ABSTRACT

WANG, ZIYU. Alkaline Pretreatment of Coastal bermudagrass for Bioethanol Production. (Under the direction of Jay J. Cheng.)

Sodium hydroxide and lime pretreatments of coastal bermudagrass for enhanced reducing sugars recovery were investigated in this study. In the study of sodium hydroxide pretreatment, coastal bermudagrass was pretreated with 0.5% to 3% (w/v) NaOH solutions for 15 min to 90 min at 121°C. For lime pretreatment, a variety of temperatures (room temperature to 121°C) at a range of residence times with different lime loadings (0.02 to 0.20 g/g dry biomass) were examined. Sodium hydroxide pretreatment at 121°C was more effective than lime pretreatment with regard to lignin removal with an average difference of 55%. After enzymatic hydrolysis with excessive cellulases and cellobiase, the best total reducing sugars yield for lime pretreatment was 78% of the theoretical maximum which is comparable to 77% for NaOH pretreatment at 121°C. The optimal conditions for NaOH pretreatment at 121°C are 15 min and 0.75% NaOH under which glucan and xylan conversion rates were approximately 91% and 65% respectively. As for lime pretreatment, the best condition is 100°C for 15 min with a lime loading of 0.1 g/g dry biomass under which 87% of glucan and 68% of xylan were converted to glucose and xylose respectively.

The coastal bermudagrass pretreated under the recommended conditions with NaOH and lime was hydrolyzated with different enzyme loadings (cellulases: 0 to 40 FPU (FPU, filter paper unit, expressed as μmol of glucose produced per minute with filter paper as a substrate) /g dry biomass; cellobiase: 0 to 70 CBU (CBU, cellobiase unit, expressed as μmol of cellobiose that is converted into glucose per minute with cellobiose as a substrate)/g dry biomass). A cellulases loading of 20 FPU/g was required to improve sugar recovery for lime-

pretreated biomass, while 15 FPU/g was sufficient for enhanced sugar yield for NaOH-pretreated biomass. The optimal cellobiase loading was found to be 10 CBU/g for the two types of pretreated biomass. The supplementation of xylanase during hydrolysis was not beneficial to higher sugar recovery for both pretreatment methods. More than 99% of glucose in the hydrolyzate was utilized by the yeast strain for ethanol production with 95% of the theoretical maximum yield for the hydrolyzate and 83% of the theoretical yield for the raw biomass. There was no significant difference in ethanol yield between NaOH and lime-pretreated coastal bermudagrass.

Alkaline Pretreatment of Coastal Bermudagrass for Bioethanol Production

by
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A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Master of Science

Biological and Agricultural Engineering

Raleigh, North Carolina

2009

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BIOGRAPHY

Ziyu Wang was born in Fuzhou, a so-called “Town of Brilliant People”, Jiangxi, P.R. China. He is not a very smart guy but just a diligent person who is willing to devote himself to his beloved engineering business. As the only child of his parents, he had a wonderful but unusual childhood with their love. He moved to Humen in Guangdong with his parents when he was 8 years old. In the following two years, he went to a Kungfu school without living together with his parents. The two years’ special experience made him an independent and indomitable teenager.

After finishing the study in the Kungfu school, he went back to Fuzhou to continue his education in a well-known middle school. Following another three years in a high school, he matriculated for undergraduate study in Beijing University of Chemical Technology with major in Bioengineering in 2002. He obtained his bachelor’s degree after four years’ happy and unforgettable time. The year of 2006, in which he came to the United States for graduate study, is one of the most important and meaningful years to him. He was initially admitted to the PhD program in the Chemistry department at NC State in fall 2006. After one month study there, he started thinking about his future career path and made a decision to change his major from chemistry to bioprocess engineering. He switched to the MS program in the Department of Biological and Agricultural Engineering at NC State in spring 2007, pursuing his Master’s degree under the guidance of Dr. Jay J. Cheng and his committee. He learned a lot in communication and scientific research during the past two years and decided to continue his graduate student life for a doctoral degree in the same department.

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Jay J. Cheng, for his guidance throughout this work. He gave me a lot of help and support not only for my study and research but also for my living in US. I am thankful to Dr. Ratna Sharma for her support to my lab work and good advice on my research. I am pleased to give my thanks to Dr. Steven Peretti for his kind help on my minor courses.

I am also grateful to Geoffrey Moxley, a senior research associate at Novozymes North America Inc., for his great help on the HPLC analysis of my experimental samples. His contribution to this thesis work is highly appreciated.

I would also like to thank my colleagues, Dr. Ye Chen, Deepak Keshwani, Arthur Redding, Jiele Xu and Ying Yang, for their friendly support and help on my course study and research work.

I am especially thankful to my parents for their encouragement and persistent support to me. Because of them, I have been able to achieve my goals step by step. Their love, understanding, and support always accompany with me as I grow up.

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Petroleum is the largest energy source in the United States and the world. Approximately 60% of petroleum consumed in the U.S. is imported (Um et al., 2003), leading to balance of trade deficits and national energy security concerns. Transportation sector represents about two thirds of the total U.S. petroleum consumption and is almost exclusively fueled by oil (Wyman et al., 2005). Demand for liquid transportation fuels is projected to increase by 17 percent from 2006 to 2030 (EIA, 2008). The need for transportation fuels is rapidly rising, while oil reserves in the U.S. are limited and the increasing dependency on a limited number of oil-providing countries creates inherent risks for national security and price distortions (Rogner, 2000). In addition, greenhouse gases such as carbon dioxide emitted mainly from burning fossil fuels for transportation are detrimental to our environment, greatly contributing to global climate changes and air pollution. Therefore, the use of biofuels is important in addressing issues with both greenhouse gas emissions of transportation and the dependency on fossil fuels.

In the transportation sector, ethanol is the most widely used liquid biofuel in the world. Fuel ethanol produces less CO, NO_x and photochemical pollutants than gasoline. By replacing gasoline with ethanol and using ethanol as an additive, the amount of gasoline pollutants can be reduced (Wheals et al., 1999). In the U.S., nearly all ethanol is blended into

gasoline at up to 10 percent by volume to produce a fuel called E10 or "gasohol". Total U.S. ethanol production was 3.9 billion gallons, or 2.9 percent of the total gasoline pool in 2005. Preliminary data for 2006 indicate that ethanol use rose to 5.4 billion gallons. Ethanol blended into gasoline is projected to account for 7.5 percent of the total gasoline pool by volume in 2012, and 7.6 percent in 2030 (EIA, 2007).

Ethanol can be produced from any feedstock that contains plentiful natural sugars or starch that can be readily converted to sugar. The United States has sufficient biorenewable resources to satisfy all of its fuel ethanol needs. Corn is currently the primary feedstock for ethanol production in the United States. Starch, a polysaccharide carbohydrate consisting of a large number of glucose units linked together by α-1,4 glycosidic bonds that constitutes about 70% of the corn kernel, is easily broken down to glucose that can be further fermented to ethanol. There are two methods called dry milling and wet milling for the production of corn-based ethanol, with wet milling accounting for more than 60% of the total ethanol production in the U.S. (Elander and Putsche, 1996). Typically, starch is gelatinized with steam, liquefied to dextrin with amylolytic enzymes, saccharified with glucoamylase to release glucose, and then fermented to ethanol (Gong et al., 1999). Major byproducts from the corn-based ethanol production process include dried distillers' grains and solubles (DDGS) that can be used as animal feed. On a smaller scale, corn gluten meal, gluten feed, corn oil, CO_2, and sweeteners are also byproducts of the corn-based ethanol production process used in the U.S. (EIA, 2007). Corn feedstock made up nearly 57 percent of the total production cost in 2002 (USDA, 2005). Fluctuations in the price of corn can have dramatic

effects on the ethanol production costs. As ethanol production increases, competition for corn supplies among the fuel, food, and export markets, along with a decline in the marginal value of ethanol co-products, is expected to make ethanol production more expensive (Baker and Zahniser, 2006). The corn-based ethanol processing technology is highly mature and there is little possibility of significantly reducing production costs further. On the other hand, lignocellulosic biomass, the most plentiful of all naturally occurring organic compounds, is particularly well-suited for energy applications due to its availability at large quantities, lower cost than corn and environmentally benign production (Lynd et al., 1999).

Conversion of lignocellulosic biomass to transportation fuels offers a tremendous opportunity to improve energy security, reduce trade deficits, and considerably reduce greenhouse gas emissions (Wyman, 1999). Lignocellulosic biomass includes wood, herbaceous crops, agricultural and forestry residues, waste paper and paper products, pulp and paper mill waste, and municipal solid waste (Gong et al., 1999). Different from starchy feedstocks, lignocellulosic biomass is structurally complex. Lignocelluloses consist of variable amounts of cellulose, hemicelluloses, lignin and other minor components such as ash, protein, and extractives. Cellulose, a linear polymer of anhydro D-glucose units linked by β-1,4 glycosidic bonds, is an important constituent of most plants. Microfibrils are formed by intra- and intermolecular hydrogen bonding in cellulose, with high packing densities resulting in highly ordered microfibrils known as crystalline cellulose. Hemicellulose, a large number of heteropolysaccharides, is mainly composed of hexoses (D-glucose, D-mannose, and D-galactose), pentoses (D-xylose, L-arabinose, and D-arabinose), and deoxyhexoses (L-

rhamnose or 6-dexoy-L-mannose and rare L-fucose or 6-deoxy-L-galactose) with small amounts of uronic acids present (Brown, 2003). A large fraction of pentoses, as opposed to hexoses from cellulose, is liberated upon hydrolysis of hemicelluloses. The chemical and thermal stability of hemicellulose is lower than that of cellulose, most likely because of its lack of crystallinity and lower degree of polymerization. Lignin, the largest non-carbohydrate fraction in lignocelluloses, is a phenylpropane-based polymer that cannot be depolymerized to its original monomers. Lignin and hemicellulose form a sheath that is embedded with cellulose in the lignocellulosic biomass (Brown, 2003).

Although lignocellulosic materials are competitive in price with oil, the complex structure of lignocellulosic biomass such as the cellulose crystallinity, the sheathing of hemicellulose, and the lignin barrier, makes it more difficult to be hydrolyzed by enzymes compared to corn starch. The key challenge to commercializing cellulosic ethanol production is to develop low-cost technology for overcoming the recalcitrance of lignocellulosic materials (Lynd et al., 1999; Wyman, 1999). The conversion of lignocellulosic biomass to ethanol involves three main steps: pretreatment, the hydrolysis of carbohydrate components present in pretreated biomass to fermentable sugars, and the fermentation of the sugars to ethanol. Due to the recalcitrant nature of cellulosic biomass, a pretreatment step is required to enhance enzymatic hydrolysis efficiency by disrupting the structure of biomass and increasing accessibility of cellulolytic enzymes to the substrates. Pretreatment is one of the most costly processes and has a major impact on the cost of prior and subsequent operations (Wooley et al., 1999). For instance, more efficient pretreatment contributes to less use of

expensive enzymes. In addition, considerable R&D by the National Renewable Energy Laboratory and its partners has significantly reduced the estimated cost of enzyme production. Although cellulosic ethanol currently is not cost-competitive with gasoline or corn-based ethanol, further significant successes in processing technologies along with large quantities of less expensive raw feedstocks, could make cellulosic ethanol a viable economic option for expanded ethanol production in the future.

1.2 OBJECTIVES OF THE STUDY

The swine industry in the southeast of the United States is expanding rapidly. To avoid environmental pollution caused by the swine wastewater, many farmers grow coastal bermudagrass, a warm-season, deep-rooted perennial pasture grass, for nitrogen and phosphorus removal to prevent potential pollution of these nutrients to the nearby watershed. The existence of cropping system makes coastal bermudagrass considered as a potential lignocellulosic feedstock for bioethanol production. Furthemore, the harvested coastal bermudagrass is usually given away or sold at very low price as animal feed. Therefore, there is a great interest to investigate the conversion of coastal bermudagrass into ethanol.

This project is affiliated with a lager project that mainly focuses on studying different pretreatment technologies including acid, alkaline, microwave, steam explosion, ammonia fiber explosion, and ozonolysis. The purpose of this specific project was to investigate the effect of alkaline pretreatment on the subsequent hydrolysis and fermentation of coastal bermudagrass for bioethanol production. This research would provide important information on the commercial utilization of coastal bermudagrass for large-scale ethanol production.

Specific objectives were: 1) Study the effect of sodium hydroxide pretreatment of coastal bermudagrass on the subsequent enzymatic hydrolysis; 2) Investigate the effect of calcium hydroxide (lime) pretreatment of coastal bermudagrass on the subsequent enzymatic hydrolysis; 3) Examine enzyme dosing levels during hydrolysis for economical ethanol production from coastal bermudagrass.

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CHAPTER 2

CONVERSION OF LIGNOCELLULOSIC MATERIALS INTO BIOETHANOL: A REVIEW

2.1 INTRODUCTION

Energy consumption has steadily risen over the last century, and is expected to continue to increase, at a steep rate globally due to the rapid development of the world. The United States energy consumption rate has also steadily been increasing with no indication of slowing down. Crude oil has been playing a critical role in meeting the continuous rise of energy demand. The decline in worldwide crude oil production before 2010 was predicted along with the decline from the current 25 billion barrels to estimated 5 billion barrels in 2050 (Campell and Laherrere, 1998). With regards to the increased energy use and the limited oil production as well as the environmental concerns, the United States has implemented energy policies that aim to develop renewable, domestic sources of fuels.

Biomass, which is a form of stored solar energy (sunlight having been converted by photosynthesis to cellulosic materials) is an abundant, renewable, domestically available energy resource (Ladisch et al., 1979). Dedicated crops of both perennial herbaceous plants and rapidly growing woody plants such as switchgrass and willow tress, respectively can provide readily available biomass for energy production (Lemus and Lal, 2005).

Ethanol, produced through fermentation of sugars, is a renewable energy resource that is used as a partial gasoline replacement. Ethanol is also a safer alternative to the toxic chemical

compound-methyl tertiary butyl ether (MTBE), the most common additive to gasoline used to provide cleaner combustion (McCarthy and Tiemann, 1998). The beginning of regulatory action to eliminate MTBE in gasoline has been announced by the US Environmental Protection Agency (Browner, 2000). The United States produced 1.77 billion gallons of ethanol in 2001, which increased approximately 10% from 1.63 billion gallons in 2000 and 20% from 1.47 billion gallons in 1999 (Sun and Cheng, 2005). Demand for fuel ethanol will rise because of the reduction of crude oil resources and the elimination of MTBE from gasoline (Sun and Cheng, 2002). Current fuel ethanol production is mainly based on the fermentation of glucose derived from corn starch, which will compete against the corn-based food and feed production. On the other hand, there are plentiful lignocellulosic materials such as crop residues, grasses, sawdust, solid animal waste and wood chips that can be utilized to substitute the equivalent of 40% of the gasoline in the current US market (Wheals et al., 1999). Lignocellulosic materials are considered as a potential source for a large amount of low-cost ethanol production.

2.2 CONVERSION OF LIGNOCELLULOSIC MATERIALS TO ETHANOL

Lignocellulosic materials have been considered as alternative energy sources because they can capture CO_2 during growth so that their combustion does not generate net CO_2 (Klass, 1998). Unlike starch-based biomass, lignocellulosic materials are structurally complex. Native lignocellulosic biomass, which is heterogeneous, is composed of cellulose, hemicellulose, lignin, protein, ash, and minor extractives. Cellulose is a linear high molecular weight polysaccharide containing two residues (β-1,4-linked glucose, known as cellobiose) in

repeated units (Gong et al., 1999). Complete hydrolysis of cellulose yields glucose that can be fermented to ethanol, however, crystalline cellulose is recalcitrant to hydrolysis. Hemicellulose, which has a much lower molecular weight than cellulose, is a heteropolysaccharide that is composed of various hexose (e.g. glucose, mannose and galactose), pentoses (D-xylose and L-arabinose), uronic acids, acetic acid, and other minor sugars (Gong et al., 1999). The hemicellulose fraction is readily hydrolyzed to pentose, but pentoses are difficult to ferment. Lignin, a complex 3-dimensional polyaromatic matrix, forms a seal around cellulose microfibrils and exhibits limited covalent association with hemicellulose, which prevents enzymes and acids from accessing some regions of the cellulose polymers (Weil et al., 1994). Table 2.1 (Sun and Cheng, 2002) shows the percentages of cellulose, hemicellulose, and lignin in some agricultural residues and wastes.

Table 2.1. The contents of cellulose, hemicellulose, and lignin in common lignocelluloses

Lignocellulosic materials	Cellullose (%)	Hemicellulose (%)	Lignin (%)
Hardwoods 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 from chemical pulps	60–70	10–20	5–10
Primary wastewater solids	8–15	NA[b]	24–29
Swine waste	6.0	28	NA[b]
Solid cattle manure	1.6–4.7	1.4–3.3	2.7–5.7
Coastal Bermuda grass	25	35.7	6.4
Switch grass	45	31.4	12.0

Extensive research has been conducted to study the conversion of lignocellulosic materials to ethanol during the past two decades. The processing of lignocellulosic biomass

to ethanol consists of four major unit operations: pretreatment, hydrolysis, fermentation, and product separation/purification (Mosier et al., 2005). The following process schematic displays the multi-unit process (Fig. 2.1).

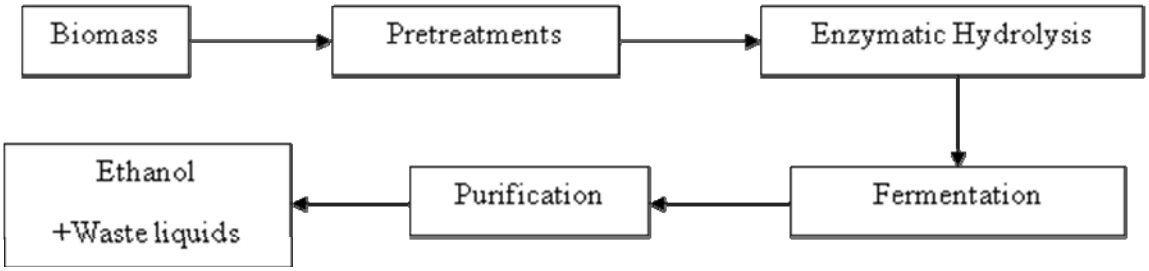

Fig. 2.1. Conversion process of lignocellulosic biomass to ethanol

There are limiting factors to the maximum possible efficiency of the conversion of ligno-cellulosic materials to ethanol. Enzymatic hydrolysis is hindered by the following substrate-related factors: cellulose contains highly resistant crystalline structure, lignin and hemicellulose surrounding cellulose form a physical barrier, and sites available for enzymatic attacks are limited (Kim et al., 2001). Pretreatment, an important tool for practical lignocellulose conversion processes, is required to alter the structure of lignocellulosic biomass to make cellulose more accessible to the enzymes as described in the schematic diagram of Fig. 2.2 (Moiser et al., 2005). The goal is to increase the accessible surface area of cellulose by breaking the lignin seal, disrupting cellulose crystallinity, solubilizing hemicellulose, and/or increasing pore volume (Weil, 1994), thus significantly improves the hydrolysis of lignocellulosic materials. The effect of pretreatment of lignocellulosic biomass has been investigated for a long time (McMillan, 1994). Pretreatment has been viewed one of the most costly steps in the conversion of lignocellulose to sugars, accounting for about 33%

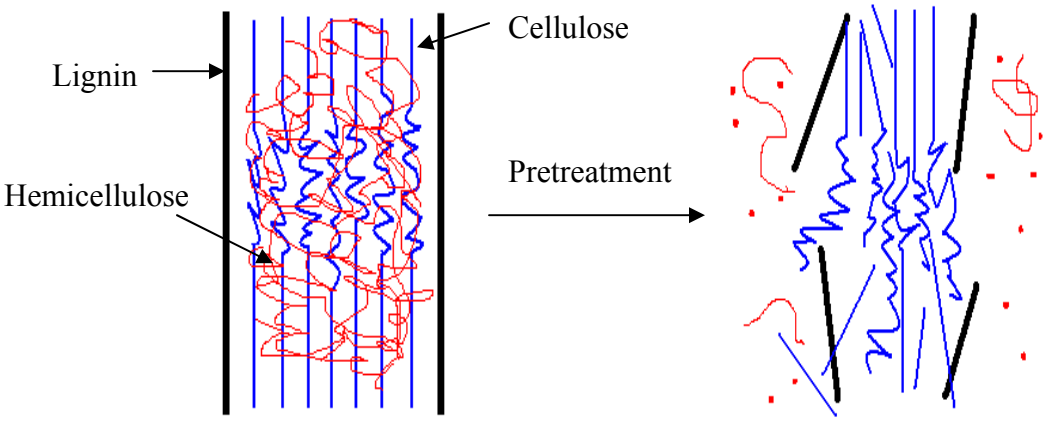

Fig. 2.2. Schematic of goals of pretreatment on lignocellulosic material

of the total operating costs (Brown, 2003). Pretreatment methods lead to complications to waste management for the developing process (Palmarola-Adrados et al., 2005; Thorsell et al., 2004). In addition, pretreatment can generate toxic byproducts that will reduce enzyme activities and growth and productivity of downstream cultures (Mussatto and Roberto, 2004; Pamqvist and Hahn-Hägerdal, 2000). The efficiency of pretreatment of lignocellulosic materials conversion to ethanol needs to be improved to meet the following requirements:(1) improve the formation of sugars or the ability to subsequently release sugars by enzymatic hydrolysis; (2) allay the degradation or loss of carbohydrate; (3) reduce the formation of byproducts inhibitory to the subsequent enzymatic hydrolysis and fermentation; and (4) be cost-effective (Sun and Cheng, 2002). Various pretreatment methods for lignocellulose conversion to ethanol can be categorized into physical, physico-chemical, chemical, and biological processes.

Another limiting factor for lignocellulosic conversion to ethanol is the utilization of five carbon sugars (pentoses) by few native microorganisms, however, six carbon sugars (hexoses)

including glucose, galactose, and mannose can be readily fermented to ethanol by common strains such as *Saccharomyces cerevisiae*. Xylulose, the ketose of xylose, is digested by *S. Pombe, S. cerevisiae, S. amucae, and Kluveromyces lactis* to form ethanol (Gong, 1983). In order to make the conversion of lignocellulosic biomass to ethanol economically feasible, a significant content of xylose and arabinose in hardwoods, agricultural residues, and grasses ought to be utilized during the fermentation process (Lynd et al., 1999). Research has been accomplished to genetically modify bacteria (Ingram et al., 1998, 1999) and yeast (Ho et al., 1998, 1999) to make them capable of co-fermenting both hexoses and pentoses to ethanol.

