model approach

Developing useful models for steam explosion of lignocellulosic biomass has seen abundant efforts during the past two decades (Conner 1984; Garrote et al. 2001, 2002; Mittal et al. 2009; Overend and Chornet 1987; Rogalinski et al. 2008; Zimbardi et al. 1999). Steam explosion can be viewed as a hydrolysis process in acidic media, since acetate present in biomass generates acetic acid during the course of hydrothermal reaction (Conner 1984). Accordingly, the pseudo-homogeneous kinetics with an Arrhenius-type temperature dependence of reaction rate constants, which has been successfully employed for acid pretreatment, is applicable to hydrothermal process (Garrote et al. 1999). The kinetic model

that combines two parallel pseudo-first order reactions delivered the best fit to the removal of xylan (Conner 1984), while both the two-parallel-reaction model and the one considering only the degradation of the reactive part of xylan accurately predicted the yields of xylooligomer, xylose, furfural, and decomposition products (Garrote et al. 2001, 2002; Mittal et al. 2009). Apart from xylan solubilization simulation, modeling of glucan hydrolysis can be achieved using the pseudo-first-order reaction kinetics (Rogalinski et al. 2008).

2.4.2 Severity factor approach

Based on the theory of H factor applied in the pulping industry, Overend and Chornet (1987) developed a reaction ordinate factor (P),

$$P = t \cdot \exp[(T_r\text{-}100)/14.75] \quad (7)$$

where T_r is the temperature of reaction, by combining the effects of steam temperature and residence time. The applicability of the P factor in simulating changes of biomass composition during steam explosion has been demonstrated for a variety of lignocellulosic materials (Overend and Chornet 1987; Ramos 2003; Zimbardi et al. 1999). Different types of reaction systems such as batch and continuous processes can result in varying P factors, which can be correlated with each other using a relationship proposed by Zimbardi et al. (1999). Considering non-isothermal conditions caused by the different temperatures during the heat-up periods, a modified severity factor that includes a temperature profile in the reaction system was developed by Rogalinski et al. (2008). This work is considerably useful for a wide range of hydrothermal pretreatment conditions, regardless of the length of heating

or cooling times. Similar to the *H* factor, the *P* factor is limited in its use for pretreatments relying on chemicals because it does not incorporate the effect of chemical concentration (Ramos 2003).

3. ENZYMATIC HYDROLYSIS PROCESS MODELING

3.1 Process overview

Enzymatic hydrolysis of lignocellulosic biomass involves the use of cellulase and hemicellulase enzymes to convert cellulose and hemicellulose into hexoses (glucose, galactose, and mannose) and pentoses (xylose and arabinose). Cellulases are a mixture of three different cellulolytic enzymes, including endoglucanase (1,4-β-D-glucan glucanohydrolase), exoglucanase (1,4-β-D-glucan cellobiohydrolase), and cellobiase (β-glucosidase), that act synergistically to convert cellulose into glucose (Ladisch et al. 1983; Taherzadeh and Karimi 2007b). Endoglucanase randomly attacks and cleaves the β-1-4 glycosidic bonds of cellulose to produce cello-oligosaccharides and glucose. Exoglucanase releases cellobiose from the nonreducing ends of cello-oligosaccharides. This particular enzyme is able to work on both amorphous and crystalline celluloses. Once cellobiose is released into the hydrolysis liquor, cellobiase will degrade cellobiose into glucose. Cellulases generally act on amorphous regions of cellulose, and very few isolated cellulases have shown the ability to hydrolyze crystalline cellulose (Shewale 1982). The inhibition of endoglucanase and exoglucanase by cellobiose (Holtzapple et al. 1984) as well as the inhibition of β-glucosidase by glucose (Gong et al. 1977) can be significant factors affecting the performance of cellulolytic enzymes in the process. In the hydrolysis of xylan, which is the

dominant hemicellulose in many lignocellulosic feedstocks, three major enzymes including endo-β-1-4-xylanase, which catalyzes the hydrolysis of the β-1-4 bonds between D-xylose residues of heteroxylans and xylo-oligosaccharides, exoxylanase which releases xylobioses, and β-xylosidase which degrades xylo-oligosaccharides and xylobiose to xylose are involved (Saha and Bothast 1999). The activity of xylanase is likely to increase the accessibility of cellulose to cellulase by enhancing the removal and solubilization of xylan (Berlin et al. 2005; Xu et al. 2011). Depending on the variety of biomass feedstock and the type of pretreatment technology used, different combinations of the aforementioned enzymes need to be carefully selected for efficient enzymatic hydrolysis (Xu et al. 2011).

In enzymatic hydrolysis, the first step is the formation of an enzyme-substrate complex, which involves the mass transfer of enzyme from bulk aqueous phase to cellulose/hemicellulose surface and the formation of an enzyme-substrate complex following enzyme adsorption. The subsequent hydrolysis of cellulose/hemicellulose has two possible modes of action with respect to the location of enzyme and substrate. One mode of action focuses on the movement of substrate and incorporates three major steps (O'Dwyer et al. 2007). First, the reactant molecules are transferred to the active site of the enzyme-substrate complex. Then reaction is catalyzed by the enzyme, followed by release of soluble products to the bulk aqueous phase. The other mode of action illustrated by Ladisch et al. (1983) refers to the location of enzyme, which either moves to the next reaction site along the surface of cellulose or desorbs and then readsorbs onto cellulose. In cellulose hydrolysis, the conversion of cellulose to cellobiose and glucose involves two heterogeneous reactions, while the

degradation of cellobiose to glucose is considered to be a homogeneous reaction (Kadam et al. 2004).

3.2 Factors affecting enzymatic hydrolysis of lignocellulosic biomass

Different pretreatments can result in various consequences involving changes in physical and chemical characteristics (cellulose crystallinity index, degree of polymerization, lignin content, pore volume and size, and surface area accessibility) of lignocellulosic materials, and the changes in these features will have impact on the kinetics of cellulolytic enzymes (Chang and Holtzapple 2000; Zhang and Lynd 2004). Substrate concentration in the hydrolysis slurry, activity of cellulolytic enzymes, and hydrolysis conditions including temperature, pH, and mixing also have impact on the effectiveness and efficiency of enzymatic hydrolysis of cellulosic biomass (Taherzadeh and Karimi 2007b). Chang and Holtzapple (2000) stated that both lignin content and crystallinity index of cellulosic biomass have remarkable impact on ultimate sugar yield, while the initial hydrolysis rate is predominantly affected by crystallinity index. Later studies by Gollapalli et al. (2002) and Laureano-Perez et al. (2005) confirmed the findings, but the overall conversion efficiency of carbohydrates was reported to be mainly dependent on the amount of residual lignin in pretreated biomass (Laureano-Perez et al. 2005). During pretreatment, hydrogen bonds that contribute to the high degree of cellulose crystallinity can be broken down, which is beneficial for enhancing the initial hydrolysis rate.

In addition to the major contributions of delignification and decrystallization to biomass digestibility, deacetylation facilitates the overall hydrolysis efficiency by improving

hemicellulose solubilization (Chang and Holtzapple 2000). As the reaction progresses, several factors including end-product inhibition, low substrate reactivity, enzyme inactivation, and loss of enzyme because of irreversible adsorption on lignin, will slow down hydrolysis rates (O'Dwyer et al. 2007). Specifically, the substrate reactivity is dependent on physical and chemical characteristics of the biomass. After pretreatment, the accessibility of reactants to the active site of enzyme tends to increase, but the degree of polymerization is likely to decrease. These changes would improve the overall substrate reactivity. A common theme for studying the effects of biomass properties on enzymatic hydrolysis efficiency is to build a mathematical correlation through statistical analysis, with some models showing good predictive ability (Chang and Holtzapple 2000; Laureano-Perez et al. 2005) but others not being able to accurately predict the digestibility of biomass (Gollapalli et al. 2002).

3.3 Adsorption based kinetic models approach

The kinetics of enzymatic hydrolysis of lignocellulosic substrates is very complex because of multiple hydrolytic enzyme activities encompassed in the process and the heterogeneous nature of substrate (Kadam et al. 2004). To successfully model the cellulase kinetics, a thorough fundamental understanding of physical and chemical properties of the reacting substrate and its relevant enzyme, and a full investigation of rate-limiting factors are required (Bansal et al. 2009). Both Zhang and Lynd (2004) and Bansal et al. (2009) have conducted comprehensive reviews on understanding the basic assumptions of a number of kinetic models that have been developed for hydrolysis of lignocellulosic substrates by cellulolytic enzymes; they identified shortcomings and potential improvements for these

models. In particular, Bansal et al. (2009) classified all the models into four categories including empirical models, Michaelis-Menten based models, adsorption based models, and soluble substrates based models. Previous work on empirical models has seen limitations on their applicability to conditions outside those used for model development. Moreover, they are useless in terms of disclosing the mechanism of enzymatic hydrolysis. Likewise, soluble substrates based models are not applicable to the enzymatic hydrolysis of insoluble lignocellulosic substrate. Therefore, adsorption based and Michaelis-Menten based kinetic models are the focus of this paper.

Adsorption of cellulases onto insoluble substrates is an essential part of the kinetics of cellulose hydrolysis (Bernardez et al. 1993; Zhang and Lynd 2004), which can be assumed to follow a Langmuir-type isotherm. The first attempt to model the kinetics of cellulose hydrolysis at high substrate and enzyme concentrations was by Wald et al. (1984) who incorporated an enzyme adsorption mechanism into their model. A typical example of the Langmuir isotherm can be given as,

$$E_B = (E_{max}\, K_{ad}\, E_f\, S)/(1+K_{ad}\, E_f) \quad (8)$$

where E_B is the adsorbed enzyme concentration (mg cellulase/L), E_{max} is the maximum adsorption capacity in the unit of mg cellulase per gram cellulose, K_{ad} is the dissociation constant for adsorption, E_f is the free enzyme concentration, and S is the substrate concentration (Bansal et al. 2009; Kadam et al. 2004). A simplified expression of Equation (8) is: [adsorbed enzyme] = constant $\times$ [free enzyme] $\times$ [substrate], according to South et al. (1995). Although the underlying assumptions (including uniform binding sites and

independence of the adsorbing molecules) for the Langmuir isotherm are invalid for cellulolytic enzymes adsorption onto lignocellulosic substrates, the Langmuir equation generally provides a good fit to the cellulase adsorption data for cellulosic substrates (Zhang and Lynd 2004), with standard microcrystalline cellulosic substrate such as Avicel showing an excellent fit (Boussaid and Saddler 1999). The capacity to bind cellulase onto Avicel was reported to be lower than that for pretreated mixed hardwood, as hemicellulose and lignin can also bind cellulase (Bernardez et al. 1994).

High cellulase adsorption capacity is critical for pretreated biomass to bind a sufficient amount of cellulase and then render high hydrolysis rate and sugar yield (Kumar and Wyman 2009a,b). According to Kumar et al. (2009) who studied cellulase adsorption capacity of corn stover and poplar solids pretreated with leading pretreatment technologies, lime pretreated corn stover and flowthrough pretreated poplar had considerably higher cellulases adsorption capacity than dilute acid pretreated corn stover and ammonia fiber explosion (AFEX) pretreated poplar. The study also found that the lignin remaining in lime-pretreated biomass has higher cellulase adsorption capacities than that left in AFEX pretreated biomass. The mechanism for cellulase adsorption onto lignin deserves extensive research efforts because lignin interferes with the performance of cellulases by acting as a competitive cellulases adsorbent that reduces the amount of cellulases avail-able to hydrolyze cellulose (Bernardez et al. 1993; Ooshima et al. 1990) or by blocking enzymatic access to the substrate (Eriksson et al. 2002).

The adsorption model parameters are believed to differ accordingly for different pretreated lignocellulosic substrates and various enzymatic reaction schemes. Table 2 summarizes the values of cellulase adsorption parameters obtained for different substrates pretreated using leading pretreatment technologies.

Table 2. Pretreatment conditions and parameter estimation for delignification kinetic models of lime pretreatment

References	Feedstock	Pretreatment	Maximum adsorption capacity (mg/g substrate)	Dissociation constant (L/g substrate)
Kumar and Wyman (2009a)	Corn stover	AFEX	99.7	1.86
		Dilute acid	90.7	2.49
		Lime	133.6	0.88
Kumar and Wyman (2009b)	Poplar wood	AFEX	107.4	0.21
		Dilute acid	170.9	0.94
		Lime	150.8	0.09
Kadam et al. (2004)	Corn stover	Dilute acid	60	2.22

These results show that the type of feedstock, the variety of pretreatment method, and the associated pretreatment conditions can result in the marked variability of adsorption parameters. As compared to pure cellulose substrates, lignocellulosic substrates tend to have more noticeable changes in adsorption attributes such as the maximum adsorption capacity (Bansal et al. 2009). Therefore, when applying adsorption isotherm equation to model kinetics of enzymatic hydrolysis of lignocellulosic biomass, it is important to validate the model against experimental values of the adsorbed enzyme concentration during the course of hydrolysis. Several studies have considered this scenario and indicated that the adsorption

characteristics can be caused by not only the properties of enzymes used but also the nature of substrates (Liao et al. 2008; Nidetzky and Steiner 1993; Shao et al. 2009). The effects of temperature and competitive sugar inhibitions also need careful consideration in developing a successful kinetic model for enzymatic hydrolysis of lignocellulose (Kadam et al. 2004).

3.4 Michaelis-Menten based kinetic models approach

The simplest enzymatic reaction is considered to be irreversible, and no product inhibition exists during the reaction, with the reaction scheme shown as,

$$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E+P \quad (9)$$

where E is the enzyme, S is the substrate, ES is the enzyme-substrate complex, P is the product, k_1 is the forward rate constant for the formation of enzyme-substrate complex, k_{-1} is the dissociation rate constant of the enzyme-substrate complex, and k_2 is the rate constant of product formation. A typical kinetic model for homogeneous enzymatic reaction follows the Michaelis-Menten equation, which is described as,

$$v = (v_{max}\,[S])/(K_M+[S]) \quad (10)$$

where v is the conversion rate of substrate, v_{max} is the maximum conversion rate of substrate, [S] is the substrate concentration, and K_M is the Michaelis constant.

The quasi-steady state assumption employed in the derivation of Equation (10) cannot be directly applied in enzymatic hydrolysis of insoluble lignocellulosic substrates since it is a heterogeneous reaction system (Bansal et al. 2009). However, the published studies have

showcased satisfying applications of Michaelis-Menten based models in simulating the complex enzymatic reaction system for both pure cellulose (Bezerra and Dias 2004; Caminal et al. 1985; Grous et al. 1985; Huang 1975; Ohmine et al. 1983) and lignocellulosic substrates (Brown et al. 2010; Kadam et al. 2004; O'Dwyer et al. 2007).

The identification of inhibition pattern of kinetic models for enzymatic hydrolysis of cellulosic biomass is of particular interest, as product inhibition can limit the sugar production from the biomass. Competitive inhibition has been the primary concern in this context, and the results show that the kinetic models incorporating competitive inhibition are able to fit experimental data very well (Bezerra and Dias 2004; Grous et al. 1985; Huang 1975; Kadam et al. 2004). Another pattern is non-competitive inhibition, which is regarded as the consequence of non-preferential and irreversible binding of enzymes to lignin (Holtzapple et al. 1984; O'Dwyer et al. 2007). This inhibition pattern has been successfully covered by the HCH-1 model, which may be expressed as,

$$V = k[\mathrm{S}][\mathrm{E}]/(\alpha + \phi[\mathrm{S}] + \varepsilon[\mathrm{E}]) \qquad (11)$$

where [S] is the substrate concentration, [E] is the enzyme concentration, ϕ is the fraction of the substrate surface that is accessible to be hydrolyzed, and k, α, and ε are parameters that represent the degree of substrate reactivity. As reviewed by Bansal et al. (2009), the decreasing rate of enzymatic hydrolysis with increasing conversion can be attributed to enzymatic deactivation, biphasic composition of cellulose, decrease in substrate reactivity and accessibility, fractal or spatially constrained reacting environment, decrease in synergism of cellulase components, and interference by lignin. All these factors need to be considered in

the development of any kinetic models for lignocellulose hydrolysis by cellulolytic enzymes in order to satisfactorily simulate the hydrolysis at high conversion levels.

Most of the cellulase kinetic models developed for hydrolysis of cellulosic substrates lack in the involvement of more than one substrate state variable and more than one hydrolyzing activity. In view of this issue, Zhang and Lynd (2006) proposed a functionally based model, taking into account multiple substrate variables and more than one solubilizing activity, for cellulase kinetics using pure substrate. They found that the fraction of accessible β-glucosidic bonds and the degree of polymerization are highly correlated with substrate reactivity, thus influencing the hydrolysis rate. The degree of synergy between endoglucanase and exoglucanase predicted by their model increases as the extents of the two substrate parameters are raised. Regardless of the significant contribution of their model to the fundamental and applied investigation on enzymatic cellulose hydrolysis, more realistic scenarios should be analyzed and included in the development of more rigorous models.

4. CONCLUSIONS AND OUTLOOK

Sugar produced from lignocellulosic materials has shown a great potential of applications in the production of biofuel and other value-added products such as xylitol, lactic acid, and vanillin. The major challenge associated with the production of these chemicals is how we can effectively release sugars as cheaply as possible from cellulose and hemicellulose present in the recalcitrant biomass. To realize this goal, factors that hinder the conversion of cellulosic biomass to sugar need to be well understood, which will require wisely linking computer simulations with experimental results. Research efforts on modeling

the conversion process have been focused on the kinetic behavior of cellulose, xylan, and lignin present in the biomass. Modeling compositional changes of the biomass for the pretreatment process presents variability due to the nature of the heterogeneous materials and the complex reactions involved in the system. The following three points appear to be important for comparison of different models applied in pretreatment of lignocellulosic biomass.

1. In the development of kinetic models for simulating pretreatment processes, the impact of pretreatment conditions such as temperature, chemical concentration or pH value, and residence time, as well as the interactions between lignin and carbohydrates on the reaction rate of each major component needs to be considered. A deeper understanding of pretreatment mechanisms is useful in building robust models that are appropriate for a wide range of pretreatment technologies.

2. A severity factor that combines all pretreatment conditions into one variable can be integrated into kinetic or empirical models to simplify the inputs for saving efforts in process optimization.

3. Non-kinetic models that exclude mathematical formulas, such as artificial neural network and fuzzy-logic-based inference systems, tend to be more efficient than kinetic models in modeling the changes of cellulose, hemicellulose, and lignin in a variety of pretreatment processes.

In light of the inherent complexity of enzymatic reaction of lignocellulose and its associated inhibition patterns, Langmuir-type adsorption and Michaelis-Menten based models have shown good performance in simulating kinetics of cellulolytic enzymes for heterogeneous cellulosic substrates. Factors including substrate reactivity, enzyme activity and accessibility, irreversible binding of enzymes to lignin, and enzyme deactivation at high conversion levels, which considerably influence the kinetics of enzymatic hydrolysis, ought to be investigated and considered in model development. Inclusion of multiple substrate state variables and more than one hydrolyzing activity into a functionally based model development is also believed to be the trend for simulating enzymatic hydrolysis of lignocellulosic biomass. Until the present, however, most of the process models developed in the context of biochemical conversion of lignocellulose to sugar cannot be applied to the entire conversion scheme. In order to accurately predict the behavior of the entire conversion system for efficient process design and optimization, different existing models should be integrated. To make that take place, a thorough understanding of the conversion system as to physical and chemical properties of biomass, pretreatment reaction mechanism, and enzyme kinetics is also required.

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CHAPTER 3: SODIUM HYDROXIDE PRETREATMENT OF GENETICALLY MODIFIED SWITCHGRASS FOR IMPROVED ENZYMATIC RELEASE OF SUGARS

ABSTRACT

Overcoming biomass recalcitrance to bioconversion is crucial for cellulosic biofuels commercialization. In this study, switchgrass (cv. Alamo) was genetically transformed to suppress the expression of 4-coumarate-CoA ligase (4CL). The transgenic plants were determined to have lignin content reductions of up to 8.5%, while the ratios of acid soluble lignin (ASL) to acid insoluble lignin (AIL) and syringyl/guaiacyl (S/G) were remarkably higher in the transgenic plants than those in the conventional biomass. Both conventional and transgenic plants were pretreated with 0.5, 1, and 2% (w/v) NaOH for 15, 30, and 60 min at 121 ℃, followed by enzymatic hydrolysis. At the optimal conditions of 1% NaOH and 30 min, the glucan and xylan conversion efficiency in the best transgenic plants were 16 and 18% higher than the conventional plant, respectively. The results suggest that higher ASL/AIL and S/G ratios may compromise the negative influence of high lignin content on biomass saccharification.

