ABSTRACT

XU, JIELE. Alkaline Pretreatment of Switchgrass for Ethanol Production. (Under the direction of Jay J. Cheng.)

Lignocellulose-to-ethanol conversion is a promising technology to supplement corn-based ethanol production. However, the recalcitrant structure of lignicellulosic materials necessitates a pretreatment step to break up the lignocellulosic matrix, thus improving the accessibility of carbohydrates to hydrolytic enzymes for fermentable sugar production. Switchgrass (*Panicum virgatum L.*) is regarded as a potential feedstock for ethanol production because of its excellent growth in various soil and climate conditions, and low requirements for agricultural inputs. The general objective of this research is to explore alkaline pretreatment of switchgrass for improved enzymatic hydrolysis.

Sodium hydroxide (NaOH) pretreatment of switchgrass was investigated at 121, 50, and 21 ^{o}C respectively for 0.25-1 h, 1-48 h, and 1-96 h at different NaOH concentrations (0.5, 1.0, and 2.0%, w/v). At the best pretreatment conditions (50 ^{o}C, 12 h, and 1.0% NaOH), the yield of total reducing sugars in the subsequent enzymatic hydrolysis reached 453.4 mg/g raw biomass, which was 3.78 times that of untreated biomass. Although using reduced temperatures resulted in lower carbohydrate and higher lignin percentages of the pretreated biomass, better carbohydrate preservations achieved at such milder conditions contributed to high sugar productions that were comparable with those obtained using elevated

pretreatment temperatures. The optimum cellulase and cellobiase loadings applied in enzymatic hydrolysis were respectively 15 FPU/g and 20 CBU/g.

Lime pretreatment of switchgrass was investigated at 121, 50, and 21 ^{o}C respectively for 0.25-1 h, 1-48 h, and 1-168 h, and the effects of lime loading (0.05-0.20 g/g raw biomass) and biomass washing (100 and 300 ml water/g raw biomass) on the sugar production efficiency were also studied. At the best pretreatment conditions (50 ^{o}C, 24 h, 0.10 g $Ca(OH)_2$/g raw biomass, and wash intensity of 100 ml water/g raw biomass), the yield of total reducing sugars reached 433.4 mg/g raw biomass, which was 3.61 times that of untreated biomass. Calcium ions from $Ca(OH)_2$ dissociation could extensively crosslink lignin molecules within the biomass, resulting in low lignin reductions. However, as long as the chemical bonds stiffening lignocellulose were disrupted and biomass porosities increased, the enzymatic digestibilities of biomass could still be substantially improved even in the presence of great lignin contents. The optimum cellulase and cellobiase loadings applied in enzymatic hydrolysis were respectively 20 FPU/g and 20 CBU/g.

Pretreatment of switchgrass using the combination of sodium hydroxide and lime was invented to improve the cost-effectiveness of alkaline pretreatment at room temperature (21 ^{o}C). The effects of residence time (3, 6, and 9 h), NaOH loading (0.05, 0.10, and 0.20 g/g raw biomass), time point for NaOH addition (adding NaOH after 0, 1/3, or 2/3 of the residence time elapses), lime loading (0-0.10 g/g raw biomass), and biomass washing (100 and 200 ml water/g raw biomass) on the sugar

production efficiency were investigated. At the best pretreatment conditions (6 h, 0.10 g NaOH/g raw biomass, start point NaOH addition, 0.02 g $Ca(OH)_2$/g raw biomass, and wash intensity of 100 ml water/g raw biomass), the yield of total reducing sugars reached 386.4 mg/g raw biomass, which was 3.22 times that of untreated biomass. The sugar production of the pretreatment using the combination of 0.10 g NaOH/g raw biomass and 0.02g $Ca(OH)_2$/g raw biomass was comparable with that of using 0.20 g NaOH/g raw biomass, while its cost was barely higher than that of using 0.10 g NaOH/g raw biomass only, considering the low cost of lime and the minor loading required. The optimum cellulase and cellobiase loadings applied in enzymatic hydrolysis were respectively 20 FPU/g and 10 CBU/g.

Alkaline Pretreatment of Switchgrass for Ethanol Production

by
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DEDICATION

This dissertation is dedicated to my dear wife Ying for her constant love and support along the way.

BIOGRAPHY

Jiele Xu was born on May 11, 1980 in Shanghai, China, and had lived there ever since until he came to the U.S. with his wife in 2006. After finishing high school, he attended Tongji University in 1999, and spent four blessed and joyful years there. Jiele Xu received his Bachelor's degree in Water and Wastewater in July 2003, and matriculated in Department of Environmental Engineering at East China University of Science and Technology for graduate study. During the days in graduate school, Jiele Xu developed his interest in converting waste materials to value-added products, and decided to pursue his career in this area. In the third year of graduate school, Jiele Xu married Ying Ji, and now they have a lovely daughter, Elizabeth Xu. After getting his Master's degree in Environmental Engineering in March 2006, he came to the U.S. to study as a Ph.D. student in Department of Biological and Agricultural Engineering at North Carolina State University, under the guidance of Dr. Jay J. Cheng. He dedicated himself to the research on biofuel production from lignocellulosic biomass in the past three years, and received great professional training in the program. Jiele Xu's ultimate career goal is to begin his own business in China someday to use biomass-based renewable energy technologies to create good jobs in rural China and help people there lead a better life.

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Energy shortage and global warming are the two big challenges faced by the world today. According to a report from the Energy Information Administration, the world energy consumption was projected to grow by 44 percent from 472 quadrillion British thermal units (Btu) to 678 quadrillion Btu over the 2006 to 2030 period (EIA, 2009). However, it is unlikely that the production of fossil fuels especially crude oil can meet this ever-growing demand. It was predicted that the world oil production would peak sometime between 2030 and 2050 (Jackson, 2007). Moreover, burning of fossil fuels causes the emissions of greenhouse gases, which is the major contributor to global warming. The Intergovernmental Panel on Climate Change (2007) reported that the linear warming trend over the 50 years from 1956 to 2005 was nearly twice that over the 100 years from 1906 to 2005, which was directly related to the growth of carbon dioxide emission from fossil fuel usage.

Biomass refers to any biological materials derived from natural or human activities and is considered as a promising renewable energy source for many reasons. First, biomass such as trees, grasses, agricultural residues, and forestry wastes, is produced on a renewable basis, thus contributing to the development of a sustainable fuel industry. Second, the CO_2 emitted during the consumption of biomass-based energy source can be balanced by the CO_2 absorbed from the atmosphere for biomass growth (Spatari et al., 2005). Third, since biomass is locally available, the development of biomass-based energy industry will be beneficial to

the social aspect of sustainability by creating good jobs in local areas (Lin and Tanaka, 2006). In addition, using biomass for energy purposes also provides a profitable way to dispose some biomass waste such as agricultural residues, yard wastes, forestry wastes, and municipal wastes.

Biomass-based ethanol is one of the best alternative fuels for the transportation sector. It is now widely used in the U.S. as a partial gasoline replacement to reduce petroleum usage and tailpipe emissions. In 2008, the U.S. ethanol industry produced 9 billion gallons of ethanol, with an increase of 5.4 times over the past decade (RFA, 2009). Corn grain is currently the predominant feedstock for ethanol production in the United States. However, since corn is also a food source, its conversion for energy purposes will inevitably impact corn supply to food markets, thus resulting in a food versus fuel competition. Lignocellulose-to-ethanol conversion is an alternative process to supplement corn-based ethanol production. Lignocellulosic biomass is abundant, has a great number of raw materials, and requires less agricultural inputs for its prodution.

Switchgrass (*Panicum virgatum L.*) is a perennial native warm-season grass species in North America. Because of its excellent growth in various soil and climate conditions, as well as low nitrogen fertilizer and herbicide requirements for the production, great interest has been developed in converting switchgrass to ethanol (Jensen et al., 2007; Keshwani and Cheng, 2009). It was reported that, with newer varieties of switchgrass, yields in excess of 20 Mg ha^{-1} had been realized for test

plots (Keshwani and Cheng, 2009). Switchgrass is relatively rich in cellulose and hemicellulose, while contains less lignin and other non-carbohydrate components, making it a good feedstock (Dien et al., 2006; van den Oever et al., 2003). Moreover, switchgrass also serves as a carbon storage sink, making its cultivation beneficial for the reduction of CO_2 in atmosphere (Sladden et al., 1991).

Lignocellulosic materials consist of three major components: cellulose, hemicellulose, and lignin. Cellulose and hemicellulose are polysaccharides that can be used for ethanol production, while lignin is a complex aromatic polymer that stiffens and surrounds the fibers of polysaccharides (Fan et al., 1987). The extensive interactions between these three components render a recalcitrant structure of lignocellulose, which necessitates a pretreatment step to break it up, thus making cellulose and hemicellulose more accessible to hydrolytic enzymes for fermentable sugar production. Pretreatment technologies including comminution, pyrolysis, steam explosion, ammonia fiber explosion, acid pretreatment, and alkaline pretreatment have been intensively studied to improve the enzymatic digestibility of lignocellulose (Alizadeh et al., 2005; Chang et al., 1997; Negro et al., 2003; Piskorz et al., 1989; Sun and Cheng, 2005). Among all the pretreatment methods, alkaline pretreatment has received great attention because it is relatively inexpensive and less energy intensive (Chang et al., 2001). The major mechanism of alkaline pretreatment is believed to be the saponification of intermolecular ester bonds that crosslink hemicellulose and other components. Alkaline solutions also lead to a

disruption of lignin structure, an increase in internal surface area, and a decrease in cellulose crystallinity (Sun and Cheng, 2002).

1.2 RESEARCH OBJECTIVES

Previous reports supported using switchgrass as the feedstock for ethanol production and alkaline pretreatment has been proven effective on various feedstocks especially forages and agricultural residues (Belkacemi et al., 1998; Chang et al., 1997; Chang et al., 2001; Chen et al., 2007; Hu and Wen, 2008; Silverstein et al, 2007; Xu et al., 2008). However, studies on the alkaline pretreatment of switchgrass for enzymatic hydrolysis improvement are scarce. The general objective of this research is to explore alkaline pretreatment of switchgrass to improve its potential for ethanol production, and lay the ground work for the future scale-up applications. Specific objectives are as follows:

1) Investigate and optimize the processing conditions (temperature, residence time, alkali loading, and biomass washing) of pretreatments using sodium hydroxide and lime.

2) Explore pretreatment using the combination of sodium hydroxide and lime to improve the cost-effectiveness of alkaline pretreatment at room temperature.

3) Elucidate the compositional and structural changes of biomass cause by pretreatments.

4) Study and optimize the enzyme loadings applied in the subsequent enzymatic hydrolysis.

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CHAPTER 2

CONVERSION OF LIGNOCELLULOSIC MATERIALS TO ETHANOL:

A LITERATURE REVIEW

2.1 INTRODUCTION

Due to the growing concerns over oil consumption and reserves, and climate change, the usage of alternative renewable energy sources has become highly preferred. In recent years, more and more attention has been devoted to ethanol, one of the best alternative fuels to power the transportation sector. Actually, ethanol has been blended up to 10% (E10) into nearly half of the gasoline sold in the U.S. (DOE, 2009). Ethanol can be produced on a renewable basis from a variety of biomass, which attracted great interests for several reasons. First, since biomass is locally available, biomass-based ethanol production can lead to the sustainable development of fuel industry (Lin and Tanaka, 2006; Monique et al., 2003). Second, since carbon dioxide is used for biomass growth, much less net carbon dioxide is added to the atmosphere during the production and consumption of ethanol, making it an environmentally friendly energy source. Third, the establishment and operation of energy plantations and ethanol plants create new jobs in rural areas, which contributes to a lot of social benefits (Lin and Tanaka, 2006).

Today, ethanol is predominantly produced from sugar cane or starch-rich materials (Öhgren et al., 2006). In the U.S., almost all fuel ethanol is produced by fermentation of corn glucose. However, depending ethanol production on corn starch conversion is unsustainable because the corn cultivation for ethanol production will compete for the limited agricultural land for food and feed production (Sun and Cheng, 2002). Compared with corn grain, lignocellulosic materials such as trees,

grasses, agricultural residues, and forestry wastes, are more promising feedstocks for low-cost ethanol production. The National Commission on Energy Policy reported that, compared with corn ethanol, cellulosic ethanol had a number of benefits. For example, because it took less fertilizer and energy to plant cellulosic biomass, greenhouse gas emissions from producing cellulosic ethanol were considerably lower. The commission also reported that the abundance of cellulosic biomass made cellulosic ethanol preferable to corn ethanol. According to the report, even if all of the current U.S. corn crop were used for ethanol production, it would only displace 25% of current gasoline usage, while with improvements in the production and utilization of cellulosic ethanol, half of the gasoline could be displaced (Weeks, 2005).

Although lignocellulosic biomass is a very promising alternative feedstock for ethanol production, its conversion to ethanol is more difficult than that of sugar or starch (Öhgren et al., 2006). Three basic steps are involved in the converison: 1) pretreatment of raw biomass, 2) enzymatic hydrolysis for fermentable sugar production, and 3) ethanol fermentation. The biggest challenge of the conversion comes from the recalcitrant structure of lignocellulosic biomass. This recalcitrance is not only due to the composition of lignocellulose but also the way specific components interact with each other. To improve the biomass digestibility, a pretreatment step is required to break up the lignocellulosic matrix, thus making the carbohydrate fraction more accessible to hydrolytic enzymes for fermentable sugar production. Challenges in the subsequent hydrolysis and fermentation include high

cost of hydrolytic enzymes (cellulases), substrate and product inhibition to enzymes, enzyme inactivity, pentose fermentation, and compounds inhibitory to fermentation in lignocellulosic hydrolysate. All these challenges need to be addressed before the potential of ethanol production from lignocellulosic biomass is fully realized.

2.2 NATURE OF LIGNICELLULOSIC MATERIALS

Lignocellulosic materials consist of three major components: cellulose, hemicellulose, and lignin. Cellulose and hemicellulose are structural carbohydrates that can be depolymerized through enzymatic hydrolysis for ethanol production, while lignin is a complex aromatic polymer which forms a crust surrounding the carbohydrate fraction and performs as a barrier limiting the accessibility of carbohydrates to hydrolytic enzymes. There are extensive interactions among these three components, which further improves the stiffness of biomass structure. The contents of cellulose, hemicellulose, and lignin in common agricultural residues and wastes are shown in Table 2.1 (Sun and Cheng, 2002).

Cellulose is the major structural component of any lignocellulosic biomass. It is a linear chain of several hundred to over ten thousand β-1, 4-linked D-glucopyranose units. Due to the existence of many equatorially oriented hydroxyl groups, cellulose molecules have a strong tendency to form intra-molecular hydrogen bonds which are responsible for the stiff and rigid nature of the cellulose

Table 2.1 Contents of cellulose, hemicellulose, and lignin in common agricultural residues and wastes.

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood stems	40-55	24-40	18-25
Softwood stems	45-50	25-35	25-35
Nut shells	25-30	25-30	30-40
Corn cobs	45	35	15
Grasses	25-40	35-50	10-30
Paper	85-99	0	0-15
Wheat straw	30	50	15
Sorted refuse	60	20	20
Leaves	15-20	80-85	0
Cotton seed hairs	80-95	5-20	0
Newspaper	40-55	25-40	18-30
Waste papers form chemical pulps	60-70	10-20	5-10
Primary wastewater solids	8-15	NA	24-29
Swine waste	6.0	28	NA
Solid cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
Coastal Bermuda grass	25	35.7	6.4
Switchgrass	45	31.4	12

NA: not available.

molecule. Also, hydroxyl groups from one chain form inter-molecular hydrogen bonds with oxygens on another chain, holding the chains firmly together side-by-side and forming microfibrils with high tensile strength. The semicrystalline structure of cellulose confers rigidity to plant cell wall (Roberts, 1996).

Hemicellulose is a non-structural polysaccharide with low molecular weight. It has amorphous, heterogeneous, and branched structure, thus having low crystallinity. Monosaccharide residues constituting hemicelluloses include pentose (xylose and arabinose) and hexose (glucose, galactose, and mannose). Great amounts of hemicellulose in the grass is arabinoxylans (Wilkie, 1979), which

consists of a xylan backbone made up of β-1, 4-linked D-xylopyranose units with arabinose side chains. Glucomannan, copolymer of glucose and mannose, can also be found in the grass. Until now, the function of hemicellulose in plant wall is still not well understood. During the biomass conversion, since acetyl and various uronic acid substitutions on hemicellulose are considered as impediments to hydrolytic enzymes, their removals are required in the pretreatment (Chang and Holzapple, 2000).

Lignin is a three dimensional complex aromatic polymer which forms a sheath surrounding cellulose and hemicellulose, stiffening and holding together the fibers of polysaccharides (Fan et al., 1987). It strengthens the cell wall structure, aids in conducting water in the plant stem, and provides protection against microbial attack and decay. Lignin is a polymer of p-Coumaryl alcohol, Coniferyl alcohol, and Sinapyl alcohol, and great amounts of C-C or C-O-C bonds involve both the aromatic rings and the three carbon atoms in the side chain of the alcohols, resulting in an extreme complicated structure (Roberts, 1996). Lignin limits enzyme access to carbohydrates not only through posing physical barrier, but also by causing unproductive binding of enzymes (Palonen, 2004). Therefore, quite a few pretreatment technologies were developed targeting at lignin removal.

2.3 PRETREATMENT OF LIGNOCELLULOSIC MATERIALS

The purpose of the pretreatment is to break up lignocellulosic matrix, thus improving the enzymatic digestibility of biomass (Figure 2.1). An effective pretreatment is supposed to meet the following requirements: 1) substantially improve sugar production in enzymatic hydrolysis; 2) avoid the degradation or loss of carbohydrates; 3) avoid the formation of inhibitors to downstream processes; 4) be cost-effective (Sun and Cheng, 2002). Pretreatment is believed to have great potential for efficiency improvement and cost lowering through research and development (Mosier et al. 2005), and a number of technologies have been developed for the effective pretreatment of a variety of lignocellulosic biomass.

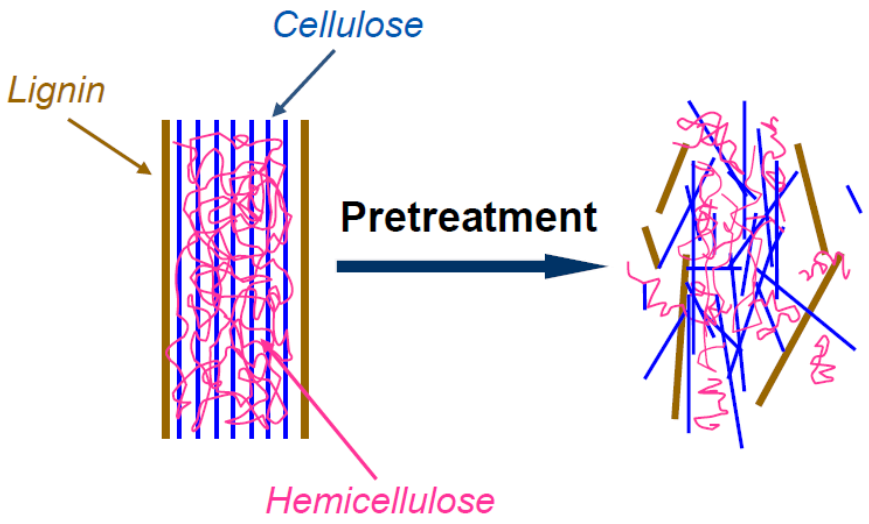

Figure 2.1 Effect of pretreatment (adapted from Mosier et al. 2005).

2.3.1 Physical pretreatment

2.3.1.1 Mechanical comminution

Mechanical comminution reduces the size of raw biomass and is usually the first step of lignocellulose-to-ethanol conversion. Chipping can reduce the size of raw materials to 10-30 mm, and grinding or milling can further reduce the size to 0.2-2 mm. Sidiras and Koukios (1989) reported that, due to the reduction of crystallinity by ball milling, saccharification of more than 50% of straw cellulose with minimal glucose degradation was accomplished in the subsequent hydrolysis. The ball attrition of either wet or dry mode can be used to comminute a variety of materials to ultrafine particles. Although the effects of milling in wet or dry mode could be very different in terms of crystallinity index and particle size changes, both modes were expected to produce substrates with increased conversion susceptibility (Rivers and Emert, 1987). Compression-milling was also studied for the destruction of the structural integrity of lignocellulose, and its effectiveness has been demonstrated for a wide variety of substrate resources (Tassinari et al. 1980, 1982). The energy consumption of comminution is a function of final particle size, raw material, and comminution equipment. It was reported that, for a multiple feedstock plant processing lignocellulosic wastes for the production of ethanol, through an adequate selection of equipment, the energy input for comminution could be kept below 30 kWh per tonne of processed raw material when the final particle size was kept in the range of 3-6 mm (Cadoche and López, 1989).

2.3.1.2 Microwave irradiation

Although microwave irradiation has been widely used for its high heating efficiency and easy operation in many areas such as food processing and chemistry, its application in the pretreatment of lignocellulosic materials has just started. Several studies have been conducted so far with satisfactory results. It was reported that, if rice straw and bagasse were irradiated with microwave at 2450 MHz respectively at 170 ^{o}C for 5 min and 200 ^{o}C for 5min, the accessibilities of the cellulosic materials for enzymatic hydrolysis were enhanced by 1.6 and 3.2 times (Ooshima et al. 1984). When ground rice straw or sugarcane bagasse was treated with 240 W of microwave irradiation for 10 min at atmospheric pressure, the amount of reducing sugars produced from enzyme saccharification was doubled compared with that of control (Kitchaiya et al. 2003). Microwave irradiation can also be applied along with some chemical pretreatment methods. Zhu et al. (2005) reported that microwave-assisted alkali pretreated rice straw resulted in a higher hydrolysis rate and glucose content in the hydrolysate in comparison with the one pretreated using alkali alone.

2.3.1.3 Hydrothermolysis

Hydrothermal processing of lignocellulosic materials could cause various effects including hemicellulose hydrolysis, lignin and extractive removal, and cellulose digestibility improvement (Garrote et al. 1999; Liu and Wyman, 2003).

Under high temperature and pressure, critical water maintained in liquid state was past through raw biomass materials, resulting in the collapse of biomass structure. The study on the pretreatment of sugar-cane bagasse and leaves (190-230 ^{o}C, $P>P_{sat}$) showed that over 50% of the biomass was solubilized after hydrothermal process. All of the hemicellulose and most of the acid-insoluble lignin in the bagasse (>60%) was solubilized, while less than 10% of the cellulose was dissolved (Allen et al. 1996). In another research, several woody and herbaceous biomass species were treated using hydrothermolysis for 0-15 min at 200-230 ^{o}C. After the treatment, all of the hemicellulose was solubilized, of which 90% was recovered as monomeric sugar, and 4-22% of the cellulose and 35-60% of the lignin were also solubilized (Mok and Antol, 1992). There are three types of hydrothermolysis configurations: co-current, countercurrent, and flow through. In co-current process, water and biomass are simultaneously heated to a high temperature and held in certain reaction condition. In countercurrent process, water and biomass move in opposite direction in the reactor. In flow through process, hot water passes through the biomass which is kept in a stationary bed and dissolved biomass components are carried away (Mosier et al. 2005).

2.3.2 Physico-chemical pretreatment

2.3.2.1 Steam explosion

Steam explosion is one of the most intensively studied technologies in the pretreatment of lignocellulosic materials (Avellar and Glasser, 1998; Ballesteros et al., 2000; Brownell, 1986; Glasser and Wright, 1998; Heitz, et al., 1991). In the treatment, chipped biomass is heated with high-pressure steam (160-260 ^{o}C, 0.69-4.83 MPa) for a set of time. At high temperature, hemicellulose hydrolyzes and lignin converts while cellulose remains intact. Then steam is swiftly vented from the reactor, making the biomass undergo an explosive decompression, which opens up the particle structure of biomass and improves the accessibility of cellulose to enzymes. Particle size is one of the major parameters that should be considered in steam explosion. Ballesteros et al. (2002) evaluated the effect of chip size (2-5, 5-8 and 8-12 mm) on steam-explosion pretreatment of *Brassica carinata*. The results showed that using large particle size (8-12 mm) resulted in higher cellulose and hemicellulose recoveries, as well as better enzymatic digestibility. Biomass sources also have an impact on the effectiveness of steam explosion. Different biomass responds differently to the process, which was closely related to chemical, morphological, and anatomical features of the biomass (Glasser and Wright, 1998). The major disadvantage of steam explosion is the release of products that are inhibitory to downstream processes (Mackie et al., 1985). The inhibiting effects of components released during steam-explosion (formic, acetic, and levulinic acids,

furfural, 5-hydroxymethyl furfural, syringaldehyde, 4-hydroxy benzaldehyde, and vanillin) were studied at different concentrations, and it was reported that formic acid (11.5 g/L) could inactivate the enzymes and levulinic acid (29.0 g/L) partially affected the cellulase (Cantarella et al. 2004). Since the pretreated biomass needs to be washed with water to remove the inhibitory compounds, soluble sugars that primarily generated from hemicellulose hydrolysis are also removed, thus lowering the overall saccharification yields (Sun and Cheng, 2002).

2.3.2.2 Ammonia fiber explosion (AFEX)

In the AFEX process, lignocellulosic biomass is treated with liquid ammonia at moderate temperature and under high pressure for 10-15 min, followed by an explosive pressure release (Reshamwala et al., 1995). Instantly releasing the pressure in the AFEX process disrupts the fibrous structure of biomass and increases the accessible surface area, thus improving the digestibility of the biomass. AFEX pretreatment also reduces lignin content, decrystallize cellulose, and prehydrolyze hemicellulose (Teymouri et al., 2005). A research showed that rye-grass straw, corn fiber, and switchgrass could be pretreated with AFEX process so effectively that theoretical sugar yields were achieved using low cellulase enzyme levels (1-5 IU/g) (Dale et al., 1995). The optimum AFEX pretreatment conditions depend on various feedstocks. The best conditions for corn stover pretreatment were found to be 90 ^{o}C, ammonia : dry biomass ratio of 1:1, 60% moisture content of

biomass (dry weight basis), and residence time of 5 min, at which the AFEX-treated sample showed 98% glucan conversion (Teymouri et al. 2005). The optimum pretreatment conditions for switchgrass were 100 ^{o}C, ammonia : dry biomass ratio of 1:1, 80% moisture content of biomass (dry weight basis), and residence time of 5 min, at which the AFEX-treated sample showed 93% glucan conversion (Alizadeh et al. 2005). Economic evaluation estimated that it cost $10-15 to treat one dry ton of biomass (Dale et al. 1995). To reduce the cost as well as protect the environment, ammonia should be recycled after the process (Sun and Cheng, 2002). Because almost no inhibitors are produced during AFEX process, water wash is not necessary and sugar loss can be prevented, which improves the overall cost-effectiveness of the process.

2.3.2.3 Carbon dioxide explosion

Supercritical CO_2 explosion, an alternative method to steam explosion and AFEX, has been developed for efficient pretreatment of lignocellulose. Under high pressure (critical pressure 73 atm), carbon dioxide is maintained in its supercritical status. CO_2 molecules, comparable in size to those of water or ammonia, can penetrate small pores of biomass. CO_2 will form carbonic acid in water and help to increase the hydrolysis rate of cellulose and hemicellulose. When carbon dioxide pressure is swiftly released, biomass undergos an explosive decompression and more surface area is produced for cellulase access (Zheng et al., 1998). Research

showed that, after CO_2 explosion pretreatment, the glucose release from alfalfa reached 75% of the theoretical level after 24 h enzymatic hydrolysis (Dale and Moreira, 1982). It was also reported that CO_2 explosion pretreatment could substantially remove and solubilize the hemicellulose fraction of the raw materials (Puri and Mamers, 1983). Compared with steam explosion and AFEX, CO_2 explosion has flowing advantages: 1) CO_2 explosion applies a lower temperature than steam explosion, which could avoid sugar decomposition; 2) CO_2 can be regarded as a free and environmentally friendly resource; 3) CO_2 explosion doesn't produce inhibitors that impede the following processes.

2.3.3 Chemical pretreatment

2.3.3.1 Acid prehydrolysis

Dilute acid pretreatment has been widely used and most approaches were based on sulfuric acid (Israilides et al., 1978; Kim and Lee, 2002; Nguyen et al,. 2000; Saha et al., 2005; Sun and Cheng, 2005). The dilute acid prehydrolysis can effectively reduce the crystallinity of cellulose and solubilize hemicellulose into monomeric sugars and soluble oligomers. Meanwhile, the digestibility of cellulose in the biomass will also be further imporved (Kim et al., 2002; Mosier et al., 2005; Sun and Cheng, 2005). When the concentration of sulfuric acid was higher than 1.2% and pretreatment time was longer than 60 min, about 50-66% of xylan was converted to xylose. With the elevation of acid concentration, both of the

hemicellulose solubilization and cellulose digestibility were increased (Sun and Cheng, 2005). Varga et al. (2004) reported that the conversion rate of cellulose to glucose of corn stover impregnated in dilute sulfuric acid (0.5% and 2%) before steam explosion was much higher than that of the control sample (without acid application). Diffusion experiment showed that the diffusivity of sulfuric acid in agricultural residues was much higher than that in hardwood, making agricultural residues more suitable for acid pretreatment. The applications of other acids (hydrochloride acid and phosphoric acid) were also studied and the result showed that the effect of hydrochloride acid pretreatment of ryegrass straw was comparable with that of sulfuric acid pretreatment (Israilides et al., 1978). However, acid can cause corrosion to reactors and must be neutralized before the sugars are subjected to biological steps. Some degradation products and fermentation inhibitors are formed in acid prehydrolysis although their adverse impact seemed limited based on some researches (Varga et al., 2004).

2.3.3.2 Alkaline prehydrolysis

Alkaline prehydrolysis is another intensively studied chemical pretreatment method. Alkali breaks the intercellular bonds crosslinking hemicellulose and other compounds (lignin and cellulose), resulting in increased porosity and internal surface of biomass, and decreased crystallinity and polymerization degree of carbohydrates (Sun and Cheng, 2002). Sodium hydroxide is one of most effective alkaline reagents.

Research showed that at 150 ^{o}C, 65% of the corn stover was dissolved after 5 min in 2% sodium hydroxide solution and, after enzymatic hydrolysis, the saccharification rate of the residual and dissolved solids reached 52% (MacDonald et al., 1983). At 120 ^{o}C, over 55% reduction in the lignin content could be achieved within the first half hour of pretreatment using 0.5% sodium hydroxide solution (Soto et al., 1994). Lime (calcium hydroxide) also received much attention because it is very inexpensive and safe to handle. Lime pretreatment was reported to increase the enzymatic hydrolysis of corn stover by 9 times compared with untreated sample (Kaar and Holtzapple, 2000). Systematic researches have been conducted using aqueous ammonia as the alkaline reagent to improve biomass digestibility (Kim et al., 2003, 2006; Kim and Lee, 2005). The results showed that, after the corn stover was soaked in aqueous ammonia over an extended period (10-60 d) at room temperature, 55-74% of the lignin was removed, while nearly 100% of the glucan and 85% of the xylan was retained. When ammonia recycled percolation (ARP) process was applied, 70-85% of the lignin and 40-60% of the hemicellulose in corn stover were removed, while cellulose was left intact. Like acid pretreatment, reagent recovery and solution neutralization are required after the process.