Fermentation step has two common categories. One is separate hydrolysis and fermentation (SHF) in which enzymatic hydrolysis is performed separately from the fermentation. The other is simultaneous saccharification and fermentation (SSF) during which cellulose hydrolysis is carried out in the presence of the fermentative microbes (Moiser et al., 2005). A innovative process that is so-called SSCF would be operated by combining saccharification of both cellulose and hemicellulose with co-fermentation of both hexoses and pentoses using genetically engineered microorganisms to further lower the costs (Wright et al., 1987). At the last step of conversion of lignocellulose to ethanol, ethanol is purified from the fermentation broth by distillation and dehydration. The residual lignin, unreacted cellulose and hemicellulose, ash, enzyme, microbes, other components in the bottom of the distillation column may be concentrated and burned as fuel to power the process, or converted to different types of coproducts (Wyman, 1995a; Hinman et al., 1992; Wooley et al., 1999).

2.3 PROMISING PRETREATMENT TECHNOLOGIES

The comparisons for various pretreatment options are based on not only the efficiency of the pretreatment methods themselves, but also on their impact on the cost of the downstream steps and the trade-off between operating costs, capital costs, and biomass costs (Lynd et al., 1996; Wyman, 1995b, 1996, 1999; Palmqvist and Hahn-Hagerdal, 2000). The optimum pretreatment process options for a particular feedstock and product opportunity with the available experimental data can be determined by a rigorous process economic analysis (Aden et al., 2002). The pretreatment technologies that are promising for cost effective biological conversion of lignocellulosic materials to fuels are reviewed.

Pretreatment methods can be physical, chemical, physico-chemical, and biological processes. Physical pretreatment technologies include mechanical comminution (Millet et al., 1976; Cadoche and López, 1989) and pyrolysis(Kilzer and Brodio, 1965; Shafizadeh and Bradbury, 1979; Fan et al., 1987). Steam explosion (autohydrolysis), ammonia fiber explosion (AFEX), and CO_2 explosion (Dale and Moreira, 1982; Zheng et al., 1998; Kim and Hong, 2001) are the three common physico-chemical pretreatment methods. Among the chemical pretreatment options, the most commonly used technologies are acid hydrolysis and alkaline hydrolysis. Ozonolysis is one of the chemical pretreatments in which ozone can be used to degrade lignin and hemicellulose in wheat straw (Ben-Ghedalia and Miron, 1981), cotton straw (Ben-Ghedalia and Shefet, 1983), bagasse, green hay, pine (Neely, 1984) and poplar sawdust (Vidal and Molinier, 1988). Hydrogen peroxide delignification of cane bagasse (Azzam, 1989) and wet oxidation with alkaline pretreatment can greatly enhance the

susceptibility of enzymatic hydrolysis. Some organic solvents including methanol, ethanol, acetone, ethylene glycol, triethylene glycol and tetrahydrofurfuryl alcohol (Chum et al., 1988; Thring et al., 1990), and organic acids such as oxalic, acetylsalicylic and salicylic acid (Sarkanen, 1980) were used as catalysts in the organosolv process. Microorganisms such as brown-, white- and soft-rot fungi can be used to degrade lignin and hemicellulose in biological pretreatment processes. The delignification of Bermuda grass by white-rot fungi was reported to be improved by 29-32% using *Ceriporiopsis subvermispora* and 63-77% using *Cyathus stercoreus* after 6 weeks (Akin et al., 1995).

Of the above pretreatment methods, steam explosion, liquid hot water, ammonia, dilute acid, and lime pretreatments are potentially cost-effective and are discussed in this review. Table 2.2 (Mosier et al., 2005) outlines key features of different pretreatment methods.

Table 2.2. Effect of pretreatment methods on characteristics of lignocellulosic biomass

	Increases accessible surface area	Decrystalizes cellulose	Removes hemicellulose	Removes lignin	Alters lignin structure
Uncatalyzed steam explosion	■		■		▪
Liquid hot water	■	ND	■		▪
pH controlled hot water	■	ND	■		ND
Flow-through liquid hot water	■	ND	■	▪	▪
Dilute acid	■		■		■
Flow-through acid	■		■	▪	■
AFEX	■	■	▪	■	■
ARP	■	■	▪	■	■
Lime	■	ND	▪	■	■

■: Major effect.
▪: Minor effect.
ND: Not determined.

2.3.1 Steam explosion

Steam explosion, one of the most commonly used method for pretreatment of lignocellulosic materials, refers to a technique in which biomass is rapidly heated by high-

pressure steam with or without addition of any chemicals. This process is typically operated at a temperature of 160-260 ^{o}C for a period of time to cause hemicellulose degradation and lignin transformation, and terminated by exposed to atmospheric pressure (Brownell and Saddler, 1984; Heitz et al., 1991; Ramos et al., 1992; Avellar and Glasser, 1998).

In steam explosion, the lignocellulosic materials are pretreated through the removal of hemicellulose, thus to increase the accessibility of the enzymes to the cellulose. Reduction in particle size and open of the particulate structure contribute weakly to the improvement of the digestibility of the pretreated biomass (Brownell et al., 1986; Biermann et al., 1984). Residence time, temperature, chip size and moisture content are considered to affect the steam explosion pretreatment (Duff and Murray, 1996). Although high temperature and short residence time or lower temperature and longer residence time both enable the optimal hemicellulose solubilization, lower temperature and longer residence time are reported to be more favorable (Wright, 1998). Compared to uncatalyzed steam explosion, steam explosion with addition of H_2SO_4 or CO_2 can effectively remove hemicellulose, reduce the amount of inhibitory compounds, and lead to more complete enzymatic hydrolysis with the optimal conditions as following: 220 ^{o}C; 30s residence time; water-to-solids ratio, 2; and 1% H_2SO_4 (Morjanoff and Gray, 1987).

Steam explosion requires 70% less energy than the conventional mechanical methods to achieve the same size reduction of biomass (Holtzapple et al., 1989), which is one of the advantages of steam explosion. Furthermore, there is no recycling or environmental costs for this pretreatment method compared to mechanical comminution. However, the processing of

steam explosion destructs a part of the xylan in biomass, incompletely solubilizes of lignin-carbohydrate matrix, and generates inhibitory compounds that may affect the growth of microorganisms used in downstream processes (Mackie et al., 1985). Water can be used to wash the pretreated biomass to remove the inhibitory compounds (McMillan, 1994) with the cost of decreasing the overall saccharification yields. Previous studies indicate that this inhibition can be overcome by fermentation of the prehydrolysate prior to enzymatic hydrolysis (Tengborg et al., 2001).

2.3.2 Liquid hot water pretreatment

The goal of liquid hot water pretreatment of lignocellulosic biomass is to hydrate the more chemically resistive regions of cellulose in order to improve enzymatic hydrolysis to sugars (Weil et al., 1998). During the process, water is maintained in the liquid state by pressurizing at elevated temperatures (Bobleter, 1994; Kohlman et al., 1995; Allen et al., 1996). The liquid hydrolyzate can be fermented to ethanol (Lynd et al., 1996; van Walsum et al., 1996). The results of liquid hot water pretreatment of biomass for up to 15 min at temperatures of 200-230 ^{o}C showed that around 50% of the total biomass is dissolved in the process, with 4-22% of the cellulose, 35-60% of the lignin and all of the hemicellulose being removed (Mok and Antal, 1992, 1994). The pH of water at 200 ^{o}C is nearly 5.0 (Weil et al., 1998). Controlled pH, liquid hot water pretreatment of a 16% slurry of corn stover has been optimized for enzyme digestibility with the optimal conditions to be 190 ^{o}C for 15 min (Mosier et al., 2005). Industrial scale-up of pH-controlled liquid hot water pretreatment of corn fiber for ethanol production was found to be economically feasible (Mosier et al., 2005).

There are three types of reactors for the liquid hot water pretreatment step, including co-current, countercurrent, and flow through. Co-current pretreatment is used to heat a slurry of biomass and water to the desired temperature and kept at the pretreatment conditions for a controlled residence time. In counter-current pretreatments, water and lignocellulosic feedstock are moved in opposite directions inside the reactor. Flow-through pretreatment is designed to pass hot water that hydrolyzes and dissolves lignocellulosic components and carries them out of the reactor over a stationary bed of biomass (Mosier et al., 2005).

Neutralization and conditioning chemicals are not required in liquid hot water pretreatments due to no addition of acid. Despite the fact that size reduction of the biomass is not needed when cooked in water (Kohlman et al., 1995; Weil et al., 1997), liquid hot water pretreatment was demonstrated to be able to cause ultrastructural changes and formation of micron-sized pores that make the cellulose more accessible to enzymes (Zeng et al., 2007). The cleavage of *O*-acetyl and uronic acid substitutions from hemicellulose to generate acetic and other organic acids not only helps but also hinders the liquid hot water pretreatment. These acids can assist in reducing oligosaccharides, however, hemicellulose, in the acidic environment, may be degraded to aldehydes which are inhibitory to microbial fermentation (Palmqvist and Hahn-Hagerdal, 2000).

2.3.3 Ammonia pretreatment

In ammonia fiber/freeze explosion (AFEX) pretreatment, lignocellulosic materials are exposed to liquid ammonia at high temperature and pressure for a period of time after which the pressure is quickly reduced (Sun and Cheng, 2002). Herbaceous and agricultural residues

such as wheat straw, wheat chaff (Mes-Hartree et al., 1988), barley straw, corn stover, rice straw (Vlasenko et al., 1997), coastal Bermudagrass, switchgrass (Reshamwala et al., 1995; Alizadeh et al., 2005), bagasse (Holtzapple et al., 1991), and municipal solid waste (Holtzapple et al., 1992a) are well suited for AFEX. However, this method is moderately efficient for hardwoods, and not useful for softwoods (McMillan, 1994).

AFEX was found to not significantly degrade hemicellulose while steam explosion solubilized the hemicellulose (Mes-Hartree et al., 1988). AFEX pretreatment of Bermuda grass and bagasse was implemented to yield 90% hydrolysis of cellulose and hemicellulose (Holtzapple et al., 1991). In addition, lignin can react with aqueous ammonia and depolymerized, which leads to the cleavage of lignin-carbohydrate linkages in hardwood (Yoon et al., 1995) and agricultural residues (Iyer et al., 1996), for 14 min at 160-180 ^{o}C. Some modifications were attemped in the process to improve the extent of delignification and to actualize fractionation of biomass (Kim and Lee, 1996; Kim et al., 2002).

To make AFEX economical and protect the environment, a superheated ammonia vapor with a temperature up to 200 ^{o}C can be utilized to separate and recover the residual ammonia from the pretreated biomass (Holtzapple et al., 1992b). Two advantages of AFEX are that it does not form inhibitors for the downstream processes (Dale et al., 1984; Mes-Hartree et al., 1988), and it does not require reducing particle size (Holtzapple et al., 1990).

2.3.4 Dilute acid pretreatment

Considerable research attention has been paid to dilute acid (0.7-3.0%) pretreatment of lignocellulosic materials for ethanol production. In dilute acid pretreatment process, the acid

is mixed with the biomass in the vessels, heated up to over 100 ^{o}C. Two types of dilute acid pretreatment methods include low temperature less than 160 ^{o}C with batch process for high solids loading (10-40%) (Cahela et al., 1983; Esteghlalian et al., 1997) and high temperature greater than 160 ^{o}C with continuous-flow process for low solids loading (5-10%) (Brennan et al., 1986; Converse et al., 1989). Dilute sulfuric acid is most widely used in this process (Grohmann et al., 1985; Torget et al., 1992; Nguyen et al., 2000; Kim et al., 2000), while some other acids such as hydrochloric acid (Israilides et al., 1978; Goldstein et al., 1983; Goldstein and Easter, 1992), and phosphoric acid (Israilides et al., 1978) have also been studied for the pretreatment of lignocellulosic biomass.

Addition of sulfuric acid during pretreatment of biomass can remove hemicellulose to enhance digestibility of lignocellulose (Knappert et al., 1981; Brownell and Saddler, 1984; Converse and Grethlein, 1985). A wide range of biomass from hardwoods to herbaceous grasses and agricultural residues (Lee et al., 1978; Knappert et al., 1981; Grous et al., 1985; Torget et al., 1990, 1991, 1992) has been pretreated by acid for removal of hemicellulose. A kinetics was established to model the acid hydrolysis in which acid catalyzes breakdown of cellulose to glucose followed by HMF and other degradation products formation (Saeman, 1945), based on which the hydrolysis of lignocellulosic materials was described by modification (Kwarteng, 1983; Ladisch, 1989; Esteghlalian et al., 1997; Lee et al., 1999; Mosier et al., 2002). The yield of xylan to xylose conversion can be improved with the increase of sulfuric acid concentration and residence time (Sun and Cheng, 2005) in order to achieve favorable process economics.

Although the dilute sulfuric acid pretreatment enables the improvement of the cellulose hydrolysis, it has some limitations including the higher cost than steam explosion or AFEX, neutralization of pH before proceeding to fermentation, formation of degradation products, and release of natural fermentation inhibitors (Mosier et al., 2005). A highly dilute sulfuric acid (about 0.07%) pretreatment, flow-through sulfuric acid pretreatment, was used to hydrolyze cellulose followed by subsequent enzyme hydrolysis with up to 90% digestion (Torget et al., 1996, 1998, 1999).

2.3.5 Lime pretreatment

Compared to other pretreatment technologies, alkaline pretreatment is typically operated for longer residence time at lower temperature and pressure. Saponification of intermolecular ester bonds crosslinking xylan hemicellulose and other components is believed to be the mechanism of alkaline pretreatment (Sun and Cheng, 2002). The major effect of alkaline pretreatment is the delignification of lignocellulosic biomass, thus enhancing the reactivity of the remaining carbohydrates. Delignification kinetics of corn stover in lime pretreatment was studied to indicate that the activation energies for delignification in the oxidative lime pretreatment reactions are similar to the Kraft delignification of bagasse, but much less than in Kraft delignification of wood (Kim and Holtzapple, 2006). Alkaline pretreatments also remove acetyl and different kinds of uronic acid substitutions on hemicellulose, which increases the extent of enzymatic hydrolysis of cellulose and hemicellulose (Chang and Holtzapple, 2000). The lignin contents of the biomass influence the effect of alkaline pretreatment (Fan et al., 1987).

Lime pretreatment typically mixes the slurry of lime and water with the biomass and then stores the material in a pile for hours or weeks. Studies has been carried out on the lime pretreatment for optimizing pretreatment conditions: switchgrass, 100°C, 2 h (Chang et al., 1997); wheat straw/bagasse, 85°C/120°C, 3 h/1 h (Chang et al., 1998); corn stover, 100°C, 13 h (Karr and Holtzapple, 1998, 2000); poplar wood/ newspaper, 150°C/140°C, 6 h/3 h, 14-atm oxygen/7.1-atm oxygen (Chang et al., 2001). Adding air/oxygen to the reaction system can significantly improve the delignification of the biomass (Chang and Holtzapple, 2000). Chang et al. (2001) performed oxidative lime pretreatment of poplar wood at 150 °C for 6h with 78% removal of lignin and 71% improvement of the glucose yield from enzymatic hydrolysis. Lime (0.5g lime/g raw biomass) was used to pretreat corn stover in non-oxidative and oxidative conditions at 25ºC, 35ºC, 45ºC, and 55°C. The optimal condition was found to be 55°C for 4 weeks with aeration (Kim and Holtzapple, 2005).

The disadvantage of alkaline pretreatments is that some of the alkali is converted to irrecoverable salts or incorporated as salts into the biomass (Mosier et al., 2005). On the other hand, low reagent cost and safety, and the recovery of lime from water as insoluble calcium carbonate by reaction with carbon dioxide benefit the lime pretreatment method (Playne, 1984; Chang et al., 1997). Lime kiln technology can be used to convert calcium carbonate to lime (Chang et al., 1998). In addition to lime pretreatments, there are other alkaline pretreatments using sodium hydroxide, potassium hydroxide, and ammonium hydroxide among which sodium hydroxide has been investigated most (MacDonald et al., 1983; Fox et al., 1989; Soto et al., 1994; Sharmas et al., 2002).

2.4 HYDROLYSIS AND FERMENTATION PROCESSES

Cellulases enzymes, comprised of endoglucanase, exoglucanase and β-glucosidase, hydrolyze pretreated lignocellulosic materials to reducing sugars including glucose. Glucose is then fermented to ethanol by naturally occurring microorganisms such as *Saccharomyces cerevisiae*. Although hydrolysis and fermentation processes involve mature technologies relative to the pretreatment step, there are still many challenges that need to be further investigated in order to make the conversion of lignocellulosic biomass to ethanol more economically feasible. Research should be focused on the improvement of hydrolysis efficiency, and the development of co-fermentation of glucose and xylose.

2.4.1 Biomimetic hydrolysis of lignocellulose

Utilizing either cellulolytic enzymes or sulfuric acid to degrade cellulose into glucose h-as been examined over the years. The largest shortcoming to using sulfuric acid is that it easily degrades glucose during the hydrolysis process (Mosier et al., 2001). The degradation of glucose not only decreases the yield of ethanol from fermentable sugars but also produces inhibitory compounds including hydroxymethyl furfural, levulinic acid, and formic acid to microbial fermentation.

Dicarboxylic acids have the potential to act as catalytic domains in cellulolytic enzymes for developing organic macromolecules that mimic the function of enzymes in the hydrolysis of lignocellulose. Dilute maleic acid was used to hydrolyze cellulose as a comparison with sulfuric acid hydrolysis to draw a conclusion that maleic acid does not catalyze the degradation of glucose, thus yielding a higher amount of glucose (Mosier et al., 2001, 2002).

With regard to the efficient and economical process for hydrolysis of lignocellulose, the biomimetic approach with dicarboxylic acid catalyst mimicking the catalytic sites in natural enzymes has been studies for hemicellulose hydrolysis in corn stover. Under optimized reaction conditions, maleic acid hydrolysis was found to render minimal xylose degradation, while sulfuric acid resulted in 3-10% times more xylose degradation (Lu and Mosier, 2007). Furthermore, about 95% monomeric xylose was produced with trace contents of furfural by using optimal maleic acid hydrolysis of hemicellulose (Lu and Mosier, 2007).

2.4.2 Co-fermentation of glucose and xylose

Genetically engineered *Saccharomyces* yeasts that are capable of fermenting xylose has been being developed by worldwide researchers since 1980 (Ho et al., 1999). The first generation of genetically engineered *Saccharomyces* yeasts was successfully developed to coferment both glucose and xylose to ethanol simultaneously in 1993 (Ho et al., 1999). Two years later, another breakthrough was achieved by creating super-stable genetically engineered glucose-xylose-cofermenting *Saccharomyces* yeasts which contain multiple copies of the three xylose-metabolizing genes including a xylose reductase gene, a xylitol dehydrogenase gene, and a xylulokinase gene (Ho et al., 1999; Sedlak and Ho, 2004; Ho et al., 1998). As genetically engineered yeasts become more and more stable and efficient in cofermenting glucose and xylose, the bioconversion of lignocellulosic biomass to ethanol approaches the commercialization (Toon et al., 1997).

In order to improve the economics of ethanol yield from lignocellulose, glucose, xylose, and galactose need to be fermented by genetically engineered yeasts. The ethanol production

merely from xylose by using a xylose-fermenting yeast mutant is greater under aerobic than fermentative conditions (Gong et al., 1981), while anaerobic conditions yield higher ethanol production from all sugars including glucose, xylose, arabinose, and galactose (Moniruzzaman et al., 1997). From a mixture of glucose (31g/l), xylose (15.2g/l), arabinose (10.5g/l), and galactose (2g/l), a recombinant *Saccharomyces* yeast strain produced 22g/l ethanol, equivalent to 90% of the theoretical yield (excluding the arabinose in the calculation since it is not fermented), in 24 h (Moniruzzaman et al., 1997).

The genetically engineered yeasts have some extraordinary characteristics that the absence of xylose does not affect the synthesis of xylose-metabolizing enzymes directed by the cloned genes, and the presence of glucose in the medium does not inhibit the synthesis (Ho et al., 1998). Ethanol inhibition has more impact on xylose fermentation than on glucose fermentation (Krishnan et al., 1999). Compared to glucose, xylose is digested less efficiently by glucose-xylose-cofermenting yeasts, thus, xylose fermentation should be optimized by ways through carefully analyzing gene expression during co-fermentation process (Sedlak et al., 2003). One of the factors limiting the fermentation of xylose was found to be the transport of xylose in the fermentation system (Sedlak and Ho, 2004). Integrating all the above aspects such as substrate inhibition, product inhibition, and inoculum size into kinetic studies enable developing a fermentation model to improve the ethanol yield (Krishnan et al., 1999).

2.4.3 Consolidated bioprocessing of lignocellulose

The common lignocellulosic biomass processing schemes involving hydrolysis and fer-

mentation contain four biologically mediated conversions: (1) the production of cellulases and hemicellulases enzymes; (2) the hydrolysis of carbohydrates in pretreated biomass to reducing sugars; (3) the fermentation of hexoses (glucose, galactose dn mannose); (4) the fermentation of pentoses (xylose and arabinose) (Lynd et al., 2005). These four converseon processes can be integrated in a single step to form an alternative approach called consolidated bioprocessing (CBP). There are two strategies involved in the development of CBP-enabling microorganisms. One of them is to engineer naturally occurring cellulolytic microbes to improve product-related properties such as yield and titer; the other method is to engineer non-cellulolytic microorganisms with high product yields and titers to creat a heterologous cellulases system (Lynd et al., 2005). By synergizing enzyme and microbe, higher rates (2.7- to 4.7-fold) of cellulose hydrolysis were obtained for growing cultures of *Clostridium thermocellum* than purified cellulasess prepared from this organism (Lu et al., 2006). Therefore, the microbial conversion of lignocellulosic biomass to ethanol can be realized without adding saccharolytic enzymes.

2.5 CONCLUSIONS

Ethanol produced from lignocellulosic materials has a great potential to replace the current petroleum-based gasoline for transportation. The topics reviewed above include the pretreatment efficiency on the subsequent hydrolysis and the development of cost-effective hydrolysis and fermentation processes. The hydrolysis efficiency of lignocellulose is affected by the substrate reactivity and the enzyme activity (Ladisch et al., 1983). Effective pretreatment technologies are able to improve the substrate reactivity by removing lignin,

decreasing cellulose crystallinity and increasing accessible surface area. Lignin content and crystallinity indices (CrIs) are considered to have the greatest impact on the lignocellulosic biomass digestibility (Chang and Holtzapple, 2000). Promising pretreatment technologies need to be modeled by understanding the fundamentals of the chemical and physical mechanisms occurring during pretreatment as well as the effect of the change of the chemical composition and physico-chemcial structure of lignocellulose on the digestibility of cellulose and hemicellulose (Mosier et al., 2005). Biomimetic hydrolysis is another potential technology, compared to enzymatic hydrolysis, for converting carbohydrates in pretreated lignocelluloisc biomass to reducing sugars. Xylose utilization as a substrate by genetically engineered microorganisms to produce ethanol contributes to the economical feasibility of conversion of lignocellulosic materials to ethanol. Simultaneous saccharification and fermentation (SSF) has been applied to lower the product inhibition to the hydrolysis. With the development of new technologies in pretreatment, hydrolysis and fermentation, the consolidated bioprocessing of lignocellulosic biomass is capable of integrating two or more steps into a single step, therefore, commercializing the ethanol production from lignocellulose. Three major problems that need to be further investigated include developing cost-effective pretreatment technologies, further reducing the cost of enzymes, and improving the ability of microorganisms for co-fermentation of hexoses and pentoses.