Keywords: Genetic modification; Lignin; Switchgrass; Sodium hydroxide pretreatment, Sugar

1. INTRODUCTION

Current fuel ethanol production in the United States (US) is mainly based on the fermentation of glucose derived from corn starch. This approach, however, is limited by the arable land and the food and feed applications for corn (Elobied et al., 2007; Sun and Cheng, 2002). Using corn to produce ethanol also incurs environmental concerns as to the increase of greenhouse gas (GHG) emissions (Searchinger et al., 2008). To sustainably meet the increased demand for energy consumption in transportation sector across the globe, there is a need to manufacture more biofuels from lignocellulosic biomass such as agricultural residues, forest wastes, and herbaceous bioenergy crops. These cellulosic feedstocks are considered as promising alternative energy sources because they are abundant, non-edible, renewable, and have the potential to replace the equivalent of 30% of petroleum consumption in the US (Perlack et al., 2005).

While lignocellulosic materials are advantageous in terms of their availability, cost of production, and feedstock diversity for fuel ethanol production, the intrinsic complex nature of biomass structure makes them more difficult to break down than starchy biomass in the hydrolysis to simple sugars (Mosier et al., 2005; Sun and Cheng, 2002). Lignin, together with the way it interacts with cellulose and hemicellulose in lignocellulosic biomass (Kumar et al., 2009), is a major hurdle for efficient biomass conversion (Himmel et al., 2007). The presence of lignin, as a natural barrier to protect plants against insect and pathogen attacks, hinders enzyme accessibility to inner polysaccharides resulting in poor yields of fermentable sugars which in turn negatively impacts the subsequent microbial fermentation (Kumar and Wyman,

2009). In this regard, a pretreatment step is necessary to open up the biomass structure and improve the susceptibility of carbohydrates to enzymatic saccharification. The impact of effective pretreatment processes typically includes reducing cellulose crystallinity and degree of polymerization, increasing biomass porosity and surface area, and removing lignin and/or hemicellulose (Moiser et al., 2005). Of the many promising pretreatment technologies, alkaline pretreatment has received much attention (Xu et al., 2011; Yang and Wyman, 2008). Alkaline pretreatment works primarily by removing lignin, altering lignin structure, and increasing accessible surface area for increased biomass reactivity, which leads to enhanced enzymatic hydrolysis efficiency (Mosier et al., 2005; Wang and Cheng, 2011; Xu et al., 2011). The potential of using sodium hydroxide (NaOH) as a promoting agent in biomass pretreatment has been examined in a number of studies among which most cases demonstrated the great effectiveness of NaOH pretreatment on a variety of lignocellulosic feedstocks under various conditions (Bjerre et al., 1996; Macdonald et al., 1983; Wang et al., 2010; Xu et al., 2010).

Another promising approach to enable easy access to plant polysaccharides in lignocellulose conversion is to rationally modify lignin formation in the development of plants using genetic engineering approach (Hisano et al., 2009). Down-regulation of central genes governing lignin biosynthesis in attempts to reduce lignin content or to alter its composition (mainly ratio of syringyl (S) to guaiacyl (G) units) or structure has been realized in various plant materials such as alfalfa (Chen and Dixon, 2007), poplar (Stewart et al., 2009), and switchgrass (Fu et al., 2011). In the above research efforts, the individual genes

were manipulated to suppress the expression of lignin biosynthetic enzymes including caffeic acid O-methyltransferase (COMT), caffeoyl CoA 3-O-methyltransferase (CCoAOMT), cinnamate 4-hydroxylase (C4H), cinnamyl alcohol dehydrogenase (CAD), cinnamoyl CoA reductase 1 (CCR1), 4-coumarate-CoA ligase (4CL), coniferaldehyde 5-hydroxylase (CAld5H), ferulate 5-hydroxylase (F5H), and hydroxycinnamoyl CoA:shikimate hydroxycinnamoyl transferase (HCT), which could remarkably facilitate lignocellulose conversion for improved fermentable sugar yields. This is due to the fact that lignin content and its composition, especially S/G ratio, are significant factors affecting biomass saccharification efficiency (Studer et al., 2011), and the changes of the two variants via genetic transformation can positively influence biofuel production from lignocellulosic biomass by potentially reducing or even eliminating the costs associated with pretreatment and enzymatic hydrolysis (Chen and Dixon, 2007; Fu et al., 2011).

Switchgrass (*Panicum virgatum L.*) is a perennial warm-season C4 grass and identified as a dedicated bioenergy crop by the US Department of Energy (McLaughlin, 1992). It can be cultivated widely across the US due to its high yield potential, low agricultural inputs, and overall positive environmental benefits (Keshwani and Cheng, 2009). Renewable energy generated from switchgrass could be 540% more than fossil-based energy consumed in the process, and an estimated reduction of GHG emissions of 94% was achieved from burning switchgrass-derived ethanol as compared with petroleum-based gasoline (Schmer et al., 2008). In this study, switchgrass (cv. Alamo) was genetically engineered through the manipulation of 4-coumarate:coenzyme A ligase (4CL) gene in

efforts to reduce lignin content and possibly change its composition for favorable pretreatment requirements. The objectives of this research were to: (1) examine the impact of 4CL suppression on lignin content, syringyl/guaiacyl (S/G) ratio, and carbohydrates composition in switchgrass (cv. Alamo); (2) investigate the effects of lignin down-regulation on the NaOH pretreatment effectiveness for improved carbohydrates conversion efficiency; and (3) identify optimized pretreatment conditions for fermentable sugar yields from conventional and transgenic switchgrass plants.

2. MATERIALS AND METHODS

2.1 Biomass preparation

Callus of switchgrass (cv. Alamo) was transformed (Li and Qu, 2011) with an RNAi construct of switchgrass 4-coumarate:coenzyme A ligase gene (4CL) isolated from young stems of switchgrass (cv. Alamo) (Li et al., in preparation). Presence of the transgene was analyzed by Southern blot analysis. The level of 4CL mRNA expression was measured by qRT-PCR.

Conventional and genetically-engineered switchgrass (cv. Alamo) (harvested in October, 2010) were obtained from the air-conditioned roof-top greenhouse at the North Carolina State University Phytotron in Raleigh, NC. The conventional and five transgenic plants are denoted as ALRCK, AL10/9-33, AL10/9-40, AL11/5-47, AL10/9-28, and AL11/5-41, respectively. The collected biomass was size reduced to pass a 2-mm sieve using a Wiley Laboratory Mill (Thomas, Model No. 4) and stored in sealed plastic bags at room temperature until use for composition analysis and NaOH pretreatment.

2.2 Composition analysis of raw biomass

Prior to pretreatment, the prepared raw switchgrass was analyzed for its chemical composition. The moisture and ash contents of the biomass were determined based on the National Renewable Energy Laboratory (NREL) laboratory analytical procedures (LAP) "Determination of Total Solids in Biomass" and "Determination of Ash in Biomass" (Sluiter, 2005a,b). The structural carbohydrates including glucan, xylan, galactan, and arabinan, as well as acid insoluble lignin (AIL) and acid soluble lignin (ASL) were examined using a two-stage sulfuric acid hydrolysis procedure outlined in the NREL LAP " Determination of Structural Carbohydrates and Lignin in Biomass" (Sluiter, 2006).

2.3 Determination of S/G ratio

Lignin composition was determined using nitrobenzene oxidation following the procedure of Iiyama and Lam (1990) and Hatfield et.al (2009) with modifications. Briefly 4 ml of 2 M NaOH and 0.25 ml of nitrobenzene were added to accurately weighed (~20-25 mg) cell wall sample in stainless steel reaction vessels. Reaction vessels were sealed and heated (170 ℃) for 3 h in a forced air oven. At the completion of the heating cycle, reaction vessels were cooled under cold water and ice. Individual reaction vessels were opened and contents were quantitatively transferred to Pyrex culture tubes (20 x 250 mm, with Teflon lined caps) with dH_2O (2 x 2 ml) and 100 mg of 4,4'-ethylidenebisphenol (EDB; Aldrich, 50 ml of 2mg/ml in 95% ethyl alcohol) added with mixing as internal standard. The reaction mixtures were extracted once with chloroform (5 ml), followed by acidification with 12 M HCl (pH < 2), and extraction continued with (5 ml 2x) dichloromethane and diethyl ether (5

ml 1x). The ether and dicholormethane extracts were combined and half the sample volume was evaporated to dryness in reaction vials under filtered air. Nitrobenzene oxidation products were identified and quantified as trimethylsilane derivatives (40 ml TMSI, Pierce and 10 ml pyridine) using GLC-FID on a ZB-5ms column (Zebron; 30 m x 0.25 mm, 0.25 micron film). The GLC conditions were injector 315 ℃, detector 300 ℃, and a temperature program of 150 ℃ for 5 min, 4 ℃ /min to 200 ℃, 10 ℃ /min to 240 ℃, 30 ℃/min to 300 ℃ and hold for 10 min.

2.4 Pretreatment

Since NaOH pretreatment was proved to be effective in terms of sugar recovery from switchgrass (Performer) at temperatures ranging from room temperature (21 ℃) to 121 ℃ , the pretreatment experiments in this study were employed at 121 ℃ in an autoclave (Model 3021, Amsco) to shorten the residence time from days/hours to minutes. Both conventional and transgenic switchgrass samples (3 g per replicate) were immersed in dilute NaOH solutions (solid to liquid ratio of 1:10) in glass serum bottles and mixed thoroughly before the bottles were sealed. The pretreatment liquor was vacuum filtered through a porcelain Buchner funnel and the pretreated biomass recovered by filtration was washed with 150 ml of deionized (DI) water (50 ml DI water/g raw biomass) to neutralize the substrate for preventing enzyme activity from abnormal pH values. The wet solids were completely transferred to a preweighed plastic bag, weighed, and stored sealed at 4 ℃ for the subsequent enzymatic hydrolysis experiment. A small portion of the wet pretreated biomass was weighed and dried at 105 ℃ to determine solid recovery. The NaOH solution concentrations

of 0.5, 1, and 2% (w/v), along with a range of residence times at 15, 30, and 60 min, were investigated herein.

2.5 Enzymatic hydrolysis

Cellic CTec2 (cellulase complex blended with aggressive cellulases, high level of β-glucosidase, and hemicellulase) and Cellic HTec2 (endoxylanse with cellulase background) were acquired from Novozymes North America Inc. (Franklinton, NC). The densities of CTec2 and HTec2 were reported to be 1.203 and 1.238 g/ml respectively according to the enzyme manufacturer.

To evaluate the effectiveness of NaOH pretreatment on sugar yields from conventional and transgenic switchgrass (cv. Alamo), enzymatic hydrolysis experiments (triplicates per sample) were conducted in 50 ml plastic tubes in a controlled environment reciprocal shaking bath (Model C76, New Brunswick Scientific) at 50 ℃ and 150 rpm. The wet pretreated biomass equivalent to 0.5 g dry basis was immersed in 0.05 M sodium citrate buffer to maintain a pH of 4.8 with a total liquid volume of 15 ml. The excessive dosage of CTec 2 and HTec 2 was 40% and 6% (g enzyme/g dry biomass), respectively, to enable the comparison of different pretreatment conditions free of the impact of enzyme limitation. Sodium azide (0.3% (w/v)) was added to the hydrolysis mixture to prevent microbial growth. The hydrolysis was carried out for 72 hours after which the hydrolysate was centrifuged (Model 5810R, Eppendorf) at 4 ℃ and 4000 rpm for 15 min and the supernatant was stored at -80 ℃ for further analysis. Enzymatic hydrolysis of untreated biomass was conducted as a control.

2.6 Sugar analysis

Total reducing sugars in the enzymatic hydrolysates were determined by the dinitrosalicylic acid (DNS) method (Miller, 1959). In DNS assay, each sample of 0.025 ml diluted in 1.475 ml citrate buffer (0.05 M) was mixed with 3 ml DNS reagent in each assay glass tube which was boiled at 100 ℃ for 5 min. A glucose solution of 2 mg/mL was used for the calibration, hence the reducing sugar was measured as "equivalent glucose". Monosaccharides (glucose, xylose, galactose, and arabinose) generated from the composition analysis of raw biomass and from the enzymatic hydrolysis of pretreated biomass were measured with a high performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) (Dionex ICS-5000, Dionex Corporation, Sunnyvale, CA, USA). The HPAE-PAD was equipped with a CarboPac PA1 (100μeq per 4 x 250 mm) analytical column operated at 18 ℃ with 0.018 M potassium hydroxide as the mobile phase at a flow rate of 0.9 ml/min, a CarboPac PA1 guard column (4 x 50 mm), a thermostatted autosampler, and a quaternary pump. An internal standard (fucose) solution of 1 g/l was added to each sugar sample for the determination of monomeric sugars based on a proportional relationship between the ratio of fucose to each simple sugar concentrations and the ratio of fucose to individual sugar peak areas.

2.7 Statistical analysis

Experimental data were statistically analyzed using the GLM procedure in SAS 9.1 software. Significant ($P<0.05$) and nonsignificant differences between treatments were

evaluated by Tukey adjustment for comparisons. All treatments were conducted in triplicate. The sample SAS code used to analyze data is shown in Appendix A.

3. RESULTS AND DISCUSSION

3.1 Impact of 4CL suppression on lignin content and S/G ratio

Transgenic plants of switchgrass (cv. Alamo) with the RNAi construct of 4CL gene were obtained via Agrobacterium-mediated transformation. Presence of the transgene was confirmed by Southern blot analysis. Up to ninety percent reduction of the 4CL mRNA expression was observed for transgenic plants. The whole tillers (including stem, leaf, and sheath) of conventional and transgenic switchgrass plants were examined for both acid soluble lignin and acid insoluble lignin contents. In all five transgenic plants, the acid insoluble lignin (AIL) content was significantly ($P<0.05$) lower than that of the conventional plant, with the transgenic plant AL11/5-41 showing the highest reduction of 8.5% (Table 1) which was relatively less than the data reported in other research work on genetic transformation of switchgrass (Fu et al., 2011). Contrarily, the acid soluble lignin (ASL) content in the transgenics was considerably increased from that of the conventional plant. Although the combined content of ASL and acid AIL was essentially identical in both conventional and transgenic plants due to the opposite directions of changes of the two component contents, the ratio of ASL to AIL contents was greatly raised by 21.4-64.3% in the transgenic biomass.

In this study, the S/G ratio in the non-transgenic control plants was averaged at 0.93 (n=3), while the ratios of the five transgenic plants analyzed in this report ranged from 1.04

Table 1. Chemical composition of conventional and transgenic switchgrass (cv. Alamo) plants

Plant line	Mass percentage of chemical components[a] (wt%, dry basis)							ASL/AIL
	ASL	AIL	Glucan	Xylan	Galactan	Arabinan	Ash	
AL RCK	2.71 ±0.014	18.75 ±0.148	40.11 ±0.963	22.07 ±0.747	0.97 ±0.045	2.49 ±0.074	2.74 ±0.024	0.14 ±0.001
AL 10/9-33	3.57 ±0.006	17.53 ±0.141	40.76 ±0.638	22.53 ±0.182	1.47 ±0.039	3.29 ±0.032	3.68 ±0.006	0.20 ±0.002
AL 10/9-40	2.96 ±0.052	17.26 ±0.222	41.42 ±0.857	22.21 ±0.322	1.15 ±0.020	3.18 ±0.074	2.68 ±0.003	0.17 ±0.002
AL 11/5-47	3.57 ±0.029	17.60 ±0.010	40.19 ±0.511	21.39 ±0.300	1.35 ±0.045	3.53 ±0.075	3.23 ±0.156	0.20 ±0.002
AL 10/9-28	3.02 ±0.014	17.27 ±0.026	40.19 ±0.764	22.64 ±0.264	1.39 ±0.033	3.81 ±0.079	3.18 ±0.039	0.17 ±0.001
AL 11/5-41	3.97 ±0.054	17.15 ±0.206	40.34 ±0.469	21.72 ±0.642	1.24 ±0.051	2.81 ±0.100	3.35 ±0.029	0.23 ±0.006

[a] Values are means ± standard deviation (n=3).

to 2.46. Acid soluble lignin (ASL) is considered to consist of "low-molecule-weight degradation products and hydrophilic derivatives of lignin" (Yasuda et al., 2001). It is suggested that ASL is related to syringyl lignin because there is little ASL in gymnosperm wood which consists of only guaiacyl lignin and no S lignin (Yasuda et al., 2001; Vanholme et al., 2010), higher ASL from syringyl lignin-rich woods (Yasuda et al., 2001), and other experimental results (Matsushita et al., 2003). According to the published results (Fu et al., 2011), the S/G ratio in transgenic switchgrass was reported to be less than that of wild-type plant, which was opposite to the findings in this research. This is probably because lignin biosynthetic genes targeted in our study are different from those in the research by Fu et al. (2011).

3.2 Impact of 4CL suppression on carbohydrates composition

The composition of carbohydrates (including glucan, xylan, galactan, and arabinan) was determined to evaluate the potential impact of 4CL suppression on availability of fermentable sugars. Glucan and xylan accounted for over 90% of total carbohydrates in switchgrass, and their contents in transgenic plants were essentially the same as those in the conventional plant (Table 1). Unlike glucan and xylan, the contents of galactan and arabinan were both significantly ($P<0.05$) higher in the transgenic plants. Nevertheless, the overall content of carbohydrates in switchgrass was maintained at the same level after genetic manipulation of lignin because of the negligible amounts of galactan and arabinan in the biomass. In light of the above results, the reduced lignin content and increased ASL/AIL and S/G ratios achieved by 4CL down-regulation did not affect the carbohydrates contents.

3.3 Effects of lignin down-regulation on NaOH pretreatment effectiveness

Lignin content and S/G ratio have been demonstrated to have significant influence on sugar release from biomass through pretreatment and enzymatic hydrolysis (Chen and Dixon, 2007; Fu et al., 2011; Studer et al., 2011). Compared with conventional plants, the transgenic plants AL11/5-47, AL10/9-28, and AL11/5-41 showed significant ($P<0.05$) increases in total reducing sugar yield without alkali pretreatment (Figure 1a). At 0.5% NaOH loading, as shown in Figure 1a, remarkable improvements of 23.9-26.4% in total sugar production were noted in AL 11/5-47, AL 10/9-28, and AL 11/5-41 pretreated for 15 min, but no such substantial gains were seen in AL10/9-33 and AL10/9-40. When the residence time was extended to 30 and 60 min, however, the plants AL10/9-33 and AL10/9-40 started to exhibit variously enhanced levels of total sugar recovery as compared with the conventional plant. This was presumably caused by the increased biomass digestibility at more severe pretreatment conditions.

Similarly, the total sugar yields in all five transgenic plants were found to be markedly higher than that in the conventional line with pretreatment at 1% NaOH for 15-60 min (Figure 1b). In particular, the transgenic plant AL10/9-33 had an increase of 21.7% in total sugar recovery compared with the conventional plant, when the biomass was pretreated with 1% NaOH for 30 min under which the maximum total sugar yield was obtained. However, increases of less than 10% in total sugar release from switchgrass using transgenic plants were observed for pretreatments at 2% NaOH (Figure 1c), because the corresponding

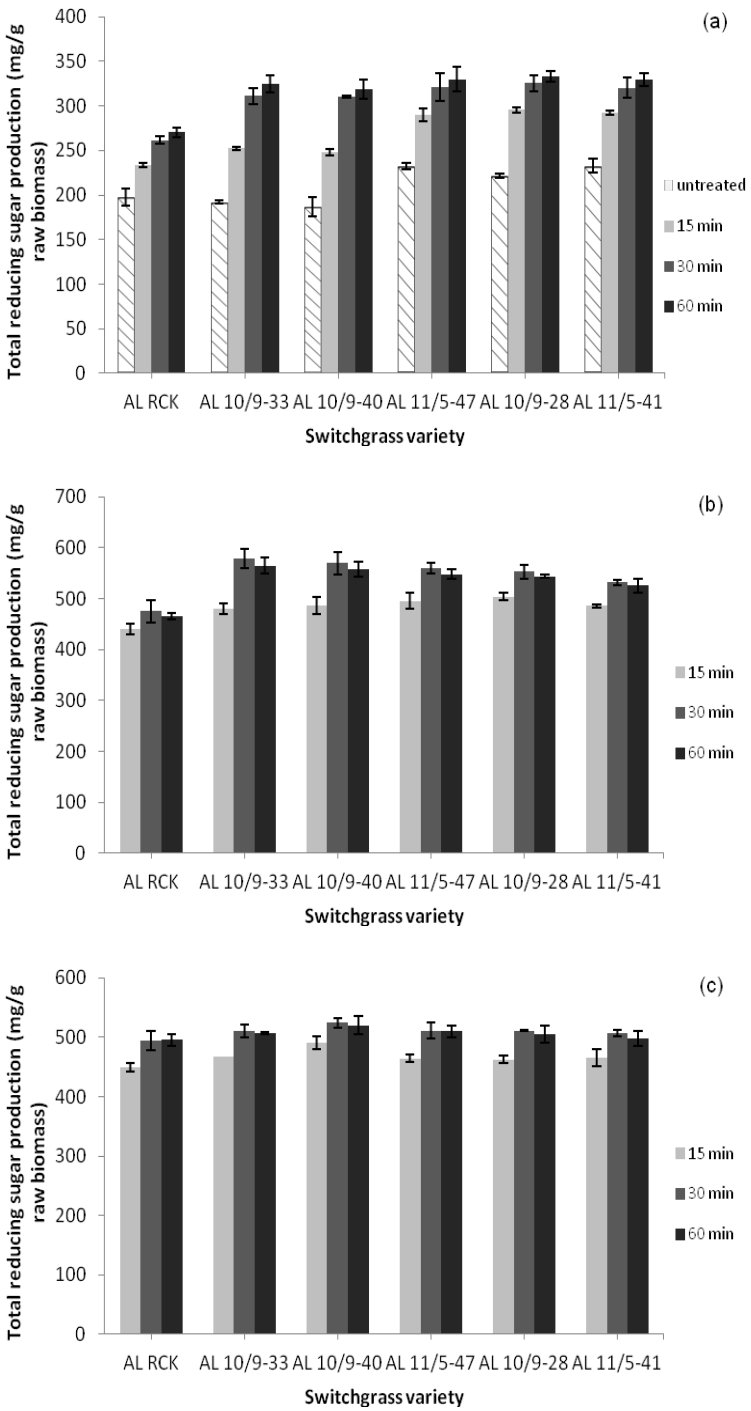

Figure 1. Total reducing sugar production from conventional and transgenic switchgrass pretreated with NaOH of 0.5 (a), 1 (b), and 2% (w/v) (c) for 15, 30, and 60 min at 121 ℃.

pretreatment severity was overly high to lead to loss of carbohydrates (data not shown) during the process which could make the positive impact of lignin modification on pretreatment effectiveness less noticeable (Chen and Dixon, 2007).

