ABSTRACT

SUN, YE. Enzymatic Hydrolysis of Rye Straw and Bermudagrass for Ethanol Production. (Under the direction of Jiayang Cheng and Philip W. Westerman.)

Dilute sulfuric acid pretreatment of rye straw and bermudagrass was investigated under acid concentrations of 0.6% to 1.5% (w/w) and residence time 30 min to 90 min. The pretreatment effectively solubilized the hemicellulose components to monomeric sugars such as xylose, arabinose, and galactose. Cellulose in the rye straw was not hydrolyzed into glucose during the acid pretreatment, while part of the cellulose in the bermudagrass was converted into glucose by the acid pretreatment. After enzymatic hydrolysis with excessive cellulases, the glucose yields of 30% to 52% and 46% to 81% of the theoretical potentials were obtained for rye straw and bermudagrass, respectively.

The pretreated solid residues were hydrolyzed with cellulases supplemented with β-glucosidase. The addition of β-glucosidase greatly improved the glucose production rate. There was no cellobiose accumulation when the β-glucosidase loading was up to 25 CBU/g (CBU, cellobiase unit, expressed as μmol of cellobiose that is converted into glucose per minute). The conversion rate was 45% for bermudagrass hydrolyzed with cellulases of 10 FPU/g (FPU, filter paper unit, expressed as μmol of glucose produced per min with filter paper as a substrate) and β-glucosidase of 25 CBU/g. The further increase of enzyme loadings did not significantly improve the glucose yield. Rye straw was more resistant to enzymatic hydrolysis than bermudagrass. The conversion rate was 38% with the additions of cellulases at 15 FPU/g and β-glucosidase at 25 CBU/g rye straw.

The production of cellulase enzymes in transgenic plants has the potential to significantly reduce enzyme costs. Expression of cellulase enzyme, *Acidothermus cellulolyticus* E1 endoglucanase, in transgenic duckweed *Lemna minor* was examined. The recombinant E1 protein was biologically active with the expression level of 0.24% of total soluble protein. HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) buffer (pH = 8) extracted more proteins including E1 from duckweed fronds than sodium citrate buffer (pH = 4.8) and sodium acetate buffer (pH = 5). The E1 protein was thermotolerant and exhibited the optimal enzyme activity at temperature 80°C and pH 5. The E1 activity remained unchanged after heating at 60°C for 6 h. However, it was inactivated after 15-min heating at 90°C.

ENZYMATIC HYDROLYSIS OF RYE STRAW AND BERMUDAGRASS FOR ETHANOL PRODUCTION

by

YE SUN

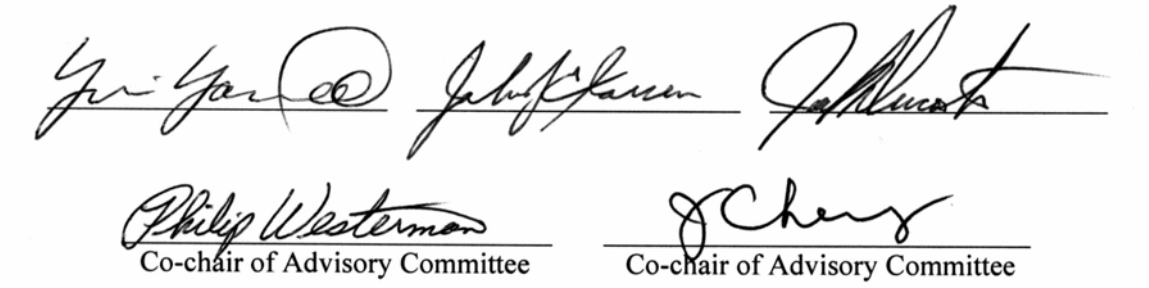
A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

BIOLOGICAL AND AGRICULTURAL ENGINEERING

Raleigh, North Carolina

2002

APPROVED BY:



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BIOGRAPHY

Ye Sun was born on January 13, 1973 in Shenyang, P.R. China. As the first daughter of her parents, she spent a happy childhood with their care. Both of her parents are mechanical engineers who dedicated themselves to the research of machine design and development. Affected by her parents, she was strongly attracted by knowledge and wanted to be an engineer.

Following the secondary education in Shenhe District, Shenyang, Ye Sun matriculated in Food Engineering Department at Tianjin University of Light Industry with major Fermentation Engineering in 1991. She received her Bachelor's degree and entered the graduate school in 1995. After getting her Master's degree in Fermentation Engineering in 1998, she came to US to study at Department of Agricultural and Biosystems Engineering at South Dakota State University. During that time, she married Jun Qu, who she has known for over 13 years. Now they have a lovely son, Steven Qu.

She entered North Carolina State University in the fall of 1999 to pursue her doctoral degree under the guidance of her advisors Dr. Jiayang Cheng, Dr. Philip Westerman, and her committee. She dedicated herself to the research during the past three years and improved her capability to learn, think and work independently.

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Jiayang Cheng, for his guidance, help and support throughout this work. I am thankful to Dr. Yuri Yamamoto for her helpful suggestions and criticisms in the course of the study. Her efforts, enthusiasm, and scientific attitudes towards research taught me a lot. I am grateful to Dr. Philip Westerman, Dr. John Classen, and Dr. Joel Ducoste for their kind advice and valuable comments.

I am also thankful to Dr. John Singleton for his assistance in the use of HPLC and Rachel Huie, Christine Hayes and Tracey Daly for their help and cooperation in the lab work.

I am grateful to my parents for their encouragement, support, and patience that accompany me all through my life. Without their help to take care of my son during the last year of my Ph.D. study, I could not focus on the research work. I would like to express my special appreciation to my husband, Jun Qu. His love, understanding and companionship are always the power for my study.

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INTRODUCTION

1.1 BACKGROUND

1.1.1 Corn-based Ethanol Production

Use of ethanol as fuel can be dated back to 1908 when Henry Ford first designed his Model T automobile (U.S. Department of Energy, Biofuels program). The early efforts to sustain ethanol production in USA did not succeed because of the availability of petroleum at low cost and high corn price. Interest in the ethanol fuel resumed in the 1970s, when oil embargo from the Middle East caused the concerns of national security and ethanol was used as a gasoline octane booster instead of lead (Dipardo, 2000). The ethanol production in USA escalated from 175 million gallons in 1980 to 1.77 billion gallons in 2001 (Renewable Fuels Association, ethanol report, 2002). Demand for ethanol could increase further with the phaseout of methyl tertiary butyl ether (MTBE) in gasoline and the reduction of non-renewable fossil fuels.

Currently, corn is the primary raw material for ethanol production in USA. Approximately 655 million bushels of corn were used for ethanol production in 2001, which accounted for 92% of the total feedstocks in ethanol industry (Francl, 2002). Starch, an α-1,4-linked glucose polymer that constitutes about 70% of the corn kernel, is easy to be broken down to glucose that can be further fermented to ethanol. There are basically two methods for corn-based ethanol production: dry milling and wet milling (Fig. 1.1). α-Amylase enzyme is used at the liquefaction stage to cleave the α-1,4

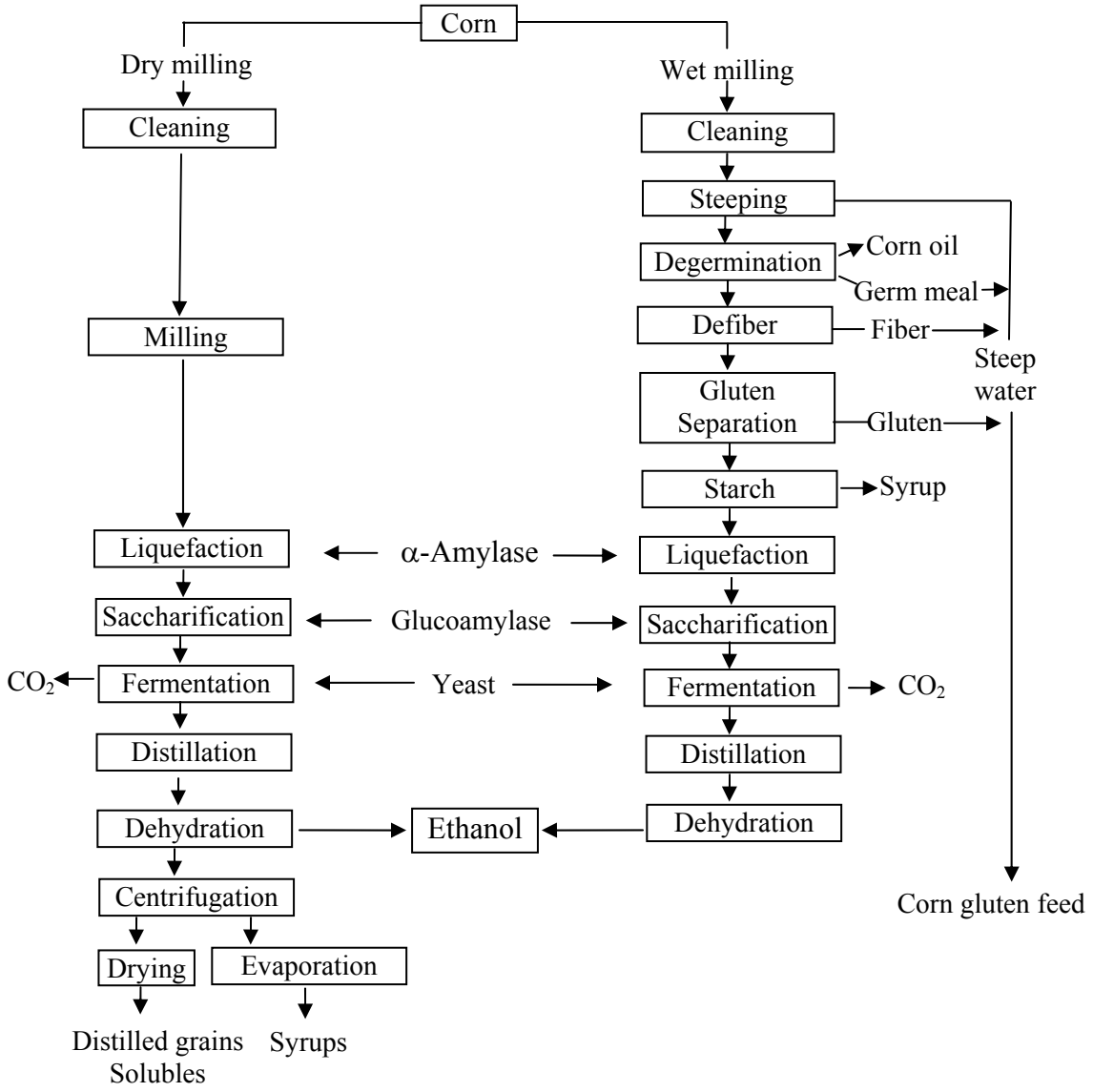

Fig. 1.1. The flowchart of corn dry milling and wet milling processes.

linkages in amylose and amylopectin to produce maltose and higher oligomers with various chain length in both methods. In the subsequent saccharification, glucoamylase is used to liberate glucose molecules from the nonreducing end of maltose and higher oligomers. Wet milling requires steeping to soften the corn hull and the separation of

germ, fiber, gluten and starch before liquefaction, while dry milling uses the entire mash for enzymatic hydrolysis. Compared to dry milling, wet milling is more elaborate and capital intensive because of its complex process. However, wet milling produces more valuable co-products such as corn oil, gluten feed, and germ meal, etc. More than 60% of the ethanol in USA is produced by wet milling (Elander and Putsche, 1996).

The ethanol production technology has developed rapidly over the last 30 years. It was reported that the production cost of corn-based ethanol had dropped from $2.47/gallon in 1978 to $1.43/gallon in 1994, and the production cost in 1999 was estimated to be $0.88/gallon with the assumptions that ethanol yield was 114 gallons per dry ton of corn and corn price was $0.68/gallon of ethanol produced (McAloon, 2000). The cost reduction is caused by several factors: the improvement of conversion technology that increased ethanol yield from 2.5 gallons/bushel corn to more than 2.7 gallons/bushel corn; the technical advances in downstream processing including the use of molecular sieves for ethanol dehydration that eliminated the need to replace desiccant annually and the development of the energy integration techniques that enhanced the energy efficiency during distillation, dehydration, and evaporation; the steady reduction of corn price from $3.24/bushel in 1995 to $1.82/bushel in 1999. The corn-to-ethanol technology has become mature based on the current technology and it is not likely to significantly reduce production cost further. With the current status of corn price as the dominant cost factor, the development of low-cost feedstock is the key to further reduce the cost.

1.1.2. Cellulose-based Ethanol Production

Lignocellulosic biomass has the potential to substantially reduce the ethanol production cost because it is less expensive than corn and available at large quantities. Cellulosic feedstock includes agricultural wastes (wheat straw, corn stover, rice straw, bagasse, grasses, etc.), forest residues, and other low-value biomass such as municipal wastes. The lignocellulosic materials are mainly composed of cellulose, hemicellulose, lignin and other minor components such as ash and protein. Cellulose is a linear polymer of anhydro D-glucose units connected by β-1,4 glycosidic bonds (Fig. 1.2). Native cellulose exists in the form of microfibrils (Fig. 1.3a), which are paracrystalline assemblies of several dozen (1→4) β-D-glucan chains with hydrogen bonds connected to one another (Carpita and McCann, 2000). The cellulose microfibrils are embedded in a matrix of noncellulosic polysaccharides, mainly hemicellulose and pectic substances (Fig. 1.3b).

Hemicellulose is a complex, heterogeneous mixture of sugars and sugar derivatives that form a highly branched network (Hopkins, 1999). The monomers that comprise hemicellulose are hexoses (glucose, galactose, and mannose) and pentoses (arabinose and xylose). Some monomers are acetylated. Hemicellulose can be classified into three groups, namely, xylans, mannans, and galactans based on the polymer backbone that is very often homopolymeric with β-1,4 linkage. Xylan is by far the most important component because of its large quantities in the biomass. It was reported that grasses contain 20-40% of arabinoxylans (Fig. 1.4), while the principal hemicellulose in hardwood is glucomannan and methylglucuronoxylan (Brigham et al., 1996).

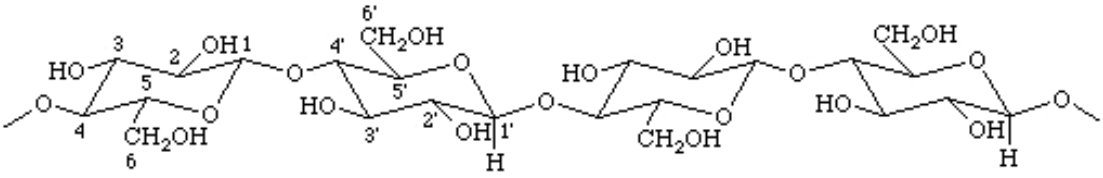

Fig. 1.2. Linkage structures of cellulose.

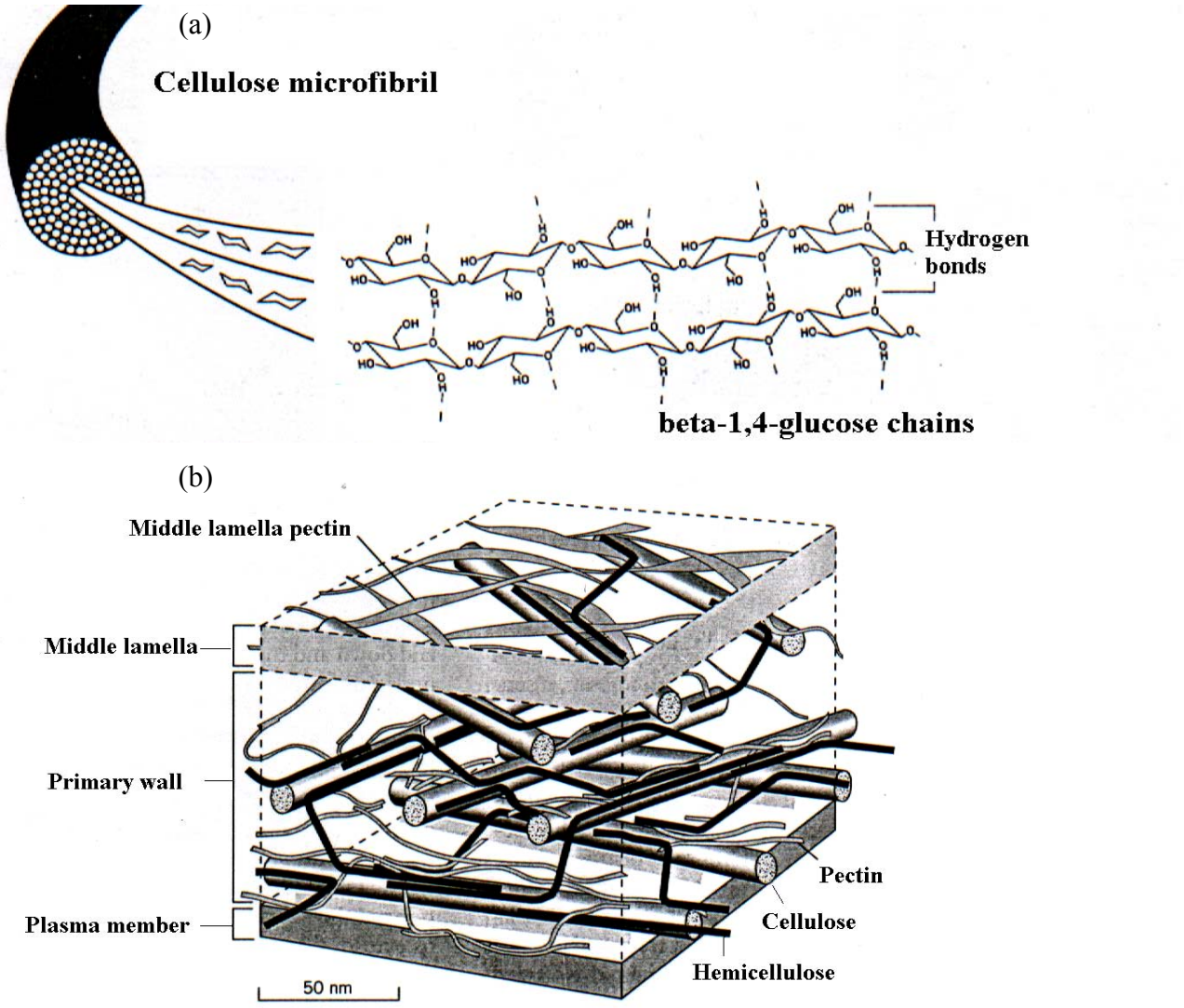

Fig. 1.3. A simplified model to illustrate the cross-linking of cellulose microfibrils and hemicellulose in the lignocellulosic biomass. Source: Hopkins, W.G., 1999. Introduction to Plant Physiology, second edition. John Wiley & Sons, Inc., New York.

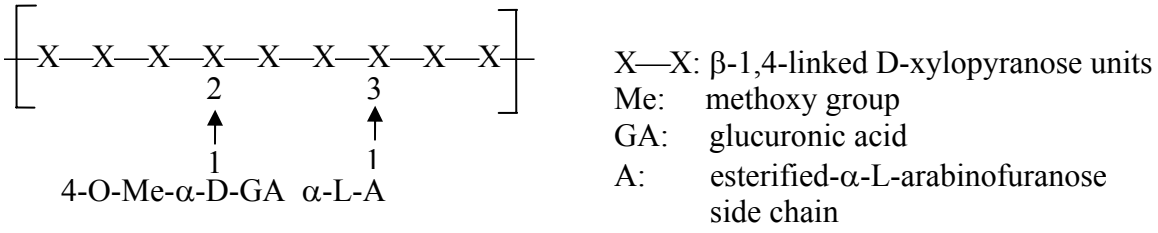

Fig. 1.4. Hemicellulose structure (arabinoxylans).

Cellulosic materials also contain lignin, a three dimensional phenylpropane polymer with phenylpropane units held together by ether and carbon-carbon bonds. When the plant matures and the cell growth ceases, the middle lamella (the cement between the primary walls of adjacent cells) and the secondary cell wall (inside of primary wall) have a large degree of lignin. The lignin strengthens the cell structures by stiffening and holding the fibers of polysaccharides together (Fan et al., 1987).

The cellulose-based bioethanol production includes two steps:

(1) Enzymatic hydrolysis (saccharification):

$$\underset{\text{Cellulose}}{(C_6H_{10}O_5)_n} + nH_2O \xrightarrow{\text{cellulases}} \underset{\text{Glucose}}{nC_6H_{12}O_6}$$

(2) Fermentation:

$$\underset{\text{Glucose}}{C_6H_{12}O_6} \xrightarrow{\text{microbes}} \underset{\text{Ethanol}}{2C_2H_5OH} + 2CO_2$$

The structures of the lignocellulosic biomass, especially cellulose crystallinity, the sheathing of hemicellulose, and the lignin barrier, make it more recalcitrant to enzymatic hydrolysis compared to corn starch. Mechanical or chemical pretreatment is used to break down the hemicellulose and lignin structures in order to improve the substrate digestibility. The flowchart of biomass-to-ethanol conversion process is shown

in Fig. 1.5. Although the biomass feedstock is less expensive than corn feedstock, the cellulose-based ethanol production is more costly. Compare to corn-to-ethanol process, the biomass-to-ethanol production requires extensive processes: the feedstock needs to be baled, washed, shredded, and then milled to small particles before conveyed to the process, which increase the cost for feed handling; the high-temperature pretreatment and long residence time for saccharification and fermentation (6-7 days) compared to two-day starch conversion require more energy. The cost of cellulase enzymes is about \$0.5/gallon of ethanol produced which is more expensive than the cost of α-amylase and glucoamylase (\$0.044/gallon of ethanol produced). In addition, the by-products from corn such as corn gluten feed and corn oil have a higher value than lignin from biomass-ethanol production (DiPardo, 2000; McAloon et al., 2000). These factors listed above contribute to the high cost of ethanol production from lignocellulosic biomass.

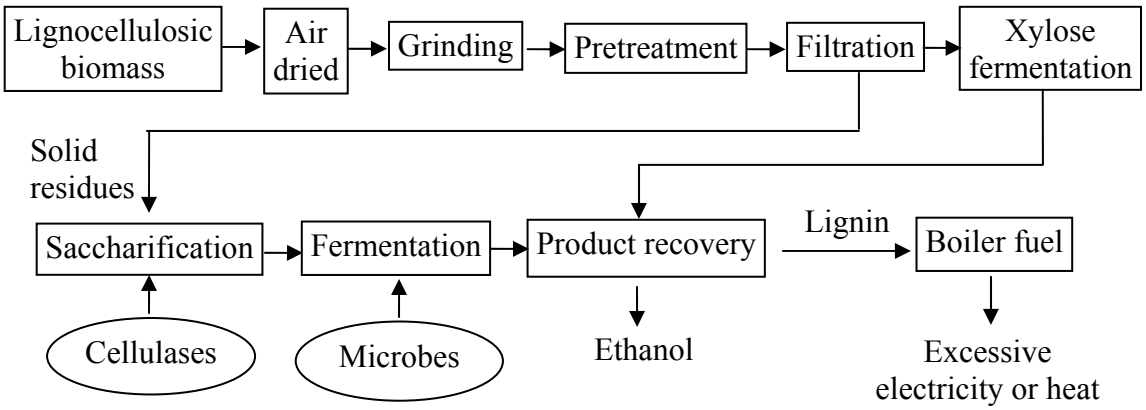

Fig. 1.5. Schematic overview of biomass-to-ethanol conversion process.

The production cost of biomass-based ethanol is estimated to be between \$1.16 and \$1.44 per gallon based on current status, making it a higher-cost alternative to corn-

based ethanol (Wooley et al., 1999). Among all the expenses, the cellulase cost of $0.5/gallon of ethanol produced is especially high for commercial use. With the development of biotechnology and availability of sufficient market, it was estimated that the cellulase cost could be reduced to less than $0.10/gallon of ethanol and the ethanol cost could be as low as $0.6/gallon by 2015 (DiPardo, 2000). Therefore, cellulosic materials have the capability for cost-effective ethanol production.

1.2 OBJECTIVES OF THE STUDY

With the rapid expansion of swine production in the southeast of USA, the management of nutrients in the swine wastewater is essential to avoid environmental pollution. Many farmers grow rye in the winter and bermudagrass in the summer and spray the stabilized swine waste in the grass field for nitrogen and phosphorus removal. The harvested rye straw and bermudagrass are usually given away or sold at very low price as animal feed due to the low nutrient values. There is a great interest to utilize the harvested grass for making value-added products. Bioconversion of cellulose and hemicellulose in the biomass into ethanol provides a good potential in that direction.