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CHAPTER 3

HIGH TEMPERATURE SODIUM HYDROXIDE PRETREATMENT OF COASTAL BERMUDAGRASS

3.1 ABSTRACT

Lignocellulosic materials are regarded as an alternative energy source for bioethanol production to reduce our reliance on fossil fuels. Pretreatment is important for improving the enzymatic digestibility of lignocelluloses to increase the yield of fermentable sugars. Sodium hydroxide pretreatment of coastal bermudagrass for enhanced reducing sugars recovery was investigated in this study. The effect of NaOH pretreatment at 121°C using 1%, 2% and 3% (w/v) NaOH for 15, 30, 60 and 90 minutes was evaluated first. Lower NaOH concentrations (0.5% and 0.75%) and lower temperatures (50, 80 and 100°C) were then examined. Total lignin reduction, total reducing sugars production, glucose and xylose yields were analyzed. NaOH pretreatment can remove up to 86% of lignin in the coastal bermudagrass. The optimal NaOH pretreatment conditions at 121°C for total reducing sugars production as well as glucose and xylose production were 15 min and 0.75% NaOH. The highest reducing sugars yield reached up to 77% of theoretical maximum for NaOH pretreatment at 121°C. Under the recommended conditions, glucan and xylan conversion rates were approximately 91% and 65% respectively.

Keywords: Sodium hydroxide; Pretreatment; Reducing sugars; Glucose; Xylose; Costal bermudagrass

3.2 INTRODUCTION

Current fuel ethanol production in the United States is mainly based on the fermentation of glucose derived from corn starch, which competes with the corn-based food and feed production. On the other hand, there are plentiful lignocellulosic materials such as crop residues, grasses, sawdust, solid animal waste and wood chips that can be utilized to substitute the equivalent of 40% of gasoline in the current US market (Wheals et al., 1999). Lignocellulosic materials can capture CO_2 during growth so that their combustion does not generate net CO_2 (Klass, 1998). Therefore, lignocellulosic materials are considered as potential feedstocks for a large amount of low-cost ethanol production.

There are limiting factors to the maximum possible efficiency of the conversion of lignocellulosic materials to ethanol. Enzymatic hydrolysis is hindered by the following substrate-related factors: cellulose contains highly resistant crystalline structure, lignin and hemicellulose surrounding cellulose form a physical barrier, and sites available for enzymatic attacks are limited (Kim et al., 2001). Pretreatment, an important tool for practical lignocellulose conversion processes, is required to alter the structure of lignocellulosic biomass to make cellulose more accessible to the enzymes that are responsible for the conversion of polymeric carbohydrates to fermentable monosaccharides (Moiser et al., 2005). Pretreatment methods can be physical, chemical, physico-chemical, and biological processes.

Of the promising pretreatment technologies, alkaline pretreatment has received much attention. Saponification of intermolecular ester bonds crosslinking hemicellulose and other components (cellulose and lignin) is believed to be the mechanism of alkaline pretreatment

(Sun and Cheng, 2002). Breaking up the crosslinks among lignin, hemicelluloses and cellulose would increase the porosity of lignocellulosic biomass (Tarkow and Feist, 1969). The major effect of alkaline pretreatment is the delignification of lignocellulosic biomass, thus enhancing the reactivity of the remaining carbohydrates. The lignin content of the biomass influences the effect of alkaline pretreatment (Fan et al., 1987). Alkaline pretreatments also remove acetyl and different kinds of uronic acid substitutions on hemicellulose, which improves the extent of enzymatic hydrolysis of cellulose and hemicellulose (Chang and Holtzapple, 2000). Sodium hydroxide effectively enhances lignocellulose digestibility by increasing internal surface area, decreasing the degree of polymerization and the crystallinity of cellulose, and separating structural linkages between lignin and carbohydrates (Fan et al., 1987). NaOH pretreatment increased the digestibility of hardwood from 14% to 55% as the lignin content decreased from 24-55% to 20% (Millet et al, 1976). Corn stover was pretreated with 2% NaOH for 5 min at 150°C and 52% sugar conversion was achieved compared to only 20% for untreated sample (Macdonald et al., 1983). A cellulose solvent composed of a ferric sodium tartrate complex in 1.5N NaOH solution and a 1.5N NaOH solution alone were used to treat the corn residue prepared by agitating the biomass in 5% H_2SO_4 for 4 hours at 90°C (Hamilton et al., 1984). Sodium hydroxide pretreatment was also effective for enhancing the digestibility of wheat straw (Bjerre et al., 1996).

To avoid environmental pollution caused by swine wastewater, many hog farmers grow coastal bermudagrass for nitrogen and phosphorus removal to prevent potential pollution of

these nutrients to the nearby watershed. The existence of a cropping system makes coastal bermudagrass a potential lignocellulosic feedstock for bioethanol production. Furthermore, the harvested coastal bermudagrass is usually given away or sold at very low price as animal feed. Therefore, there is a great interest to investigate the conversion of coastal bermudagrass into ethanol. The purpose of this study was to investigate the impacts of sodium hydroxide pretreatment on subsequent hydrolysis and fermentation of coastal bermudagrass for bioethanol production. This research can provide important information on the commercial utilization of coastal bermudagrass for large-scale ethanol production.

3.3 MATERIALS AND METHODS

3.3.1 Biomass preparation

Air-dried coastal bermudagrass (harvested in June, 2007) was obtained from North Carolina State University Central Crops Research Station in Clayton, NC. The biomass was size reduced to pass a 2-mm sieve using a Thomas Wiley Laboratory Mill (model no. 4) and stored in sealed plastic bags at room temperature until use for characterization and pretreatment.

3.3.2 Pretreatment

Pretreatments were done in an autoclave (for pretreatments at 121°C) or a water bath (for pretreatments up to 100°C). Biomass samples were immersed in dilute sodium hydroxide solutions (solid to liquid ratio of 1:10) in sealed serum bottles. After pretreatment, the biomass was washed with 200 ml of deionized (DI) water and the solid residues were stored at 4°C for enzymatic hydrolysis.

Four sets of experiments were conducted. The first set of experiments evaluated the effect of pretreatment at 121°C using 1%, 2% and 3% (w/v) NaOH for 15, 30, 60 and 90 minutes. The second set examined pretreatment at 121°C with lower NaOH concentrations (0.5% and 0.75%) for 15 and 30 minutes. The last two sets were conducted at 50, 80 and 100°C with the optimal NaOH concentrations and pretreatment times determined for maximized total reducing sugars production and maximized glucose and xylose production, respectively, obtained at 121°C.

3.3.3 Enzymatic hydrolysis

Enzymatic hydrolysis was carried out in 250 ml Erlenmeyer flasks in a controlled environmental incubator shaker set at 55°C and 150 rpm. One g (dry basis) of pretreated biomass (untreated biomass as a control) was immersed in 0.05 M sodium citrate buffer to maintain a pH of 4.8 with the total liquid volume of 30 ml. Prior to hydrolysis, activity of cellulases and cellobiase was determined to be 76.44 FPU/ml and 283.14 CBU/ml respectively (Ghose, 1987). The dosage of cellulases and cellobiase was 40 FPU and 70 CBU per gram of dry biomass. Sodium azide (0.3% (w/v)) was added to the hydrolysis mixture to inhibit microbial growth. The hydrolysis was carried out for 72 hours after which the hydrolyzate was centrifuged and the supernatant was stored at -20°C for sugar analysis.

3.3.4 Analytical methods

Moisture content and ash content of the biomass were determined based on the NREL laboratory procedures (Sluiter, 2005). The biomass was analyzed for extractives with 2:1 toluene-ethanol mixture in a Soxhlet apparatus with a reflux time of 24 hours (Silverstein,

2004). The extractive-free biomass and the pretreated biomass were both analyzed for lignin using a two-stage sulfuric acid hydrolysis procedure recommended by the National Renewable Energy Laboratory (Sluiter, 2006). Total reducing sugars in the enzymatic hydrolyzates were determined by the DNS (dinitrosalicylic acid) method using glucose as the standard (Miller, 1959). Monosaccharides (glucose and xylose) from composition analysis and in the hydrolyzates were measured with a high performance liquid chromatography (HPLC) system. The HPLC system was equipped with a Bio-Rad Aminex HPX-87P column (300mm × 7.8mm) tailored for analysis of hexoses and pentoses in lignocellulosic materials, a Bio-Rad Micro-Guard column, a thermostatted autosampler, a quaternary pump, and a refractive index detector. The standards used were monomeric sugars at concentrations of 0.5, 2.0, 5.0, 7.5, 10.0 g/L. The analytical column was operated at 80°C with Milli-Q water (0.2 µm filtered) 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. To allow for late-eluting compounds to come off the column, a post-run time of 25 min was included between injections.

3.3.5 Statistical analysis

Experimental data were statistically analyzed using the GLM procedure in SAS 8.02 software. Significant ($P<0.05$) and nonsignificant differences between treatments were evaluated by Tukey adjustment for comparisons. All treatments were conducted in triplicate.

3.4 RESULTS AND DISCUSSION

3.4.1 Biomass characterization

The coastal bermudagrass was analyzed for carbohydrates, lignin, ash and extractives. T-

he weight percentages of each component per gram of dry biomass are presented in Table 3.1. Glucan was the major component followed by xylan and acid insoluble lignin. There were o-

Table 3.1. Chemical composition of coastal bermudagrass.

Component	wt%, dry basis in biomass
Glucan	25.59
Xylan	15.88
Arabinan	1.95
Galactan	1.46
Acid insoluble lignin	15.37
Acid soluble lignin	3.96
Extractives	4.17
Ash	6.60

nly a small amount of arabinan and galactan in coastal bermudagrass. No mannan was detected in the biomass. Xylan was the main component of hemicellulose in coastal bermudagrass. The chemical composition of the coastal bermudagrass in this study was slightly different from that reported by Sun and Cheng (2005). Cultivation and harvest time would affect the chemical composition of the biomass. In addition, the carbohydrates analysis reported here was carried out on extractive free biomass while the previous report by Sun and Cheng (2005) used biomass with extractives for sugar analysis.

3.4.2 Total reducing sugars yields

The major effect of sodium hydroxide pretreatment of lignocellulosic materials is to remove the lignin in the biomass. Lignin analysis of the solid residues after pretreatment was examined to evaluate the effects of pretreatment conditions on lignin removal and how the lignin removal relates with total reducing sugars production after enzymatic hydrolysis. Lignin reduction increased with the increase of sodium hydroxide concentration from 1% to

3% (w/v) and the increase of pretreatment time from 15 min to 90 min (Fig. 3.1a). Pretreatment time and sodium hydroxide concentration both affected total lignin reduction significantly. However, the effect of pretreatment time on removing total lignin does not depend on the sodium hydroxide concentration. Pretreatment time of 30 min was sufficiently long to achieve the maximum total lignin reduction no matter what sodium hydroxide conce-

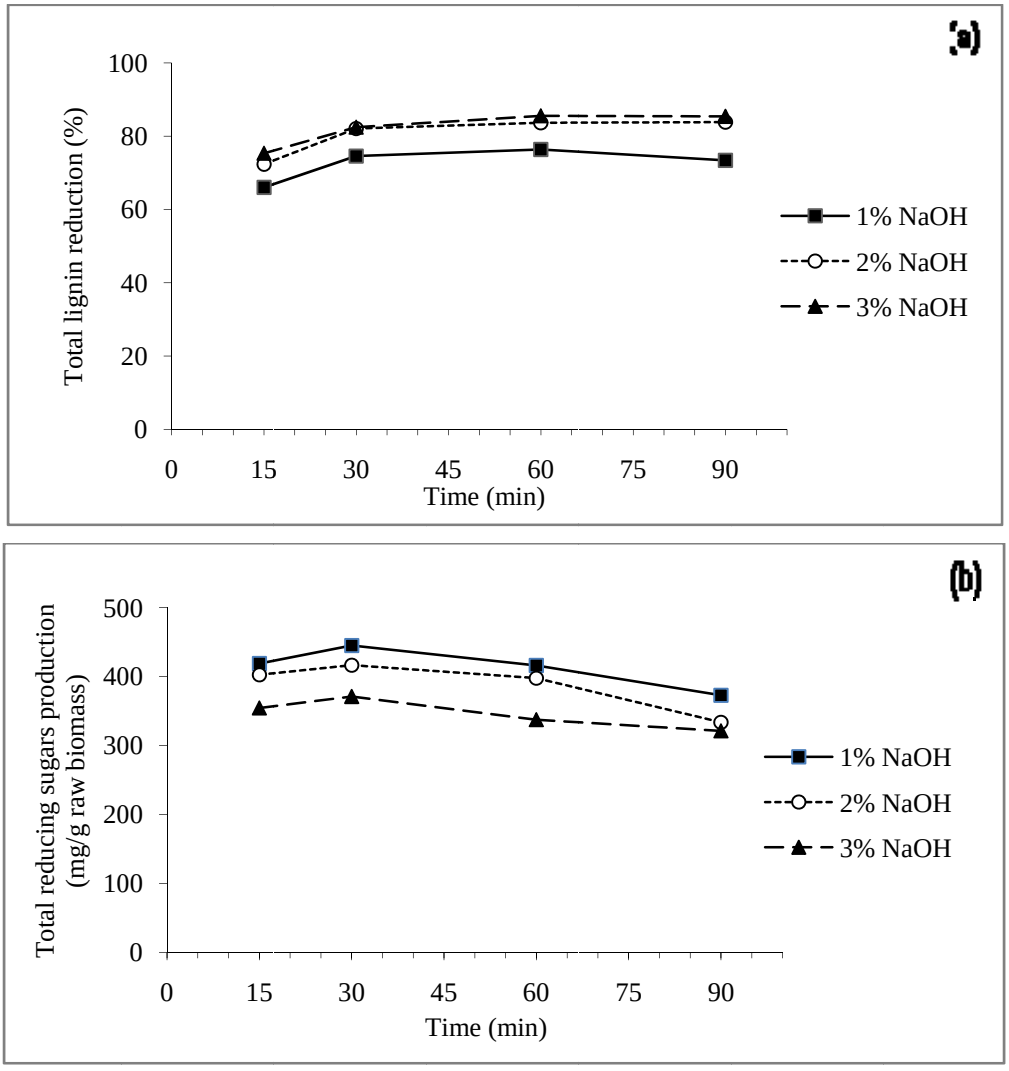

Fig. 3.1. Total lignin reduction (a) and total reducing sugars production (b) from pretreated coastal bermudagrass with NaOH of 1%, 2% and 3% for 15, 30, 60 and 90 min at 121°C.

ntration was. Further extending pretreatment time longer than 30 min did not significantly improve total lignin removal. There was no significant difference in lignin removal between 2% NaOH and 3% NaOH. As the results from total lignin reduction for sodium hydroxide pretreatment indicated, sodium hydroxide pretreatment can significantly remove lignin in lignocellulosic materials.

As the total lignin reduction increased, the total reducing sugars production was enhanced (Fig. 3.1). Pretreatment with NaOH of 1% and 2% yielded significantly higher total reducing sugars than 3% (Fig. 3.1b). Higher NaOH concentrations gave higher total solid loss although they had higher lignin reduction, thus leading to less reducing sugars yield. Extending pretreatment time beyond 30 min reduced total reducing sugars production because of high solid loss. Based on this experiment, the highest total reducing sugars yield (approximately 77% of the theoretical maximum) was obtained with 1% NaOH for a pretreatment time of 30min. The results above prompted the use of lower NaOH concentrations at 121°C. Decreasing sodium hydroxide concentration from 1% to 0.5% significantly reduced total lignin removal (Fig. 3.2a), which could have a negative impact on total reducing sugars yield. There was a significant difference in lignin reduction between pretreatment time of 15 min and 30 min (Fig. 3.1a and Fig. 3.2a). A similar trend as that in lignin reduction was noticed in reducing sugars production for lower sodium hydroxide concentrations (Fig. 3.2b) and no statistical difference was observed among 0.75%, 1% NaOH for pretreatments at 15 min and 30 min. Therefore, 0.75% NaOH with a pretreatment time of 15 min are the optimal conditions for NaOH pretreatment of coastal bermudagrass at

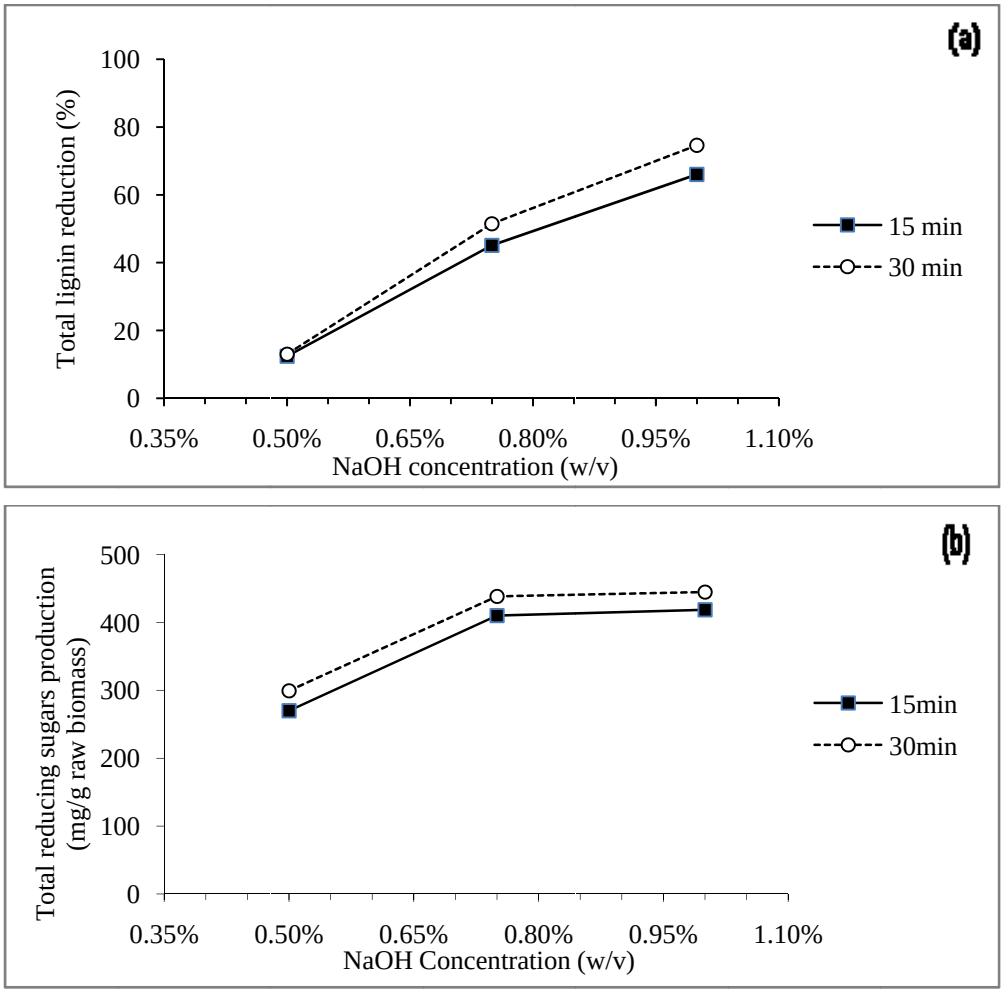

Fig. 3.2. Total lignin reduction (a) and total reducing sugars production (b) from pretreated coastal bermudagrass with NaOH of 0.5%, 0.75% and 1% for 15 and 30 min at 121°C.

121°C. Because higher lignin reduction correlates with higher solid loss, the increased rate of reducing sugars production was lower than that of lignin removal. The results indicate that high lignin reduction does not guarantee improved reducing sugars yield because of high solid loss.

Lower temperature pretreatments with 0.75% NaOH at 15 min were examined for the purpose of cost savings. Total lignin removal was tremendously reduced by decreasing the p-

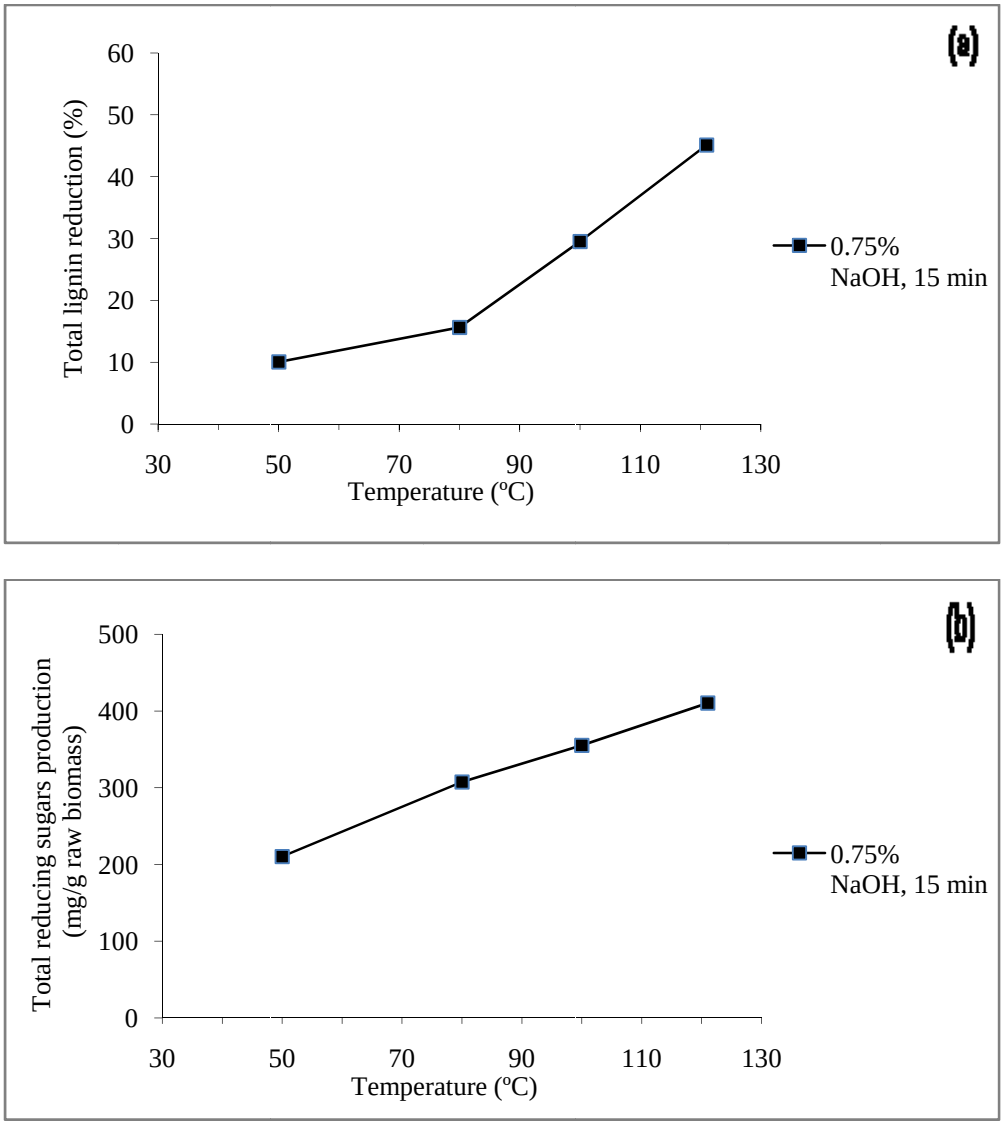

Fig. 3.3. Total lignin reduction (a) and total reducing sugars production (b) from pretreated coastal bermudagrass with NaOH of 0.75% for 15 min at 50°C, 80°C, 100°C and 121°C.

retreatment temperature from 121°C to 50°C (Fig. 3.3a). A significantly smaller amount of intermolecular ester bonds was disrupted at temperatures lower than 121°C because of lower reaction rate at lower temperatures, therefore, fewer lignin was separated from hemicelluloses and cellulose. As a result of more lignin remaining in the biomass at lower te-

mperature, total reducing sugars yield was diminished from 70.8% of the theoretical maximum at 121°C to 36.3% at 50°C (Fig. 3.3b). The results revealed that the existence of lignin could inhibit the enzymatic hydrolysis of polysaccharides to monosaccharides. It is concluded that lower temperatures for NaOH pretreatment was not favorable to enhanced sugar yield because the crosslinkages between lignin and carbohydrates were not disrupted sufficiently to reach a high sugar production.