The overall improvement in total sugar recovery from switchgrass using transgenic plants was speculated to be attributed to the reduced lignin content and increased ASL/AIL and S/G ratios. Lignin content reduction has been successfully achieved by down-regulating lignin biosynthesis in a variety of plants such as alfalfa, aspen, and switchgrass, and the resultant lower lignin content in transgenic plants may help to some extent overcome biomass recalcitrance to biological conversion (Chen and Dixon, 2007; Fu et al., 2011). On the other hand, lignin composition, especially S/G ratio, can be altered after genetic modification (Chen and Dixon, 2007).

Chemical analysis revealed that the transgenic switchgrass plants had higher S/G ratios. The shift to higher S/G ratio in the lignin, even without an obvious reduction of total lignin content, could result in altered lignin structure and/or altered structure of the lignin-carbohydrate complexes (Albersheim et al., 2011), in which the lignin is more of linear type and less branched due to the added syringyl type residues or reduced guaiacyl residues in the total lignin molecules. This in turn may explain why there were higher levels of acid soluble lignin in these transgenic samples. This may also explain why there were greater released sugars from the transgenic grasses as a result of NaOH pretreatment: more linear lignin molecules may result in looser structure with less cross-links between the two units, and a total wall matrix that is a bit more open (less condensed from cross linking of the lignin) after

pretreatment, that would allow greater digestibility of not only cellulose but also hemicellulose. This finding is also corroborated by a recent study (Studer et al., 2011) which concluded that high S/G ratios in *populus* variants enable sugar release less susceptible to the large amount of lignin present in the biomass (Studer et al., 2011).

Examination of glucan conversion efficiency revealed that the increases in glucose release (shown in Table 2) from switchgrass that was genetically transformed had similar trend as those in total reducing sugar production with or without NaOH pretreatment. Compared with conventional plants, the transgenic biomass presented 12.6-17.5% and 9.8-16% improvements in glucan conversion efficiency for pretreatments with 0.5 and 1% NaOH, respectively. Further increasing the NaOH concentration to 2% significantly ($P<0.05$) compromised the favorable influence of modified lignin content and ASL/AIL and S/G ratios on glucan conversion. Table 2 also indicates that the transgenic plants AL10/9-33 and AL10/9-40 outperformed AL 11/5-47, AL10/9-28, and AL11/5-41 in terms of glucose recovery from the biomass under pretreatment conditions at 1% NaOH for 30 and 60 min.

Apart from glucan conversion efficiency, the effectiveness of releasing xylose from the biomass is another factor to consider in a comparison between conventional and transgenic plants. Table 3 shows the xylan conversion efficiency of switchgrass after pretreatment and enzymatic saccharification for both conventional and transgenic plants. Variously increased levels of xylan conversion efficiency were observed in all five transgenic plants regardless of the involvement of pretreatment in biomass conversion into sugars. At 0.5 and 1% NaOH loadings, the improvements reached 11.2-42.6% and 7-18% respectively,

Table 2. Glucan conversion efficiency after enzymatic hydrolysis of conventional and transgenic switchgrass pretreated at 121 ℃

Time (min)	NaOH concentration (%, w/v)	Glucan conversion[a] (%)					
		AL RCK	AL 10/9-33	AL 10/9-40	AL 11/5-47	AL 10/9-28	AL 11/5-41
15	0.5	39.08	38.57	38.64	45.32	45.66	45.90
	1.0	67.67	71.74	71.02	73.81	74.30	72.87
	2.0	69.14	72.63	74.35	72.25	70.72	70.88
30	0.5	45.13	50.81	50.80	50.40	50.38	50.71
	1.0	74.59	86.50	85.54	83.70	82.55	81.17
	2.0	78.76	80.58	82.59	80.35	79.87	77.35
60	0.5	46.38	50.18	49.25	52.45	52.53	50.62
	1.0	73.07	84.65	83.36	81.16	81.88	80.77
	2.0	76.80	75.41	78.64	76.66	78.41	76.30
Control[b]		37.40	38.01	37.99	42.01	39.74	41.43

[a] Glucan conversion efficiency = (g glucose produced in the hydrolyzate) × (0.9) / (g glucan in raw biomass); Values are means of three replicates.

[b] Untreated switchgrass sample hydrolyzed with excessive loadings of CTec2 and HTec2.

Table 3. Xylan conversion efficiency after enzymatic hydrolysis of conventional and transgenic switchgrass pretreated at 121 ℃

Time (min)	NaOH concentration (%, w/v)	Xylan conversion[a] (%)					
		AL RCK	AL 10/9-33	AL 10/9-40	AL 11/5-47	AL 10/9-28	AL 11/5-41
15	0.5	22.10	24.70	24.57	28.32	30.54	29.12
	1.0	52.39	56.08	56.52	61.26	59.17	59.35
	2.0	52.25	50.54	54.33	53.06	53.68	55.26
30	0.5	24.06	29.02	29.76	32.06	34.30	33.41
	1.0	58.22	68.67	68.68	68.06	64.13	65.04
	2.0	56.11	56.41	55.39	55.76	51.90	55.44
60	0.5	26.69	31.08	31.77	35.36	37.48	33.90
	1.0	55.86	64.47	64.51	63.78	61.77	62.42
	2.0	54.69	51.29	55.53	54.57	52.09	54.21
Control[b]		5.52	5.63	5.66	6.51	6.60	6.92

[a] Xylan conversion efficiency = (g xylose produced in the hydrolyzate) × (0.88) / (g xylan in raw biomass); Values are means of three replicates.

[b] Untreated switchgrass sample hydrolyzed with excessive loadings of CTec2 and HTec2.

while there was no significant ($P>0.05$) differences in xylose release from the biomass at 2% NaOH between transgenic and conventional plants. The aforementioned results suggest that lignin modification not only increases the susceptibility of cellulose to enzymatic saccharification, but also more favorably improves hemicellulose conversion.

3.4 Optimization of pretreatment conditions

The impact of NaOH concentration and residence time on pretreatment efficiency was statistically evaluated. For both conventional and transgenic switchgrass plants, as shown in Fig. 1 and Table 2 and 3, pretreatments at 15 min gave significantly ($P<0.05$) lower sugar release from the biomass than those at 30 and 60 min for all NaOH loadings tested. Since no significant ($P>0.05$) changes in sugar recovery were observed between 30 and 60 min, the best pretreatment time was determined to be 30 min. Likewise, pretreatments with 1 and 2% NaOH loadings lead to significantly ($P<0.05$) higher carbohydrates conversion efficiency than those with 0.5% NaOH loading for all three levels of residence time. The difference in both glucan and xylan conversion efficiency between 1 and 2% NaOH loadings was not obvious in conventional plants, but was remarkable in the transgenic plants except for that in glucan conversion efficiency at a residence time of 15 min. This indicates that transgenic plants were more susceptible to the change of NaOH loading during pretreatment, which further proved that lignin modification could facilitate biomass conversion by reducing the severity of pretreatment (Chen and Dixon, 2007). Therefore, a residence time of 30 min and 1% NaOH loading were identified as the optimal pretreatment conditions for switchgrass (cv. Alamo) including conventional and transgenic plants in this study. At the

optimized pretreatment conditions, the total reducing sugar production of all five transgenic lines showed 12-21.7% increases as compared with the conventional plant, with both AL10/9-33 and AL10/9-40 presenting the best glucan and xylan conversion efficiency of about 16 and 18% respectively higher than those of conventional plants.

4. CONCLUSIONS

Lignin modification through genetic transformation of switchgrass enabled reduced lignin content and considerably increased ASL/AIL and S/G ratios. The changes of lignin content and composition effectively overcame biomass recalcitrance to enhance the accessibility of cellulose and hemicellulose to enzymatic release, which could potentially result in improved cellulosic biofuel production. Future research directions include a deeper understanding of cell wall structure to investigate factors in addition to lignin content and composition that govern sugar release, genetic transformation of biomass for increased carbohydrates content, and evaluation of genetic engineering, pretreatment, and biological conversion approaches for their impact on economics of cellulosic biofuels.

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CHAPTER 4: IMPROVEMENT OF SUGAR PRODUCTION FROM TRANSGENIC SWITCHGRASS WITH LOW TEMPERATURE ALKALI PRETREATMENT

ABSTRACT

Genetically modified switchgrass (cv. Alamo) and its conventional plant both were pretreated using two groups of conditions: lime at 50 ℃ and the combination of lime and NaOH at ambient temperature. The results show that the transgenic plant (with altered lignin content and composition) was more susceptible to alkali pretreatment than the conventional plant. At the recommended conditions (0.1 g/g raw biomass and 12 h) for lime pretreatment at 50 ℃, the glucan and xylan conversions of transgenic switchgrass were 12 and 10%, respectively, higher than those of conventional plant. These increases were reduced to 7 and 8% for glucan and xylan conversions, respectively, when the best conditions (0.025 g lime/g raw biomass, 0.1 g/g raw biomass, and 6 h) for combined alkali pretreatment at ambient temperature were employed. The advantage of transgenics over conventional plant in sugar production could be maximized if proper pretreatment conditions were used.

Keywords: Lime pretreatment; Genetic modification; Lignin; Switchgrass; Sugar

1. INTRODUCTION

Advanced biofuels derived from lignocellulosic materials are believed to have a great potential to replace a large amount of petroleum-based fuels and slow down global climate change (Sun and Cheng, 2002; Xu et al., 2011). Bioethanol, a popular type of biofuel, made from lignocellulosic feedstocks is a promising alternative to gasoline in the transportation sector. Switchgrass (Panicum virgatum L.), a perennial grass, has been identified as a dedicated energy crop due to its high biomass yield, limited fertilization requirements, and positive environmental impacts (Keshwani and Cheng, 2009). Although there are abundant lignocellulosic sources for bioethanol production, the recalcitrant nature of these feedstocks makes the production of cellulosic ethanol more costly than that of first generation ethanol from sugary food crops such as corn and sugarcane (Himmel et al., 2007). Thus, a key step towards achieving cost-effective cellulosic ethanol has been taken by many researchers to focus on effectively releasing fermentable sugars as cheaply as possible from cellulosic biomass.

High lignin content, together with the complex plant cell wall structure constructed with lignin, cellulose, and hemicellulose, is a major hurdle for inexpensive production of cellulosic sugars (Himmel et al., 2007; Kumar et al, 2009). Modifying lignin content and lignin-carbohydrate structure using genetic techniques, and innovating efficient biomass conversion approaches, are considered as two potent strategies for overcoming biomass recalcitrance. A number of plant materials such as switchgrass (Fu et al., 2011; Li and Qu, 2011), alfalfa (Chen and Dixon, 2007), and poplar (Stewart et al., 2009) has been genetically

manipulated in attempts to reduce lignin content and/or alter the structure of lignin-carbohydrate complex. A variety of lignin biosynthetic enzymes were targeted in the genetic transformation of these lignocellulosic feedstocks, with the outcome of successfully suppressing the expression of these enzymes while maintaining normal biomass growth. Genetic modification of switchgrass could lead to reduced amounts of lignin and altered ratios of syringyl/guaiacyl (S/G), and consequently enhanced the conversion of biomass to fermentable sugars which can be synthesized into fuels (Fu et al., 2011; Wang et al., 2012; Zhou et al., 2011). The results of these studies present a potential to avoid severe pretreatment conditions for generating comparable quantities of sugars from transgenics with those from wild-type plants, or yield significantly more sugars on a per acre basis under identical process conditions.

A pretreatment step is still required to unlock the intrinsic recalcitrance of biomass for better accessibility of cellulose and hemicellulose to enzymes despite the progress made in genetic modification of lignin. The effects of reduced lignin content and/or changed S/G ratio on the effectiveness of pretreatment are of great interest, with acid pretreatment (Chen and Dixon, 2007; Fu et al., 2011; Zhou et al., 2011) explored most. Apart from dilute sulfuric acid pretreatment, alkaline pretreatment has been investigated extensively in the past two decades, and is one of the most promising pretreatment technologies for making cellulosic ethanol economically competitive (Sun and Cheng, 2002; Yang and Wyman, 2008). Our previous study has examined the impact of lignin down-regulation on the efficiency of sodium hydroxide (NaOH) pretreatment of switchgrass, and reported that the transgenic

plants, when pretreated under the optimal conditions at 121 ℃, exhibited glucan and xylan conversions of up to 16 and 18% higher than the wild-type biomass, respectively. (Wang et al., 2012). In order to further cut down the cost of pretreatment, there is a need to explore other cheaper alkali reagents (eg. lime) at milder temperatures. Lime ($Ca(OH)_2$) is cheaper, safer, and easier to recover as compared with NaOH (Chang et al., 2001). Lime pretreatment at 50 ℃ can be as effective as that at high temperature (>100 ℃) in terms of carbohydrate conversion, as long as appropriate residence times (normally less than 1 day) are employed (Xu et al., 2010a; Wang and Cheng, 2011). At ambient temperature, however, using lime alone requires much longer residence time (over 3 days) (Xu et al., 2010a) while the use of only NaOH necessitates fairly high chemical loading (0.2 g NaOH/g raw biomass) (Xu and Cheng, 2011). This implies that ambient temperature pretreatment of cellulosic biomass with the use of either lime or NaOH is not economically feasible. Nevertheless, the combined use of lime and NaOH enables greater cost-effectiveness of pretreatment at ambient temperature than the individual uses of these two chemicals. This is because: (1) the use of cheaper lime in place of part of the NaOH alkalinity can reduce the chemical cost; (2) Calcium ions form linkages within the biomass to protect carbohydrates being severely lost during the pretreatment; and (3) the gradual dissolution of lime is able to sustain the pH value at a high level throughout the pretreatment (Xu and Cheng, 2011; Zhang et al., 2011).

Based on the results of a previous study on lime pretreatment of switchgrass (Xu et al., 2010a), genetically modified switchgrass (cv. Alamo) and its wild-type plant were pretreated using lime alone at 50 ℃, to investigate the effects of lime loading and residence

time on carbohydrate conversion. In addition to lime pretreatment, the combined alkali pretreatment using both lime and NaOH at ambient temperature was explored regarding the effects of NaOH loading, residence time, and lime loading on the efficiency of releasing fermentable sugars in the subsequent enzymatic hydrolysis. Materials balances for the pretreatments were performed and used along with carbohydrate conversion to compare the efficiency of the two pretreatment methods between conventional and transgenic plants.

2. MATERIALS AND METHODS

2.1 Biomass preparation

Genetic transformation of switchgrass (cv. Alamo) was performed to suppress the expression of 4-coumarate:coenzyme A ligase gene (4CL) (Li and Qu, 2011; Li et al., in preparation). Conventional and transgenic switchgrass (harvested in October, 2010) were obtained from the air-conditioned roof-top greenhouse at the North Carolina State University Phytotron in Raleigh, NC. Five transgenic plants were previously evaluated based on sugar release from the biomass after NaOH pretreatment and enzymatic hydrolysis (Wang et al., 2012). In this study, both conventional (denoted as AL RCK) and one of the best transgenic plants (denoted as AL10/9-40) were subjected to LTL pretreatment. The collected biomass was size reduced to pass a 2-mm sieve using a Wiley Laboratory Mill (Thomas, Model No. 4) and stored in sealed plastic bags at room temperature until use for composition analysis and low temperature lime pretreatment.

2.2 Composition analysis of raw/pretreated biomass

The prepared raw and pretreated switchgrass were analyzed for their chemical composition. The moisture and ash contents of the biomass were determined based on the National Renewable Energy Laboratory (NREL) laboratory analytical procedures (LAP) "Determination of Total Solids in Biomass" and "Determination of Ash in Biomass" (Sluiter, 2005a,b). The structural carbohydrates including glucan, xylan, galactan, and arabinan, as well as acid insoluble lignin (AIL) and acid soluble lignin (ASL) were examined using a two-stage sulfuric acid hydrolysis procedure outlined in the NREL LAP " Determination of Structural Carbohydrates and Lignin in Biomass" (Sluiter, 2006). The syringyl/guaiacyl (S/G) ratio in the raw biomass was also determined (Wang et al., 2012).

2.3 Pretreatment

A previous study on lime pretreatment of switchgrass (cv. Performer) showed that lime exhibits the most effectiveness in facilitating sugar release from the biomass at 50 ℃ (Xu et al., 2010a). In the first stage of this study, the pretreatment experiments were employed at 50 ℃ in a water bath using lime as a pretreatment reagent (Model 205, Fisher Scientific). In the second stage of investigation on low temperature alkali pretreatment, lime and NaOH were used together at room temperature (21 ℃). The effects of NaOH loading and residence time on biomass carbohydrate conversion at a constant lime loading of 0.1 g/g raw biomass were examined first, followed by an evaluation of various lime loadings at the optimized NaOH loading and residence time. Table 1 shows all the pretreatment conditions investigated in this study. Both conventional and transgenic switchgrass samples (3 g per

Table 1. Experimental conditions investigated in low temperature lime pretreatment of switchgrass with/without NaOH addition

Pretreatment method	Temperature (℃)	Lime loading (g/g raw biomass)	NaOH loading (g/g raw biomass)	Residence time (h)
lime pretreatment	50	0.05, 0.10, 0.15	N/A	1, 3, 6, 12, 24, 48
lime pretreatment with NaOH addition	21 (1^{st} step)	0.10	0.05, 0.075, 0.10, 0.15	3, 6, 9
	21 (2^{nd} step)	0.025, 0.05, 0.075, 0.10	optimized condition from 1st step	optimized condition from 1st step

replicate) were mixed thoroughly with lime ($Ca(OH)_2$) powder alone or with lime and NaOH together in deionized (DI) water in a solid to liquid ratio of 1:10 in glass serum bottles, before the bottles were sealed and placed in the water bath or on a flat counter for pretreatments.

After pretreatment, the biomass slurry was vacuum filtered across Fisherbrand P8 filter paper in a porcelain Buchner filter funnel, and the pretreated biomass recovered by filtration was washed with DI water in the amount of 100 ml DI water/g raw biomass to neutralize the substrate for preventing enzyme activity from being inhibited at abnormal pH values. The wet solids were completely transferred to a preweighed plastic bag, weighed, and stored sealed at 4 ℃ for the subsequent enzymatic hydrolysis experiment. A small portion of the wet pretreated biomass was weighed and dried in a convection oven (Isotemp, Fisher Scientific) at 105 ℃ to determine solid recovery. After a certain amount of the pretreated biomass was used for enzymatic hydrolysis, the remainder was dried in a vacuum oven at 40 ℃ to constant weight for composition analysis.