2.3.3.3 Organosolv process

Organosolv process has been utilized to isolate lignin from lignocellulosic materials (Lee et al., 1987). It induces the fractionation of lignocellulosic biomass

into three major components: sugar from hemicellulose, cellulosic fibers, and lignin of low molecular weight (Sidiras and Koukios, 2004). Organic solvent, an aqueous/solvent/catalyst mixture, hydrolyzes the lignin-lignin and lignin-carbohydrate bonds, and dissolves lignin in an organophilic environment created by the solvent (Holtzapple and Humphrey, 1984). The organic solvents used in the process include methanol, ethanol, butanol, ethylenediamine, and ethylene glycol (Lee et al., 1987). Organic acids (oxalic, acetylsalicylic and salicylic acid) are common catalyst utilized in organosolv processes (Chum et al., 1988; Sun and Cheng, 2002). At high temperatures (>180 ^{o}C), the acids released from the wood act as catalyze for the delignification reactions (Holtzapple and Humphrey, 1984). According to the kinetic model proposed by Sidiras and Koukios (2004), at 140 ^{o}C and catalyst concentration of 0.2 N H_2SO_4, pulp yield, maximum lignin solubilization, and total soluble sugar yield could respectively reach 42, 96, and 27% after organosolv process. After pretreatment, solvents need to be recycled to lower the cost and removed from the system to prevent inhibition to the sequent procedures (Sun and Cheng, 2002).

2.3.3.4 Ozonolysis

Ozone has been studied for the pretreatment of ligncellulosic materials because it can substantially degrade lignin and hemicellululose while hardly affects cellulose (Vidal and Molinier, 1988). Ozonolysis has been explored in the

pretreatment of a number of lignocellulosic materials including pone, oak, poplar, wheat straw, peanut shells, corn stover, and bagasse (Ben-ghedalia and Miron, 1981; Neely, 1984). The results showed that a contact time of 1-2 h with an ozone consumption of 4-6% of the dry weight of biomass was required to obtain a good pretreatment effectiveness, while size reduction of raw biomass (<5mm) was not necessary (Neely, 1984). The advantages of ozonolysis can be included as: 1) Ozone can be generated on-site as needed; 2) the decomposition of ozone is so fast that it will not induce pollution; 3) the ozonization reaction takes place at room temperature and pressure. However, ozonolysis is expensive if the usage of a large amount of ozone is required (Neely, 1984; Sun and Cheng, 2002).

2.3.3.5 Oxidative pretreatment

Oxidative pretreatments including peroxide pretreatment and wet oxidation have been extensively studied due to their outstanding delignification capability (Bjerre et al., 1996; Gould and Freer, 1984; Klinke et al., 2003; Schmidt and Thomsen, 1998; Takagi, 1987; Wei and Cheng, 1985). At appropriate conditions, H_2O_2 can delignify wheat straw and other crop residues so effectively that the cellulose can be easily converted to glucose in enzymatic hydrolysis (Gould and Freer, 1984). When peroxide pretreatment was conducted at alkaline conditions, >90% of theoretical saccharification efficiency can be attained from big bluestem (*Andropogon ferardi*) and Indian grass (*Sorghastrum mutans*) (Gould, 1985). Wet

oxidation fractionates lignocellulose into a cellulose-rich solid fraction and a hemicellulose-rich liquid fraction, with lignin degraded (Bjerre et al., 1996; Schmidt and Thomsen, 1998). Research showed that, at the optimum conditions (60 g l^{-1} straw, 185 ^{o}C, 6.5 g l^{-1} Na_2CO_3, 12 bar O_2 with a 15 min reaction time), 55% of the lignin and 80% of the hemicellulose were solubilized, while 95% of the cellulose remained in the solid fraction (Schmidt and Thomsen, 1998).

2.3.4 Biological pretreatment

Although biological pretreatments of lignocellulose avoid high energy requirements for heating and other chemical expenses, they have not been studied as extensively as other physical or chemical methods due to the low reaction rates. With the deeper understanding of the mechanisms behind and the development of biotechnologies, however, the future of biological pretreatment is definitely promising. White-rot fungi, which belongs to *Basidiomycetes*, is one of the best organisms used in lignocellulose pretreatment (Akin et al., 1995; Blanchette, 1991; Hatakka, 1983; Waldner et al., 1988). Although white-rot fungi may degrade lignin and cell wall carbohydrate simultaneously, some species can preferentially remove lignin with cellulose intact (Blanchette, 1991). Much work has been done with *Phanerochaete chrysosporium*, and lignin peroxidases and manganese-dependent peroxidases were proven to be the two major components of its lignin-degrading enzyme system (Boominathna and Reddy, 1992). Other fungi also showed great potential. Hatakka

(1983) tested 19 white-rot fungi and found that *Pleurotus ostreatus*, *Pleurotus ap. 535*, *Pycnoporus cinnabarinus 115* and *Ischnoderma benzoinum 108* increased the enzymatic saccharification rate. Within 5 weeks, the conversion rate of straw to reducing sugar reached 35%, compared with 12% of the control sample. It was also reported that, after 6-week treatment with *C. subvermispora* and *C. stercoreus*, the biodegradation of Bermuda grass stems was improved by 29-32% and 63-77% respectively (Akin et al., 1995).

2.4 ENZYMATIC HYDROLYSIS OF CARBOHYDRATES

Hydrolysis of carbohydrates is the second step of the lignocellulose-to-ethanol conversion, during which fermentable sugars such as glucose and xylose are produced. Since enzymatic hydrolysis is much cheaper than the hydrolysis using chemicals, it is more extensively studied and applied. Cellulases, responsible for the hydrolysis of cellulose, include three major groups: endoglucanase, exoglucanase and ß-glucosidase. Endoglucanase randomly attacks ß-(1-4) glycosidic bonds of cellulose to create free chair-ends, exoglucanase release cellobiose from the free chain-ends, and ß-glucosidase degrades cellobiose into glucose (Sun and Chen, 2002). All of these three cellulases can be produced by a large variety of bacteria and fungi (Duff and Murray, 1996; Jatinder et al., 2006; Jørgensen and Olsson, 2006). Other enzyme such as xylanases, ß-xylosidase plays an important role in the hydrolysis of hemicellulose. The major factors that affect the efficiency and the cost

of hydrolysis process involves: substrate inhibition, end-product inhibition, cellulase activity, and cellulases combination. Much valuable work has been done in these areas.

2.4.1 Substrate inhibition

At low substrate levels, the rate of enzymatic hydrolysis increases with the elevation of substrate concentration, while at high substrate levels, substrate inhibition happens, which is attributed to the dead-end complex formation between the substrate and enzyme (Huang and Penner, 1991). It was reported that Van Dyke was the first scientist to find the existence of substrate inhibition by excess cellulose in his work in 1972 (Howell and Stuck, 1975). Huang and Penner (1991) reported that, in a *Trichoderma reesi* cellulase system, after the rate of saccharification reached maximum, further increases in substrate concentration resulted in a decrease in the rate of saccharification, and substrate inhibition was only observed at substrate/enzyme ratios greater than 5. Similar phenomena were observed in other researches (Howell and Stuck, 1975; Lee and Fan, 1982). The inhibiting effect of substrate differs based on substrate sources. It was reported that, at relatively high substrate concentration, the rate of saccharification of Avical cellulose decreased by 35% compared with the maximum rate observed in a *Trichoderma viride* system, while substrate inhibition was not observed with the Solka-Floc cellulose under the same reaction conditions (Liaw and Penner, 1990).

2.4.2 End-product inhibition

In enzymatic hydrolysis, cellulose is converted to cellobiose by endoglucanase and exoglucanase, and cellobiose is further converted to glucose by β-glucosidase. The product inhibition happens in each step (Ooshima et al., 1985). To solve this problem, a promising technology, simultaneous saccharification and fermentation (SSF), has been developed. In SSF, since enzymatic hydrolysis and fermentation are conducted simultaneously in one reactor, the glucose produced in hydrolysis step is immediately converted to ethanol by fermentation, so that the accumulation of cellobiose and glucose in the reactor is prevented and product inhibition alleviated. However, several problems must be solved before SSF can meet the requirements of industry. One outstanding problem is the different operational temperatures of enzymatic hydrolysis and fermentation. The optimum temperature used in SSF is around 38 ^{o}C, which is a compromise between hydrolysis (45-50 ^{o}C) and fermentation (30 ^{o}C) (Philippidis, 1996). Since the ethanol production rate in an SSF process is controlled by the enzymatic hydrolysis which requires a higher temperature, extended researches have been devoted to the exploitation of thermotolerant yeast and bacteria (Sun and Cheng, 2002). Hacking et al. (1984) reported that, of all 55 yeast strains tested at a high temperatures (40 ^{o}C), strains of *C. pserdotropicalis*, *S. cerevisiae*, and *S. uvarm* resulted in best ethanol yields. Kadam and Schmidt (1997) found that *C. acidothermophilum* could produce 80% of the theoretical ethanol yield at 40 ^{o}C, and its performance was much better than that

of *S. cerevisiae* D_5A at either 37 ^oC or 40 ^oC. In addition, some “superstrains” capable of cellulose and xylan hydrolysis along with fermentation are expected to be created via genetic engineering in the future (Lin and Tanaka, 2006). However, a challenge stemming from SSF is that the accumulated ethanol, which is the end-production of fermentation, not only harms microbes but also inhibits enzymes. It was reported that ethanol inhibited cellulase from *Trichoderma reesi* linearly up to 65g/L (Wu and Lee, 1997). Vallander and Eriksson (1985), and Moritz and Duff (1996) also found similar ethanol inhibition in their studies. To solve this problem, Roychoudnury et al. (1992) developed a novel method to eliminate the impact of high ethanol concentration on enzymes and microbes in SSF system. With a vacuum cycling reactor, excessive ethanol was removed from the flash chamber, thus, alleviating the accumulation of ethanol in the system.

2.4.3 Cellulase activity

During the enzymatic hydrolysis, cellulase may lose its activity due to its irreversible binding on cellulose. Surfactants, amphiphilic molecules which tend to adsorb onto cellulose surfaces, can help to alter the surface and interfacial properties of the reaction system by lowering the surface tension or the free energy between different phases, providing a way to prevent cellulase inactivity (Park et al., 1992). Various kinds of surfactants have been used in enzymatic hydrolysis (Castanon and Wilke, 1981; Converse et al., 1988; Helle et al., 1993; Ooshima et al.,

1986; Park et al., 1992; Wu and Ju, 1998). Castanon and Wilke (1981) reported that Tween 80 (0.1%) increased the rate of enzyme usage by 33% in the hydrolysis of newspaper, and a higher enzyme recovery was achieved. Park et al. (1992) studied the effects of 5 nonionic surfactants on the hydrolysis of newspaper and found that all the surfactant enhanced the saccharification rate. The saccharification rate of the system using POG phenyl ether was 2 times higher than that of the control sample. It was also reported that if surfactants were used in cellulose hydrolysis, the enzyme dosage can be halved, which could dramatically decrease the cost of lignocellulose-to-sugar process (Helle et al. 1993). However, surfactants are sometimes inhibitory to microorganisms used for ethanol fermentation. Wu and Ju (1998) found that Tween 20 was strongly inhibitory to *D. clausenii* even at a low concentration (0.1%).

2.4.4 Enzyme combination

Using the combination of enzymes in enzymatic hydrolysis has been proven to be able to substantially enhance saccharification rate in some circumstances. Extensive studies have been conducted in this area (Beldman et al., 1984, 1988; Manonmani and Sreekantiah, 1988). β-glucosidases is one of the most common supplementary enzymes. By swiftly converting cellobiose to glucose, β-glucosidases effectively reduce the inhibition of cellobiose to cellulase. It was reported that the application of cellulase (1,4-(1,3;1,4)- β-D-glucan 4-glucanohydrolase, EC 3.2.1.4) from *Trichoderma viride* and pectimase (poly (1,4-α-D-galacturonide)

glycanohydrolase, EC 3.2.1.15) from *Aspergillus niger* could significantly increase the cellulose conversion (Beldman et al., 1984). Ghose and Bisaria (1979) reported that, due to the creation of more accessible cellulosic regions for cellulase by xylanase pretreatment, the hydrolysis was enhanced. Manonmani and Sreekantiah (1987) also found that 90% conversion could be achieved when a mixture of cellulases obtained from *Aspergillus ustus* and *Trichoderma viride* was used (1.0 U ml^{-1}). The effect of enzyme combination should not be explained by simple sequential attack of different components (Beldman et al., 1988), and further studies on the mechanism of enzyme synergism are required.

2.5 FERMENTATION OF SUGARS

Fermentation of sugars to ethanol by microorganisms is the last step of the lignocellulose-to-ethanol process. Although sugar fermentation is far from a new technology used in our daily life, there are still some problems encountered in the fermentation step of lignocellulose converison. Pentoses fermentation and inhibition removal are the two major challenges need to be addressed.

2.5.1 Microorganisms producing ethanol

Many bacteria, yeasts and fungi have been reported to be used in ethanol production (Dien et at., 1998; Olsson and Hahn-Hägerdal, 1996; Skoog and Hahn-Hägerdal, 1988). The most commonly used microbe for ethanol production is

Saccharomyces cerevisiae. They could produce ethanol to a concentration as high as 18% of the fermentation broth and were proven to be quite robust and less sensitive to inhibitors (Varga et al. 2004; Lin and Tanaka, 2006). To improve the economic promise of the lignocellulose-to-ethanol conversion, hemicellulose hydrolysates containing high proportion of pentoses should also be utilized for ethanol production. Unlike hexoses, pentoses can not be fermented by *Saccharomyces cerevisiae* and other commonly used microorganisms. The main pentose component in hemicellulose is xylose (Skoog and Hahn-Hägerdal, 1996). Quite a few microorganisms (natural as well as recombinant) have been studied to convert xylose to ethanol. For yeasts, *Brettanomyces*, *Candida*, *Clavisporam*, *Kluyveromyces*, *Pachysolen*, *Pichia* and *Schizosaccharomyces* have been studied and the highest ethanol concentration of 27 g l^{-1} was reported for *Kluyveromyces cellobiovorus KY 5199* (Morikawa et al., 1985). *Zymomonas mobilis* is an exceptional bacterium with high ethanol yield and productivity. When two operons encoding xylose assimilation (xylose isomerase and xylulocinase) and pentose phosphate pathway enzymes (transaldolase and transketolase) were constructed and transformed into it, the recombinant *Z. mobilis* grew only on xylose and produced ethanol with a productivity of 0.57 g l^{-1} h^{-1} and a xylose yield of 0.44 g g^{-1} (Olsson and Hahn-Hägerdal, 1996). Since fungi are able to to use a broad range of substrates, they are promising in a process where pretreatment, saccharification, and fermentation take place simultaneously. However, the ethanol productivity on

xylose is too low (0.04-0.24 g l^{-1} h^{-1}) to raise commercial attention (Olsson and Hahn-Hägerdal, 1996).

2.5.2 Inhibition compounds in lignocellulosic hydrolysate

Depending on pretreatment and hydrolysis procedures, inhibitors in lignocellulosic hydrolysates may include: 1) fermentation products such as ethanol, acetic acid, glycerol, and lactic acid; 2) by-products of sugar degradation from pretreatment and hydrolysis such as furfural, 5-hydroxymethyl furfural, levulinic acid, formic acid, and humic substance; 3) acetic acid released from hemicellulose degradation and extractives such as terpenes, alcohols, and aromatic compounds; 4) a wide range of aromatic and polyaromatic compound produced from lignin degradation; and 5) metals released from the equipment and additives (Frazer and Mccaskey, 1989; Maiorella et al., 1983; Olsson and Hahn-Hägerdal, 1996). Many methods such as overliming, supplementing activated carbon, ion exchange have been investigated for inhibitor removal (Olsson and Hahn-Hägerdal, 1996; Weil et al., 2002). Overliming has been one of the most widely used inhibitor removal methods. With the addition of excessive calcium hydroxide, the precipitate, which was dominated by calcium sulfate, formed at high pH and was removed along with acidic compounds (Olsson and Hahn-Hägerdal, 1996). Furfural can strongly inhibit fermentation. It was reported that applying polymeric adsorbents was effective in furfural removal. After the treatment of XAD-4, a polymeric adsorbent, the furfural

concentration decreased to less that 0.01 g/L from the initial concentrations that were in the range of 1-5 g/L (Weil et al., 2002). Bioabatement is a novel method to remove inhibitory compounds from lignocellulose hydrolysates. In the research of Nichols et al. (2005), *Coniochaeta ligniaria* NRRL30616, an Ascomycete that metabolizes furfural and 5-hydroxymethylfurfural, was selected from 24 strains as the best fungal strain for the removal of inhibitors from hydrolysate.

2.6 CONCLUSIONS

Although lignocellulose-to-ethanol conversion is a promising technique for the sustainable production of biofuel, major efforts should be made to enhance its economic competitiveness. Ethanol production from lignocellulosic materials involves three steps: pretreatment of raw biomass materials, hydrolysis of carbohydrates to sugars, and ethanol production by sugar fermentation. So far, much valuable work has been done to optimize the technical conditions of these steps and great improvement has been made. However, deeper understanding of the mechanisms of various methods used to improve the performance of each step is required for better operation control and output prediction. Moreover, since almost all of the explorations discussed in this review were performed in labs, the conclusions and optimum conditions proposed can not be directly used. Further modifications are required when experiments are scaled up. In addition, a comprehensive economic analysis for the lignocellulose-to-ethanol conversion is of

great importance. Since the performances of different steps are closely interrelated with each other, the adoption of a cost-effective method in one step may have a negative impact on other steps. Thus, trade-off is required sometimes to make the entire process economically optimized.

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CHAPTER 3

SODIUM HYDROXIDE PRETREATMENT OF SWITCHGRASS FOR ETHANOL PRODUCTION

ABSTRACT

Lignocellulose-to-ethanol conversion is a promising technology to supplement corn-based ethanol production. However, the recalcitrant structure of lignocellulosic materials is a major obstacle to the efficient conversion. In this study, NaOH pretreatment of switchgrass for enzymatic saccharification improvement was investigated. At 121, 50, and 21 ^{o}C, raw switchgrass biomass at a solid : liquid ratio of 0.1 g/ml was pretreated respectively for 0.25-1 h, 1-48 h, and 1-96 h at different NaOH concentrations (0.5, 1.0, and 2.0%, w/v). Pretreatments were evaluated based on the yields of lignocellulose-derived sugars in the subsequent enzymatic hydrolysis. At the best pretreatment conditions (50 ^{o}C, 12 h, and 1.0% NaOH), the yield of total reducing sugars was 453.4 mg/g raw biomass, which was 3.78 times that of untreated biomass, and the glucan and xylan conversions reached 74.4 and 62.8% respectively. Lignin reduction was closely related to the degree of pretreatment. The maximum lignin reductions were 85.8% at 121 ^{o}C, 77.8% at 50 ^{o}C, and 62.9% at 21 ^{o}C, all of which were obtained at the combinations of the longest residence times and the greatest NaOH concentration. Cellulase and cellobiase loadings of 15 FPU/g dry biomass and 20 CBU/g dry biomass were sufficient to maximize sugar production.

3.1 INTRODUCTION

Ethanol is one of the most promising alternatives to fossil fuels to power the U.S. transportation sector. It not only can be produced on a renewable basis from various biomass sources, including sugarcane, corn, trees, grasses, yard wastes, agricultural residues, and forestry wastes, but will also cause less net greenhouse gas (GHG) emissions during combustion. Corn is currently the predominant feedstock for conventional ethanol conversion processes in the United States. In 2008, 3.2 billion bushels of U.S. corn was consumed to produce 9 billion gallons of ethanol. This is an increase of 5.4 times of that produced over a decade ago (RFA, 2009). However, corn-based ethanol production diverts corn away from food markets, thus inevitably resulting in food-fuel competition (Sun and Cheng, 2002). Lignocellulosic biomass offers a better choice because of its abundance and diverse raw materials. Moreover, its lower requirements for agricultural inputs contribute to higher net energy values of feedstocks and less GHG emissions from ethanol combustion. The conversion of lignocellulose to ethanol, however, is more challenging than that of corn due to the recalcitrant structures of lignocellulosic materials. Lignocellulose-to-ethanol conversion involves three major steps: 1) pretreatment of raw biomass, 2) enzymatic hydrolysis for fermentable sugar production, and 3) ethanol fermentation. Pretreatment is essential to the conversion as it breaks up lignocellulosic matrix, thus making the carbohydrates (cellulose and hemicelluloses) more accessible to hydrolytic enzymes in the following hydrolysis. A

successful pretreatment must effectively improve the enzymatic digestibility of biomass, avoid carbohydrate loss or formation of byproducts inhibitory to the subsequent processes, and be cost-effective as well (Sun and Cheng, 2002).

Many physical (mechanical comminution, microwave irradiation, pyrolysis), chemical (acid, alkali, ozone, and solvent pretreatments), physico-chemical (steam explosion, ammonia fiber explosion, carbon dioxide explosion) and biological pretreatment methods have been studied to improve the enzymatic digestibility of lignocellulose. Sun and Cheng (2002), and Mosier et al. (2005) have provided good reviews on this topic. Among all the pretreatment methods, alkaline pretreatment has received more attention because it is relatively inexpensive, less energy intensive, and effective on many feedstocks especially forages and agricultural residues (Belkacemi et al., 1998; Chang et al., 2001; Chen et al., 2007; Xu et al., 2008). The application of alkaline solutions leads to removal of lignin barrier, disruption of structural linkages, reduction of cellulose crystallinity, and decrease in polymerization degree of carbohydrates (Mosier et al., 2005; Sun and Cheng, 2002). NaOH is one of the most effective alkaline reagents and has been used to treat a variety of lignocellulosic feedstocks (Fox et al., 1989; MacDonald et al., 1983; Sharmas et al., 2002; Silverstein et al., 2007; Soto et al., 1994). Silverstein et al. (2007) investigated chemical pretreatment of cotton stalks and reported that, among four pretreatment methods (NaOH, H_2SO_4, H_2O_2, and ozone pretreatments), NaOH pretreatment resulted in the highest level of delignification (65.63% at 2% NaOH, 90

min, 121 ^{o}C) and cellulose conversion (60.8%). Soto et al. (1994) studied NaOH pretreatment of sunflower hulls and reported saccharification values of over 50% after pretreatment using 0.5% alkali solution at 121 °C for 1.5 h. MacDonald et al. (1983) reported that almost all the lignin in corn stover was dissolved after pretreatment using 2% NaOH at 150 ^{o}C for 15 min, and 80% of the potential glucose in raw biomass was recovered.

Switchgrass (*Panicum virgatum L.*) is a perennial native warm-season grass to North America and is well adapted to the continental United States with the exception of the extent Northwest. It is regarded as a promising feedstock for ethanol production because of its biomass yield, adaptation to various soil and climate conditions, and low fertilizer, herbicide, and pesticide requirements for its prodution (Jenson et al., 2007; Keshwani and Cheng, 2009). With annual biomass yields ranging from 5.2 to 11.1 Mg ha^{-1}, switchgrass produced 540% more renewable energy than nonrenewable energy consumed, while the combustion of switchgrass-based ethanol produced 94% less GHG emissions than that of gasoline (Schmer et al., 2008). Moreover, the extensive and deep root system of switchgrass serves as a carbon storage sink, making switchgrass production beneficial for the reduction of CO_2 in atmosphere (Sladden et al., 1991). Other environmental benefits associated with switchgrass growth include improving surface water quality through nutrient uptake and providing a suitable wild life habitat for endangered grassland species (Lee et al., 1998; Murray et al., 2003).

Almost all previous studies on NaOH pretreatment involved the application of elevated temperatures (>100 ^{o}C). However, alkaline reagents, including lime and ammonia, have been proven quite effective even at much lower temperatures (room temperature-55 ^{o}C), whereas extended residence times were required (Kim and Holtzapple, 2005; Kim and Lee, 2005). NaOH is a stronger alkali than lime and ammonia, thus potentially more promising to work at reduced temperatures. In this research, NaOH pretreatment of switchgrass was explored at 121, 50, and 21 ^{o}C, with combinations of residence times and NaOH concentrations at each temperature. Material balances were performed to investigate the compositional changes of biomass caused by pretreatments, and enzyme loadings in hydrolysis were optimized.

3.2 MATERIALS AND METHODS

3.2.1 Biomass preparation

"Performer" switchgrass, a cultivar recently released (Burns et al., 2008) as an animal feed of improved quality, was used as feedstock in this research. Switchgrass biomass was obtained from Central Crops Research Station near Clayton, North Carolina. The plants were harvested in July 2007, oven dried at 50 ^{o}C for 72 hours, and ground using a Wiley mill fitted with a 2 mm screen. The prepared biomass was collected in plastic bags, sealed, and delivered to the Bio-products Development Lab in the Department of Biological & Agricultural Engineering at North

Carolina State University. The biomass was stored at room temperature and its chemical composition was analyzed before pretreatment.

3.2.2 Pretreatment

Switchgrass was pretreated at 121 ^{o}C in an autoclave, at 50 ^{o}C in a water bath, and at 21 ^{o}C (room temperature). Four g of biomass sample and 40 ml of NaOH solution of a specific concentration were placed in a serum bottle and mixed using a glass rod, forming a slurry at a solid : liquid ratio of 0.1 g/ml. All serum bottles were sealed and crimped before pretreatment. Table 3.1 shows the conditions explored. Longer residence times were applied at lower temperatures to offset the impact of reduced chemical reaction rates. Pretreated biomass was recovered by filtration and washed with 400 ml of deionized water to remove excess alkali and dissolved byproducts that might inhibit enzymes in the subsequent hydrolysis. About 1 g (dry basis) of the pretreated biomass was dried at 45 ^{o}C to constant weight for composition analysis, and the rest was stored in a sealed plastic bag at 4 ^{o}C for enzymatic hydrolysis.

Table 3.1 Conditions explored in NaOH pretreatment of switchgrass.

Temperature (^{o}C)	Residence time (h)	NaOH concentration (%)
121	0.25, 0.5, 1	0.5, 1.0, 2.0
50	1, 3, 6, 12, 24, 48	
21	1, 3, 6, 12, 24, 48, 96	

3.2.3 Enzymatic hydrolysis

The hydrolysis was carried out in an air bath shaker at 55 ^{o}C, 150 rpm for 72 h. One g of pretreated biomass (dry basis) was mixed with 30 ml of 50 mM sodium citrate buffer (pH 4.8) in a 250 ml Erlenmeyer flask. Cellulase from *Trichoderma reesei* (E.C. 3.2.1.4) was added at an enzyme loading of 35 FPU (filter paper unit)/g dry biomass. This was supplemented with cellobiase from *Aspergillus niger* (E.C. 3.2.1.21) at an enzyme loading of 61.5 CBU (cellobiase unit)/g dry biomass to prevent cellobiose inhibition to cellulase. FPU is defined as the amount of enzyme that produces 1 μmol of glucose from filter paper per minute, and CBU is defined as the amount of enzyme that produces 2 μmol of glucose from cellobiose per minute. The activities of cellulase and cellobiase were respectively 80 FPU/ml and 277 CBU/ml. Both enzymes were obtained from Novozymes North America, Inc. (Franklinton, North Carolina, USA). Sodium azide (0.3%, w/v) was added into the mixture to inhibit microbial growth. After the hydrolysis, the flasks were immediately transferred to an ice bath to avoid further reaction. The hydrolysate was collected by centrifugation at 10000 rpm for 5 min and the supernatant was stored at -80 ^{o}C for sugar analysis.

3.2.4 Analytical methods

Laboratory Analytical Procedures (LAP) established by National Renewable Energy Laboratory (NREL) (Sluiter et al., 2005a; Sluiter et al., 2005b; Sluiter et al.,

2008) were used for the measurement of total solids, ash, structural carbohydrates, and lignin in raw and pretreated biomass. Total reducing sugar in hydrolysate was measured using 3, 5-dinitrosalicylic acid method adapted from Miller (1959) and Ghose (1987). The carbohydrates in biomass and the sugar profile of hydrolysate were determined by measuring cellulose and hemicellulose derived monosaccharides (glucose, xylose, galactose, arabinose, and mannose) using high performance liquid chromatography (HPLC). The HPLC system was equipped with a Bio-Rad Aminex HPX-87P column (300mm×7.8mm) tailored for analysis of lignocellulose-derived sugars, a Bio-Rad Micro-Guard column, a thermostatted autosampler, a quaternary pump, and a refractive index detector. The standards used were glucose, xylose, galactose, arabinose, and mannose at concentrations of 0.5, 2.0, 5.0, 7.5, 10.0 g/L. The analytical column was operated at 80 °C with HPLC grade water as the mobile phase at a flow rate of 0.6 ml/min. The samples were injected at 10 µl and the acquisition time was 35 min. A post-run time of 25 min was included between injections to allow for late-eluting compounds to come off the column.

3.2.5 Statistical analysis

The GLM procedure in SAS 9.1 software (SAS Institute Inc., Cary, NC) was used for all data analysis. Analysis of variance (ANOVA) was used to determine the effects of various factors on pretreatments and Tukey simultaneous tests were

conducted to determine the statistical differences between treatments. All treatments in this study were conducted in triplicate and a 95% confidence level was applied in all analysis.

3.3 RESULTS AND DISCUSSION

3.3.1 Characterization of “Performer” switchgrass

Because glucan and xylan are the backbones of structural carbohydrates (cellulose and hemicellulose) in the plant cell wall, they accounted for almost 50% of the total switchgrass biomass (Table 3.2). The contents of galactan and arabinan were relatively low in switchgrass, while mannan was not detected. The carbohydrate fraction of switchgrass feedstock was 53.5% of the total biomass and its composition corresponded to the previous reports that cellulose made up the majority of plant’s carbohydrates, and xylose, galactose, and arabinose were the major building blocks of hemicellulose in grasses (Chen et al., 2007; Sun and Cheng, 2005). In raw switchgrass, lignin (including acid soluble lignin and acid insoluble lignin) was 21.4% of the total biomass, which was comparable with the typical lignin contents (10-20%) of herbaceous species and agricultural residues (McMillan, 1994). The composition of “Performer” switchgrass used in this study agreed with those of other switchgrass varieties like Alamo and Cave-in-Rock reported in the Biomass Feedstock Composition and Property Database of DOE (DOE, 2004). The feedstock also contained some undefined components, which were mainly nonstructural

compounds including but were not limited to protein, waxes, fats, resins, gums, and chlorophyll (Kuhad and Singh, 1993; Sluiter et al., 2005c).

Table 3.2 Chemical composition of “Performer” switchgrass.