3.4.3 Monosaccharides yields

Hexoses are easily fermented into ethanol, however, pentoses are still difficult to be fermented by microorganisms for the commercial ethanol production. In order to make ethanol production from lignocellulosic materials economically feasible, both hexose and pentose in lignocellulose need to be utilized by microorganisms for ethanol production.

Analysis of monomeric reducing sugars after hydrolysis reveals that both pretreatment time and NaOH concentration have impacts on glucose and xylose yields (Fig. 3.4). Because of the fairly small amount of galactose and arabinose in the hydrolyzates, glucose and xylose production were considered for optimizing pretreatment conditions based on monomeric reducing sugars yield. As pretreatment time and NaOH concentration increased, glucose yield slightly decreased (Fig. 3.4a). Statistical analysis indicated that there was no significant difference in glucose production between 2% and 3% NaOH pretreatments. Extending pretreatment time beyond 30 min did not significantly affect the glucose yield. By comparing total reducing sugars production in Fig. 3.1b and xylose yield in Fig. 3.4b, it is noted that the amount of reducing sugars released after hydrolysis was highly associated with the xylose yi-

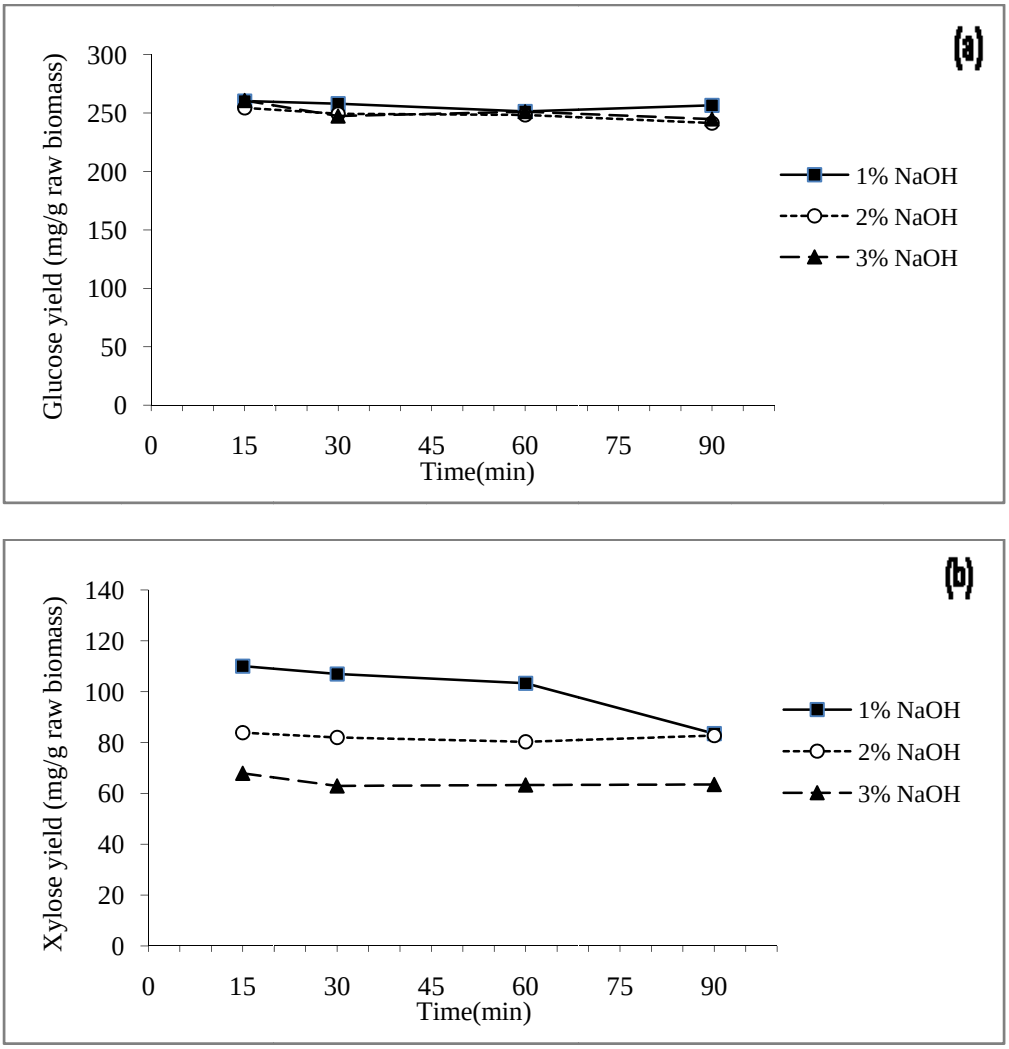

Fig. 3.4. Monosaccharides production from pretreated coastal bermudagrass with NaOH of 1%, 2% and 3% for 15, 30, 60 and 90 min at 121°C.
(a) Glucose yield; (b) Xylose yield.

eld. With the increase of NaOH concentration from 1% to 3%, the xylose yield was significantly reduced while the glucose yield was fairly constant, thus leading to the decrease of total reducing sugars production. The same trend as in total reducing sugars yield when conducting pretreatments for over 30 min was observed in xylose yield, which further indicated the correlation between total reducing sugars production and xylose released after

hydrolysis. According to the total lignin reduction and total reducing sugars productionn shown in Fig. 3.1, it was noticed that the increase of lignin removal enhanced total reducing sugars yield for the increase of pretreatment time from 15 min to 30 min. However, the results from monosaccharides yields showed that the glucose and xylose yields were not improved although lignin reduction increased. As mentioned before, higher NaOH concentra-

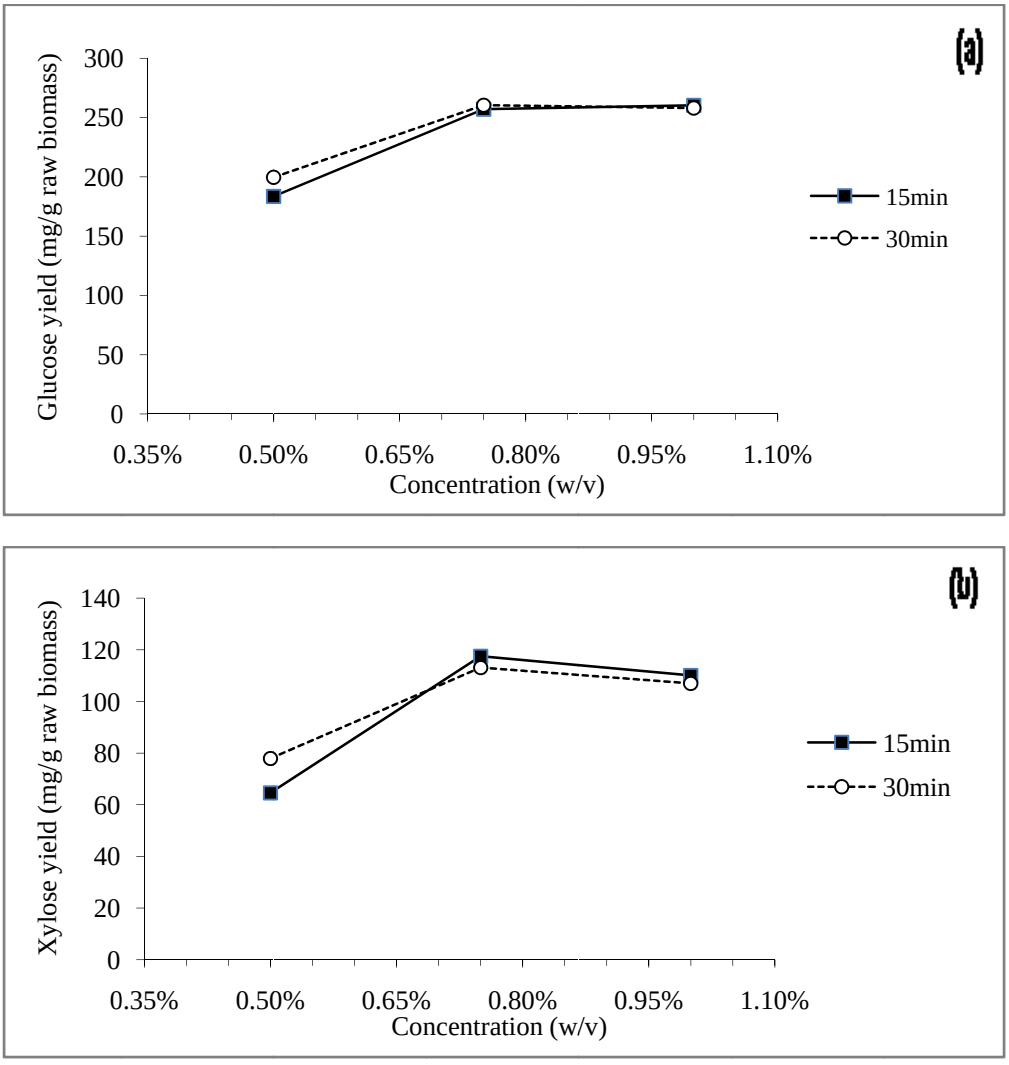

Fig. 3.5. Monosaccharides production from pretreated coastal bermudagrass with NaOH of 0.5%, 0.75% and 1% for 15, 30 min at 121°C.
(a) Glucose yield; (b) Xylose yield.

tions and longer pretreatment times resulted in higher solid loss, consequently, lower reducing sugars were produced. The same trend was noticed in xylose yield but not in glucose yield, which implied that lignin removal and xylose degradation after pretreatment account for the major part of total solid loss. Therefore, it was proven that hemicellulose is easier to be degraded than cellulose during pretreatment.

The above results showed that for NaOH pretreatment with 1%, 2% and 3% for 15, 30, 60 and 90 min at 121°C, 1% NaOH with pretreatment time of 15 min is good enough for maximized glucose and xylose production. The pretreatment with 0.75% NaOH generated statistically similar glucose and xylose yields compared to the pretreatment with 1% NaOH for both 15 and 30 min at 121°C, whereas glucose and xylose production for the pretreatment with 0.5% NaOH were significantly lower than that obtained for the pretreatment with 0.75% and 1% NaOH (Fig. 3.5). Based on these results, optimal pretreatment conditions at 121°C for glucose and xylose production are 15 min and 0.75% NaOH.

The previous results showed that pretreatments at lower temperatures reduced total reducing sugars production after hydrolysis because of less lignin removal. Much smaller amounts of glucose and xylose were released at lower pretreatment temperatures with the optimal pretreatment conditions (0.75% NaOH, 15 min) obtained at 121°C for maximized glucose and xylose yields (Fig. 3.6). Glucan conversion rate was reduced from 90.4% at 121°C to 58.8% at 50°C, while xylan conversion rate decreased from 65.1% to 28.2%. As mentioned before, the crosslinkages among lignin, cellulose and hemicellulose were not sufficiently broken up at lower temperatures to reach a high sugar yield, which was further e-

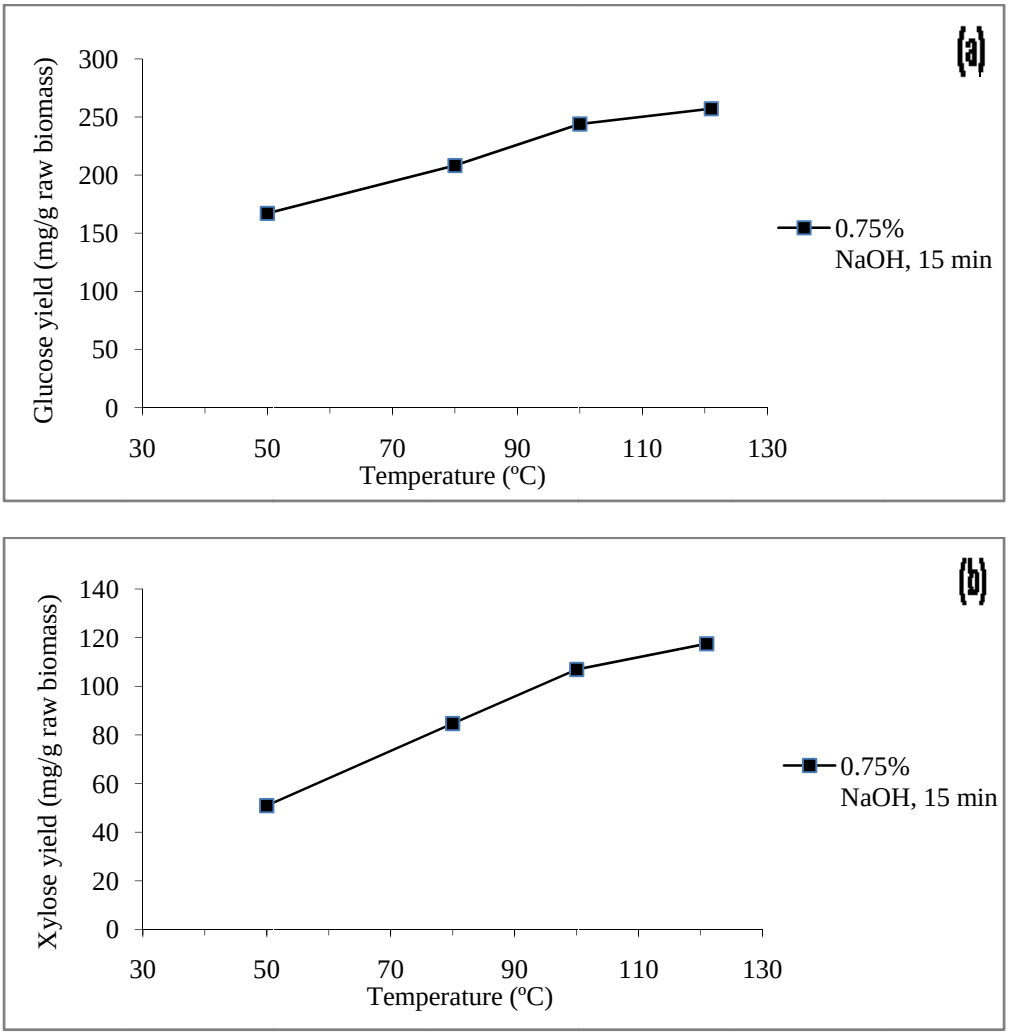

Fig. 3.6. Monosaccharides production from pretreated coastal bermudagrass with NaOH of 1% for 30 min at 50°C, 80°C, 100°C and 121°C.
(a) Glucose yield; (b) Xylose yield.

xplained by the results that glucose and xylose conversion rates were greatly lessened at temperatures lower than 121°C. During NaOH pretreatment, cellulose was more difficult to degrade than hemicellulose so that hemicellulose was easier to be lost in the prehydrolyzate than cellulose, which might contribute to the fact that glucan conversion rate was much higher than xylan conversion rate.

The glucan conversion rate was tremendously lower at 0.5% NaOH than other higher NaOH concentrations. There was no significant difference in glucan conversion rate between 2% and 3% NaOH. The conversion of glucan to glucose did not vary significantly by increasing the pretreatment time from 30 min to 90 min. The xylan conversion rate significantly decreased with the increase of NaOH concentration from 1% to 3%, which was

Table 3.2. Glucan conversion rate after 72-h enzymatic hydrolysis of NaOH pretreated coastal bermudagrass.

NaOH concentration (%)	Time (min)	Temperature (°C)	Glucan conversion rate (%)
0.5	15	121	64.56
0.75	15	50/80/100/121	58.79/73.26/85.77/90.43
1	15	121	91.56
2	15	121	89.51
3	15	121	91.68
0.5	30	121	70.19
0.75	30	121	91.61
1	30	121	90.73
2	30	121	87.72
3	30	121	87.02
0.5	60	121	N/A
0.75	60	121	N/A
1	60	121	88.43
2	60	121	87.37
3	60	121	88.36
0.5	90	121	N/A
0.75	90	121	N/A
1	90	121	90.27
2	90	121	84.91
3	90	121	86.12
Control			31.22

Table 3.3. Xylan conversion rate after 72-h enzymatic hydrolysis of NaOH pretreated coastal bermudagrass.

NaOH concentration (%)	Time (min)	Temperature (°C)	Xylan conversion rate (%)
0.5	15	121	35.79
0.75	15	50/80/100/121	28.19/46.89/59.22/65.11
1	15	121	60.98
2	15	121	46.45
3	15	121	37.61
0.5	30	121	43.18
0.75	30	121	62.66
1	30	121	59.27
2	30	121	45.41
3	30	121	34.85
0.5	60	121	N/A
0.75	60	121	N/A
1	60	121	57.24
2	60	121	44.47
3	60	121	35.06
0.5	90	121	N/A
0.75	90	121	N/A
1	90	121	46.25
2	90	121	45.84
3	90	121	35.16
Control			6.73

similar to the variation of total reducing sugars yield from 1% to 3%. Further reducing concentration to 0.75% did not impair the xylose yield, but NaOH concentration of 0.5% greatly reduced the xylan conversion rate from approximately 60% to 39%. All the conversion rates for glucan and xylan were listed in Table 3.2 and Table 3.3. The maximum glucan and xylan conversion rates could reach up to approximately 92% and 65%

respectively, while the non-pretreated control sample showed much lower conversion rates of roughly 31% for glucan and 7% for xylan. Based on the results above, it is indicated that sodium hydroxide pretreatment at 121°C significantly improved the digestibility of coastal bermudagrass for sugars recovery.

3.5 CONCLUSIONS

The efficiency of sodium hydroxide pretreatment of coastal bermudagrass for bioethanol production was evaluated in this study. Dilute sodium hydroxide pretreatment at high temperature (121°C) was effective in removing lignin from the biomass. Glucose and xylose were the major components in total reducing sugars produced after hydrolysis with a fairly small amount of galactose and arabinose. The optimal NaOH pretreatment conditions at 121°C for total reducing sugars production as well as glucose and xylose yields are 15 min and 0.75% NaOH. The highest reducing sugars yield can reach up to 77% of theoretical maximum for sodium hydroxide pretreatment at 121°C. Approximately 91% of glucan and 65% of xylan from coastal bermudagrass were converted into glucose and xylose when coastal bermudagrass was treated with 0.75% NaOH for 15 min at 121°C. Pretreatments with temperature below 121°C did not perform as efficiently as 121°C pretreatment on reducing sugars production as well as glucose and xylose yields. The total lignin reduction of 60-80% was observed at 121°C, while lower temperature pretreatments are not favorable to lignin degradation. Removal of lignin from the biomass did facilitate the digestibility of polymeric carbohydrates, however, higher lignin reduction means more solid loss which might cause the decrease of reducing sugars yield. The change of total reducing sugars production with

the pretreatment severity had the same trend as the xylan conversion rate, which indicated that xylan conversion rate is more sensitively affected by the variation of NaOH pretreatment conditions than glucan conversion rate. For future study, the filtrate after NaOH pretreatment is necessary for composition analysis to understand how much carbohydrate is degraded during pretreatment. Enzyme dosing will be optimized for economical ethanol production.

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CHAPTER 4

LIME PRETREATMENT OF COASTAL BERMUDAGRASS

4.1 ABSTRACT

The steady increase of energy consumption along with current dependence on crude oil to satisfy energy demands, calls for renewable energy sources development all over the world. Coastal bermudagrass is regarded as a potential lignocellulosic feedstock for bioethanol production in the southeast of the United States. In this study, coastal bermudagrass was pretreated with various lime loadings (0.02, 0.05, 0.08, 0.10, 0.15, 0.20 g/g dry biomass) at different temperatures (room temperature, 50, 80, 100, and 121 ^{o}C) for a range of residence times. During pretreatment, 10-20% of lignin was removed. After enzymatic hydrolysis with excessive enzyme doses, the efficiency of pretreatment factors such as temperature, residence time and lime loading on the biomass digestibility was examined. The recommended condition is 100 ^{o}C for 15 min with a lime loading of 0.1 g/g dry biomass. Under the optimal condition, the total reducing sugars production was 78% of the theoretical maximum which is over two times that of untreated biomass, with 87.4% of glucan and 67.5% of xylan converted into glucose and xylose respectively.

Keywords: Lime; Pretreatment; Reducing sugars; Glucose; Xylose; Costal bermudagrass

4.2 INTRODUCTION

Ethanol, as a transportation fuel, has the potential to replace gasoline use for the reason that the use of fuel ethanol can alleviate the dependency on fossil fuel and reduce net CO_2 emissions and global warming (Chang et al., 2001). The most widely commercialized conversion process for ethanol production in the United States is starch-based processing technology in which corn is used as the feedstock. Continued increase in ethanol production by using corn-based technology may not be sustainable because of the low density of biomass and limited agricultural land needed for food and feed production (Chen et al., 2007; Sun and Cheng, 2002). Enormous amounts of renewable biomass are available for fuel ethanol production in addition to corn.