2.4 Enzymatic hydrolysis

To evaluate the effectiveness of LTL pretreatment on biomass conversion for sugar production, enzymatic hydrolysis experiments (triplicates per sample) were carried out in 50 ml plastic tubes in a controlled environment reciprocal shaking bath (Model C76, New Brunswick Scientific) at 50 ℃ and 150 rpm. The wet pretreated biomass equivalent to 0.5 g dry basis was immersed in 0.05 M sodium citrate buffer to maintain a pH of 4.8 with a total liquid volume of 15 ml. Cellic CTec2 (enzyme complex of aggressive cellulases, high level

of β-glucosidase, and hemicellulase) and Cellic HTec2 (endoxylanse with cellulase background) donated by Novozymes North America Inc. (Franklinton, NC) were used in this study. The densities of CTec2 and HTec2 were reported to be 1.203 and 1.238 g/ml respectively according to the enzyme manufacturer. Excessive dosage of CTec 2 and HTec 2 of 40% and 6% (g enzyme/g dry biomass), respectively, were employed to avoid the impact of enzyme limitation on comparison of different pretreatment conditions. Anti-microbial agent, sodium azide (0.3% (w/v)) was added to the hydrolysis mixture to prevent microbial growth. The hydrolysis was carried out for 72 hours after which the hydrolysate was centrifuged (Model 5810R, Eppendorf) at 4 ℃ and 4000 rpm for 15 min and the supernatant was collected in 15 ml plastic storage tubes and stored at -80 ℃ for further analysis.

2.5 Sugar analysis

Total reducing sugar in the enzymatic hydrolysates was determined using the dinitrosalicylic acid (DNS) method (Miller, 1959). In DNS assay, each sample of 0.025 ml diluted in 1.475 ml citrate buffer (0.05 M) was mixed with 3 ml DNS reagent in each assay glass tube which was boiled at 100 ℃ for 5 min. A glucose solution of 2 mg/mL was used for the calibration, thus the reducing sugar was measured as "equivalent glucose". A high performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) (Dionex ICS-5000, Dionex Corporation, Sunnyvale, CA, USA) was used to measure monosaccharides (glucose, xylose, galactose, and arabinose) generated from the composition analysis of raw/pretreated biomass and from the enzymatic hydrolysis of pretreated biomass. The HPAE-PAD system was equipped with a CarboPac PA1 (100μeq per 4 x 250 mm)

analytical column operated at 18 ℃ with 0.018 M potassium hydroxide as the mobile phase at a flow rate of 0.9 ml/min, a CarboPac PA1 guard column (4 x 50 mm), a thermostatted autosampler, and a quaternary pump. An internal standard (fucose) solution of 1 g/l was added to each sugar sample for the determination of amounts of monomeric sugars based on a proportional relationship between the ratio of fucose to each simple sugar concentrations and the ratio of fucose to individual sugar peak areas. The conversion efficiencies of carbohydrates were calculated using the following formulas.

$$\text{Overall carbohydrate conversion (\%)} = \frac{\text{g total reducing sugar produced in the hydrolyzate}}{\text{g total reducing sugar in raw biomass}} \times 100$$

$$\text{Glucan conversion} = \frac{\text{g glucose produced in the hydrolyzate}}{\text{g glucan in raw biomass}} \times 0.9 \times 100$$

$$\text{Xylan conversion} = \frac{\text{g xylose produced in the hydrolyzate}}{\text{g xylan in raw biomass}} \times 0.88 \times 100$$

2.6 Statistical analysis

Experimental data (triplicates) were statistically analyzed using the GLM procedure in SAS 9.1 software. Significant ($P<0.05$) and nonsignificant differences between treatments were evaluated by Tukey adjustment for comparisons.

3. RESULTS AND DISCUSSION

3.1 Biomass characterization

The goal of suppressing the expression of 4CL gene in switchgrass was to reduce lignin content and/or modify lignin structure for better carbohydrate conversion. The content of total lignin including acid soluble lignin (ASL) and acid insoluble lignin (AIL) was

analyzed for both conventional and transgenic switchgrass plants. The AIL content in the transgenic plant AL 10/9-40 was approximately 8% less than that (18.8%, dry basis) in the conventional plant. Contrarily, the ASL content in the transgenics was about 9% higher than that (2.7%, dry basis) in the conventional plant. Because of the opposite directions of changes in the amounts of these two components, the total lignin content in the transgenic plant was only slightly (around 6%) less than that of the conventional plant. In despite of the relatively less lignin reduction observed in this study as opposed to that reported in other research work on genetic transformation of switchgrass (Fu et al., 2011), the ratio of ASL to AIL contents was remarkably raised by 21.4% in the transgenic biomass. The ratio of syringyl (S) to guaiacyl (G) lignin units in the conventional plant was determined to be 0.93, while the transgenic plants feature significantly ($P<0.05$) increased S/G ratios (1.0-2.46) (Wang et al., in preparation). These results demonstrate the positive correlation between ASL/AIL ratio and S/G ratio as ASL is related to syringyl lignin, which is in agreement with the study by Yasuda et al. (2001).

The composition of carbohydrates in switchgrass was supposedly changed marginally, if any, after genetic transformation since the lignin contents in the two plants were comparable. The glucan and xylan contents in the conventional plant were 40.1 and 22.1%, respectively, which accounted for more than 90% of total carbohydrates in switchgrass. The transgenic plant contained almost the same amounts of glucan and xylan as those in the control. Unlike glucan and xylan, the contents of galactan and arabinan in the transgenic plant were 1.2% and 3.2%, respectively, which were significantly ($P<0.05$) higher

than those in the conventional plant. Regardless of the changes in galactan and arabinan, the overall carbohydrate content in the transgenic switchgrass was about the same as that in the control plant due to the negligible amounts of galactan and arabinan in the biomass.

3.2 Lime pretreatment

Effective carbohydrate preservation during pretreatment of lignocellulosic biomass is desirable for releasing as much fermentable sugars as possible in the subsequent enzymatic hydrolysis. Solid recovery implies the potential amount of carbohydrates in the pretreated biomass that can be converted to sugar for bioethanol production. In general, biomass components can be preserved well in lime pretreatment as calcium ions are able to form linkages with lignin and carbohydrate in the biomass to prevent their loss during the pretreatment (Torre et al., 1992; Wang and Cheng, 2011; Xu et al., 2010a). In this study, the solid recoveries (data not shown) of switchgrass after lime pretreatment at 50 ℃ ranged from 80 to 90%, which is considered to be high as compared with those (50-75%) reported for sodium hydroxide pretreatment (Wang et al., 2010; Xu et al., 2010b). Insignificant ($P>0.05$) difference in solid recovery was observed between the conventional and transgenic plants. The impact of lime loadings on solid recovery was marginal due to the increased linkages between calcium ions and biomass components for biomass preservation at higher lime loadings. With the extension of residence time from 1 to 48 h, the solid recoveries for both conventional and transgenic switchgrass slightly decreased. Regardless of good capability of lime pretreatment in solid recovery, its overall effectiveness still needs to be evaluated based on sugar production in the following enzymatic hydrolysis. Since the concentrations of

arabinose and galactose in hydrolysates were very low, the yields of these two individual sugars were not reported herein.

At a lime loading of 0.05 g/g raw biomass, the total reducing sugar released from switchgrass in enzymatic hydrolysates was generally low (Figure 1a), with the maximal reducing sugar yields for both conventional and transgenic plants reaching 1.52 and 1.81 times those of untreated biomass, respectively. This is comparable with other studies showing that lime loadings less than 0.1 g/g raw biomass usually are not sufficient for maximizing sugar yield of cellulosic biomass (Wang and Cheng, 2011; Xu et al., 2010a). As the residence time was extended from 1 to 48 h, the reducing sugar production at this lime loading was found to steadily increase, with the possibility of continuing its improvement beyond 48 h. Likewise, the glucose and xylose yields exhibited continued boost with the increase of residence time (Figure 1b and 1c). It was also noted that the xylose yield showed steeper increasing rate than the glucose yield because of higher degradability of hemicellulose than cellulose. At 48 h, the transgenic plant AL 10/9-40 had glucan and xylan conversions of 46.0 and 41.4%, respectively. These values were 10-11% higher than those for AL RCK, indicating that lignin modification enabled more effective biomass carbohydrate conversion.

To greatly improve carbohydrate conversion of switchgrass, a lime loading of 0.1 g/g raw biomass was applied at a wide range of residence times. At this specific lime loading, the total reducing sugar yield was noticeably improved as the residence time increased from 1 to 12 h at which it reached the best production of 2.48 and 2.93 times those of untreated

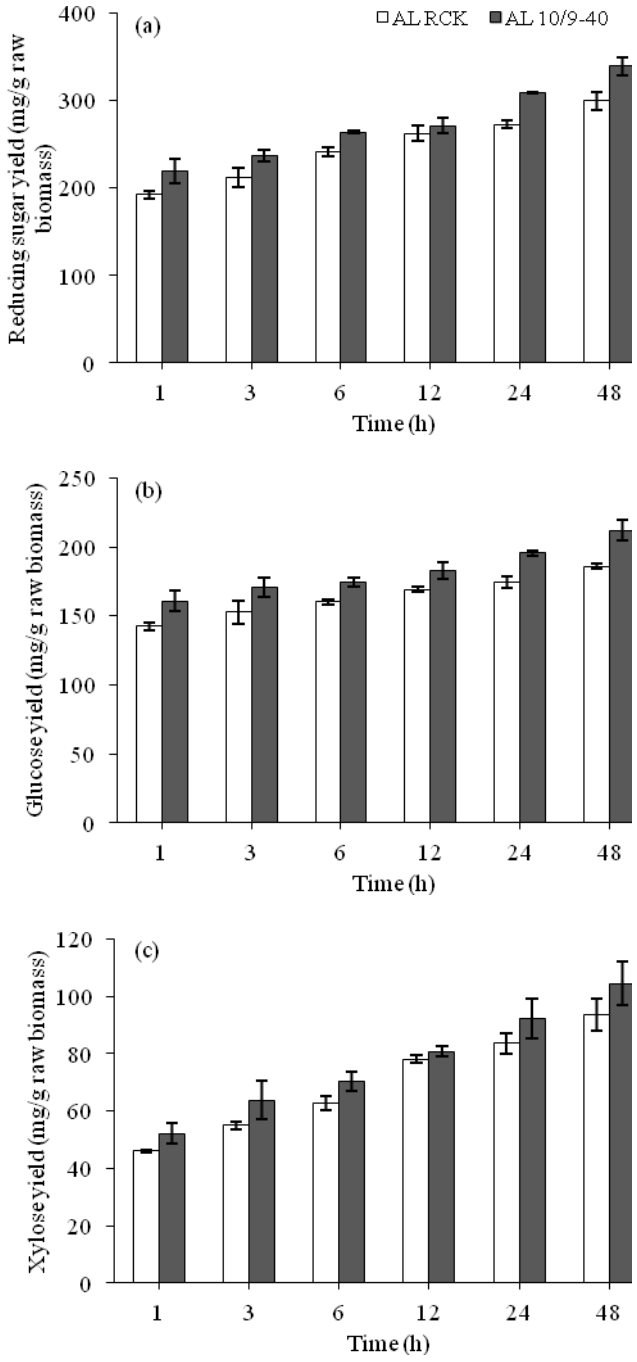

Figure 1. Reducing sugar yield (a), glucose yield (b), and xylose yield (c) from conventional and transgenic switchgrass pretreated with lime (0.05 g/g raw biomass) at 50 ℃.

biomass for AL RCK and AL 10/9-40, respectively (Figure 2a). The glucose and xylose yields both had the same optimal residence time (12 h) as the total reducing sugar yield (Figure 2b and 2c). Previous results (Xu et al., 2010a) reported that a residence time of 24 h was required to maximize the sugar production from switchgrass for lime pretreatment at 50 ℃. The reason that resulted in the difference in the optimal residence time between this study and the previous one, lies in the fact that different varieties of switchgrass were used in the two studies. As with the improvement in carbohydrate conversion of switchgrass achieved at 0.05 g lime/g raw biomass using the transgenic plant, both glucan and xylan conversions for AL 10/9-40 were considerably higher than those for AL RCK at the use of 0.1 g lime/g raw biomass. This further demonstrated the positive impact of lignin modification through genetic transformation on pretreatment efficiency.

When a lime loading of 0.15 g/g raw biomass was employed, the total reducing sugar, glucose, and xylose yields for both conventional and transgenic switchgrass plants started to level off at 6 h beyond which the sugar yields were not significantly ($P>0.05$) improved (Figure 3). The total reducing sugar yields at 6 h were 2.54 and 2.99 times those of untreated biomass for AL RCK and AL 10/9-40, respectively (Figure 3a). At this recommended residence time for the loading of 0.15 g lime/g raw biomass, the glucan conversion for the transgenic plant was 80.0%, an increase of 10% as compared with that for the conventional plant (Figure 3b). This improvement was comparable with those obtained at lime loadings of 0.05 and 0.10 g/g raw biomass. Unlike glucan conversion, the xylan conversion was enhanced by only 5% using the transgenic plant (Figure 3c). This was caused

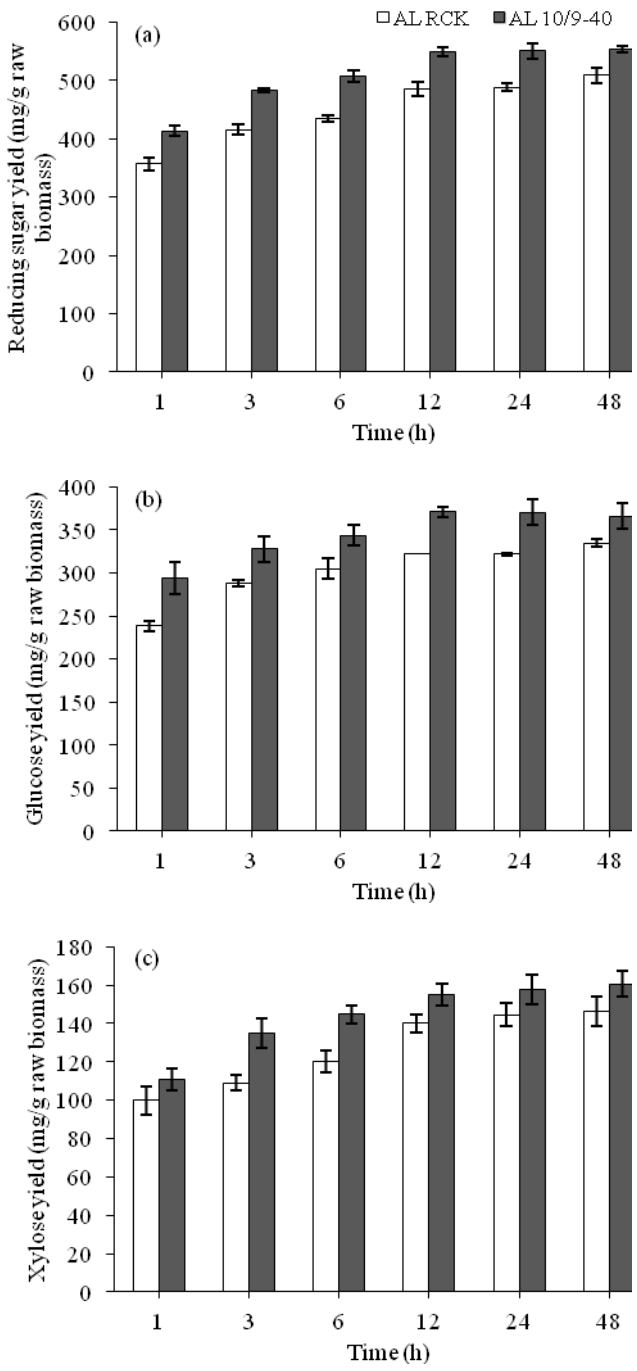

Figure 2. Reducing sugar yield (a), glucose yield (b), and xylose yield (c) from conventional and transgenic switchgrass pretreated with lime (0.1 g/g raw biomass) at 50 ℃.

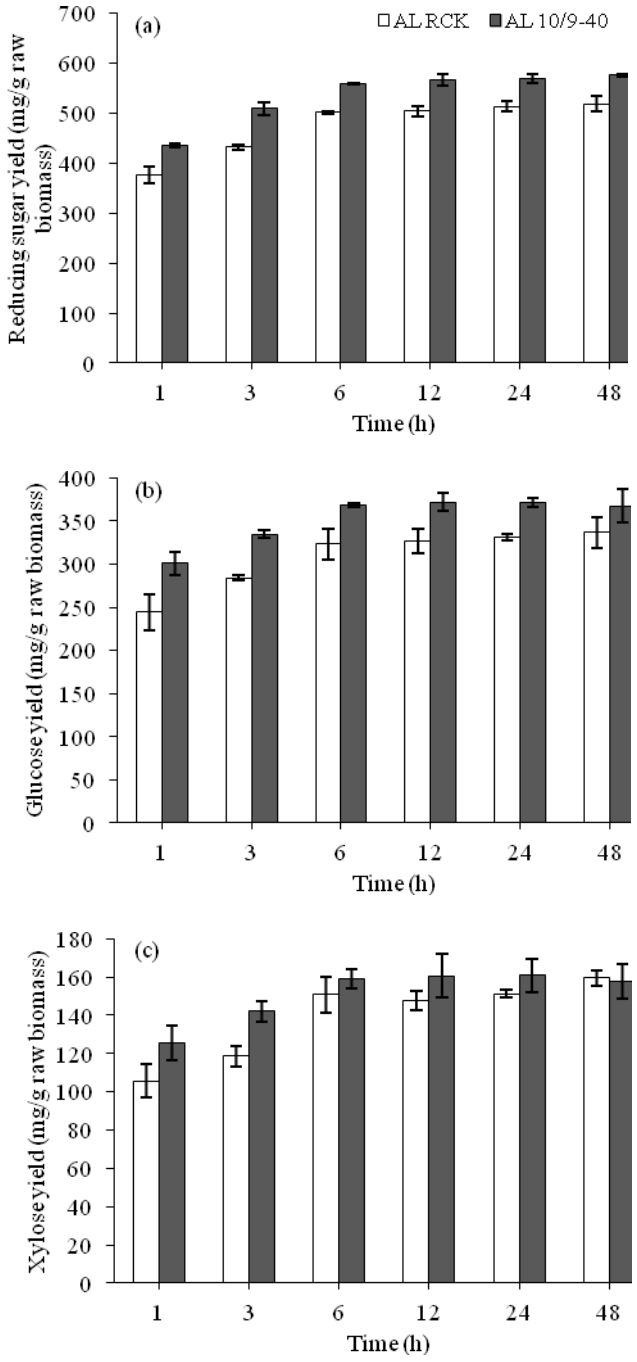

Figure 3. Reducing sugar yield (a), glucose yield (b), and xylose yield (c) from conventional and transgenic switchgrass pretreated with lime (0.15 g/g raw biomass) at 50 ℃.

by the continued increase of sugar yield in the conventional biomass but the unchanged sugar production in the transgenic plant when the lime loading was raised from 0.1 to 0.15 g/g raw biomass. As pretreatment severity increases, the enhancement of sugar release from biomass using transgenic plants may become less noticeable (Chen and Dixon, 2007), because more severe pretreatment conditions may lead to greater carbohydrate loss for transgenic biomass during the pretreatment (Wang et al., 2012), while better sugar yield could be attained for non-transgenic plant at these harsh conditions. The results imply that the effect of genetically engineering lignin biosynthesis on enhancing sugar release from the biomass can be maximized when proper pretreatment conditions are used. Moreover, the evidence that the carbohydrate conversion was yet improved irrespective of high lignin content in the transgenic plant, corroborates previous findings (Studer et al., 2011; Wang et al., 2012) that higher S/G and/or ASL/AIL ratios in cellulosic biomass may overcome the recalcitrance of lignin-carbohydrate complex to its biochemical conversion even in the presence of high lignin content.

3.3 Pretreatment with combination of lime and NaOH

The aforementioned results suggest that two sets of conditions (0.1 g lime/g raw biomass, 12 h; 0.15 g lime/g raw biomass, 6 h) were favorable for best sugar yields of switchgrass with lime pretreatment at 50 ℃. With the goal of achieving effective alkali pretreatment at ambient temperature within reasonable periods of residence time, a stronger base, NaOH, was used together with lime to maintain high alkalinity throughout the pretreatment. A previous study on pretreatment of switchgrass (cv. Performer) using the

combination of lime and NaOH has revealed the major role of NaOH in improving biomass digestibility (Xu and Cheng, 2011). Hence, the first part of the study on the combined alkali pretreatment was an investigation of the effects of NaOH loading and residence time on pretreatment efficiency, after which the impact of lime loading was then explored.