Component	Dry weight (%)
Glucan	31.99 (1.69)
Xylan	17.90 (1.04)
Galactan	1.73 (0.09)
Arabinan	1.87 (0.11)
Lignin*	21.37 (0.34)
Ash	3.77 (0.12)
Other	21.37 (2.45)

*Including acid soluble lignin and acid insoluble lignin

3.3.2 Study on pretreatment conditions

3.3.2.1 Sugar production

Biomass solubilization caused by pretreatment could have substantial effect on total sugar production in enzymatic hydrolysis. Table 3.3 showed that the solid recovery decreased with the elevation of pretreatment severity (temperature, residence time, and NaOH concentration), and 19.5-53.9% of the biomass was lost during pretreatment.

Table 3.3 Solid recoveries after pretreatments.

Temperature (°C)	Residence time (h)	Percent solid recovery (%)		
		NaOH concentration (%)		
		0.5	1.0	2.0
121	0.25	75.12 (0.77)	57.46 (0.85)	48.86 (0.59)
	0.5	74.39 (0.67)	55.14 (0.43)	48.11 (0.90)
	1	72.99 (0.68)	52.30 (0.22)	46.14 (0.28)
50	1	78.06 (0.13)	75.57 (0.68)	68.84 (1.59)
	3	77.67 (0.55)	72.90 (0.55)	59.99 (0.97)
	6	77.11 (0.46)	68.89 (0.41)	57.66 (0.81)
	12	75.86 (0.31)	68.21 (0.32)	54.70 (0.16)
	24	75.27 (0.91)	64.46 (0.83)	53.83 (0.52)
	48	75.24 (0.91)	62.47 (0.57)	52.12 (0.44)
21	1	80.55 (0.41)	80.02 (0.22)	76.77 (0.19)
	3	78.93 (0.69)	78.89 (0.41)	72.51 (0.29)
	6	79.15 (0.46)	76.89 (0.82)	68.58 (0.59)
	12	79.62 (0.36)	76.17 (0.40)	67.07 (0.55)
	24	79.41 (0.71)	73.60 (0.33)	63.52 (0.93)
	48	79.33 (0.33)	70.51 (0.47)	60.69 (0.51)
	96	78.68 (0.14)	69.61 (0.20)	57.71 (0.96)

After enzymatic hydrolysis of pretreated biomass, glucose and xylose in the hydrolysate were measured by HPLC, while galactose and arabinose were measured but not reported due to their low concentrations. The overall pretreatment effectiveness was evaluated using the total reducing sugar yield based on raw biomass. At 121 °C, NaOH concentration had significant ($P<0.05$) effect on glucose yield at all the residence times, while residence time didn't significantly ($P>0.05$) influenced glucose yield at 2.0% NaOH (Figure 3.1a). At 0.5 h and 2.0% NaOH, glucose yield reached the maximum value of 279.1 mg/g raw biomass, with the glucan conversion of 78.5% which was comparable with those of barley straw, triticale hay, triticale straw, and wheat straw pretreated using 2.0% NaOH at 121 °C

for 1 h (70.9-81.3%) (Chen et al., 2007). Since hemicelluloses are a group of amorphous, low molecular weight, heterogeneous and branched polysaccharides, they are more susceptible to alkaline attack than semicrystalline cellulose. However, the increased pretreatment severities didn't necessarily lead to higher xylose yields due to the greater solubilization of hemicellulose (Figure 3.1b). Increasing residence time favored xylose yields at 0.5% NaOH, while it didn't have significant ($P>0.05$) impact on xylose yields at 1.0% NaOH. Xylose yields at 2.0% NaOH were lower than those at 1.0% NaOH, and decreased with the extension of residence time after 0.5 h. At 0.5 h and 1.0% NaOH, the xylose yield reached the maximum value of 114.7 mg/g raw biomass, with the xylan conversion of 56.4%. The effects of residence time and NaOH concentration on biomass digestibility improvement are clear as shown in Figure 3.1c. Due to the more serious carbohydrate solubilization at high NaOH concentration, using 2.0% NaOH didn't significantly ($P>0.05$) increase total reducing sugar yield. Further, 0.5 h was sufficient to maximize sugar production. At the best combination of residence time and NaOH concentration (0.5 h and 1.0% NaOH), the total reducing sugar yield reached 425.4 mg/g raw biomass, which was 3.55 times that of untreated biomass. The total reducing sugar yields were significantly ($P<0.05$) higher than the sums of glucose and xylose yields, which was attributed to the existence of other reducing sugars such as galactose, arabinose, mannose, and cellobiose in the hydrolysate.

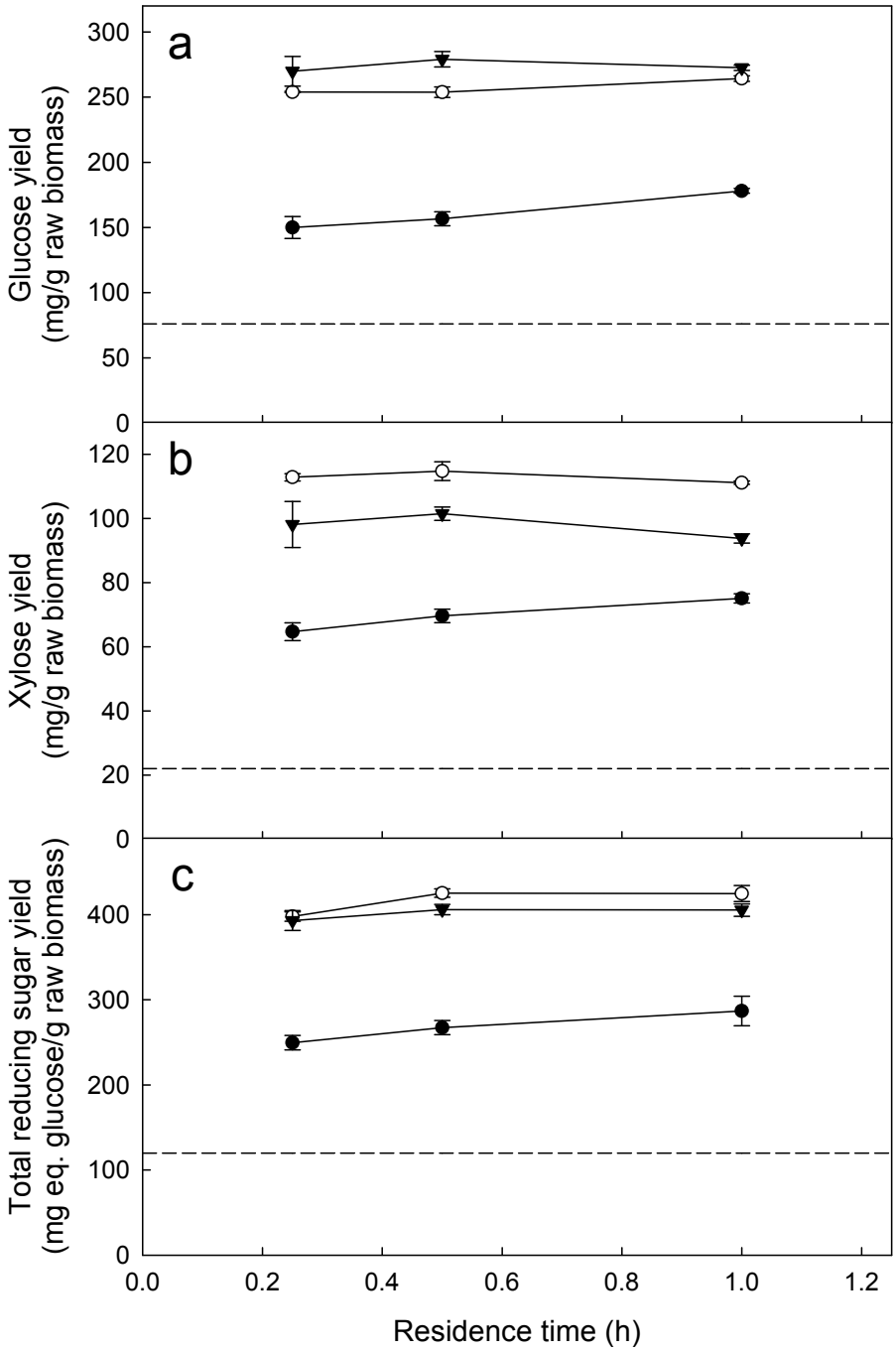

Figure 3.1 Glucose, xylose, and total reducing sugar yields of (---) raw switchgrass biomass and biomass pretreated at (●) 0.5%, (○) 1.0%, and (▼) 2.0% NaOH at 121 ^{o}C.

At 50 ^{o}C, glucose yields at 1.0 and 2.0% NaOH were comparable and started to level off respectively at 6 h and 3 h (Figure 3.2a). At 12 h and 2.0% NaOH, the glucose yield reached the maximum value of 276.1 mg/g raw biomass, with the glucan conversion of 77.7%. Using 2.0% NaOH resulted in lower xylose yields than using 1.0% NaOH after 3 h (Figure 3.2b). With the extension of residence time from 1 h to 48 h, the xylose yield increased by 24.7% at 1.0% NaOH while decreased by 7.2% at 2.0% NaOH. At 12 h and 1.0% NaOH, the xylose yield reached the maximum value of 127.8 mg/g raw biomass, with the xylan conversion of 62.8%. The maximum glucose yield at 50 ^{o}C was comparable with that at 121 ^{o}C, while the maximum xylose yield was considerably higher at 50 ^{o}C due to the better hemicellulose preservation at the reduced temperature. Figure 3.2c shows that 1.0% NaOH performed better than 2.0% NaOH in terms of total sugar production. At the best combination of residence time and NaOH concentration (12 h and 1.0% NaOH), the total reducing sugar yield reached 453.4 mg/g raw biomass, which was 3.78 times that of untreated biomass and 7% greater than the maximum yield at 121 ^{o}C.

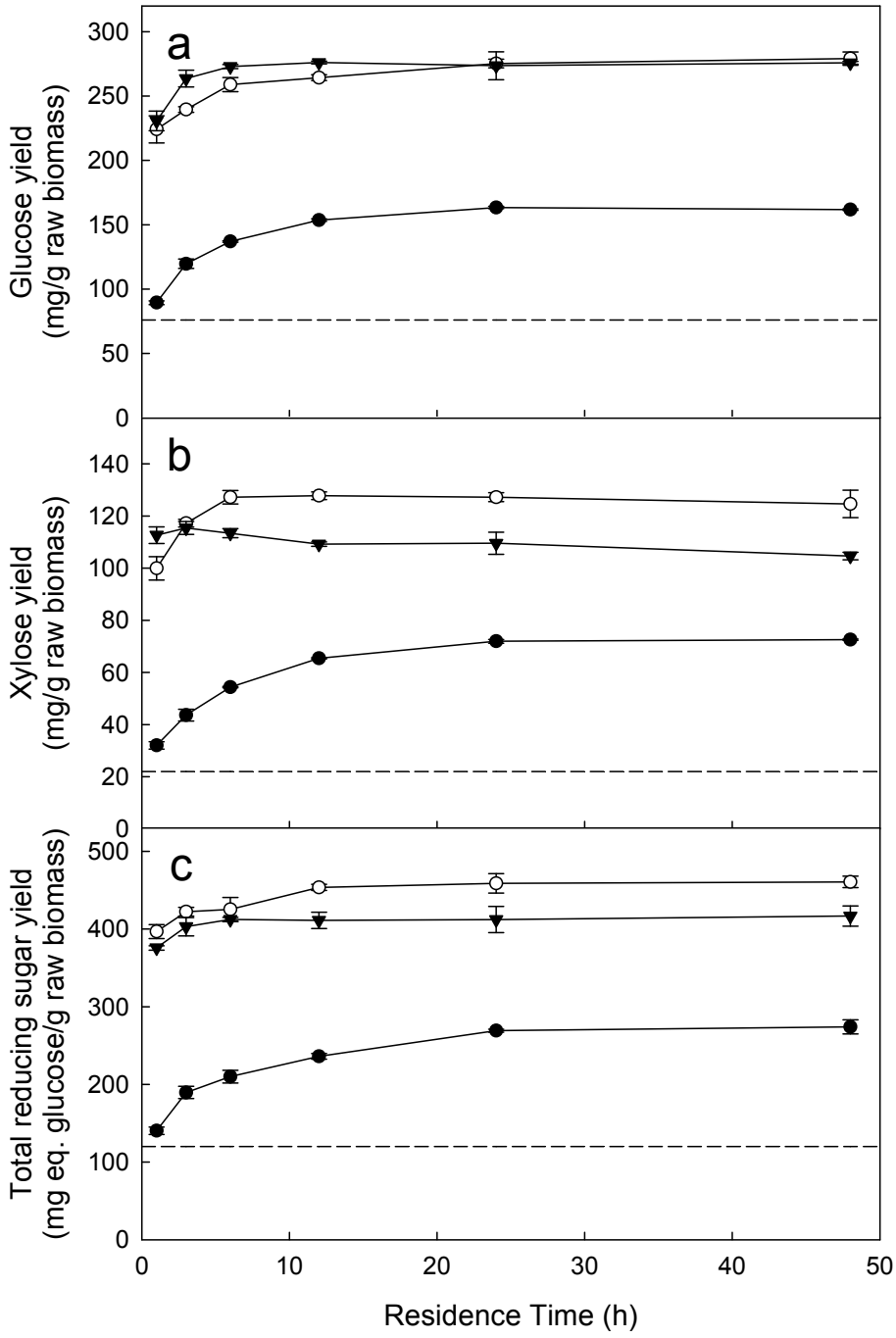

Figure 3.2 Glucose, xylose, and total reducing sugar yields of (---) raw switchgrass biomass and biomass pretreated at (●) 0.5%, (○) 1.0%, and (▼) 2.0% NaOH at 50 ^{o}C.

At 21 ^{o}C, longer residence times or greater chemical loadings were required for effective pretreatments. Glucose yields at 0.5 and 1.0% NaOH were considerably less than those at 2.0% NaOH (Figure 3.3a). At 96 h and 2.0% NaOH, the glucose yield reached the maximum value of 262.9 mg/g raw biomass, with glucan conversions of 74.0%. Unexpectedly, when using 0.5% NaOH, the glucose yield of the biomass pretreated for 1 h was even less than that of the untreated biomass. This is probably because that in the hydrolysate of untreated biomass, a part of the glucose was derived from nonstructural carbohydrates, such as starch and sucrose (Dien et al., 2006), while using 0.5% NaOH, although barely improved the biomass digestibility at the initial stage of pretreatment, caused considerable solubilization of nonstructural carbohydrates, thus resulting in an even less glucose recovery. At 0.5 and 1.0% NaOH, xylose yield increased with the extension of residence time, while at 2.0% NaOH, xylose yield increased within the first 6 h, and decreased thereafter from 111.5 to 94.6 mg/g raw biomass (Figure 3.3b). This was associated with the enhancement of hemicellulose solubilization. At 2.0% NaOH, the total reducing sugar yield leveled off at 6 h, while it took much longer to achieve comparable sugar yields using 1.0% NaOH (Figure 3.3c). At the best combination of residence time and NaOH concentration (6 h and 2.0% NaOH), the total reducing sugar yield was 406.2 mg/g raw biomass, which was 3.39 times that of untreated biomass but 5 and 10% less than the maximum yields respectively obtained at 121 and 50 ^{o}C. Based on total reducing sugar yield, 50 ^{o}C, 12 h, and 1.0% NaOH was determined as the best conditions for NaOH pretreatment of switchgrass.

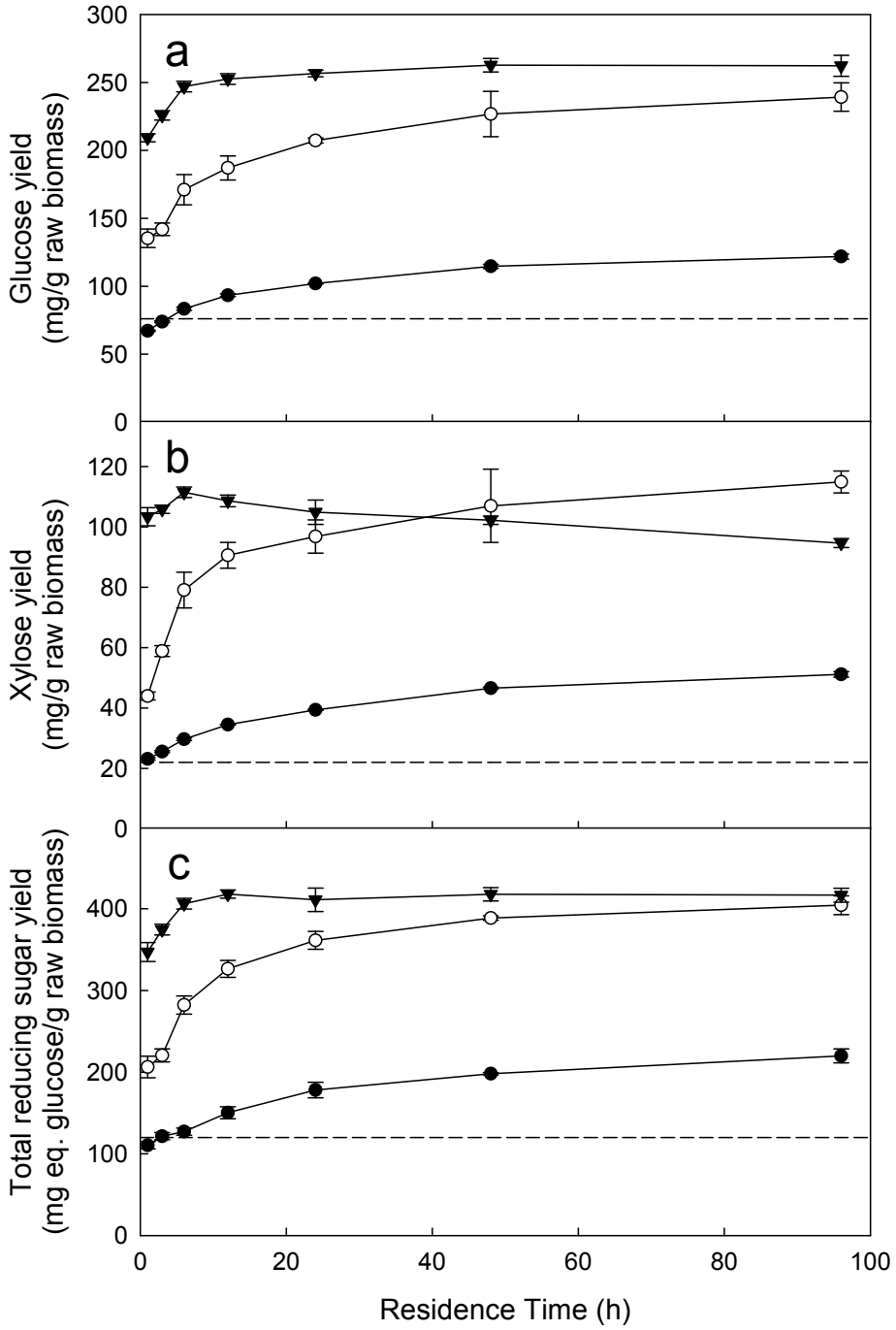

Figure 3.3 Glucose, xylose, and total reducing sugar yields of (---) raw switchgrass biomass and biomass pretreated at (●) 0.5%, (○) 1.0%, and (▼) 2.0% NaOH at 21 ^{o}C.

3.3.2.2 Lignin reduction

Lignin is a three dimensional complex aromatic polymer which forms a sheath surrounding cellulose and hemicellulose, stiffening and holding together the fibers of polysaccharides (Fan et al., 1987). Since it is a major barrier limiting the accessibility of carbohydrates to hydrolytic enzymes, its reduction is crucial to the improvement of biomass digestibility. NaOH pretreatment has shown great effectiveness in removing lignin from various biomass feedstocks. Chen et al. (2007) reported that 75.1-84.5% of the lignin in barley straw, triticale hay, triticale straw, and wheat straw were removed after pretreatment at 121 ^{o}C, 60 min, and 2.0% NaOH. In this study, NaOH concentration significantly ($P<0.05$) affected lignin reduction at all temperatures (Figure 3.4), which was in agreement with Silverstein et al. (2007). At 121 ^{o}C, residence time had significant ($P<0.05$) impact on lignin reduction at 1.0 and 2.0% NaOH, and the maximum lignin reduction of 85.8% was obtained at 1 h and 2.0% NaOH. At 50 and 21 ^{o}C, residence time had significant ($P<0.05$) impact on lignin reduction at all the NaOH concentrations, and the greatest lignin reductions at 50 ^{o}C and room temperature were 77.8 and 62.9%, which were respectively obtained at 48 h, 2.0% NaOH and 96 h, 2.0% NaOH. The maximum lignin reductions at different temperatures were all obtained at the combinations of the longest residence times and the greatest NaOH concentration, which indicated a close relationship between lignin reduction and pretreatment severity. However,

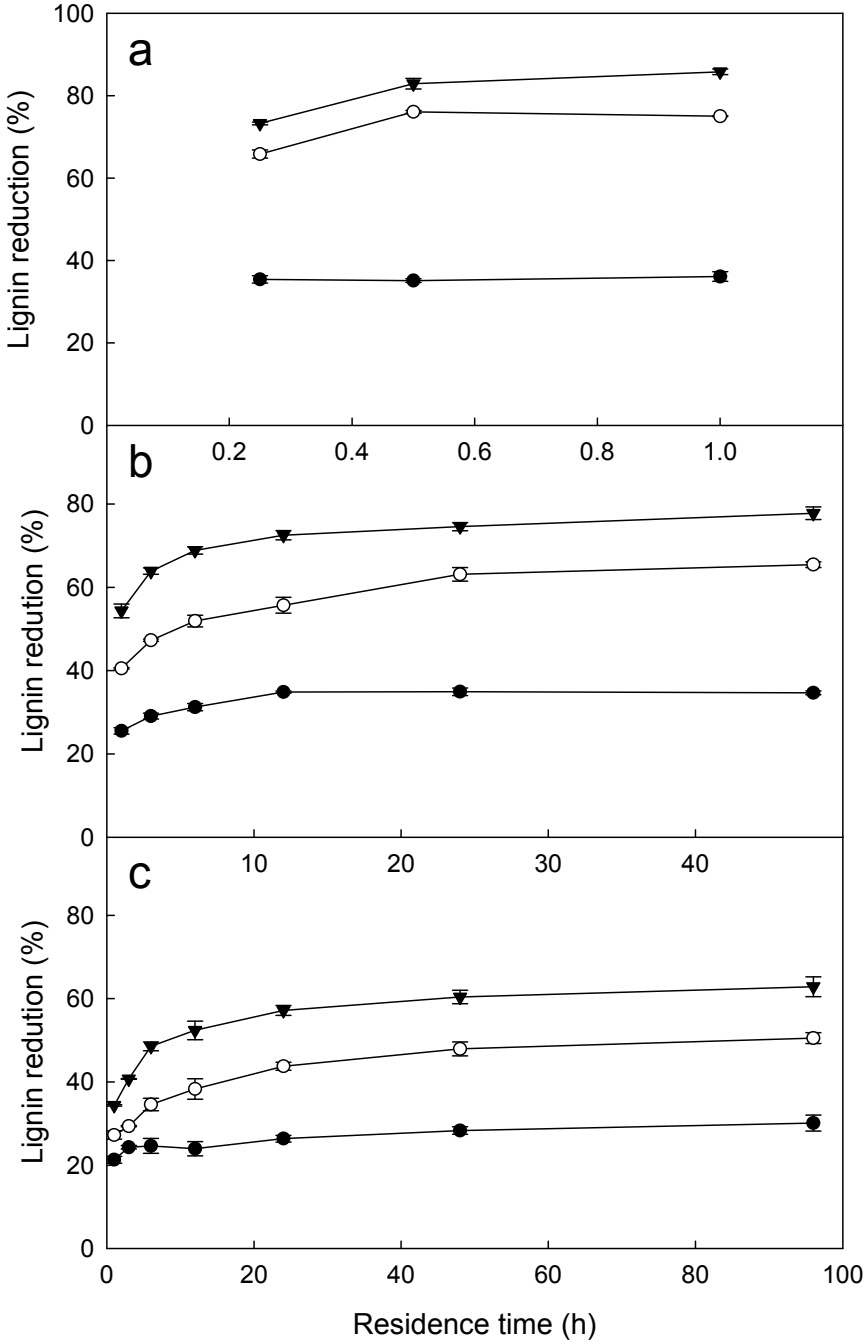

Figure 3.4 Lignin reduction of switchgrass pretreated at (●) 0.5%, (○) 1.0%, and (▼) 2.0% NaOH at (a) 121 ^{o}C, (b) 50 ^{o}C, and (c) 21 ^{o}C.

since increasing pretreatment severity doesn't necessarily lead to more sugar production due to the greater biomass solubilization, lignin reduction may not be an accurate indicator for overall pretreatment effectiveness.

3.3.3 Material balances

Material balances were performed on the biomass pretreated using the best combinations of residence time and NaOH concentration at different temperatures (0.5 h, 1.0% NaOH at 121 ^{o}C; 12 h, 1.0% NaOH at 50 ^{o}C; 6 h, 2.0% NaOH at 21 ^{o}C). The total dry weight of the sample was measured after pretreatment, and the compositions of pretreated biomass (glucan, xylan, galactan, arabinan, lignin, ash, and other) were determined and compared with that of the raw biomass. At 121 ^{o}C, considerable solubilization occurred to all the biomass components, including carbohydrates, as rated by material balances (Figure 3.5). At 50 and 21 ^{o}C, glucan was well preserved, while xylan recoveries remained low due to the extent of hemicellulose solubilizaiton, even at the reduced temperatures. The lignin reduction increased from 48.8% at 21 ^{o}C to 76.1% at 121 ^{o}C, indicating a substantial impact of temperature on lignin removal. After pretreatment at the best conditions at 121, 50, and 21 ^{o}C, the carbohydrate content of the biomass, respectively, increased from 53.5% to 72.4, 66.1, and 64.1% (Figure 3.6). On the other hand, the lignin content decreased from 21.4% to 9.2, 14.1, and 16.1% respectively. The above results clearly show that the pretreatments using 50 and 21 ^{o}C resulted in inferior

compositions (reduced carbohydrate but greater lignin percentages) than the pretreatment using 121 ^{o}C. However, better carbohydrate preservations achieved at the reduced temperatures contributed to comparable maximum total sugar productions at different temperatures, which indicated that NaOH pretreatment could maintain its effectiveness even at low temperatures.

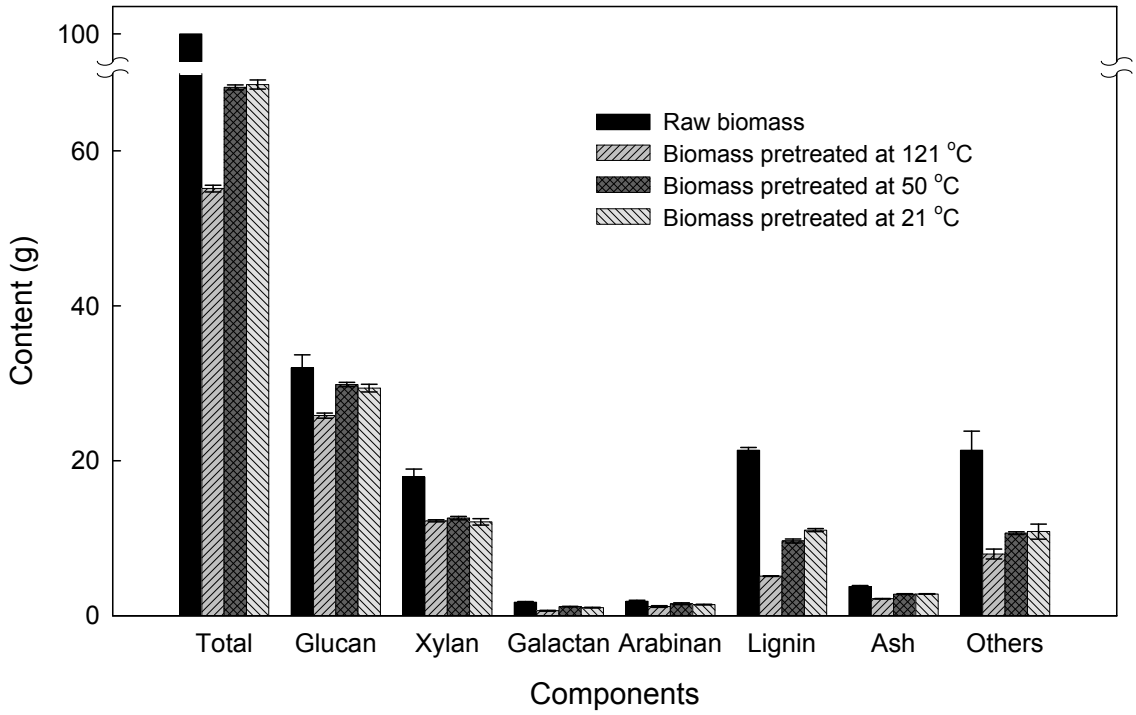

Figure 3.5 Material balances for raw switchgrass biomass and biomass pretreated at the best combinations of residence time and NaOH concentration at different temperatures (0.5 h, 1.0% NaOH at 121 ^{o}C; 12 h, 1.0% NaOH at 50 ^{o}C; 6 h, 2.0% NaOH at 21 ^{o}C).