Lignocellulose, the most abundant biomass, has been considered as a potentially inexpensive feedstock for ethanol production (Klyosov, 1986; Duff and Murray, 1996). It was projected that the equivalent of 40% of the US gasoline consumption could be replaced by converting agricultural, forest and municipal wastes alone to ethanol, and the rest could be substituted by growing energy crops (Lynd, 1991). A recently signed act requires the use of 16 billion gallons of ethanol produced from cellulosic biomass annually by 2022 (RFA, 2008). Therefore, there is a great interest to investigate cost-effective conversion processes for cellulosic ethanol production.

The digestibility of lignocellulosic biomass during enzymatic hydrolysis is hindered by its structural features such as cellulose crystallinity, lignin content, hemicelluloses acetylation, and inaccessible surface area (Kong et al. 1992). Pretreatments are essential to enhance the

susceptibility of lignocellulose to enzymatic hydrolysis by removing lignin and hemicellulose, reducing cellulose crystallinity, and increasing the porosity of the materials. Extensive research has been done on various pretreatment methods such as steam explosion, liquid hot water, ammonia, dilute acid, and alkaline pretreatments (Avellar and Glasser, 1998; Weil et al., 1998; Reshamwala et al., 1995; Sun and Cheng, 2005; Fan et al., 1987; Chang et al., 1997). The most commonly used alkalis are sodium hydroxide (NaOH), ammonia (NH_3), and lime ($Ca(OH)_2$). Fundamental studies indicated that alkalis remove lignin and acetate groups from hemicellulose, thus improving the reactivity of the remaining carbohydrates (Kong et al., 1992). Compared to sodium hydroxide and ammonia, lime is cheaper, safer, and can be recovered by carbonating wash water with CO_2 (Chang et al., 2001). On the other hand, lime seems to be less effective than other alkalis because it is a weak base and has a low solubility in water (Ibrahim and Pearce, 1983). To make lime as efficient as other alkalis in enhancing the digestibility of lignocellulose, appropriate pretreatment conditions need to be employed. Lime pretreatment typically mixes the slurry of lime and water with the biomass and then stores the material in a pile for hours or weeks. Delignification kinetics of corn stover in lime pretreatment was studied to indicate that the activation energies for delignification in the oxidative lime pretreatment reactions are similar to the Kraft delignification of bagasse, but much less than in Kraft delignification of wood (Kim and Holtzapple, 2006). Studies have been carried out with lime pretreatment for optimizing pretreatment conditions for various feedstocks: switchgrass, 100^{o}C, 2 h (Chang et al., 1997); wheat straw/bagasse, 85^{o}C/120^{o}C, 3 h/1 h (Chang et al., 1998); corn stover, 100^{o}C, 13 h (Karr and Holtzapple, 1998, 2000);

poplar wood/ newspaper, 150°C/140°C, 6 h/3 h, 14-atm oxygen/7.1-atm oxygen (Chang et al., 2001). Adding air/oxygen to the reaction system can significantly improve the delignification of the biomass (Chang and Holtzapple, 2000). Chang et al. (2001) performed oxidative lime pretreatment of poplar wood at 150°C for 6 h with 78% removal of lignin and 71% improvement of the glucose yield from enzymatic hydrolysis. Lime (0.5 g lime/g raw biomass) was also used to pretreat corn stover in non-oxidative and oxidative conditions at 25ºC, 35ºC, 45ºC, and 55°C. The optimal condition was found to be 55°C for 4 weeks with aeration (Kim and Holtzapple, 2005).

Coastal bermudagrass is widely grown in the southeast of the United States to remove some nutrients such as nitrogen and phosphrous for pollution prevention and feed animals. In this study, the effects of lime pretreatment on the enzymatic hydrolysis of coastal bermudagrass were investigated at different pretreatment temperatures, residence times and lime loadings. The optimal conditions were generated based on the efficiency of lime pretreatment on improved sugars production.

4.3 MATERIALS AND METHODS

4.3.1 Biomass preparation

Coastal bermudagrass was supplied from North Carolina State University Central Crops Research Station (Clayton, NC) in June, 2007. The bermudagrass was already air dried in the field. The biomass was ground in a Thomas Wiley Laboratory Mill (model no. 4) with sieve diameter of 2 mm and then stored in sealed plastic bags at room temperature until use for pretreatment.

4.3.2 Pretreatment

Coastal bermudagrass was mixed with lime (calcium hydroxide) in deionized (DI) water (solid to liquid ratio of 1:10) in sealed serum bottles, and pretreated in an autoclave (for pretreatments at 121°C) and a water bath (for pretreatments up to 100°C). After pretreatment, the biomass was washed with 200 ml of deionized water/0.1 g lime used and then the solid residues were stored at 4°C for enzymatic hydrolysis.

Two sets of experiments were conducted. The first set of experiments were performed at room temperature, 50°C, 80°C, 100°C, and 121°C for various residence times with a lime loading of 0.1 g/g of dry biomass. The second set examined pretreatments with different lime loadings (0.02, 0.05, 0.08, 0.15, 0.20 g/g of dry biomass) at the above temperatures for the optimal residence times obtained in the first set.

4.3.3 Enzymatic hydrolysis

Enzymatic hydrolysis of pretreated biomass was carried out in 250 ml Erlenmeyer flasks in a controlled environmental incubator shaker set at 55°C and 150 rpm. 0.5 g (dry basis) of pretreated biomass was immersed in 0.05 M sodium citrate buffer to maintain a pH of 4.8 with the total liquid volume of 15 ml. Prior to hydrolysis, activity of cellulases and cellobiase was determined to be 76.44 FPU/ml and 283.14 CBU/ml respectively (Ghose, 1987). The dosage of cellulases and cellobiase for all hydrolysis experiments was 40 FPU and 70 CBU per gram of dry biomass. Sodium azide (0.3% (w/v)) was added to the hydrolysis mixture to inhibit microbial growth. The hydrolysis was carried out for 72 hours after which the hydrolyzate was centrifuged and the supernatant was stored at -20°C for sugar analysis.

4.3.4 Analytical methods

Moisture content of the biomass was measured by drying the sample at 105°C in an oven to constant weight (Sluiter, 2005). The biomass was analyzed for extractives with 2:1 toluene-ethanol mixture in a Soxhlet apparatus with a reflux time of 24 hours (Silverstein, 2004). The extractive free biomass and the pretreated biomass were both analyzed for lignin using a two-stage sulfuric acid hydrolysis procedure recommended by the National Renewable Energy Laboratory (Sluiter, 2006). Total reducing sugars in the enzymatic hydrolyzates were determined by the DNS (dinitrosalicylic acid) method using glucose as the standard (Miller, 1959). Monosaccharides (glucose and xylose) from composition analysis and in the hydrolyzates were measured with a high performance liquid chromatography (HPLC) system. The HPLC system was equipped with a Bio-Rad Aminex HPX-87P column (300mm × 7.8mm) tailored for analysis of hexoses and pentoses in lignocellulosic materials, a Bio-Rad Micro-Guard column, a thermostatted autosampler, a quaternary pump, and a refractive index detector. The standards used were monomeric sugars at concentrations of 0.25, 2.5, 5.0, 7.5, 10.0 g/L. The analytical column was operated at 80°C with Milli-Q water (0.2 µm filtered) 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. To allow for late-eluting compounds to come off the column, a post-run time of 25 min was included between injections.

4.3.5 Statistical analysis

Experimental data were statistically analyzed using the GLM procedure in SAS 8.02 software. Significant ($P<0.05$) and nonsignificant differences between treatments were evalu-

ated by Tukey adjustment for comparisons. All treatments were conducted in triplicate.

4.4 RESULTS AND DISCUSSION

4.4.1 High temperature pretreatment

The effects of sodium hydroxide pretreatment of coastal bermudagrass on enzymatic digestibility of the biomass were investigated prior to the study of lime pretreatment. The results indicated that NaOH pretreatment at high temperature (121°C) performed much better on enhancing sugars production than at lower temperatures (< 121°C). For lime pretreatment study, high temperature (121°C) pretreatments were explored first. Lime loading of 0.1 g/g dry biomass was used according to the findings in a previous study on switchgrass (Chang et al., 1997). Comparisons between lime (0.1 g/g dry biomass) pretreatment and NaOH (1%) pretreatment at 121°C were made in the study. NaOH pretreatment was more effective in removing lignin than lime pretreatment, with an average difference of 55% lignin removal (Fig. 4.1a). This is because sodium hydroxide is a stronger base than lime, and it breaks up more crosslinks among lignin, hemicelluloses, and cellulose. There was no significant difference on lignin removal among residence times of 15, 30, and 60 min for lime pretreatment at 121°C. Although the extent of delignification for lime pretreatment was much lower than that for NaOH pretreatment, there was no significant difference in total reducing sugars production between the two pretreatements above at 121°C (Fig. 4.1b). The results suggest that it is not necessary to remove up to 60-80% lignin in order to enhance sugars production. There is a possibility that the digestibility of the biomass can be improved, as long as the porosity is increased to let the carbohydrates become more accessible to enzymes

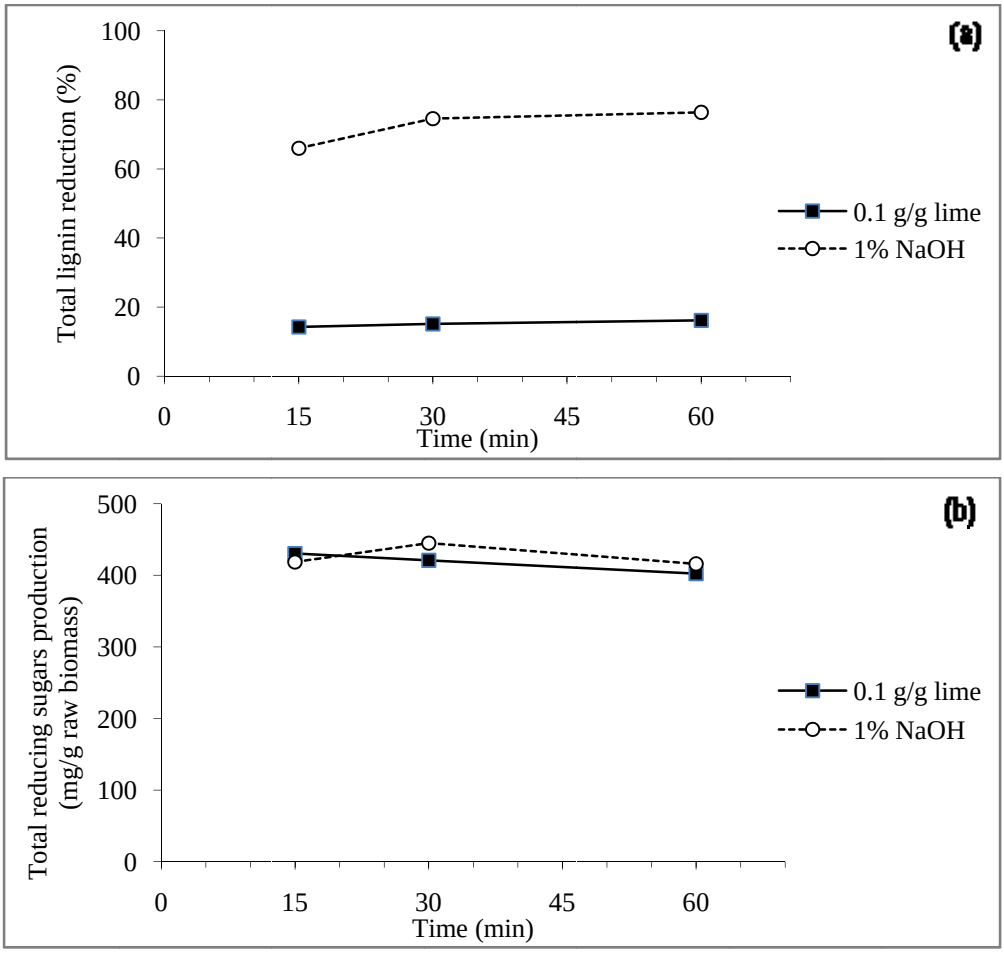

Fig. 4.1. Total lignin reduction (a) and total reducing sugars production (b) from coastal bermudagrass pretreated at 121°C with lime (0.1 g/g dry biomass) and 1% (w/v) NaOH respectively.

during the hydrolysis without high lignin removal. As residence time increased from 15 min to 60 min, total reducing sugars production remained at the same level, which is similar to the trend for total lignin reduction. This indicates that delignification has an impact on the digestibility of carbohydrates.

Monomeric sugars analysis of hydrolyzates is shown in Fig. 4.2. Glucose yield for lime (0.1 g/g dry biomass) pretreated biomass was slightly less than that obtained for NaOH (1%)

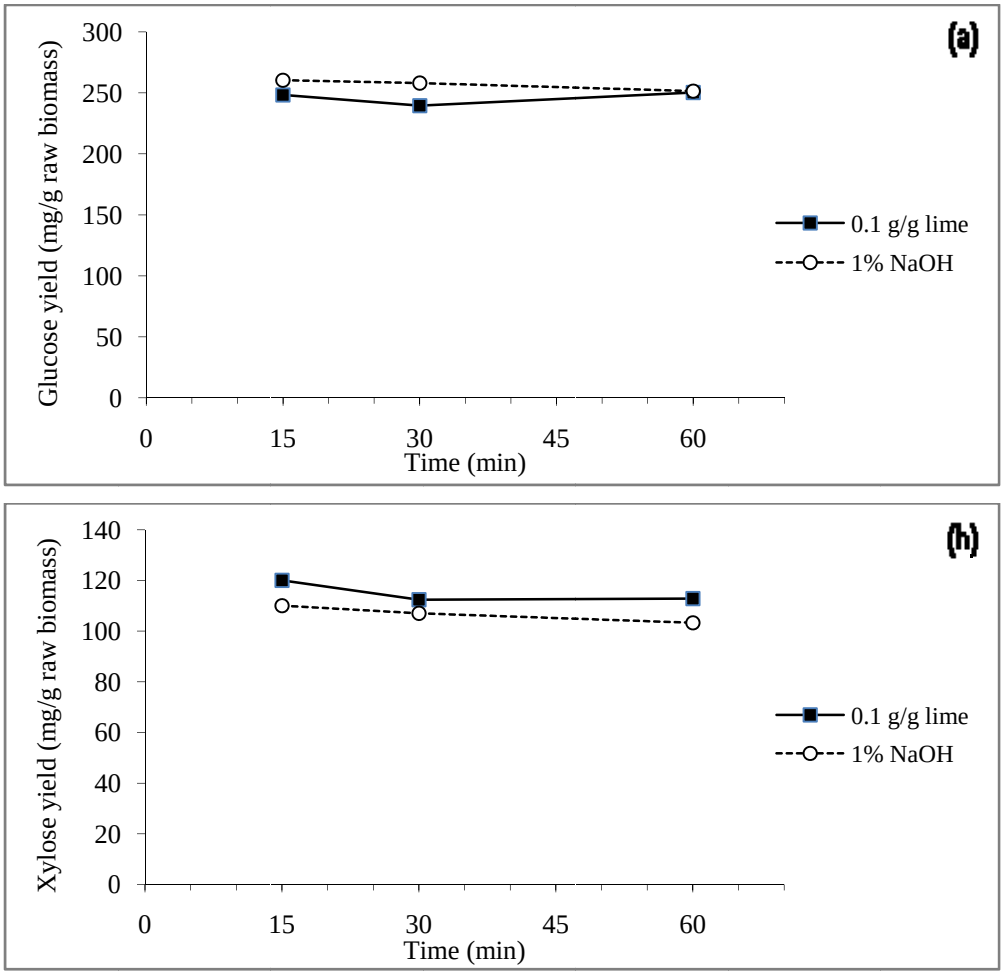

Fig. 4.2. Monosaccharides production for coastal bermudagrass pretreated at 121°C with lime (0.1 g/g dry biomass) and 1% (w/v) NaOH respectively.
(a) Glucose yield; (b) Xylose yield.

pretreatment at 121°C. Glucose production after enzymatic hydrolysis is mainly from the degradation of crystalline cellulose. The crystallinity of cellulose is more easily disrupted by NaOH than lime during pretreatment process probably due to the higher pH in NaOH solution than in lime water at the same concentration, thereby, more glucose can be released from crystalline cellulose for NaOH pretreatment. Because of higher extent of delignification and decrystallization for NaOH pretreatment (Fan et al., 1987), hemicelluloses in the biomass

have a greater chance to be degraded into oligomers and monomers (mainly xylose) that goes into the pretreatment liquor. This may explain why lime pretreatment, to some extent, liberated more xylose than NaOH pretreatment after enzymatic hydrolysis, perhaps owing to less xylose loss during lime pretreatment.

4.4.2 Low temperature pretreatment

The solubility of calcium hydroxide (lime) in water decreases with the increase of temperature. Despite no statistically significant difference in reference to total reducing sugars yield between NaOH and lime pretreatments at 121°C, lime pretreatment overall yielded less total reducing sugars production than NaOH pretreatment. Two reasons may contribute to this phenomenon. One is that lime is a medium strength base while NaOH is a high strength base. The other is that the solubility of lime is very low compared to NaOH, especially at high temperatures. On the other hand, high temperature offsets negative impacts of the two reasons above. Total reducing sugars yield for lime pretreatment at 121°C reached up to 74% of theoretical maximum, which is comparable to 77% for NaOH pretreatment. Therefore, it is favorable to study lime pretreatment at temperatures below 121°C with potential pretreatment cost savings as lime is more soluble in water at lower temperatures.

The effect of room temperature on lime pretreatment efficiency was examined, as showed in Fig. 4.3. Lime pretreatment at room temperature did not achieve adequate total reducing sugars production at residence times less than 34 h (Fig. 4.3c). When considering monomeric reducing sugar yields, 24 h is sufficient for glucose production (Fig. 4.3a), while 34 h is needed to enhance releasing xylose (Fig. 4.3b). In order to maximize total reducing s-

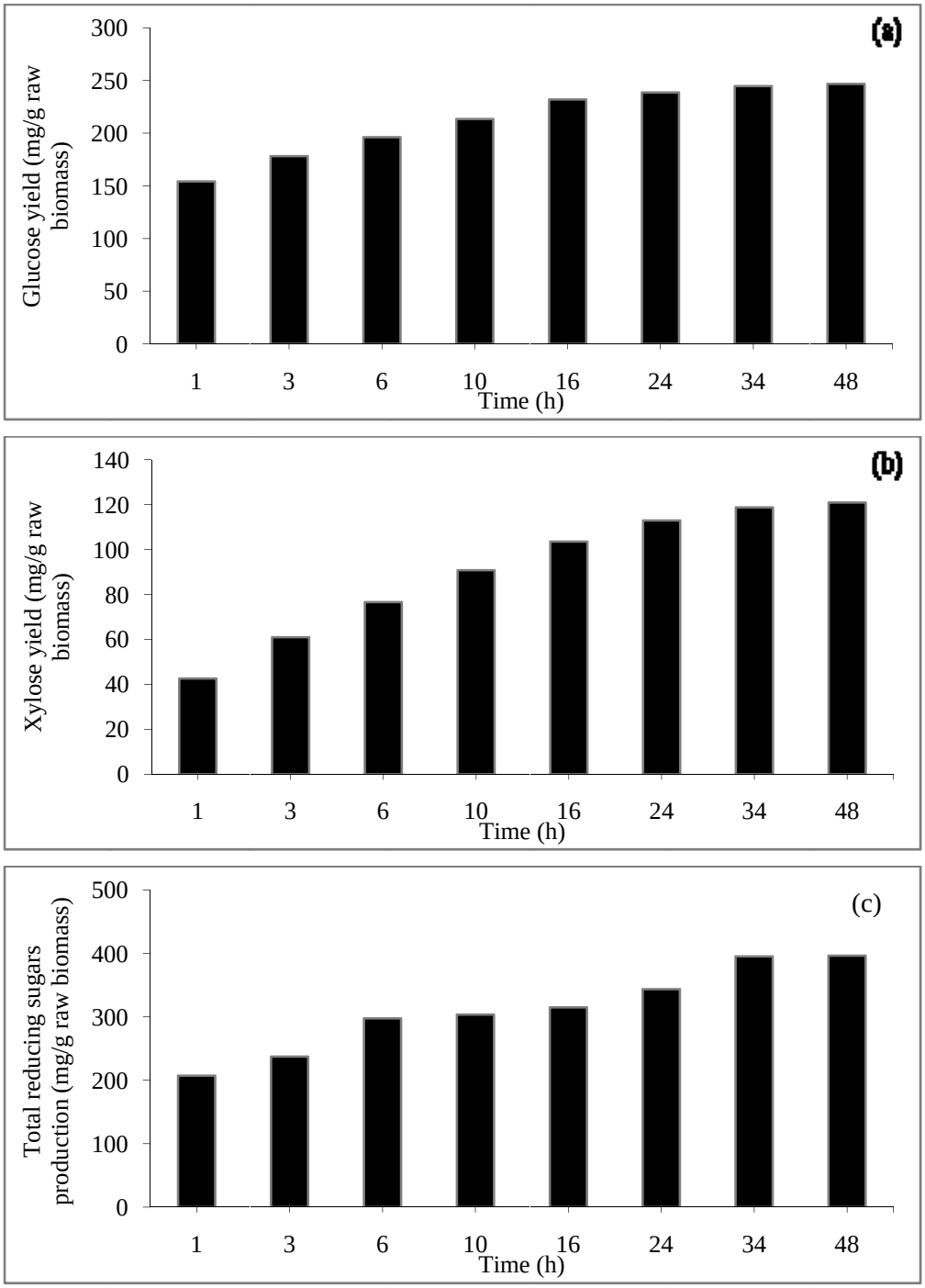

Fig. 4.3. Monomeric and total reducing sugars production from coastal bermudagrass pretreated with lime (0.1 g/g dry biomass) at room temperature.
(a) Glucose yield; (b) Xylose yield; (c) Total reducing sugars production.

ugars yield, 34 h is required. Whether to choose 24 h or 34 h as residence time for lime pretreatment of coastal bermudagrass at room temperature needs economic analysis for final fermentable sugars yield.

Total reducing sugars production for lime pretreatment at 50°C was presented in Fig. 4.4c. Compared to room temperature, much less residence time (6 h) is desirable for increased digestibility of the biomass at 50°C. The results showed that increasing pretreatment temperature from room temperature to 50°C could improve sugars production by 8% as well as shorten optimal residence time from 34 h to 6 h. Both glucose and xylose yields have the same trend as total reducing sugars production with the increase of residence time, which suggests that the increase of total reducing sugars production is mainly attributed to the increase of glucose and xylose yields (Fig. 4.4a, b).