It was reported that the growth of duckweed on the swine wastewater could remove the N and P effectively (Bergmann, 2000; Cheng et al., 2002). The production of recombinant proteins from transgenic plants has the potential to significantly reduce the enzyme cost (Herbers and Sonnewald, 1996). Duckweed has the characteristics of fast multiplication, high biomass and protein yields, and easy to transform, which make it a good bioreactor for the production of recombinant proteins (Landolt, 1986). Therefore,

development of transgenic duckweed to produce cellulase enzymes has dual applications: nutrient removal from swine wastewater and cellulase production from the harvested duckweed.

An on-site ethanol production system based on the use of rye straw and bermudagrass as feedstocks, and cellulase production from genetically engineered duckweed that utilizes the excessive nutrients in swine wastewater for its biomass accumulation may provide a cost-effective ethanol production process, solve the environmental concerns caused by the disposal of these agricultural wastes, and supply energy for the farm operation.

Although many lignocellulosic materials have been studied, the ethanol production from rye straw and bermudagrass by dilute sulfuric acid pretreatment and enzymatic hydrolysis was neglected. In addition, the expression of thermostable E1 cellulase enzyme in transgenic duckweed provides a novel bioreactor for cellulase production despite the previous study on the cellulase production from transgenic tobacco, potato, and alfafa (Ziegelhoffer et al., 1999; Hooker et al., 2001).

The objectives of this study were:

(1) To investigate the dilute sulfuric acid pretreatment of rye straw and bermudagrass for effective enzymatic hydrolysis of the lignocellulosic materials.

(2) To study the hydrolysis of the pretreated rye straw and bermudagrass using commercial cellulases supplemented with β-glucosidase.

(3) To examine the expression level, activity, extraction conditions, pH and temperature characteristics, and heat stability of recombinant endoglucanase E1 in transgenic duckweed *Lemna minor* 8627.

This thesis consists of one journal paper as chapter 2 (Sun and Cheng, 2002) and three manuscripts (chapters 3-5) with each objective as one topic in each chapter.

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HYDROLYSIS OF LIGNOCELLULOSIC MATERIALS FOR ETHANOL PRODUCTION: A REVIEW

Published at "Bioresource Technology", 83 (2002), 1–11.
Author: Ye Sun, Jiayang Cheng
Received 6 November 2000; received in revised form 30 October 2001; accepted 31 October 2001

2.1 ABSTRACT

Lignocellulosic biomass can be utilized to produce ethanol, a promising alternative energy source for the limited crude oil. There are mainly two processes involved in the conversion: hydrolysis of cellulose in the lignocellulosic biomass to produce reducing sugars, and fermentation of the sugars to ethanol. The cost of ethanol production from lignocellulosic materials is relatively high based on current technologies, and the main challenges are the low yield and high cost of the hydrolysis process. Considerable research efforts have been made to improve the hydrolysis of lignocellulosic materials. Pretreatment of lignocellulosic materials to remove lignin and hemicellulose can significantly enhance the hydrolysis of cellulose. Optimization of the cellulase enzymes and the enzyme loading can also improve the hydrolysis. Simultaneous saccharification and fermentation effectively remove glucose, which is an inhibitor to cellulase activity, thus increase the yield and rate of cellulose hydrolysis.

2.2 INTRODUCTION

Energy consumption has increased steadily over the last century as the world population has grown and more countries have become industrialized. Crude oil has been the major resource to meet the increased energy demand. Campbell and Laherrere (1998) used several different techniques to estimate the current known crude oil reserves and the reserves as yet undiscovered and concluded that the decline in worldwide crude oil production will begin before 2010. They also predicted that annual global oil production would decline from the current 25 billion barrels to approximately 5 billion barrels in 2050. Because the economy in the United States and many other nations depends on oil, the consequences of inadequate oil availability could be severe. Therefore, there is a great interest in exploring alternative energy sources.

Unlike fossil fuels, ethanol is a renewable energy source produced through fermentation of sugars. Ethanol is widely used as a partial gasoline replacement in the U.S. Fuel ethanol that is produced from corn has been used in gasohol or oxygenated fuels since the 1980s. These gasoline fuels contain up to 10% ethanol by volume. As a result, the U.S. transportation sector now consumes about 4,540 million liters of ethanol annually, about 1% of the total consumption of gasoline (Wang et al., 1999). Recently, U.S. automobile manufacturers have announced plans to produce significant numbers of flexible-fueled vehicles that can use an ethanol blend – E85 (85% ethanol and 15% gasoline by volume) – alone or in combination with gasoline. Using ethanol-blended fuel for automobiles can significantly reduce petroleum use and exhaust greenhouse gas

emission (Wang et al., 1999). Ethanol is also a safer alternative to methyl tertiary butyl ether (MTBE), the most common additive to gasoline used to provide cleaner combustion (McCarthy and Tiemann, 1998). MTBE is a toxic chemical compound and has been found to contaminate groundwater. The U.S. Environmental Protection Agency recently announced the beginning of regulatory action to eliminate MTBE in gasoline (Browner, 2000). However, the cost of ethanol as an energy source is relatively high compared to fossil fuels. A dramatic increase in ethanol production using the current cornstarch-based technology may not be practical because corn production for ethanol will compete for the limited agricultural land needed for food and feed production. A potential source for low-cost ethanol production is to utilize lignocellulosic materials such as crop residues, grasses, sawdust, wood chips, and solid animal waste.

Extensive research has been completed on conversion of lignocellulosic materials to ethanol in the last two decades (Dale et al., 1984; Wright, 1988; Azzam, 1989; Cadoche and López, 1989; Reshamwala et al., 1995; Bjerre et al., 1996; Duff and Murray, 1996). The conversion includes two processes: hydrolysis of cellulose in the lignocellulosic materials to fermentable reducing sugars, and fermentation of the sugars to ethanol. The hydrolysis is usually catalyzed by cellulase enzymes, and the fermentation is carried out by yeasts or bacteria. The factors that have been identified to affect the hydrolysis of cellulose include porosity (accessible surface area) of the waste materials, cellulose fiber crystallinity, and lignin and hemicellulose content (McMillan, 1994). The presence of lignin and hemicellulose makes the access of cellulase enzymes to cellulose difficult, thus reducing the efficiency of the hydrolysis. The contents of

cellulose, hemicellulose, and lignin in common agricultural residues are listed in Table 2.1. Removal of lignin and hemicellulose, reduction of cellulose crystallinity, and increase of porosity in pretreatment processes can significantly improve the hydrolysis (McMillan, 1994).

Table 2.1. The contents of cellulose, hemicellulose, and lignin in common agricultural residues and wastes*.

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**	24-29
Swine waste	6.0	28	NA**
Solid cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
Coastal Bermudagrass	25	35.7	6.4
Switch grass	45	31.4	12.0

*Source: Reshamwala et al., 1995; Cheung and Anderson, 1995; Boopathy, 1998; Dewes and Hünsche, 1998.
**NA: not available.

2.3 PRETREATMENT OF LIGNOCELLULOSIC MATERIALS

The effect of pretreatment of lignocellulosic materials has been recognized for a long time (McMillan, 1994). The purpose of the pretreatment is to remove lignin and hemicellulose, reduce cellulose crystallinity, and increase the porosity of the materials. Pretreatment must meet the following requirements: (1) improve the formation of sugars or the ability to subsequently form sugars by enzymatic hydrolysis; (2) avoid the degradation or loss of carbohydrate; (3) avoid the formation of byproducts inhibitory to the subsequent hydrolysis and fermentation processes; and (4) be cost-effective. Physical, physico-chemical, chemical, and biological processes have been used for pretreatment of lignocellulosic materials.

2.3.1 Physical Pretreatment

2.3.1.1 Mechanical comminution

Waste materials can be comminuted by a combination of chipping, grinding and milling to reduce cellulose crystallinity. The size of the materials is usually 10 to 30 mm after chipping and 0.2 to 2 mm after milling or grinding. Vibratory ball milling has been found to be more effective in breaking down the cellulose crystallinity of spruce and aspen chips and improving the digestibility of the biomass than ordinary ball milling (Millet et al., 1976). The power requirement of mechanical comminution of agricultural materials depends on the final particle size and the waste biomass characteristics (Cadoche and López, 1989). A comparison is shown in Table 2.2.

Table 2.2. Energy requirement of mechanical comminution of agricultural lignocellulosic materials with different size reduction (Cadoche and López, 1989).

Lignocellulosic Materials	Final Size mm	Energy Consumption, kWh/ton Knife mill	Hammer mill
Hardwood	1.60	130	130
	2.54	80	120
	3.2	50	115
	6.35	25	95
Straw	1.60	7.5	42
	2.54	6.4	29
Corn stover	1.60	NA*	14
	3.20	20	9.6
	6.35	15	NA*
	9.5	3.2	NA*

*NA: not available.

2.3.1.2 Pyrolysis

Pyrolysis has also been used for pretreatment of lignocellulosic materials. When the materials are treated at temperatures greater than 300°C, cellulose rapidly decomposes to produce gaseous products and residual char (Kilzer and Broido, 1965; Shafizadeh and Bradbury, 1979). The decomposition is much slower, and less volatile products are formed at lower temperatures. Mild acid hydrolysis (1N H_2SO_4, 97°C, 2.5 h) of the residues from pyrolysis pretreatment has resulted in 80 to 85% conversion of cellulose to reducing sugars with more than 50% glucose (Fan et al., 1987). The process can be enhanced with the presence of oxygen (Shafizadeh and Bradbury, 1979). When zinc chloride or sodium carbonate is added as a catalyst, the decomposition of pure cellulose can occur at a lower temperature (Shafizadeh and Lai, 1975).

2.3.2 Physico-Chemical Pretreatment

2.3.2.1 Steam explosion (autohydrolysis)

Steam explosion is the most commonly used method for pretreatment of lignocellulosic materials (McMillan, 1994). In this method, chipped biomass is treated with high-pressure saturated steam and then the pressure is swiftly reduced, which makes the materials undergo an explosive decompression. Steam explosion is typically initiated at a temperature of 160 to 260°C (corresponding pressure 0.69 to 4.83 MPa) for several seconds to a few minutes before the material is exposed to atmospheric pressure. The process causes hemicellulose degradation and lignin transformation due to high temperature, thus increasing the potential of cellulose hydrolysis. Ninety percent efficiency of enzymatic hydrolysis has been achieved in 24 hours for poplar chips pretreated by steam explosion, compared to only 15% hydrolysis of untreated chips (Grous et al., 1986). The factors that affect steam explosion pretreatment are residence time, temperature, chip size and moisture content (Duff and Murray, 1996). Optimal hemicellulose solubilization and hydrolysis can be achieved by either high temperature and short residence time (270°C, 1 min) or lower temperature and longer residence time (190°C, 10 min) (Duff and Murray, 1996). These two conditions had the same effect on the solubilization of hemicellulose, while high temperature resulted in the formation of inhibitory compounds, and the overcooking of the outside of the lignocellulosic materials and incomplete autohydrolysis in the interior. Therefore, recent studies indicated that lower temperature and longer residence time were more favorable (Wright, 1988).

Addition of H_2SO_4 (or SO_2) or CO_2 in steam explosion can effectively improve enzymatic hydrolysis, decrease the production of inhibitory compounds, and lead to more complete removal of hemicellulose (Morjanoff and Gray, 1987). The optimal conditions of steam explosion pretreatment of sugarcane bagasse have been found to be as following: 220°C; 30 s residence time; water-to-solids ratio, 2; and 1% H_2SO_4 (Morjanoff and Gray, 1987). Sugar production was 65.1 g sugars/100 g starting bagasse after steam explosion pretreatment.

The advantages of steam explosion pretreatment include the low energy requirement compared to mechanical comminution and no recycling or environmental costs. The conventional mechanical methods require 70% more energy than steam explosion to achieve the same size reduction (Holtzapple et al., 1989). Steam explosion is recognized as one of the most cost-effective pretreatment processes for hardwoods and agricultural residues, but it is less effective for softwoods (Clark and Mackie, 1987). Limitations of steam explosion include destruction of a portion of the xylan fraction, incomplete disruption of the lignin-carbohydrate matrix, and generation of compounds that may be inhibitory to microorganisms used in downstream processes (Mackie et al., 1985). Because of the formation of degradation products that are inhibitory to microbial growth, enzymatic hydrolysis, and fermentation, pretreated biomass needs to be washed by water to remove the inhibitory materials along with water-soluble hemicellulose (McMillan, 1994). The water wash decreases the overall saccharification yields due to the removal of soluble sugars, such as those generated by hydrolysis of hemicellulose.

Typically, 20 to 25% of the initial dry matter is removed by water wash (Mes-Hartree et al., 1988).

2.3.2.2 Ammonia fiber explosion (AFEX)

AFEX is another type of physico-chemical pretreatment in which lignocellulosic materials are exposed to liquid ammonia at high temperature and pressure for a period of time, and then the pressure is swiftly reduced. The concept of AFEX is similar to steam explosion. In a typical AFEX process, the dosage of liquid ammonia is 1 to 2 kg ammonia/kg dry biomass, temperature 90°C, and residence time 30 minutes. AFEX pretreatment can significantly improve the saccharification rates of various herbaceous crops and grasses. It can be used for the pretreatment of many lignocellulosic materials including alfalfa, wheat straw, wheat chaff (Mes-Hartree et al., 1988), barley straw, corn stover, rice straw (Vlasenko et al., 1997), municipal solid waste, softwood newspaper, kenaf newspaper (Holtzapple et al., 1992a), coastal Bermuda grass, switchgrass (Reshamwala et al., 1995), aspen chips (Tengerdy and Nagy, 1988), and bagasse (Holtzapple et al., 1991). The AFEX pretreatment does not significantly solubilize hemicellulose compared to acid pretreatment (to be discussed in following section) and acid-catalyzed steam explosion (Mes-Hartree et al., 1988; Vlasenko et al., 1997). Mes-Hartree et al. (1988) compared the steam and ammonia pretreatment for enzymatic hydrolysis of aspenwood, wheat straw, wheat chaff, and alfalfa stems, and found that steam explosion solubilized the hemicellulose, while AFEX did not. The composition of the materials after AFEX pretreatment was essentially the same as the original materials. Over 90% hydrolysis of cellulose and hemicellulose has been obtained after AFEX

pretreatment of Bermuda grass (approximately 5% lignin) and bagasse (15% lignin) (Holtzapple et al., 1991). However, the AFEX process was not very effective for the biomass with high lignin content such as newspaper (18 to 30% lignin) and aspen chips (25% lignin). Hydrolysis yield of AFEX-pretreated newspaper and aspen chips was reported as only 40% and below 50%, respectively (McMillan, 1994).

To reduce the cost and protect the environment, ammonia must be recycled after the pretreatment. In an ammonia recovery process, a superheated ammonia vapor with a temperature up to 200°C was used to vaporize and strip the residual ammonia in the pretreated biomass and the evaporated ammonia was then withdrawn from the system by a pressure controller for recovery (Holtzapple et al., 1992b). The ammonia pretreatment does not produce inhibitors for the downstream biological processes, so water wash is not necessary (Dale et al., 1984; Mes-Hartree, 1988). AFEX pretreatment does not require small particle size for efficacy (Holtzapple et al., 1990).

2.3.2.3 CO_2 explosion

Similar to steam and ammonia explosion pretreatment, CO_2 explosion is also used for pretreatment of lignocellulosic materials. It was hypothesized that CO_2 would form carbonic acid and increase the hydrolysis rate. Dale and Moreira (1982) used this method to pretreat alfalfa (4 kg CO_2/kg fiber at the pressure of 5.62 MPa) and obtained 75% of the theoretical glucose released during 24 hours of the enzymatic hydrolysis. The yields were relatively low compared to steam or ammonia explosion pretreatment, but high compared to the enzymatic hydrolysis without pretreatment. Zheng et al. (1998) compared CO_2 explosion with steam and ammonia explosion for pretreatment of recycled

paper mix, sugarcane bagasse, and repulping waste of recycled paper, and found that CO_2 explosion was more cost-effective than ammonia explosion and did not cause the formation of inhibitory compounds that could occur in steam explosion.

2.3.3 Chemical Pretreatment

2.3.3.1 Ozonolysis

Ozone can be used to degrade lignin and hemicellulose in many lignocellulosic materials such as wheat straw (Ben-Ghedalia and Miron, 1981), bagasse, green hay, peanut, pine (Neely, 1984), cotton straw (Ben-Ghedalia and Shefet, 1983), and poplar sawdust (Vidal and Molinier, 1988). The degradation was essentially limited to lignin and hemicellulose was slightly attacked, but cellulose was hardly affected. The rate of enzymatic hydrolysis increased by a factor of 5 following 60% removal of the lignin from wheat straw in ozone pretreatment (Vidal and Molinier, 1988). Enzymatic hydrolysis yield increased from 0 to 57% as the percentage of lignin decreased from 29% to 8% after ozonolysis pretreatment of poplar sawdust (Vidal and Molinier, 1988). Ozonolysis pretreatment has the following advantages: (1) it effectively removes lignin; (2) it does not produce toxic residues for the downstream processes; and (3) the reactions are carried out at room temperature and pressure (Vidal and Molinier, 1988). However, a large amount of ozone is required, making the process expensive.

2.3.3.2 Acid hydrolysis

Concentrated acids such as H_2SO_4 and HCl have been used to treat lignocellulosic materials. Although they are powerful agents for cellulose hydrolysis, concentrated acids are toxic, corrosive and hazardous and require reactors that are resistant to corrosion. In

addition, the concentrated acid must be recovered after hydrolysis to make the process economically feasible (Sivers and Zacchi, 1995).

Dilute acid hydrolysis has been successfully developed for pretreatment of lignocellulosic materials. The dilute sulfuric acid pretreatment can achieve high reaction rates and significantly improve cellulose hydrolysis (Esteghlalian et al., 1997). At moderate temperature, direct saccharification suffered from low yields because of sugar decomposition. High temperature in dilute acid treatment is favorable for cellulose hydrolysis (McMillan, 1994). Recently developed dilute-acid hydrolysis processes use less severe conditions and achieve high xylan to xylose conversion yields. Achieving high xylan to xylose conversion yields is necessary to achieve favorable overall process economics because xylan accounts for up to a third of the total carbohydrate in many lignocellulosic materials (Hinman et al., 1992). There are primarily two types of dilute acid pretreatment processes: high temperature (T greater than 160°C), continuous-flow process for low solids loading (5% to 10% [weight of substrate/weight of reaction mixture]) (Brennan et al., 1986; Converse et al., 1989), and low temperature (T less than 160°C), batch process for high solids loading (10 to 40%) (Cahela et al., 1983; Esteghlalian et al., 1997). Although dilute acid pretreatment can significantly improve the cellulose hydrolysis, its cost is usually higher than some physico-chemical pretreatment processes such as steam explosion or AFEX. A neutralization of pH is necessary for the downstream enzymatic hydrolysis or fermentation processes.

2.3.3.3 Alkaline hydrolysis

Some bases can also be used for pretreatment of lignocellulosic materials and the effect of alkaline pretreatment depends on the lignin content of the materials (Fan et al., 1987; McMillan, 1994). The mechanism of alkaline hydrolysis is believed to be saponification of intermolecular ester bonds crosslinking xylan hemicelluloses and other components, for example, lignin and other hemicellulose. The porosity of the lignocellulosic materials increases with the removal of the crosslinks (Tarkow and Feist, 1969). Dilute NaOH treatment of lignocellulosic material caused swelling, leading to an increase in internal surface area, a decrease in the degree of polymerization, a decrease in crystallinity, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure (Fan et al., 1987). The digestibility of NaOH-treated hardwood increased from 14% to 55% with the decrease of lignin content from 24-55% to 20%. However, no effect of dilute NaOH pretreatment was observed for softwoods with lignin content greater than 26% (Millet et al., 1976). Dilute NaOH pretreatment was also effective for the hydrolysis of straws with relatively low lignin content of 10 to 18% (Bjerre et al., 1996). Chosdu et al. (1993) used the combination of irradiation and 2% NaOH for pretreatment of corn stalk, cassava bark and peanut husk. The glucose yield of corn stalk was 20% in untreated samples compared to 43% after treatment with electron beam irradiation at the dose of 500 kGy and 2% NaOH, but the glucose yields of cassava bark and peanut husk were only 3.5% and 2.5%, respectively.

Ammonia was also used for the pretreatment to remove lignin. Iyer et al. (1996) described an ammonia recycled percolation process (temperature, 170°C; ammonia

concentration, 2.5 to 20%; reaction time, 1 h) for the pretreatment of corn cobs/stover mixture and switchgrass. The efficiency of delignification was 60 to 80% for corn cobs and 65 to 85% for switchgrass.

2.3.3.4 Oxidative delignification

Lignin biodegradation could be catalyzed by the peroxidase enzyme with the presence of H_2O_2 (Azzam, 1989). The pretreatment of cane bagasse with hydrogen peroxide greatly enhanced its susceptibility to enzymatic hydrolysis. About 50% lignin and most hemicellulose were solubilized by 2% H_2O_2 at 30°C within 8 hours, and 95% efficiency of glucose production from cellulose was achieved in the subsequent saccharification by cellulase at 45°C for 24 hours (Azzam, 1989). Bjerre et al. (1996) used wet oxidation and alkaline hydrolysis of wheat straw (20 g straw/l, 170°C, 5 to 10 min), and achieved 85% conversion yield of cellulose to glucose.

2.3.3.5 Organosolv process

In the organosolv process, an organic or aqueous organic solvent mixture with inorganic acid catalysts (HCl or H_2SO_4) is used to break the internal lignin and hemicellulose bonds. The organic solvents used in the process include methanol, ethanol, acetone, ethylene glycol, triethylene glycol and tetrahydrofurfuryl alcohol (Chum et al., 1988; Thring et al., 1990). Organic acids such as oxalic, acetylsalicylic and salicylic acid can also be used as catalysts in the organosolv process (Sarkanen, 1980). At high temperatures (above 185°C), the addition of catalyst was unnecessary for satisfactory delignification (Sarkanen, 1980; Aziz and Sarkanen, 1989). Usually, a high yield of xylose can be obtained with the addition of acid. Solvents used in the process

need to be drained from the reactor, evaporated, condensed and recycled to reduce the cost. Removal of solvents from the system is necessary because the solvents may be inhibitory to the growth of organisms, enzymatic hydrolysis, and fermentation.

2.3.4 Biological Pretreatment

In biological pretreatment processes, microbes such as brown-, white- and soft-rot fungi are used to degrade lignin and hemicellulose in waste materials (Schurz, 1978). Brown rots mainly attack cellulose, while white and soft rots attack both cellulose and lignin. White-rot fungi are the most effective basidiomycetes for biological pretreatment of lignocellulosic materials (Fan et al., 1987). Hatakka (1983) studied the pretreatment of wheat straw by 19 white-rot fungi and found that 35% of the straw was converted to reducing sugars by *Pleurotus ostreatus* in five weeks. Similar conversion was obtained in the pretreatment by *Phanerochaete sordida* 37 and *Pycnoporus cinnabarinus* 115 in four weeks. In order to prevent the loss of cellulose, cellulase-less mutant of *Sporotrichum pulverulentum* was developed for the degradation of lignin in wood chips (Ander and Eriksson, 1977). Akin et al. (1995) also reported the delignification of bermudagrass by white-rot fungi. The biodegradation of bermudagrass stems was improved by 29 to 32% using *Ceriporiopsis subvermispora* and 63 to 77% using *Cyathus stercoreus* after 6 weeks.

The white-rot fungus *Phanerochaete chrysosporium* produces lignin-degrading enzymes, lignin peroxidases and manganese-dependent peroxidases, during secondary metabolism in response to carbon or nitrogen limitation (Boominathan and Reddy, 1992). Both enzymes have been found in the extracellular filtrates of many white-rot fungi for

the degradation of wood cell walls (Kirk and Farrell, 1987; Waldner, et al., 1988). Other enzymes including polyphenol oxidases, laccases, H_2O_2 producing enzymes and quinone-reducing enzymes can also degrade lignin (Blanchette, 1991). The advantages of biological pretreatment include low energy requirement and mild environmental conditions. However, the rate of hydrolysis in most biological pretreatment processes is very low.