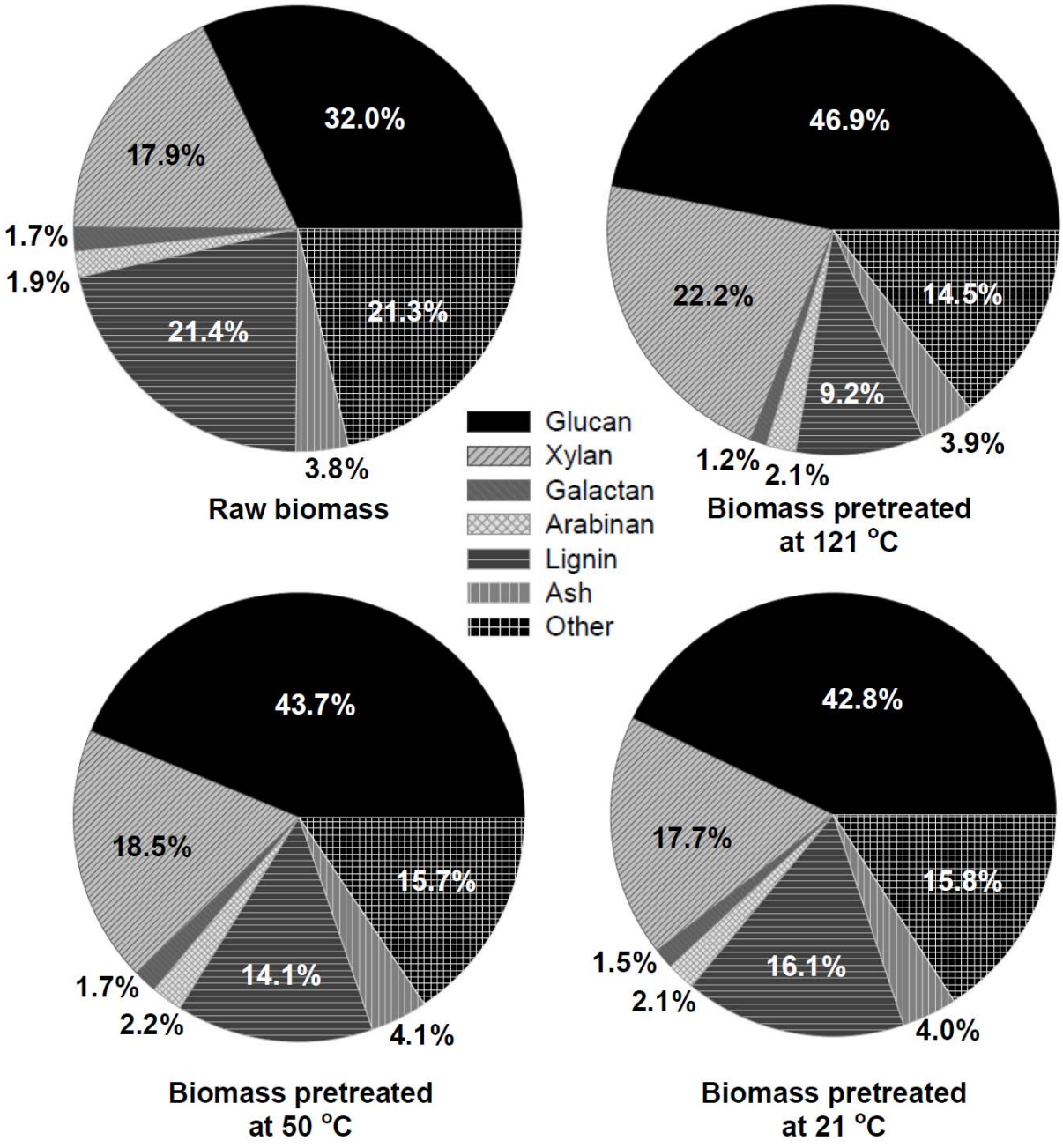

Figure 3.6 Compositions of raw switchgrass biomass and biomass pretreated at the best combinations of residence time and NaOH concentration at different temperatures (0.5 h, 1.0% NaOH at 121 °C; 12 h, 1.0% NaOH at 50 °C; 6 h, 2.0% NaOH at 21 °C).

3.3.4 Enzyme loading study

To study the effects of enzyme loadings on biomass saccharification, cellulase loadings of 0-25 FPU/g dry biomass and cellobiase loadings of 0-50 CBU/g dry biomass were investigated. The study on enzyme loadings was based on the pretreatment at the best conditions (50 ^{o}C, 12 h, and 1.0% NaOH). In the cellulase loading test, the cellobiase loading was kept excessive at 61.5 CBU/g biomass to eliminate the impact of cellobiase limitation. At the excessive cellobiase loading, no inhibition of cellobiose to cellulase was observed. With the increase of cellulase loading from 0 to 15 FPU/g dry biomass, the total reducing sugar yield elevated by 5.84 times from 62.6 to 428.4 g/g raw biomass. Further increasing cellulase loading didn't favor the improvement of sugar production (Figure 3.7a). Similar trends were observed for both glucose and xylose yields. A cellulase loading of 15 FPU/g dry biomass was sufficient to maximize sugar yields. Cellobiase loadings were studied based on the best cellulase loading. Supplementing cellobiase enhanced sugar production not only by eliminating cellobiose inhibition to cellulase, but also through the hydrolysis of cellobiose to glucose. Figure 3.7b shows that supplementing cellobiase significantly ($P<0.05$) increased sugar yields and a cellobiase loading of 20 CBU/g dry biomass was sufficient.

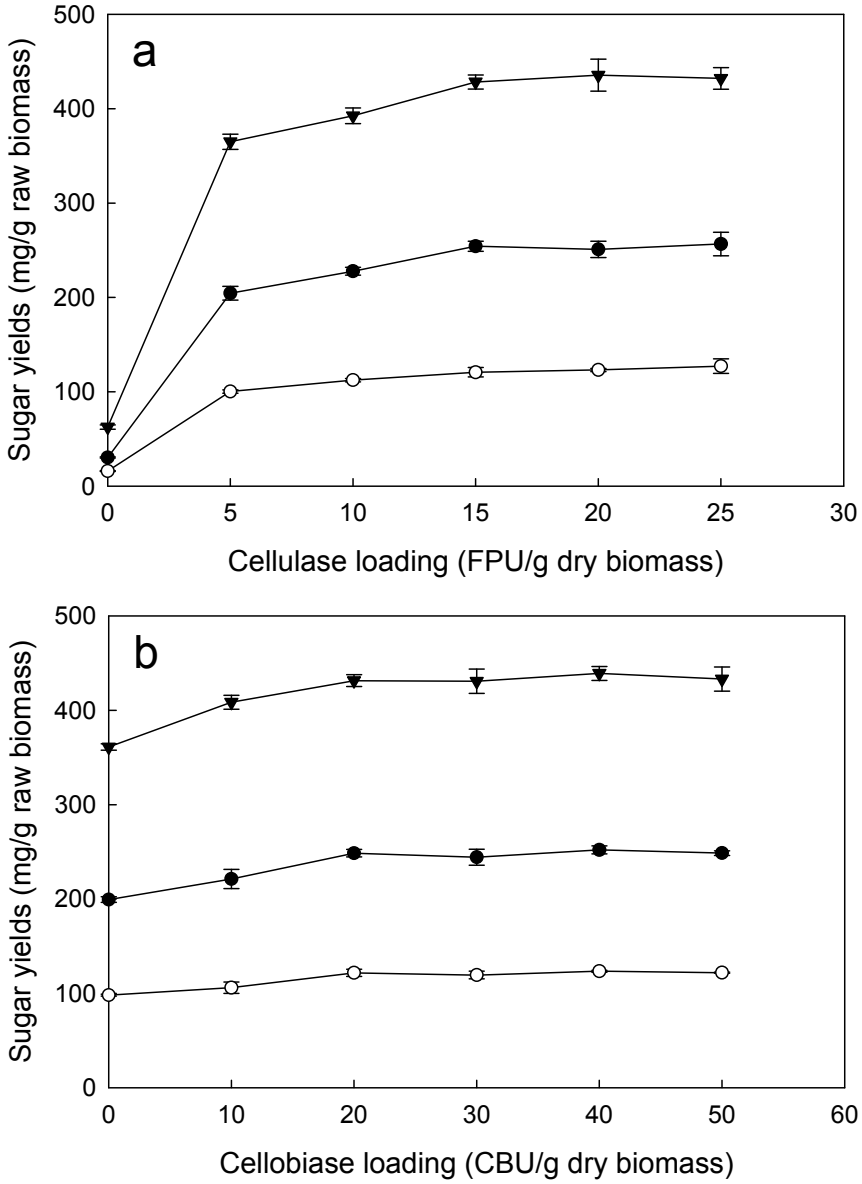

Figure 3.7 Effects of cellulase and cellobiase loadings on (●) glucose, (○) xylose, and (▼) total reducing sugar yields of switchgrass pretreated at the best conditions (50 ^{o}C, 12 h, and 1.0% NaOH).

3.4 CONCLUSIONS

NaOH pretreatment was effective in improving the enzymatic digestibility of switchgrass at all the temperatures studied (121, 50, and 21 ^{o}C). At the best combinations of residence time and NaOH concentration at different temperatures (1.0% NaOH, 0.5 h at 121 ^{o}C; 1.0% NaOH, 12 h at 50 ^{o}C; 2.0% NaOH, 6 h at 21 ^{o}C), the total reducing sugar yields were respectively 425.4, 453.4, and 406.2 mg/g raw biomass, which were 3.55, 3.78 and 3.39 times that of untreated biomass. Based on total reducing sugar yield, the best conditions for switchgrass pretreatment were: 50 ^{o}C, 12 h, and 1.0% NaOH, at which the enzymatic conversion of switchgrass to sugars was 74.4% of glucan, 62.8% of xylan, and 70.8% of the total available carbohydrates. NaOH pretreatment showed outstanding delignification capacity, and the removal of lignin barrier was closely related to the pretreatment severity applied. However, lignin reduction should not be regarded as an accurate indicator for pretreatment effectiveness due to greater carbohydrate loss at more intense pretreatment conditions. NaOH is a strong base and has shown great potential to work at reduced temperatures. Nevertheless, extended residence times or greater chemical loadings required at reduced temperatures would offset the cost reduction from decreased heating requirement. Therefore, primary cost-benefit analysis is necessary prior to initiating any scale-up studies.

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CHAPTER 4

LIME PRETREATMENT OF SWITCHGRASS FOR ETHANOL PRODUCTION

ABSTRACT

Lignocellulosic biomass is a promising alternative feedstock for ethanol production. However, its recalcitrant structure necessitates a pretreatment step to break up the lignocellulosic matrix, thus improving the accessibility of carbohydrates to hydrolytic enzymes for sugar production. In this study, lime pretreatment of switchgrass was explored at 121, 50, and 21 ^{o}C, and the effects of residence time, lime loading, and biomass washing on the sugar production efficiency investigated. Pretreatments were evaluated based on the yields of biomass-derived sugars in the following enzymatic hydrolysis. Under the best pretreatment conditions (50 ^{o}C, 24 h, 0.10 g $Ca(OH)_2$/g raw biomass, and wash intensity of 100 ml water/g raw biomass), the yields of glucose, xylose, and total reducing sugars reached 239.6, 127.2, and 433.4 mg/g raw biomass, which were respectively 3.15, 5.78, and 3.61 times those of untreated biomass. The study on calcium-lignin bonding showed that calcium ions crosslinked lignin molecules at alkaline conditions, which substantially decreased lignin solubilization in pretreatment, but the resulting high lignin contents of the pretreated biomass did not compromise the improvement of enzymatic digestibility.

4.1 INTRODUCTION

The world's ever-increasing demand for energy, inevitable depletion of fossil fuels, and growing concerns over global warming have stimulated the exploration for alternative energy sources. Ethanol has attracted much attention and is now widely used in the U.S. transportation sector as a partial gasoline replacement to reduce petroleum usage and tailpipe emissions. For example, nearly half of the U.S. gasoline contains up to 10% ethanol (E10) (DOE, 2009). Ethanol can be produced on a renewable basis from sources of high-carbohydrate biomass such as sugarcane, corn, sweet potato, and lignocellulosic biomass. In the United States, corn grain is currently the predominant feedstock for ethanol production. In 2008, the U.S. ethanol industry produced a record 9 billion gallons of corn-based ethanol, at an increase of 38% over the previous year (RFA, 2009). However, corn-based ethanol production will compete with food and feed production for limited agricultural land (Sun and Cheng, 2002). Moreover, corn cultivation has high requirements for agricultural inputs, and causes substantial environmental pollution and soil erosion (Pimentel et al., 2003). Lignocellulosic biomass, which includes trees, grasses, agricultural residues, and forestry wastes, is an alternative feedstock. The lignocellulosic biomass is abundant and its production imposes much less negative impacts on economics, energy balance, and environment.

Compared with corn-based ethanol production, however, lignocellulose-to-ethanol conversion is more challenging due to the complex structure of

lignocellulosic materials, which necessities a pretreatment step to break up the lignocellulosic matrix, thus making cellulose and hemicellulose more accessible to hydrolytic enzymes for fermentable sugar production. Pretreatment technologies including comminution, pyrolysis, steam explosion, ammonia fiber explosion, acid pretreatment, and alkaline pretreatment have been extensively studied (Alizadeh et al., 2005; Chang et al., 1997; Negro et al., 2003; Piskorz et al., 1989; Sun and Cheng, 2005). Among all the pretreatment methods, alkaline pretreatment has received more attention because it is relatively inexpensive and less energy intensive (Chang et al., 2001). The major mechanism of alkaline pretreatment is believed to be the saponification of intermolecular ester bonds that extensively exist in the lignocellulosic matrix. Alkaline conditions also cause disruption of lignin structure, an increase in internal surface area, and a decrease in cellulose crystallinity and degree of polymerization (Sun and Cheng, 2002). Lime ($Ca(OH)_2$) has been proven effective in treating various biomass feedstocks and is much cheaper than other commonly used alkaline reagents such as sodium hydroxide, ammonia, and potassium hydroxide (Chang et al., 1997; Kaar and Holtzapple, 2000). It is also safe to handle and can be easily recovered by carbonating wash water using CO_2 (Chang et al., 1998). Chang et al. (1998) studied lime pretreatment of bagasse and wheat straw and reported that under the conditions of 120 ^{o}C, 1 h, 0.1 g $Ca(OH)_2$/g dry biomass, and 100 ml water/g dry biomass, the 3-d reducing sugar yield of pretreated bagasse was 4.3 times of that of untreated biomass. Under the pretreatment conditions of 50 ^{o}C, 24 h, 0.10 g $Ca(OH)_2$/g dry biomass, and 15 ml

water/g dry biomass, the pretreated wheat straw produced the reducing sugars which were 9 times greater than untreated biomass. Kaar and Holtzapple (2000) reported that at the recommended pretreatment conditions of 120 ^{o}C, 4 h, 0.075 g $Ca(OH)_2$/g dry biomass, and 5 g water/g dry biomass, the enzymic conversion of corn stover to monomeric sugars was about 60% cellulose, 47% xylan, and 53% total available polysaccharide. Chang et al. (2001) reported that the 3-d reducing sugar yield of the poplar wood pretreated at 150 ^{o}C, 6 h, 0.10 g $Ca(OH)_2$/g dry biomass, and 9 ml water/g dry biomass with oxidation was 9.1 times that of untreated biomass.

Switchgrass (*Panicum virgatum L.*) is a perennial native warm-season grass species in North America. It is regarded as a potential feedstock for ethanol production because of its excellent growth in various soil and climate conditions, and low nitrogen fertilizer and herbicide requirements for cultivation (Jensen et al., 2007; Keshwani and Cheng, 2009). According to the study by Schmer et al. (2008), with an annual biomass yield reaching up to 11.1 Mg ha^{-1}, switchgrass was capable of producing 5.4 times more renewable energy than nonrenewable energy consumed, while greenhouse gas emissions from switchgrass-based ethanol were 94% less than those from gasoline. Moreover, since conventional agricultural equipments for seeding, crop management, and harvesting can be used for switchgrass production, it is easy to integrate switchgrass into the existing farming operations (Lewandowski et al., 2003).

Lime pretreatment of switchgrass was first studied by Chang et al. (1997). The investigation was conducted at 60-130 ^{o}C and the results showed that, at the recommended conditions (2 h, 100-120 ^{o}C, 0.10 g $Ca(OH)_2$/g dry biomass, and 9 ml water/g dry biomass), the 3-d reducing sugar yield of pretreated switchgrass biomass was 5 times of that of untreated biomass. However, pretreatments at temperatures lower than 60 ^{o}C have never been explored. Although lime is considered as a weak base due to its poor solubility in water, lime pretreatment at reduced temperatures has potential promise. This is attributed to the inverse relationship between lime solubility and temperature, which enables lime to provide more alkalinities at lower temperatures. It is desirable, therefore, to study the lime pretreatment of switchgrass within a broader temperature range to obtain a more comprehensive understanding of its application perspective.

In this research, “Performer” switchgrass, a cultivar developed and recently released (Burns et al., 2008) for improved quality as an animal feed, was subjected to lime pretreatment at 121, 50 and 21 ^{o}C. The effects of residence time, lime loading, and biomass washing were studied. Material balances were performed to investigate the compositional changes of biomass caused by pretreatments, and cellulase and cellobiase loadings applied in hydrolysis were optimized. Many studies showed that divalent calcium ions had high affinity for lignin and could effectively crosslink lignin molecules (Duong et al., 2005; Sundin and Harlter, 2000a, 200b; Torre et al., 1992). However, its effect on lignocellulose pretreatment has not been

determined. Because lignin is a major barrier limiting the accessibility of carbohydrates to hydrolytic enzymes, the mitigated lignin removal due to the formation of lignin-calcium-lignin linkages within biomass could potentially effect biomass digestibility improvement. Therefore, the interaction between calcium ions and lignin in alkaline pretreatment conditions, and its impact on biomass digestibility improvement were also investigated in this research.

4.2 MATERIALS AND METHODS

4.2.1 Biomass preparation

Switchgrass plants, harvested in late July, 2007, were obtained from Central Crops Research Station near Clayton, North Carolina. A harvest strip was taken randomly from each quarter of the field, and four strips were combined to form one bulk sample. The biomass sample was oven dried at 50 ^{o}C for 72 hours, ground to pass 2 mm sieve in a Wiley mill and stored in sealed plastic bags at room temperature. The chemical composition of raw biomass was analyzed before pretreatment.

4.2.2 Pretreatment

Switchgrass was pretreated at 121 ^{o}C in an autoclave, at 50 ^{o}C in a water bath, and at 21 ^{o}C (room temperature). Four g of biomass sample, 40 ml of deionized water, and a specific amount of $Ca(OH)_2$ were all mixed in a serum bottle, forming a

slurry of biomass and lime milk. All serum bottles were sealed and crimped before pretreatment. After pretreatment, the residual biomass was recovered by filtration and washed with 400 ml of deionized water to remove excess alkali, dissolved biomass components and byproducts that might inhibit enzymes. About 1 g (dry basis) of the pretreated biomass was dried at 45 ^{o}C to constant weight for composition analysis, and the rest was stored in a sealed plastic bag at 4 ^{o}C for enzymatic hydrolysis.

4.2.3 Enzymatic hydrolysis

Wet pretreated biomass (1 g, dry basis) was immersed in 30 ml of 50 mM sodium citrate buffer (pH 4.8) in a 250 ml Erlenmeyer flask. Cellulase from *Trichoderma reesei* (E.C. 3.2.1.4) was added, supplemented with cellobiase from *Aspergillus niger* (E.C. 3.2.1.21) to prevent cellobiose inhibition. To eliminate the impact of enzyme limitation on sugar yields, excessive cellulase of 35 FPU/g dry biomass and cellobiase of 61.5 CBU/g dry biomass were added. Enzymes were obtained from Novozymes North America, Inc. (Franklinton, North Carolina, USA). The activities of cellulase and cellobiase were 80 FPU/ml and 277 CBU/ml, respectively. Sodium azide (0.3%, w/v) was added into the mixture to mitigate microbial contamination. The flasks were incubated at 55 ^{o}C, and shaken at 150 rpm in an air bath shaker for 72 h. After the hydrolysis, the flasks were immediately chilled in an ice bath to avoid further reaction. Hydrolysate was collected by

centrifugation at 10000 rpm for 5 min. The supernatant was stored at -80 ^{o}C for sugar analysis.

4.2.4 Study on the interaction between calcium ions and lignin

Four g of raw switchgrass biomass was treated using the combination of a specific amount of $Ca(OH)_2$ and 40 ml of 3% NaOH. Three levels of calcium loadings consisted of 0, 0.05, and 0.10 g $Ca(OH)_2$/g raw biomass. 3% NaOH was used to create an elevated pH environment, which not only caused the effective breaking up of the biomass structure but also isolated the impact of calcium ions by minimizing the alkalinity contribution from $Ca(OH)_2$. $CaCl_2$ has been commonly used as the calcium source in studies on calcium-lignin interaction (Duong et al, 2005; Sundin and Hartler, 2000a, 2000b; Torre et al., 1992), but its use is not appropriate in this study. At high pH conditions, a portion of calcium ions from $CaCl_2$ dissociation will associate with free hydroxyl groups to form a $Ca(OH)_2$ precipitate, which inevitably decreases the alkalinity in the system. Since both the decrease of alkalinity and the formation of lignin-calcium-lignin linkages may lead to mitigated lignin solubilization, the effect from calcium bonding can not be isolated. However, using $Ca(OH)_2$ as the calcium source slightly increases the alkalinity (this increase is minimized by 3% NaOH), which potentially promotes lignin solubilization, thus making lignin preservation by calcium bonding outstanding. The treatment in this study was conducted at 121 ^{o}C for 0.5 h and 21 ^{o}C for 6 h to investigate the effect of different

conditions. Lignin contents of the treated biomass were analyzed to determine if the interaction between calcium ions and switchgrass lignin caused mitigated lignin reduction.

4.2.5 Study on the effect of high lignin content on biomass digestibility improvement

When extensive lignin-calcium-lignin linkages are formed within the biomass, the pretreated biomass is expected to have high lignin content. To understand the effect of increased lignin content on biomass digestibility improvement, Simons' stain method (Simon, 1950) was used to evaluate the porosity improvement of biomass pretreated at various pretreatment severities while having similar high lignin contents. Simons' stain is a semiquantitative method that can provide useful information on the overall porosity of the pretreated biomass and has been proven effective in evaluating the enzymatic digestibility of pretreated lignocellulosic substrates (Chandra et al., 2008; Esteghlalian et al., 2001). This method is based on the competitive adsorption of two dyes (Direct Blue 1 and Direct Orange 15) in an aqueous environment. Blue dye molecules are small and can penetrate small pores within the biomass, while the larger orange dye molecule has greater affinity for the hydroxyl groups of cellulose, thus replacing blue dye molecules in large pores. Therefore, the amount of orange and blue dyes adsorbed by the biomass can effectively reflect the improvement of biomass porosity caused by pretreatment. The protocol used in this study was adapted from Esteghlalian et al. (2001).

4.2.6 Analytical methods

Total reducing sugars were measured using 3, 5-dinitrosalicylic acid method adapted from Miller (1959) and Ghose (1987). The sugar contents in cellulase and cellobiase were measured and subtracted to determine the actual reducing sugar content in hydrolysis supernatant (Chang et al., 1997). Total solids, ash, structural carbohydrates, and lignin in raw and pretreated biomass were measured according to Laboratory Analytical Procedures (LAP) established by National Renewable Energy Laboratory (NREL) (Sluiter et al., 2005a; Sluiter et al., 2005b; Sluiter et al., 2008). The sugars in hydrolysate and the carbohydrates in biomass were determined by measuring cellulose and hemicellulose derived monosaccharides (glucose, xylose, galactose, arabinose, and mannose) using high performance liquid chromatography (HPLC). The HPLC system consisted of a Bio-Rad Aminex HPX-87P column tailored for analysis of lignocellulose-derived sugars, a Bio-Rad Micro-Guard column, a thermostatted autosampler, a quaternary pump, and a refractive index detector. The analytical column was operated at 80 °C with HPLC grade water as the mobile phase at a flow rate of 0.6 ml/min. The samples were injected at 10 µl and the acquisition time was 35 min. A post-run time of 25 min was included between injections to allow for late-eluting compounds to come off the column. The standards used were glucose, xylose, galactose, arabinose, and mannose at concentrations of 0.5, 2.0, 5.0, 7.5, 10.0 g/L.

4.2.7 Statistical analysis

All treatments in this study were conducted in triplicate. The GLM procedure in SAS 9.1 software (SAS Institute Inc., Cary, NC) was used for all data analysis. Analysis of variance (ANOVA) was used to determine the effects of various factors on pretreatments. Tukey simultaneous tests were conducted to determine the statistical differences between treatments. A 95% confidence level was applied for all analysis.

4.3 RESULTS AND DISCUSSION

4.3.1 Study on pretreatment conditions

4.3.2.1 Sugar production

In lime pretreatment of lignocellulosic biomass, temperature and residence time were reported to have the most impact on biomass digestibility, whereas lime loading had minor effect (Chang et al., 1997; Chang et al., 1998). Since the solubility of lime in water is poor, increasing lime usage beyond the amount required for maintaining the saturated lime solution is unnecessary. Studies showed that, regardless of the feedstock properties or the changes of other pretreatment conditions, lime loading generally had a critical value of approximately 0.10 g $Ca(OH)_2$/g raw biomass (Chang et al., 1997, Chang et al., 1998; Kaar and Holtzapple, 2000; Kim and Holtzapple, 2005). Below this value, the biomass digestibility substantially decreased, while above it, the digestibility barely changed.

In this study, residence time was first investigated at different temperatures, with lime loading kept constant at 0.10 g/g raw biomass, and after that, the low-impact lime loading was investigated. Because lime loading might affect the amount of water required to wash away the excess lime, wash intensity was investigated concurrently with the lime loading study. After pretreatment and enzymatic hydrolysis, glucose, xylose, and total reducing sugars in hydrolysate were measured, but galactose and arabinose were not reported due to their low concentrations. The overall pretreatment effectiveness was evaluated using the total reducing sugar yield based on raw biomass.

At 121 ^{o}C, 0.5 h was sufficient to maximize sugar yields, with the total reducing sugar yield averaging 415.0 mg/g raw biomass, and 3.46 times that of untreated biomass, and the glucan and xylan conversions were respectively 66.3 and 53.4% (Figure 4.1a). The conversion of xylan was much lower than that of glucan due to the serious solubilization of hemicellulose during pretreatment. Hemicellulose has amorphous, heterogeneous and branched structure with little strength, which makes it more susceptible to solubilization than semicrystalline cellulose at severe conditions. The sums of glucose and xylose yields were significantly ($P<0.05$) lower than total reducing sugar yields due to the existence of other reducing sugars such as galactose, arabinose, mannose, and cellobiose in the hydrolysate. At 50 ^{o}C, the yields of glucose, xylose, and total reducing sugar started to level off at 6 h (Figure 4.1b). At 24 h, the total reducing sugar yield reached the maximum value of 433.4

mg/g raw biomass, and 3.61 times that of untreated biomass, and the glucan and xylan conversions were also maximized, reaching 67.4 and 62.5% respectively. The maximum total reducing sugar yield at 50 ^{o}C was significantly ($P<0.05$) greater than that at 121 ^{o}C, which was largely due to the better hemicellulose preservation at the lower temperature. At 21 ^{o}C, much longer residence times were required for high sugar yields. The xylose yield was maximized at 48 h, and the glucose and total reducing sugar yields were maximized at 96 h (Figure 4.1c). The maximum total reducing sugar yield was 411.7 mg/g raw biomass, which was 3.43 times of that of untreated biomass and comparable with those obtained at 121 ^{o}C and 50 ^{o}C, and the maximum glucan and xylan conversions were 65.2 and 63.7% respectively.

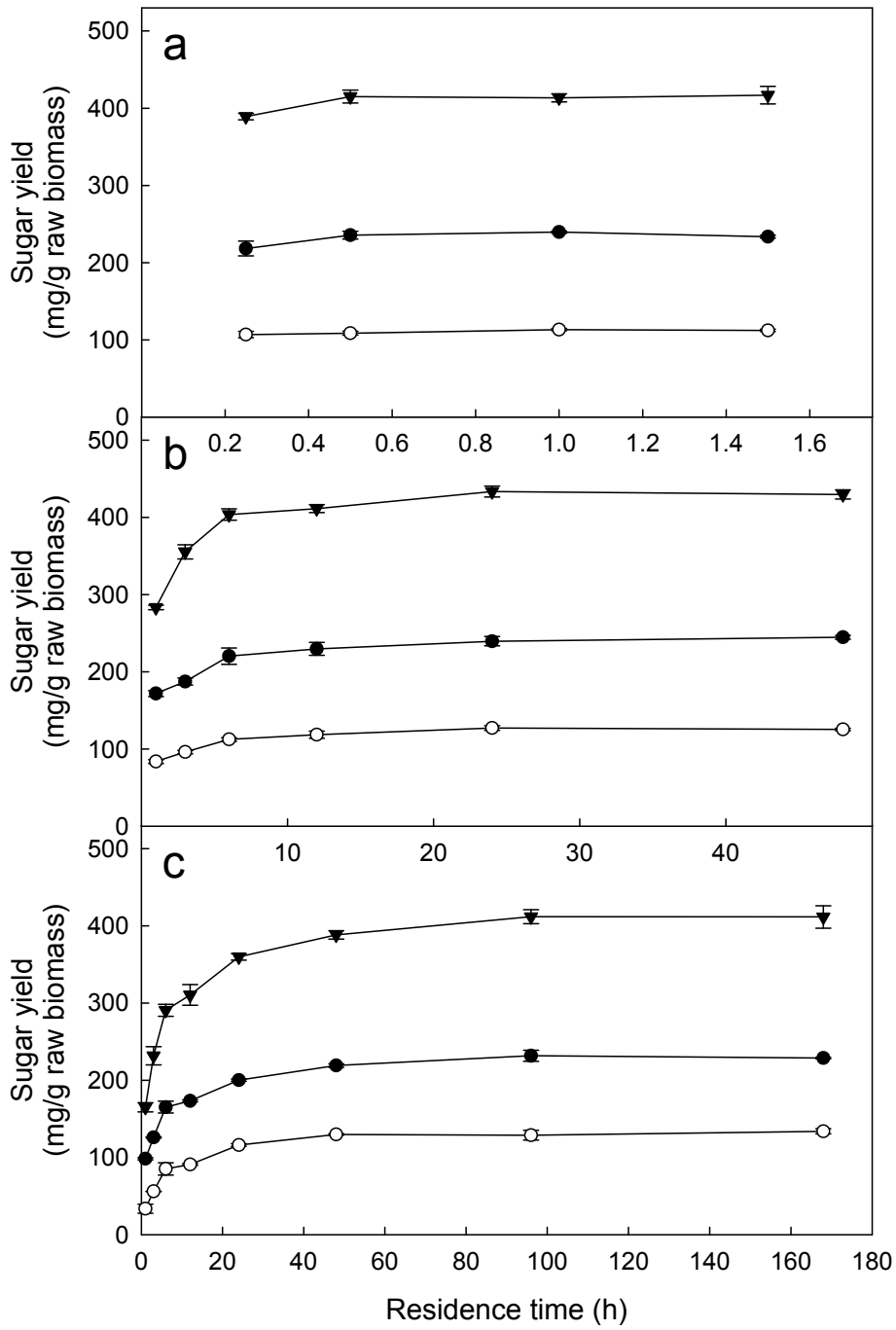

Figure 4.1 Effect of residence time on (●) glucose, (○) xylose, and (▼) total reducing sugar yields of switchgrass at (a) 121 °C, (b) 50 °C, and (c) 21 °C.