Since both NaOH (Wang et al., 2012) and lime pretreatments showed similar sugar improvements of switchgrass using the transgenic plants as compared with the conventional biomass, the study on ambient temperature pretreatment with the combination of lime and NaOH was solely carried out for the transgenic plant AL 10/9-40, and the optimized pretreatment conditions were then applied to the conventional plant. As displayed in Figure 4a, with the increase of NaOH loading from 0.05 to 0.1 g/g raw biomass, the total reducing sugar yield was significantly ($P<0.05$) enhanced. The extension to 0.15 g NaOH/g raw biomass did not favor a continued increase of total sugar production. This is presumably because the solid recoveries at the use of 0.15 g NaOH/g raw biomass were lower than those at 0.1 g NaOH/g raw biomass, and more carbohydrates were lost during the pretreatment correspondingly (data not shown). At all the NaOH loadings, the total reducing sugar yields at 3 h were significantly ($P<0.05$) less than those obtained at 6 and 9 h, whereas there was no significant ($P>0.05$) difference in the sugar production between 6 and 9 h. The glucose and xylose yields exhibited alike trends as that of total reducing sugar yield with the changes of NaOH loading and residence time (Figure 4b and 4c). A closer observation of the production of these two monomeric sugars shows that the extent of increase in xylose yield was greater than that in glucose yield as the pretreatment severity increased. The amorphous and

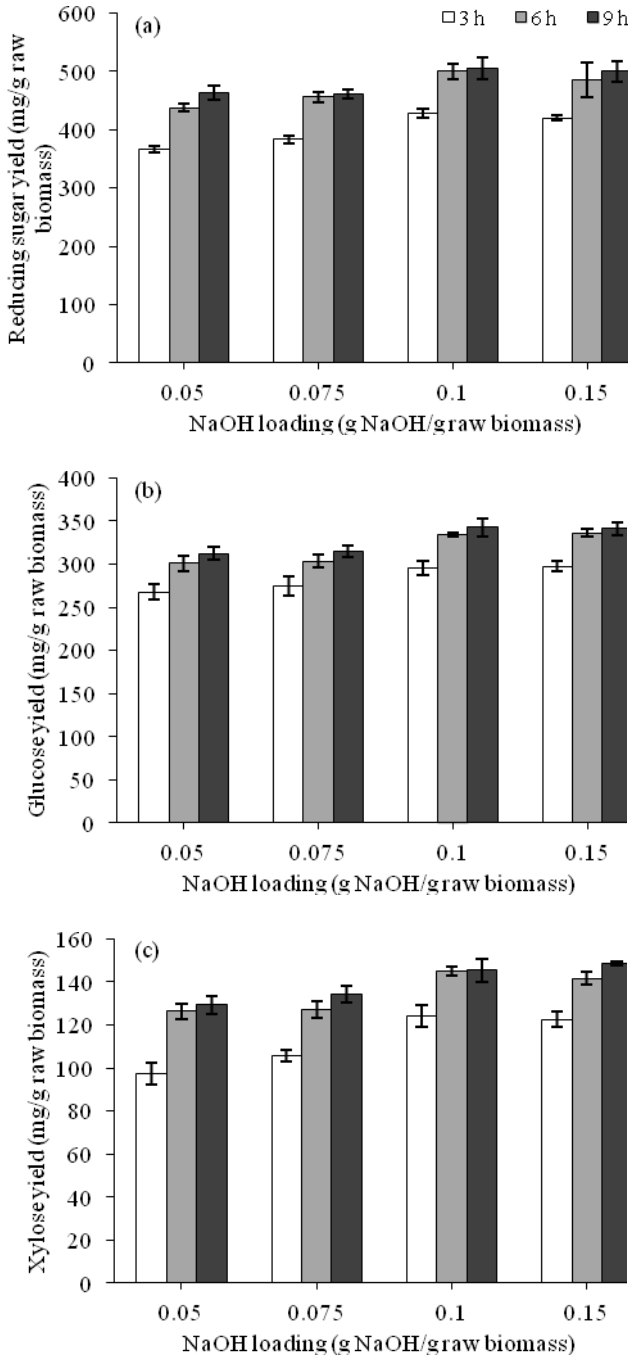

Figure 4. Sugar yield from transgenic switchgrass pretreated with lime (0.1 g/g raw biomass) and NaOH (0.05, 0.075, 0.1, and 0.15 g/g raw biomass) at 21 ℃.

branched structure of hemicellulose is easier to break down than the semicrystalline cellulose, which makes hemicellulose more susceptible to alkali attack. At the best conditions of 0.1 g NaOH/g raw biomass and 6 h for the combined alkali pretreatment with a lime loading of 0.1 g/g raw biomass at ambient temperature, the total reducing sugar yield of the transgenic plant reached 65.9% of theoretical maximum, 2.67 times that of untreated biomass, and the glucan and xylan conversions were 72.7 and 57.5%, respectively.

Based on the optimized NaOH loading and residence time stated above, lime loadings ranging from 0 to 0.1 g/g raw biomass were studied. The total reducing sugar, glucose, and xylose yields at 0 g lime/g raw biomass were all markedly lower than those at the other lime loading levels, while no significant ($P>0.05$) difference in the sugar production was observed among lime loadings of 0.025, 0.05, and 0.075 g/g raw biomass (Figure 5). Two factors are believed to have contributed to the occurrence of this phenomenon. First, the combined use of lime and NaOH can sustain relatively higher pH than the pretreatment using NaOH alone (Xu and Cheng, 2011), because more lime particles will dissolve into the solution to provide more hydroxide ions as they are being consumed by biomass during the pretreatment (Wang and Cheng, 2011). As a result, such prevention from substantial pH drop throughout the pretreatment with the combination of lime and NaOH enabled better pretreatment effectiveness. This in turn explains why the sugar yields per gram of pretreated biomass with the use of lime loadings at or above 0.025 g/g raw biomass were found to be considerably higher than those without lime dosed. Another important cause is that the use of lime leads to better preservation of biomass constituents after pretreatment as calcium ions can form

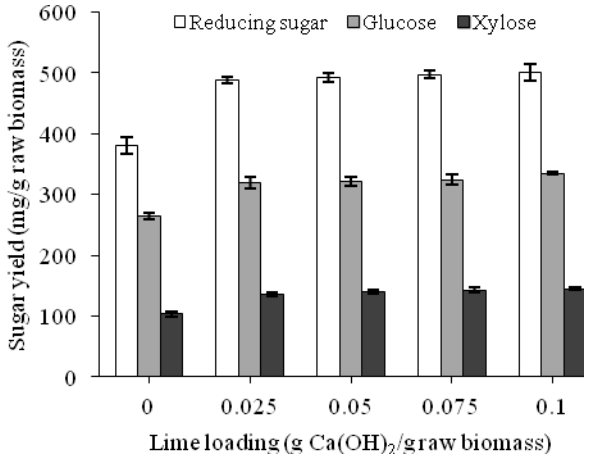

Figure 5. Sugar yield from transgenic switchgrass pretreated with lime (0, 0.025, 0.05, 0.075, and 0.1 g/g raw biomass) and NaOH (0.1 g/g raw biomass) at 21 ℃.

linkages with lignin and carbohydrate within the biomass (Torre et al., 1992; Xu et al., 2010a). This is supported by the results (data not shown) of solid recoveries showing that, with the increase of lime loading, the solid recovery was enhanced. Although the total reducing sugar, glucose, and xylose yields at the dose of 0.1 g lime/g raw biomass were 2.5, 4.9, and 7.1% respectively higher than those at the use of 0.025 g lime/g raw biomass, an increase of lime loading by 3 times would apparently not be an economical strategy in commercial scale. Therefore, a lime loading of 0.025 g/g raw biomass, a NaOH loading of 0.1 g/g raw biomass, and 6 h were recommended as the best conditions for the pretreatment of transgenic switchgrass using the combination of lime and NaOH at ambient temperature. At these optimal conditions, the total sugar production from switchgrass was improved by 11% after the biomass was genetically transformed.

3.4 Material balances

To evaluate the effects of the two pretreatment technologies on the compositional changes of switchgrass, material balances were performed on biomass pretreated under the optimal conditions (0.1 g lime/g raw biomass, 12 h, 50 ℃; 0.025 g lime/g raw biomass, 0.1 g NaOH/g raw biomass, 6 h, 21 ℃). Table 2 shows the changes of total solids, lignin, carbohydrates, and other components in both conventional and transgenic switchgrass after they were subjected to the pretreatment using lime alone and the combination of lime and NaOH. For lime pretreatment without NaOH addition, total solids of more than 80% were recovered after the pretreatment. The solid loss is mainly caused by the reductions of lignin, xylan, and other components such as organic compounds with uronic acid and acetyl groups, waxes, fats, resins, minerals, and gums (Samson et al., 2005). Contrarily, the glucan reduction was marginal due to its semicrystalline structure. In the case of pretreatment with the combination of lime and NaOH, fewer amounts of total solids were recovered as compared with the pretreatment using lime alone, simply because NaOH is stronger than lime in terms of alkalinity, leading to higher extent of biomass solubilization. As with lime pretreatment, the majority of solid loss occurred in the combined alkali pretreatment was associated with the removal of lignin, xylan, and others.

In a comparison of compositional changes between conventional and transgenic switchgrass, it was noted the solid recovery of the transgenic plant was slightly higher than that of the conventional plant under the two sets of pretreatment conditions. In particular, the lignin reduction was even less for the transgenic biomass after the pretreatment using lime

Table 2. Material balances from raw switchgrass to low temperature alkali pretreatment at the corresponding optimal conditions

Plant line	Pretreatment conditions	Total solids[a] (g/100 g initial dry biomass)	Composition of solids fractions[a] (g/100 g initial dry biomass)						
			Lignin	Glucan	Xylan	Galactan	Arabinan	Ash	Others
AL RCK	raw biomass	100	21.46 (0.16)	40.11 (0.96)	22.07 (0.75)	0.97 (0.05)	2.49 (0.07)	2.74 (0.02)	11.91 (0.86)
	12 h, 0.1 g $Ca(OH)_2$/g raw biomass at 50 ℃	82.01 (0.23)	14.95 (0.45)	37.27 (0.32)	17.88 (0.25)	0.48 (0.01)	1.93 (0.02)	1.43 (0.14)	8.07 (1.07)
	6 h, 0.025 g $Ca(OH)_2$/g raw biomass, 0.1 g NaOH/g raw biomass at 21 ℃	76.37 (0.36)	14.32 (0.12)	34.43 (0.80)	16.93 (0.48)	0.47 (0.01)	1.78 (0.08)	1.05 (0.07)	7.39 (0.72)
AL 10/9-40	raw biomass	100	20.21 (0.26)	41.42 (0.86)	22.21 (0.32)	1.15 (0.02)	3.18 (0.07)	2.68 (0.03)	9.15 (1.07)
	12 h, 0.1 g $Ca(OH)_2$/g raw biomass at 50 ℃	85.57 (1.40)	15.02 (0.33)	39.35 (1.27)	18.84 (0.24)	0.51 (0.02)	2.09 (0.03)	1.53 (0.55)	8.23 (0.91)
	6 h, 0.025 g $Ca(OH)_2$/g raw biomass, 0.1 g NaOH/g raw biomass at 21 ℃	78.60 (0.68)	13.58 (0.17)	36.18 (0.33)	17.84 (0.03)	0.57 (0.01)	2.00 (0.06)	1.13 (0.10)	7.3 (0.63)

[a] Data are means of three replicates with standard deviation included in the parenthese.

alone, but it was comparable between the two plants after the combined alkali pretreatment because of more severe attack of NaOH to lignin than lime. Regardless of the extent of lignin reduction, the carbohydrate conversion efficiency was greatly improved using the transgenic plant. These further imply that, while genetic modification of lignin did not significantly reduce the lignin content in raw biomass, the probable alteration of structure of lignin and/or lignin-carbohydrate complex due to the increased S/G ratio (Albersheim et al., 2011) could be beneficial for structure disruption after pretreatment, thus leading to better digestibility of cellulose and hemicellulose.

3.5 Comparison of pretreatment effectiveness

Together with our previous study on NaOH pretreatment of transgenic switchgrass (Wang et al., 2012), the two pretreatment methods explored in this study were compared with one another with respect to the efficiency of these pretreatments as well as the impact of genetic transformation on pretreatment effectiveness. Table 3 summarizes the overall carbohydrate, glucan, and xylan conversions of both conventional and transgenic switchgrass plants after the biomass underwent pretreatment and enzymatic hydrolysis. The carbohydrate conversions for NaOH pretreatment, as shown in Table 3, were mostly higher than those for lime pretreatment, while both of them gave much greater conversions of carbohydrate than the combined alkali pretreatment. The major difference between the three pretreatment technologies is temperature which is believed to play a crucial role in achieving high pretreatment efficiency. Further examination of carbohydrate conversions of both conventional and transgenic plants reveals that the overall carbohydrate conversion for

Table 3. Comparisons of carbohydrate conversion efficiencies after enzymatic hydrolysis of conventional and transgenic switchgrass pretreated under various alkali conditions

Plant line	Pretreatment reagent	Overall carbohydrate conversion (%)	Glucan conversion (%)	Xylan conversion (%)
AL RCK	NaOH[a]	63.3 (3.01)[d]	74.6 (5.64)	58.2 (5.31)
	lime[b]	64.7 (1.71)	72.2 (2.24)	55.8 (1.91)
	lime + NaOH[c]	58.4 (0.72)	65.1 (1.84)	49.8 (2.78)
AL 10/9-40	NaOH[a]	75.1 (4.20)	85.5 (5.30)	68.7 (4.31)
	lime[b]	72.3 (2.72)	80.6 (1.85)	61.4 (0.92)
	lime + NaOH[c]	64.3 (0.96)	69.3 (2.41)	53.7 (1.45)

[a] Pretreatment with 1% (w/v) NaOH at 121 ℃ for 30 min; Data were obtained from Wang et al. (2012).

[b] Pretreatment with 0.1 g lime/g raw biomass at 50 ℃ for 12 h.

[c] Pretreatment with 0.025 g lime/g raw biomass and 0.1 g NaOH/g raw biomass at 21 ℃ for 6 h.

[g] The number in parenthese is standard deviation of three replicates.

AL 10/9-40, from the ambient temperature pretreatment to the pretreatments at higher temperatures, was raised by 12-17%, which was considerably greater than that for AL RCK. This phenomenon suggests that the transgenic plant could be more susceptible to biochemical conversion, and this also explains the general improvement in sugar release from switchgrass after its lignin was genetically modified. For the lime pretreatment at 50 ℃, the glucan and xylan conversions of the transgenic plant were respectively 12 and 10% higher than those of the wild-type plant. Similarly, when the combined alkali pretreatment at ambient temperature was employed, the improvement of glucan and xylan conversions from the conventional to transgenic plant was 7 and 8%, respectively. As a result of lower reactivity of biomass pretreated at milder temperatures, the degree of these increases was less than that in the case of applying high temperature (121 ℃) NaOH pretreatment to switchgrass.

4. CONCLUSIONS

Low temperature alkali pretreatment was effective in releasing fermentable sugars from both conventional and transgenic switchgrass after enzymatic hydrolysis. In general, the lime pretreatment at 50 ℃ was able to yield more sugars than the combined alkali pretreatment at ambient temperature due to the significant impact of temperature on pretreatment efficiency. Although genetic transformation of switchgrass did not considerably reduce lignin content, the increased S/G ratio was believed to alter biomass structure for easier chemical attack during pretreatment, thus enhancing sugar production in hydrolysate. The impact of lignin down-regulation on enzyme loading requirement needs to be explored in future work.

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CHAPTER 5: MODELING SUGAR PRODUCTION FROM SWITCHGRASS AFTER ALKALI PRETREATMENT AND ENZYMATIC HYDROLYSIS

ABSTRACT

The yields of glucose and xylose after lime pretreatment and enzymatic hydrolysis of switchgrass were predicted using two different modeling approaches: multiple linear regression (MLR) and a modified SIR (mSIR) model. The amount of alkali loading and residence time applied in the pretreatment were used as model inputs in both approaches. For the MLR model, the values of correlation coefficient R^2 (ranging from 0.90 to 0.97) for both glucose and xylose yields were comparable between the training and testing data sets, while the corresponding root mean square error (RMSE) values for the testing data were considerably higher than those obtained with the training data. The differences between the predicted and experimental values for glucose and xylose yields were within 3-6% and 3-10%, respectively. The mSIR model yielded comparable R^2 and RMSE values for glucose yield with the MLR model. However, the predictions for xylose yield by the mSIR model were more accurate than those obtained with the MLR model. Further validation of the mSIR model for two other alkali pretreatments (NaOH, combined lime and NaOH) show that the mSIR model had the best performance for combined alkali pretreatment.

Keywords: Modeling; Alkali pretreatment; Switchgrass; Enzymatic hydrolysis; Sugar

1. INTRODUCTION

The worldwide energy consumption continues to increase rapidly, especially in the developing countries. In the meantime, non-renewable petroleum-based energy reserve is facing a progressive depletion and most countries' unavoidable dependence on petroleum imported from the politically unstable Middle East makes the global economy vulnerable to oil supply disruption and price hikes (Sanchez and Cardona, 2008; Yang and Wyman, 2008). On top of all these issues, negative environmental impact such as global climate change, influenced by the largest contributor-burning petroleum-based fuels into polluting gases, is another concern that needs to be addressed when resorting to alternatives for replacing fossil fuels (Sanchez and Cardona, 2008). Ethanol produced from lignocellulosic materials has a great potential to partially replace the current petroleum-based gasoline use in the transportation sector (Sun and Cheng, 2002; Xu et al., 2011). Switchgrass (Panicum virgatum L.), a perennial grass, is considered a promising energy crop due to its high biomass yield across a wide geographic range, low agricultural inputs including water and nutritional requirements, and positive environmental impacts such as carbon sequestration and soil remediation (Keshwani and Cheng, 2009).

Unlike the relatively easy conversion of sugary food crop feedstocks to fermentable sugars, the conversion of lignocellulosic biomass still remains challenging because of the recalcitrant nature of biomass components (Himmel et al., 2007). This recalcitrance is mainly caused by high lignin content, high crystallinity of cellulose, and the limited number of sites that are available for enzyme attack (Kumar et al, 2009). All these intrinsic factors reduce the

efficiency of carbohydrates conversion in enzymatic hydrolysis. As a result, pretreatment is required to alter the structure of lignocellulosic biomass to make carbohydrates more accessible to hydrolytic enzymes.

The major goals of pretreatment are to remove lignin from the biomass, reduce cellulose crystallinity, and increase the surface area and porosity of biomass. Detailed reviews of various pretreatment technologies including physical, chemical, physical-chemical, and biological processes can be found in previously published work (Moiser et al., 2005; Sun and Cheng, 2002). Of the promising pretreatment technologies, alkali pretreatment has received much attention due to its potentially low cost and relatively high effectiveness in removing lignin from the biomass and increasing the biomass porosity (Wang et al., 2010; Xu et al., 2010a). Alkali pretreatment also removes acetyl and different kinds of uronic acid substitutions on hemicellulose, which in turn improves the efficiency of enzymatic hydrolysis of cellulose and hemicellulose (Chang and Holtzapple, 2000).

The current cost associated with pretreatment and enzymatic hydrolysis is still a major contributor to the costly cellulosic ethanol production (Lynd et al. 2008; Yang and Wyman 2008). The cost-effectiveness of cellulosic sugar can be potentially achieved by developing highly efficient conversion technologies based on the information regarding what factors are critically important for impeding sugar release from the biomass (Lynd et al., 2008). Simulating the conversion process with robust models is believed to be useful in identifying any physical and/or chemical properties that may inhibit the desirable reactions for sugar production. In addition, valid predictions for meaningful outputs such as sugar yield

in the hydrolysate can provide significant value in economic analysis of a cellulosic ethanol plant.

Pretreatment and enzymatic hydrolysis are the two technologically and economically critical steps that have been extensively studied for modeling purposes. A comprehensive review on modeling biochemical conversion of lignocellulosic materials for sugar production has been reported by Wang et al. (2011), who discussed the advantages and disadvantages of a number of important models that have been developed for predicting the changes of biomass composition during pretreatment and the sugar yields in enzymatic hydrolysis. Statistical model such as multiple linear regression equation is relatively easy to develop and optimize but this type of model does not offer insight on kinetic mechanism and is prone to exhibit over-prediction for the outputs. The foremost valuable approach for process simulation is the kinetic modeling which has been widely explored. Because it is developed based on inherent reaction mechanisms, the kinetic model provides useful knowledge for a thorough understanding of the process for effective process manipulation and optimization. Apart from the numerous studies on kinetic modeling (Conner et al. 1985; Dang and Nguyen 2006, 2007; Fuentes et al. 2011; Jacobsen and Wyman 2000; Kadam et al. 2004; Kim and Holtzapple 2006; Keshwani and Cheng 2010; South et al. 1995), some non-kinetic or fuzzy inference models attract particular interest in modeling the complex conversion of lignocellulosic biomass (O'Dwyer et al. 2008; Redding 2009; Keshwani and Cheng 2010).