Further increase of pretreatment temperature to 80°C reduced optimal residence time to 3 h, with a small increase of total reducing sugars production but glucose and xylose yields (Fig. 4.5). It is obvious that there is an inverse correlation between pretreatment temperature and residence time. It is a trade-off when choosing either high temperature pretreatment for short residence time or low temperature for long residence time. When looking into total reducing sugars production at 80°C, lower sugars yield was observed after 16 h because of higher solid loss. The same trend was noticed for xylose yield but not for glucose yield. Higher solid loss implies more severe pretreatment, thus causing higher loss of hemicelluloses due to its higher digestibility than cellulose.

To maximize total reducing sugars production as well as glucose and xylose yields for

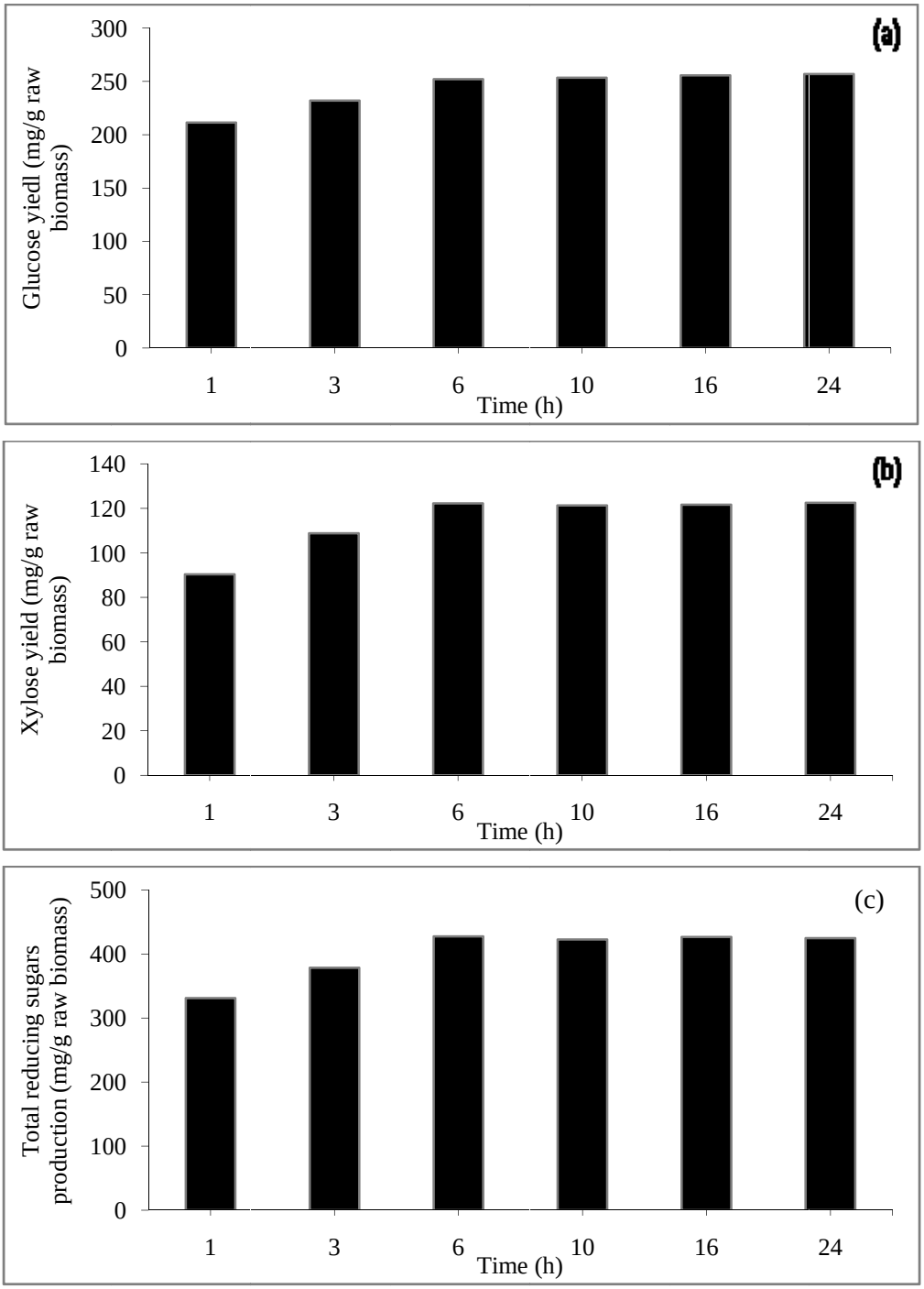

Fig. 4.4. Monomeric and total reducing sugars production from coastal bermudagrass pretreated with lime (0.1 g/g dry biomass) at 50°C.
(a) Glucose yield; (b) Xylose yield; (c) Total reducing sugars production.

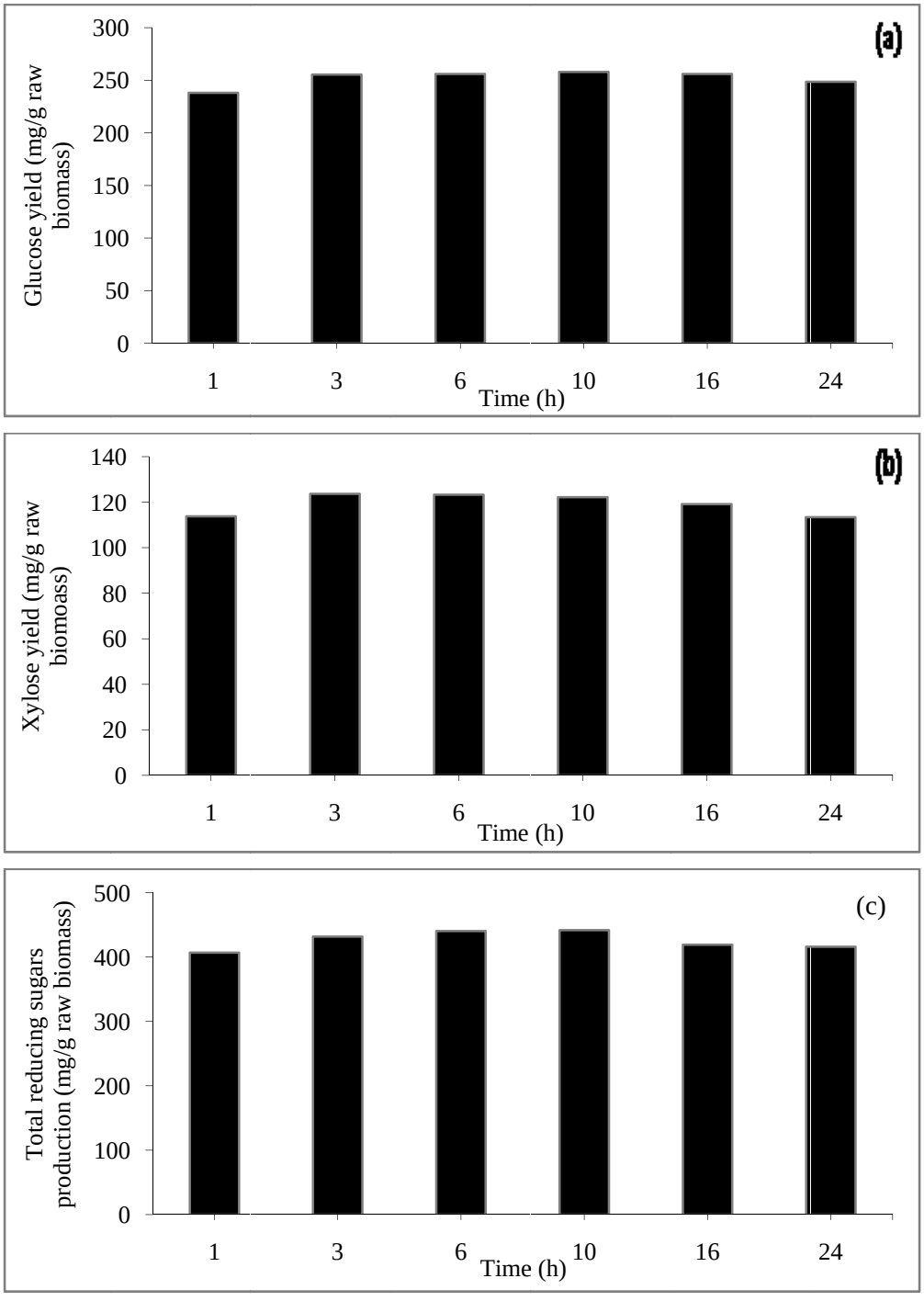

Fig. 4.5. Monomeric and total reducing sugars production from coastal bermudagrass pretreated with lime (0.1 g/g dry biomass) at 80°C.
(a) Glucose yield; (b) Xylose yield; (c) Total reducing sugars production.

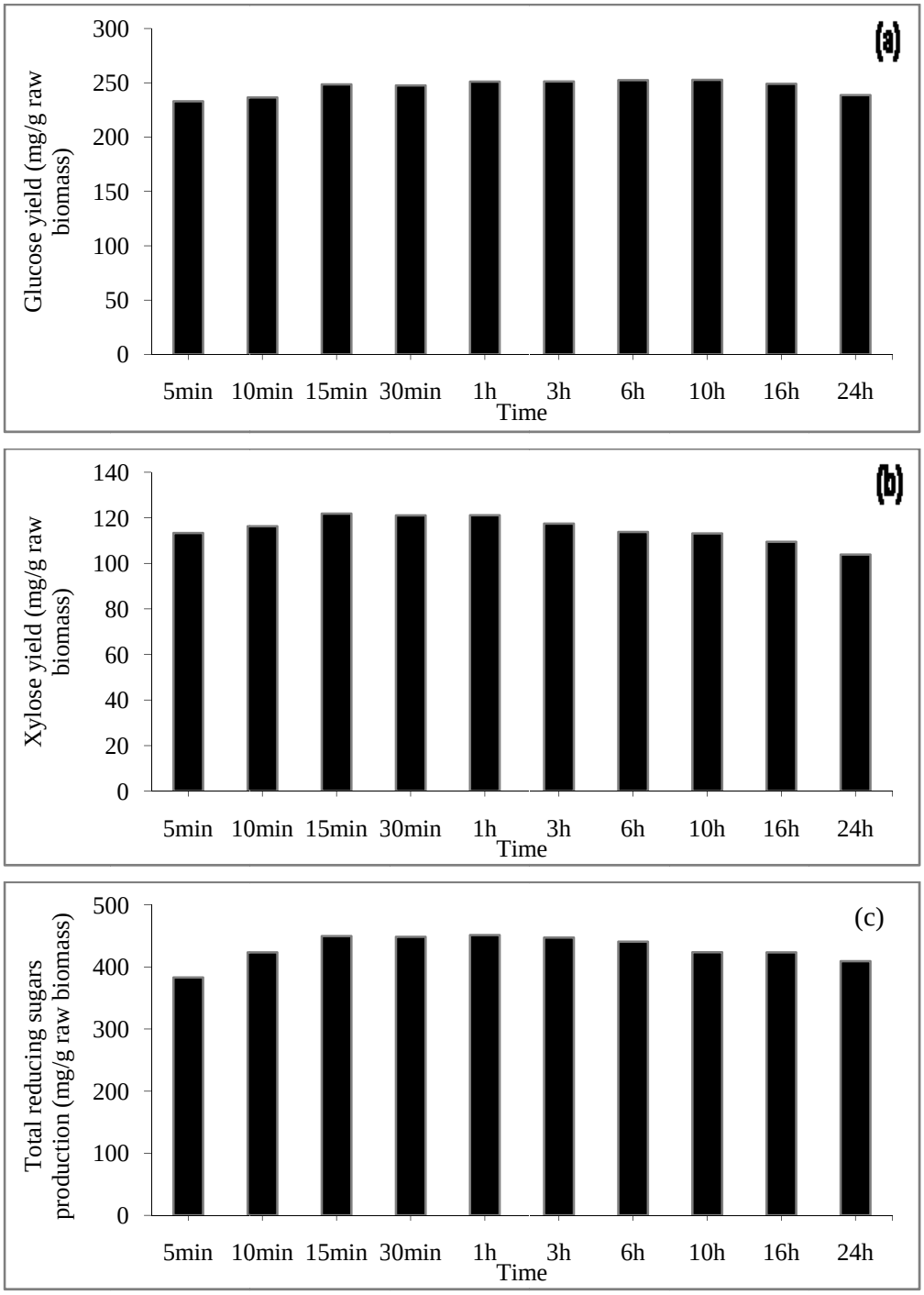

Fig. 4.6. Monomeric and total reducing sugars production from coastal bermudagrass pretreated with lime (0.1 g/g dry biomass) at 100°C.
(a) Glucose yield; (b) Xylose yield; (c) Total reducing sugars production.

lime pretreatment of coastal bermudagrass at 100°C, residence time of 15 min is sufficiently long to meet the goal. As it was found at 80°C, total reducing sugars and xylose yields both decreased significantly after 10 h because of high solid loss. The highest total reducing sugars yield was improved by 13% as the pretreatment temperature increased from room temperature to 100°C. With lime loading of 0.1 g/g dry biomass, total reducing sugars yield was maximized at 100°C for 15 min with approximately 78% of theoretical maximum, which is comparable to 77% for NaOH pretreatment. However, total lignin reduction for lime pretreatment was around 10-20%, much less than that for NaOH pretreatment. This outcome signifies that delignification plays a part in enhancing the digestibility of biomass, but not the key one, because less than 20% of lignin removal appears to be satisfactory for sugars production. Besides, lignin reduction higher than 75% may not be beneficial for sugars yield (calculated based on raw biomass) because of low solid recovery.

As shown in Table 4.1, glucan and xylan conversion rates obtained at optimal residence times for each lime (0.1 g/g dry biomass) pretreatment temperature were presented here. The glucan conversion rate varied from 86.05% to 89.83% which is about 4% less than that achieved under optimal NaOH pretreatment conditions at 121°C. The range of xylan conversion rate from 65.78% to 68.54% is slightly higher than that gained for NaOH pretreatment conducted at optimal conditions. The comparison suggested that the digestibility of cellulose is higher in NaOH pretreatment, while the hemicellulose is well preserved to avoid a large extent of degradation in lime pretreatment. Given the fact that each pretreatment temperature with optimal residence time reached similar glucan and xylan

conversion rates, 50, 80, and 100°C with their respective optimal residence times were selected for lime loading studies.

Table 4.1. Glucan and xylan conversion rates after 72-h enzymatic hydrolysis of lime pretreated coastal bermudagrass.

Temperature (°C)	Residence time	Lime loading (g/g dry biomass)	Glucan conversion rate (%)
room temp	34 h	0.1	86.05
50	6 h	0.1	88.64
80	3 h	0.1	89.83
100	15 min	0.1	87.38
121	15 min	0.1	87.32
Temperature (°C)	Residence Time	Lime loading (g/g dry biomass)	Xylan conversion rate (%)
room temp	34 h	0.1	65.78
50	6 h	0.1	67.71
80	3 h	0.1	68.54
100	15 min	0.1	67.48
121	15 min	0.1	66.49

4.4.3 Lime loadings studies

Lime loading of 0.1 g/g dry biomass was recommended for switchgrass (Chang et al., 1997) and poplar wood (Chang et al., 2001). With the goal of developing cost-effective pretreatment technologies for ethanol production, it is necessary to study how different lime dosing affects pretreatment efficiency and optimize lime loading for enhanced sugar recovery. For the three optimal residence times at each pretreatment temperature, no significant improvement of total reducing sugars production with the increase of lime loading (from 0.1 to 0.20 g/g dry biomass) was observed (Fig. 4.7). As a result, there is no benefit from further increasing lime loading beyond 0.1 g/g dry biomass. Total reducing sugars yield was reduced by 8-9% when decreasing lime loading from 0.1 to 0.08 g/g dry biomass. Lime

loading of 0.02 and 0.05 g/g dry biomass did not work at all in terms of sugar recovery simply because there was not enough lime for reacting with biomass during the pretreatment.

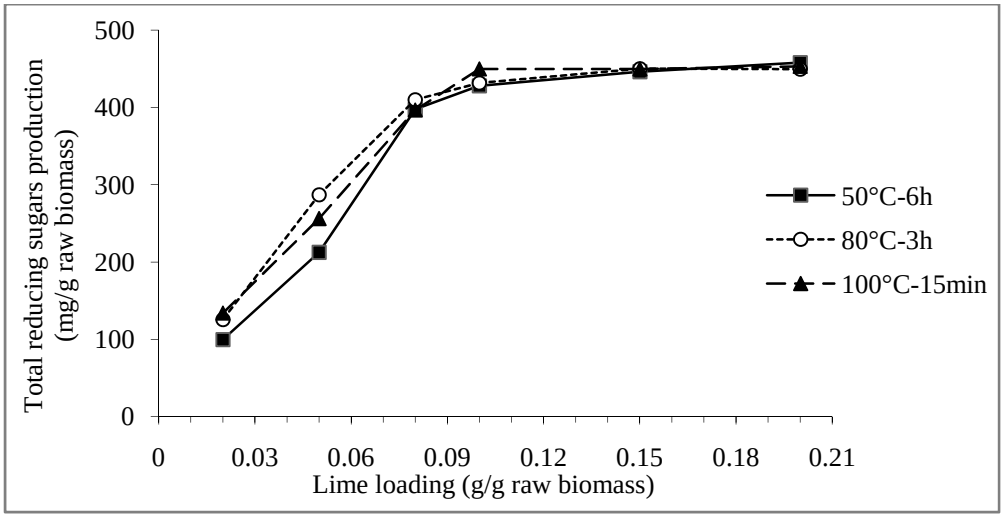

Fig. 4.7. Total reducing sugars production from coastal bermudagrass pretreated with different lime loadings (0.02, 0.05, 0.08, 0.10, 0.15, and 0.20 g/g dry biomass) at 50°C for 6 h, 80°C for 3 h, and 100°C for 15 min.

Lime is slightly soluble in water. The suspended lime particles have a very high total surface area which means that, as the lime in solution is used up during pretreatment, more lime will quickly dissolve into the solution. From Fig. 4.7, it seems that 0.1 g lime was used up during the whole pretreatment process, and any amount of lime more than 0.1 g was excessive for 1 g dry biomass. It was unexpectedly noted that as lime loading increased from 0.1 to 0.20 g/g dry biomass, glucose and xylose yields both declined (Fig. 4.8). This is probably related to the interactions of calcium ions with lignin, cellulose and hemicellulose in the biomass. Calcium ions, each carrying two positive charges, are able to adsorb onto the surface of cellulosic biomass components that are negatively charged due to the ionization of hydroxyl groups and acid groups in alkaline condition, and to crosslink with these compoun-

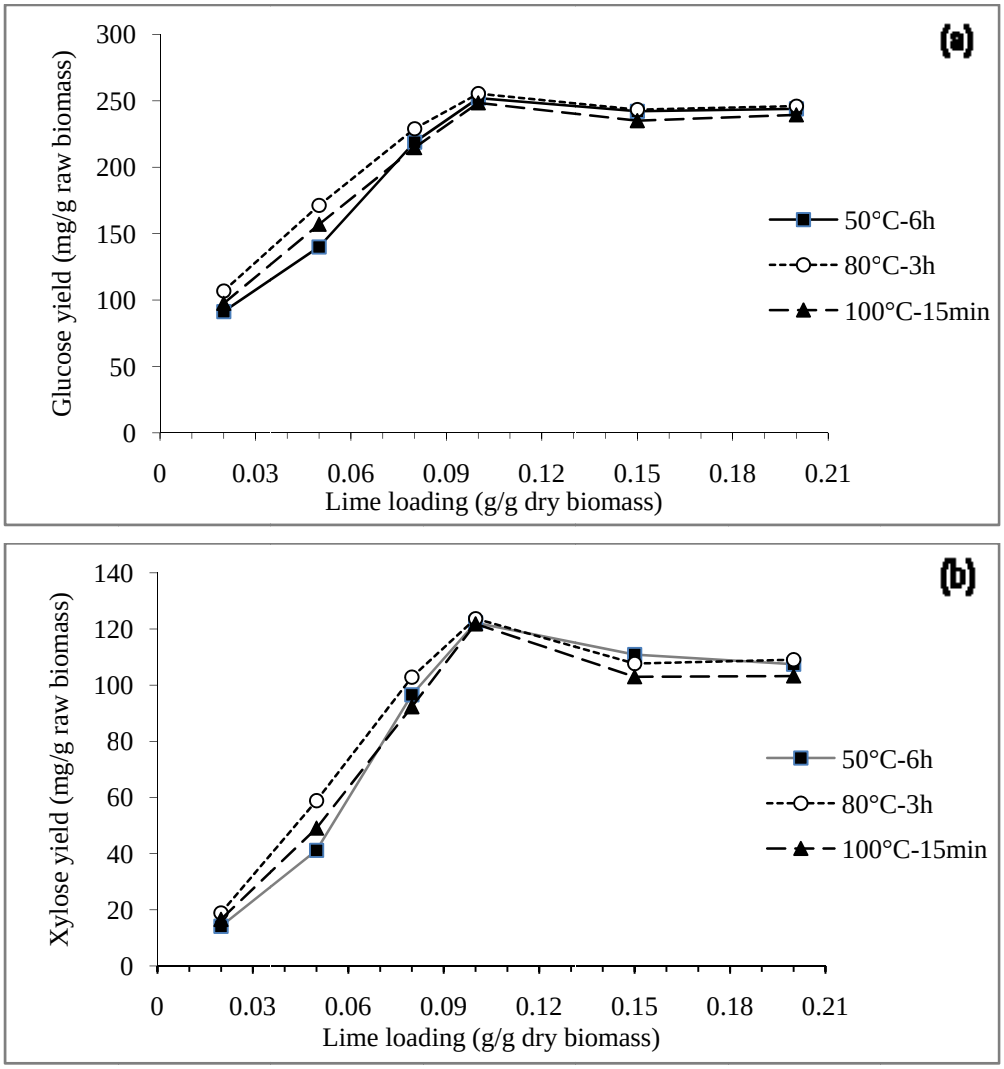

Fig. 4.8. Monomeric reducing sugars production from coastal bermudagrass pretreated with different lime loadings (0.02, 0.05, 0.08, 0.10, 0.15, and 0.20 g/g dry biomass) at 50°C for 6 h, 80°C for 3 h, and 100°C for 15 min.
(a) Glucose yield; (b) Xylose yield.

ds such as lignin. It was reported that lignin exhibits a high affinity to calcium ions in solution, whereas the interaction of cellulose with calcium ions was weaker (Torre et al., 1992). This is probably why low lignin removal occurred in lime pretreatment. Calcium ions could also congregate and precipitate lignin in a base medium (Jonas and Niles, 2000).