Based on the optimum residence times at different temperatures (0.5 h at 121 ^{o}C, 24 h at 50 ^{o}C, and 96 h at 21 ^{o}C), lime loadings of 0.05, 0.10, 0.15, and 0.20 g/g raw biomass were studied and their impacts on pretreatment were determined. Two wash intensities (100 and 300 ml water/g raw biomass) were applied at each lime loading to study their effects on sugar production. The use of 0.10 g $Ca(OH)_2$/g raw biomass was sufficient to maximize total reducing sugar yield (Figure 4.2), which corresponded to previous reports (Chang et al., 1997, Chang et al., 1998; Kaar and Holtzapple, 2000; Kim and Holtzapple, 2005). When lime loadings were greater than 0.10 g/g raw biomass, 100 ml water/g raw biomass was not sufficient to wash away excess lime, thus resulting in reduced sugar yields due to the abnormal hydrolysis pHs. Although sodium citrate buffer (pH 4.8) was used to maintain the hydrolysis pH at the optimum level, the dissolution of residual lime particles still increased the pH. After 72 h incubation of the biomass pretreated at 0.20 g $Ca(OH)_2$/g raw biomass and washed with 100 ml water/g raw biomass, the pHs of the hydrolysate solutions rose to 6.5-7.0, while the pHs were kept lower than 5.5 for the samples pretreated at lime loadings not exceeding 0.10 g/g raw biomass. Increasing the wash intensity to 300 ml water/g raw biomass effectively eliminated pH inhibition to enzymes, which contributed to significant ($P<0.05$) increases in the total reducing sugar yields of biomass pretreated at 0.20 g $Ca(OH)_2$/g raw biomass. However, more intensive washing inevitably caused greater biomass loss, resulting in reduced sugar yields at lime loadings of 0.05 and 0.10 g/g raw biomass. When the wash intensity was tripled, the solid recoveries decreased by 6.8-7.3%,

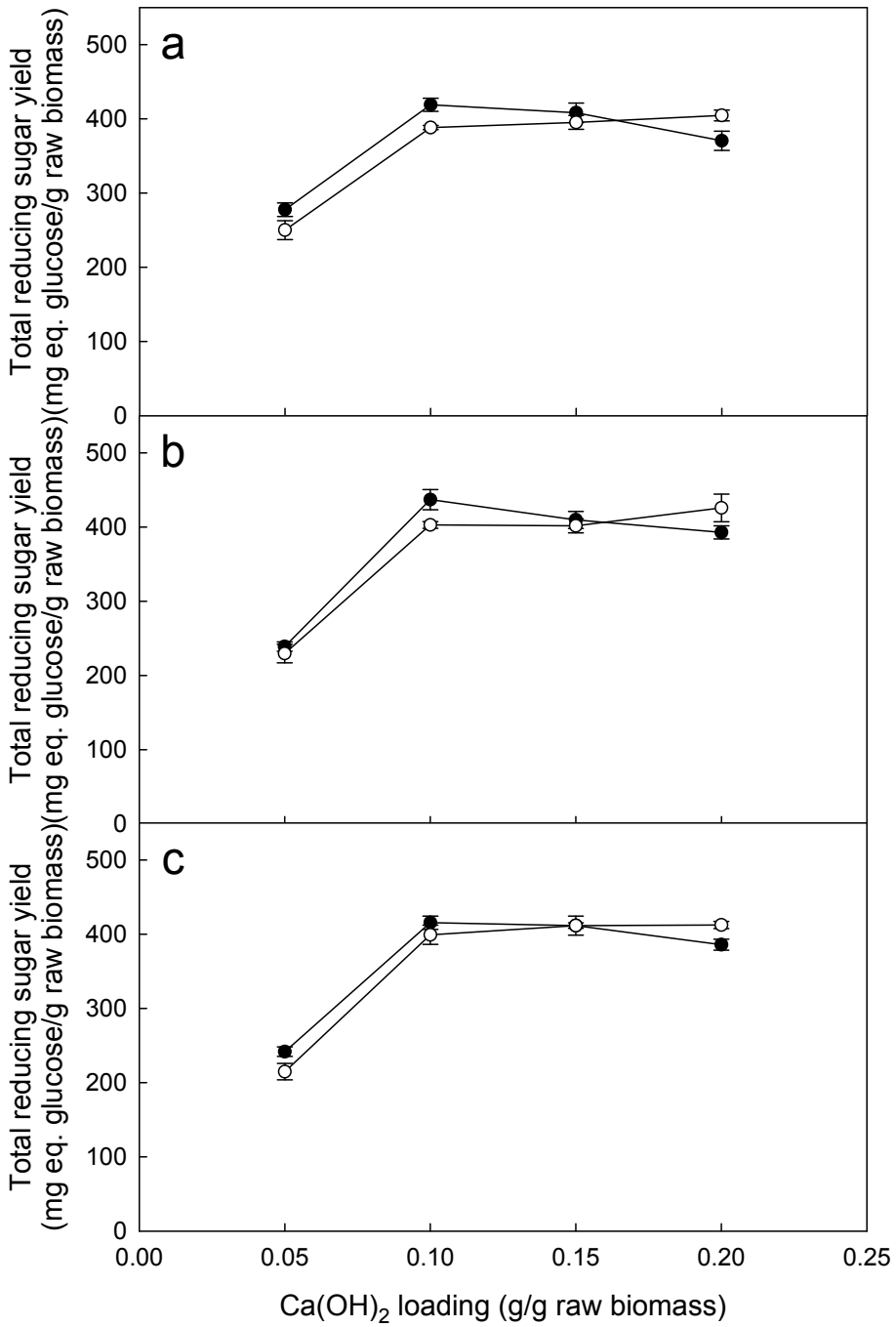

Figure 4.2 Effect of lime loading on total reducing sugar yield of switchgrass at the wash intensities of (●) 100 ml water/g raw biomass and (○) 300 ml water/g raw biomass at (a) 121 ^{o}C, (b) 50 ^{o}C, and (c) 21 ^{o}C.

most of which could be from carbohydrate loss. Applying optimum lime loading is important not only for reducing chemical requirement but also for avoiding intensive washing that would increase sugar loss and water expense.

Lime pretreatment effectively improved the enzymatic saccharificaiton of switchgrass at all the temperatures studied. Based on total reducing sugar yield, 50 ^{o}C, 24 h, 0.10 g $Ca(OH)_2$/g raw biomass, and a wash intensity of 100 ml water/g raw biomass were determined as the best conditions for lime pretreatment of switchgrass.

4.3.2.2. Lignin reduction

Lignin is a three dimensional complex aromatic polymer which forms a sheath surrounding cellulose and hemicellulose, stiffening and holding together the fibers of polysaccharides (Fan et al., 1987). Alkaline pretreatment can effectively remove lignin barriers, thus exposing the carbohydrates to enzymes. Chen et al. (2007) reported that 75.1-84.5% of the lignin in barley straw, triticale hay, triticale straw, and wheat straw were removed after pretreatment at 121 ^{o}C, 60 min, and 2.0% NaOH. Kim et al. (2003) treated corn stover using aqueous ammonia and reported that the lignin reduction reached 84.7% at 170 ^{o}C, 90 min, and 15 wt.% of ammonia concentration. Lime pretreatment resulted in much less lignin reduction, ranging from 16.7-35.5% (Figure 4.3), although extending residence time did favor delignification. The reduced lignin reduction was probably due to the formation of a

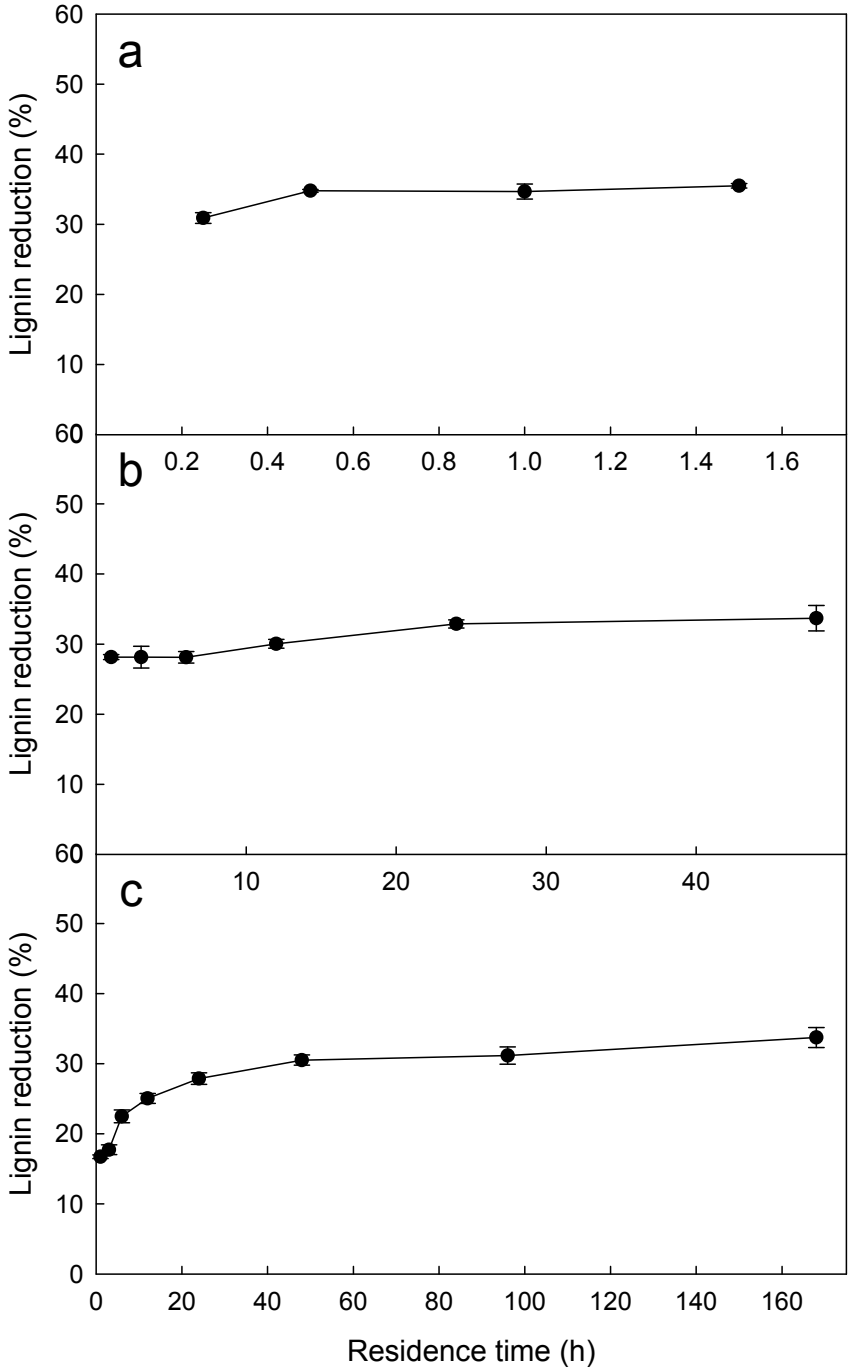

Figure 4.3 Effect of residence time on lignin reduction of switchgrass at (a) 121 ^{o}C, (b) 50 ^{o}C, and (c) 21 ^{o}C.

calcium-lignin complex. Calcium ions, eachcarrying two positive charges, tended to crosslink lignin molecules which are negatively charged under alkaline conditions due to the ionization of functional groups including carboxyl, methoxy, and hydroxyl through the formation of stoichiometric bonds, thus preventing serious lignin solubilization during pretreatment (Xu et al., 2008; Torre et al., 1992). Sundin and Hartler (2000a) reported that calcium ions could efficiently congregate and precipitate lignin, especially high molecular mass lignin, in alkaline solutions. And the affinity between calcium ions and lignin was strengthened with the increase of initial calcium concentration, pH, and quantity of lignin (Torre et al., 1992).

4.3.3 Interaction between calcium ions and lignin

Increasing the calcium loading resulted in a significant ($P<0.05$) decrease in lignin reduction under different treatment conditions (Figure 4.4). With the increase of calcium loading from 0 to 0.10 g $Ca(OH)_2$/g raw biomass, the lignin reductions decreased by 13.9% at 121 ^oC, 0.5 h, and 14.3% at 21 ^oC, 6 h. Moreover, the biomass treated with $Ca(OH)_2$ was much darker in color, which was probably due to the precipitation of a large amount of chromophoric group-containing lignin on the biomass surface. Calcium bonding did have an effect on lignin removal during pretreatment. Unexpectedly, sugar loss was also mitigated by the presence of calcium ions. Compared with the control (without $Ca(OH)_2$ addition), the loss of total reducing sugars at 0.10 g $Ca(OH)_2$/g raw biomass decreased by 10.2% at 121 ^oC,

0.5 h, and 16.5% at 21 ^{o}C, 6 h. A plausible explanation is that calcium ions also crosslinked with carbohydrates providing protection. Torre et al. (1992) reported that the interaction of Ca^{2+} to cellulose and pectin was weak at pH 3.5-7.5. However, at greater pH, ionizable groups carried by cellulose and hemicellulose become binding sites to calcium ions, thus resulting in the formation of extensive carbohydrate-calcium-carbohydrate and carbohydrate-calcium-lignin linkages within biomass.

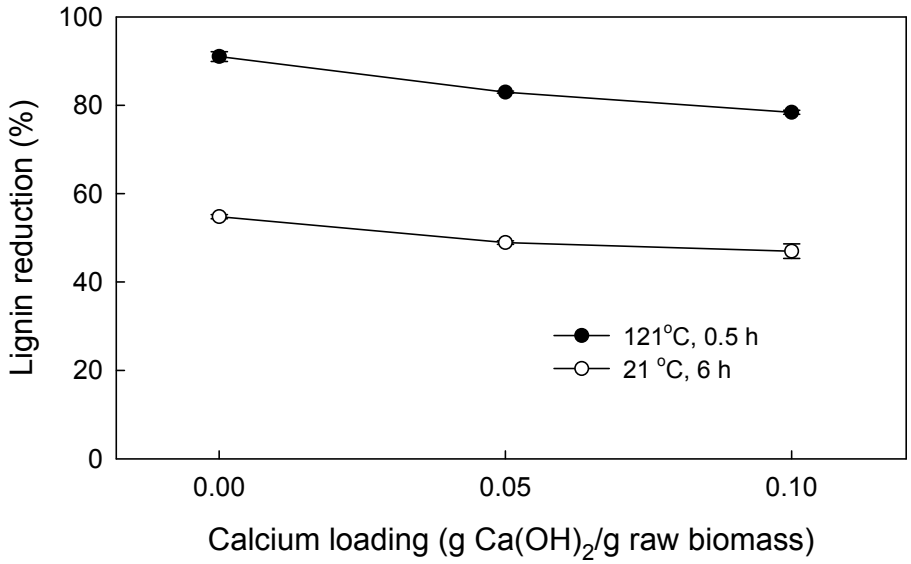

Figure 4.4 Effect of calcium loading on lignin reduction of switchgrass at the alkaline conditions.

4.3.4 Effect of high lignin content on biomass digestibility improvement

Lignin is identified as a major deterrent to enzyme attack on cellulose because of its close association with cellulose microfibrils (Zhu et al., 2008). Due to calcium bonding, the lignin contents of switchgrass remained high after lime pretreatments. However, based on hydrolysis results, the lignin present did not result in low enzymatic saccharification. It seemed that as long as the chemical bonds holding the biomass components together were removed by alkaline attack, and the biomass structure loosened, the enzymes have good access to the carbohydrates even in the presence of lignin. To support this hypothesis, Simons' stain method was used to quantify changes in biomass porosity as a result of pretreatment. The biomass pretreated for 0.25, 0.5, and 1 h at 121^{o}C, and 6, 24, and 96 h at 21 ^{o}C in the first part of this study on pretreatment conditions were subjected to Simons' staining procedure. The biomass samples were selected based on their similar high lignin contents (18.5-19.6%). The pretreated biomass became more porous with the extension of residence time and the sugar yield from the biomass increased correspondingly (Figure 4.5). This indicates that the enzymatic digestibility of biomass was directly proportional to its porosity, while high lignin contents did not inhibit saccharification improvement. Therefore, lignin reduction may not be an appropriate indicator for the effectiveness of lime pretreatment.

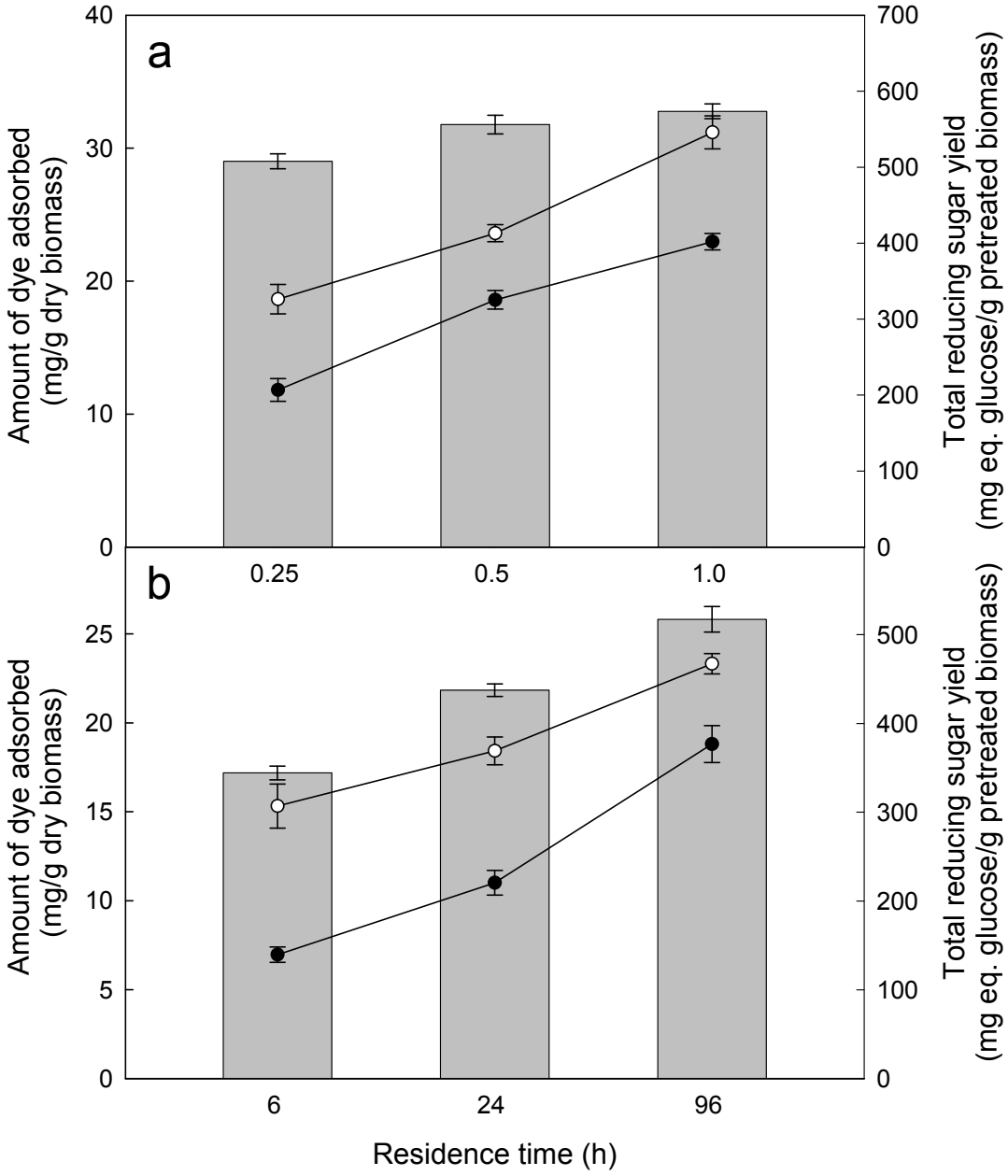

Figure 4.5 Adsorption of (●) orange and (○) blue dyes, and (column) total reducing sugar yields of biomass pretreated for (a) 0.25, 0.5, and 1 h at 121 ^{o}C, and (b) 6, 24, and 96 h at 21 ^{o}C.

4.3.5 Material balances

Material balances were performed on the biomass pretreated at the optimum conditions at different temperatures (0.5 h at 121 ^{o}C, 24 h at 50 ^{o}C, 96 h at 21 ^{o}C, 0.10 g $Ca(OH)_2$/g raw biomass, and wash intensity of 100 ml water/g raw biomass). The total dry weight of the sample was measured after pretreatment, and the compositions of pretreated biomass (glucan, xylan, galactan, arabinan, lignin, ash, and other) were determined and compared with that of raw biomass. The solid recovery at 121 ^{o}C was less than that at 50 or 21 ^{o}C due to the more solubilization of the biomass components at the high temperature (Figure 4.6). Glucan (cellulose) was the best preserved carbohydrate at all temperatures because of its semicrystilline structure. Lignin contents remained high after pretreatments, with 32.9-34.8% of the lignin in raw biomass removed. Ash contents were just slightly decreased, which was probably due to the increase of calcium content in the pretreated biomass. Chang et al. (2001) reported that the calcium content of poplar wood after oxidative lime pretreatment was 4 times that of raw biomass.

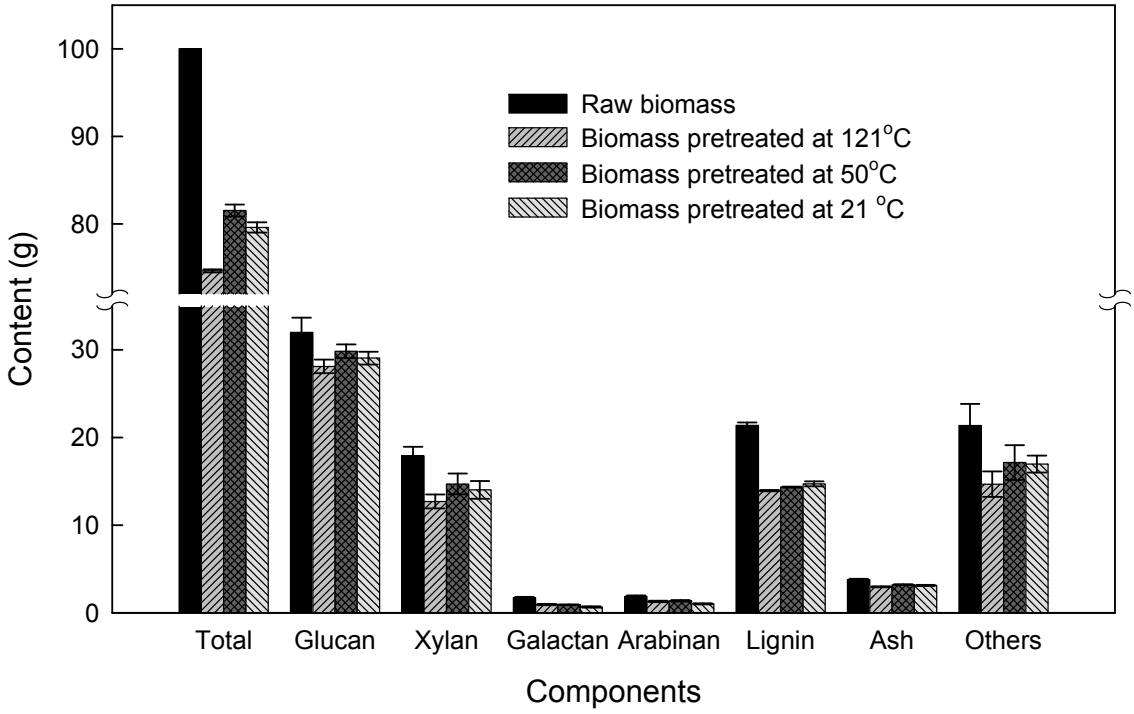

Figure 4.6 Material balances for raw switchgrass and biomass pretreated at the optimum conditions at different temperatures (0.5 h at 121 °C, 24 h at 50 °C, 96 h at 21 °C, 0.10 g $Ca(OH)_2$/g raw biomass, and wash intensity of 100 ml water/g raw biomass).

4.3.6 Study on enzyme loading

To study the effects of enzyme loadings on biomass saccharification, cellulase loadings of 0-35 FPU/g dry biomass and cellubiase loadings of 0-50 CBU/g dry biomass were investigated. In the study of cellulase loadings, the cellobiase loading was kept constant at 61.5CBU/g dry biomass. At the excessive cellobiase loading, no inhibition of cellobiose to cellulase was observed. Sugar yields from the

switchgrass pretreated using the best conditions (50 ^{o}C, 24 h, 0.10 g $Ca(OH)_2$/g raw biomass, and wash intensity of 100 ml water/g raw biomass) at different cellulase loadings were determined (Figure 4.7a). With the increase of cellulase loading from 0 to 20 FPU/g dry biomass, the total reducing sugar yield elevated by 5.14 times from 69.7 to 427.7 g/g raw biomass. Cellulase loading of 20 FPU/g dry biomass was sufficient to maximize sugar yields, which was comparable with the optimum loading (25 FPU/g dry biomass) obtained by Chang et al. (1997). The study on cellobiase loadings, based on the optimum cellulase loading, showed that supplementing cellobiase significantly ($P<0.05$) increased sugar yields and a cellobiase loading of 20 CBU/g was sufficient (Figure 4.7b).

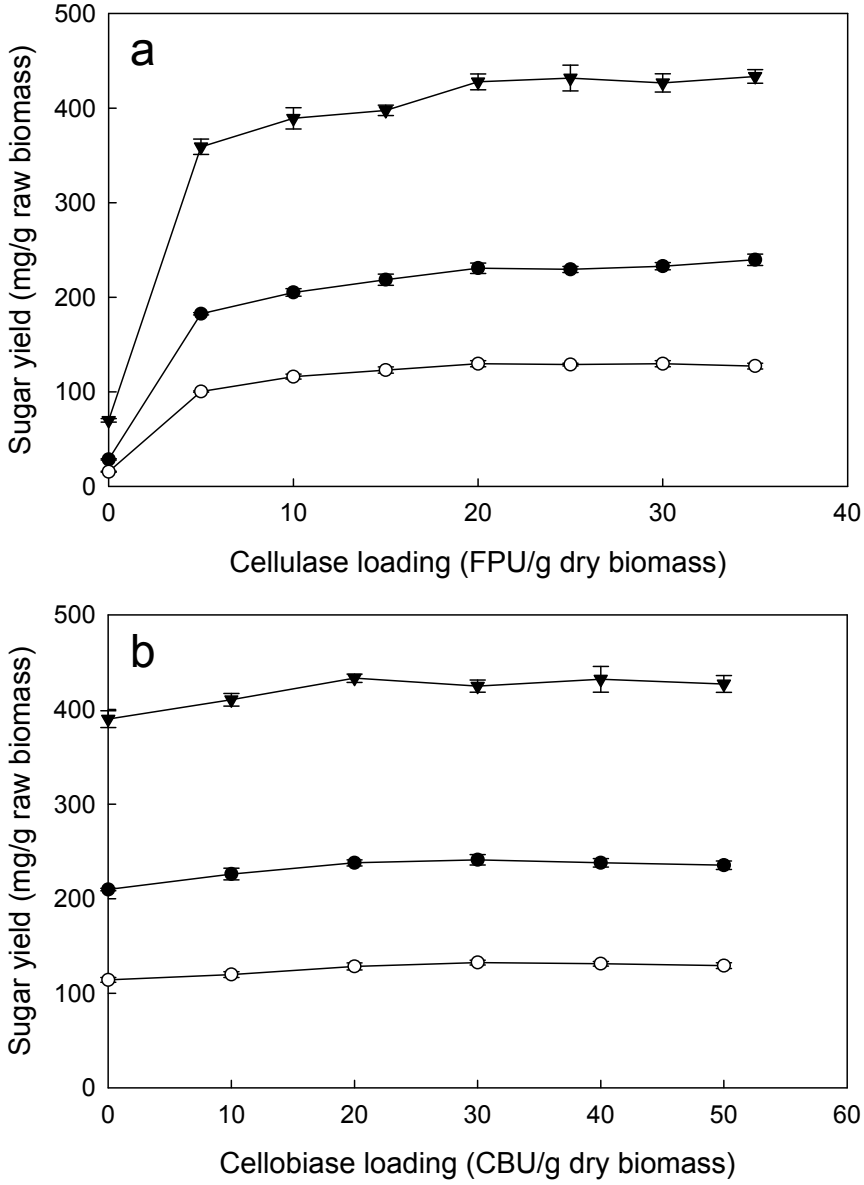

Figure 4.7 Effects of cellulase and cellobiase loadings on (●) glucose, (○) xylose, and (▼) total reducing sugar yields of switchgrass pretreated at the best conditions (50 ^{o}C, 24 h, 0.10 g $Ca(OH)_2$/g raw biomass, and wash intensity of 100 ml water/g raw biomass).

4.4 CONCLUSIONS

Lime pretreatment effectively improved the enzymatic digestibility of switchgrass at all temperatures studied. With the decrease of temperature from 121 to 21 ^{o}C, the residence time required to maximize total reducing sugar yield increased from 0.5 h to 96 h. Calcium ions extensively crosslinked lignin molecules under alkaline pretreatment conditions, which resulted in little lignin reduction. However, as long as the chemical bonds stiffening lignocellulose were disrupted and the biomass porosities were improved, the biomass enzymatic digestibilities could be substantially increased even in the presence of high lignin contents. Calcium bonding also led to better carbohydrate preservation, which was beneficial for total sugar production. Compared with other pretreatment methods, lime pretreatment is relatively inexpensive, safe to handle, and environmentally friendly, thus more promising for industrial application. This research provides important information for the future scale-up conversion studies.

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CHAPTER 5

PRETREATMENT OF SWITCHGRASS FOR ETHANOL PRODUCTION BY USING THE COMBINATION OF SODIUM HYDROXIDE AND LIME

ABSTRACT

NaOH and lime are the two commonly used chemical reagents in the alkaline pretreatment of lignocellulosic biomass. At room temperature, however, high chemical loadings or extended residence times are required for effective pretreatments. This inevitably reduces the overall cost-effectiveness of the process. In this study, switchgrass was used as feedstock, and NaOH and lime were innovatively applied together to improve the economic promise of alkaline pretreatment at room temperature. Effects of residence time, NaOH loading, time point for NaOH addition, lime loading, and biomass washing on the sugar production efficiency were investigated, and pretreatments were evaluated based on the yields of biomass-based sugars in the subsequent enzymatic hydrolysis. At the best pretreatment conditions (6 h, 0.10g NaOH/g raw biomass, start point NaOH addition, 0.02 g $Ca(OH)_2$/g raw biomass, and wash intensity of 100 ml water/g raw biomass), the yields of glucose, xylose, and total reducing sugars reached 211.4, 116.6 and 379.4 mg/g raw biomass, which were respectively 2.78, 5.30, and 3.16 times those of untreated biomass. The pretreatment effectiveness of using the combination of 0.1 g NaOH/g raw biomass and 0.02g $Ca(OH)_2$/g raw biomass was comparable with that of using 0.2 g NaOH/g raw biomass, while its cost was barely higher than that of using 0.1 g NaOH/g raw biomass considering the low cost of lime and the minor loading required.