In raw cellulosic biomass, cellulose and hemicellulose generally are not readily hydrolyzable, with only a small portion of the carbohydrates being susceptible to enzymatic

attack. After the pretreatment process, these carbohydrates become more susceptible to cellulolytic enzymes and the susceptibility depends on the environment which is defined herein as pretreatment condition. Most or all of the carbohydrates in the pretreated solids can then be "recovered" in the form of (or converted into) fermentable sugars such as glucose and xylose. This phenomenon bears an analogy with the behavior of an epidemic disease that is normally predicted by a SIR (S: susceptible; I: infectious; R: recovered) model.

The objective of this study was to develop models to predict sugar yield after alkali pretreatment and enzymatic hydrolysis of switchgrass. Two different modeling approaches including statistical multiple linear regression (MLR) and the modified SIR (mSIR) models were used to predict both glucose and xylose yields generated in enzymatic hydrolysis of switchgrass pretreated with lime. The MLR model was developed based on the statistical point of view on the conversion system, while the development of the mSIR model was based on the similarities between the spread of epidemic disease and the behavior of carbohydrates during the conversion process, and a kinetic modeling approach for describing the changes of biomass components during pretreatment (Dang and Nguyen, 2006, 2007). In both approaches, the inputs for the models are expressed as C (alkali loading) and t (residence time), while the output is expressed as B (sugar yield in the hydrolysate). In addition to its application to lime pretreatment, the mSIR model was evaluated as to its validity for two other alkali pretreatments (NaOH and combined lime and NaOH) on switchgrass.

2. MODELING APPROACHES

2.1 Multiple linear regression model

The general form of the multiple linear regression (MLR) models that were developed from experimental data to predict glucose and xylose yields of switchgrass after lime pretreatment and enzymatic hydrolysis is shown as:

$$B = a_0 + a_c \cdot C + a_t \cdot t + a_{ct} \cdot C \cdot t + a_{cc} \cdot C^2 + a_{tt} \cdot t^2 \quad (1)$$

where B represents the yield of a particular sugar (glucose or xylose) at any given chemical loading (C) and residence time (t) employed in the pretreatment. The coefficients (a_0, a_c, a_t, a_{ct}, a_{cc}, a_{tt}) for the constant, linear, interaction, and quadratic terms and the significance of each term for both glucose and xylose yields were determined using the REGSTATS function in MATLAB (Version R2011a, Mathworks, Cambridge, MA). The MATLAB code used to obtain the MLR model coefficients and relevant p-values is shown in Appendix D.

2.2 Modified SIR model

The second modeling approach used in this study is a modified SIR (mSIR) model which was developed based on the similarity of system behavior between the spread of epidemic disease and the sugar release from lignocellulosic biomass after pretreatment and enzymatic hydrolysis. The general form of a simple SIR (S: susceptible; I: infectious; R: recovered) model for predicting the behavior of disease spread among a certain group of people can be described as follows:

$$\frac{dS}{dt} = -\beta IS$$
$$\frac{dI}{dt} = \beta IS - vI \quad (2)$$
$$\frac{dR}{dt} = vI$$

where β is the contact rate and v is the recovery rate.

During biomass pretreatment, the amount of carbohydrates in the solids continuously decreases as the pretreatment time increase. The rate of this change is dependent on both pretreatment conditions and the remaining carbohydrates in pretreated biomass (Keshwani and Cheng, 2010). After the subsequent enzymatic hydrolysis, the yield of fermentable sugars normally exhibits an increase as the pretreatment time is raised but it levels off after a certain period of time (Wang and Cheng, 2011; Xu et al., 2010b). The dynamic change of sugar yield is subject to the quantity of carbohydrates in the pretreated biomass and the susceptibility of these carbohydrates to enzyme attack. In light of the description of the entire conversion process including pretreatment and enzymatic hydrolysis, for the first time, this phenomenon was simulated using the following mSIR model:

$$\frac{dB_1}{dt} = -\mu(t, C, T)B_1$$
$$\frac{dB_2}{dt} = h(B_1 - B_2) \quad (3)$$

where B_1 is the amount of monosaccharide (glucose or xylose, mg/g raw biomass) of the corresponding polysaccharide (cellulose or xylan) present in the pretreated biomass, B_2 is the

yield of sugars (glucose or xylose, mg/g raw biomass) after enzymatic hydrolysis of pretreated biomass, μ is a rate coefficient of pretreatment reaction that is related to pretreatment conditions including residence time (t), chemical loading/concentration (C), and temperature (T), and h is a constant that is based on the accessibility of carbohydrates in pretreated biomass to hydrolytic enzymes.

The effect of temperature on the rate coefficient μ was not considered in this study since lime pretreatment that was used for model development was carried out at constant temperature. The general form of μ used in the mSIR model is based on previously published work by Dang and Nguyen (2006, 2007), who derived the expression shown in Equation (4) to relate the pretreatment rate coefficient to residence time and chemical concentration for developing a kinetic model to predict carbohydrate losses of lignocellulosic materials.

$$\mu = knt^{n-1}C^{b} \tag{4}$$

In Equation (4), k, n, and b are constant that are based on the intrinsic properties of lignin-carbohydrate complex and the heterogeneous reactions involved in pretreatment. The effect of chemical loading on μ is assumed to be consistent as residence time increases for lime pretreatment, thus the rate of change of chemical concentration with respect to time is assumed to be zero. This assumption is based on the fact that the concentration of hydroxide ions (OH^-) rendered by lime in the beginning of pretreatment accounts for a small percentage of total amount of OH^- loaded due to the poor solubility of lime, and the suspended lime particles will continuously dissolve into the solution as OH^- is reacted with biomass

components, and consequently the pH can be presumed to maintain at a relatively stable level (Wang and Cheng, 2011). If the chemical loading is assumed constant during the course of the pretreatment, the mSIR model indicated in Equation (3) can be simplified to Equation (5) by substituting μ with Equation (4).

$$\frac{dB_2}{dt} = h(B_{1i} \cdot exp(-kt^n C^b) - B_2) \quad (5)$$

Note that B_{1i} is the amount of monosaccharide (glucose or xylose, mg/g raw biomass) of the corresponding polysaccharide (cellulose or xylan) present in the raw biomass. The model parameters were determined using the optimization toolbox (FMINSEARCH) in MATLAB (Version R2011a, Mathworks, Cambridge, MA). The MATLAB code used to obtain the model parameters is provided in Appendix B.

3. MATERIALS AND METHODS

3.1 Biomass preparation

Conventional (denoted as AL RCK) and transgenic (denoted as AL 10/9-40) switchgrass (cv. Alamo) (harvested in October, 2010) were obtained from the air-conditioned roof-top greenhouse at the North Carolina State University Phytotron in Raleigh, NC. The collected biomass was size reduced to pass a 2-mm sieve using a Wiley Laboratory Mill (Thomas, Model No. 4) and stored in sealed plastic bags at room temperature until use for composition analysis and alkali pretreatments.

3.2 Pretreatment

Lime and combined alkali (lime+NaOH) pretreatments were carried out in a water bath (Model 205, Fisher Scientific) at 50 ℃ and on a flat counter at 21 ℃, respectively. For each pretreatment, switchgrass samples (3 g per replicate) were mixed thoroughly with particular chemicals in deionized (DI) water in a solid to liquid ratio of 1:10 in glass serum bottles before the bottles were sealed for pretreatments. After pretreatment, the biomass slurry was vacuum filtered across Fisherbrand P8 filter paper in a porcelain Buchner filter funnel, and the pretreated biomass recovered by filtration was washed with DI water in the amount of 100 ml DI water/g raw biomass to remove excess chemicals and/or other pretreatment byproducts. The wet solids were completely transferred to a preweighed plastic bag, weighed, and stored sealed at 4 ℃ for the subsequent enzymatic hydrolysis experiment. A small portion of the wet pretreated biomass was weighed and dried in a convection oven (Isotemp, Fisher Scientific) at 105 ℃ to determine solid recovery.

Two-thirds of the experimental data (12 experimental conditions: 0.05, 0.1, and 0.15 g lime/g raw biomass for 1, 6, 12, and 48 h) generated from lime pretreatment and enzymatic hydrolysis of switchgrass was used as a training data set to develop both MLR and modified SIR models, and the remaining one-third (6 experimental conditions: 0.05, 0.1, and 0.15 g lime/g raw biomass for 3 and 24 h) was used as a testing data set for model validation. The validity of modified SIR models was then further evaluated by applying the models to sodium hydroxide (NaOH) pretreatment and combined alkali pretreatment. The data for

Table 1. Experimental conditions for three different types of alkali pretreatment of switchgrass

Switchgrass variety	Pretreatment reagent	Temperature (℃)	Lime loading (g/g raw biomass)	NaOH loading (g/g raw biomass)	Residence time (h)
Alamo (this study)	lime	50	0.05, 0.10, 0.15	N/A	1, 3, 6, 12, 24, 48
	lime + NaOH	21	0.10	0.05, 0.075, 0.10, 0.15	3, 6, 9
Performer (Xu et al., 2010b)	NaOH	50	N/A	0.05, 0.10, 0.20	1, 3, 6, 12, 24, 48
		21	N/A	0.05, 0.10, 0.20	1, 3, 6, 12, 24, 48, 96

NaOH pretreatment was obtained from a previous study by Xu et al. (2010b). Table 1 shows the conditions employed in all three alkali pretreatments.

3.3 Enzymatic hydrolysis

Enzymatic hydrolysis experiments (triplicates per sample) were carried out in 50 ml plastic tubes in a controlled environment reciprocal shaking bath (Model C76, New Brunswick Scientific) at 50 ℃ and 150 rpm. The wet pretreated biomass equivalent to 0.5 g dry basis was immersed in 0.05 M sodium citrate buffer to maintain a pH of 4.8 with a total liquid volume of 15 ml. Cellic CTec2 (enzyme complex of aggressive cellulases, high level of β-glucosidase, and hemicellulase) and Cellic HTec2 (endoxylanse with cellulase background) donated by Novozymes North America Inc. (Franklinton, NC) were used in this study. The densities of CTec2 and HTec2 were reported to be 1.203 and 1.238 g/ml respectively according to the enzyme manufacturer. Excessive dosage of CTec 2 and HTec 2 of 40% and 6% (g enzyme/g dry biomass), respectively, were employed to avoid the impact of enzyme limitation on the correlation between pretreatment conditions and sugar production. Sodium azide (0.3% (w/v)) was added to the hydrolysis mixture to prevent microbial growth. The hydrolysis was carried out for 72 hours after which the hydrolysate was centrifuged (Model 5810R, Eppendorf) at 4 ℃ and 4000 rpm for 15 min and the supernatant was collected in 15 ml plastic storage tubes and stored at -80 ℃ for further analysis.

3.4 Sugar analysis

Structural carbohydrates in raw switchgrass were measured using a two-stage sulfuric acid hydrolysis procedure outlined in the NREL LAP " Determination of Structural Carbohydrates and Lignin in Biomass" (Sluiter, 2006). A high performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) (Dionex ICS-5000, Dionex Corporation, Sunnyvale, CA, USA) was used to measure monosaccharides (glucose, xylose, galactose, and arabinose) generated from the composition analysis of raw biomass and from the enzymatic hydrolysis of pretreated biomass. The HPAE-PAD system was equipped with a CarboPac PA1 (100μeq per 4 x 250 mm) analytical column operated at 18 ℃ with 0.018 M potassium hydroxide as the mobile phase at a flow rate of 0.9 ml/min, a CarboPac PA1 guard column (4 x 50 mm), a thermostatted autosampler, and a quaternary pump. An internal standard (fucose) solution of 1 g/l was added to each sugar sample for the determination of amounts of monomeric sugars based on a proportional relationship between the ratio of fucose to each simple sugar concentrations and the ratio of fucose to individual sugar peak areas.

3.5 Analysis of models

The performance of the models was assessed using correlation coefficients R^2 from the regression line fitting measured values to predicted values, and root mean square errors (RMSE) associated with the predictive capability of the models for training and testing data sets. The normality of prediction residuals was evaluated using the Shapiro-Wilk test (Shapiro and Wilk, 1965) to verify that the outputs (glucose or xylose yields in the

hydrolysates) are not significantly over or under-predicted by the models. The MATLAB code used for computing R^2 and RMSE, conducting the Shapiro-Wilk test, and generating normality plots is given in Appendix B.

4. RESULTS AND DISCUSSION

4.1 Multiple linear regression model

The training data set generated from lime pretreatment and enzymatic hydrolysis was used to estimate the coefficients of multiple linear regression (MLR) models for both conventional and transgenic switchgrass. The estimated values of these coefficients are shown in Table 2. The significance analysis (at an alpha level of 0.05) of constant, linear (C and t), interaction ($C\ t$), and quadratic (C^2 and t^2) terms indicates that only the interaction term was not significant. Hence, the MLR models without the interaction term were evaluated in terms of their performance in predicting the yields of glucose and xylose in hydrolysate based on the inputs including residence time and lime loadings.

For conventional switchgrass, the training-R^2 values for the prediction of glucose and xylose yields were 0.95 and 0.97, respectively, and the corresponding RMSE values show that the MLR models were capable to predict the outputs with accuracy within 4.1 and 2.6% (Table 3). The testing-R^2 values in both cases were found to be slightly lower than those obtained with the training data. While the testing-RMSE value for glucose yield was comparable to the RMSE value for the training data, the RMSE value for xylose yield considerably increased for the testing data, resulting in prediction for xylose yield off by 6.8%. This is probably because that xylan is easier to degrade during the pretreatment than

Table 2. Estimates of coefficients for the MLR model (Eq.1) for glucose and xylose yields from Alamo (cv.) switchgrass after lime pretreatment and enzymatic hydrolysis

Plant line	Model	a_0 (*p*-value)	a_c (*p*-value)	a_t (*p*-value)	a_{ct} (*p*-value)	a_{cc} (*p*-value)	a_{tt} (*p*-value)
AL RCK	glucose yield	-140.7 (<0.05)	6.47E3 (<0.05)	7.03 (<0.05)	4.41 (0.5795)	-2.56E4 (<0.05)	-0.12 (<0.05)
	xylose yield	-61.04 (<0.05)	2.42E3 (<0.05)	4.48 (<0.05)	-1.10 (0.7436)	-8.46E3 (<0.05)	-0.069 (<0.05)
AL 10/9-40	glucose yield	-177.4 (<0.05)	7.83E3 (<0.05)	6.85 (<0.05)	-2.41 (0.7240)	-3.05E4 (<0.05)	-0.11 (<0.05)
	xylose yield	-84.94 (<0.05)	3.15E3 (<0.05)	4.77 (<0.05)	-6.10 (0.0840)	-1.16E4 (<0.05)	-0.067 (<0.05)

Table 3. R^2 and RMSE values for the MLR and mSIR models applied to training and testing data sets generated from lime pretreatment and enzymatic hydrolysis of Alamo (cv.) switchgrass

			Training data set		Testing data set	
Plant line	Modeling approach	Model output	R^2	RMSE (mg/g)	R^2	RMSE (mg/g)
AL RCK	MLR	glucose yield	0.95	18.28	0.90	24.79
		xylose yield	0.97	6.61	0.95	17.03
	mSIR	glucose yield	0.94	17.99	0.89	26.44
		xylose yield	0.97	6.63	0.92	11.17
AL 10/9-40	MLR	glucose yield	0.98	13.66	0.93	28.41
		xylose yield	0.92	17.31	0.87	24.98
	mSIR	glucose yield	0.95	18.71	0.92	24.99
		xylose yield	0.94	9.33	0.93	9.44

cellulose, which in turn leads to less variability of xylose yield in the hydrolysate with respect to the change of pretreatment conditions as the majority of xylan present in pretreated biomass is readily hydrolyzable. The finding that the MLR model performs better in predicting glucose yield than xylose yield in enzymatic hydrolysis was also reported by Redding (2009) for acid pretreatment. Figure 1 shows the correlation between predicted and

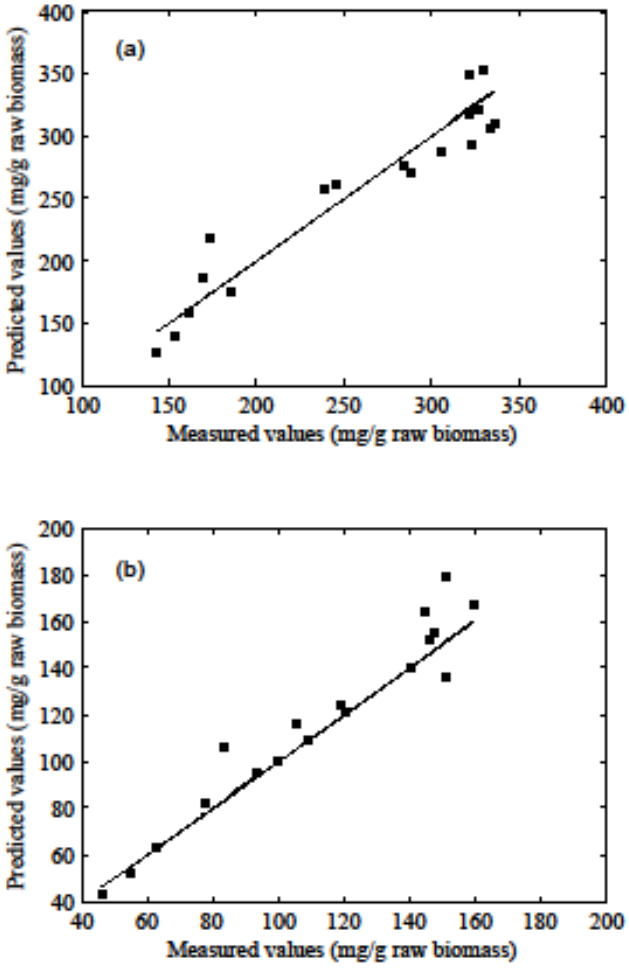

Figure 1. Correlation between predicted and measured values for the multiple linear regression models of glucose (a) and xylose (b) yields applied to the combined training and testing data sets for conventional switchgrass (AL RCK).

measured values of glucose and xylose yields for conventional switchgrass for the combined training and testing data sets.

When the MLR model was applied to transgenic switchgrass, it was observed that the model for glucose yield exhibited comparable R^2 and RMSE values with those for conventional biomass. Noticeably, the MLR model for xylose yield in transgenic switchgrass had weaker performance than that developed for the conventional plant based on the R^2 and RMSE values, with the prediction being off by 9.9% for the testing data. A possible reason for the less accuracy of prediction for xylose yield in transgenic switchgrass is that the lignin-carbohydrate complex could be more favorable for structure disruption during the pretreatment due to the increased syringyl/guaiacyl (S/G) ratio found in the lignin of transgenic plant (Wang et al., 2012; Zhou et al., 2011), and this change could cause xylan remaining in the pretreated biomass more vulnerable to enzyme attack even after mild pretreatments, and thus the yield of xylose in hydrolysates is less changeable as pretreatment conditions vary.

The results of Shapiro-Wilk test show that the values of test statistic for the prediction residuals of all MLR models were higher than 0.9 and were significant at an alpha level of 0.05. The normality plots for the prediction residuals of the MLR models for glucose and xylose yields from conventional switchgrass are shown in Figure 2. These results indicate that the MLR models did not significantly over or under-predict the sugar production and the prediction residuals follow a normal distribution.

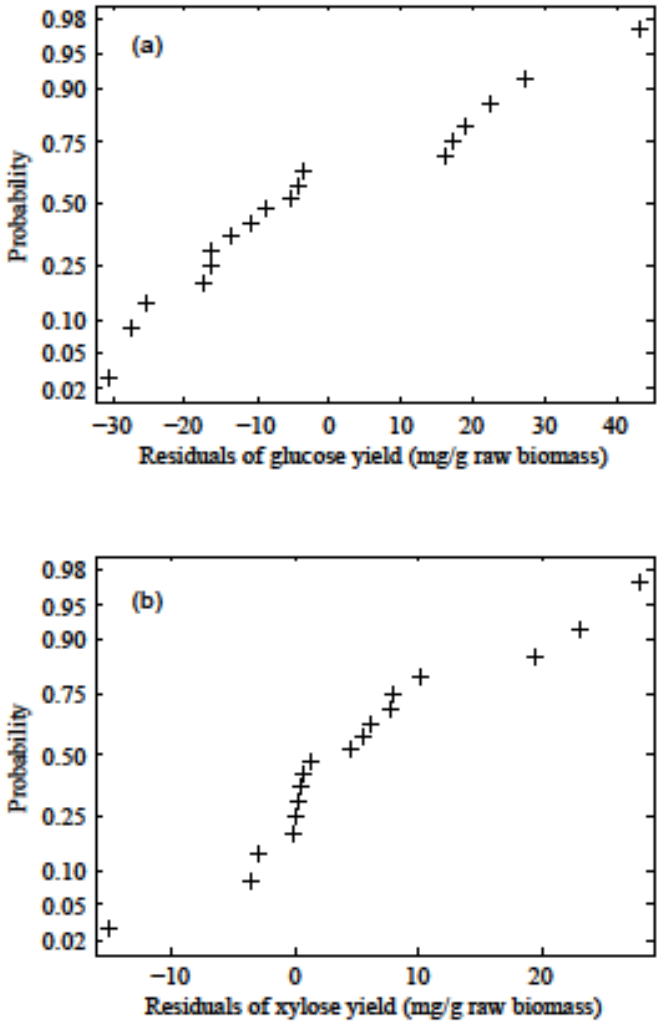

Figure 2. Normal probability plots for prediction residuals from the multiple linear regression models of glucose (a) and xylose (b) yields for conventional switchgrass (AL RCK).