2.4 ENZYMATIC HYDROLYSIS OF CELLULOSE

Enzymatic hydrolysis of cellulose is carried out by cellulase enzymes which are highly specific (Béguin and Aubert, 1994). The products of the hydrolysis are usually reducing sugars including glucose. Utility cost of enzymatic hydrolysis is low compared to acid or alkaline hydrolysis because enzyme hydrolysis is usually conducted at mild conditions (pH 4.8 and temperature 45 to 50°C) and does not have a corrosion problem (Duff and Murray, 1996). Both bacteria and fungi can produce cellulases for the hydrolysis of lignocellulosic materials. These microorganisms can be aerobic or anaerobic, mesophilic or thermophilic. Bacteria belonging to *Clostridium, Cellulomonas, Bacillus, Thermomonospora, Ruminococcus, Bacteriodes, Erwinia, Acetovibrio, Microbispora,* and *Streptomyces* can produce cellulases (Bisaria, 1991). *Cellulomonas fimi* and *Thermomonospora fusca* have been extensively studied for cellulase production. Although many cellulolytic bacteria, particularly the cellulolytic anaerobes such as *Clostridium thermocellum* and *Bacteroides cellulosolvens* produce cellulases with high

specific activity, they do not produce high enzyme titres (Duff and Murray, 1996). Because the anaerobes have a very low growth rate and require anaerobic growth conditions, most research for commercial cellulase production has focused on fungi (Duff and Murray, 1996).

Fungi that have been reported to produce cellulases include *Sclerotium rolfsii, Phanerochete chrysosporium* and species of *Trichoderma, Aspergillus, Schizophyllum* and *Penicillium* (Sternberg, 1976; Fan et al., 1987; Duff and Murray, 1996). Of all these fungal genera, *Trichoderma* has been most extensively studied for cellulase production (Sternberg, 1976).

Cellulases are usually a mixture of several enzymes. At least three major groups of cellulases are involved in the hydrolysis process: (1) endoglucanase (EG, endo-1,4-D-glucanohydrolase, or EC 3.2.1.4. EC 3.2.1.4 is the enzyme nomenclature of International Union of Biochemistry and Molecular Biology for endoglucanase) which attacks regions of low crystallinity in the cellulose fiber, creating free-chain ends, (2) exoglucanase or cellobiohydrolase (CBH, 1,4-β-D-glucan cellobiohydrolase, or EC 3.2.1.91) which degrades the molecule further by removing cellobiose units from the free-chain ends, (3) β-glucosidase (EC 3.2.1.21) which hydrolyzes cellobiose to produce glucose (Coughlan and Ljungdahl, 1988). In addition to the three major groups of cellulase enzymes, there are also a number of ancillary enzymes that attack hemicellulose, such as glucuronidase, acetylesterase, xylanase, β-xylosidase, galactomannanase and glucomannanase (Duff and Murray, 1996). During the enzymatic hydrolysis, cellulose is degraded by the cellulases to reducing sugars that can be fermented by yeasts or bacteria to ethanol.

2.5 IMPROVING ENZYMATIC HYDROLYSIS

The factors that affect the enzymatic hydrolysis of cellulose include substrates, cellulase activity, and reaction conditions (temperature, pH, as well as other parameters). To improve the yield and rate of the enzymatic hydrolysis, research has focused on optimizing the hydrolysis process and enhancing cellulase activity (Cantwell et al., 1988; Durand et al., 1988; Orpin, 1988).

2.5.1 Substrates

Substrate concentration is one of the main factors that affect the yield and initial rate of enzymatic hydrolysis of cellulose. At low substrate levels, an increase of substrate concentration normally results in an increase of the yield and reaction rate of the hydrolysis (Cheung and Anderson, 1997). However, high substrate concentration can cause substrate inhibition, which substantially lowers the rate of the hydrolysis. The extent of substrate inhibition was dependent on the ratio of total enzyme to total substrate and the optimum enzyme to substrate ratio was observed to be 0.8 FPU (FPU, filter paper unit, is defined as the micromole of reducing sugar as glucose produced by 1 ml of enzyme per minute) of cellulase from *T. reesei* per gram of microcrystalline substrate Avicel pH105 (Penner and Liaw, 1994). Huang and Penner (1991) reported that the substrate inhibition occurred when the ratio of microcrystalline substrate Avicel pH101 to cellulase from *T. reesei* (grams of cellulose/FPU of enzyme) is greater than 5. The susceptibility of cellulosic substrates to cellulases depends on the structural features of the substrate including cellulose crystallinity, degree of cellulose polymerization, surface

area, and content of lignin. Lignin interferes with hydrolysis by blocking access of cellulases to cellulose and by irreversibly binding hydrolytic enzymes. Therefore, removal of lignin can dramatically increase the hydrolysis rate (McMillan, 1994).

2.5.2 Cellulases

Increasing the dosage of cellulases in the process, to a certain extent, can enhance the yield and rate of the hydrolysis, but would significantly increase the cost of the process. Cellulase dosage of 10 FPU/g cellulose is often used in laboratory studies because it provides a hydrolysis profile with high levels of glucose yield in a reasonable time (48 to 72 h) at a reasonable enzyme cost (Gregg and Saddler, 1996). Cellulase enzyme loadings in hydrolysis vary from 7 to 33 FPU/g substrate, depending on the type and concentration of substrates.

Enzymatic hydrolysis of cellulose consists of three steps: adsorption of cellulase enzymes onto the surface of the cellulose, the biodegradation of cellulose to fermentable sugars, and desorption of cellulases. Cellulase activity decreases during the hydrolysis. The irreversible adsorption of cellulase on cellulose is partially responsible for this deactivation (Converse et al., 1988). Addition of surfactants during hydrolysis is capable of modifying the cellulose surface property and minimizing the irreversible binding of cellulases on cellulose. The surfactants used in the enzymatic hydrolysis include nonionic Tween 20, 80 (Wu and Ju, 1998), polyoxyethylene glycol (Park et al., 1992), Tween 81, Emulgen 147, amphoteric Anhitole 20BS, cationic Q-86W (Ooshima et al., 1986), sophorolipid, rhamnolipid, and bacitracin (Helle et al., 1993). Inhibitory effects have been observed with cationic Q-86W at high concentration and anionic surfactant

Neopelex F-25 (Ooshima et al., 1986). Nonionic surfactants are therefore believed to be more suitable for enhancing the cellulose hydrolysis. The rate of enzymatic hydrolysis was improved by 33% using Tween 80 as a surfactant in the hydrolysis of newspaper (Castanon and Wilke, 1981). Wu and Ju (1998) tested Pluronic F68 and F88 (BASF) and Tween 20 and 80 for enhancing the enzymatic hydrolysis of pretreated newsprint (Table 2.3). The cellulose conversion with 2% (w/v) F68 and 2 g/l cellulase reached 52%, which was about the same as 48% conversion in a surfactant-free system with much higher cellulase loading (10 g/l). However, Tween 20 was highly inhibitory to *D. clausenii* even at a low concentration of 0.1%.

Table 2.3. Effects of different surfactants on hydrolysis of cellulose newsprint* (Wu and Ju, 1998).

Surfactants		Cellulose Conversion (%)			
Type	Concentration (%)	10 h	15 h	44.5 h	123.5 h
Control	0	11.9	17.5	20.7	27.5
Tween 20	0.5	14.1	21.6	27.2	43.6
	2.0	16.0	24.7	32.1	46.8
Tween 80	0.5	14.5	22.0	28.0	43.1
	2.0	14.2	24.7	29.6	43.6
F68	0.5	17.3	26.7	34.4	51.0
	2.0	16.6	27.5	34.0	56.5
F88	0.5	15.4	24.7	32.8	47.8
	2.0	14.5	24.6	33.9	51.2

*Enzyme loading: 2 g/l; Solid substrate concentration: 10%.

Use of a cellulase mixture from different microorganisms or a mixture of cellulases and other enzymes in the hydrolysis of cellulosic materials has been extensively studied (Beldman et al., 1988; Excoffier et al., 1991; Xin et al., 1993). The addition of β-glucosidase into the *T. reesei* cellulases system achieved better saccharification than the system without β-glucosidase (Excoffier et al., 1991; Xin et al., 1993). β-Glucosidase hydrolyzes the cellobiose which is an inhibitor of cellulase activity. A mixture of hemicellulases or pectinases with cellulases exhibited a significant increase in the extent of cellulose conversion (Ghose and Bisaria, 1979; Beldman et al., 1984). A cellulose conversion yield of 90% was achieved in the enzymatic saccharification of 8% alkali-treated sugar-cane bagasse when a mixture of cellulases (dose, 1.0 FPU/g substrate) from *Aspergillus ustus* and *Trichoderma viride* was used (Mononmani and Sreekantiah, 1987). A nearly complete saccharification of steam-explosion pretreated *Eucalyptus viminalis* chips (substrate concentration of 6% and enzyme loading of 10 FPU/g cellulose) was obtained using a cellulase mixture of commercial Celluclast and Novozym preparations (Ramos et al., 1993). Baker et al. (1994) found that a new thermostable endoglucanase, *Acidothermus cellulolyticus* E1, and another bacterial endoglucanase, *Thermomonospora fusca* E5 exhibited striking synergism with *Trichoderma reesei* CBH1 in the saccharification of microcrystalline cellulose.

Cellulases can be recovered from the liquid supernatant or the solid residues and most recycled cellulases are from the liquid supernatant. Enzyme recycling can effectively increase the rate and yield of the hydrolysis and lower the enzyme cost (Mes-

Hartree et al., 1987). Ramos et al. (1993) reported that the enzyme mixture of the commercial Celluclast and Novozym preparation was successfully recycled for five consecutive steps with an elapsed time of 48 h between each recycling step. The efficiency of cellulose hydrolysis decreased gradually with each recycling step.

2.5.3 End-Product Inhibition of Cellulase Activity

Cellulase activity is inhibited by cellobiose and to a lesser extent by glucose. Several methods have been developed to reduce the inhibition, including the use of high concentrations of enzymes, the supplementation of β-glucosidase during hydrolysis, and the removal of sugars during hydrolysis by ultrafiltration or simultaneous saccharification and fermentation (SSF). The SSF process has been extensively studied to reduce the inhibition by end products of hydrolysis (Takagi et al., 1977; Blotkamp et al., 1978; Szczodrak and Targonski, 1989; Saxena et al., 1992; Philippidis et al., 1993; Zheng et al., 1998). In the process, reducing sugars produced in cellulose hydrolysis or saccharification are simultaneously fermented to ethanol, which greatly reduces the product inhibition to the hydrolysis.

The microorganisms used in the SSF are usually fungi *T. reesei* and yeast *S. cerevisiae*. The optimal temperature for SSF is around 38°C, which is a compromise between the optimal temperatures for hydrolysis (45 to 50°C) and fermentation (30°C) (Philippidis, 1996). Hydrolysis is usually the rate-limiting process in SSF (Philippidis and Smith, 1995). Thermotolerant yeasts and bacteria have been used in the SSF to raise the temperature close to the optimal hydrolysis temperature. Ballesteros et al. (1991) have identified *Kluyveromyces marxianus* and *Kluyveromyces fragilis* that have the

highest ethanol productivity at 42°C from 27 yeast strains. *Kluyveromyces marxianus* has an ethanol yield of 0.5g/g cellulose in 78 h using Solka Floc 200 as substrate at 42°C. Kadam and Schmidt (1997) found that a thermotolerant yeast, *Candida acidothermophilum,* produced 80% of the theoretical ethanol yield at 40°C using dilute-acid-pretreated poplar as substrate. *Kluyveromyces* strains have been found to be more thermotolerant than *Candida* and *Saccharomyces* strains (Hacking et al., 1984).

Compared to the two-stage hydrolysis-fermentation process, SSF has the following advantages: (1) increase of hydrolysis rate by conversion of sugars that inhibit the cellulase activity; (2) lower enzyme requirement; (3) higher product yields; (4) lower requirements for sterile conditions since glucose is removed immediately and ethanol is produced; (5) shorter process time; and (6) less reactor volume because a single reactor is used. However, ethanol may also exhibit inhibition to the cellulase activity in the SSF process. Wu and Lee (1997) found that cellulase lost 9%, 36% and 64% of its original activity at ethanol concentrations of 9, 35 and 60 g/l, respectively, at 38°C during SSF process. The disadvantages which need to be considered for SSF include: (1) incompatible temperature of hydrolysis and fermentation; (2) ethanol tolerance of microbes; and (3) inhibition of enzymes by ethanol.

2.6 FUTURE PROSPECTS

The U.S. fuel ethanol industry produced more than 6.2 billion liters of ethanol in 2000, most of which was produced from corn (MacDonald et al., 2001). However, an

increase of ethanol production from corn will compete for the limited land against corn-based food and feed production. On the other hand, there is a huge amount of low-value or waste lignocellulosic materials that are currently burned or wasted. Utilization of lignocellulosic materials can replace the equivalent of 40% of the gasoline in the U.S. market (Wheals et al., 1999). Using lignocellulosic materials such as agricultural residues, grasses, forestry wastes and other low-cost biomass can significantly reduce the cost of raw materials (compared to corn) for ethanol production. A reduction of the cost of ethanol production can be achieved by reducing the cost of either the raw materials or the cellulase enzymes. It was predicted that the use of genetically engineered raw materials with higher carbohydrate content combined with the improvement of conversion technology could reduce the cost of ethanol by $0.11 per liter over the next ten years (Wooley et al., 1999). Reducing the cost of cellulase enzyme production is a key issue in the enzymatic hydrolysis of lignocellulosic materials. Genetic techniques have been used to clone the cellulase coding sequences into bacteria, yeasts, fungi and plants to create new cellulase production systems with possible improvement of enzyme production and activity. Wood et al. (1997) reported the expression of recombinant endoglucanase genes from *Erwinia chrysanthemi* P86021 in *Escherichia coli* KO11 and the recombinant system produced 3,200 IU endoglucanase/l fermentation broth (IU, international unit, defined as a micromole of reducing sugars released per minute using carboxymethyl cellulose as substrate). The thermostable endoglucanase E1 from *Acidothermus cellulolyticus* was expressed in *Arabidopsis thaliana* leaves (Ziegler et al., 2000), potato (Dai et al., 2000), and tobacco (Hooker et al., 2001). Using genetically

engineered microorganisms that can convert xylose and/or pentose to ethanol can greatly improve ethanol production efficiency and reduce the cost of the production. The constructed operons encoding xylose assimilation and pentose phosphate pathway enzymes were transformed into the bacterium *Zymomonas mobilis* for the effective fermentation of xylose to produce ethanol (Zhang et al., 1995). The recombinant strain of *E. coli* with the genes from *Zymomonas mobilis* for the conversion of pyruvate into ethanol has been reported by Dien et al. (2000). The recombinant plasmids with xylose reductase and xylitol dehydrogenase genes from *Pichia stipitis* and xylulokinase gene from *Saccharomyces cerevisiae* have been transformed into *Saccharomyce spp.* for the co-fermentation of glucose and xylose (Ho et al., 1998). Although bioethanol production has been greatly improved by new technologies, there are still challenges that need further investigations. These challenges include maintaining a stable performance of the genetically engineered yeasts in commercial scale fermentation operations (DiPardo, 2000), developing more efficient pretreatment technologies for lignocellulosic biomass, and integrating the optimal components into economic ethanol production systems.

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DILUTE ACID PRETREATMENT OF RYE STRAW AND BERMUDAGRASS FOR ETHANOL PRODUCTION

3.1 ABSTRACT

Ethanol production from lignocellulosic materials provides an alternative energy production system. Rye and bermudagrass that are used in hog farms for nutrient uptake from swine wastewater have the potential for fuel ethanol production because they have a relatively high cellulose and hemicellulose content. Dilute sulfuric acid pretreatment of rye straw and bermudagrass for the enzymatic hydrolysis of cellulose in the materials have been investigated in this study. The biomass was pretreated by dilute sulfuric acid with solid loading of 10% at 121°C. Different sulfuric acid concentrations (0.6, 0.9, 1.2 and 1.5%, w/w) and residence time (30, 60 and 90 min) were used during the pretreatment. The prehydrolyzates were analyzed for total reducing sugars, arabinose, galactose, glucose and xylose. The solid residues were hydrolyzed by cellulases to investigate the enzymatic digestibility. The amount of arabinose and galactose in the filtrates increased with the increasing acid concentration and residence time. The glucose concentration in the prehydrolyzates of rye straw was not significantly influenced by the sulfuric acid concentration and residence time, but it increased in the prehydrolyzates of bermudagrass with the increase of pretreatment severity. The xylose concentration in the filtrates increased with the increase of sulfuric acid concentration and residence time. Most of the arabinan, galactan and xylan in the biomass were hydrolyzed during the acid

pretreatment. Cellulose remaining in the pretreated feedstock was highly digestible by cellulases from *Trichoderma reesei*.

3.2 INTRODUCTION

Ethanol is a renewable energy resource that is used as a partial gasoline replacement. Ethanol is also an environmentally-friendly alternative gasoline additive to methyl tertiary butyl ether (MTBE) that will be eliminated from gasoline in California by 2002 (Hanson, 1999). The United States produced 1.77 billion gallons of ethanol in 2001, which increased nearly 10% from 1.63 billion gallons in 2000 and 20% from 1.47 billion gallons in 1999 (Francl, 2002). Demand for ethanol will increase with the reduction of crude oil resource and the elimination of MTBE from gasoline. About 655 million bushels of corn were utilized in fuel ethanol industry that accounted for 92% of the feedstock in 2001 (Francl, 2002). The increase of ethanol production from corn will compete for the corn-based food and feed production. The cost can be significantly reduced if cellulose-based agricultural residues such as crop residues, herbaceous crops, sawdust, and wood chips are used instead of corn.

The bioconversion of lignocellulosic materials to ethanol contains two steps: the hydrolysis of cellulose to reducing sugars and the following fermentation by yeast or bacteria to convert fermentable sugars to ethanol. The hydrolysis process currently used is either acid hydrolysis or enzymatic hydrolysis. Compared to acid hydrolysis, enzymatic hydrolysis is milder and more specific, but it requires pretreatment to improve

the enzymatic digestibility. The pretreatment process can remove hemicellulose, reduce cellulose crystallinity, and increase the porosity of the materials. Comminution, steam explosion, ammonia fiber explosion, and acid or alkaline pretreatment processes have been extensively investigated (Morjanoff and Gray, 1987; Cadoche and López, 1989; Holtzapple et al., 1991; Torget et al., 1991; Bjerre et al., 1996). Among all the pretreatment methods, dilute acid pretreatment has been widely studied because it is effective and inexpensive. The dilute sulfuric acid pretreatment can effectively solubilize the hemicellulose into monomeric sugars (arabinose, galactose, glucose, mannose, and xylose) and soluble oligomers, and thus improves the cellulose conversion. Compared to other pretreatment methods, it is especially useful for the conversion of xylan in hemicellulose to xylose that can be further fermented to ethanol by many microorganisms (McMillan, 1996). Grohmann et al. (1985) reported the sulfuric acid pretreatment of wheat straw and aspen wood. About 80% of xylan was removed at 140°C for 1 h and enzymatic digestibility of cellulose was nearly 80%. Torget et al. (1990) investigated the dilute sulfuric acid pretreatment of short rotation hardwoods and herbaceous crops. About 92% of the xylan was solubilized and 75% enzymatic digestibility was obtained when switchgrass and weeping lovegrass were pretreated by 0.5% (v/v) sulfuric acid at 140°C for 60 min or 160°C for 10 min. Three hardwoods (Silver maple, sycamore, black locust), corn cobs, and corn stover were pretreated with dilute sulfuric acid (0.45-0.5%, v/v) at 160°C for 5-10 min and more than 90% of the xylan was solubilzed (Torget et al., 1991). Torget and Hsu (1994) studied the two-temperature (140/170°C) dilute acid prehydrolysis of hybrid poplar using a percolation process and found soluble xylose to be

92% of the theoretical value and 2% of the xylan being degraded to furfural. Acid-impregnated steam explosion (0.4% sulfuric acid, 200-230°C, 1-5 min) was reported to solubilize 90-95% of the hemicellulose from softwood and 90% of the remaining cellulose can be hydrolyzed by cellulase enzymes (Nguyen et al., 1998). The total sugar yields were improved by 10% and the net enzyme requirement was reduced by about 50% if two-stage dilute-acid pretreatment was used instead of one-stage (Nguyen et al., 2000). Although the temperature as high as 170°C produced more solubilized xylan, a large portion of the glucose was degraded to hydroxymethyl furfural (HMF) and xylose was degraded to furfural that was inhibitory to microbial growth and xylose fermentation (Grohmann et al., 1984; Lee et al., 1997). Therefore, Saha and Bothast (1999) reported the dilute sulfuric acid (0.5-1.0%, v/v) pretreatment of corn fiber at 121°C and found that the monomeric sugar yield was 85-100% of the theoretical yields.

Many hog farmers in the Southeast of United States grow rye and bermudagrass to remove the nitrogen and phosphorus from stabilized swine wastes to prevent potential pollution of these nutrients to the nearby watershed. After harvest, the rye straw is usually given away because of its low nutrient value. Bermudagrass hay is sold at a very low price as animal feed or given away in some cases. The use of rye straw and bermudagrass for ethanol production not only makes use of these agricultural residues, but also reduces the ethanol production cost. Although different lignocellulosic materials have been investigated for their potential of ethanol production, the dilute sulfuric acid pretreatment and saccharification of rye straw and bermudagrass have not been reported. The purpose of this study was to investigate the effect of dilute sulfuric acid pretreatment

on the solubilization of cellulose and hemicellulose in rye straw and bermudagrass, and the following enzymatic hydrolysis of the cellulose by cellulases and β-glucosidase. This research would provide important information on the commercial utilization of rye straw and bermudagrass for large-scale ethanol production.

3.3 MATERIALS AND METHODS

3.3.1 Biomass

Rye (scientific name: *Secale cereale*) straw and bermudagrass (scientific name: *Cynodon dactylon*) were obtained from Barham Farm (Zebulon, NC). The rye grew in the winter from November to May and was irrigated with swine wastewater once during that time. The rye straw was harvested in May. The bermudagrass was collected in August after cultured for 5-6 weeks and irrigated with wastewater for two times. The biomass was air dried in the field for one week and ground in a Wiley mill with sieve diameter of 3.13 mm. The ground biomass was then stored in sealed plastic bags at 4°C.

3.3.2 Pretreatment

The biomass was mixed with dilute sulfuric acid (final concentration: 0.6, 0.9, 1.2, 1.5% (w/w)) at solid loading 10% (w/w) and pretreated in an autoclave at 121°C with residence time of 30, 60 and 90 min. The collected solids were washed with 100 ml of hot water for three times. The filtrate and washes were collected for the analysis of monomeric sugars using high performance anion chromatography with pulsed

amperometric detection. The total reducing sugars were analyzed by spectrophotometer. The solid residues were stored at 4°C for enzymatic hydrolysis.

3.3.3 Enzymatic Hydrolysis

The solid residues recovered after sulfuric acid pretreatment were hydrolyzed by cellulases and β-glucosidase at 50°C and 100 rpm for 48 hours in a water bath shaker with a solid loading of 5%. β-Glucosidase was used to supplement the insufficient β-glucosidase activity in the cellulases from *Trichoderma reesei*. The sodium citrate buffer was used to maintain the pH at 4.8. Sodium azide (0.3%, (w/v)) was contained in the mixture to inhibit the microbial infections. The enzymes used were cellulases from *Trichoderma reesei* (E.C. 3.2.1.4) and Novozyme 188 purchased from Sigma Company. The enzyme activity was 1.08 FPU/mg of cellulases and 321.7 CBU/ml of Novozyme 188. The enzyme loadings were excessive: cellulases 25 FPU/g dry biomass, β-glucosidase 75 IU/g dry biomass. Synthetic α-cellulose Sigmacell 20 containing the same amount of cellulose as rye straw was used as a control of the enzymatic hydrolysis. The glucose and the total reducing sugars were analyzed after enzymatic hydrolysis for 48 hours.

3.3.4 Analytical Methods

Moisture content of the biomass was measured by drying the sample at 105°C in an oven to constant weight (Ehrman, 1994). Carbohydrate composition was determined by two-stage sulfuric acid hydrolysis (Ruiz and Ehrman, 1996). Total reducing sugars were determined by the DNS (dinitrosalicylic acid) method using glucose as the standard

(Miller, 1959). The color formed was measured at 550 nm with a spectronic® 401 spectrophotometer (Spectronic Instruments Inc., Rochester, NY).

Monosaccharides in the hydrolyzates were separated using a Dionex DX-300 chromatography system (Dionex Corporation) equipped with a CarboPac™ PA10 (4×250 mm) anion exchange column, CarboPac™ PA10 (4×50 mm) guard column, gradient pump, automated sampler and pulsed amperometric detector with flow-through detector, gold working electrode. Voltage was applied to the electrochemical cell by a potentiostat in a series of three potentials: E1 = 0.05 V, E2 = 0.6 V, E3 = - 0.6 V, three pulse durations: t1 = 480 ms, t2 = 120 ms, t3 = 60 ms. The separation was achieved by operating in an isocratic elution with a binary solvent system of 99% H_2O and 1% 200 mM NaOH solution for 21 min and ramping a linear gradient to 100% NaOH solution over the next 10 min. The NaOH concentration was maintained for 7 min, then the solvent composition was gradually changed to 99% H_2O and 1% 200 mM NaOH solution to equilibrate the column for the next injection.