5.1 INTRODUCTION

Lignocellulose-to-ethanol conversion is a promising technology for sustainable ethanol production because lignocellulosic biomass is abundant and has diverse raw materials including trees, grasses, agricultural residues, and forestry wastes. Switchgrass (*Panicum virgatum L.*), a perennial warm-season grass native to North America, is considered as one of the best feedstocks for ethanol production. It not only gives excellent biomass yield but also contains high carbohydrates. Parrish and Fike (2005) reported that, annual yields of over 15 Mg ha^{-1} could be expected for well-adapted switchgrass cultivars grown on the lands that receive annual rainfall of at least 70 cm with nitrogen applications of 50 kg N ha^{-1} $year^{-1}$. Keshwani and Cheng (2009) reported that, due to the outstanding biomass yield and high content of total available sugars in biomass, the theoretical ethanol yield from switchgrass was likely to be between 2000 and 4000 l ha^{-1}, which was comparable with that from corn starch. Switchgrass is also preferred for its suitability for marginal land quality, adaptation to many climate conditions, and low water and nutritional requirements (Keshwani and Cheng, 2009; McLaughlin et al., 1999).

However, the recalcitrant structure of lignocellulosic materials necessitates a pretreatment step to break up lignocellulosic matrix to make the cellulose and hemicelluloses more accessible to hydrolytic enzymes. Alkaline pretreatment has been intensively studied because it is potentially inexpensive, less energy intensive, and effective on various feedstocks especially forages and agricultural residues

(Belkacemi et al., 1998; Chang et al., 2001; Chen et al., 2007; Silverstein et al, 2007; Xu et al., 2008). The application of alkaline solutions causes the swell of the biomass through solvation and saponification reactions, making it more porous for better access to hydrolytic enzymes (Hendriks and Zeeman, 2009). Sodium hydroxide (NaOH) and lime ($Ca(OH)_2$) are the two commonly used chemical reagents in the alkaline pretreatment of lignocellulosic biomass. NaOH is a strong base and has outstanding delignification capability. Silverstein et al. (2007) compared NaOH, H_2SO_4, H_2O_2, and ozone pretreatments of cotton stalks and found that NaOH pretreatment resulted in the highest level of delignification (65.63% at 2.0% NaOH, 90 min, 121 ^oC) and cellulose conversion (60.8%). Although lime is not as strong as NaOH due to its poor solubility in water, the performances of lime pretreatment at high temperatures (>100 ^oC) are still satisfying. Kaar and Holtzapple (2000) reported that at the recommended pretreatment conditions of 120 ^oC, 4 h, 0.075 g $Ca(OH)_2$/g dry biomass, and 5 g water/g dry biomass, the enzymic conversion of corn stover to monomeric sugars was about 60% cellulose, 47% xylan, and 53% total available polysaccharide. In addition, lime is very inexpensive and can be easily recovered by carbonating wash water using CO_2 (Chang et al., 1998).

Although NaOH and lime pretreatments have been intensively studied, room temperature was scarcely investigated in such studies. This is probably because that the substantially reduced chemical reaction rates at room temperature inevitably necessitate the application of much higher alkali loadings or much longer residence

times for effective pretreatments, which would considerably offset the cost reduction from reduced heating requirement at room temperature. The previous study on NaOH pretreatment of switchgrass showed that, at room temperature, to achieve a high sugar production comparable with that obtained at 121 or 50 ^{o}C, the NaOH loading must be increased from 0.10 to 0.20 g/g raw biomass. Since NaOH is expensive, doubling NaOH loading might be unacceptable in the commercial application. The application of lime at room temperature was seriously limited by its poor solubility. Regardless of the feedstock properties or the changes of other pretreatment conditions, increasing lime loading beyond 0.10 g/g raw biomass didn't improve sugar production (Chang et al., 1997, Chang et al., 1998; Kaar and Holtzapple, 2000; Kim and Holtzapple, 2005). The previous study on lime pretreatment of switchgrass showed that, since increasing lime loading beyond 0.10 g/g raw biomass didn't help, extending residence time to as long as several days was required for effective pretreatments at room temperature. Therefore, it can be concluded that, although NaOH still works at room temperature, great chemical loadings are required, while lime is too weak to achieve effective pretreatments within a reasonable period of time. Considering the high cost of NaOH, it seems neither of these two alkaline pretreatment technologies is promising to work at room temperature.

However, based on the unique properties of NaOH and lime, it is possible to manipulate these two alkali reagents to make them work together to achieve high

cost-effectiveness. First, lime is much cheaper than NaOH (Chang et al., 1997; Kaar and Holtzapple, 2000), thus being able to replace part of the NaOH alkalinity at a very low cost. Second, due to its poor solubility, a considerable part of lime exists as solid and will gradually dissolve to supplement the alkalinity consumed by the biomass, thus stabilizing the pH at a high level throughout the pretreatment. Third, calcium ions, each carrying two positive charges, tended to provide linkages within biomass which were negatively charged at alkaline conditions due to the ionization of some functional groups including carboxyl, methoxy, and hydroxyl, thus preventing serious solid loss (Xu et al., 2008; Torre et al., 1992). Therefore, lime, although not strong enough by itself, can be used as a supplementary chemical to strong but expensive NaOH to improve the economic promise of the alkaline pretreatment at room temperature.

In this research, pretreatment of switchgrass using the combination of NaOH and lime was explored at room temperature (21 ^{o}C). The effects of residence time, NaOH loading, time point for NaOH addition, lime loading, and biomass washing on pretreatment effectiveness were studied. The best pretreatment conditions were determined and the results were compared with those of the pretreatment using NaOH alone. Material balances were performed to investigate the compositional changes of biomass caused by pretreatments, and enzyme loadings in hydrolysis were optimized.

5.2 MATERIALS AND METHODS

5.2.1 Biomass preparation

The biomass feedstock used in this research was “Performer” switchgrass, a cultivar cooperatively developed by the USDA-Agricultural Research Service and the North Carolina Agricultural Service, North Carolina State University for improved forage quality (Burns et al., 2008). Switchgrass plants were harvested in late July, 2007, from Central Crops Research Station near Clayton, North Carolina. A harvest strip was taken randomly from each quarter of the field, and four strips were combined to form one bulk sample. The biomass was oven dried at 50 ^{o}C for 72 hours, ground to pass 2 mm sieve in a Wiley mill and stored in sealed plastic bags at room temperature. The chemical composition of raw biomass was analyzed before pretreatment.

5.2.2 Pretreatment

Four g of biomass sample and 40 ml of DI water were mixed in a serum bottle, forming a slurry at a solid : liquid ratio of 0.1 g/ml. NaOH and lime at specific chemical loadings were added into the bottle and mixed with the slurry. As shown in Table 5.1, three major factors which were residence time, NaOH loading, and time point for NaOH addition, and two minor factors which were lime loading and wash intensity were studied. The ranges of residence time and NaOH loading investigated were determined based on the previous studies on NaOH and lime pretreatment.

Time point for NaOH addition was studied because of its potential impact on sugar recovery. Since NaOH is a strong base and could cause serious carbohydrate solubilization, postponing its addition would probably improve carbohydrate preservation by giving lime more time to dissolve and form calcium linkages within biomass before harsh NaOH attack occurred. However, overly delaying NaOH addition would have negative impact on biomass digestibility improvement by reducing the exposure time for the biomass to the higher pH too much. Therefore, determining the best time point for NaOH addition was necessary. Lime loading and wash intensity were studied as minor factors because they would not have much interaction with other factors. Since the solubility of lime is poor, to provide as much cheap alkalinity as possible, an excessive lime loading was required anyway to maintain the saturated lime solution throughout the pretreatment. Wash intensity was investigated because lime loading might affect the amount of water required to wash away the excess lime. In the 3×3 factorial study of the three major factors, lime loading and wash intensity were held constant at 0.10 g/g raw biomass and 200 ml water/g raw biomass respectively, while the subsequent studies on lime loading and wash intensity were based on the best combination of the three major factors. The pretreated biomass was recovered by filtration and about 1 g (dry basis) of it was dried at 45 ^{o}C to constant weight for composition analysis, the rest stored in a sealed plastic bag at 4 ^{o}C for enzymatic hydrolysis.

Table 5.1 Factors studied in the pretreatment of swithgrass using the combination of NaOH and lime.

Major Factors			**Minor Factors**	
Residence time (h)	NaOH addition point	NaOH loading (g/g raw biomass)	Lime loading (g/g raw biomass)	Wash intensity (ml water/g raw biomass)
3, 6, 9	0, 1/3, 2/3*	0.05, 0.10, 0.20	0, 0.02, 0.04, 0.06, 0.08, 0.10	100, 200

* 0, 1/3, 2/3 mean adding NaOH at the start of pretreatment, after 1/3 of pretreatment time elapses, and after 2/3 of pretreatment time elapses.

5.2.3 Enzymatic hydrolysis

One g of pretreated biomass (dry basis) was immersed in 30 ml of 50 mM sodium citrate buffer (pH 4.8) in a 250 ml Erlenmeyer flask. Cellulase from *Trichoderma reesei* (E.C. 3.2.1.4) was added at an enzyme loading of 35 FPU/g dry biomass. To prevent cellobiose inhibition to cellulase, cellobiase from *Aspergillus niger* (E.C. 3.2.1.21) at an enzyme loading of 61.5 CBU/g dry biomass was supplemented. Enzymes were obtained from Novozymes North America, Inc. (Franklinton, North Carolina, USA), and the activities of cellulase and cellobiase were respectively 80 FPU/ml and 277 CBU/ml. To mitigate microbial contamination, sodium azide (0.3%, w/v) was added into the mixture. The incubation was carried out at 55 ^{o}C, 150 rpm in an air bath shaker for 72 h. After hydrolysis, the flasks were immediately chilled in an ice bath to avoid further reaction. The hydrolysate was collected by centrifugation at 10000 rpm for 5 min, and the supernatant stored at -80 ^{o}C for sugar analysis.

5.2.4 Analytical methods

Total solids, structural carbohydrates, lignin, and ash in raw and pretreated biomass were measured according to Laboratory Analytical Procedures (LAP) established by National Renewable Energy Laboratory (NREL) (Sluiter et al., 2005a; Sluiter et al., 2005b; Sluiter et al., 2008). Total reducing sugars in hydrolysate was measured using DNS method adapted from Miller (1959) and Ghose (1987). Cellulose and hemicellulose derived monomeric sugars (glucose, xylose, galactose, arabinose, and mannose) in hydrolysate and liquor from composition analysis were measured by high performance liquid chromatography (HPLC). The HPLC system consisted of a Bio-Rad Aminex HPX-87P column tailored for analysis of lignocellulose-derived sugars, a Bio-Rad Micro-Guard column, a thermostatted autosampler, a quaternary pump, and a refractive index detector. The analytical column was operated at 80 °C with HPLC grade water as the mobile phase at a flow rate of 0.6 ml/min. The samples were injected at 10 µl and the acquisition time was 35 min. A post-run time of 25 min was included between injections to allow for late-eluting compounds to come off the column. The standards used were glucose, xylose, galactose, arabinose, and mannose at concentrations of 0.5, 2.0, 5.0, 7.5, 10.0 g/L.

5.2.5 Statistical analysis

All treatments in this study were conducted in triplicate and a 95% confidence level was applied for data analysis. The GLM procedure in SAS 9.1 software (SAS Institute Inc., Cary, NC) was used for all statistical analysis. Analysis of variance (ANOVA) was used to determine the effects of various factors on pretreatments and Tukey simultaneous tests were conducted to determine the statistical differences between treatments.

5.3 RESULTS AND DISCUSSION

5.3.1 Solid recovery

Alkaline pretreatment effectively alters biomass structure for better enzyme access. However, biomass solubilization caused by alkaline attack is inevitable especially at severe conditions, which could substantially affect total sugar production in the subsequent enzymatic hydrolysis. As shown in Figure 5.1, 71.6-86.1% of the total solids were recovered after pretreatments at room temperature, which was considerably higher than those from alkaline pretreatments at elevated temperatures (Silverstein et al., 2007; Xu et al., 2009). NaOH loading has significant ($P<0.05$) effect on solid recovery at all the combinations of residence time and time point for NaOH addition with the exception of 3 h and 2/3 point NaOH addition, at which the biomass was exposed to NaOH attack for just an hour, while residence time had significant ($P<0.05$) effect on solid recovery at all the combinations of

NaOH loading and time point for NaOH addition. The results clearly showed that postponing NaOH addition was crucial for the mitigation of biomass solubilization especially at high NaOH loadings. Adding NaOH after 1/3 of residence time elapsed significantly ($P<0.05$) increased solid recovery, which was plausibly due to the avoidance of harsh NaOH attack before the formation of sufficient calcium linkages within biomass structure. In the absence of NaOH, the dissolution of lime was promoted due to a relative lower environmental pH, so more calcium ions would be available for biomass preservation. However, postponing NaOH addition till 2/3 of residence time elapsed didn't necessarily result in further increase of solid recovery, which indicated that major calcium linkages were probably formed at the initial stage of pretreatment.

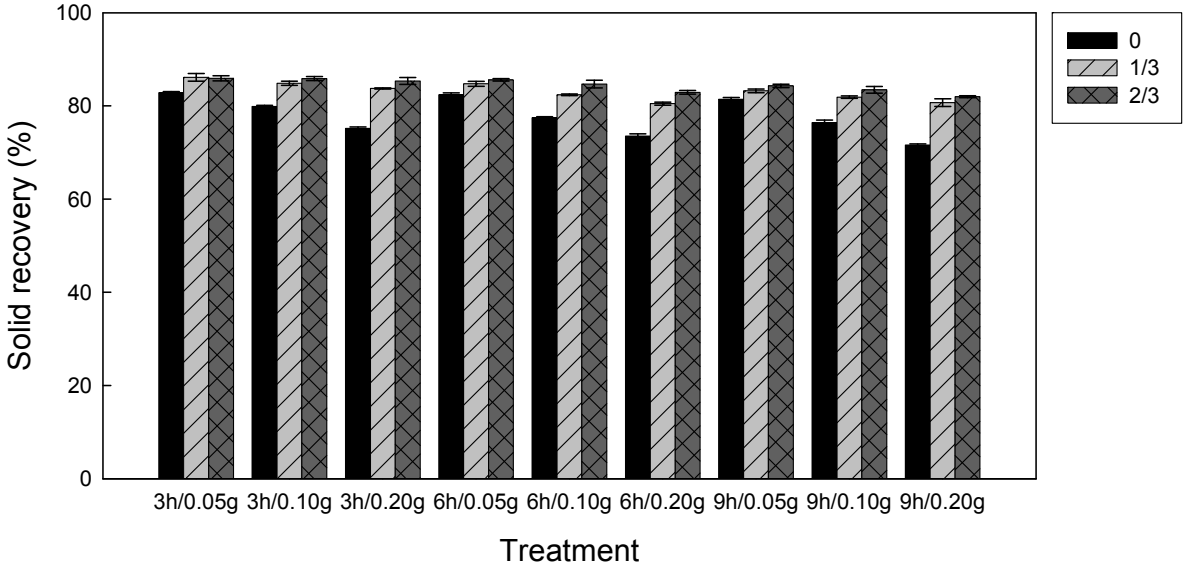

Figure 5.1 Solid recoveries after pretreatments at different combinations of residence time, NaOH loading, and time point for NaOH addition.

5.3.2 Sugar production

After pretreatment and enzymatic hydrolysis, sugars in hydrolysate were analyzed. The yields of glucose and xylose were reported to understand the impact of pretreatments on cellulose and hemicellulose conversion. Galactose and arabinose, the two minor hemicellulose constituents, were measured but not reported in this paper due to their low concentrations in the hydrolysate. The overall pretreatment effectiveness was evaluated using the total reducing sugar yield based on raw biomass.

As shown in Figure 5.2a, glucose yields were significantly ($P<0.05$) increased with the elevation of residence time and NaOH loading. However, it was found that postponing NaOH addition didn't favor the improvement of glucose prodution. This is probably because that cellulose has semicrystalline structure which makes it less susceptible to solubilization under alkaline attack while necessitates intense pretreatment conditions for its digestibility improvement. Although allowing the formation of more calcium linkages by postponing NaOH addition could potentially mitigate cellulose solubilization, reducing the exposure time for the biomass to harsh NaOH attack substantially compromised the improvement of biomass digestibility. The maximum glucose yield of 263.0 mg /g raw biomass was obtained at the fiercest pretreatment conditions of 9 h, 0.20g NaOH/g raw biomass, and start point NaOH addition, at which the overall glucan conversion reached 74.0%, 3.46 times than that of untreated biomass. Hemicellulose has amorphous, heterogeneous and branched

structure with little strength, which makes it more susceptible to solubilization than semicrystalline cellulose at alkaline conditions. Figure 5.2b shows that, at 3 h, increasing NaOH loading significantly ($P<0.05$) increased xylose yield while postponing NaOH didn't result in higher yields. The relationships between xylose yield and various factors were more complicated at elevated pretreatment severities. At 6 and 9 h, increasing NaOH loading didn't necessarily lead to higher xylose yields. At NaOH loading of 0.20 g/g raw biomass, there was no significant ($P<0.05$) difference between xylose yield at start point NaOH addition and that at 1/3 point NaOH addition, which indicated that postponing NaOH addition might favor the hemicellulose preservation. The maximum xylose yield of 135.3 mg /g raw biomass was obtained at 3 h, 0.20g NaOH/g raw biomass, and start point NaOH addition, at which the xylan conversion reached 66.5%, 5.15 times that of untreated biomass. The effects of pretreatment conditions on biomass digestibility improvement are clear shown in Figure 5.2c. NaOH loading had significant ($P<0.05$) effect on the total reducing sugar yield at all the combinations of residence time and time point for NaOH addition, while extending residence time from 6 to 9 h didn't favor the sugar production at start and 1/3 point NaOH addition due to the more serious carbohydrate loss at extended exposure times to NaOH attack. Similar to xylose yield, although postponing NaOH addition could result in better carbohydrate preservation, reducing the exposure time of biomass to higher pH substantially compromised the biomass digestibility improvement, which indicated that postponing

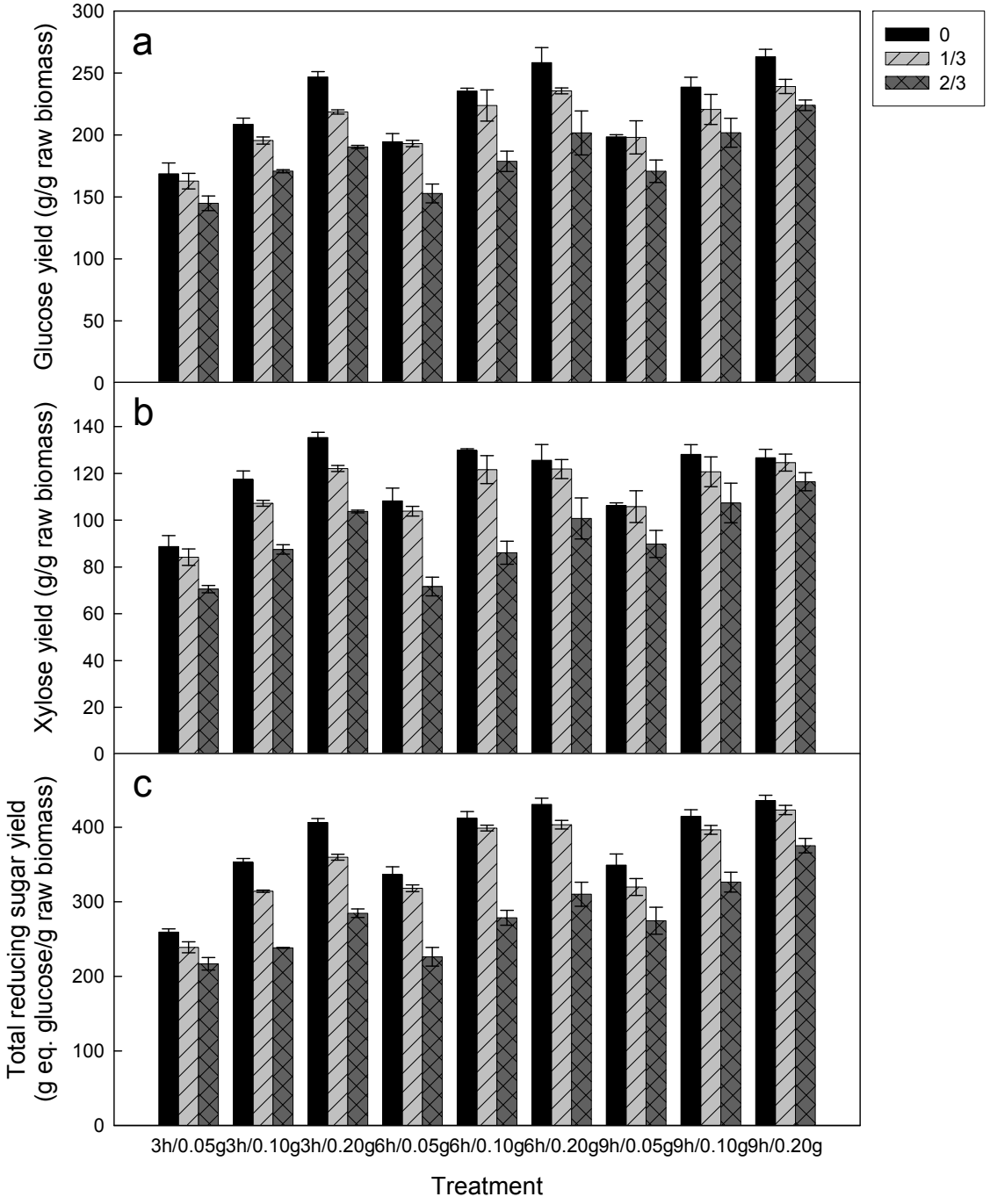

Figure 5.2 Yields of (a) glucose, (b) xylose, and (c) total reducing sugars of switchgrass biomass pretreated at different combinations of residence time, NaOH loading, and time point for NaOH addition.

NaOH addition might not be necessary for higher sugar production. The maximum total reducing sugar yield of 435.8 mg /g raw biomass was obtained at 9 h, 0.20g NaOH/g raw biomass, and start point NaOH addition, at which the conversion of total available carbohydrate reached 68.1%, 3.63 times that of untreated biomass. However, cutting the residence time to 6 h and applying half the NaOH loading only resulted in a 5% reduction in sugar production. Therefore, after cost-benefit consideration, 6 h, 0.10g NaOH/g raw biomass, and start point NaOH addition were used for the following lime loading and biomass washing studies.

Based on the best combination of residence time, NaOH loading, and time point for NaOH addition, lime loadings of 0, 0.02, 0.04, 0.08 and 0.10 g/g raw biomass and wash intensities of 100 ml and 200 ml water/g raw biomass were studied. Figure 5.3 shows that 200 ml water/g raw biomass was sufficient to wash away residual lime and the sugar production was maximized at the lime loading of 0.04 g $Ca(OH)_2$/g raw biomass. Using 100 ml water/g raw biomass at lime loadings of 0.04-0.10 g/g raw biomass caused significant ($P<0.05$) reduction of sugar production due to abnormal hydrolysis pHs. Although sodium citrate buffer (pH 4.8) was used according to the standard procedure to maintain the hydrolysis pH at the optimum level, the dissolution of residual lime particles still substantially increased the pH (the pH could rise to 6.5 at 0.10 g $Ca(OH)_2$/g raw biomass). However, there was no significant ($P<0.05$) decrease in sugar production at the lowest lime loading of 0.02 g $Ca(OH)_2$/g raw biomass, at which the total reducing sugar yield was 30%

higher than that of the biomass treated without lime supplementation. Although using the combination of 0.04 g $Ca(OH)_2$/g raw biomass and 200 ml water/g raw biomass resulted in a 6% higher total reducing sugar yield than using the combination of 0.02 g $Ca(OH)_2$/g raw biomass and 100 ml water/g raw biomass, doubling both lime loading and wash intensity could potentially compromise the cost-effectiveness of the process. Therefore, primary economic analysis is necessary before initiating any scale-up studies. Based on total sugar production and cost-benefit considerations, 6 h, 0.10 g NaOH/g raw biomass, start point NaOH addition, 0.02 g $Ca(OH)_2$/g raw biomass, and wash intensity of 100 ml water/g raw biomass were determined as the best conditions of the room temperature pretreatment of switchgrass in this study.

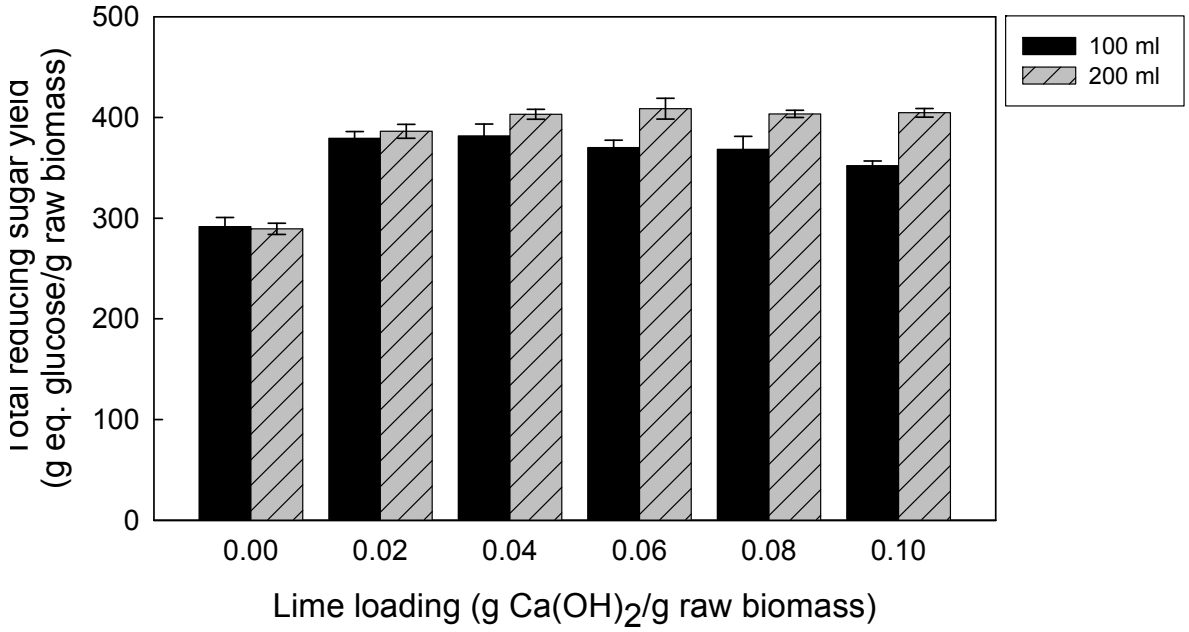

Figure 5.3 Total reducing sugar yield of switchgrass biomass pretreated at different combinations of lime loading and wash intensity.

5.3.3 Lignin reduction

Lignin is a three dimensional complex aromatic polymer which forms a sheath surrounding cellulose and hemicellulose, stiffening and holding together the fibers of polysaccharides (Fan et al., 1987). A number of studies showed that NaOH can effectively remove lignin barriers, thus exposing the carbohydrates to enzymes (Chen et al., 2007; MacDonald et al., 1983; Silverstein et al., 2007). However, the delignification capability of lime was much lower. Wang (2009) report that, even at 121 ^{o}C, the total lignin reductions from lime pretreatment of coastal bermudagrass was around 10-20%, which were much lower than that from NaOH pretreatment at similar conditions. This was probably because of the preservation of lignin by calcium ions through linkage formation. Many studies showed that divalent calcium ions had high affinity for lignin and could effectively crosslink lignin molecules especially at alkaline conditions (Duong et al., 2005; Sundin and Harlter, 2000a, 200b; Torre et al., 1992). As shown in Figure 5.4, all of the three parameters (residence time, NaOH loading, and time point for NaOH addition) had significant ($P<0.05$) effects on lignin reduction. Elevating residence time, NaOH loading, and the exposure time of biomass to higher pH increased the removal of lignin barrier. It was also found that postponing NaOH addition till 1/3 of residence time elapsed caused a remarkable mitigation in lignin reduction, which corresponded to the previous reports that calcium ions crosslink lignin molecules, thus resulting in better resistance of lignin against solubilization during alkaline pretreatment (Wang, 2009;

Xu et al., 2008). Lignin reductions ranged from 23.8% at 3 h, 0.05g NaOH/g raw biomass, 2/3 point NaOH addition, to 45.1% at 9 h, 0.2 g NaOH/g raw biomass, start point NaOH addition, and were directly proportional to the pretreatment severity.

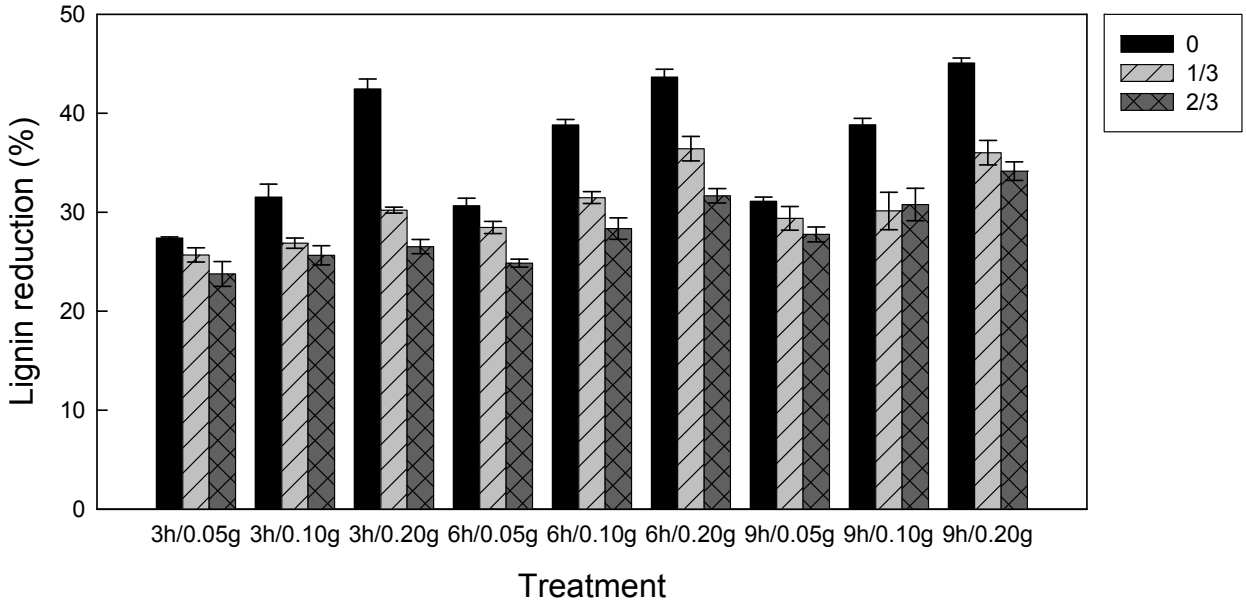

Figure 5.4 Lignin reduction after pretreatment at different combinations of residence time, NaOH loading, and time point for NaOH addition.