Therefore, calcium ions, under excessive dosing conditions, form more linkages with biomass constituents, which consequently would have a negative impact on the digestibility of cellulose and hemicellulose. This may explain why glucan and xylan conversion rates decreased when using excessive lime to treat biomass.

4.5 CONCLUSIONS

In this study, lime pretreatment of coastal bermudagrass was investigated at different temperatures, residence times and lime loadings. Lime pretreatment was not as effective as NaOH pretreatment with regard to lignin removal. However, total reducing sugars yield for lime pretreatment was comparable to that for NaOH pretreatment. Increasing pretreatment temperature could reduce optimal residence time for enhanced sugar recovery. The maximal reducing sugars yield was improved by 13% with the increase of pretreatment temperature from room temperature (22°C) to 100°C. There was no statistical difference on sugar recovery between 100°C and 121°C. The highest total reducing sugars yield for lime pretreatment was achieved at 78% of theoretical maximum, which is over two times that of untreated biomass. Further increasing lime loading to more than 0.1 g/g dry biomass did not enhance sugars production, whereas the use of lime loadings less than 0.1 g/g impaired the production of total reducing sugars. Excessive lime loading should be avoided because more linkages formed in the biomass would inhibit the digestibility of carbohydrates. The optimal condition was found to be 100°C for 15 min with a lime loading of 0.1 g/g dry biomass. Under the recommended condition, 87.4% of glucan and 67.5% of xylan were converted to glucose and xylose respectively. For future work, it is favorable to analyze the

prehydrolyzate after lime pretreatment to examine the degradation of carbohydrates during pretreatment. The impact of enzyme dosing on sugars recovery needs to be investigated.

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CHAPTER 5

ENZYMATIC HYDROLYSIS OF COASTAL BERMUDAGRASS FOR ETHANOL PRODUCTION

5.1 ABSTRACT

Enzymatic hydrolysis is an indispensable step for converting cellulosic polysaccharides to monosaccharides that can be further fermented to ethanol. Cellulases and cellobiase are the two key enzymes for the conversion. In this study, coastal bermudagrass was pretreated under optimal conditions with NaOH and lime. The pretreated biomass was then hydrolyzed with different enzyme loadings (cellulases: 0, 5, 10, 15, 20, 40 FPU/g dry biomass; cellobiase: 0, 10, 20, 30, 40, 50, 70 CBU/g dry biomass). A cellulases loading of 20 FPU/g was required to improve sugar recovery for lime-pretreated biomass, while 15 FPU/g was sufficient for enhanced sugar yield for NaOH-pretreated biomass. The optimal cellobiase loading was found to be 10 CBU/g for the two types of pretreated biomass. The supplementation of xylanase did not significantly increase sugar yield. Ethanol yield was detected as 95% of theoretical maximum yield for the hydrolyzate and 83% for the raw biomass. There was no significant difference in ethanol yield between NaOH and lime-pretreated coastal bermudagrass.

Keywords: Enzymatic hydrolysis; Cellulases; Cellobiase; Ethanol yield; Costal bermudagrass

5.2 INTRODUCTION

Lignocellulosic biomass is an abundant and renewable source of fermentable sugars that can be used for ethanol production. Lignocellulose fibers contain three major components including cellulose, hemicelluloses, and lignin. The complex structure formed by the combination of these fractions in the biomass is able to protect the fibers from physical and microbial attack. A pretreatment is required to open the biomass structure, remove the lignin, and make the polysaccharides accessible for conversion to sugars. The five carbon and six carbon sugars from the cellulose and hemicellulose are then liberated through enzymatic hydrolysis. The fermentation of hexoses and pentoses is finally carried out to produce ethanol.

Enzymatic hydrolysis of pretreated lignocellulosic biomass is usually carried out at around 50°C and pH of 4.8, which is milder than conventional acid hydrolysis. Cellulose is typically hydrolyzed by cellulases which can be obtained from many organisms including aerobic bacteria (Mohagheghi et al., 1986), anaerobic bacteria (Lamed et al., 1985), white rot fungi (Eriksson et al., 1980), soft rot fungi (Watson et al., 1984), and anaerobic fungi (Barichievich and Calza, 1990). Fugal cellulases are the most promising cellulolytic enzymes for ethanol production in a commercial scale, especially those from soft rot fungus *Trichoderma reesei* (Duff and Murray, 1996; Kadam, 1996). Cellulases activity is generally confined to amorphous regions of cellulose because very few isolated cellulases have been shown to hydrolyze crystalline cellulose (Shewale, 1982).

Cellulases are a mixture of three enzymes, 1,4-β-D-glucan glucanohydrolase (EC 3.2.1.3), 1,4-β-D-glucan cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21),

that act synergistically to hydrolyze cellulose (Ladisch et al., 1983). These components in cellulases enzyme system are normally referred to as endoglucanase (C_x), exoglucanase (C_1), and cellobiase (β-glucosidase) respectively. Endoglucanases randomly attack and cleave the β-1,4 glycosidic bonds of cellulose to produce cello-oligosaccharides and glucose. Exoglucanases release cellobiose from the nonreducing ends of a cellulosic substrate. Cellobiases hydrolyze cellobiose to glucose. The supplementation of β-glucosidase in hydrolysis is required due to its insufficient amount from *T. reesei*, to prevent cellulases inhibition resulted from cellobiose accumulation (Ryu and Mandels, 1980). The inhibition of endoglucanase and exoglucanase activities by cellobiose (Holtzapple et al., 1984) as well as the inhibition of β-glucosidase action by glucose (Gong et al., 1977), can be significant factors in enzyme cost in the saccharification process.

There are three main enzymes involved in the hydrolysis of xylan which is a major part of hemicellulose in the lignocellulosic biomass. These enzymes are endo-β-1,4-xylanase which catalyzes the hydrolysis of the β-1,4 bonds between D-xylose residues of heteroxylans and xylo-oligosaccharides, exoxylanase that releases xylobioses, and β-xylosidase that hydrolyzes xylo-oligosaccharides to xylose (Saha and Bothast, 1999). In addition to the three major enzymes, some accessory enzymes such as α-L-arabinofuranosidase, α-glucuronidase, acetylxylan esterase, ferulic acid esterase, and ρ-coumaric acid esterase are necessary for the hydrolysis of different substituted xylans (Saha, 2003). It was reported that with the increase of the dosages of xylanase and β-xylosidase, more pentosans could be removed from unbleached sulfite pulps (Christov and Prior, 1994). Unlike the tightly packed crystalline

structure of cellulose, hemicellulose is more accessible to enzymes during hydrolysis (Gilbert and Hazlewood, 1993). Hemicellulases were identified by 2D electrophoresis in the *T. reesei* cellulases system (Vinzant et al., 2001). To make ethanol production from lignocelluloses economically feasible, the improvement of hydrolysis efficiency and the development of co-fermentation of hexoses and pentoses as well as cost-effective pretreatmentt technologies are required.

This study was aimed to investigate the impacts of enzyme dosing on the enhanced sugars recovery from NaOH-pretreated and lime-pretreated coastal bermudagrass respectively. The effect of xylanase supplementation on the hydrolysis efficiency was examined. Final ethanol yield from the optimal pretreatment conditions and enzyme dosing was detected to estimate potential ethanol production in a larger scale.

5.3 MATERIALS AND METHODS

5.3.1 Biomass preparation and pretreatment

Coastal bermudagrass was provided by North Carolina State University Central Crops Research Station (Clayton, NC). The bermudagrass was harvested in June, 2007 and air dried in the field. The biomass was ground in a Thomas Wiley Laboratory Mill (model no. 4) with sieve diameter of 2 mm and then stored in sealed plastic bags at room temperature.

For sodium hydroxide pretreatment, 3 gram of coastal bermudgrass were immersed in 0.75% (w/v) sodium hydroxide solution (solid to liquid ratio of 1:10) in sealed serum bottles, and pretreated in an autoclave at 121°C for 15 min. The biomass was then washed with 200 ml of deionized (DI) water and the solid residues were stored at 4°C for the enzymatic hydro-

lysis test later.

For lime pretreatment, coastal bermudagrass was mixed with lime (0.1 g/g dry biomass) in deionized (DI) water (solid to liquid ratio of 1:10) in sealed serum bottles, and pretreated in a water bath at 100°C for 15 min. After that, the biomass was washed with 200 ml of deionized water/0.1 g lime used and then the solid residues were stored at 4°C for the following enzymatic hydrolysis.

5.3.2 Enzymatic hydrolysis

Cellulases (NS50013) produced by *Trichoderma reesei*, cellobiase (NS50010) produced by *Aspergillus niger*, and xylanase (NS50014) of *T. reesei* origin were obtained from Novozymes North America Inc. (Franklinton, NC). The cellulases and cellobiase activities were determined to be 76.44 FPU/ml (FPU, filter paper unit, expressed as μmol of glucose produced per minute with filter paper as a substrate) and 283.14 CBU/ml (CBU, cellobiase unit, expressed as μmol of cellobiose that is converted into glucose per minute with cellobiose as a substrate), respectively (Ghose, 1987). The xylanase activity was 870 FXU/ml (FXU, fungal xylanase unit) according to Novozymes Biomass Kit.

Enzymatic hydrolysis of the pretreated biomass was carried out in 50 ml plastic tubes in a controlled environment reciprocal shaking bath at 55°C and 150 rpm. 0.5 g (dry basis) of pretreated biomass was immersed in 0.05 M sodium citrate buffer to maintain a pH of 4.8 with the total liquid volume of 15 ml. Sodium azide (0.3% (w/v)) was added to the hydrolysis mixture to inhibit microbial growth. For each pretreated biomass, cellulases loadings (0, 5, 10, 15, 20, and 40 FPU/g dry biomass) test was conducted first with an excessive cellobiase

loading of 70 CBU/g dry biomass. Based on the optimal cellulases loading obtained under the excessive cellobiase dosage, cellobiase loadings (0, 10, 20, 30, 40, and 50 CBU/g dry biomass) test was then accomplished. Two xylanase loadings (10 and 68 FXU/g dry biomass) were studied with optimal cellulases and cellobiase loadings as well as excessive cellulases and cellobiase loadings, respectively. The hydrolysis was carried out for 72 hours after which the hydrolyzate was centrifuged and the supernatant was stored at -20°C for sugar analysis.

5.3.3 Fermentation

Yeast (*Saccharomyces cerevisiae*, ATCC 24859) supplied from the Agricultural and Biological Engineering Department at Pennsylvania State University was aerobically grown at 30°C in 100 ml medium (consisting of 20 g glucose, 8.5 g yeast extract, 1.32 g NH_4Cl, 0.11 g $MgSO_4$, and 0.06 g $CaCl_2$, per liter of deionized water) in a shaker incubator with 150 rpm for 24 h. The yeast cells were collected by centrifugation at 4,000×g at 4°C for 10 min and washed three times with 0.1% peptone water, and then resuspended in 30 ml peptone before use. The dry matter (%) content was measured to determine the volume of yeast used to inoculate the hydrolyzate.

After enzymatic hydrolysis, five milliliter of supernatant of the harvested hydrolyzate from each hydrolysis sample was added into 100 ml serum bottles for fermentation. The hydrolyzate was then adjusted to pH 7 by adding 2 N NaOH and inoculated with *S. cerevisiae* at a cell concentration of 10 g dry matter/l (Chen et al., 2007). All samples were then incubated at 30°C for 72 h. The collected liquid samples were analyzed for ethanol content after fermentation.

5.3.4 Analytical methods

Moisture content of the biomass was measured by drying the sample at 105°C in an oven to constant weight (Sluiter, 2005). Total reducing sugars in the enzymatic hydrolyzates were determined by the DNS (dinitrosalicylic acid) method using glucose as the standard (Miller, 1959). Monosaccharides (glucose and xylose) in the hydrolyzates were measured using HPLC as described in "Materials and Methods" section of Chapter 2 & 3. Ethanol in the fermentation liquor was determined with a HPLC system equipped with a Bio-Rad Aminex HPX-87H column (300mm × 7.8mm) used for analysis of carbohydrates in solution with alcohols, a Bio-Rad Micro-Guard column, and a refractive index detector. The analytical column was operated at 65°C with 0.005 M H_2SO_4 (0.2 μm filtered) as the mobile phase at a flow rate of 0.7 ml/min. The samples were injected at 10 μl and the acquisition time was 25 min.

5.3.4 Statistical analysis

Experimental data were statistically analyzed using the GLM procedure in SAS 8.02 software. Significant ($P<0.05$) and nonsignificant differences between treatments were evaluated by Tukey adjustment for comparisons. All treatments were conducted in triplicate.

5.4 RESULTS AND DISCUSSION

5.4.1 Sodium hydroxide pretreatment

The optimal NaOH pretreatment conditions obtained under excessive cellulases and cellobiase loadings are 0.75% (w/v) NaOH at 121°C for 15 min. These conditions were used for cellulases loadings test with an excessive cellobiase loading of 70 CBU/g dry biomass.

The main reaction products of cellulose hydrolysis with the cellulases (NS50013) are cellobiose and glucose. Since cellobiase was loaded excessively, there was no cellobiose accumulated in the hydrolyzate. As showed in Fig. 5.1b, only 10% of total reducing sugars in the raw biomass was converted with no cellulases added in the hydrolysis but excessive cell-

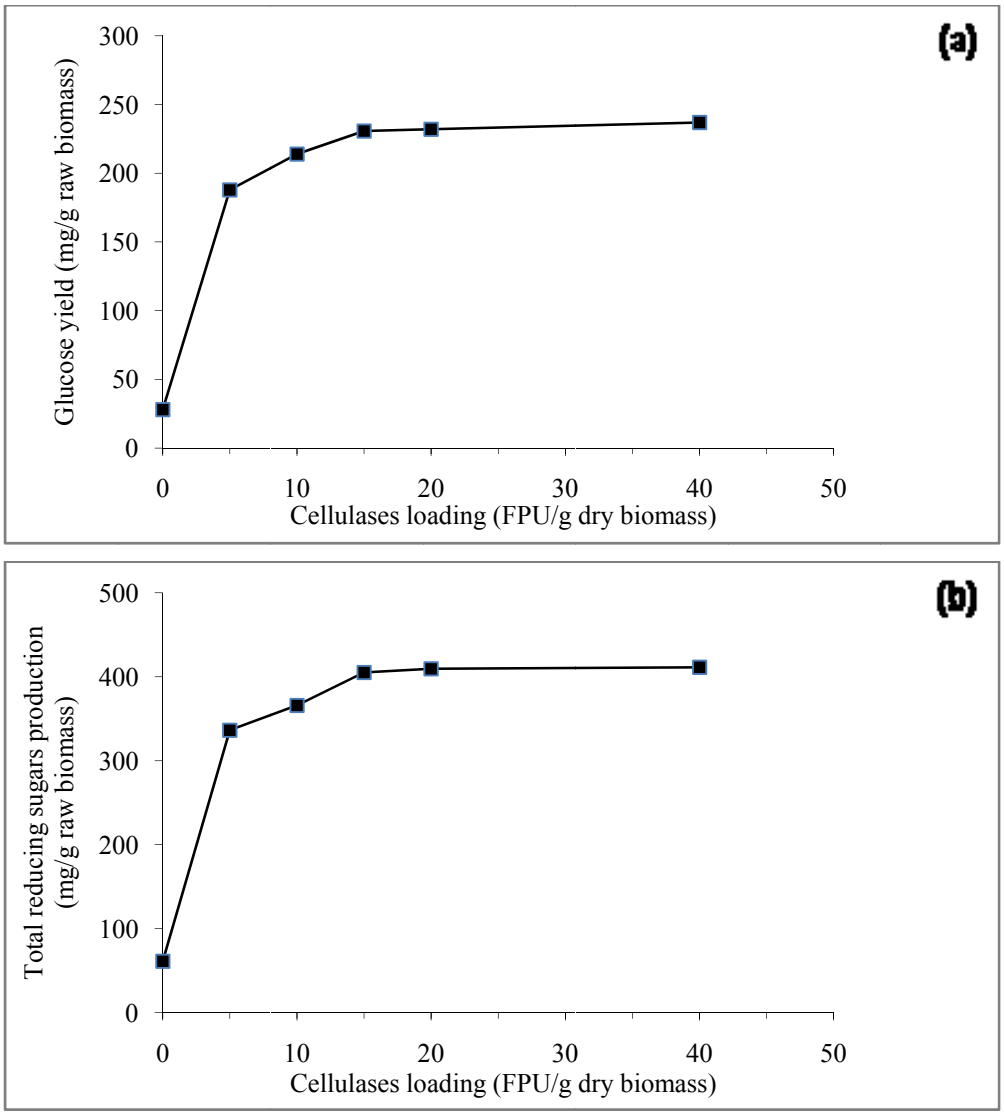

Fig. 5.1. Glucose (a) and total reducing sugars (b) production from NaOH-pretreated coastal bermudagrass (0.75% (w/v) NaOH pretreatment at 121°C for 15 min) under different cellulases loadings with excessive cellobiase of 70 CBU/g dry biomass.

obiase loading. The enzymatic hydrolysis was performed at a pH around 4.8, which could be considered as acid hydrolysis. This may explain why 10% sugar conversion rate occurred even without any cellulases during the hydrolysis. With the increase of cellulases loading from 0 FPU/g to 15 FPU/g dry, total reducing sugars conversion rate was improved by 60%

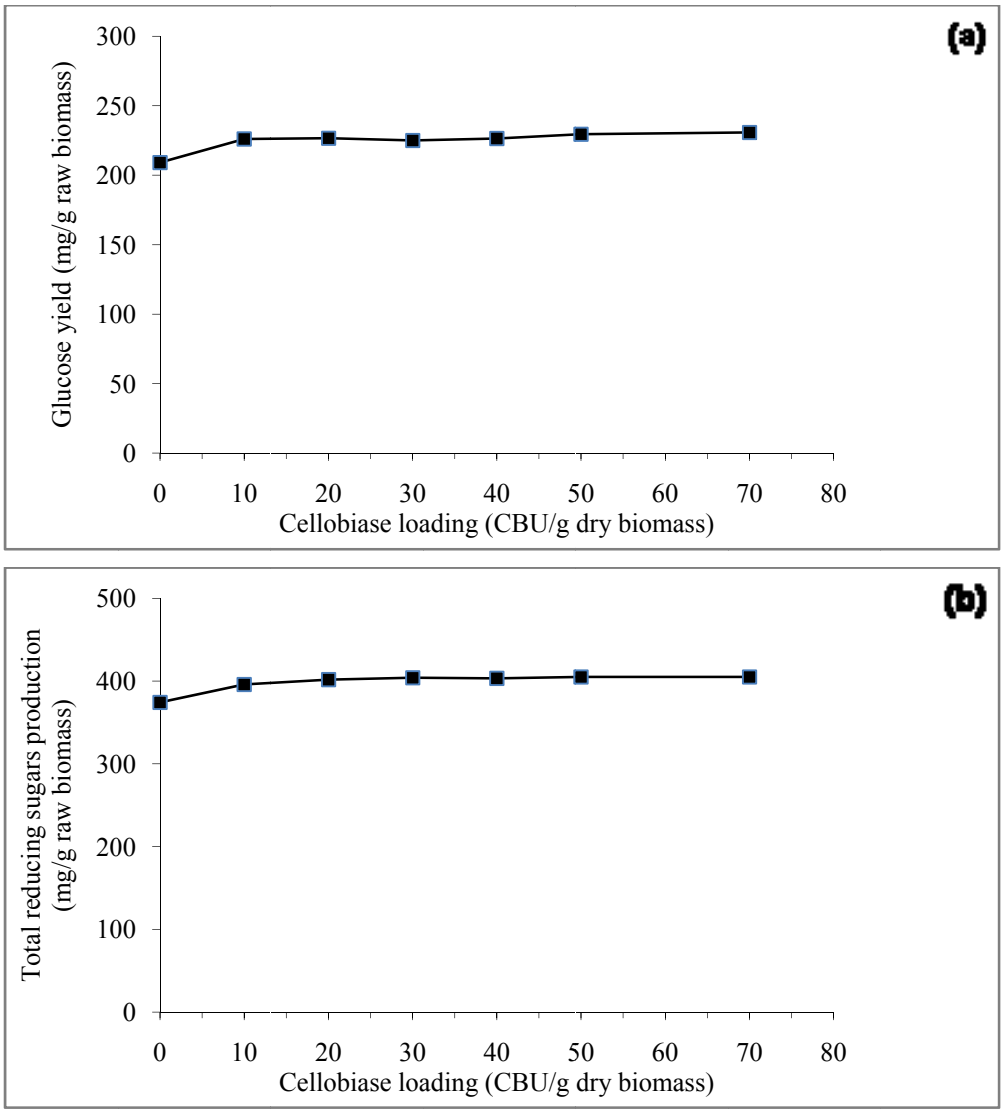

Fig. 5.2. Glucose (a) and total reducing sugars (b) production from NaOH-pretreated coastal bermudagrass (0.75% (w/v) NaOH pretreatment at 121°C for 15 min) under different cellobiase loadings with optimal cellulases loading of 15 FPU/g dry biomass.

of theoretical maximum. Further raising cellulases dosage did not enhance the sugar production. The same trend was observed for glucose yield with no significant difference among 15, 20, and 40 FPU/g cellulases loadings (Fig. 5.1a). It is clear that a cellulases loading of 15 FPU/g is sufficient for improved sugar recovery in excess of cellobiase dosage.

Under the cellulases loading of 15 FPU/g, a range of cellobiase dose from 0 CBU/g to 70 CBU/g was examined for its impact on sugar, specifically glucose, production. Fig. 5.2a indicated that there was a statistically significant improvement on glucose yield when cellobiase loading increased from 0 CBU/g to 10 CBU/g, after that, no significant increase of glucose production was noted as more cellobiase was added. The same phenomenon was found in the total reducing sugars production for cellobiase loading test. For the reason that cellobiase specifically hydrolyzes cellobiose to glucose, the enhancement of total reducing sugars production, when increasing cellobiase loading, should be only contributed by the increase of glucose yield. The results suggest that a cellulases loading of 15 FPU/g and a cellobiase loading of 10 CBU/g are required for improved sugars recovery of coastal bermudagrass pretreated with 0.75% (w/v) NaOH at 121°C for 15 min.

It was noted that adding xylanase during hydrolysis did not enhance total reducing sugars production at all. Significant amounts of xylose in the hydroyzate was detected even without adding xylanase into the hydrylosis system. This implies that the cellulases complex used in this research contains hemicellulases such as xylanase. It was reported that cellulases (Celluclast) and β-glucosidase (Novozyme 188) had xylanase activity (Saha et al., 2005). Saddler et al. (1983) found that the xylanase activity was even higher than that of β-

glucosidase in cellobiase. Therefore, the cellulases system with addition of cellobiase was sufficient for improved total reducing sugars production.