Cellulase activity was assayed as filter paper units (FPU) (Ghose, 1987).

3.3.5 Statistical analysis

Experimental data were statistically analyzed using Proc GLM (SAS Institute, Cary, NC). The effects of pretreatment time and residence time on the yields of monomeric sugars and reducing sugars after acid pretreatment and enzymatic hydrolysis were analyzed using t test (LSD, $P<0.05$). Second-order polynomial equations were used with the level of significance $P<0.05$ to predict the relationship of monomeric sugar yields in the prehydrolyzates with acid concentration and pretreatment time.

3.4 RESULTS AND DISCUSSION

3.4.1 Rye straw

The chemical composition of rye straw is shown in Table 3.1. Glucan was the major component followed by acid-insoluble lignin and xylan. Arabinan and Galactan accounted for only a small amount of the biomass composition. No mannan component was detected in the biomass. The arabinan, galactan, xylan, and mannan are the major types of hemicellulose (McMillan, 1994). The complex structure of hemicellulose varies with plant species. It was reported that wheat straw and grasses contained arabinan, galactan, and xylan (Grohmann et al., 1984; Torget et al., 1990), while hardwood and softwood have one more component, mannan, in the hemicellulose composition (Torget et al., 1990; Brigham et al., 1996). From the experimental results, the hemicellulose of rye straw includes mainly arabinan, galactan, and xylan with xylan as the dominant carbohydrate. It was reported that rye straw contained 27.2% of cellulose and 34.0% of hemicellulose (Sun and Cheng, 2002), and bermudagrass contained 25.0% of cellulose and 35.7% of hemicellulose (Salo, 1965).

Table 3.1. Chemical composition of rye straw and bermudagrass (wt%, dry basis).

	Rye straw	Bermudagrass
Arabinan	2.47	4.33
Galactan	0.31	1.09
Glucan	33.12	32.36
Xylan	19.46	19.37
Acid-insoluble lignin	19.80	20.33
Ash	6.15	4.17
Other	18.69	18.34

After being pretreated at different sulfuric acid concentrations and times, the prehydrolyzates were collected. The separation of monosaccharides arabinose, galactose, glucose and xylose in the prehydrolyzates by high performance anion chromatography is shown in Fig. 3.1.

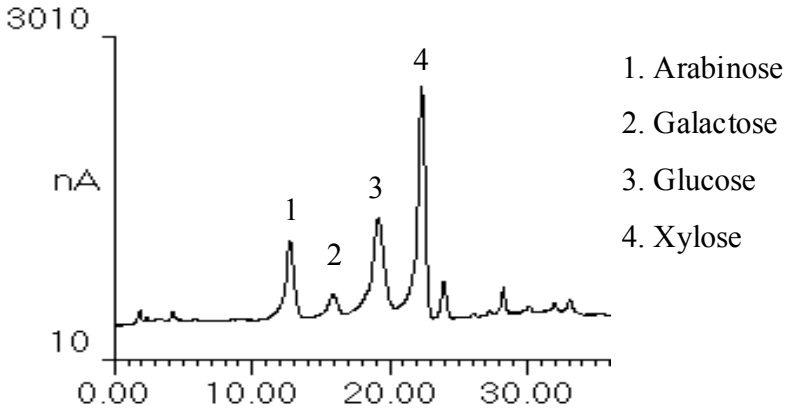

Fig. 3.1. Chromatograph of monomeric sugars in the prehydrolyzates of acid pretreatment of rye straw and bermudagrass using a high performance anion chromatography.

Fig. 3.2 showed the amount of monosaccharides and total reducing sugars released from rye straw. The prehydrolyzates contained monomeric sugars arabinose, galactose, glucose, and xylose with xylose as the major component. The yields of these monomeric sugars in the filtrate have shown the hemicellulose removal after acid pretreatment. The pretreatment time did not significantly influence the release of arabinose from the biomass ($P>0.05$), while the arabinose concentration in the filtrate increased with the increase of sulfuric acid concentration up to 1.2% (Fig. 3.2a). The monomeric arabinose solubilized from rye straw was 50% to 70% of the arabinan. The galactose concentration increased with the increase of acid concentration when the

pretreatment time was 30 min, while the galactan was completely hydrolyzed when sulfuric acid concentration increased to 0.9% and residence time to 60 min (Fig. 3.2b). About 35 mg glucose/g dry rye straw was released by acid pretreatment, which accounted for 10% of the glucan content in the biomass. The acid concentration and pretreatment time had no significant effect on the glucose content in the prehydrolyzates (Fig. 3.2c). Xylose yield was significantly influenced by the acid concentration and pretreatment time (Fig. 3.2d). The xylose increased with the increase of sulfuric acid concentration and pretreatment time. The monomeric xylose in the liquid fraction accounted for about 55% of the xylan in the biomass when pretreated with 0.9% sulfuric acid for 90 min and increased to 66% when pretreated with 1.5% sulfuric acid for 90 min. Other xylan in the biomass may be solubilized in the prehydrolyzates as oligomeric xylose, degraded into furfural or other byproducts, or remained in the solid residues. The results indicate that residence time of 30 min was not enough for the solubilization of hemicellulose in the biomass. The increased severity of the pretreatment conditions resulted in more solubilization of hemicellulose. More than 50% of the hemicellulose was solubilized into monomeric sugars when pretreated with 1.2% sulfuric acid for 60 min or 0.9% sulfuric acid for 90 min. The total reducing sugar content in the prehydrolyzates is shown in Fig. 3.2e. Fig. 3.2e clearly shows the effect of dilute sulfuric acid concentration and reaction time on the hydrolysis and solubilization of the biomass. From the experimental data, the total reducing sugars in the prehydrolyzates measured with the DNS method were higher than the sum of arabinose, galactose, glucose and xylose measured using an anion chromatography system. The difference may be partially caused by other reducing sugars

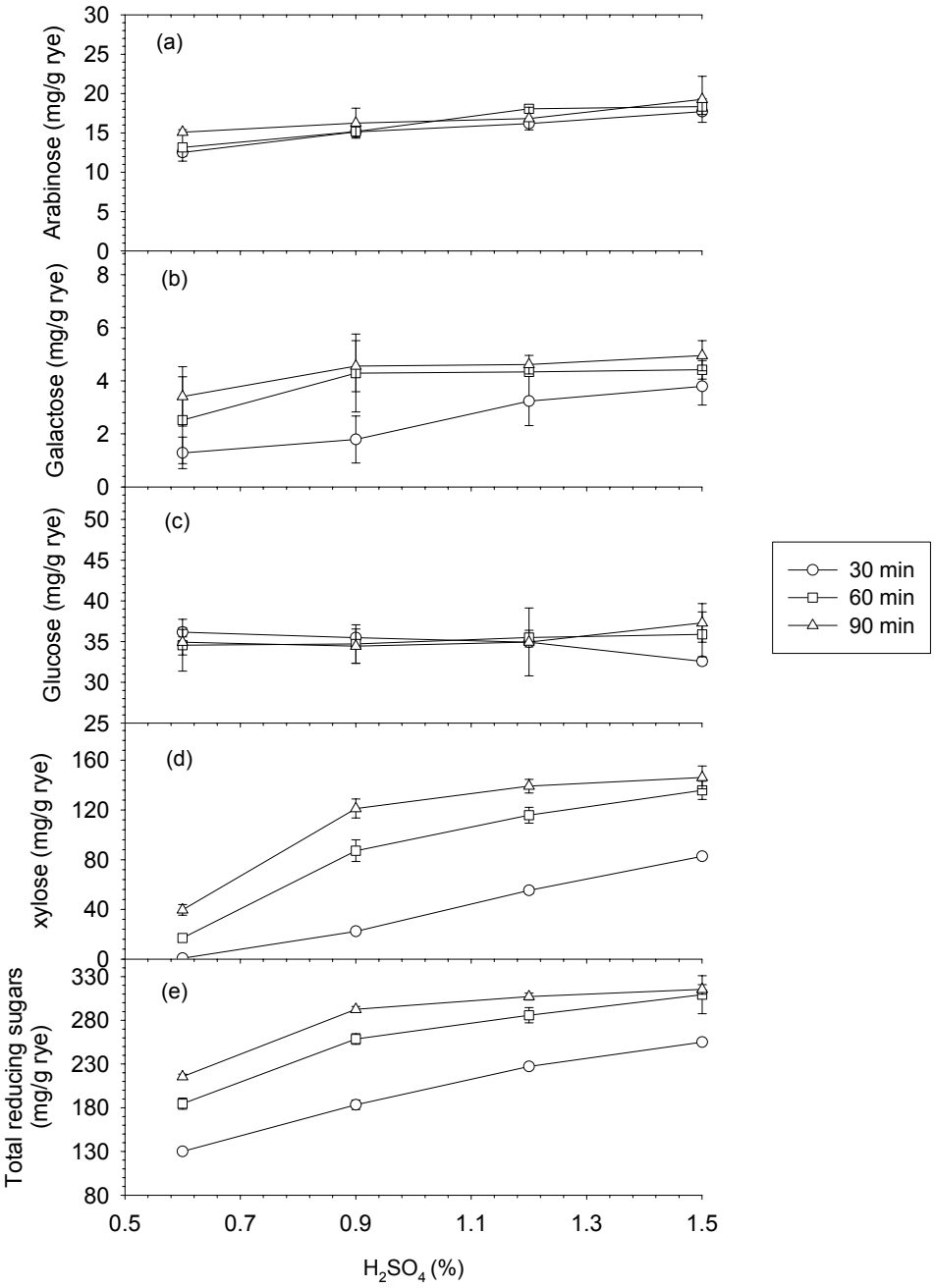

Fig. 3.2. The release of monomeric and total reducing sugars from rye straw after dilute sulfuric acid pretreatment.
Data are means ± SD of two replicates.
(a) Arabinose yield; (b) Galactose yield; (c) Glucose yield; (d) Xylose yield; (e) Total reducing sugar yield.

(cellobiose, etc.) and non-sugar reducing compounds that dissolved in the prehydrolyzates and were measured as reducing sugars.

An empirical model was built to establish the relationship of monomeric sugar content in the prehydrolyzates with dilute sulfuric acid concentration and pretreatment time:

$$Y_{MS} = K + \alpha_1 C + \alpha_2 T + \beta_1 CT + \beta_2 C^2 + \beta_3 T^2 \quad (1)$$

where Y_{MS} is monomeric sugar yield (mg/g substrate); K is a constant; C is acid concentration (%); T is reaction time (min); and α_1, α_2, β_1, β_2, β_3 are coefficients. The polynomial equation was chosen because it effectively exhibited the interaction of experimental variables. After eliminating the insignificant terms from Equation 1, the coefficients in the model are shown in Table 3.2. There was no interaction between acid concentration and pretreatment time for the arabinose and galactose content in the prehydrolyzates. The glucose yield was not significantly influenced by the acid concentration and reaction time. R^2 of xylose and reducing sugar yields was 0.95 and 0.98 respectively, which indicates that the model fits the data well. The established empirical model is appropriate in the range of sulfuric acid concentration and residence time used in this experiment.

Sigmacell 20 was used as a control for the digestibility experiment of the pretreated biomass. After 48 h of enzymatic hydrolysis at 50°C, 95% of Sigmacell 20 was converted into glucose. This indicates that the enzyme loading (cellulases 25 FPU/g dry biomass, β-glucosidase 75 IU/g dry biomass) was not the limiting factor. Therefore,

Table 3.2. Second-order polynomial predictive equations for the effect of acid concentration and reaction time on the yields of monomeric sugars and total reducing sugars from rye straw.

Sugars	K	α_1	α_2	β_1	β_2	β_3	$R^{2\,a}$
Arabinose	9.04	5.34	0.025	N [b]	N	N	0.77
Galactose	-0.52	2.23	0.030	N	N	N	0.60
Glucose	N	N	N	N	N	N	N
Xylose	-260.53	342.76	2.93	N	-110.19	-0.015	0.95
Reducing sugars	-201.68	442.89	4.53	-0.58	-134.24	-0.021	0.98

a: Coefficient of determination.
b: N means that factor is not significant ($p>0.05$).

the glucose produced after the enzymatic hydrolysis reflected the digestibility of the pretreated biomass. The solid residue pretreated at 121°C for 30 min produced much less glucose after 48 h of saccharification, while the glucose produced under the pretreatment time of 60 min and 90 min was similar (Fig. 3.3). The digestibility increased quickly with the increase of dilute sulfuric acid concentrations from 0.6% to 1.2% and glucose yield reached 135-155 mg/g dry biomass when acid concentration was 1.2-1.5% and reaction time 60 – 90 min. The total reducing sugars data showed the same trend as the glucose curves (Table 3.3). The glucose in the hydrolysis liquor accounted for about 70-80% of total reducing sugars. The conversion rate of cellulose to glucose ranged from 30-52% under different pretreatment conditions (Table 3.3).

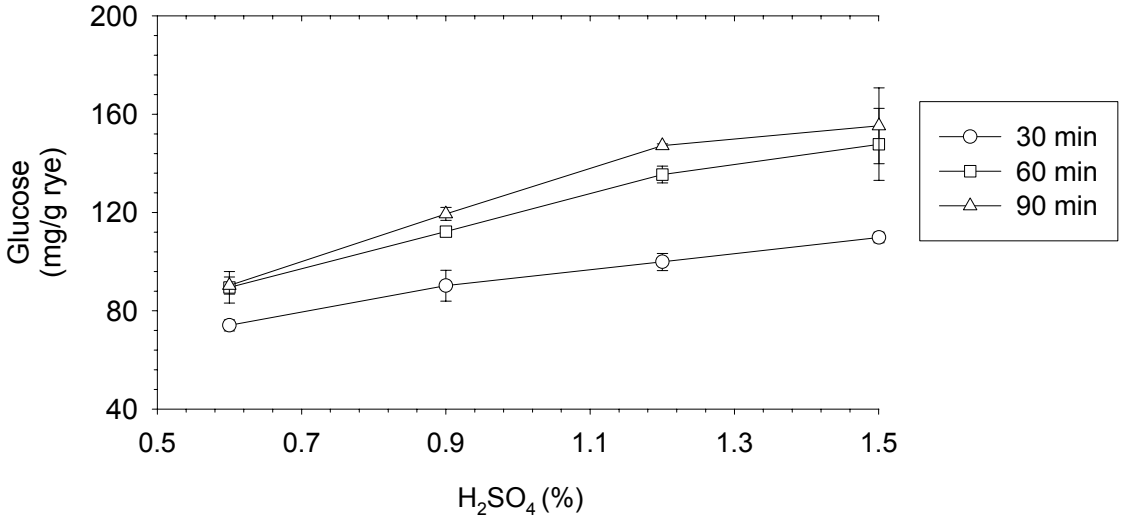

Fig. 3.3. Glucose yields after 48-h enzymatic hydrolysis of pretreated rye straw. Data are means ± SD of two replicates.

Table 3.3. Yield of total reducing sugars and conversion rate after 48-h enzymatic hydrolysis of pretreated rye straw [a].

Acid concentration (%)	Time (min)	Total reducing sugars (mg/g dry biomass)	Conversion rate[b] (%)
0.6	30	125.0	29.9
0.9	30	135.3	34.1
1.2	30	146.2	36.6
1.5	30	159.7	38.7
0.6	60	133.7	33.7
0.9	60	161.9	39.9
1.2	60	177.4	46.5
1.5	60	192.9	49.9
0.6	90	136.1	34.0
0.9	90	169.2	41.8
1.2	90	187.1	49.5
1.5	90	197.1	52.3

[a] Data are means ± SD of two replicates.
[b] Conversion rate was calculated as follows: % conversion rate = glucose produced after pretreatment and enzymatic hydrolysis × 0.9 × 100/glucan.

3.4.2 Bermudagrass

The chemical composition of bermudagrass was similar to that of rye straw except that bermudagrass had slightly more arabinan and galactan and less ash (Table 3.1). The composition of rye straw and bermudagrass corresponds to the previous report that large amount of hemicellulose in the grass was arabinoxylans (20-40%) (Wilkie, 1979).

Fig. 3.4 shows the monomeric sugar yields (arabinose, galactose, glucose and xylose) in the prehydrolyzates. The pretreatment time did not significantly influence the arabinose yield ($p>0.05$). The release of arabinose increased when the dilute acid concentration increased from 0.6% to 0.9% and did not show significant difference when the acid concentration was higher than 0.9% (Fig. 3.4a). The arabinose yield at 0.9 or 1.2% sulfuric acid and 60 min was higher than that of 90 min at the same acid loading. The arabinose released decreased when pretreated at 60°C with acid concentration changed from 1.2% to 1.5%. This may be caused by the degradation of arabinose at long residence time and high acid concentration. The acid concentration and pretreatment time showed the same effect on the release of galactose and xylose from bermudagrass as rye straw. More than 90% of the galactan was hydrolyzed into galactose when pretreated by 1.5% sulfuric acid for 90 min (Fig. 3.4b). As shown in Fig. 3.4d, the xylose released increased from 0.85% of the total xylan in the bermudagrass (0.6% sulfuric acid, 30 min) to 62% (1.5% sulfuric acid, 60 min). When acid concentration was up to 1.2%, xylose yields were almost the same for the pretreatment time of 60 min and 90 min. More than 60% of the xylan was hydrolyzed into monomeric xylose at the most severe condition.

Xylan is the major component in the hemicellulose structure, so the xylose in the filtrate can be used as an indication of the solubilization level of hemicellulose. Based on the experimental results, the pretreatment time of 30 min was not sufficient for the removal of hemicellulose.

The experimental data indicate that the glucose concentration in the prehydrolyzates of bermudagrass was significantly influenced by the acid concentration and pretreatment time (Fig. 3.4c). The percentage of monomeric glucose solubilized increased from 9% (0.6% sulfuric acid, 30 min) to 33% (1.2% sulfuric acid, 90 min). It was reported that the rate of cleavage of β-1,4 glucosidic bonds in glucans was approximately one fifth of the rate of cleavage of the linkage in β-xylan (Grohmann et al., 1984), so the glucose should be released at lower rate than xylose. However, the glucose in the prehydrolyzate of rye straw was stable despite the change of pretreatment severity. This phenomenon might be explained that the glucose released was from the free glucose in the rye straw or from the minor hemicellulosic component that was hydrolyzed together with the solubilization of xylan. The glucose released during the pretreatment of bermudagrass accounted for as high as 33% of the glucan in the biomass. It might consist of the minor hemicellulosic glucan and cellulosic glucan together. The solubilization of sugars during the acid pretreatment does not represent any actual loss. The solubilized glucan can be recovered from the liquid fraction and further utilized for ethanol production. The total reducing sugar yields in the prehydrolyzates of bermudagrass were higher than that of rye straw (Fig. 3.4e).

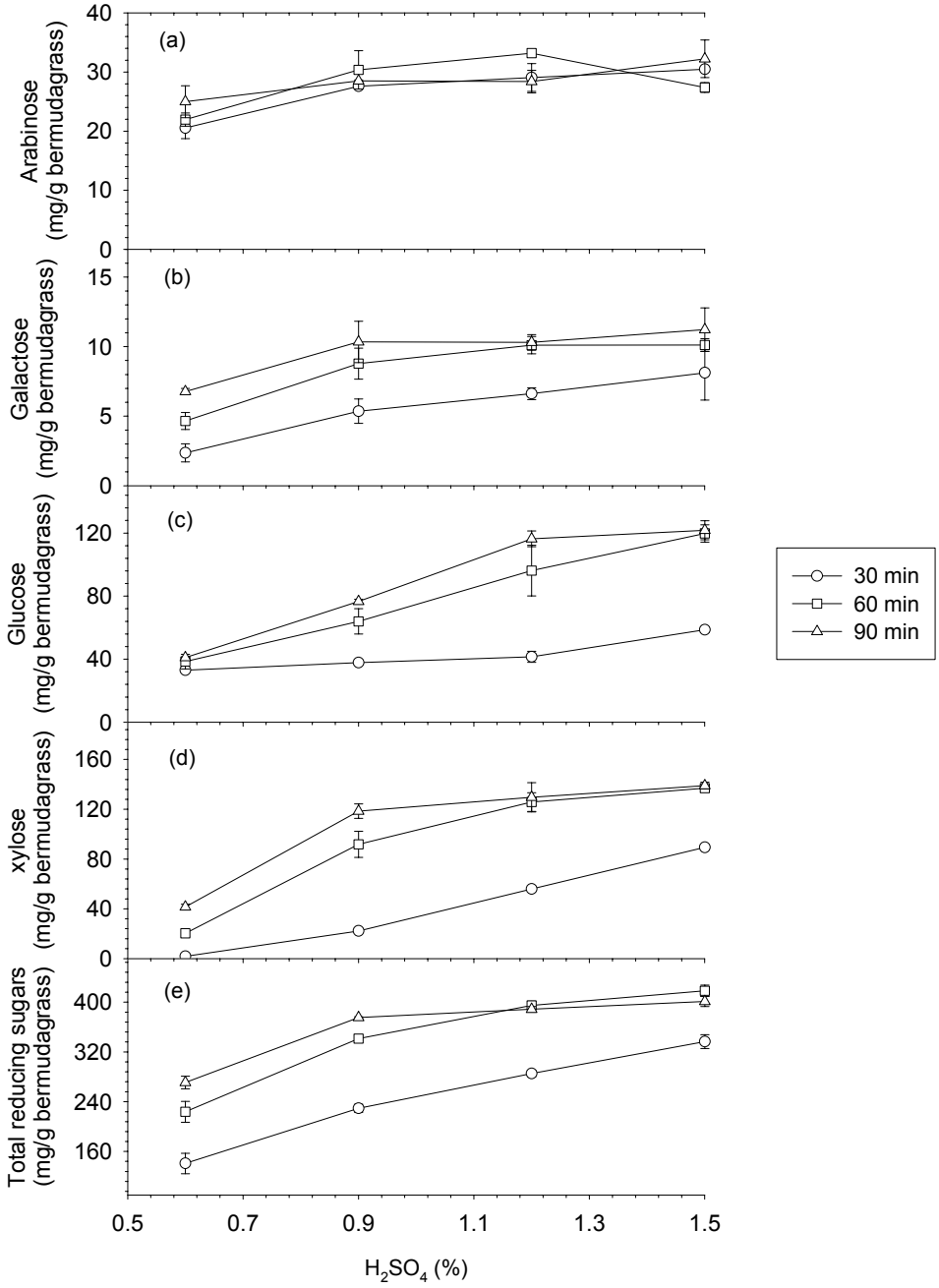

Fig. 3.4. The release of monomeric and total reducing sugars from bermudagrass after sulfuric acid pretreatment.

Data are means ± SD of two replicates.

(a) Arabinose yield; (b) Galactose yield; (c) Glucose yeld; (d) Xylose yield; (e) Total reducing sugar yield.

The same predictive model was proposed to establish the relationship of monomeric sugar content in the prehydrolyzates with dilute sulfuric acid concentration and pretreatment time. The coefficients were shown in Table 3.4. The coefficients of determination (R^2) were 0.89-0.98 except for arabinose, which indicate the effectiveness of the model.

Table 3.4. Second-order polynomial predictive equations for the effect of acid concentration and reaction time on the yields of monomeric sugars and total reducing sugars from bermudagrass.

Sugars	K	α_1	α_2	β_1	β_2	β_3	$R^{2\,a}$
Arabinose	1.52	46.07	N [b]	N	-18.12	N	0.65
Galactose	-13.86	23.62	0.20	N	-8.57	-0.00114	0.89
Glucose	-25.86	3.97	1.43	1.12	N	-0.0153	0.94
Xylose	-267.02	332.46	3.61	N	-106.24	-0.021	0.94
Reducing sugars	-415.72	701.11	8.72	-1.33	-206.77	-0.046	0.98

a: Coefficient of determination.
b: N means that factor is not significant ($p>0.05$).

The pretreated solid residue was used for saccharification. The glucose yields with pretreatment time of 60 min and 90 min did not show significant difference after 48 h of enzymatic hydrolysis (Fig. 3.5). The glucose yield from bermudagrass was higher, compared to rye straw pretreated under the same condition. The digestibility increased with the increasing sulfuric acid concentration. Although the conversion rate was improved at high acid concentration, approximately 1% (w/w) sulfuric acid concentration was usually used for the pretreatment process to be cost-effective (McMillan, 1994).