5.3.4 Comparison with NaOH pretreatment

According to the previous study on lime pretreatment of switchgrass, lime was too weak to effectively improve biomass digestibility within a reasonable period of time at room temperature. Therefore, the pretreatment using the combination of NaOH and lime at the best conditions (0.10 g NaOH/g raw biomass, 0.02 g $Ca(OH)_2$/g raw biomass, 6 h) was compared with the NaOH pretreatment at the best

conditions obtained in the previous study (0.20 g NaOH/g raw biomass, 6h). The water loading (10 ml water/g raw biomass) and the intensity of biomass washing (100 ml water /g raw biomass) applied in both pretreatments were the same. Considering the comparable expenses of using 0.10 g NaOH/g raw biomass and using the combination of 0.10 g NaOH/g raw biomass and 0.02 g $Ca(OH)_2$/g raw (due to the low cost of $Ca(OH)_2$ and the minor loading required), NaOH pretreatment at 0.10 g NaOH/g raw biomass and 6h was also included for comparision. The overall carbohydrates conversions, lignin reductions, pH changes from three pretreatment strategies are shown in Table 5.2. After pretreatment and enzymatic hydrolysis, the overall glucan conversion of the biomass treated with lime supplementation was 23.5% higher than that of the biomass treated just using 0.10 g NaOH/g raw biomass, but 14.5% lower than that of the biomass treated using 0.20 g NaOH/g raw biomass. The xylan conversion of the biomass treated with lime supplementation was respectively 47.3% and 4.6% higher than those of the biomass treated using 0.10 and 0.20 g NaOH/g raw biomass, indicating better hemicellulose preservations from calcium bonding. As a result, the total carbohydrate conversion of the biomass treated with lime supplementation was 34.5% higher than that of the biomass treated using 0.10 g NaOH/g raw biomass, and comparable with that of the biomass treated using 0.20 g NaOH/g raw biomass. Since the cost of supplementing 0.02 g $Ca(OH)_2$/g raw biomass was tremendously lower than that of doubling the loading of expensive NaOH, using the combination of NaOH and lime showed great

Table 5.2 Comparison of carbohydrate conversions, lignin reductions, and pH changes of NaOH pretreatment and the pretreatment using the combination of NaOH and lime.

Chemical loading (g raw biomass^{-1})	Glucan conversion (%)	Xylan conversion (%)	Total carbohydrate conversion (%)	Lignin reduction (%)	Initial pH	Final pH	
						6h	24h
0.20 g NaOH	69.5 (1.09)	54.8 (0.87)	63.5 (1.01)	48.6 (1.03)	12.92 (0.02)	12.81 (0.02)	12.66 (0.04)
0.10 g NaOH	48.1 (3.12)	38.9 (2.90)	44.1 (1.73)	34.6 (1.51)	12.84 (0.02)	12.59 (0.02)	12.32 (0.03)
0.10 g NaOH + 0.02g $Ca(OH)_2$	59.4 (0.79)	57.3 (0.79)	59.3 (1.04)	37.8 (0.55)	12.86 (0.01)	12.77 (0.03)	12.65 (0.04)

economic promise. Assuming the market prices of NaOH and lime are respectively $400 and $80/ton, the chemical costs of pretreating one ton of dry switchgrass biomass will be $80.0, $40.0 and $41.6 for using 0.20 g NaOH, 0.10 g NaOH, and the combination of 0.10 g NaOH and 0.02 g lime. Lignin reduction from the pretreatment with lime supplementation was comparable with that from the pretreatment using 0.10 g NaOH/g raw biomass. Although lignin was better preserved in the presence of calcium ions, the higher pH level still resulted in a slight increase in lignin reduction. The pH changes indicated that lime did help to avoid serious pH drop during pretreatment. After 6 h, the decline of pH at 0.10 g NaOH/g raw biomass without lime supplementation was significantly ($P<0.05$) greater than that with lime supplementation. To make the differences more outstanding, the pretreatment time was extended to 24 h. The final pH from the pretreatment with lime supplementation was not significantly ($P<0.05$) different from that using 0.20 g NaOH/g raw biomass, while considerably higher than that using 0.10 g NaOH/g raw biomass.

5.3.5 Material balances

Material balances were performed on the biomass pretreated at the best conditions (6 h, 0.10g NaOH/g raw biomass, and start point NaOH addition, 0.02 g $Ca(OH)_2$/g raw biomass, and wash intensity of 100 ml water/g raw biomass). The biomass pretreated using 0.10 and 0.20 g NaOH/g raw biomass was also analyzed

for comparison. The total dry weight of the sample was measured after pretreatment, and the compositions of pretreated biomass (glucan, xylan, galactan, arabinan, lignin, ash, and other) were determined and compared with that of raw biomass. The solid recovery significantly ($P<0.05$) decreased with the increase of NaOH loading (Figure 5.5). However, lime supplementation, although contributed to greater alkalinity, didn't cause greater solid loss due to the better biomass preservation in the presence of calcium linkages. Glucan (cellulose) was the best preserved

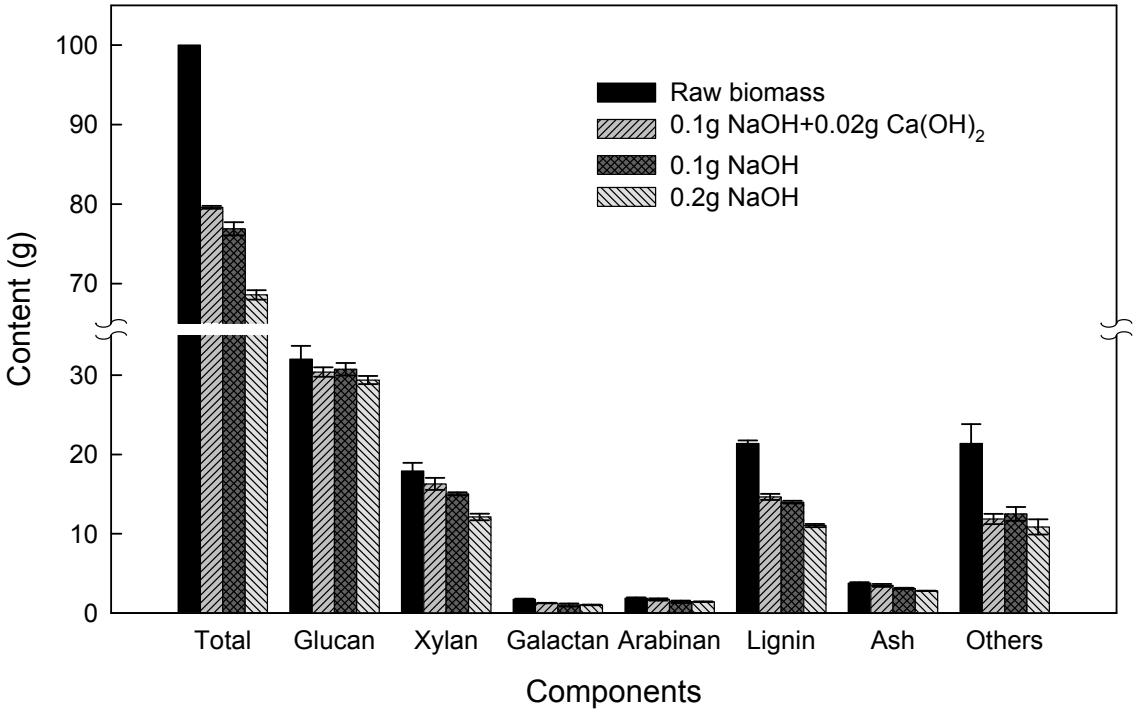

Figure 5.5 Material balances for raw switchgrass, the biomass pretreated using the combination of NaOH and lime at the best conditions (6 h, 0.1g NaOH/g raw biomass, start point NaOH addition, 0.02 g $Ca(OH)_2$/g raw biomass, and wash intensity of 100 ml water/g raw.

carbohydrate at all pretreatment conditions because of its semicrystilline structure. The impacts of pretreatment on xylan and lignin were similar to that on total solids. Better xylan and lignin preservations were achieved with lime supplementation. The biomass pretreated using the combination of 0.10g NaOH and 0.02g $Ca(OH)_2$/g raw biomass was more comparable with that of the biomass pretreated using 0.20 g NaOH/g raw biomass in terms of sugar production, however, its compositional changes was more similar to that of the biomass pretreated using 0.10 g NaOH/g raw biomass. This is because that although the higher pH level maintained by lime substantially improved biomass digestibility, the calcium linkages prevented serious solubilization of biomass components.

5.3.6 Study on enzyme loading

To determine the best enzyme loadings, cellulase loadings of 0-35 FPU/g dry biomass and cellobiase loadings of 0-50 CBU/g dry biomass were investigated. The cellobiase loading was kept constant at 61.5 CBU/g biomass in the cellulase loading test to eliminate the impact of cellobiase limitation on sugar production. At the excessive cellobiase loading, no inhibition of cellobiose to cellulase was observed. The yields of glucose, xylose, and total reducing sugar of switchgrass pretreated at the best conditions (6 h, 0.10 g NaOH/g raw biomass, start point NaOH addition, 0.02 g $Ca(OH)_2$/g raw biomass, and wash intensity of 100 ml water/g raw biomass) at different cellulase loadings were determined (Figure 5.6a). With the increase of

cellulase loading from 0 FPU/g dry biomass to 20 FPU/g dry biomass, the total reducing sugar yield increased by 6.52 times, while further increasing cellulase

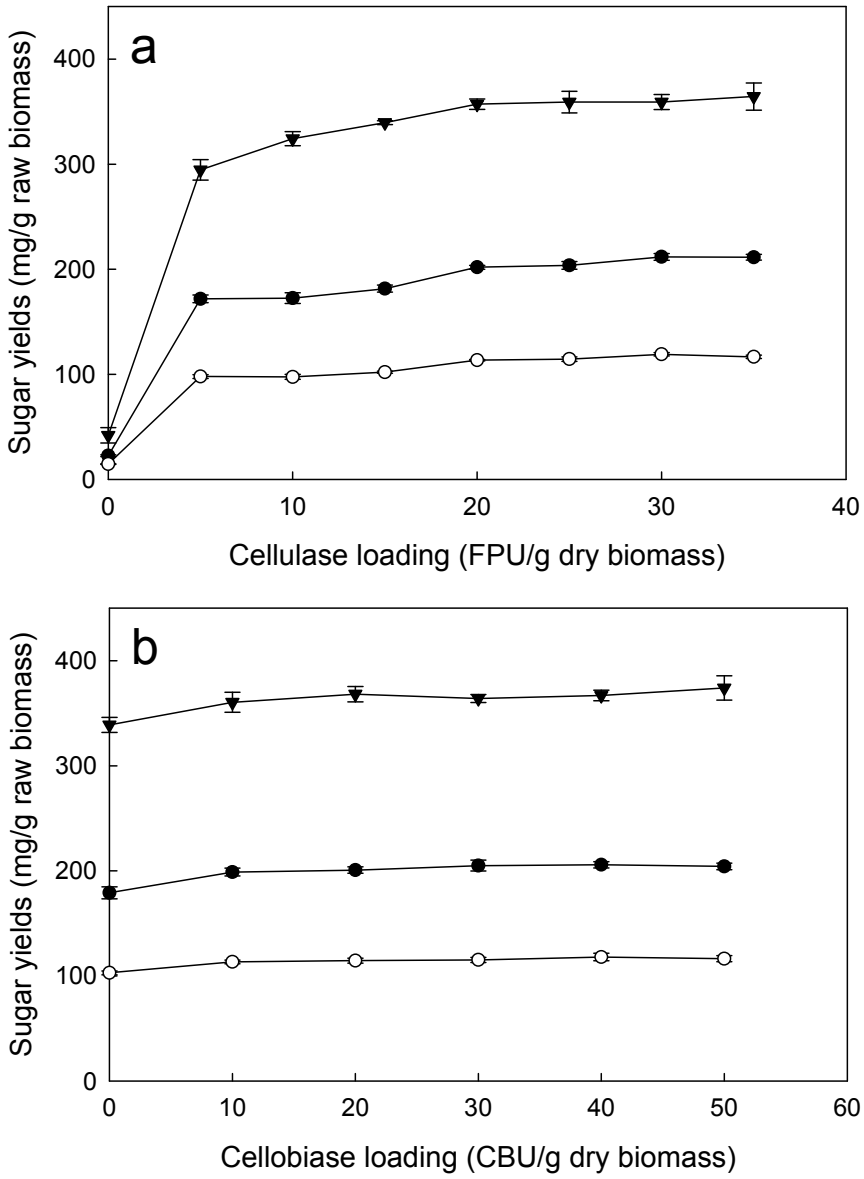

Figure 5.6 Effects of cellulase and cellobiase loadings on (●) glucose, (○) xylose, and (▼) total reducing sugar yields of switchgrass pretreated at the best conditions (6 h, 0.1g NaOH/g raw biomass, start point NaOH addition, 0.02 g $Ca(OH)_2$/g raw biomass, and wash intensity of 100 ml water/g raw biomass).

loading didn't improve sugar production. A cellulase loading of 20 FPU/g dry biomass was sufficient to maximize sugar yields. Similar trends were observed for both glucose and xylose yields. Cellobiase loadings were studied based on the best cellulase loading. Figure 5.6b shows that supplementing cellobiase significantly ($P<0.05$) increased sugar yields and a cellobiase loading of 10 CBU/g dry biomass was sufficient.

5.4 CONCLUSIONS

In this study, NaOH and lime, two commonly studied alkalis, were innovatively used together to improve the cost-effectiveness of alkaline pretreatment at room temperature. Lime, which is very inexpensive but too weak to work alone at room temperature, managed to replace the requirement for strong but expensive NaOH by providing cheap alkalinity, maintaining high pH during pretreatment, and mitigating solubilization of carbohydrates especially hemicellulose. At the best pretreatment conditions (6 h, 0.10g NaOH/g raw biomass, start point NaOH addition, 0.02 g $Ca(OH)_2$/g raw biomass, and wash intensity of 100 ml water/g raw biomass), total reducing sugar yield in enzymatic hydrolysis was 3.16 times that of untreated biomass, which was almost 30% higher than that of NaOH pretreatment at the same conditions without lime supplementation, while the chemical cost was barely increased considering the low cost of lime and the small amount applied. Achieving alkaline pretreatment at room temperature leads to a major reduction in the cost of

pretreatment by avoiding high energy requirement for heating, which might have a significant impact on the economic success of lignocellulose-to-ethanol conversion, and this research provides a novel strategy to achieve effective room temperature pretreatment at reduced costs. Future work will be focused on scale-up studies and cost-benefit analysis to evaluate the perspective for industrial application.

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CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

6.1 CONCLUSIONS

Switchgrass is a promising feedstock for ethanol production. However, the recalcitrant structure of lignicellulosic materials necessities a pretreatment step to break down lignocellulosic matrix, thus making the carbohydrates more accessible to hydrolytic enzymes for fermentable sugar production in the subsequent hydrolysis. In this research, alkaline pretreatment was explored to improve the enzymatic saccharification of switchgrass biomass. Pretreatments were conducted using sodium hydroxide and lime, and processing conditions including temperature, residence time, alkali loading, and biomass washing were investigated and optimized. NaOH and lime were also innovatively used together to improve the cost-effectiveness of alkaline pretreatment at room temperature.

NaOH pretreatment substantially improved enzymatic digestibility of switchgrass at all the temperatures studied (121, 50, and 21 ^{o}C). With the decrease of temperature, longer residence times or higher NaOH concentrations were required for effective pretreatments. At the best combinations of residence time and NaOH concentration at different temperatures (1.0% NaOH, 0.5 h at 121 ^{o}C; 1.0% NaOH, 12 h at 50 ^{o}C; 2.0% NaOH, 6 h at 21 ^{o}C), the total reducing sugar yields were respectively 3.55, 3.78 and 3.39 times that of untreated biomass. Based on total reducing sugar yield, the best conditions for lime pretreatment of switchgrass were: 50 ^{o}C, 12 h, and 1.0% NaOH, at which 74.4% of glucan, 62.8% of xylan, and 70.8% of the total available carbohydrates in raw biomass were converted to monomeric

sugars in enzymatic hydrolysis. The results also showed that although the biomass pretreated at reduced temperatures contained reduced carbohydrate but higher lignin, better carbohydrate preservations at such milder conditions led to high sugar productions which were comparable with those obtained from high temperature pretreatments. NaOH pretreatment showed outstanding delignification capability. All of the maximum lignin reductions at different temperatures were obtained at the combinations of the longest residence times and the greatest NaOH concentration, indicating a close relationship between lignin removal and pretreatment severity. However, lignin reduction should not be regarded as an accurate indicator for pretreatment effectiveness due to the more serious carbohydrate loss at more severe pretreatment conditions. Cellulase and cellobiase loadings of 15 FPU/g dry biomass and 20 CBU/g dry biomass were sufficient to maximize sugar production.

Lime pretreatment could also substantially improve the enzymatic digestibility of switchgrass at different temperatures (121, 50, and 21 ^{o}C). However, since lime is a much weaker base due to its poor solubility in water, the residence time required to maximize the total reducing sugar yield increased from 0.5 to 96 h with the reduction of temperature from 121 to 21 ^{o}C. At the best residence times at different temperatures (0.5 h at 121 ^{o}C, 24 h at 50 ^{o}C, and 96 h at 21 ^{o}C), the total reducing sugar yields were respectively 3.46, 3.61 and 3.43 times that of untreated biomass. Lime loading of 0.10 g $Ca(OH)_2$/g raw biomass and wash intensity of 100 ml water/g raw biomass were the optimums for sugar production. Based on total reducing sugar

yield, the best conditions for switchgrass pretreatment were: 50 ^{o}C, 24 h, 0.1 g $Ca(OH)_2$/g raw biomass, and wash intensity of 100 ml water/g raw biomass, at which 67.4% of glucan, 62.5% of xylan, and 67.7% of the total available carbohydrates were converted to monomeric sugars in enzymatic hydrolysis. Calcium ions extensively crosslinked lignin in the biomass under alkaline conditions, which resulted in low lignin reductions. However, as long as the interactions between biomass components were effectively disrupted and the biomass porosity improved, the enzymes could still get good access to the carbohydrates even in the presence of high lignin. Carbohydrate solubilization was also mitigated by calcium bonding, which could potentially increase overall sugar recovery. Cellulase and cellobiase loadings of 20 FPU/g dry biomass and 20 CBU/g dry biomass were sufficient to maximize sugar production.

Pretreatment using the combination of NaOH and lime has been proven to be a very promising technology which could substantially increase the cost-effectiveness of alkaline pretreatment at room temperature. Lime, which is very inexpensive but too weak to work alone at room temperature, managed to performce as a supplementary chemical to reduce the requirement for strong but expensive NaOH by providing cheap alkalinity, maintaining high pH during pretreatment, and mitigating solubilization of carbohydrates especially hemicellulose. Using the best pretreatment conditions (6 h, 0.10 g NaOH/g raw biomass, start point NaOH addition, 0.02 g $Ca(OH)_2$/g raw biomass, and wash intensity of 100 ml water/g raw biomass),

59.4% of glucan, 57.3% of xylan, and 59.3% of the total available carbohydrates in raw biomass were converted to monomeric sugars in enzymatic hydrolysis. The total reducing sugar yield was 3.16 times that of untreated biomass, which was almost comparable with the maximum sugar production obtained in NaOH pretreatment at doubled NaOH loading, while the chemical expense of using the combination of 0.10 g NaOH/g raw biomass and 0.02 g $Ca(OH)_2$/g raw biomass was barely higher than that using 0.10 g NaOH/g raw biomass, where the sugar production was about 30% lower. This invention was developed based on the unique properties of two alkalis, and could potentially lead to a major reduction in the cost of pretreatment by avoiding high energy requirement for heating and high chemical expense at the same time. Cellulase and cellobiase loadings of 20 FPU/g dry biomass and 10 CBU/g dry biomass were sufficient to maximize sugar production.

Assuming the annual yield of switchgrass is 15 Mg ha^{-1} and all the reducing sugars in hydrolysate are converted to ethanol after fermentation, the theoretical ethanol yields from switchgrass after pretreatments using NaOH, lime, and the combination of the two alkalis at the respective best pretreatment conditions and enzymatic hydrolysis using the optimal enzyme doses were respectively 448.1, 449.9, and 374.4 gallon/acre, showing the great promise of alkaline pretreatment of switchgrass for ethanol production.

6.2 RECOMMENDATIONS FOR FUTURE WORK

One of the major advantages of alkaline pretreatment is that effective pretreatments can be expected even at reduced temperatures. However, unlike other pretreatment methods requiring high temperatures, at which considerable carbohydrates are solubilized and dissolve into liquid phase, after alkaline pretreatment at reduced temperatures and enzymatic hydrolysis, much carbohydrate remains in solid residues which necessitates further disposal. Therefore, investigation is required to understand the composition and structure of the residue biomass, and figure out appropriate ways to utilize it.

Lime is very inexpensive, safe to handle, and can be easily recovered. However, since the solubility of lime in water is poor, much more water is required to wash away the residual lime particles to prevent them from inhibiting the following enzymatic hydrolysis, which not only causes greater carbohydrate loss but also results in higher water expenses and operational cost. To solve this problem, a specially designed two-chamber reaction vessel would be ideal to prevent the mixing of biomass and solid lime. Although a lot of challenges need to be addressed before any sound designs come out, it is worthwhile to make this effort not only for cost reduction but also for envirometnal benefits.

Using the combination of NaOH and lime is very promising in achieving effective pretreatment of switchgrass at room temperature and reduced cost, while

its performances at other temperatures is unknown. Based on the mechnisms discussed, it is promising to apply this technology at elevated temperatures for higher sugar production. Besides, since the effectiveness of alkaline pretreatment is quite substrate specific, it is necessary to test this innovative technology on other feedstocks.

APPENDICES

APPENDIX A: ORIGINAL DATA

Table 1 Solid recovery, glucose, xylose, and total reducing sugar yields, and lignin reduction for NaOH pretreatment of switchgrass at 121 °C.

NaOH concentration (%)	Residence time (h)	Solid recovery (%)	Sugar production (mg/g raw biomass)			Lignin reduction (%)
			Glucose	Xylose	Reducing sugars	
0.5	0.25	74.98	159.59	67.62	243.99	35.95
		75.95	146.52	64.15	259.32	34.41
		74.42	143.89	62.13	245.52	35.97
	0.5	75.17	161.41	71.46	257.74	34.62
		74.03	157.67	70.01	272.02	35.39
		73.99	150.87	67.31	272.16	35.41
	1	73.22	176.05	73.39	294.21	34.95
		73.51	178.92	75.79	267.06	36.17
		72.22	179.34	75.97	299.43	37.27
1.0	0.25	58.35	254.47	111.68	392.47	64.71
		56.64	253.81	113.99	403.54	66.35
		57.39	253.60	112.75	397.10	66.49
	0.5	54.99	251.93	115.05	431.15	76.03
		55.63	258.43	117.40	423.15	75.77
		54.80	251.07	111.62	421.93	76.46
	1	52.44	266.72	111.23	435.25	74.89
		52.41	262.62	110.56	417.14	75.11
		52.05	263.51	111.59	421.96	75.09
2.0	0.25	48.40	275.32	102.47	397.64	73.64
		49.52	277.51	102.00	401.75	73.23
		48.66	256.75	89.80	379.78	72.96
	0.5	48.46	280.16	103.16	405.12	83.64
		48.78	284.31	102.07	412.51	81.41
		47.09	272.67	99.14	400.53	83.65
	1	46.42	274.79	95.20	409.43	85.31
		46.12	271.55	92.33	409.70	85.50
		45.87	271.20	93.65	397.14	86.59

Table 2 Solid recovery, glucose, xylose, and total reducing sugar yields, and lignin reduction for NaOH pretreatment of switchgrass at 50 °C.

NaOH concentration (%)	Residence time (h)	Solid recovery (%)	Sugar production (mg/g raw biomass)			Lignin reduction (%)
			Glucose	Xylose	Reducing sugars	
0.5	1	78.14	87.82	30.51	140.23	24.84
		77.91	89.88	33.36	145.18	26.36
		78.13	90.53	32.16	135.43	25.42
	3	77.04	115.64	41.23	171.87	29.87
		78.05	122.82	45.75	187.27	28.84
		77.91	120.60	43.93	181.58	28.50
	6	76.89	136.51	54.29	217.45	31.35
		77.64	137.09	54.19	211.26	30.31
		76.80	137.45	54.69	201.32	32.04
	12	75.65	153.01	65.37	237.69	35.09
		75.72	153.16	65.22	238.48	34.60
		76.21	154.74	65.71	231.88	34.92
	24	76.32	163.25	72.80	269.00	34.13
		74.84	164.49	71.62	267.22	34.74
		74.66	162.66	71.49	271.18	35.90
	48	74.48	161.23	72.69	272.23	35.11
		76.25	161.80	72.33	265.84	34.16
		74.98	162.38	72.81	283.82	34.77
1.0	1	75.99	231.12	102.30	406.34	40.46
		74.80	211.94	94.78	388.48	40.73
		75.94	229.25	102.75	395.02	40.46
	3	73.24	241.78	118.94	428.26	47.56
		73.19	239.26	116.05	421.35	47.00
		72.26	237.58	116.85	416.99	47.36
	6	68.42	262.01	129.75	408.49	53.54
		69.06	262.20	127.42	438.20	51.01
		69.18	252.51	124.57	429.68	51.24
	12	68.58	262.54	127.46	448.84	56.03
		68.00	266.88	129.43	455.65	53.70
		68.06	263.59	126.50	455.55	57.41
	24	65.25	276.50	127.68	472.31	62.03
		63.60	277.79	128.72	447.69	62.32
		64.54	271.52	125.40	456.27	65.01
	48	62.51	279.31	125.78	456.83	65.10
		61.88	284.21	129.27	469.08	66.26
		63.03	273.88	118.93	455.74	64.99

Table 2 (continued)

2.0	1	67.01	221.88	108.99	372.65	53.34
		69.84	235.52	114.59	375.93	53.49
		69.67	234.65	114.45	378.27	56.22
	3	59.20	256.10	112.63	391.33	63.83
		60.78	267.11	116.61	402.21	63.15
		60.97	267.64	117.10	415.13	64.71
	6	58.59	271.13	111.44	411.04	68.03
		57.12	273.91	114.74	410.40	69.81
		57.26	273.77	114.13	415.35	68.81
	12	54.74	275.05	109.06	407.49	73.27
		54.84	275.91	108.47	422.92	73.01
		54.53	277.26	110.19	402.60	71.24
	24	53.30	263.13	105.31	395.95	74.19
		54.33	284.79	113.79	429.45	75.70
		53.85	272.88	109.60	410.81	73.84
	48	51.68	275.09	105.13	429.82	76.39
		52.14	275.32	103.05	403.73	79.44
		52.56	277.16	105.84	416.20	77.59

Table 3 Solid recovery, glucose, xylose, and total reducing sugar yields, and lignin reduction for NaOH pretreatment of switchgrass at 21 °C.

NaOH concentration (%)	Residence time (h)	Solid recovery (%)	Sugar production (mg/g raw biomass)			Lignin reduction (%)
			Glucose	Xylose	Reducing sugars	
0.5	1	80.30	67.70	23.58	110.73	21.76
		81.02	67.42	22.86	114.82	20.37
		80.33	66.60	23.05	106.15	21.79
	3	78.33	74.04	25.91	123.01	24.71
		79.68	74.36	25.34	116.74	23.89
		78.77	73.20	25.43	125.21	24.31
	6	79.63	82.17	29.23	127.98	23.58
		78.72	84.39	29.72	131.16	23.63
		79.11	83.62	30.08	122.49	26.68
	12	79.78	94.53	34.57	150.24	23.43
		79.87	92.90	34.51	143.06	22.60
		79.21	92.70	34.39	157.66	25.87
	24	80.21	101.60	39.67	189.12	25.59
		79.17	102.26	39.33	172.13	27.22
		78.85	102.27	39.29	173.23	26.32
	48	79.21	115.62	46.99	199.49	28.79
		79.72	115.46	46.36	197.72	28.89
		79.09	112.80	46.37	196.78	27.28
	96	78.75	123.96	52.06	212.29	28.69
		78.77	120.57	50.21	218.29	32.32
		78.52	120.91	51.29	229.01	29.39
1.0	1	80.12	139.16	44.89	219.05	26.44
		80.17	130.83	42.49	192.68	26.96
		79.77	135.89	44.58	207.05	28.35
	3	79.16	136.88	57.37	225.44	29.53
		78.41	145.99	60.84	224.51	29.21
		79.09	142.96	58.40	211.30	29.40
	6	77.79	183.80	85.85	279.89	32.99
		76.17	163.79	75.36	272.33	35.98
		76.70	165.52	75.98	294.19	34.75
	12	75.71	180.52	89.08	327.49	40.55
		76.49	197.03	95.40	315.64	35.68
		76.29	183.61	87.29	336.42	38.66
	24	73.27	208.11	94.87	352.25	43.93
		73.93	208.53	103.03	358.25	42.73
		73.60	205.03	92.54	373.57	44.67
	48	69.99	207.51	92.97	391.67	49.27
		70.67	237.79	113.51	386.27	48.46
		70.89	234.95	114.41	388.03	46.06

Table 3 (continued)

	96	69.47	227.15	110.73	394.60	51.63
		69.53	244.91	116.50	401.36	49.09
		69.83	245.67	117.45	416.99	50.84
2.0	1	76.96	212.63	104.80	353.12	34.30
		76.59	213.63	103.38	354.37	34.22
		76.76	201.43	99.02	333.72	34.55
	3	72.45	221.84	104.27	371.63	40.73
		72.25	227.96	106.41	370.03	40.62
		72.83	227.66	106.81	381.92	40.80
	6	67.99	247.67	111.70	402.35	48.86
		68.57	242.80	109.61	413.66	47.40
		69.17	250.45	113.12	402.47	49.39
	12	66.53	249.64	108.28	412.06	53.03
		67.62	257.10	110.69	421.40	54.22
		67.05	250.89	106.83	420.38	49.89
	24	64.07	257.71	107.75	396.34	56.07
		62.45	253.77	100.27	411.64	58.44
		64.04	258.42	106.62	425.04	57.02
	48	60.18	257.63	101.99	418.82	62.13
		61.21	267.47	100.98	425.49	58.94
		60.68	262.93	103.67	409.27	60.13
	96	58.80	271.02	95.45	425.40	60.43
		56.98	255.65	95.52	416.18	65.21
		57.34	262.10	92.92	408.28	62.97

Table 4 Material balances for raw switchgrass biomass and biomass pretreated using NaOH at the best combinations of residence time and NaOH concentration at different temperatures (0.5 h, 1.0% NaOH at 121 ^{o}C; 12 h, 1.0% NaOH at 50 ^{o}C; 6 h, 2.0% NaOH at 21 ^{o}C).