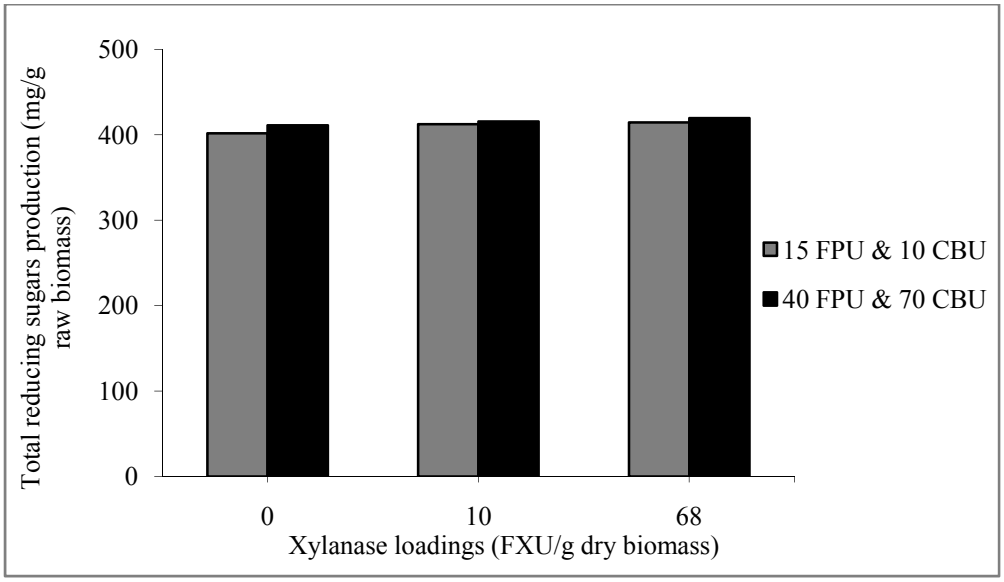

Fig. 5.3. Total reducing sugars production from NaOH-pretreated coastal bermudagrass (0.75% (w/v) NaOH pretreatment at 121°C for 15 min) under optimal and excessive cellulases and cellobiase loadings with xylanase supplementation.

5.4.2 Lime pretreatment

The recommended conditions for lime pretreatment of coastal bermudagrass were reported as 0.1 g/g lime loading, 100°C, and 15 min. Similar to enzyme loading study for NaOH-pretreated coastal bermudagrass, cellulases loading test was first investigated for lime-pretreated coastal bermudagrass. With an excessive cellobiase loading of 70 CBU/g, the increase of cellulases dose from 0 FPU/g to 20 FPU/g did result in improved total reducing sugars production for the lime-pretreated biomass. However, there was no significant difference in sugar recovery between 20 FPU/g and 40 FPU/g. The cellulases loading of 20 FPU/g was adequate for maximizing glucose yield as well (Fig. 5.4). It was noted that the op-

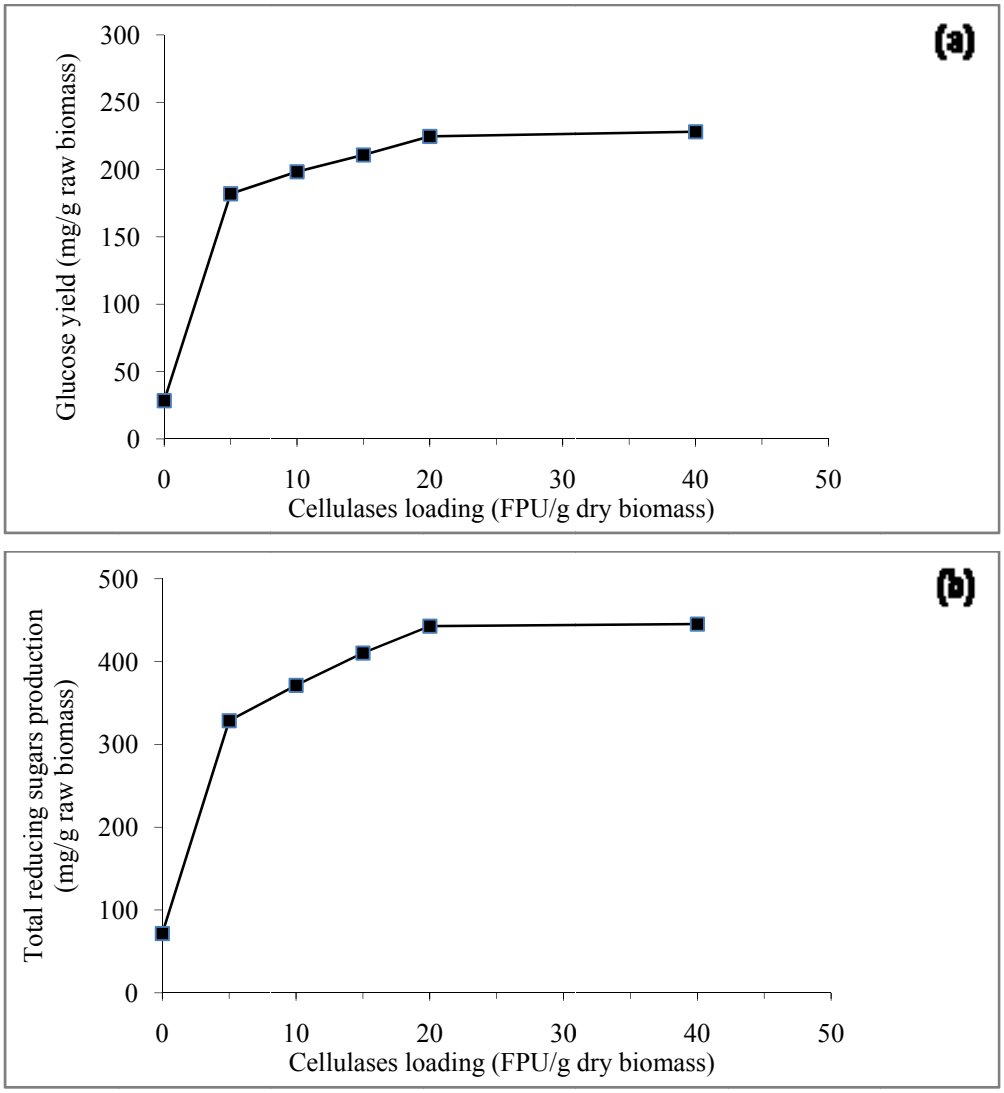

Fig. 5.4. Glucose (a) and total reducing sugars (b) production from lime-pretreated coastal bermudagrass (lime dose of 0.1 g/g dry biomass at 100°C for 15 min) under different cellulases loadings with excessive cellobiase of 70 CBU/g dry biomass.

timal dosage of cellulases (20 FPU/g) for lime-pretreated biomass was higher than that (15 FPU/g) for NaOH-pretreated biomass. The action of cellulases can be impacted by substrate properties such as crystallinity of pretreated biomass, degree of polymerization, accessible area, and lignin content which depend on the category of biomass and the choice of pretreat-

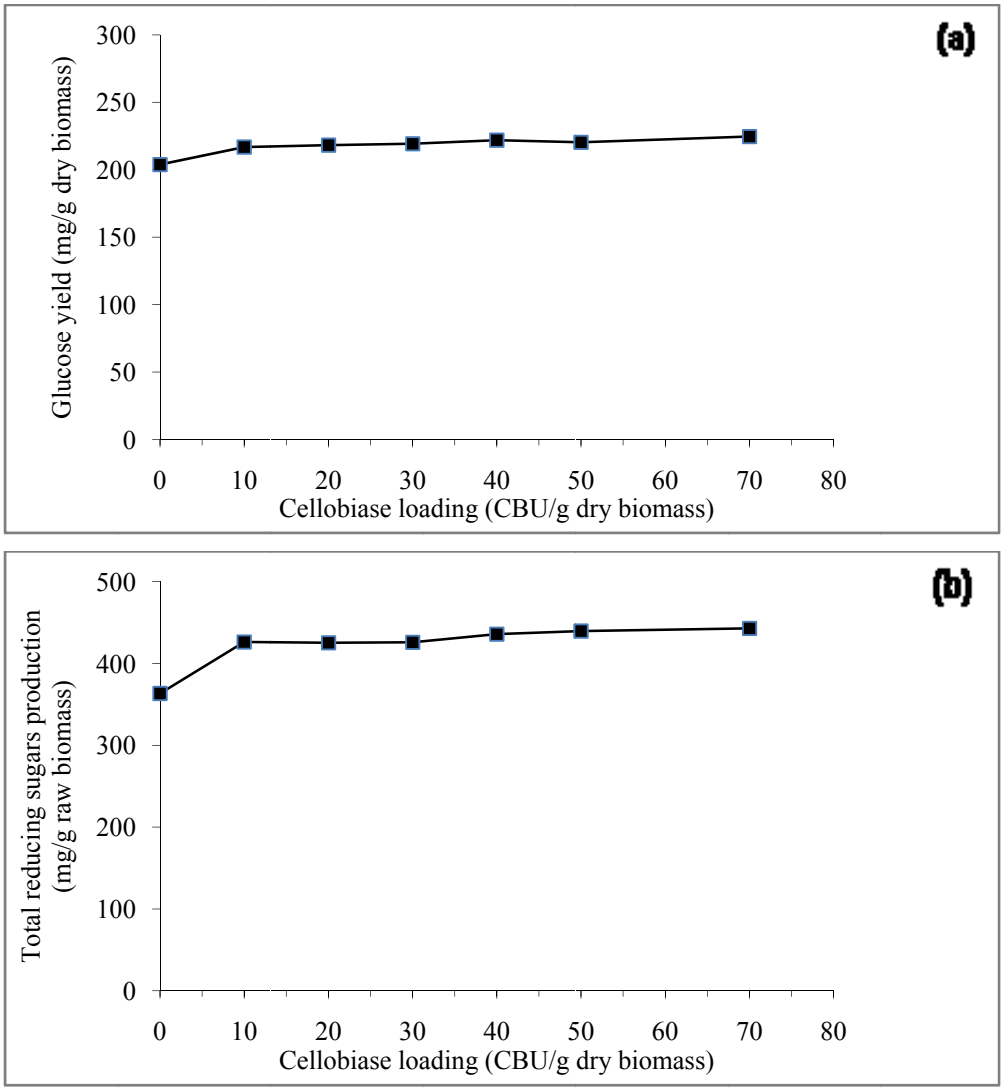

Fig. 5.5. Glucose (a) and total reducing sugars (b) production from lime-pretreated coastal bermudagrass (lime dose of 0.1 g/g dry biomass at 100°C for 15 min) under different cellobiase loadings with optimal cellulases loading of 20 FPU/g dry biomass.

ment method (Zhang and Lynd, 2004). The pretreatment method can create enzyme inhibiting products that can lower sugars yield and result in increased enzyme dose. Lignin can interfere with the cellulases performance by acting as a competitive cellulases adsorbent that reduces the amount of cellulases available to hydrolyze cellulose (Bernardez et al., 1993;

Ooshima et al., 1990) or by blocking the enzymatic access to its substrate (Eriksson et al., 2002). The previous results showed that lime is less effective in lignin removal than sodium hydroxide during pretreatment. More residual lignin was found in lime-pretreated biomass, thereby, leading to higher loading of cellulases needed for enhanced sugars recovery due to the inhibition of enzymatic hydrolysis by the remained lignin.

With the increase of cellobiase dose from 0 CBU/g to 10 CBU/g under the condition of optimal cellulases dose at 20 FPU/g, total reducing sugars production as well as glucose yield was significantly improved (Fig. 5.5). No continued increase was observed when using cellobiase loading more than 10 CBU/g. The cellulases dose of 20 FPU/g and cellobiase dose of 10 CBU/g were recommended for enhancing sugars recovery of lime-pretreated (a lime loading of 0.1 g/g at 100°C for 15 min) coastal bermudagrass. As the NaOH-pretreated bermudagrass, the supplementation of xylanase for the lime-pretreated biomass was not favo-

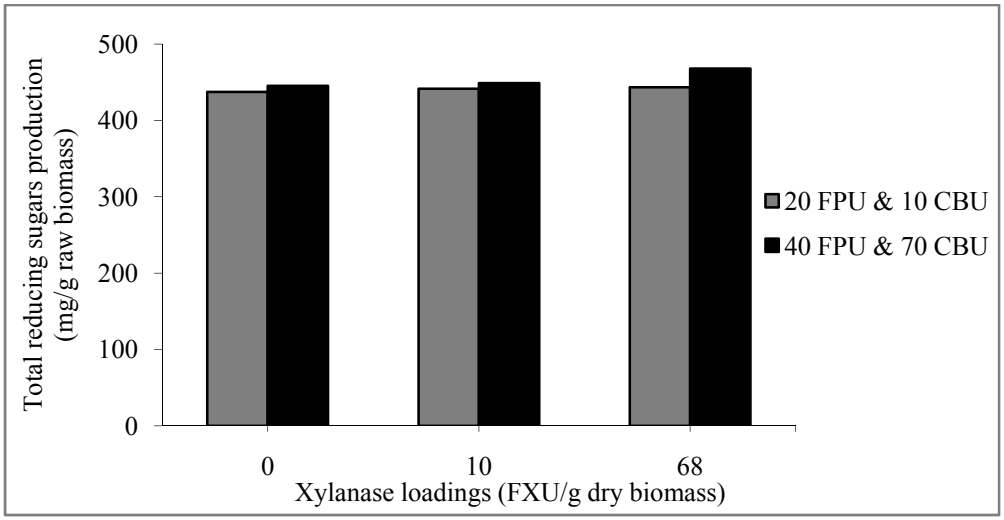

Fig. 5.6. Total reducing sugars production from lime-pretreated coastal bermudagrass (lime dose of 0.1 g/g dry biomass at 100°C for 15 min) under optimal and excessive cellulases and cellobiase loadings with xylanase supplementation.

rable to increase total reducing sugars yield significantly. The result further indicates that there is no benefit in sugar recovery from adding xylanase into the hydrolysis for the two varieties of alkaline pretreatment for coastal bermudagrass.

5.4.3 Fermentation tests

For costal bermudagrass pretreated under optimal conditions of each pretreatment method, the hydrolyzates from enzymatic hydrolysis using optimal enzymes loadings were fermented at 30°C for 72 h. The yeast strain used in this study had limited capability of utilizing pentoses of which xylose is the predominant monomeric sugar. Because of a neglectable amount of galactose and arabinose compared to glucose and xylose, ethanol yields were all calculated based on either merely glucose or the summation of glucose and xylose. Two ways for calculating ethanol yield in terms of percentage of theoretical maximum ethanol yield were reported. One was to determine ethanol yield for the hydrolyzate. The other was to estimate ethanol yield for the raw biomass by using data from

Table 5.1. Fermentation of hydrolyzate from enzymatic hydrolysis[a] of pretreated coastal bermudagrass.

Ethanol yield	NaOH pretreatment[b]	Lime pretreatment[c]
g/g glucose	0.49	0.49
% of the theoretical yield for hydrolyzate, glu	95.75	96.71
% of the theoretical yield for raw, glu	84.03	82.92
g/g glucose+xylose[d]	0.46	0.46
% of the theoretical yield for hydrolyzate, glu+xyl	90.02	90.02
% of the theoretical yield for raw, glu+xyl	68.10	67.59

[a] optimal enzyme loadings.
[b] 0.75% (w/v) NaOH at 121°C for 15 min.
[c] 0.1 g/g lime loading at 100°C for 15 min.
[d] obtained from Krishnan et al., 1999.

a published paper (Krishnan et al., 1999). The ethanol yield for the hydrolyzate based on glucose was more than 95% of theoretical yield for both pretreatment methods (Table 5.1). This indicated that the fermentation was barely inhibited during the course, with over 99% of glucose in the hydrolyzate consumed. Moreover, the ethanol yield for the raw coastal bermudagrass was around 83% of theoretical yield which is very promising with reference to the efficiency of converting sugars to ethanol.

To realize the commercial application of converting lignocellulose to ethanol, both hexoses and pentoses need to be utilized by microorganisms for ethanol production. An ethanol yield of 0.46 g/g sugar (glucose and xylose) (Krishnan et al., 1999) was used to estimate the potential ethanol yield by co-fermenting of glucose and xylose in coastal bermudagrass. The results showed that approximate 68% of theoretical ethanol yield from raw coastal bermudagrass could be achieved if both glucose and xylose were fermentable (Table 5.1). Assuming the production of coastal bermudagrass is 6 tons/acre/year, when based on the theoretical ethanol yield from raw coastal bermudagrass of 44.2 gallons/dry ton biomass for glucose fermentation, the practical ethanol yields from raw coastal bermudagrass by using the optimal pretreatment conditions and enzyme doses are 222.85 gallons/acre/year for NaOH pretreatment and 219.90 gallons for lime pretreatment, respectively.

5.5 CONCLUSIONS

Cellulases and cellobiase are essential for enhancing sugar recovery after alkaline-pretreated coastal bermudagrass. For bermudagrass pretreated with 0.75% (w/v) NaOH at 121°C for 15 min, the optimal cellulases and cellobiase doses were determined to be 15

FPU/g dry biomass and 10 CBU/g dry biomass respectively. On the other hand, for bermudagrass pretreated with a lime loading of 0.1g/g dry biomass at 100°C for 15 min, a cellulases loading of 20 FPU/g and a cellobiase loading of 10 CBU/g were required for improved sugar recovery. Addition of xylanase during hydrolysis was not beneficial for higher sugar recovery, mainly because the presence of xylanase activity in cellulases and cellobiase. More than 99% of glucose in the hydrolyzate was utilized by the yeast strain for ethanol production with 95% of theoretical maximum yield for the hydrolyzate and 83% of theoretical yield for the raw biomass. Co-fermentation of glucose and xylose was proposed using ethanol yield of 0.46 g/g sugar from a published paper, to obtain an estimated ethanol yield of 68% of theoretical maximum. The fermenting ability of pretreatment liquor needs to be examined for the purpose of maximizing ethanol yield from coastal bermudagrass.

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CHAPTER 6

CONCLUSIONS AND FUTURE WORK

6.1 SUMMARY

Coastal bermudagrass, widely grown in the southeast of the United States, has the potential for the production of ethanol which can be used for partial gasoline replacement. Alkaline (specifically sodium hydroxide and lime) pretreatment methods for converting coastal bermudagrass to ethanol were investigated in this thesis. The study focused on the effects of pretreatments on the sugar recovery after enzymatic hydrolysis with excessive enzyme doses. Each pretreatment method was optimized based on a statistical analysis of the total reducing sugars yield as well as the production of glucose and xylose in the hydrolyzate. For the purpose of cost savings, a variety of enzyme loading was evaluated for optimal conditions for both pretreatments. Ethanol yield from the hydrolyzate obtained under optimal pretreatment conditions and enzyme doses was examined with an estimation of potential ethanol yield from coastal bermudagrass including pentose fermentation.

6.2 CONCLUSIONS

Based on the experimental results in this research, a set of conclusions was drawn as follows:

1. The coastal bermudagrass used in this study contains 25.59%(wt basis) glucan, 15.88% xylan, 1.95% arabinan, 1.46% galactan, 15.37% acid insoluble lignin, 3.96% acid soluble lignin, 4.17% extractives, and 6.60% ash.

2. Dilute sodium hydroxide pretreatment at high temperature (121°C) was able to remove 60-80% of lignin from the coastal bermudagrass, while lower temperature pretreatments are not favorable to lignin removal.

3. The optimal NaOH pretreatment conditions at 121°C for total reducing sugars production as well as glucose and xylose yields are 15 min and 0.75% NaOH.

4. The highest reducing sugars yield can reach up to 77% of theoretical maximum for sodium hydroxide pretreatment at 121°C, which is over two times that of untreated biomass.

5. Approximately 91% of glucan and 65% of xylan from coastal bermudagrass were converted into glucose and xylose respectively, when the biomass was treated with 0.75% NaOH for 15 min at 121°C.

6. Sodium hydroxide pretreatment at temperatures below 121°C yielded significantly ($P<0.05$) lower total reducing sugars than at 121°C.

7. Removal of lignin from the biomass did facilitate the digestibility of polysaccharides, however, higher lignin reduction means more solid loss which might cause the decrease of reducing sugars yield in the hydrolyzate.

8. The change of total reducing sugars production with the pretreatment severity had the same trend as the xylan conversion rate.

9. Lime pretreatment was not as efficient as NaOH pretreatment with reference to lignin removal, however, total reducing sugars yield for lime pretreatment was comparable to values for NaOH pretreatment.

10. Increasing pretreatment temperature could reduce optimal residence time for enhanced

sugar recovery.

11. The highest reducing sugars yield for lime pretreatment was 78% of theoretical maximum.

12. Further increasing lime loading larger than 0.1 g/g dry biomass did not significantly (P> 0.05) enhance sugars recovery, whereas the use of lime loadings less than 0.1 g/g impaired the production of total reducing sugars.

13. For lime pretreatment, the optimal condition was found to be 100°C for 15 min with a lime loading of 0.1 g/g dry biomass. Under the recommended condition, 87.4% of glucan and 67.5% of xylan were converted to glucose and xylose respectively.

14. For coastal bermudagrass pretreated with 0.75% (w/v) NaOH at 121°C for 15 min, the optimal cellulases and cellobiase loadings were determined to be 15 FPU/g dry biomass and 10 CBU/g dry biomass respectively.

15. A cellulases loading of 20 FPU/g and a cellobiase loading of 10 CBU/g were required for improved sugar recovery for coastal bermudagrass pretreated with a lime loading of 0.1g/g dry biomass at 100°C for 15 min.

16. Addition of xylanase during hydrolysis was not beneficial to higher sugar recovery.

17. More than 99% of glucose in the hydrolyzate was utilized by the yeast strain for ethanol production with 95% of theoretical maximum yield for the hydrolyzate and 83% of theoretical yield for the raw biomass.

6.3 SUGGESTIONS FOR FUTURE WORK

There are several directions for future work on converting coastal bermudagrass into ethanol. With the experimental results obtained in this study, future possible research is

suggested as follows. It was noted that both sodium hydroxide and lime pretreatments solubilized partial polysaccharides during pretreatment. Composition analysis of the pretreatment liquor and the feasibility of fermenting it for ethanol production are needed for maximizing potential ethanol yield from coastal bermudagrass. Enzymes produced by genetically engineered microorganisms for hydrolyzing lignocellulose are proposed to be applied in the conversion of coastal bermudagrass, and to be compared with the conventional enzymes used in this study. The indication of relationship between pretreatment conditions and sugar recovery addressed in the thesis would be used to develop a predictive pretreatment model that could enable the design of pretreatment factors and the pretreatment process control for the desired sugar production. A variety of pretreatment methods are being investigated for converting coastal bermudagrass into ethanol, therefore, it is highly recommended that an economic analysis of different pretreatment technologies is essential for realizing ethanol production from coastal bermudagrass in a commercial scale.