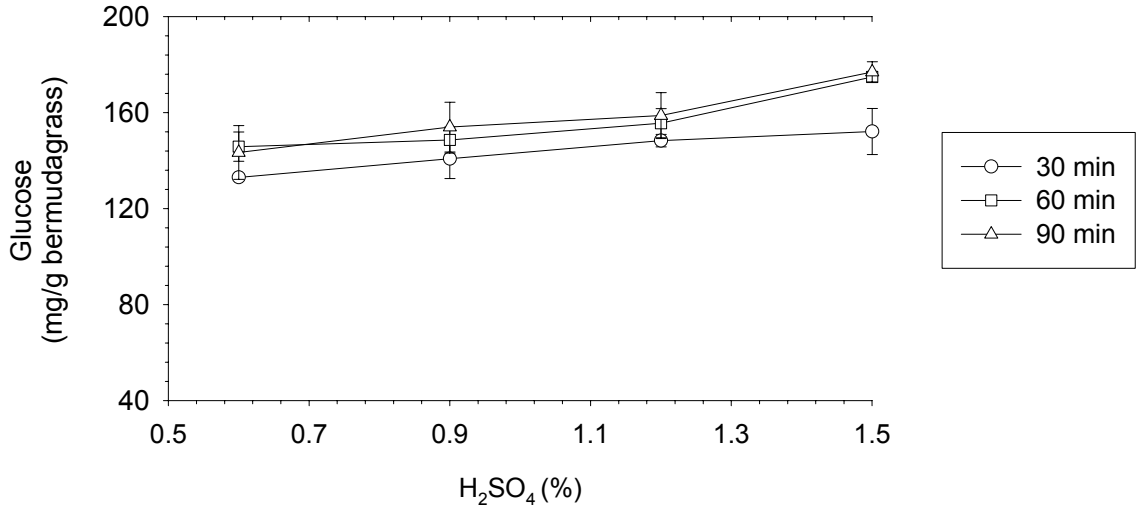

Fig. 3.5. Glucose yields after 48-h enzymatic hydrolysis of pretreated bermudagrass. Data are means ± SD of two replicates.

The conversion rate from glucan to glucose was listed in Table 3.5. The digestibility increased with the severity of the pretreatment condition and reached 70-83% when pretreated with sulfuric acid concentrations higher than 1.2% and pretreatment time 60 min or longer. The data of total reducing sugar yield (Table 3.5) had the same trend as the glucose curves (Fig. 3.5).

3.5 CONCLUSIONS

Rye straw and bermudagrass have the potential for fuel ethanol production. Dilute sulfuric acid pretreatment is effective for the solubilization of hemicellulose in the biomass with arabinose, galactose, and xylose as the major monomeric sugars produced. About 50%-66% of xylan in the biomass was hydrolyzed into monomeric xylose for acid

Table 3.5. Yield of total reducing sugars and conversion rate after 48-h enzymatic hydrolysis of pretreated bermudagrass [a].

Acid concentration (%)	Time (min)	Total reducing sugars (mg/g dry biomass)	Conversion rate[b] (%)
0.6	30	207.2	46.1
0.9	30	205.2	49.6
1.2	30	218	52.8
1.5	30	216.9	58.6
0.6	60	195.7	51.2
0.9	60	208.2	59.1
1.2	60	204.1	70.0
1.5	60	217.3	81.9
0.6	90	203.7	51.3
0.9	90	206.4	64.1
1.2	90	219.2	76.5
1.5	90	229.3	83.1

[a] Data are means ± SD of two replicates.
[b] Conversion rate was calculated as follows: % conversion rate = glucose produced after pretreatment and enzymatic hydrolysis × 0.9 × 100/glucan.

concentration higher than 1.2% and pretreatment time longer than 60 min. The xylose yields in the filtrate of the two agricultural residues were similar under the same pretreatment conditions. Pretreatment time of 30 min did not provide an adequate solubilization of hemicellulose. The monomeric glucose yield in the prehydrolyzates of bermudagrass increased with the increase of acid concentration and pretreatment time. About 27-33% of glucan from bermudagrass was converted into glucose when acid concentration and pretreatment time were up to 1.2% and 60 min, respectively. However, the monomeric glucose in the prehydrolyzates of rye straw was only 10% of glucan and kept constant under different pretreatment conditions. The higher glucose

content in the prehydrolyzates of bermudgrass made the glucose recovery from liquid fraction important. The enzymatic digestibility of the pretreated solid residues increased with the severity of pretreatment. The solubilization of the hemicellulose was an indication of the enzymatic accessibility to cellulose in the pretreated solid residues. Rye straw is harder to be hydrolyzed compared to bermudagrass. The glucose yield in the range of 30% to 52% of the theoretical potential was obtained for rye straw under different pretreatment conditions, while glucose yield of bermudagrass varied from 46% to 81% with the increase of dilute sulfuric acid concentration and pretreatment time.

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ENZYMATIC HYDROLYSIS OF RYE STRAW AND BERMUDAGRASS USING CELLULASES SUPPLEMENTED WITH β-GLUCOSIDASE

4.1 ABSTRACT

The use of agricultural residues for ethanol production can greatly reduce feedstock cost compared to corn, whereas cellulase enzymes used to convert cellulose to glucose are prohibitory expensive for an economical conversion process. Effects of cellulases supplemented with β-glucosidase on glucose yield during enzymatic hydrolysis of acid-pretreated rye straw and bermudagrass were investigated. Increase of cellulase loading from 5 FPU/g biomass to 15 FPU/g biomass enhanced the glucose production. Addition of β-glucosidase effectively improved the glucose production rate. When β-glucosidase increased to 25 CBU/g in the enzymatic reaction, there was no cellobiose accumulation in the hydrolyzates. Cellulase loading of 10 FPU/g biomass with β-glucosidase supplementation of 25 CBU/g biomass showed an adequate saccharification of pretreated bermudagrass and the conversion rate reached 45%. Rye straw is more resistant to enzymatic hydrolysis than bermudagrass. The conversion rate of pretreated rye straw was 38% when cellulase and β-glucosidase loadings were 15 FPU/g biomass and 25 CBU/g biomass, respectively.

4.2 INTRODUCTION

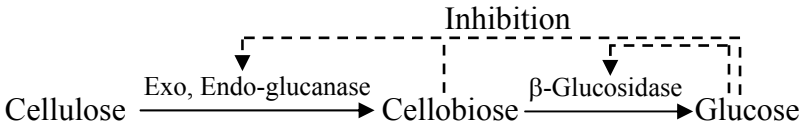
Ethanol production from lignocellulosic biomass depends on the hydrolysis of cellulose and hemicellulose into simple reducing sugars that can be fermented into ethanol by microorganisms. Both acid and enzyme can act as catalysts for the hydrolysis process. The acid hydrolysis includes dilute acid and concentrated acid hydrolyses. The dilute-acid-catalyzed hydrolysis is usually performed at two stages. The first stage is performed at relatively low temperature (140-180°C) for the maximum removal of hemicellulose, and the second high temperature stage (180-260°C) is for optimum hydrolysis of cellulose. The relatively severe hydrolysis conditions result in the formation of more by-products, such as furfural from the degradation of xylan and HMF (5-hydroxymethylfurfural) from the degradation of cellulose, which impede yeast metabolism during the fermentation process (Schell and Duff, 1996). The concentrated acid hydrolysis produces fewer toxic by-products compared to dilute acid pretreatment, but the concentrated acid needs to be recycled for the process to be economical. The separation of concentrated acid from hydrolyzate and reconcentration of the acid increase the complexity of the process (DiPardo, 2000). In addition, concentrated acid is corrosive and therefore increases the equipment cost. Compared to acid hydrolysis, enzymatic hydrolysis is milder and more specific. The cellulase-catalyzed hydrolysis is usually carried out at 40-50°C, which greatly reduces the sugar degradation at high temperature. In addition, the compromise of enzymatic hydrolysis temperature (40-50°C) and fermentation temperature (about 30°C) makes it feasible for the simultaneous

saccharification and fermentation (SSF), which will decrease end product inhibition and ethanol production cost. Therefore, the enzyme-based hydrolysis is widely studied for fuel ethanol production from lignocellulosic biomass.

Cellulases are the enzymes that act on cellulose to produce glucose by cleaving the β-1,4-glucosidic bonds in the polymer chain. It was reported that aerobic bacteria (Stoppok et al., 1982; Blackwell et al., 1985; Mohagheghi et al., 1986), anaerobic bacteria (Halliwell, 1963; Lamed et al., 1985), white rot fungi (Ander and Eriksson, 1977; Eriksson et al., 1980), soft rot fungi (Montenecourt, 1983; Watson et al., 1984), and anaerobic fungi (Barichievich and Calza, 1990) could produce cellulases. Although many organisms degrade cellulose, very few of them produce extracellular cellulases that can hydrolyze crystalline cellulose (Shewale, 1982).

Among all the cellulase-producing microorganisms, soft rot fungus *Trichoderma reesei* and its mutants are recognized as the best strain for the industrial production of cellulases due to its complete composition of cellulase complex, high productivity and stability during the saccharification process (Kadam, 1996). The cellulases from *T. reesei* comprise at least three classes of enzymes: endo-1,4-β-D-glucanase that cleaves the internal glucosidic bonds of cellulose chains at random and produces more chain ends on which exoglucanase may act; 1,4-β-D-glucan cellobiohydrolase or exoglucanase (CBH) that cleaves cellobiose units from the nonreducing end of cellulose chains; and 1,4-β-D-glucosidase that hydrolyzes the cellobiose to produce glucose. The synergism of the β-glucosidase, endo- and exo-acting enzymes on the hydrolysis of cellulose has been reported in previous studies (Henrissat et al., 1985; Coughlan et al., 1987; Woodward,

1991; Medve et al., 1994; Hoshino et al., 1997). Cellulase system is regulated by the products. The cellobiose accumulation during the hydrolysis is known to inhibit the enzyme activities of both exoglucanase and endoglucanase by reversibly binding to the enzymes to form inhibited complexes (Berghem et al., 1975; Holtzapple et al, 1984). The β-glucosidase is inhibited by glucose, so glucose inhibits the hydrolysis of cellobiose (Gong et al., 1977). The product inhibition can be expressed as:



Although *T. reesei* is one of the best microorganisms for cellulase production, its β-glucosidase is insufficient in the cellulase complex (Ryu and Mandels, 1980; Tangnu et al., 1981; Woodward, 1982). The resultant accumulation of cellobiose inhibits the endo- and exo-glucanase, thus reduces the hydrolysis rate and sugar yields (Maguire, 1977; Sternberg et al., 1977). It was reported that *Aspergillus* could produce large quantities of β-glucosidase, and the addition of *Aspergillus* β-glucosidase in the *Trichoderma* cellulases greatly improved the glucose yield (Duff et al., 1985; Duff et al., 1987; Spindler et al., 1989; Stockton et al., 1991). The supplementation of β-glucosidase within some ranges will result in enhanced glucose yield, but it also adds to the cost of cellulases.

The objective of this study is to investigate the enzymatic hydrolysis of sulfuric-acid-pretreated rye straw and bermudagrass using cellulases from *T. reesei* and β-glucosidase from *Aspergillus niger*. Despite the wide interests in ethanol production

from agricultural residues, the bioconversion of acid-pretreated rye straw and bermudagrass has not been reported. The promising development of on-site ethanol production from the harvested agricultural residues in the farm may not only solve the environmental concerns caused by waste disposal, but also provide the energy source for the farm.

4.3 MATERIALS AND METHODS

4.3.1 Biomass

Rye straw and bermudagrass were obtained from Barham Farm (Zebulon, NC). The biomass was air dried, ground in a Wiley mill with sieve diameter of 3.13 mm, and then stored in sealed plastic bags at 4°C. The moisture content of the biomass was measured before the experiment. The dilute sulfuric acid was mixed with 2.5 g of biomass (dry basis) to make the final composition of the mixture to be 1.2% (w/w) sulfuric acid and 10% (w/w) solid loading. The mixture was sealed in a flask with a rubber stopper and pretreated in an autoclave at 121°C for 60 min. The solids were then collected and washed with 100 ml of hot water for three times to remove the sugar residues. The filtrate and washes were removed by filtration. The solid residues were collected and stored in a refrigerator at 4°C for the following enzymatic hydrolysis.

4.3.2 Enzymatic Hydrolysis

The enzymes used were cellulases from *T. reesei* (E.C. 3.2.1.4) and β-glucosidase (Novozyme 188, Novozymes Biotech, Inc.) from *A. niger* purchased from Sigma

Company. Novozyme 188 was used to supplement the insufficient β-glucosidase activity in the cellulases. The enzyme activity was measured to be 1.08 FPU/mg of cellulases (FPU, filter paper unit, expressed as μmol of glucose produced per minute with filter paper as a substrate) and 321.7 CBU/ml of Novozyme 188 (CBU, cellobiase unit, expressed as μmol of cellobiose that is converted into glucose per minute with cellobiose as a substrate).

The collected solid residues were hydrolyzed by cellulases and β-glucosidase at 50°C, 100 rpm in a water bath shaker with a solid loading of 5% (w/w). To investigate the effects of enzyme loadings on the hydrolysis, a factorial experiment in a randomized block design with two replicates was conducted. The two factors, cellulase loading (5, 10, 15 FPU/g dry biomass) and β-glucosidase loading (0, 25, 50 CBU/g dry biomass) were used to hydrolyze the pretreated biomass, so nine treatments were performed. The cellulase powder was dissolved in 0.05 M sodium citrate buffer (pH 4.8). Sodium azide (0.3%, w/v) was contained in the mixture to inhibit microbial contamination. At each reaction time of 0 and 2 h, 1 ml of sample was taken and diluted for the glucose and total reducing sugar analysis. With the increase of reaction time, the sugar content in the hydrolyzate increased and sample was diluted for more times to get the liquid volume enough for sugar analysis. Therefore, less sample volume (0.7 ml) was taken at reaction time of 5, 8, 12, 24, 48, 72 h to reduce the influence of sampling on the system. The sample was heated at 95°C for 15 min to denature the enzyme, and then centrifuged at 10,000 × g for 5 min. The glucose, cellobiose and total reducing sugars in the supernatant were analyzed. The hydrolysis of non-pretreated biomass with enzyme

loading of cellulases 15 FPU/g biomass and β-glucosidase 50 CBU/g biomass was conducted as a control to investigate the effects of acid pretreatment.

4.3.3 Analytical Methods

Moisture content of the biomass was measured by drying the sample at 105°C in an oven to constant weight (Ehrman, 1994). Carbohydrate composition was determined by two-stage sulfuric acid hydrolysis (Ruiz and Ehrman, 1996). Total reducing sugars were determined by the DNS method using glucose as the standard (Miller, 1959).

The glucose and cellobiose in the hydrolyzate were analyzed using Dionex DX-300 chromatography system (Dionex Corporation) as described in the "Materials and Methods" section of Chapter III.

Cellulase activity was assayed as filter paper units (Ghose, 1987). β-Glucosidase activity was determined by the Novozyme standard method "Determination of Cellobiase Activity Using the Hexokinase Method (CBU)" (No.: EB-SM-0175.02/01).

4.3.4 Statistical analysis

Experimental data were statistically analyzed using Proc GLM (SAS Institute, Cary, NC). The means of total reducing sugars, glucose, and cellobiose in the hydrolyzate were analyzed using t test (LSD, $P<0.05$). The means of sugar yields after 72-h hydrolysis were compared using Pdiff (pairwise t-test).

4.4 RESULTS AND DISCUSSION

4.4.1 Rye straw

The goal of sulfuric acid pretreatment was to solubilize xylan that was the major component of hemicellulose in the biomass and impeded the access of cellulase enzymes to cellulose. In our previous study (Sun and Cheng, 2002), the pretreatment condition of 1.2% (w/w) sulfuric acid and 60 min at 121°C provided a good degradation of xylan. When the sulfuric acid concentration was higher than 1.2% and residence time was longer than 60 min at 121°C, the xylose yields in the prehydrolyzates of rye straw and bermudagrass almost leveled off. Although a further increase of acid concentration and residence time resulted in a slight increase of xylose in the prehydrolyzate, the consumption of more acid and electricity also raises the cost. Therefore, 1.2% sulfuric acid and 60-min residence time at 121°C was used for the pretreatment of rye straw and bermudagrass in this study.

The pretreated feedstock was hydrolyzed with cellulases supplemented with β-glucosidase. Glucose and cellobiose were the major products and well separated using a high performance anion chromatography (Fig. 4.1).

Figs. 4.2 and 4.3 show the production of glucose and total reducing sugars during the enzymatic hydrolysis of rye straw with different cellulase and β-glucosidase loadings. Influence of replicates on the glucose yields was not significant except for the treatment with enzyme loadings of 50 CBU/g and 5 FPU/g ($p = 0.03$) and control ($p = 0.01$). Statistical analysis has shown that the reaction time was a significant factor for the glucose and total reducing sugar yields. The concentrations of glucose and total reducing sugars increased rapidly during the first eight hours of hydrolysis, while only slight accumulation was observed after 48 hours.

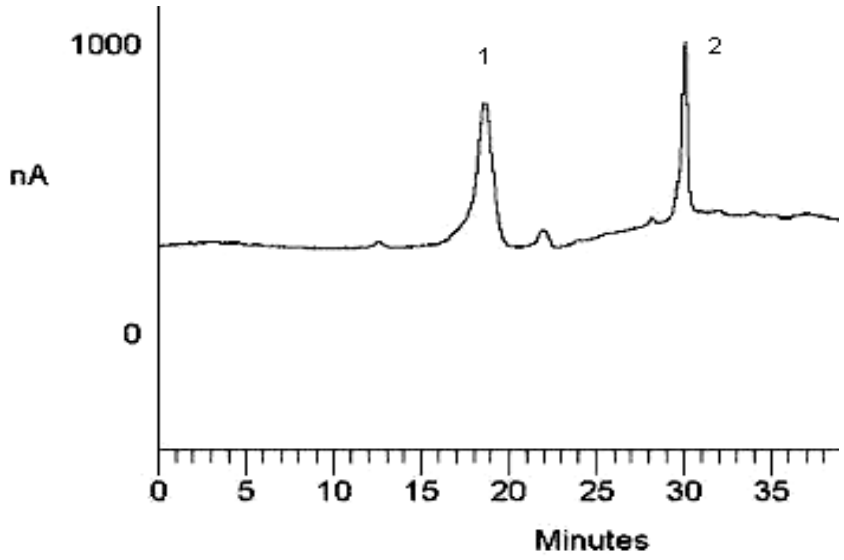


Fig. 4.1. Chromatograph analysis of sugars in the hydrolyzate of bermudagrass (cellulase loading – 10 FPU/g biomass, β-glucosidase loading – 0 CBU/g biomass) after 2-h saccharification. 1. Glucose. 2. Cellobiose.

The glucose content in the control sample was much lower than in the pretreated samples and kept constant after 24-h enzymatic hydrolysis. The control had glucose of 37 mg/g biomass and total reducing sugars of 84 mg/g biomass at time zero which was higher than the other treatments (Fig. 4.2c and Fig. 4.3c). This is probably because the free sugars in the control sample were dissolved in the liquid and contributed to the glucose content measured at time zero. In our previous study on acid pretreatment of rye straw, it was found that the glucose concentration in the prehydrolyzate was around 35 mg/g rye and remained constant despite the increased pretreatment severity (Chapter 3), which was almost the same as the glucose content of the non-pretreated control sample at the beginning of the saccharification in this study. This indicates that the cellulose in the

rye straw was not degraded into glucose by sulfuric acid pretreatment. The sugar content in the acid-pretreated samples was almost zero when the enzymatic reaction started, because soluble sugars in the dry biomass were removed with the filtrate and washes after the pretreatment. The separation of prehydrolyzate and solid residues not only effectively reduces the product inhibition that is caused by the excessive glucose in the liquid portion, but also removes the acetic acid, furfural, hydroxymethyl furfural (HMF), and phenolics that may be present as inhibitory compounds for the saccharification and/or fermentation (Hsu, 1996).

The addition of β-glucosidase improved the hydrolysis. At the cellulase loading of 5 FPU/g rye, the increase of β-glucosidase loading from 0 to 25 CBU/g rye enhanced the initial glucose production rate by 66% and the conversion rate by 14%. The supplementation of β-glucosidase accelerated the enzymatic reaction. The glucose yield after 8-h saccharification with β-glucosidase loading of 25 CBU/g was the same as that after 24-h saccharification without β-glucosidase. This means that the production cycle time was reduced by the addition of β-glucosidase.

The yield of total reducing sugars shown in Fig 4.3 exhibits the same trend as Fig. 4.2 except that the total reducing sugars of the control sample were higher than the pretreated biomass. This may be caused by the following reasons: (1) the free reducing sugars in the acid-pretreated biomass were removed with the filtrate and washes during the solid-liquid separation, but the control sample was not pretreated with acid; (2) other soluble reducing compounds released from the control sample might react with DNS reagent and be measured as reducing sugars.

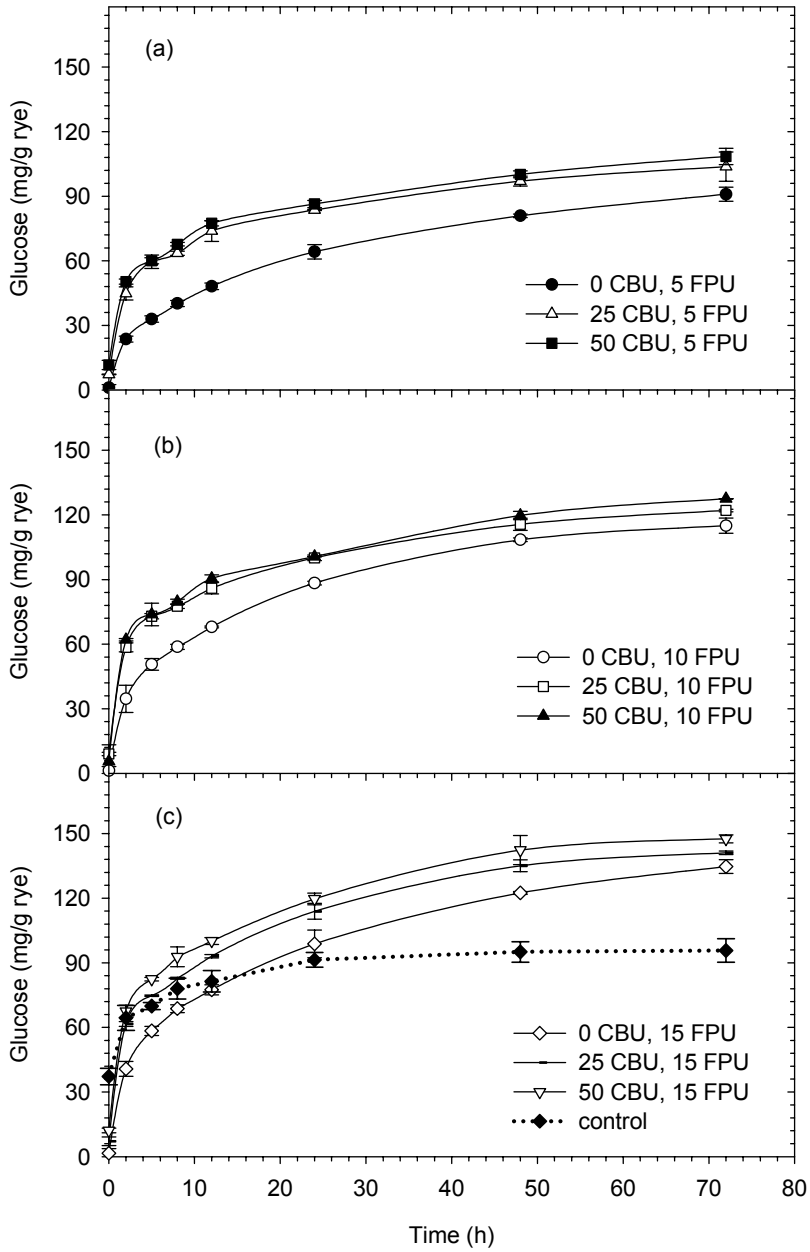


Fig. 4.2. Glucose yield during the enzymatic hydrolysis of acid-pretreated rye straw [1,2].
[1] Data are means ± SD of two replicates.
[2] CBU and FPU are β-glucosidase unit and cellulase unit based on per gram of dry biomass, respectively. Control sample was hydrolyzed with cellulases of 15 FPU/g dry biomass and β-glucosidase of 50 CBU/g dry biomass without acid pretreatment.
(a) Glucose yield with cellulase loading of 5 FPU. (b) Glucose yield with cellulase loading of 10 FPU. (c) Glucose yield with cellulase loading of 15 FPU.