Component	Content (g/100 g dry biomass)			
	Raw biomass	121 ^{o}C	50 ^{o}C	21 ^{o}C
Total solids	100.00	54.99	68.58	67.99
	100.00	55.63	68.00	68.57
	100.00	54.80	68.06	69.17
Glucan	33.85	25.63	29.86	29.37
	31.54	26.21	30.11	28.87
	30.56	25.66	29.54	29.87
Xylan	19.07	12.23	12.57	12.22
	17.51	12.38	12.84	11.65
	17.12	12.11	12.39	12.46
Galactan	1.83	0.64	1.13	1.00
	1.65	0.69	1.14	1.07
	1.71	0.59	1.19	1.00
Arabinan	1.99	1.15	1.47	1.44
	1.85	1.30	1.48	1.46
	1.78	1.08	1.66	1.38
Lignin	20.98	5.14	9.40	11.02
	21.53	5.13	9.93	11.24
	21.60	5.06	9.58	10.83
Ash	3.65	2.21	2.79	2.76
	3.77	2.19	2.72	2.80
	3.88	2.14	2.81	2.83
Other	18.62	8.14	10.46	11.74
	22.14	7.25	10.79	9.83
	23.35	8.49	10.70	10.99

Table 5 Effects of cellulase loadings on glucose, xylose, and total reducing sugar yields of switchgrass pretreated using NaOH at the best conditions (50 ^{o}C, 12 h, and 1.0% NaOH).

Cellulase loading (FPU/g dry biomass)	Sugar production (mg/g raw biomass)		
	Glucose	Xylose	Reducing sugars
0	43.04	22.42	64.77
	45.06	23.77	60.22
	44.23	23.23	62.74
5	283.96	142.21	360.81
	304.70	147.69	359.88
	304.64	148.12	374.23
10	323.67	159.60	385.17
	338.86	165.24	390.84
	332.62	165.69	401.50
15	359.33	171.58	420.13
	377.89	177.59	434.73
	373.65	178.06	430.21
20	373.26	184.70	451.37
	371.37	181.61	437.42
	351.59	171.44	417.71
25	357.99	172.80	443.81
	395.96	197.04	420.92
	367.32	185.92	431.76

Table 6 Effects of cellobiase loadings on glucose, xylose, and total reducing sugar yields of switchgrass pretreated using NaOH at the best conditions (50 ^{o}C, 12 h, and 1.0% NaOH).

Cellobiase loading (CBU/g dry biomass)	Sugar production (mg/g raw biomass)		
	Glucose	Xylose	Reducing sugars
0	292.33	143.41	364.50
	292.70	143.77	357.76
	286.04	141.52	360.97
10	303.76	143.41	416.25
	334.70	159.69	401.58
	328.64	160.12	407.58
20	358.43	176.97	438.53
	370.06	183.24	428.94
	357.82	171.69	426.75
30	361.48	176.98	438.99
	363.80	177.39	437.35
	342.03	167.26	415.81
40	362.14	177.83	447.02
	364.53	179.65	432.58
	374.81	181.64	437.61
50	360.98	177.02	440.06
	366.78	178.94	418.27
	358.70	176.63	440.80

Table 7 Solid recovery, glucose, xylose, and total reducing sugar yields, and lignin reduction for lime pretreatment of switchgrass at 121 ^{o}C.

Residence time (h)	Solid recovery (%)	Sugar production (mg/g raw biomass)			Lignin reduction (%)
		Glucose	Xylose	Reducing sugars	
0.25	76.07	208.73	102.93	393.82	31.73
	77.34	227.73	111.15	385.37	30.22
	76.54	218.34	106.50	388.23	30.76
0.5	74.83	230.65	105.94	406.17	34.26
	74.58	240.65	111.07	422.92	34.34
	74.49	235.21	108.93	415.74	33.96
1	72.69	240.66	114.95	412.19	33.43
	71.65	240.04	112.81	419.02	35.18
	71.87	237.79	112.01	408.69	35.38
1.5	68.64	233.05	112.09	412.53	35.67
	69.02	231.96	111.04	408.15	35.12
	69.44	235.60	113.76	429.68	35.70

Table 8 Solid recovery, glucose, xylose, and total reducing sugar yields, and lignin reduction for lime pretreatment of switchgrass at 50 ^{o}C.

Residence time (h)	Solid recovery (%)	Sugar production (mg/g raw biomass)			Lignin reduction (%)
		Glucose	Xylose	Reducing sugars	
1	88.09	175.53	85.88	281.03	28.56
	87.49	167.92	81.01	282.07	27.94
	87.98	171.83	84.34	286.61	27.93
3	87.80	182.07	93.50	362.66	29.44
	87.66	189.62	98.02	345.01	28.54
	87.70	190.06	96.37	357.99	26.44
6	85.51	219.94	109.59	411.22	27.63
	84.43	209.94	113.95	396.52	29.06
	85.65	231.11	113.38	403.20	27.65
12	81.68	220.54	113.85	409.59	29.41
	81.89	231.43	118.77	417.13	30.67
	81.80	236.87	122.98	407.14	30.04
24	80.86	233.53	124.20	433.97	33.22
	82.25	245.55	130.39	440.19	32.21
	81.49	239.68	126.92	426.06	33.19
48	80.47	243.06	124.88	424.06	31.63
	80.59	247.68	127.10	435.85	35.02
	79.63	243.24	124.03	428.88	34.44

Table 9 Solid recovery, glucose, xylose, and total reducing sugar yields, and lignin reduction for lime pretreatment of switchgrass at 21 ^{o}C.

Residence time (h)	Solid recovery (%)	Sugar production (mg/g raw biomass)			Lignin reduction (%)
		Glucose	Xylose	Reducing sugars	
1	84.62	97.48	30.46	162.59	17.02
	85.97	97.49	30.43	160.58	16.69
	86.30	99.94	39.87	171.77	16.50
3	86.76	125.97	55.70	223.81	16.97
	86.06	126.50	56.28	245.07	18.35
	84.79	125.15	55.96	225.88	17.89
6	83.80	172.96	92.52	282.27	23.54
	84.85	157.82	77.38	291.66	22.12
	84.47	164.72	85.15	297.48	21.83
12	83.31	173.20	91.32	310.81	25.37
	84.16	171.78	89.60	323.45	25.55
	84.22	174.56	91.79	296.77	24.25
24	82.94	201.53	116.94	364.44	27.16
	80.91	199.51	114.08	358.62	28.79
	82.93	199.03	116.93	356.17	27.69
48	79.67	216.56	129.07	386.11	30.11
	78.92	221.12	130.68	384.24	31.37
	79.72	219.07	129.14	394.25	30.08
96	80.24	239.77	123.26	406.68	31.41
	79.42	226.35	135.33	406.16	29.81
	79.12	228.82	127.70	422.19	32.26
168	77.95	229.64	137.50	412.95	32.85
	76.64	228.55	131.52	396.06	35.38
	77.98	227.85	132.41	425.05	32.96

Table 10 Effects of lime loading and wash intensity on solid recovery and sugar production in lime pretreatment of switchgrass at 121 ^{o}C.

Wash intensity (ml water/g raw biomass)	Lime loading (g/g raw biomass)	Solid recovery (%)	Sugar production (mg/g raw biomass)
100	0.05	79.38	285.12
		78.75	267.57
		79.96	280.10
	0.10	74.83	412.23
		74.58	428.95
		74.49	415.74
	0.15	73.46	393.98
		75.01	410.77
		74.14	419.80
	0.20	75.69	379.02
		74.10	355.47
		75.61	376.78
300	0.05	77.31	252.04
		76.82	261.65
		76.13	236.50
	0.10	71.78	391.34
		72.41	385.41
		71.12	388.31
	0.15	71.26	385.08
		71.27	397.24
		71.35	403.43
	0.20	72.19	397.67
		72.88	403.82
		71.58	412.24

Table 11 Effects of lime loading and wash intensity on solid recovery and sugar production for lime pretreatment of switchgrass at 50 °C.

Wash intensity (ml water/g raw biomass)	Lime loading (g/g raw biomass)	Solid recovery (%)	Sugar production (mg/g raw biomass)
100	0.05	81.19	238.45
		81.78	245.47
		83.65	232.80
	0.10	80.86	421.23
		82.25	444.43
		81.49	444.95
	0.15	80.56	404.67
		82.55	401.36
		81.12	422.58
	0.20	80.80	398.08
		81.51	397.61
		82.98	382.61
300	0.05	78.31	243.30
		79.17	226.74
		78.83	218.77
	0.10	75.97	408.05
		76.46	400.80
		76.81	399.53
	0.15	76.62	409.69
		77.49	391.17
		77.58	404.15
	0.20	76.81	415.65
		78.65	447.24
		76.46	414.42

Table 12 Effects of lime loading and wash intensity on solid recovery and sugar production in lime pretreatment of switchgrass at 21 ^{o}C.

Wash intensity (ml water/g raw biomass)	Lime loading (g/g raw biomass)	Solid recovery (%)	Sugar production (mg/g raw biomass)
100	0.05	81.63	234.45
		81.36	245.52
		81.99	245.43
	0.10	80.24	418.68
		79.42	405.39
		79.12	422.42
	0.15	79.64	431.64
		78.64	416.04
		79.98	405.67
	0.20	81.05	392.76
		83.40	386.57
		80.61	378.22
300	0.05	78.33	211.66
		77.79	205.81
		78.03	227.27
	0.10	76.84	400.92
		75.76	411.22
		74.46	385.47
	0.15	76.46	408.20
		76.24	415.65
		75.53	411.16
	0.20	75.27	407.33
		75.44	416.80
		77.39	413.16

Table 13 Effect of calcium loading on solid loss, lignin reduction, and carbohydrate solubilization at different alkaline pretreatment conditions.

Pretreatment condition	Calcium loading (g $Ca(OH)_2$/g raw biomass)	Solid loss (%)	Lignin reduction (%)	Carbohydrate solubilization (%)
121 °C, 0.5 h	0	56.23	90.64	35.70
		56.99	92.29	37.83
		56.76	90.21	35.60
	0.05	53.80	83.09	36.18
		54.24	83.13	35.98
		53.63	82.70	34.25
	0.10	52.89	78.91	33.03
		51.52	78.27	32.23
		51.87	78.04	32.77
21 °C, 6 h	0	36.34	54.46	19.86
		37.94	54.64	21.72
		37.70	55.34	21.05
	0.05	34.49	48.84	20.72
		35.47	49.38	17.23
		34.47	48.59	17.82
	0.10	33.41	47.51	16.00
		33.04	48.30	17.85
		33.04	45.15	18.41

Table 14 Adsorption of orange and blue dyes to the biomass pretreated for 0.25, 0.5, and 1 h at 121 ^{o}C, and 6, 24, and 96 h at 21 ^{o}C in Simons' stain procedure.

Pretreament condition		Dye adsorption (mg/g dry biomass)	
Temperature	Residence time (h)	Orenge	Blue
121 ^{o}C	0.25	10.88	17.39
		12.57	19.45
		11.99	19.10
	0.5	17.89	22.90
		18.61	23.78
		19.27	24.13
	1	22.43	32.62
		22.82	30.46
		23.65	30.49
Room temperature	6	7.37	14.30
		7.01	14.96
		6.52	16.70
	24	10.54	18.47
		10.69	17.62
		11.80	19.18
	96	17.63	22.78
		19.22	23.92
		19.57	23.27

Table 15 Material balances for raw switchgrass and biomass pretreated at the optimum conditions of lime pretreatment at different temperatures (0.5 h at 121 ^{o}C, 24 h at 50 ^{o}C, 96 h at 21 ^{o}C, 0.10 g $Ca(OH)_2$/g raw biomass, and wash intensity of 100 ml water/g raw biomass).

Component	Content (g/100 g dry biomass)			
	Raw biomass	121 ^{o}C	50 ^{o}C	21 ^{o}C
Total solids	100.00	74.83	80.86	80.24
	100.00	74.58	82.25	79.42
	100.00	74.49	81.49	79.12
Glucan	33.85	28.89	29.15	29.89
	31.54	27.33	30.71	28.73
	30.56	28.11	29.64	28.52
Xylan	19.07	12.95	13.71	14.02
	17.51	11.81	16.02	13.00
	17.12	13.33	14.36	15.03
Galactan	1.83	0.86	0.94	0.71
	1.65	0.95	0.96	0.73
	1.71	0.99	0.87	0.60
Arabinan	1.99	1.33	1.44	1.10
	1.85	1.35	1.40	0.96
	1.78	1.22	1.28	1.04
Lignin	20.98	13.89	14.39	14.54
	21.53	13.92	14.36	15.03
	21.60	14.02	14.28	14.56
Ash	3.65	3.02	3.28	3.20
	3.77	2.92	3.19	3.11
	3.88	2.98	3.17	3.10
Other	18.62	13.70	18.63	16.14
	22.14	16.35	14.90	18.04
	23.35	13.98	17.93	16.73

Table 16 Effects of cellulase loadings on glucose, xylose, and total reducing sugar yields of switchgrass pretreated using lime at the best conditions (50 ^{o}C, 24 h, 0.1 g $Ca(OH)_2$/g raw biomass, and wash intensity of 100 ml water/g raw biomass).

Cellulase loading (FPU/g dry biomass)	Sugar production (mg/g raw biomass)		
	Glucose	Xylose	Reducing sugars
0	28.78	15.25	71.85
	27.79	15.25	68.04
	29.02	15.99	69.31
5	183.66	101.01	356.68
	181.36	99.86	368.13
	182.51	99.86	352.21
10	207.84	118.06	393.94
	206.61	116.83	376.19
	200.46	113.15	397.17
15	225.06	126.67	403.92
	216.45	121.75	395.02
	213.99	120.52	393.75
20	224.40	125.91	424.92
	234.38	132.15	437.17
	233.13	130.90	421.05
25	231.88	129.66	418.39
	225.65	127.16	431.28
	230.64	129.66	445.46
30	236.87	133.40	437.73
	231.88	127.16	421.61
	229.39	128.41	420.33
35	233.53	124.20	433.97
	245.55	130.39	440.19
	239.68	126.92	426.06

Table 17 Effects of cellobiase loadings on glucose, xylose, and total reducing sugar yields of switchgrass pretreated using lime at the best conditions (50 ^{o}C, 24 h, 0.10 g $Ca(OH)_2$/g raw biomass, and wash intensity of 100 ml water/g raw biomass).

Cellobiase loading (CBU/g dry biomass)	Sugar production (mg/g raw biomass)		
	Glucose	Xylose	Reducing sugars
0	210.85	112.78	387.23
	208.40	117.69	389.76
	209.63	111.56	392.30
10	231.69	122.59	416.81
	219.44	116.46	410.48
	226.79	120.14	403.50
20	234.15	125.04	430.51
	240.28	127.49	430.51
	239.05	132.40	438.12
30	246.41	134.85	417.75
	241.50	132.40	430.43
	235.37	129.95	425.99
40	232.92	128.72	446.91
	239.05	131.17	428.53
	241.50	133.62	420.29
50	231.69	126.27	419.66
	234.15	128.72	436.77
	240.28	132.40	424.09

Table 18 Solid recovery, glucose, xylose, and total reducing sugar yields, and lignin reduction for the pretreatment of switchgrass using the combination of NaOH and lime at room temperature (21 ^{o}C).

Residence time (h)	NaOH loading (g/g raw biomass)	Time point for NaOH addition	Solid recovery (%)	Sugar production (mg/g raw biomass)			Lignin reduction (%)
				Glucose	Xylose	Reducing sugars	
3	0.05	0	82.72	158.58	83.53	255.36	27.48
			83.12	173.71	90.22	258.22	27.43
			82.70	173.73	92.45	264.10	27.23
		1/3	85.28	160.72	81.51	232.75	26.00
			86.23	157.86	82.89	237.01	26.19
			86.91	169.89	88.23	246.99	24.87
		2/3	85.56	146.92	72.07	210.32	25.19
			85.67	138.33	68.93	213.93	23.23
			86.54	149.55	70.57	226.14	22.87
	0.10	0	80.17	214.30	121.65	356.51	30.05
			79.81	206.43	115.49	347.35	31.91
			79.59	205.01	115.19	355.59	32.60
		1/3	84.82	193.74	105.80	313.17	26.87
			85.34	194.02	107.84	316.01	26.34
			84.43	198.78	108.05	313.51	27.39
		2/3	85.42	171.12	87.64	238.90	26.27
			86.25	171.87	89.43	237.90	26.13
			85.95	169.40	85.40	237.89	24.53
	0.20	0	75.33	245.28	135.04	408.23	42.17
			74.80	251.65	137.74	400.57	43.57
			75.42	243.53	133.17	410.54	41.62
		1/3	83.70	217.85	120.68	362.54	29.97
			83.67	217.32	122.44	355.40	30.11
			83.88	220.59	123.20	361.57	30.53
		2/3	85.80	190.89	104.25	291.15	26.81
			85.77	191.29	103.75	282.20	27.03
			84.49	188.89	103.11	280.44	25.69

Table 18 (continued)

	0.5	0	82.87	198.25	111.88	343.20	29.85
			82.21	198.43	110.98	342.03	31.39
			82.07	187.02	101.93	324.79	30.73
		1/3	84.82	195.59	106.27	315.18	28.16
			84.17	190.44	102.72	316.00	29.16
			85.24	193.31	102.64	323.30	28.05
		2/3	85.47	150.45	70.61	226.63	24.57
			85.46	146.76	68.30	213.40	24.67
			85.89	161.41	76.07	238.56	25.32
	0.10	0	77.54	234.47	130.63	404.66	38.69
			77.17	233.77	129.59	421.82	38.34
			77.64	238.12	129.12	410.30	39.41
		1/3	82.62	210.13	115.55	395.08	30.79
6			82.29	226.18	121.75	402.86	31.85
			82.26	234.98	127.48	398.53	31.79
		2/3	85.63	169.23	80.46	271.30	27.10
			84.43	183.29	88.45	274.59	28.76
			84.02	183.75	89.38	289.98	29.15
	0.20	0	73.25	244.44	117.87	424.48	44.24
			73.27	263.92	129.39	427.71	43.95
			74.07	266.77	129.58	439.97	42.73
		1/3	80.83	236.57	123.08	409.26	35.12
			80.21	237.35	125.17	403.54	37.60
			80.45	232.85	117.29	397.74	36.51
		2/3	82.48	202.20	100.65	328.72	31.35
			83.15	183.64	92.05	301.79	31.14
			83.17	219.16	109.58	300.10	32.49

Table 18 (continued)

			81.39	200.42	107.24	353.60	30.69
		0	81.06	197.40	105.05	361.54	31.49
			81.83	197.95	106.49	332.53	31.16
			84.69	182.82	97.95	307.78	28.96
	0.05	1/3	83.99	203.38	109.34	321.63	28.44
			84.20	207.93	110.00	330.04	30.73
			79.05	181.10	96.50	295.02	27.04
		2/3	78.65	165.09	87.08	268.22	28.53
			79.23	165.96	85.93	260.74	27.69
			76.74	242.83	130.53	424.86	38.14
		0	76.71	243.58	130.49	411.05	38.94
			75.84	229.75	123.27	408.28	39.41
			83.66	206.63	113.51	402.52	31.75
9	0.10	1/3	84.03	227.77	122.95	390.60	30.58
			82.68	227.44	125.62	396.46	28.06
			77.99	196.96	105.71	321.16	29.29
		2/3	77.64	193.31	99.83	316.45	30.50
			77.29	215.19	116.53	341.32	32.55
			71.57	263.18	126.37	442.43	44.50
		0	71.87	268.94	130.40	436.88	45.17
			71.35	256.99	123.29	427.98	45.50
			82.06	242.06	128.07	422.52	36.74
	0.20	1/3	82.12	232.48	120.82	429.82	36.70
			81.73	242.76	125.04	417.30	34.58
			74.78	222.46	115.66	371.80	33.10
		2/3	75.41	228.81	120.61	386.33	34.87
			74.46	220.68	112.99	367.73	34.47

Table 19 Effects of lime loading and wash intensity on solid recovery and sugar production for the pretreatment of switchgrass using the combination of NaOH and lime at room temperature (21 ^{o}C).

Wash intensity (ml water/g raw biomass)	Lime loading (g/g raw biomass)	Solid recovery (%)	Sugar production (mg/g raw biomass)
100	0	76.55	282.72
		75.92	300.56
		75.57	291.87
	0.02	79.70	384.92
		79.74	381.25
		79.42	372.03
	0.04	80.80	373.29
		80.13	376.67
		82.12	395.31
	0.06	80.90	377.68
		81.42	363.04
		80.60	369.79
	0.08	82.52	355.28
		81.50	381.11
		81.49	368.61
	0.10	81.49	356.76
		82.29	352.32
		82.26	347.55
200	0	74.97	284.72
		73.98	288.13
		75.59	295.62
	0.02	78.40	381.18
		77.98	394.22
		77.09	383.51
	0.04	78.32	402.88
		79.18	398.37
		78.87	408.24
	0.06	78.33	397.85
		78.38	418.34
		79.66	410.39
	0.08	80.27	401.25
		78.65	407.75
		79.10	401.79
	0.10	81.42	401.11
		80.49	409.48
		80.44	403.38

Table 20 Comparison of pH changes for NaOH pretreatment and the pretreatment using the combination of NaOH and lime.

Chemial loading (g^{-1} raw biomass)	Initial pH	pH at 6h	pH at 24h
0.10 g NaOH	12.85	12.61	12.32
	12.85	12.57	12.35
	12.82	12.60	12.30
0.20 g NaOH	12.93	12.81	12.67
	12.94	12.83	12.69
	12.90	12.80	12.62
0.10 g NaOH + 0.02 g $Ca(OH)_2$	12.86	12.74	12.66
	12.87	12.79	12.61
	12.85	12.75	12.68

Table 21 Material balances for raw switchgrass, the biomass pretreated using the combination of NaOH and lime at the best conditions (6 h, 0.10 g NaOH/g raw biomass, start point NaOH addition, 0.02 g $Ca(OH)_2$/g raw biomass, and wash intensity of 100 ml water/g raw biomass), and the biomass pretreated using NaOH at two loadings for 6 h.

Component	Content (g/100 g dry biomass)			
	Raw biomass	Chemial loading (g^{-1} raw biomass)		
		0.10 g NaOH + 0.02 g $Ca(OH)_2$	0.10 g NaOH	0.20 g NaOH
Total solids	100.00	79.70	77.79	67.99
	100.00	79.74	76.17	68.57
	100.00	79.42	76.70	69.17
Glucan	33.85	29.69	29.89	29.37
	31.54	30.73	31.41	28.87
	30.56	30.69	30.89	29.87
Xylan	19.07	15.42	14.84	12.22
	17.51	16.54	15.23	11.65
	17.12	16.88	15.03	12.46
Galactan	1.83	1.29	0.98	1.00
	1.65	1.23	0.84	1.07
	1.71	1.27	1.22	1.00
Arabinan	1.99	1.85	1.35	1.44
	1.85	1.56	1.32	1.46
	1.78	1.73	1.60	1.38
Lignin	20.98	15.08	14.16	11.02
	21.53	14.52	13.81	11.24
	21.60	14.32	13.98	10.83
Ash	3.65	3.69	3.11	2.76
	3.77	3.43	3.18	2.80
	3.88	3.34	3.02	2.83
Other	18.62	12.58	13.51	11.74
	22.14	11.60	11.97	9.83
	23.35	11.38	12.01	10.99

Table 22 Effects of cellulase loadings on glucose, xylose, and total reducing sugar yields of switchgrass pretreated using the combination of NaOH and lime at the best conditions (6 h, 0.10 g NaOH/g raw biomass, start point NaOH addition, 0.02 g $Ca(OH)_2$/g raw biomass, and wash intensity of 100 ml water/g raw biomass).

Cellulase loading (FPU/g dry biomass)	Sugar production (mg/g raw biomass)		
	Glucose	Xylose	Reducing sugars
0	23.38	14.35	34.43
	22.68	14.35	48.99
	21.76	15.04	42.32
5	169.76	96.86	292.94
	169.76	96.86	305.08
	176.01	99.98	285.66
10	166.64	94.77	325.05
	176.01	98.94	317.21
	174.97	98.94	330.56
15	185.38	103.11	341.49
	180.17	102.06	339.06
	179.13	101.02	337.84
20	200.82	113.09	358.87
	200.82	113.09	360.72
	203.99	114.15	351.48
25	203.99	115.21	369.96
	207.16	116.26	357.64
	199.76	112.03	349.63
30	212.44	119.43	357.64
	208.22	117.32	366.88
	214.56	120.49	352.71
35	214.56	115.21	349.60
	209.27	118.38	369.79
	210.33	116.26	373.57

Table 23 Effects of cellobiase loadings on glucose, xylose, and total reducing sugar yields of switchgrass pretreated using the combination of NaOH and lime at the best conditions (6 h, 0.10 g NaOH/g raw biomass, start point NaOH addition, 0.02 g $Ca(OH)_2$/g raw biomass, and wash intensity of 100 ml water/g raw biomass).

Cellobiase loading (CBU/g dry biomass)	Sugar production (mg/g raw biomass)		
	Glucose	Xylose	Reducing sugars
0	176.34	100.93	344.59
	185.62	104.41	340.85
	175.18	103.25	330.87
10	196.06	111.37	367.65
	203.02	114.85	363.91
	197.22	113.69	349.57
20	198.38	113.69	375.75
	204.18	117.17	361.42
	199.54	112.53	367.03
30	209.98	117.17	360.79
	205.34	112.53	368.27
	199.54	116.01	362.66
40	208.82	121.81	364.53
	203.02	117.17	372.64
	205.34	114.85	363.29
50	200.70	113.69	362.66
	205.34	116.01	385.73
	206.50	119.49	373.26

APPENDIX B: PROCEDURE FOR SIMONS' STAIN METHOD

1. Preparation of the dye solutions:

1.1 Prepare 50 ml of 1% (w/v) Blue Dye (Direct Blue 1) solution.

1.2 Prepare 50 ml of 1% (w/v) Orange Dye (Direct Orange 15) solution. The orange dye contains two fractions based on molecular weight: high and low. For this procedure, the high molecular weight fraction must be isolated.

1.3 Pour 15 ml of the 1% Orange Dye solution into a 50 ml ultracentrifugation tube (fitted with a 100 K membrane). Spin at 4000 rpm for 10 minutes. At the end of the cycle, approximately 25-30% of the original volume should be retained.

1.4 Measure the density of the retentate and dilute appropriately to 0.2% w/v.

1.5 Prepare 100 ml of 1:1 staining solution mixture of the 1% Blue Dye and 0.2% Orange Dye (high MW).

1.6 NaCl is added to a final concentration of 1%.

2. Staining of the biomass sample

2.1 Weigh out 50 mg of the biomass (dry weight) into a 125 ml Erlenmeyer flask and add 30 ml of the staining solution.

2.2 Incubate in a water bath set at 75 °C for 48 hours.

2.3 After 48 hours, filter each sample through a crucible. Make sure all solids are transferred from the flask to the crucible.

2.4 Wash the recovered solids with 30 ml of cold DI water.

3. Stripping of the dye molecules from stained biomass

3.1 Transfer the recovered solids into a 125 ml Erlenmeyer flask and add 40 ml of 25% Pyridine stripping solution.

3.2 Incubate in a water bath set of 45 °C for 18 hours.

3.3 After 18 hours, filter each sample though a crucible and store approximately 10 ml of the filtrate in storage tubes.

4. Calculation

4.1 Measure of the absorbance of the filtrate at 450 nm and 621.5 nm using 25% pyridine as a blank.

4.2 Use the following equations to calculate the concentration (g/L) of the blue (C_B) and orange (C_O) dyes in the filtrates that were stripped from the biomass:

$A_{450} = \varepsilon_{O/450}*L*C_O + \varepsilon_{B/450}*L*C_B$

$A_{621.5} = \varepsilon_{O/621.5}*L*C_O + \varepsilon_{B/621.5}*L*C_B$

In the above equations, A_{450} and $A_{621.5}$ are the absorbances of the filtrate measured at 450 nm and 621.5 nm respectively, L is the width of the cuvette (1 cm) and ε is the extinction coefficient of each dye in the staining mixture at the respective wavelengths. From literature review: $\varepsilon_{O/450}$ = 50.67 L/g cm, $\varepsilon_{B/450}$ = 1.97 L/g cm, $\varepsilon_{O/621.5}$ = 0.075 L/g cm, $\varepsilon_{B/621.5}$ = 15.65 L/g cm.

APPENDIX C: SAMPLE SAS CODE

*This SAS program evaluates the effects of concentration (conc), time, and
their interaction on solid recovery (SR), glucose yield (Glu), xylose
yield (Xyl), total reducing sugar yield (TRS), and lignin reduction (LR) for NaOH pretreatment of
switchgrass at 121 ^{o}C. Tukey adjustment is used to perform multiple comparison of treatment means
at an α level of 0.05.

```
data switchgrass;
input conc time SR Glu Xyl TRS LR;
datalines;
0.5     0.25    74.98   159.59  67.62   243.99  35.95
0.5     0.25    75.95   146.52  64.15   259.32  34.41
0.5     0.25    74.42   143.89  62.13   245.52  35.97
0.5     0.5     75.17   161.41  71.46   257.74  34.62
0.5     0.5     74.03   157.67  70.01   272.02  35.39
0.5     0.5     73.99   150.87  67.31   272.16  35.41
0.5     1       73.22   176.05  73.39   294.21  34.95
0.5     1       73.51   178.92  75.79   267.06  36.17
0.5     1       72.22   179.34  75.97   299.43  37.27
1.0     0.25    58.35   254.47  111.68  392.47  64.71
1.0     0.25    56.64   253.81  113.99  403.54  66.35
1.0     0.25    57.39   253.60  112.75  397.10  66.49
1.0     0.5     54.99   251.93  115.05  431.15  76.03
1.0     0.5     55.63   258.43  117.40  423.15  75.77
1.0     0.5     54.80   251.07  111.62  421.93  76.46
1.0     1       52.44   266.72  111.23  435.25  74.89
1.0     1       52.41   262.62  110.56  417.14  75.11
1.0     1       52.05   263.51  111.59  421.96  75.09
2.0     0.25    48.40   275.32  102.47  397.64  73.64
2.0     0.25    49.52   277.51  102.00  401.75  73.23
2.0     0.25    48.66   256.75  89.80   379.78  72.96
2.0     0.5     48.46   280.16  103.16  405.12  83.64
2.0     0.5     48.78   284.31  102.07  412.51  81.41
2.0     0.5     47.09   272.67  99.14   400.53  83.65
2.0     1       46.42   274.79  95.20   409.43  85.31
2.0     1       46.12   271.55  92.33   409.70  85.50
2.0     1       45.87   271.20  93.65   397.14  86.59
;
proc glm;
class conc time;
model SR=conc|time;
lsmeans conc|time / pdiff adjust=tukey;
run;

proc glm;
class conc time;
model Glu=conc|time;
lsmeans conc|time / pdiff adjust=tukey;
run;

proc glm;
class conc time;
```

```
model Xyl=conc|time;
lsmeans conc|time / pdiff adjust=tukey;
run;

proc glm;
class conc time;
model TRS=conc|time;
lsmeans conc|time / pdiff adjust=tukey;
run;

proc glm;
class conc time;
model LR=conc|time;
lsmeans conc|time / pdiff adjust=tukey;
run;
```