4.2 Modified SIR model

The parameters of the mSIR models for glucose and xylose yields estimated from the training data set of lime pretreatment are shown in Table 4. These parameters were determined to be significant (P<0.05) for all the models. Unlike those positive values obtained for the kinetic models for predicting the changes of biomass composition during

Table 4. Optimized values of parameters for the mSIR model (Eq.5) for glucose and xylose yields from Alamo (cv.) switchgrass after lime pretreatment and enzymatic hydrolysis

Plant line	Model	*h*	*k*	*n*	*b*
AL RCK	glucose yield	1.53	0.030	-0.030	-1.15
	xylose yield	2.37	0.146	-0.109	-0.779
AL 10/9-40	glucose yield	1.92	0.012	-0.039	-1.44
	xylose yield	2.84	0.112	-0.112	-0.844

pretreatment (Dang and Nguyen, 2006, 2007; Keshwani and Cheng, 2010), it was noted that the constants *n* and *b* optimized herein were negative. A plausible explanation is that the yields of glucose and xylose in the hydrolysate usually rise with the increase of pretreatment time and chemical concentration, whereas the amounts of cellulose and xylan present in pretreated biomass exhibit reversed changes as pretreatment severity is enhanced. Regardless of the difference in the signs of the two parameters between the kinetic pretreatment model and the mSIR model, the absolute values of the estimates for *n* and *b*, and the value of *k* obtained in this study are still consistent with the nature of the biomass carbohydrates and the reactions involved in pretreatment and enzymatic hydrolysis.

Since these parameters were determined in the context of modeling sugar production after enzymatic hydrolysis of pretreated biomass, the discussion of their estimated values was based on the reflection of these parameters in real biomass conversion situation. As indicated in Equation 5, the rate of change in sugar yield consists of the increasing and decreasing parts. The parameters *k*, *n*, and *b* are involved in the increasing rate, while the parameter *h*

contributes to both increasing and decreasing rates. The value of *n* is of primary interest because it reflects the dependence of the change of increasing rate on pretreatment time. If the value of *n* is close to zero (observed for glucose), then the increasing rate does not vary significantly as residence time is extended. When the value of *n* is away from zero (noted for xylose) the increasing rate will present notable variability with the change of residence time. In lime pretreatment, a significant amount of acetyl groups in hemicellulose can be removed, which leads to great accessibility of xylan to xylanase in the following hydrolysis (Chang and Holtzapple, 2000). This may explain why the estimated value of *n* for xylose yield model was farther from zero than that for glucose yield, which implies that for lime pretreatment the increasing part of the rate for xylose yield is more susceptible to the change of residence time than that for glucose yield.

Another important parameter for mSIR model is *h* since it is responsible for both the increasing and decreasing parts of the rate for sugar yield. As shown in Table 4, the value of *h* for xylose yield model was higher than that for glucose yield, indicating that xylose yield was more susceptible to the change of pretreatment conditions in the case of lime pretreatment. An examination of the values of *k* and *b* in the mSIR models for glucose and xylose yields reveals that the impact of constants *k* and *b* on the increasing part of the rate was more obvious for glucose yield. Although the optimized values of all the parameters between conventional and transgenic switchgrass exhibit similar changes from the glucose yield model to that for xylose yield, the estimates for the transgenic plant indicate greater variability of sugar production with respect to pretreatment conditions than the non-

transgenic biomass. This further supports the result reported by Wang et al. (2012) that genetically engineered switchgrass could be more open to pretreatment reactions to facilitate sugar release from the biomass in the subsequent hydrolysis.

The R^2 and RMSE values for the training and testing data sets for the mSIR models for glucose and xylose yields are shown in Table 3. For conventional switchgrass, the training-R^2 values for glucose and xylose yields were 0.94 and 0.97 respectively, and the corresponding RMSE values were 17.99 and 6.63 mg/g raw biomass. The testing-R^2 and RMSE values were comparable to those obtained with the training data. In particular, for xylose yield the testing-RMSE value for the mSIR model was much less than that for the MLR model, indicating better representation of xylose yield in the hydrolysate with the use of mSIR model. This improvement in model performance was even more remarkable for the transgenic biomass for which both training and testing-RMSE values were considerably reduced with the predictions within 4% of the actual values as compared to 6.9-9.9% for the MLR model. The correlation between predicted and measured values of glucose and xylose yields for the combined training and testing data sets for conventional switchgrass obtained by the modified SIR model is shown in Figure 3.

The normality plots for the prediction residuals of the mSIR models for glucose and xylose yields in conventional switchgrass are shown in Figure 4. Based on the results of Shapiro-Wilk test, the assumption of normality for the prediction residuals of all modified SIR models was accepted.

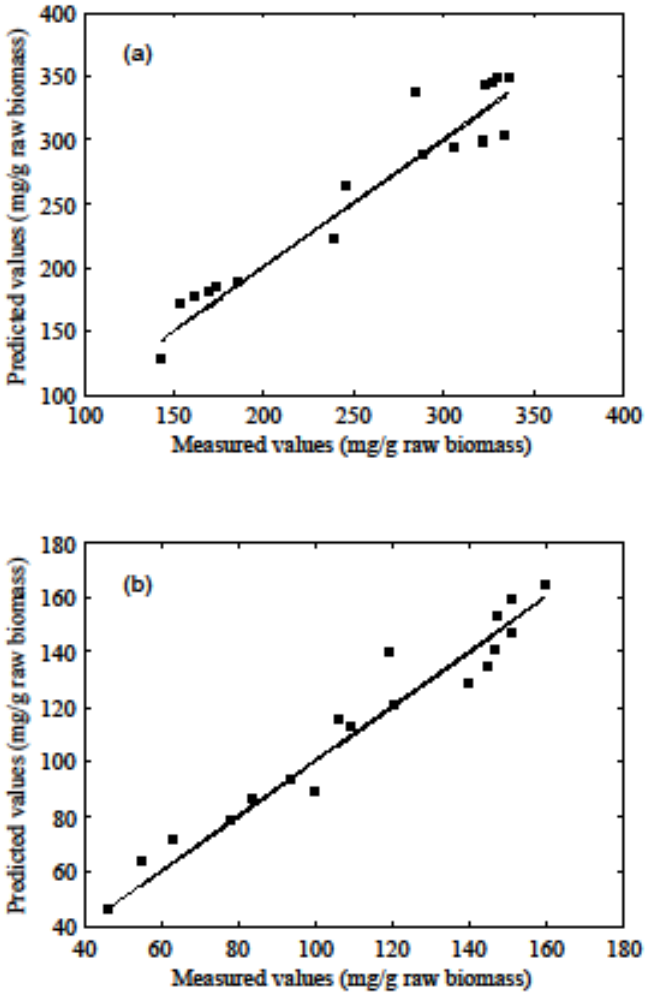

Figure 3. Correlation between predicted and measured values for the modified SIR models of glucose (a) and xylose (b) yields applied to the combined training and testing data sets for conventional switchgrass (AL RCK).

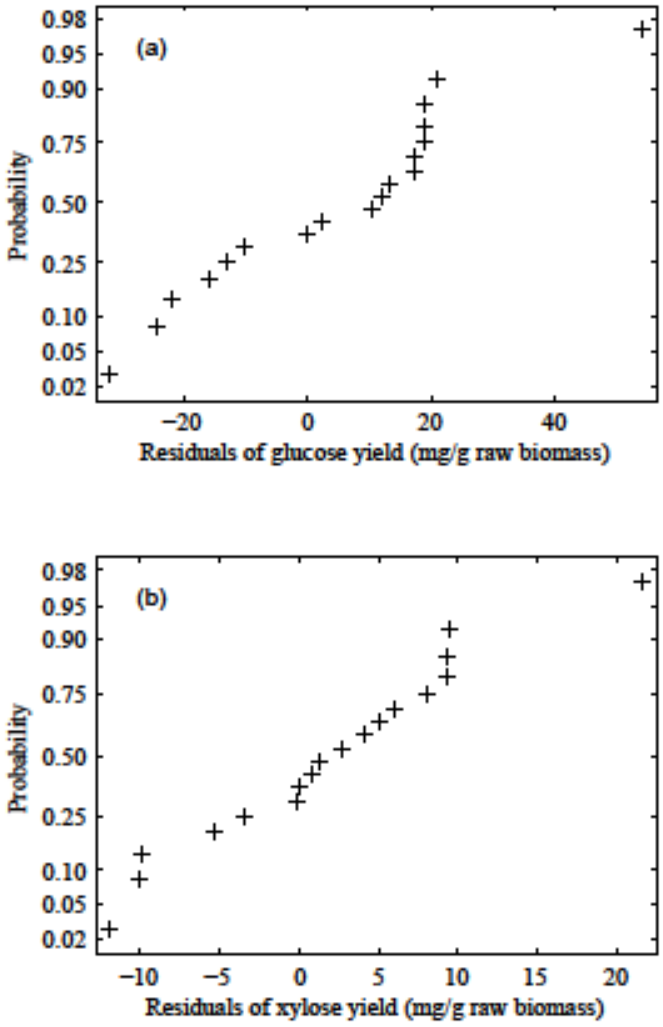

Figure 4. Normal probability plots for prediction residuals from the modified SIR models of glucose (a) and xylose (b) yields for conventional switchgrass (AL RCK).

4.3 Validation of modified SIR model for different alkali pretreatments

To further evaluate the validity of the mSIR model developed in this study, the model was applied to the data obtained from two other alkali pretreatments. The estimates of model parameters and the associated R^2 and RMSE values for these data sets for glucose and xylose yields are provided in Table 5. For the combined lime and NaOH pretreatment at 21 ℃, the model for glucose yield exhibited comparable values of all parameters, R^2, and RMSE with those for xylose yield prediction. This is presumably because both cellulose and xylan are not easily affected by combined alkali pretreatment for relatively short residence times at ambient temperature (Xu and Cheng, 2011). Hence, the two carbohydrate components remaining in pretreated biomass have similar sugar yields. While the R^2 values for glucose and xylose yields for combined alkali pretreatment were comparable to those for lime pretreatment, the corresponding RMSE values were much lower with the predictions within 2% of the actual values.

The results obtained from fitting the mSIR model to the data for NaOH pretreatment (Xu et al., 2010b) show that the R^2 and RMSE values for glucose yield were comparable with those for lime pretreatment. However, the performance of modified SIR model on predicting xylose yield of switchgrass for NaOH pretreatment, especially for the pretreatment at 50 ℃, was impaired as opposed to that for lime and combined alkali pretreatments. This is probably because the mSIR model shown in Equation 5 may not be able to capture the considerable decrease of NaOH concentration over pretreatment time. Unlike those obtained for lime pretreatment, the value of *h* for glucose yield was higher than that for xylose yield and the

Table 5. Optimized values of parameters and the values of R^2 and RMSE for the mSIR model (Eq.5) applied to different alkali pretreatments of switchgrass

Switchgrass	Pretreatment	Model	*h*	*k*	*n*	*b*	R^2	RMSE (mg/g)
Alamo	Lime + NaOH (21 ℃)	glucose yield	0.837	0.209	-0.111	-0.289	0.93	6.53
(this study)		xylose yield	0.756	0.400	-0.117	-0.252	0.91	4.54
Performer	NaOH (50 ℃)	glucose yield	2.59	0.045	-0.113	-1.07	0.89	20.82
(Xu et al., 2010b)		xylose yield	1.56	0.178	-0.017	-0.606	0.60	19.11
	NaOH (21 ℃)	glucose yield	3.18	0.098	-0.133	-0.972	0.98	10.53
		xylose yield	1.30	0.222	-0.058	-0.693	0.82	13.97

corresponding value of *n* was farther from zero for NaOH pretreatment. These results indicate that glucose yield was more susceptible to the change of pretreatment conditions than xylose yield when NaOH pretreatment was applied. By contrast with lime pretreatment, NaOH pretreatment is more effective in disrupting biomass structure and decreasing cellulose crystallinity for better accessibility of cellulose to enzyme attack because of higher alkalinity of NaOH solution. Nevertheless, more xylan was solubilized during NaOH pretreatment (Wang and Cheng, 2011). These effects result in a physical situation in which cellulose in pretreated biomass solids becomes more readily to be hydrolyzed as pretreatment severity increases, and the remaining xylan in solids is already hydrolyzable even at mild pretreatment conditions. Additionally, the values of *k* and *b* for the mSIR models present the same behavior of their influence on glucose and xylose yields among all three alkali pretreatment technologies. According to the results of Shapiro-Wilk test, the values of test statistic for the prediction residuals of all mSIR models for both combined alkali and NaOH pretreatments were higher than 0.9 and were significant at an alpha level of 0.05, indicating the normality was not rejected.

5. CONCLUSIONS

Modeling pretreatment and enzymatic hydrolysis separately has been the major focus of effort made in the field of cellulosic biomass conversion simulation. There are few studies reported on modeling the entire conversion process to predict sugar production in the hydrolysate by inputting pretreatment conditions. This study examined two different approaches to model glucose and xylose yields after enzymatic hydrolysis of switchgrass

pretreated with different alkali reagents. The first approach was a multiple linear regression (MLR) model which was developed and validated using the data sets obtained for lime pretreatment. Based on the R^2 and RMSE values for both training and testing data sets, the MLR models did not accurately predict xylose yield and the predictions were off the actual values by up to 10%. The second approach was a modified SIR (mSIR) model which was developed based on a simple SIR model and the kinetic model used for predicting the changes of biomass composition during pretreatment (Dang and Nguyen, 2006, 2007). The mSIR model had comparable predictive ability for glucose yield with the MLR model. More importantly, it exhibited higher accuracy of predictions for xylose yield than the MLR model.

In addition to its appropriateness for lime pretreatment, the mSIR model yielded more accurate predictions for both glucose and xylose yields that were within 2% of the experimental values for the combined lime and NaOH pretreatment. However, this modeling approach was shown less effective in predicting sugar yield for NaOH pretreatment because the substantial decrease of chemical concentration during the course of pretreatment was not reflected in the mSIR model. Future research work include careful incorporation of the time-dependence of chemical concentration and temperature effect into the mSIR model and validation of the model for other promising pretreatment technologies such as acid pretreatment, ammonia fiber explosion, and ionic liquid pretreatment.

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CHAPTER 6: CONCLUSIONS AND FUTURE RESEARCH

Lignocellulosic materials are considered as promising renewable energy sources worldwide to enhance energy security, slow down climate change, create green jobs, and develop rural economy. Switchgrass has drawn extensive interest for energy production due to its high biomass yield, low nutritional requirements, and positive environmental benefits. Wild-type switchgrass can be genetically transformed to reduce its lignin content and/or alter the structure of lignin-carbohydrate complex for improved sugar release. The overall goal of this study was to gain new insight into the impact of alkali pretreatment on hydrolysis efficiency of carbohydrates in genetically-engineered switchgrass. A comprehensive review of published research on modeling various pretreatment processes and enzymatic hydrolysis of lignocellulosic biomass for sugar production was conducted. The impact of lignin down-regulation on biomass composition and effectiveness of high temperature sodium hydroxide pretreatment of switchgrass was examined, followed by an investigation of the effects of low temperature alkali pretreatment on sugar release from transgenic switchgrass. Models were developed to predict sugar production from switchgrass after alkali pretreatment and enzymatic hydrolysis. This chapter summarizes important results of the study presented in this dissertation and makes suggestions for future research.

1. SUMMARY OF RESULTS

Sugar derived from lignocellulosic materials has shown a great potential of applications in the production of biofuel and other value-added products. Cost-competitive cellulosic sugar is the key to commercialize the production of fuels and chemicals from lignocellulosic biomass. To deeply understand factors that impede the conversion of

cellulosic biomass to sugar, computer simulations are required to be wisely linked with experimental results. The kinetic behavior of cellulose, xylan, and lignin during the course of pretreatment and enzymatic hydrolysis has been the focus of research effort on modeling biomass-to-sugar processes.

The nature of lignocellulosic biomass and the heterogeneous reactions involved in pretreatment incur variability in modeling compositional changes of cellulosic biomass during the process. Factors including temperature, chemical concentration or pH value, residence time, and the interactions between lignin and carbohydrates, along with their impact on reaction rates need to be considered in the development of kinetic models for simulating pretreatment processes. In addition, these factors can be integrated into a single variable-severity factor that is normally used as a predictor in kinetic or empirical models for effort saving in process optimization. Non-kinetic models that exclude mathematical equations, such as artificial neural network and fuzzy-logic-based inference systems, tend to be more efficient than kinetic models in modeling changes in biomass composition for a variety of pretreatment processes.

Modeling enzymatic hydrolysis kinetics is complex due to the inherent complexity of enzymatic reaction of lignocellulose and its associated inhibition patterns. Of particular interest are Langmuir-type adsorption and Michaelis-Menten based models that have shown good performance in predicting kinetic behavior of hydrolytic enzymes on heterogeneous cellulosic substrates. Careful investigation of several key factors including substrate reactivity, enzyme activity and accessibility, irreversible binding of enzymes to lignin, and

enzyme deactivation at high conversion levels is imperative for model development. To date, few of the process models developed in the context of biochemical conversion of lignocellulose to sugar can be applied to the entire conversion scheme. Endeavor in developing robust models that are capable of predicting sugar production from cellulosic biomass through pretreatment and enzymatic hydrolysis is strongly encouraged.

Switchgrass (cv. Alamo) was genetically transformed to suppress the expression of 4-coumarate-CoA ligase (4CL) with attempts to reduce lignin content and/or alter the biomass structure. Composition analysis shows that genetic modification of lignin in switchgrass enabled marginally reduced lignin content and considerably increased acid soluble lignin/acid insoluble lignin (ASL/AIL) and syringyl/guaiacyl (S/G) ratios in lignin. The remarkable changes in lignin composition effectively overcame biomass recalcitrance to enhance the accessibility of cellulose and hemicellulose to hydrolytic enzymes, which could potentially result in improved cellulosic biofuel production, although total lignin content was not significantly reduced. Under the optimal conditions of 1% NaOH and 30 min for sodium hydroxide (NaOH) pretreatment at 121 ℃, the glucan and xylan conversion efficiency in the best transgenic plants were improved by 16 and 18%, respectively, as compared with the conventional plant. The results suggest that higher ASL/AIL and S/G ratios may compromise the negative influence of high lignin content on biomass saccharification.

Low temperature alkali pretreatment was also effective in releasing fermentable sugars from both conventional and transgenic switchgrass after enzymatic hydrolysis. In general, more sugars could be obtained from switchgrass using the lime pretreatment at 50

℃ in comparison with the combined alkali pretreatment at ambient temperature, mainly because of the significant impact of temperature on pretreatment efficiency. As with the finding for NaOH pretreatment, the increased S/G ratio in transgenic switchgrass was believed to alter lignin and/or lignin-carbohydrate complex structure for easier chemical attack during lime and combined alkali pretreatments, thus enhancing sugar production in hydrolysate. The advantage of transgenics over conventional plant in sugar production could be maximized if proper pretreatment conditions were used.

Modeling of biomass-to-sugar conversion through pretreatment and enzymatic hydrolysis can be a valuable tool for process simulations of liquid biofuels production from lignocellulosic materials. Two different models- multiple linear regression (MLR) and the modified SIR (mSIR) model were developed to predict the yields of glucose and xylose after enzymatic hydrolysis of switchgrass (conventional vs. transgenic) pretreated with lime. Based on the R^2 and RMSE values for both training and testing data sets, the MLR models were able to predict glucose yield within 3-6% of the experimental values, but did not accurately predict xylose yield and the predictions were off the actual values by up to 10%. The modified SIR (mSIR) model was developed based on a simple SIR model and the kinetic model used for predicting the changes in biomass composition during pretreatment (Dang and Nguyen, 2006, 2007). The mSIR models had comparable predictive ability for glucose yield with the MLR models. Noticeably, they exhibited higher accuracy of predictions for xylose yield than the MLR models. In addition to its appropriateness for lime pretreatment, the mSIR models yielded more accurate predictions for both glucose and xylose yields that

were within 2% of the experimental values for the combined lime and NaOH pretreatment. However, this modeling approach was less effective in predicting sugar yields for NaOH pretreatment because the substantial decrease of chemical concentration during the course of pretreatment was not reflected in the mSIR models.