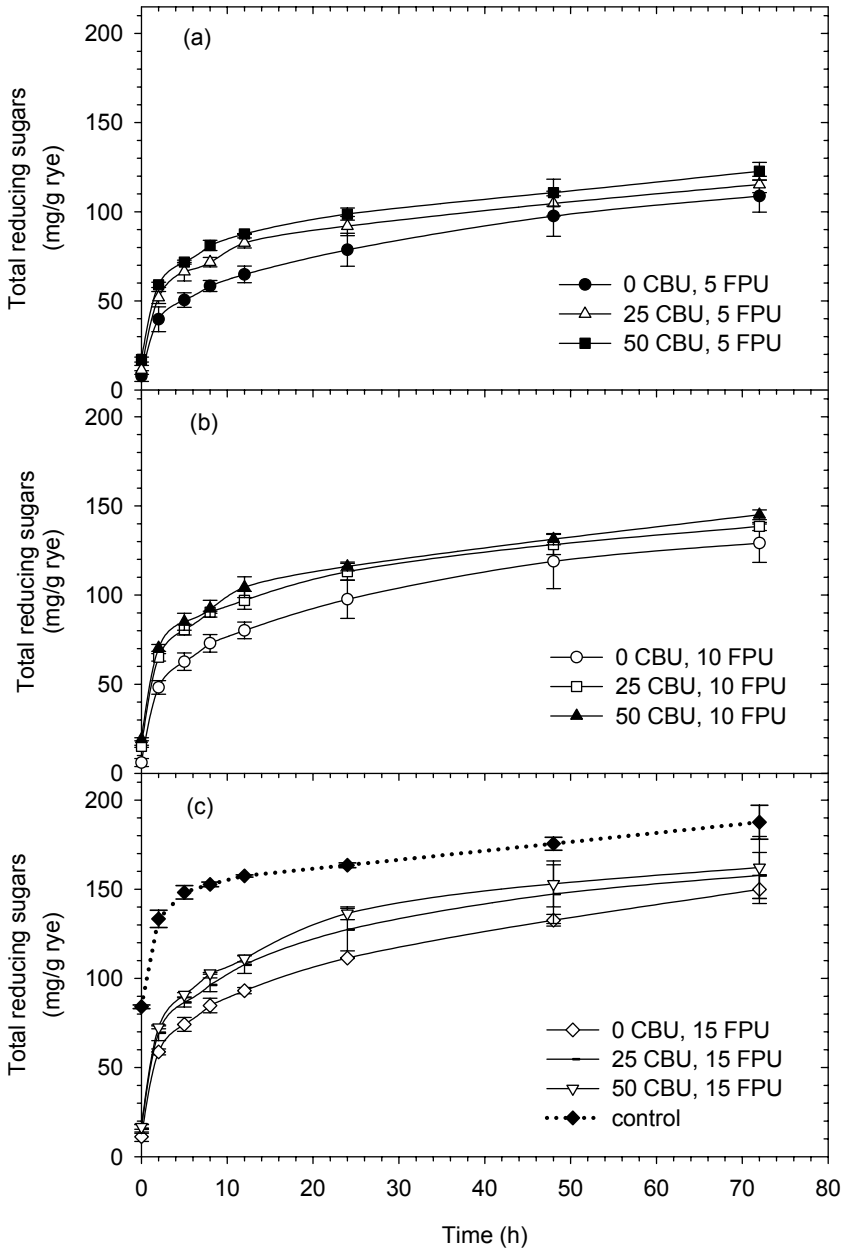


Fig. 4.3. Yield of total reducing sugars during the enzymatic hydrolysis of acid-pretreated rye straw[1,2].

[1] Data are means ± SD of two replicates.

[2] The legends represent the same meaning as those in Fig. 4.2.

(a) Total reducing sugar yield with cellulase loading of 5 FPU. (b) Total reducing sugar with cellulase loading of 10 FPU. (c) Total reducing sugar yield with cellulase loading of 15 FPU.

The conversion rate of cellulose to glucose and glucose production rate within the first two hours at different enzyme loadings are shown in Fig. 4.4 and Table 4.1. The conversion rate was calculated as glucose produced after 72-h enzymatic hydrolysis × 100 / potential glucose that can be produced from the dry biomass. Bioconversion reaction can be expressed as glucan + H_2O = glucose. Therefore, 0.9 g of glucan (molecular weight 162) is converted into 1 g of glucose (molecular weight 180) during the enzymatic hydrolysis according to their molecular weight, and the potential glucose yield is the glucan content in the biomass divided by 0.9.

Without β-glucosidase, the increase of cellulases from 5 to 15 FPU/g biomass enhanced the conversion rate (Fig. 4.4) and the glucose production rate within the first two hours (Table 4.1) by 48% and 73%, respectively. There was a linear increase in the conversion rate with the addition of β-glucosidase (Fig. 4.4). The increase of glucose yields was not significant ($p>0.05$) when β-glucosidase increased from 25 to 50 CBU/g rye (Fig. 4.2a). The cellobiose accumulated in the reactor and was gradually converted to glucose during the first two-hour hydrolysis in the sample without β-glucosidase loading (Fig. 4.5). The lower cellulase loading contained less enzyme activity and resulted in more cellobiose accumulation in the system. When the β-glucosidase loading was up to 25 CBU/g rye, the hydrolysis of cellobiose was complete and there was no cellobiose accumulation in the reactors.

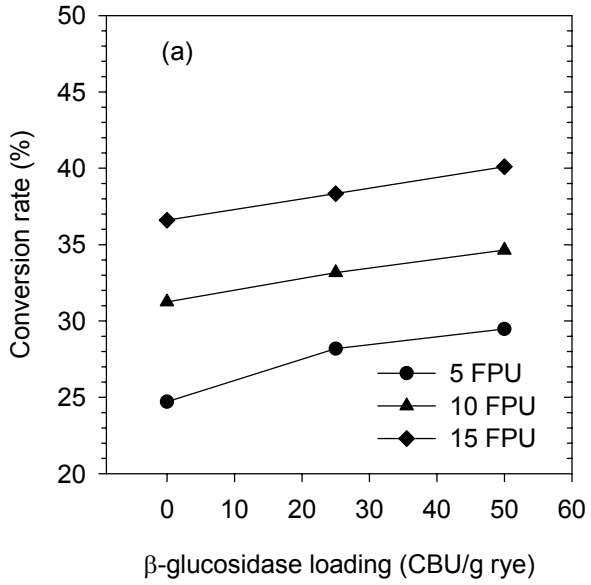


Fig. 4.4. Conversion rate after 72-h enzymatic hydrolysis of pretreated rye straw.

Table 4.1. Sugar production rate of rye straw during the initial 2-h enzymatic hydrolysis.

Cellulases (FPU/g)	β-Glucosidase (CBU/g)	Sugar production rate (mg g^{-1} h^{-1})	
		Glucose	Total reducing sugars
5	0	11.3	15.9
10	0	16.7	21.1
15	0	19.6	23.8
5	25	18.8	20.3
10	25	24.8	25.0
15	25	27.2	26.9
5	50	19.4	21.0
10	50	28.3	25.6
15	50	27.7	27.9
Control		13.6	24.7

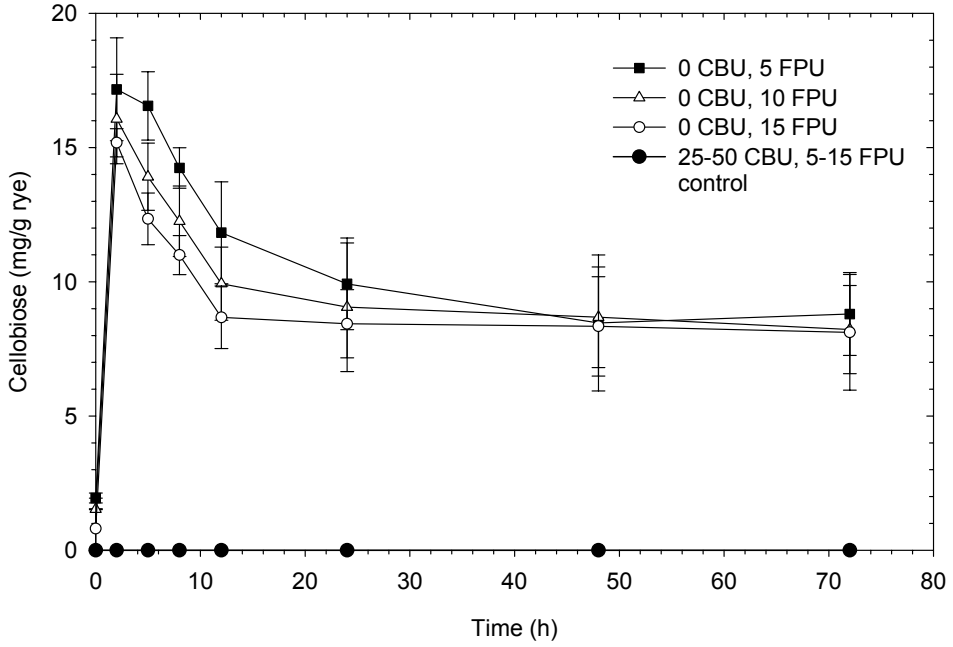


Fig. 4.5. Cellobiose yield during the enzymatic hydrolysis of pretreated rye straw [1,2].
[1] Data are means ± SD of two replicates.
[2] The legends represent the same meaning as those in Fig. 4.2.

It was reported that cellulases from *T. reesei* are deficient in the β-glucosidase enzyme and the ratio of β-glucosidase activity/filter paper activity is only 0.6, whereas the optimum hydrolysis required supplementation ratio of 0.8-2 cellobiase unit of β-glucosidase per filter paper unit of cellulases depending on the structure and composition of the lignocellulosic materials (Duff et al., 1985; Kadam, 1996). It has been known from our previous study that the glucose production reached about 135 mg/g rye when hydrolyzed with excessive enzymes (cellulases – 25 FPU/g rye, β-glucosidase – 75 CBU/g rye) for 48 hours, which equaled to that produced with cellulase loading of 15 FPU/g rye and β-glucosidase loading of 25 CBU/g rye in this study. The experimental

results demonstrate that the adequate conversion and synergism of cellulases and β-glucosidase were reached with cellulases of 15 FPU /g rye and β-glucosidase of 25 CBU/g rye and further increase of enzyme loading did not result in improved conversion rate. The supplemental ratio of β-glucosidase was 1.7 CBU/FPU at that condition.

4.4.2 Bermudagrass

Glucose produced during the enzymatic hydrolysis of acid-pretreated bermudagrass is shown in Fig. 4.6. The influence of replicates was not significant except the treatment with enzyme loading of 0 CBU/g and 15 FPU/g ($p = 0.003$). The glucose yields were significantly enhanced (Fig. 4.6a) and the glucose production rate during the first two hours increased from 8.3 to 24.8 mg/g biomass/h when β-glucosidase loading increased from 0 to 25 CBU/g biomass (Table 4.2).

When the cellulases and β-glucosidase were up to 10 FPU/g and 25 CBU/g, respectively, further increase of enzyme concentration did not significantly improve the conversion ($p>0.05$). The experimental data in chapter 3 showed that the glucose yield was about 156 mg/g dry bermudagrass when hydrolyzed with excessive enzymes, which was similar to that hydrolyzed with 10 FPU cellulases/g and 25 CBU β-glucosidase/g. It seems that cellulase loading of 10 FPU/g and supplementation ratio of 2.5 provided an adequate saccharification.

Similar effects were observed for the total reducing sugar production (Fig. 4.7). The control sample produced less glucose (Fig. 4.6c) and reducing sugars (Fig. 4.7c). The glucose content in the control sample at time zero was 25 mg/g biomass, which was much lower than that in the prehydrolyzate. The hemicellulose in grasses contains only

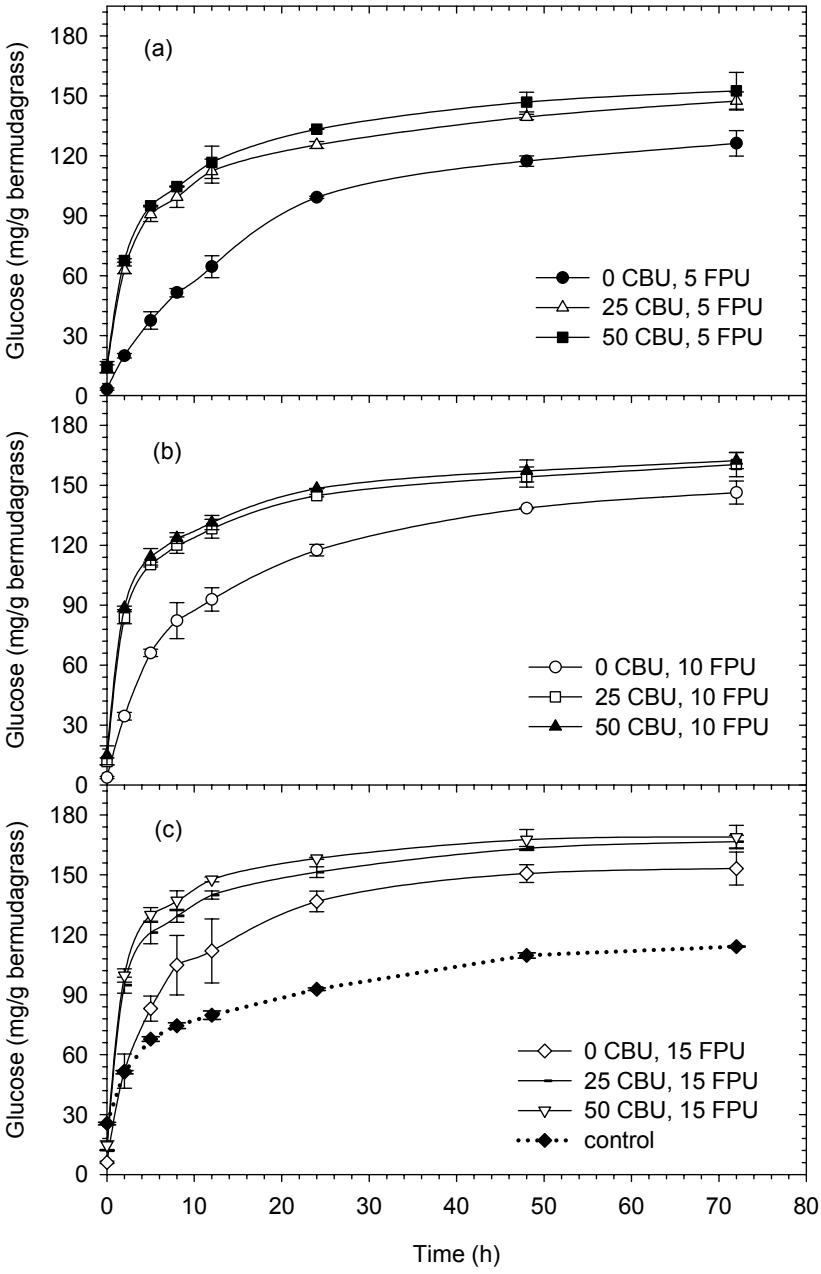


Fig. 4.6. Glucose yield during the enzymatic hydrolysis of acid-pretreated bermudagrass[1,2].

[1] Data are means ± SD of two replicates.

[2] The legends represent the same meaning as those in Fig. 4.2.

(a) Glucose yield with cellulase loading of 5 FPU. (b) Glucose yield with cellulase loading of 10 FPU. (c) Glucose yield with cellulase loading of 15 FPU.

Table 4.2. Sugar production rate of bermudagrass during the initial 2-h enzymatic hydrolysis.

Cellulases (FPU/g)	β-Glucosidase (CBU/g)	Sugar production rate (mg g^{-1} h^{-1})	
		Glucose	Total reducing sugars
5	0	8.3	23.1
10	0	15.3	31.5
15	0	22.9	37.2
5	25	24.8	28.0
10	25	36.0	40.6
15	25	41.4	44.4
5	50	26.7	30.0
10	50	36.9	39.5
15	50	42.5	45.8
Control		13.6	12.8

small amount of non-cellulosic glucan (Brigham et al, 1996). The large amount of glucose in the prehydrolyzate may be from the hydrolysis of cellulosic glucan into glucose. This verifies our previous statement that cellulose in the bermudagrass was degraded into glucose during the acid pretreatment.

The bermudagrass showed higher conversion rate compared to rye straw hydrolyzed at the same enzyme concentration (Fig 4.8). The conversion rate in the first two hours was very high when cellulose in the solid residues was converted into cellobiose and glucose. The relatively low β-glucosidase activity in the *T. reesei* cellulase system resulted in the accumulation of cellobiose during the first few hours (Fig. 4.9). When β-glucosidase reached 25 CBU/g, cellobiose was rapidly converted to glucose after formation and no accumulation was observed.

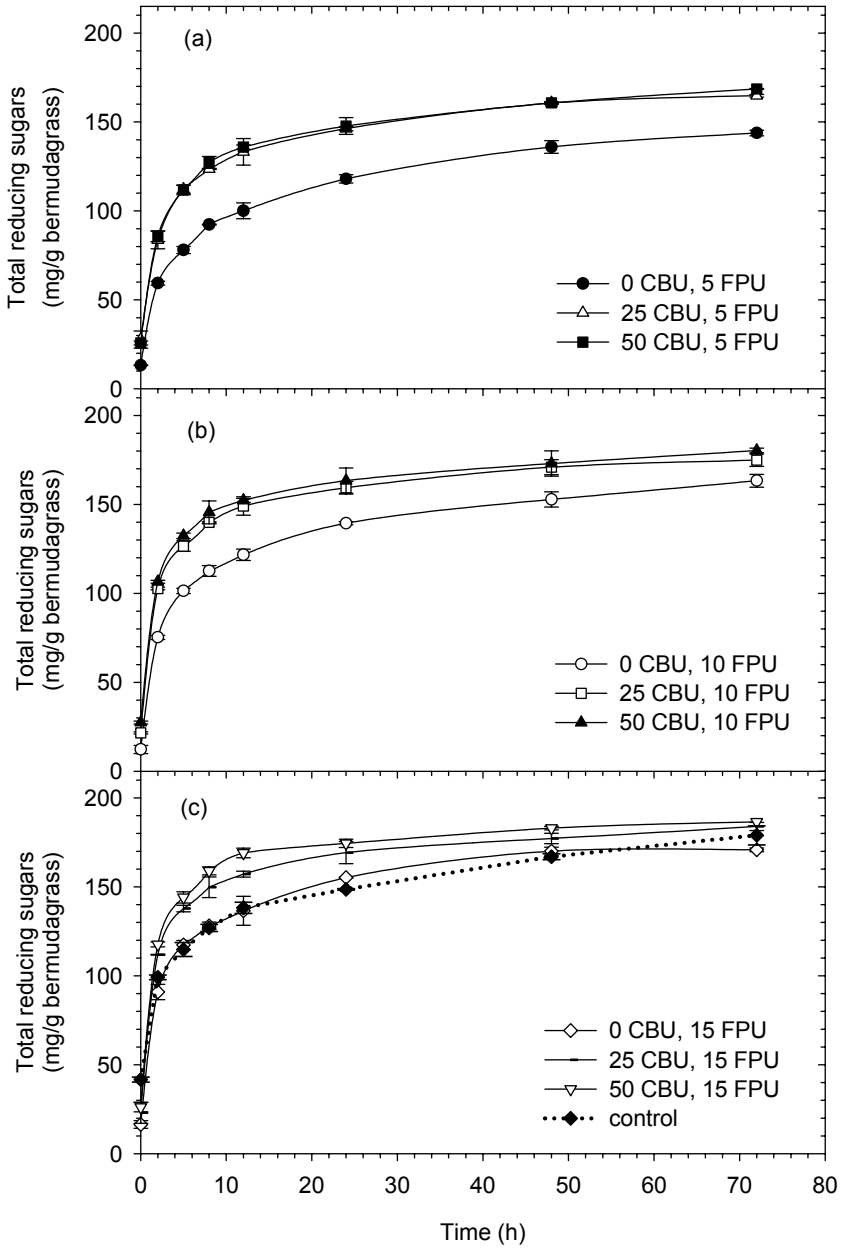


Fig. 4.7. Yield of total reducing sugars during the enzymatic hydrolysis of acid-pretreated bermudagrass[1,2].

[1] Data are means ± SD of two replicates.

[2] The legends represent the same meaning as those in Fig. 4.2.

(a) Total reducing sugar yield with cellulase of 5 FPU. (b) Total reducing sugar yield with cellulase of 10 FPU. (c) Total reducing sugar yield with cellulase of 15 FPU.

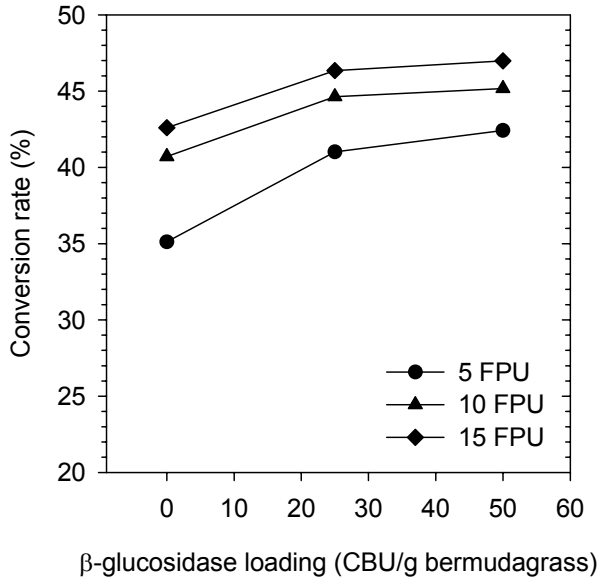


Fig. 4.8. Conversion rate after 72-h enzymatic hydrolysis of pretreated bermudagrass.

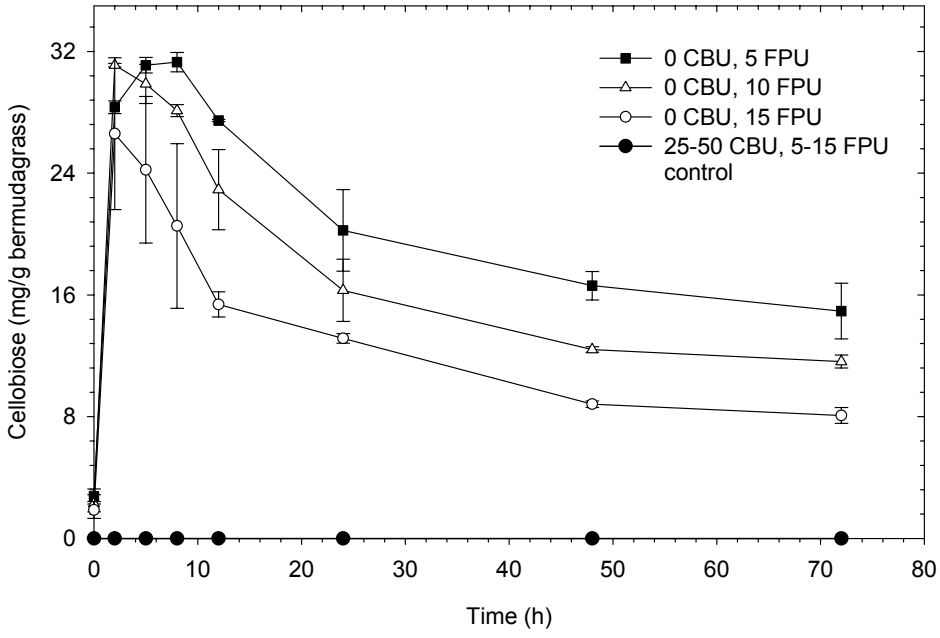


Fig. 4.9. Cellobiose yield during the enzymatic hydrolysis of pretreated bermudagrass[1,2].
[1] Data are means ± SD of two replicates.
[2] The legends represent the same meaning as those in Fig. 4.2.

4.5 CONCLUSIONS

Rye straw and bermudagrass hay, common byproducts from waste nutrient management systems on swine operations in southeastern USA, can be effectively hydrolyzed with cellulases supplemented with β-glucosidase to produce glucose. The sulfuric acid pretreatment did not solubilize the cellulose in rye straw, while part of the cellulose in bermudagrass was hydrolyzed into glucose during the acid pretreatment. Increase of cellulase loading from 5 to 15 FPU/g during the saccharification resulted in improved glucose and total reducing sugar production. The addition of β-glucosidase from *A. niger* in the cellulases from *T. reesei* improved the conversion. The cellulase loading of 10 FPU/g with supplemental ratio of β-glucosidase of 2.5 showed an adequate saccharification for pretreated bermudagrass and the conversion rate reached 45%. An adequate conversion of pretreated rye straw with conversion rate of 38% was observed when cellulase loading and β-glucosidase supplementation ratio were 15 FPU/g and 1.7, respectively. The supplemental β-glucosidase completely hydrolyzed the cellobiose accumulated in the hydrolyzate, which greatly enhanced the glucose production rate during the initial stage of the enzymatic reaction and accelerated the enzymatic conversion. Compared to bermudagrass, rye straw had relatively low conversion rate. This means rye straw is more resistant to enzymatic hydrolysis.

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EXPRESSION AND CHARACTERIZATION OF ENDOGLUCANASE E1 IN TRANSGENIC DUCKWEED *LEMNA MINOR*

5.1 ABSTRACT

Transgenic duckweed, *Lemna minor* 8627, that expresses *Acidothermus cellulolyticus* E1 endoglucanase was generated using *Agrobacterium*-mediated transformation under the control of cauliflower mosaic virus 35S promoter. The selected transgenic duckweed pCel25-IX15 exhibited no phenotypic abnormality. Two-week old transgenic duckweed fronds cultured in SH medium were ground in 50 mM sodium citrate buffer (pH 4.8), and the expression of endoglucanase E1 in the supernatant was examined using immunoblotting and enzyme assays. The E1 protein produced in the transgenic duckweed pCel25-IX15 was biologically active, and the expression level was 0.24% of total soluble protein. The E1 extract had the endoglucanase activity of 0.24 μmol glucose g^{-1} fresh duckweed min^{-1} or 0.20 μmol glucose mg^{-1} total soluble protein min^{-1} at 65°C using CMC as a substrate. HEPES buffer (50 mM, pH 8) extracted more E1 protein and other endogenous proteins than sodium citrate buffer (50 mM, pH 4.8) and sodium acetate buffer (50 mM, pH 5). A 5-min heat treatment of the crude extract at 65°C effectively removed most of other proteins without reducing the E1 enzyme activity. The thermo-stable characteristics of E1 protein may be useful for the harvest and storage of plant tissues and downstream processing. The optimal temperature and pH for E1 enzyme activity were approximately 80°C and 5, respectively. The E1 activity

remained unchanged after heating at 60°C for 6 h. However, it was inactivated after 15-min heating at 90°C.

5.2 INTRODUCTION

Lignocellulosic biomass is the most abundant biodegradable substance with an annual net yield of 1.8×10^{15} kg, of which about 40% is cellulose (Fan et al., 1987). The low cost of lignocellulosic materials makes them a promising feedstock for ethanol production. Bioconversion of cellulosic biomass to ethanol involves the breakdown of cellulose by cellulases to fermentable sugars, which are further fermented to ethanol by microorganisms. The effective enzymatic hydrolysis requires the synergism of three major groups of cellulases: endoglucanase that attacks regions of low crystallinity in the cellulose fiber and creates free-chain ends, exoglucanase that further removes cellobiose units from the free-chain ends, and β-glucosidase that hydrolyzes cellobiose to glucose.

Currently, industrial cellulases are usually produced by fungal or bacterial fermentation. Among all the cellulase-producing microorganisms, aerobic mesophilic fungus *T. reesei* is most broadly used due to its high productivity and full complement of three cellulase components. The enzymatic hydrolysis using cellulases from *T. reesei* is usually performed at 50°C and pH 5. According to the current technology, the cellulase cost is about $0.50 per gallon of ethanol produced which accounts for more than 50% of the ethanol production cost (BioTimes, 2001). This price is prohibitively expensive for a cost-effective ethanol production. The U.S. Department of Energy has a goal to reduce

the cost by ten folds for the process to be economical. Therefore, improving the cellulase productivity and reducing the cost are the key concerns.

Compared to *T. reesei,* thermophilic bacterium *Acidothermus cellulolyticus* produces thermostable cellulases with optimal temperature of 83°C (Himmel et al., 1996). Its thermotolerant property can reduce the risk of microbial contamination during saccharification and decrease the energy required to cool the process stream, which are desirable in industry (Himmel et al., 1994). The gene for the endoglucanase E1 has been isolated from *A. cellulolyticus*, and heterologous expression of cloned bacterial E1 enzyme has been carried out (Thomas et al., 1996; Adney et al., 1998).

The industrial cellulase fermentation process requires expensive culture media and controlled sterile environment including pH, temperature and dissolved oxygen levels, which are responsible for the high cost of cellulases. Because plant biomass can be produced in bulk quantities in the field, the production of recombinant enzymes in transgenic plants has the potential to significantly reduce the enzyme costs (Herbers and Sonnewald, 1996). Transgenic plants, such as potato and tobacco, have been studied for the production of cell wall degrading enzymes. Ziegelhoffer et al. (1999) reported the production and characterization of transgenic tobacco, potato and alfalfa plants that expressed the endoglucanase and cellobiohydrolase from *Thermomonospora fusca*. The recombinant cellulases in tobacco lines ranged up to about 0.1% endoglucanase and 0.02% cellobiohydrolase of total soluble protein. The expression of *A. cellulolyticus* endoglucanase E1 gene in transgenic tobacco and potato was as high as 1.35% and 2.6% of total soluble protein, respectively (Hooker et al., 2001). Ziegler et al. (2000) reported

the expression of E1 protein in the apoplast of tobacco BY-2 suspension cells and leaves of *Arabidopsis thaliana* plants. The enzyme accumulated up to 26% of the total soluble protein in leaves of primary *A. thaliana* transformants. These results indicate that transgenic plants can accumulate a large amount of E1 endoglucanase.

In this study, E1 endoglucanase from *A. cellulolyticus* was transformed into duckweed *L. minor* 8627. Duckweed (*Lemnaceae*) is a small, free-floating aquatic plant with the characteristics of fast multiplication and easy to grow (Landolt, 1998). Duckweed had a dry weight protein content of 15-45% and biomass yields of 7-20 tons dry weight per hectare of water surface per year (Landolt, 1986). This makes duckweed a good "bioreactor" for biomass and recombinant enzyme production. One way to produce large amount of duckweed with low cost may be to use nutrients in animal waste lagoon. Duckweed has been used for nutrient uptake from swine lagoon effluent (Classen et al., 2000; Cheng et al., 2002). The duckweed *Lemna minor* 8627 was selected from 41 geographic isolates and could remove 83% TKN, 100% NH_3-N, 49% P and 68% TOC when grew on 50% swine lagoon effluent within 12 days (Bergmann et al., 2000). Therefore, the development of transgenic duckweed with recombinant E1 protein may show dual applications: the nutrient removal from swine wastewater and the cellulase production from the harvested duckweed biomass. Compared to other cellulase-producing transgenic crops, the suggested dual applications have superior benefits. The expression level, enzyme activity, and biochemical characteristics of E1 protein in transgenic duckweed were examined using enzyme assay and

immunodetection in this study. The heat stability of E1 protein and effects of different extraction buffers and pH on E1 extraction were also investigated.

5.3 MATERIALS AND METHODS

5.3.1 Expression of E1 protein in Duckweed *Lemna minor* 8627

A transgenic duckweed strain expressing *A. cellulolyticus* E1 endoglucanase under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter was provided by Y. Yamamoto in Forestry Department of North Carolina State University. The genetic transformation protocol was described by Yamamoto et al. (2001). This transgenic duckweed was called pCel25-IX15. It showed the highest CMC (carboxymethyl cellulose) degrading capability among the 15 independent transgenic lines and was chosen for further study on the expression level and biochemical properties of E1 protein (Sun et al., 2002).

5.3.2 Extraction of E1 Protein

The transgenic duckweed pCel25-IX15 was cultured under sterile conditions in the liquid Schenk and Hildebrandt (SH) medium (Schenk and Hildebrandt, 1972) containing 1% sucrose at pH 5.6. After two-week culture, the duckweed fronds were collected and washed with deionized water three times to remove the medium residues. The E1 enzyme was extracted with a mortar and a pestle in 5 volumes (v/w) of 50 mM ice-cold sodium citrate buffer (pH 4.8). The crude extract was then centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was kept on ice for protein determination, SDS-

PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), immunoblot, temperature and pH optimization, and heat stability analysis.

The concentration of soluble protein in the supernatant was determined by the Bio-Rad protein assay using bovine serum albumin (BSA) as a standard (Bio-Rad Laboratories, CA).

5.3.3 Enzyme Activity Assay of E1

The activity of endoglucanase E1 was quantified at 65°C using CMC (degree of substitution = 0.7, medium viscosity) as a substrate (Ghose, 1987). The ice-cold enzyme extract was diluted with 50 mM sodium citrate buffer (pH 4.8) in the ratio of 1:4 (v/v) prior to the assay to remove the possible inhibition of the high-concentration glucose in the extract to the reaction. The enzymatic reaction was initiated by mixing 0.5 ml of diluted enzyme extract with 0.5 ml of 2% CMC substrate dissolved in 50 mM sodium citrate buffer (pH 4.8). The mixture was incubated at 65°C for 1 h in a water bath. The reaction was terminated by adding 3 ml DNS (dinitrosalicylic acid) and boiled for 15 min in a vigorously boiling water. After heating, the mixture was immediately transferred to a water bath at room temperature and the absorbance was measured at 550 nm with a spectronic® 401 spectrophotometer (Spectronic Instruments Inc., Rochester, NY). The reducing sugars generated during the reaction were calculated using glucose as a standard. The E1 activity was expressed as μmol glucose liberated from CMC substrate g^{-1} fresh duckweed min^{-1} or μmol glucose liberated from CMC substrate mg^{-1} total soluble protein min^{-1}.

5.3.4 SDS-PAGE and Immunoblot

Protein samples for gel electrophoresis were prepared by mixing three volumes of protein extracts with one volume of gel-loading buffer (200 mM Tris-HCl, pH 6.8, 400 mM dithiothreitol, 8% SDS, 0.4% bromphenol blue, 40% glycerol) and heated at 90°C for 5 min. The samples were centrifuged at 10,000 × g for 2 min. Proteins in the extracts were separated by Tris/Glycine SDS-PAGE with 10% acrylamide in the resolving gel. The proteins were then electrophoretically transferred onto nitrocellulose membranes (BioRad mini transblot cell, etc.).

Western blots were performed as recommended by ECL western blotting kit (Amersham Pharmacia Biotech Inc., NJ). The primary antibody and the second antibody were mouse monoclonal antibody against catalytic domain of E1 (original concentration 2.07 mg/ml, 1:12,000 dilution) and anti-mouse IgG conjugated with horseradish peroxidase (1:10,000), respectively. Purified E1 protein from the cultural supernatant of *Streptomyces lividans* with a plasmid containing *A. cellulolyticus* E1 gene was used as a control. The purified E1 protein and antibody were kindly provided by W. Adney (NREL, Golden, CO).

The amounts of E1 protein in the duckweed extracts were estimated from the intensities of cross-reacting bands using Molecular Dynamics Personal Densitometer SI and Molecular Dynamics Image Quant software (Molecular Dynamics, Sunnyvale, CA). Purified E1 protein (5, 25, 50 ng) from *S. lividans* was used as the standard to determine the amount of E1 protein. The molecular weight of E1 protein in the transgenic

duckweed was estimated using the broad range SDS-PAGE molecular weight standards (BIO-RAD, Hercules, CA).

5.3.5 Effects of pH and Temperature on Enzyme Activity

The effects of pH and temperature on E1 activity were tested in a triplicated 4×5 factorial experiment. The soluble proteins in the transgenic duckweed were extracted as described in section 5.3.2. In order to avoid the influence of different reaction buffers on the enzyme activity, the phosphate-citrate buffer with a broad pH range was chosen to dilute the enzyme extract and to dissolve the CMC substrate. Four phosphate-citrate buffers with pH 4, 5, 6, 7 were prepared. The enzyme extracts were diluted with 4 volumes of each phosphate-citrate buffer. Each mixture for enzyme reaction contained 0.5 ml of diluted enzyme extract and 0.5 ml of 2% CMC substrate solution dissolved in the phosphate-citrate buffer with the same pH as the enzyme extract. The mixture was incubated for one hour at 60, 70, 80, 90, or 95°C. Reducing sugars produced were calculated using a glucose standard curve. The E1 activity was expressed as μmol glucose produced per gram of fresh duckweed per min.

5.3.6 Heat Stability of the Recombinant E1 Enzyme

The protein extracts were heated at 60, 70, 80, or 90°C in a water bath. Aliquots were taken at 15, 30, 45, 60, 120, 180, 240, 300, and 360 min and cooled on ice. Insoluble materials were removed by centrifugation at 10,000 × g for 1 min at 4°C. The enzyme activity of the supernatant was assayed at 80°C at pH 5 according to the method

described in section 5.3.3. Enzyme activity was expressed as a percentage of the activity without heat treatment.

5.3.7 Effects of Extraction Media and Heating on E1 Extraction

A series of extraction buffers including sodium citrate buffer (50 mM, pH 4.8), sodium acetate (50 mM, pH 5) and HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid], 50 mM, pH 8) were prepared for the enzyme extraction. Fresh duckweed was ground in five volumes (v/w) of each extraction buffer. For heat treatment, the ground duckweed was incubated in a dry bath at 65°C for 5 min. Both samples were centrifuged at 10,000 × g for 10 min at 4°C. All the collected supernatants were analyzed for E1 amount by SDS-PAGE and immunoblot, soluble protein content, and E1 activities. The E1 activity was analyzed at 65°C, pH 5 using the method described in 5.3.3 section. Enzyme activity was expressed as μmol glucose g^{-1} fresh duckweed min^{-1} or μmol glucose mg^{-1} total soluble protein min^{-1}.

5.4 RESULTS AND DISCUSSION

5.4.1 Expression of Endoglucanase E1

The proteins in the transgenic duckweed pCel25-IX15 and wild type duckweed *L. minor* 8627 were extracted using 50 mM sodium citrate buffer at pH 4.8 as described in section 5.3.2. The CMC degrading activity, E1 amount, and total soluble protein content in the extracts are shown in Table 5.1.

Table 5.1. CMC degrading activity, total soluble protein, and E1 amount in the protein extracts of transgenic duckweed pCel25-IX15 and wild type *L. minor* 8627.

Duckweed	CMC activity[a] (μmol g^{-1} fresh duckweed min^{-1})	CMC activity[a] (μmol mg^{-1} total soluble protein min^{-1})	Total soluble protein[b] (mg/g fresh duckweed)	E1 protein (μg/g fresh duckweed)
Transgenic duckweed pCel25-IX15	0.24 ± 0.012	0.20 ± 0.057	1.337 ± 0.257	3.5
Wild type *L. minor* 8627	ND[c]	ND	1.157 ± 0.202	ND

[a]Data are means ± SD of two replicates.
[b]Data are means ± SD of three replicates.
[c]ND = not detected.

The expression level of E1 protein was estimated by gel electrophoresis and protein blot analysis (Fig. 5.1). A series of diluted *S. lividans* E1 protein were used as the standard for densitometric analysis (Fig. 5.1, lanes 3-5). The estimated amount of E1 in the extract of transgenic duckweed pCel25-IX15 was 0.24% of total soluble protein or 3.5 μg/g fresh duckweed (Table 5.1). The protein extract from wild type duckweed 8627 did not show any cross-reacting band for the immunoblot analysis (Fig. 5.1, lane 1), and no detectable CMC degrading activity was observed (Table 5.1). This has demonstrated that the observed E1 activity in the extract of transgenic duckweed pCel25-IX15 was caused by the expression of heterologous E1 enzyme in the plant tissues. The molecular weight of E1 protein from transgenic duckweed pCel25-IX15 was similar to the purified *S. lividans* E1 protein and was calculated to be 40 kDa (kilodaltons).

From the data listed above, the specific activity of endoglucanase E1 is 69 μmol glucose mg^{-1} E1 protein min^{-1}. Himmel et al. (1994) reported that the purified E1 from *A.*

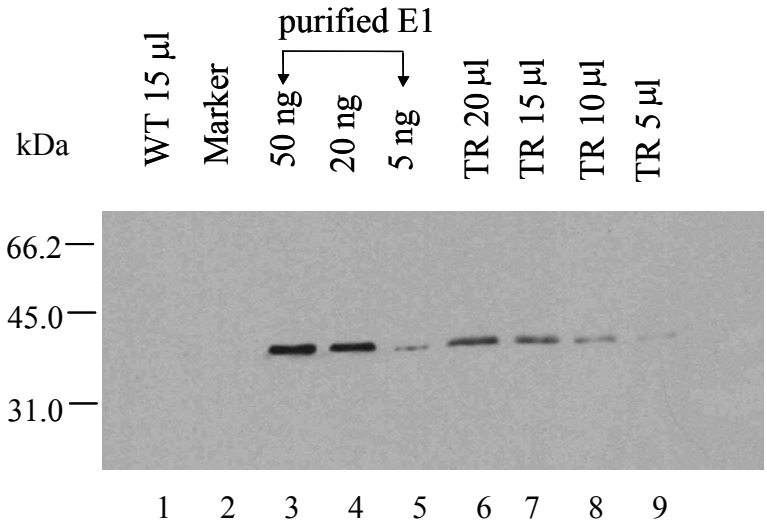

Fig. 5.1. Immnodetection of endoglucanase E1 in transgenic duckweed pCel25-IX15. Lane 1 contained 15 µl of protein extract from wild type control *L. minor* 8627. Lane 2 contained SDS-PAGE molecular weight standards. The amount used was described as ECL western blotting kit (Amersham Pharmacia Biotech Inc., NJ). Lanes 3-5 contained 50, 20, 5 ng of purified E1 protein from *S. lividans.* Lanes 6-9 contained 20, 15, 10, 5 µl of protein extract from transgenic duckweed *L. minor* pCel25-IX15.

cellulolyticus had the CMC activity of 20 µmol glucose mg^{-1} E1 protein min^{-1} at 65°C. The specific activity of E1 enzyme from transgenic duckweed was in the same range as that from *A. cellulolyticus*. This indicates that E1 enzyme activity was not inhibited by expression in duckweed or other procedures. The native E1 has the molecular weight of 72 kDa (Adney et al., 1998). The recombinant E1 gene transferred into duckweed *L. minor* was modified by PCR amplification and the produced E1 catalytic domain (E1 Cat.) had a molecular weight around 40 kDa (Andey et al., 1998). Therefore, the recombinant E1 with reduced molecular weight contains more number of E1 molecules in one mg of E1 protein compared to native E1, which may be the reason for its higher

specific activity. Another reason is experimental errors, such as the imprecision of immunodetection and CMC activity assay of E1.

The bioconversion of cellulose needs the synergism of three groups of cellulases: endoglucanase, exoglucanase and β-glucosidase. The expression of endoglucanase and exoglucanase in transgenic plants has been reported (Ziegelhoffer et al., 1999; Hooker et al., 2001). For our study, the transformation of exoglucanase and β-glucosidase into transgenic duckweed pCel25-IX15 is necessary for the efficient hydrolysis of cellulose substrate.

The expression of *A. cellulolyticus* E1 endoglucanase does not appear to influence the growth (data not shown) and phenotype of transgenic duckweed pCel25IX15. The outlook of wild type *L. minor* 8627 and transgenic duckweed pCel25IX15 is similar (Fig. 5.2.).

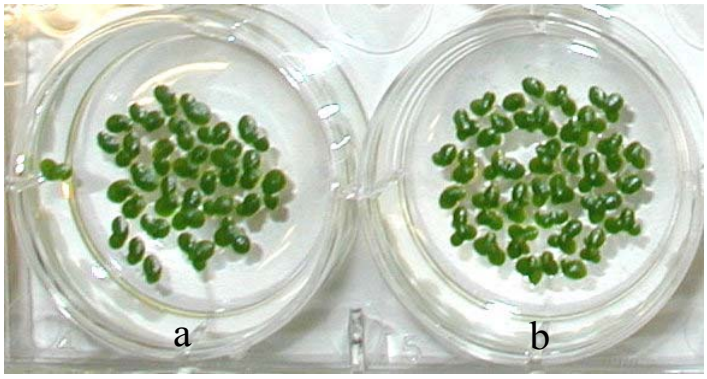

Fig. 5.2. Expression of E1 endoglucanase does not affect the growth and phenotype of *L. minor* 8627. a, Wild type duckweed *L. minor* 8627. b, Transgenic duckweed pCel25IX15.

5.4.2 Temperature & pH responses of E1

In previous study, the temperature and pH responses of E1 were studied at a certain pH or temperature, respectively (Tucker et al., 1992; Dai et al., 2000) and no factorial study on the E1 activity at different temperatures and pH has been reported. To investigate the responses of recombinant E1 to various temperatures and pH and verify that it retained the characteristics of the original *A. cellulolyticus* E1 enzyme, responses of the heterologous E1 to varying temperatures and pH were investigated by a triplicated 4×5 factorial experiment as described in section 5.3.5. The experimental results are shown in Fig. 5.3. The recombinant E1 exhibited the maximum CMC degrading activity when pH value was about 5 (Fig. 5.3), which corresponded to the reported optimum pH of *A. cellulolyticus* E1 enzyme (Tucker et al., 1989). The recombinant E1 activity dropped rapidly when pH changes from 5 to 4 at each reaction temperature. At 60°C and 70°C, the E1 activity exhibited a broad pH range. When the pH of the reaction mixture was changed from 5 to 7 at 60°C and 70°C, the E1 activity dropped only 8%. However, the E1 enzyme activity lost 30% at 80°C and 86% at 90°C when pH increased from 5 to 7. The pH response curves of E1 enzyme indicate that the E1 protein was more sensitive to pH changes when temperature was at and above 90°C.

The E1 activity increased with the increase of temperatures from 60°C to 80°C at the corresponding pH value. The E1 activity decreased dramatically at 95°C. At pH 5, the enzyme activity at 95°C dropped to be the same as that at 60°C. In addition, at pH 5, the E1 enzyme had higher CMC degrading activity at 80°C and 90°C, which was in the

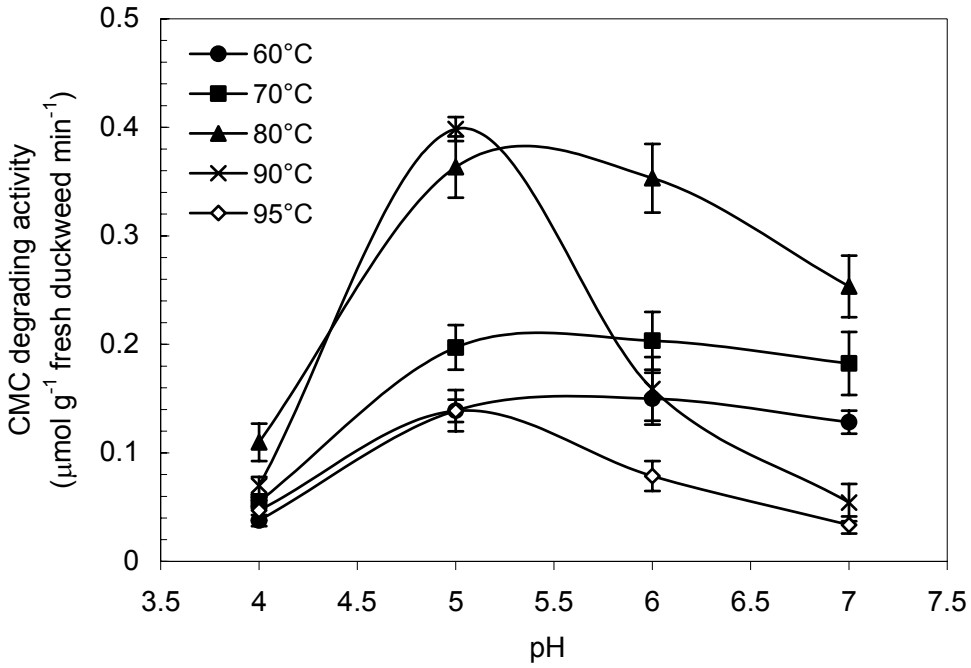

Fig 5.3. Effects of temperature and pH on E1 enzyme activity. The E1 protein was extracted using 50 mM sodium citrate buffer (pH 4.8). The CMC activity assay was performed at the different temperatures and pH. Each point is the mean ± SD of three replicates.

range of the reported optimal temperature of 83°C for endoglucanase from *A. cellulolyticus* (Tucker et al., 1989; Himmel et al., 1994). Therefore, the recombinant E1 retained the temperature and pH properties of the E1 in bacterial system.

5.4.3 Heat Stability of E1

It has been known that the native E1 has a certain degree of stability at high temperature (Tucker et al., 1989). At present, the most widely used mesophilic cellulases from *T. reesei* exhibit the optimal activity at 50°C, thus the high-temperature process stream produced from acid pretreatment requires expensive cooling process to fit the temperature for enzymatic hydrolysis (Himmel et al., 1994). The thermostable

characteristics of E1 endoglucanase are desirable for industrial use because of less energy requirement for refrigeration and reduced microbial contamination at high temperature.