2. SUGGESTIONS FOR FUTURE WORK

In this research, switchgrass was genetically transformed by suppressing the expression of 4-coumarate:coenzyme A ligase gene (4CL). The reduction of lignin content caused by the genetic modification was not as remarkable as those reported in previous studies. Moreover, the changes in S/G ratio observed in this research were completely diverted from other published results. A plausible reason for this difference would be the fact that different central genes that govern lignin biosynthesis were targeted in between this study and others. Hence, it is recommended to manipulate the expression of other genes that have also been reported to control lignin formation in cellulosic biomass, and examine the corresponding outcomes regarding lignin content and composition.

Apart from lignin content and composition, other factors such as cellulose crystallinity and biomass pore size can affect sugar release. Thus, it is of interest to compare these two physical features between conventional and transgenic switchgrass. Measurements of cellulose crystallinity and pore size for pretreated biomass are also highly recommended to study the impact of lignin down-regulation on the changes in physical properties of biomass after pretreatment. With this information, it would be more explicit to our knowledge about the major contributors for improved sugar release from transgenics as compared with

conventional plant. Use of other promising pretreatment technologies in addition to alkali pretreatment is another logical step in future work with attempts to find out the best pretreatment method along with its optimal conditions. The impact of lignin down-regulation on enzyme loading requirement in the hydrolysis needs to be explored as well for process optimization. Although many advances have been made in the field of cellulosic biofuels, a deeper understanding of cell wall structure in lignocellulosic plants is still required for developing more efficient yet cost-competitive biomass conversion technologies.

The mSIR models that were used to predict the yields of glucose and xylose after alkali pretreatment and enzymatic hydrolysis neglected the change of chemical concentration over time during pretreatment. Therefore, future research on model development includes careful incorporation of the time-dependence of chemical concentration and temperature effect into the mSIR model. The applicability of mSIR model to other potentially cost-effective pretreatment technologies such as acid pretreatment, ammonia fiber explosion, and ionic liquid pretreatment must be validated in order to develop robust models that can be tailored to particular conversion process if needed. It is anticipated that the mSIR model with further modification could be used in existing process simulations to evaluate the technical and economic feasibility of a cellulosic biorefinery plant that uses sugars generated from pretreatment and enzymatic hydrolysis to produce fuels and chemicals.

APPENDICES

APPENDIX A: SAMPLE SAS CODE FOR DATA ANALYSIS

***The following sample SAS code evaluates the effect of NaOH loading (naohload), residence time, and their interaction on total reducing sugar production (trs) for combined lime and NaOH pretreatment at a constant lime loading.**

```
DATA Transwi1010;
INPUT naohload time trs;
datalines;
naohload_data     time_data   trs_data
;

PROC GLM;

class naohload time;
model trs=naohload|time;
lsmeans naohload time naohload*time / pdiff adjust=tukey;

run;
```

APPENDIX B: MATLAB CODES FOR CHAPTER 5

```
% This MATLAB code calculates the parameters for the MLR models with
% training data and validates the models with testing data.

% input training data
datatrain = xlsread('limetranstrainsugar');

% designate conc, time, glucose and xylose yields
X=datatrain(:,1:2);
glu=datatrain(:,3);
xyl=datatrain(:,4);

% diagnose MLR for the training data
glu_stats=regstats(glu,X,'quadratic');
glu_coeff=glu_stats.beta;
glu_pvalue=glu_stats.tstat.pval;
glu_trainpred=glu_stats.yhat;

xyl_stats=regstats(xyl,X,'quadratic');
xyl_coeff=xyl_stats.beta;
xyl_pvalue=xyl_stats.tstat.pval;
xyl_trainpred=xyl_stats.yhat;

c_train=datatrain(:,1);
t_train=datatrain(:,2);
c_train2=(datatrain(:,1)).^2;
t_train2=(datatrain(:,2)).^2;
[m_train,n_train]=size(datatrain);
Xtrain=[ones(m_train,1) c_train t_train c_train2 t_train2];

glu_coeff1=[glu_coeff(1);glu_coeff(2);glu_coeff(3);glu_coeff(5);glu_coeff(
6)];
xyl_coeff1=[xyl_coeff(1);xyl_coeff(2);xyl_coeff(3);xyl_coeff(5);xyl_coeff(
6)];
glupred=Xtrain*glu_coeff1;
xylpred=Xtrain*xyl_coeff1;

% calculate R square and RMSE for the traning data
p_glu=polyfit(glu,glupred,1);
glufit=polyval(p_glu,glu);
gluresid=glupred-glufit;
SSresid_glu=sum(gluresid.^2);
SStotal_glu=(length(glupred)-1)*var(glupred);
rsq_glu=1-SSresid_glu/SStotal_glu;

p_xyl=polyfit(xyl,xylpred,1);
xylfit=polyval(p_xyl,xyl);
xylresid=xylpred-xylfit;
SSresid_xyl=sum(xylresid.^2);
SStotal_xyl=(length(xylpred)-1)*var(xylpred);
rsq_xyl=1-SSresid_xyl/SStotal_xyl;
```

```
n=length(c_train);
MSE_glu=(1/n)*sum((glupred-glu).^2);
RMSE_glu=sqrt(MSE_glu);

MSE_xyl=(1/n)*sum((xylpred-xyl).^2);
RMSE_xyl=sqrt(MSE_xyl);


% input testing data to validate model
datatest=xlsread('limetranstestsugar');
c_test=datatest(:,1);
t_test=datatest(:,2);
c_test2=(datatest(:,1)).^2;
t_test2=(datatest(:,2)).^2;
glu1=datatest(:,3);
xyl1=datatest(:,4);
[m_test,n_test]=size(datatest);
Xtest=[ones(m_test,1) c_test t_test c_test2 t_test2];

% predicting values using testing data
glu1pred=Xtest*glu_coeff1;
xyl1pred=Xtest*xyl_coeff1;

% calculate R square and RMSE for the testing data
p_glu1=polyfit(glu1,glu1pred,1);
glu1fit=polyval(p_glu1,glu1);
glu1resid=glu1pred-glu1fit;
SSresid_glu1=sum(glu1resid.^2);
SStotal_glu1=(length(glu1pred)-1)*var(glu1pred);
rsq_glu1=1-SSresid_glu1/SStotal_glu1;

p_xyl1=polyfit(xyl1,xyl1pred,1);
xyl1fit=polyval(p_xyl1,xyl1);
xyl1resid=xyl1pred-xyl1fit;
SSresid_xyl1=sum(xyl1resid.^2);
SStotal_xyl1=(length(xyl1pred)-1)*var(xyl1pred);
rsq_xyl1=1-SSresid_xyl1/SStotal_xyl1;

n1=length(c_test);
MSE_glu1=(1/n1)*sum((glu1pred-glu1).^2);
RMSE_glu1=sqrt(MSE_glu1);

MSE_xyl1=(1/n1)*sum((xyl1pred-xyl1).^2);
RMSE_xyl1=sqrt(MSE_xyl1);

% plot the experimental data and the predicted data for the combined
% training and testing data
tglu=[glu;glu1];
tglupred=[glupred;glu1pred];
txyl=[xyl;xyl1];
txylpred=[xylpred;xyl1pred];
```

```
figure(1)
subplot(2,1,1)
plot(tglu,tglupred,'ks',tglu,tglu,'k-
','linewidth',1,'markersize',4,'markerfacecolor','k');
xlabel('Measured values (mg/g raw biomass)')
ylabel('Predicted values (mg/g raw biomass)')
subplot(2,1,2)
plot(txyl,txylpred,'ks',txyl,txyl,'k-
','linewidth',1,'markersize',4,'markerfacecolor','k');
xlabel('Measured values (mg/g raw biomass)')
ylabel('Predicted values (mg/g raw biomass)')

% creating normal probability plots
resid_glu=tglupred-tglu;
resid_xyl=txylpred-txyl;
figure(2)
subplot(2,1,1)
normplot(resid_glu);xlabel('Residuals of glucose yield (mg/g raw
biomass)');
subplot(2,1,2)
normplot(resid_xyl);xlabel('Residuals of xylose yield (mg/g raw biomass)');
```

```
% This MATLAB code calculates the parameters for the mSIR models with
% training data and validates the models with testing data.

% input training data
datatrain = xlsread('limetranstrainsugar');

% designate conc, time, glucose and xylose yields
c=datatrain(:,1);
t=datatrain(:,2);
glu=datatrain(:,3);
xyl=datatrain(:,4);
n=length(c);

% set up time intervals for each conc for training data
tmin=0;
tmax=max(t);
npts=481;
dt=(tmax-tmin)/(npts-1);
t1=tmin:dt:tmax;

% set ifit to 0 and don't continue on to the fit until
% the user sets it to 1

ifit=0;
while ifit==0
disp(' Enter an initial guess for the function ')
pa=input('parameters [pa1,pa2,pa3,pa4] in vector form [...]- ')

glupred=funcfit3(pa,t1);
xylpred=funcfit4(pa,t1);

% plot the experimental testing data and the initial function guess
figure(1)
plot(glu,glupred,'ks',glu,glu,'k-
','linewidth',1,'markersize',4,'markerfacecolor','k');
xlabel('Measured values (mg/g raw biomass)')
ylabel('Predicted values (mg/g raw biomass)')
figure(2)
plot(xyl,xylpred,'ks',xyl,xyl,'k-
','linewidth',1,'markersize',4,'markerfacecolor','k');
xlabel('Measured values (mg/g raw biomass)')
ylabel('Predicted values (mg/g raw biomass)')

ifit=input(' Enter 0 to guess again, 1 to try to fit with this guess - ')
end

option=optimset('MaxFunEvals',5000,'MaxIter',5000,'TolX',1e-5);
pa_glu=fminsearch(@leastsq3,pa,option,t1,glu)
pa_xyl=fminsearch(@leastsq4,pa,option,t1,xyl)

glupred=funcfit3(pa_glu,t1);
```

```
xylpred=funcfit4(pa_xyl,t1);

% calculate standard errors for optimized parameters using
% a finite difference approximation (forward difference)
dv=10^-8;
pa_glu1=[pa_glu(1)+dv,pa_glu(2),pa_glu(3),pa_glu(4)];
glupredv=funcfit3(pa_glu1,t1);
Z_glu(:,1)=(glupredv-glupred)/dv;
dk=10^-8;
pa_glu2=[pa_glu(1),pa_glu(2)+dk,pa_glu(3),pa_glu(4)];
glupredk=funcfit3(pa_glu2,t1);
Z_glu(:,2)=(glupredk-glupred)/dk;
dn=10^-8;
pa_glu3=[pa_glu(1),pa_glu(2),pa_glu(3)+dn,pa_glu(4)];
glupredn=funcfit3(pa_glu3,t1);
Z_glu(:,3)=(glupredn-glupred)/dn;
db=10^-8;
pa_glu4=[pa_glu(1),pa_glu(2),pa_glu(3),pa_glu(4)+db];
glupredb=funcfit3(pa_glu4,t1);
Z_glu(:,4)=(glupredb-glupred)/db;
p=length(pa_glu);
s_glu=1/(n-p)*sum((glu-glupred).^2);
cov_glu = s_glu*inv(transpose(Z_glu)*Z_glu);
cov_glu_d = diag(cov_glu);
SE_glu = sqrt(cov_glu_d);

dv=10^-8;
pa_xyl1=[pa_xyl(1)+dv,pa_xyl(2),pa_xyl(3),pa_xyl(4)];
xylpredv=funcfit4(pa_xyl1,t1);
Z_xyl(:,1)=(xylpredv-xylpred)/dv;
dk=10^-8;
pa_xyl2=[pa_xyl(1),pa_xyl(2)+dk,pa_xyl(3),pa_xyl(4)];
xylpredk=funcfit4(pa_xyl2,t1);
Z_xyl(:,2)=(xylpredk-xylpred)/dk;
dn=10^-8;
pa_xyl3=[pa_xyl(1),pa_xyl(2),pa_xyl(3)+dn,pa_xyl(4)];
xylpredn=funcfit4(pa_xyl3,t1);
Z_xyl(:,3)=(xylpredn-xylpred)/dn;
db=10^-8;
pa_xyl4=[pa_xyl(1),pa_xyl(2),pa_xyl(3),pa_xyl(4)+db];
xylpredb=funcfit4(pa_xyl4,t1);
Z_xyl(:,4)=(xylpredb-xylpred)/db;
s_xyl=1/(n-p)*sum((xyl-xylpred).^2);
cov_xyl = s_xyl*inv(transpose(Z_xyl)*Z_xyl);
cov_xyl_d = diag(cov_xyl);
SE_xyl = sqrt(cov_xyl_d);

% calculate R square and RMSE for the traning data
p_glu=polyfit(glu,glupred,1);
glufit=polyval(p_glu,glu);
gluresid=glupred-glufit;
SSresid_glu=sum(gluresid.^2);
```

```
SStotal_glu=(length(glupred)-1)*var(glupred);
rsq_glu=1-SSresid_glu/SStotal_glu;

p_xyl=polyfit(xyl,xylpred,1);
xylfit=polyval(p_xyl,xyl);
xylresid=xylpred-xylfit;
SSresid_xyl=sum(xylresid.^2);
SStotal_xyl=(length(xylpred)-1)*var(xylpred);
rsq_xyl=1-SSresid_xyl/SStotal_xyl;

MSE_glu=(1/n)*sum((glupred-glu).^2);
RMSE_glu=sqrt(MSE_glu);

MSE_xyl=(1/n)*sum((xylpred-xyl).^2);
RMSE_xyl=sqrt(MSE_xyl);


% input testing data to validate model
datatest=xlsread('limetranstestsugar');
c1=datatest(:,1);
t2=datatest(:,2);
glu1=datatest(:,3);
xyl1=datatest(:,4);
n1=length(c1);

% set up time intervals for each conc for testing data
tmin=0;
tmax1=max(t2);
npts1=241;
dt1=(tmax1-tmin)/(npts1-1);
t3=tmin:dt1:tmax1;

% predict values using testing data and optimized parameters
glu1pred=funcfit3a(pa_glu,t3);
xyl1pred=funcfit4a(pa_xyl,t3);

% calculate R square and RMSE for the testing data
p_glu1=polyfit(glu1,glu1pred,1);
glu1fit=polyval(p_glu1,glu1);
glu1resid=glu1pred-glu1fit;
SSresid_glu1=sum(glu1resid.^2);
SStotal_glu1=(length(glu1pred)-1)*var(glu1pred);
rsq_glu1=1-SSresid_glu1/SStotal_glu1;

p_xyl1=polyfit(xyl1,xyl1pred,1);
xyl1fit=polyval(p_xyl1,xyl1);
xyl1resid=xyl1pred-xyl1fit;
SSresid_xyl1=sum(xyl1resid.^2);
SStotal_xyl1=(length(xyl1pred)-1)*var(xyl1pred);
rsq_xyl1=1-SSresid_xyl1/SStotal_xyl1;
```

```
MSE_glu1=(1/n1)*sum((glu1pred-glu1).^2);
RMSE_glu1=sqrt(MSE_glu1);

MSE_xyl1=(1/n1)*sum((xyl1pred-xyl1).^2);
RMSE_xyl1=sqrt(MSE_xyl1);

% plot the experimental data and the predicted data for the combined
% training and testing data
tglu=[glu;glu1];
tglupred=[glupred;glu1pred];
txyl=[xyl;xyl1];
txylpred=[xylpred;xyl1pred];

figure(3)
subplot(2,1,1)
plot(tglu,tglupred,'ks',tglu,tglu,'k-
','linewidth',1,'markersize',4,'markerfacecolor','k');
xlabel('Measured values (mg/g raw biomass)')
ylabel('Predicted values (mg/g raw biomass)')
subplot(2,1,2)
plot(txyl,txylpred,'ks',txyl,txyl,'k-
','linewidth',1,'markersize',4,'markerfacecolor','k');
xlabel('Measured values (mg/g raw biomass)')
ylabel('Predicted values (mg/g raw biomass)')

% creating normal probability plots
resid_glu=tglupred-tglu;
resid_xyl=txylpred-txyl;
figure(4)
subplot(2,1,1)
normplot(resid_glu);xlabel('Residuals of glucose yield (mg/g raw
biomass)');
subplot(2,1,2)
normplot(resid_xyl);xlabel('Residuals of xylose yield (mg/g raw biomass)');



% The following are all the function files that are required for the
script file above.

function f=funcfit3(pa,t1)
global PARAMETERS
PARAMETERS=pa;

g0=0;
[t1,glupred1]=ode45(@ODEprethydrog1,t1,g0);
[t1,glupred2]=ode45(@ODEprethydrog2,t1,g0);
[t1,glupred3]=ode45(@ODEprethydrog3,t1,g0);
f=[glupred1(11);glupred1(61);glupred1(121);glupred1(481);
    glupred2(11);glupred2(61);glupred2(121);glupred2(481);
    glupred3(11);glupred3(61);glupred3(121);glupred3(481)];
function f=funcfit4(pa,t1)
```

```
global PARAMETERS
PARAMETERS=pa;

x0=0;
[t1,xylpred1]=ode45(@ODEprethydrox1,t1,x0);
[t1,xylpred2]=ode45(@ODEprethydrox2,t1,x0);
[t1,xylpred3]=ode45(@ODEprethydrox3,t1,x0);

f=[xylpred1(11);xylpred1(61);xylpred1(121);xylpred1(481);
    xylpred2(11);xylpred2(61);xylpred2(121);xylpred2(481);
    xylpred3(11);xylpred3(61);xylpred3(121);xylpred3(481)];

function f=funcfit3a(pa,t1)
global PARAMETERS
PARAMETERS=pa;

g0=0;
[t1,glupred1]=ode45(@ODEprethydrog1,t1,g0);
[t1,glupred2]=ode45(@ODEprethydrog2,t1,g0);
[t1,glupred3]=ode45(@ODEprethydrog3,t1,g0);

f=[glupred1(31);glupred1(241);glupred2(31);glupred2(241);
    glupred3(31);glupred3(241)];

function f=funcfit4a(pa,t1)
global PARAMETERS
PARAMETERS=pa;

x0=0;
[t1,xylpred1]=ode45(@ODEprethydrox1,t1,x0);
[t1,xylpred2]=ode45(@ODEprethydrox2,t1,x0);
[t1,xylpred3]=ode45(@ODEprethydrox3,t1,x0);

f=[xylpred1(31);xylpred1(241);xylpred2(31);xylpred2(241);
    xylpred3(31);xylpred3(241)];

function dgdt=ODEprethydrog1(t,g)
global PARAMETERS
Si=460.22;
c=0.05;
z=PARAMETERS;
v=z(1);
k=z(2);
n=z(3);
b=z(4);
dgdt=v*(Si*exp(-k*t^n*c^b)-g);

function dgdt=ODEprethydrog2(t,g)
global PARAMETERS
Si=460.22;
c=0.1;
```

```
z=PARAMETERS;
v=z(1);
k=z(2);
n=z(3);
b=z(4);
dgdt=v*(Si*exp(-k*t^n*c^b)-g);

function dgdt=ODEprethydrog3(t,g)
global PARAMETERS
Si=460.22;
c=0.15;
z=PARAMETERS;
v=z(1);
k=z(2);
n=z(3);
b=z(4);
dgdt=v*(Si*exp(-k*t^n*c^b)-g);

function dxdt=ODEprethydrox1(t,x)
global PARAMETERS
Si=252.39;
c=0.05;
z=PARAMETERS;
v=z(1);
k=z(2);
n=z(3);
b=z(4);
dxdt=v*(Si*exp(-k*t^n*c^b)-x);

function dxdt=ODEprethydrox2(t,x)
global PARAMETERS
Si=252.39;
c=0.1;
z=PARAMETERS;
v=z(1);
k=z(2);
n=z(3);
b=z(4);
dxdt=v*(Si*exp(-k*t^n*c^b)-x);

function dxdt=ODEprethydrox3(t,x)
global PARAMETERS
Si=252.39;
c=0.15;
z=PARAMETERS;
v=z(1);
k=z(2);
n=z(3);
b=z(4);
dxdt=v*(Si*exp(-k*t^n*c^b)-x);

function s=leastsq3(pa,t1,R)
```

```
s=sum((R-funcfit3(pa,t1)).^2);

function s=leastsq4(pa,t1,R)
s=sum((R-funcfit4(pa,t1)).^2);
```