The heat stability of the recombinant E1 in transgenic duckweed was investigated and the heat inactivation curves of E1 protein are shown in Fig. 5.4. The E1 protein was extracted using sodium citrate buffer (50 mM, pH 4.8) and heated in a water bath at different temperatures. The E1 activity was expressed as a percentage of the initial activity. Fig. 5.4 shows that the E1 protein activity was almost the same as the original activity after 6-h heating at 60°C. However, the E1 enzyme activity suffered a 27% reduction after 30-min heating at 70°C and remained constant during the rest of the experiment. When heated at 80°C, E1 activity declined gradually to 38% of its maximum activity within one hour and almost lost all of its activity after three hours. E1 enzyme activity dropped remarkably in the first 15 minutes at 90°C and showed no activity after 45 minutes. The experimental data indicate that the heat inactivation of recombinant E1 occurred at 90°C within 15 minutes, while E1 was stable at 60°C and 70°C. Tucker et al. (1989) reported that the CMC degrading activity of concentrated *A. cellulolyticus* growth supernatant was decreased by 15% when heated at 75°C and that the complete activity loss was observed after 4-h preincubation at 90°C. The recombinant E1 protein in transgenic duckweed pCel25-IX15 exhibited the similar feature as the native E1.

The E1 enzyme produced the maximum amount of reducing sugars from CMC substrate at 90°C and pH 5 in 1-h enzyme reaction assay as demonstrated in section 5.4.2.

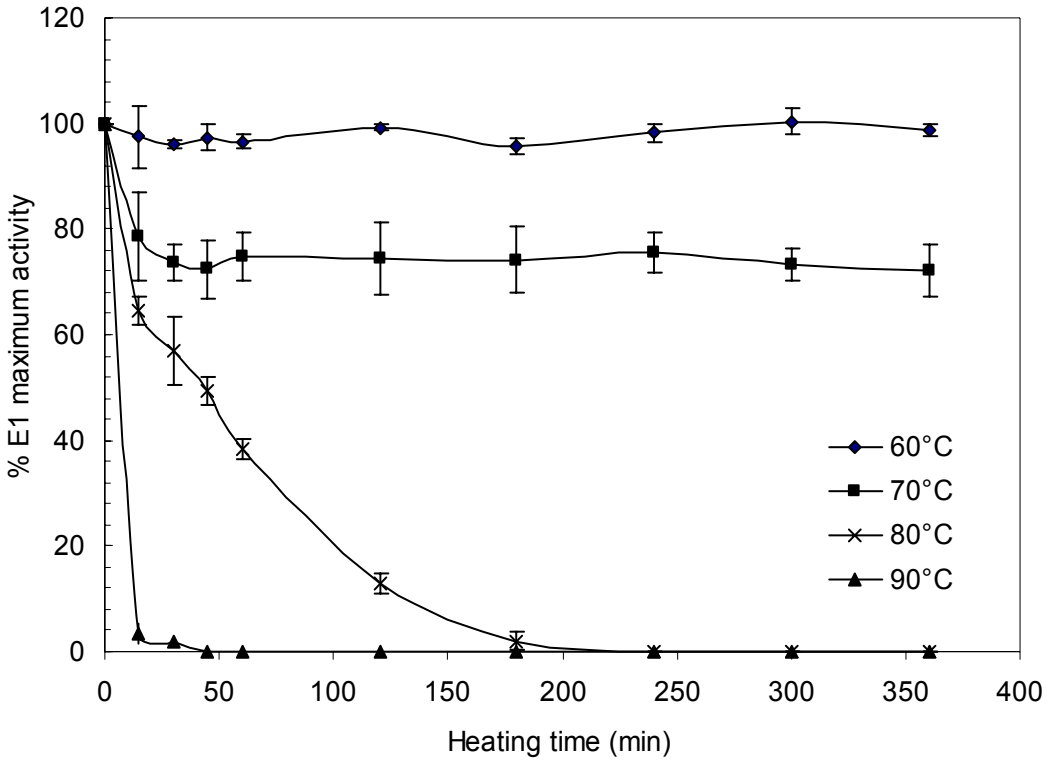

Fig. 5.4. Heat stability of E1 protein. The E1 protein extracts were heated at different temperatures for the time indicated on the graph. The E1 activity was assayed at 80°C, pH 5 using CMC as substrate. The activity was expressed as percentage of the initial activity. Each point is the mean ± SD of three replicates.

However, 96% of E1 activity was lost within 15-min preincubation at 90°C without the addition of CMC in the enzyme extract (Fig 5.4). This phenomenon might be due to the binding of CMC substrate to the E1 enzyme in the enzyme reaction mixture of section 5.4.2, which stabilized the E1 enzyme and slowed down the inactivation of E1 enzyme at 90°C. In the current ethanol production technology, the saccharification of cellulose materials by cellulases from fungi *T. reesei* is carried out at 50°C and pH 5 for 48 h during which its cellulases are stable. The recombinant E1 needs to be stable during the

saccharification process to achieve an effective bioconversion of cellulose to glucose. Although E1 protein had higher activity at 80°C or 90°C and pH 5, the heat inactivation may occur during the 48-h saccharification process. In addition, E1 activity was very sensitive to pH changes at 90°C, which made it difficult for pH control in industry. The recombinant E1 is relatively stable at 60°C and 70°C (Fig. 5.4) and not very sensitive to pH changes as observed in Fig. 5.3, which can be used for the saccharification of cellulose substrate in industry.

5.4.4 Buffer and Heating on E1 Extraction

The most commonly used buffer for enzymatic hydrolysis of lignocellulosic biomass is 50 mM sodium citrate buffer or 50 mM sodium acetate buffer with pH around 5 that is in agreement with the optimal pH of cellulase enzymes (Tucker et al., 1989; Holtzapple et al., 1994). Therefore, sodium citrate buffer (50 mM, pH 4.8) was used as E1 extraction buffer and E1 enzyme reaction buffer in this study. However, buffers with higher pH (6.5–7.2 or higher) were normally used to extract proteins from transgenic plant tissues and the buffer chemicals, its pH value, and extraction conditions are known to influence the protein extraction from plants (Hatti-Kaul and Mattiasson, 1996). Therefore, the effects of different extraction buffers on the amount and activity of E1 and total soluble protein content in the extracts were studied as described in section 5.3.7. Because most plant proteins are denatured and removed by 65°C, we also investigated the effects of heat treatment on the extraction of E1 and total soluble protein from transgenic duckweed.

The experimental results are shown in Fig. 5.5. The specific treatment conditions are listed for each lane in Fig. 5.5. The HEPES buffer at pH 8 (Fig. 5.5a, lanes 5 and 6) extracted much more soluble proteins from transgenic duckweed fronds than citrate buffer (lanes 1 and 2) and acetate buffer (lanes 3 and 4). Under no-heating conditions, the total soluble protein extracted by HEPES was 2.2 times and 7.2 times of that extracted by acetate and citrate, respectively (Fig. 5.5d). Heating dramatically reduced the soluble proteins in the extracts (40%, 73%, and 77% for HEPES, acetate, and citrate buffer, respectively), while the amount of E1 didn't show much difference between heat and no heat treatment (Fig. 5.5b, f). More E1 protein was extracted by HEPES compared to citrate and acetate buffers. The E1 extracted by acetate and citrate buffer was 75% and 70% of that by HEPES. Fig. 5.5c shows that the extracted E1 was biologically active and that the E1 activity per gram of fresh duckweed increased in the following sequence: HEPES with heating > HEPES with no heating > acetate with heating > acetate with no heating > citrate with heating > citrate with no heating.

Heating the crude extracts at 65°C for 5 min denatured a large amount of soluble proteins. The denatured protein aggregates were removed by centrifugation at 10,000 × g for 10 min at 4°C, which significantly reduced the protein content in the extracts. The E1 amount and activity increased after heat treatment. This could be due to improved degradation of cell wall by heat, and therefore resulted in more E1 released from the cell. The E1 protein is probably more heat-stable than most of the endogenous proteins in duckweed. The stability of E1 protein to heat may be useful for the biomass storage and downstream protein purification. Of the three extraction buffers, citrate buffer extracted

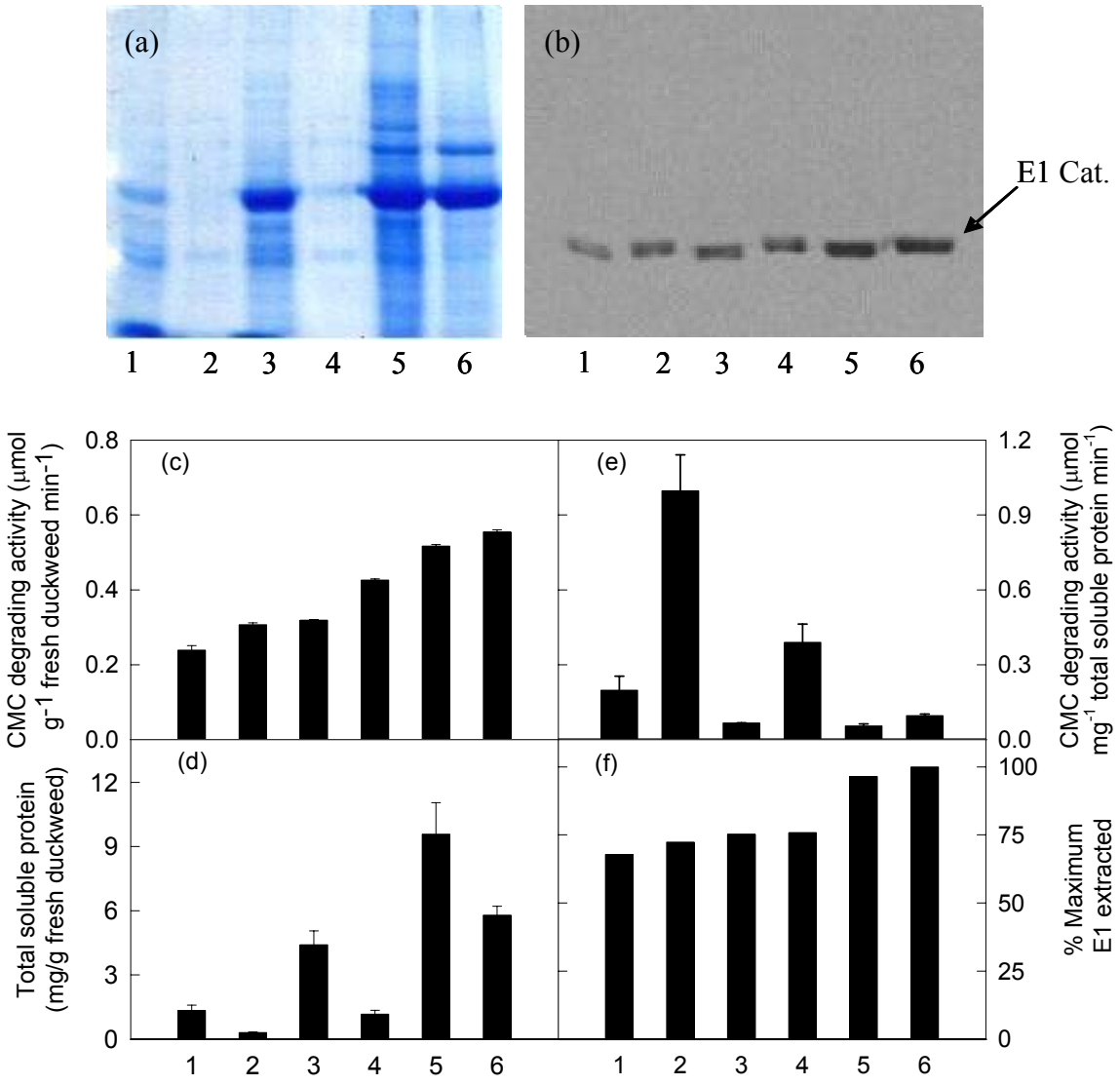

Fig. 5.5. Extraction and expression of E1 protein in transgenic duckweed pCel25-IX15 under different extraction conditions. 1, 3, 5 – E1 was extracted using 50 mM sodium citrate buffer (pH 4.8), sodium acetate (pH 5) and HEPES (pH 8), respectively. After centrifuged at 10,000 × g for 10 min at 4°C, the supernatant was used for E1 protein assay. 2, 4, 6– E1 was extracted using 50 mM sodium citrate buffer (pH 4.8), sodium acetate (pH 5) and HEPES (pH 8), respectively. The mixtures were heated at 65°C for 5 min before the analysis. (a) SDS-PAGE. 15 µl of protein extracts was loaded on the gel. (b) Western blot of E1 protein. (c) E1 activity, expressed as µmol glucose g^{-1} fresh duckweed min^{-1} (±SD of two replicates). (d) Total soluble protein extracted (±SD of two or three replicates). (e) E1 activity, expressed as µmol glucose mg^{-1} total soluble protein min^{-1} (±SD of two replicates). (f) Percentage of E1 extracted, expressed as % of E1 extracted by HEPES (50 mM, pH 8) with heat treatment at 65°C for 5 min.

the minimum amount of E1 protein and total soluble protein, while HEPES extracted the maximum amount of E1 and other proteins in the plant tissues (Fig. 5.5d, f). This could be due to the precipitation of proteins at the low pH of citrate buffer. Experimental results indicate that the pH or buffer chemicals influenced the protein extraction of transgenic duckweed. Fig. 5.4e shows that the duckweed extract prepared with citrate buffer and heat treatment had the maximum CMC activity per mg of soluble protein, which is 10 times of the extract prepared with HEPES without heat treatment. The purification of recombinant protein from transgenic plants requires the separation of other soluble proteins from the extracts using ultrafiltration and ion exchange, etc. Although HEPES extracted more E1 protein and resulted in improved E1 activity in the extract, the total extracted protein also increased, which may complicate the E1 separation and purification and result in increased cost for downstream processing of cellulase production. In addition, the use of HEPES buffer produces a protein extract with pH 8, which needs to be adjusted to the optimal pH of 5 for enzyme reaction. It may also raise the cost of enzymatic hydrolysis.

5.5 CONCLUSIONS

The endoglucanase E1 gene from bacteria *A. cellulolyticus* was successfully expressed in the transgenic duckweed *Lemna minor* 8627. The expression of E1 enzyme did not influence the growth and phenotype of selected transgenic duckweed pCel25-IX15. Grinding and heat treatment favored the release of E1 protein from duckweed cell.

The recombinant E1 protein was found to be biologically active and has the CMC degrading activity of 0.24 μmol g^{-1} fresh duckweed min^{-1} or 0.20 μmol mg^{-1} total soluble protein min^{-1}. The accumulation of E1 enzyme in transgenic duckweed pCel25-IX15 was up to 0.24% of total soluble protein or 3.5 μg/g fresh duckweed. The extraction buffers and pH influenced the amount of E1 extracted. HEPES buffer (50 mM, pH 8) extracted more E1 enzyme than sodium citrate buffer (50 mM, pH 4.8) and sodium acetate buffer (50 mM, pH 5). Therefore, the E1 activity in the HEPES extract was much higher than that in the other two buffers. Heating the crude extract denatured a large amount of endogenous proteins that were then removed by centrifugation, but E1 activity remained stable. The thermo-tolerant characteristics of E1 enzyme may simplify the E1 separation and purification processes and therefore reduce production cost of E1 enzyme. The E1 enzyme extracted from the transgenic duckweed retained the similar properties with respect to pH, temperature and heat stability as the native E1. The enzyme activity assay demonstrated that E1 protein extract had higher CMC degrading activity at pH 5 and temperature 80°C. The E1 protein was more pH sensitive at 90°C and dramatic activity reduction was observed when pH was changed from 5 to 4 or 6. The E1 activity was more stable to pH changes at relatively low temperatures (60°C and 70°C). At pH 4, E1 activity was obviously decreased compared to pH 5. The E1 enzyme activity was rather stable at 60°C and 70°C, but E1 activity was completely lost after 3-h heating at 80°C and 15-min heating at 90°C. The heat stability provides an easy way for the harvest and storage of duckweed fronds and the E1 purification.

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CONCLUSIONS AND FUTURE WORK

6.1 CONCLUSIONS

Rye straw and bermudagrass have the potential for fuel ethanol production. Hemicellulose in the biomass was solubilized by dilute sulfuric acid pretreatment with arabinose, galactose, and xylose as the major monomeric sugars produced. Xylose accounted for the largest amount of monomeric sugars in the prehydrolyzate. The increase of pretreatment severity resulted in more hemicellulose removal and thus enhanced the accessibility of cellulase enzymes to cellulose. More than 50% of the xylan was hydrolyzed into xylose when pretreated with 1.2% (w/w) sulfuric acid at 121°C for 60 min. The cellulose in the rye straw was not hydrolyzed into glucose during acid pretreatment. However, about 27% of the glucan from bermudagrass was hydrolyzed into glucose when pretreated with 1.2% sulfuric acid for 60 min and the glucose yield increased with the increased acid concentration and time. The higher glucose content in the prehydrolyzate of bermudgrass made the glucose recovery from liquid fraction important. Most of the total reducing sugars produced from the breakdown of polysaccharides in the biomass were monomeric sugars from the solubilization of hemicellulose. Cellulose remaining in the pretreated feedstock was highly digestible by cellulase enzymes and the conversion rate of cellulose to glucose increased with the pretreatment severity. The effective hemicellulose removal and enzyme hydrolysis was achieved under the pretreatment conditions of 1.2% sulfuric acid, 60 min, and 121°C.

The enzymatic hydrolysis of acid-pretreated solid residues by cellulases from *T. reesei* was greatly accelerated by the addition of β-glucosidase from *Aspergillus niger*. The cellobiose as the intermediate product was rapidly converted into glucose by the supplemental β-glucosidase and no cellobiose accumulation was observed when β-glucosidase loading was up to 25 CBU/g. The cellulase loading of 10 FPU/g with β-glucosidase supplementation ratio of 2.5 (β-glucosidase activity: filter paper acitivity of cellulases) showed an adequate saccharification of pretreated bermudagrass and the conversion rate reached 45%. The rye straw is more resistant to cellulases compared to bermudagrass. The optimal conversion rate of pretreated rye straw was observed to be 38% when the enzymatic reaction was conducted with cellulase loading and β-glucosidase supplementation ratio of 15 FPU/g and 1.7, respectively. If the glucose in the prehydrolyzate was counted, the glucose yields of 48% and 72% of the theoretical potentials were obtained for rye straw and bermudagrass, respectively.

The endoglucanase E1 gene from bacteria *Acidothermus cellulolyticus* was successfully transformed and expressed in duckweed *Lemna minor* 8627 using *Agrobacterium*-mediated transformation under the control of cauliflower mosaic virus 35S promoter. The transgenic duckweed pCel25-IX15 exhibited no phenotypic abnormality. The accumulation of E1 enzyme in pCel25-IX15 was up to 0.24% of total soluble protein or 3.5 μg/g fresh duckweed. The recombinant E1 protein was biologically active with the CMC activity of 0.24 μmol g^{-1} fresh duckweed min^{-1} or 0.20 μmol mg^{-1} total soluble protein min^{-1}. The grinding of duckweed fronds and a 5-min heating of the crude extracts at 65°C favored the E1 release from the cell. The heat

treatment denatured the endogenous proteins, but E1 activity remained stable. The extraction buffers and pH influenced the amount of E1 extracted. HEPES buffer (50 mM, pH 8) extracted more E1 protein and other endogenous proteins than sodium citrate buffer (50 mM, pH 4.8) and sodium acetate buffer (50 mM, pH 5). The E1 enzyme extracted from the transgenic plant pCel25-IX15 retained similar thermotolerant property to *A. cellulolyticus*. The CMC degrading activity of recombinant E1 was rather stable at 60°C and 70°C, but complete activity loss occurred after 3-h heating at 80°C and 15-min heating at 90°C. The heat stability may simplify the E1 separation and purification processes, and provide an easy way for the harvest and storage of plant tissues. The E1 protein exhibited the optimal CMC activity at temperature 80°C and pH 5. In addition, the E1 activity was stable to pH changes at relatively low temperatures of 60°C and 70°C and sensitive to pH at 90°C. The E1 activity decreased at pH 4 regardless of the reaction temperature.

6.2 SUGGESTIONS FOR FUTURE WORK

Based on the experimental results in this thesis, the following work can be investigated for future study:

(1) Identify the ethanol production capability of rye straw and bermudagrass by conducting fermentation of the hydrolyzates from sulfuric acid pretreatment and the subsequent saccharification. This thesis developed the dilute sulfuric acid pretreatment and enzymatic hydrolysis processes. The yields of monomeric sugars in the hydrolyzates, such as arabinose, galactose, xylose and glucose, were measured to identify

the conditions for the sufficient pretreatment and saccharification, which will provide useful information for the following fermentation. It was reported that the xylose in the prehydrolyzate can be directly fermented to ethanol by xylose-fermenting yeast and bacteria (McMillan, 1996). The glucose in the hydrolyzates of acid pretreatment and enzymatic hydrolysis can be converted to ethanol by yeast *Saccharomyces cerevisiae*. The fermentation of those reducing sugars by yeast will give the actual ethanol yields from the bioconversion of rye straw and bermudagrass.

(2) Transfer the genes of thermostable exoglucanase and β-glucosidase into transgenic duckweed pCel25-IX15 for the production of cellulase components with full complement. There are at least three major groups of cellulases involved in the conversion of cellulose to glucose: endoglucanase, exoglucanase and β-glucosidase. Although the transgenic duckweed pCel25-IX15 contained endoglucanase activity, the transformation of the other two cellulase components is needed for the efficient hydrolysis of lignocellulosic biomass. The production of endoglucanase E1, and exoglucanase in the transgenic tobacco and potato has been reported (Ziegelhoffer et al., 1999; Hooker et al., 2001).

(3) Improve the expression level of recombinant cellulase enzymes in the transgenic duckweed pCel25-IX15 using molecular biology techniques. It was estimated that the cellulase expression level up to 10-15% of the total protein in transgenic potato will provide the cellulase production cost in the range of $0.104-0.329/ gallon ethanol produced (Hooker et al., 2001). The accumulation of E1 enzyme up to 26% of the total soluble protein in leaves of primary *A. thaliana* transformants was reported (Ziegler,

2000). The high expression level was due to the expression of E1-cat gene in the apoplast of *A. thaliana* that can accumulate large quantities of heterologous proteins without affecting the growth of transgenic plants. This indicates that a large amount of recombinant cellulase production is achievable. The expression level of E1 protein in transgenic duckweed pCel25-IX15 was 0.24% of total soluble protein in this study. Further improvement is needed for the cost-effective cellulase production from transgenic duckweed.

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EXPERIMENTAL DATA

Table 1. Monomeric sugars and total reducing sugars in the prehydrolysate of dilute-sulfuric-acid-pretreated rye straw and bermudagrass[1].

	Rye Straw				
Treatment [2]	Arabinose	Galactose	Glucose	Xylose	Total Reducing Sugars
11	12.500	1.284	36.157	0.665	129.937
21	15.127	1.791	35.487	22.407	183.380
31	16.168	3.238	34.924	55.258	227.172
41	17.687	3.795	32.575	82.824	254.964
12	13.145	2.518	34.557	17.027	184.694
22	15.170	4.294	34.719	87.245	258.719
32	18.056	4.340	35.505	115.806	285.846
42	18.328	4.415	35.888	135.837	309.319
13	15.097	3.410	34.926	39.587	215.525
23	16.229	4.555	34.439	121.185	292.551
33	16.807	4.611	34.957	139.254	307.035
43	19.257	4.949	37.302	146.213	315.288
	Bermudagrass				
11	20.520	2.361	32.913	1.872	140.734
21	27.581	5.359	37.790	22.176	229.364
31	29.063	6.623	41.465	55.873	285.130
41	30.480	8.119	58.692	89.372	336.581
12	21.936	4.651	38.413	20.299	223.806
22	30.361	8.779	63.983	91.831	341.324
32	33.196	10.105	96.163	125.785	394.671
42	27.366	10.114	119.741	136.989	418.229
13	24.964	6.777	40.931	41.489	270.704
23	28.487	10.338	76.543	118.494	375.388
33	28.377	10.302	116.339	129.682	388.471
43	32.229	11.226	121.744	139.079	400.979

[1] Data units are mg/g biomass.

[2] The first digit denotes dilute sulfuric acid concentration (w/w). 1 = 0.6%; 2 = 0.9%; 3 = 1.2%; 4 = 1.5%; The second digit denotes residence time (min). 1 = 30; 2 = 60; 3 = 90.

Table 2. Glucose and total reducing sugars yields from the saccharification of pretreated rye straw and bermudagrass with enzyme loading of cellulases 25 FPU/g and β-glucosidase 75 CBU/g [1].

	Rye Straw	
Treatment [2]	Glucose	Total Reducing Sugars
11	74.035	124.953
21	90.163	135.289
31	99.872	146.228
41	109.789	159.718
12	89.519	133.678
22	112.227	161.933
32	135.434	177.369
42	147.732	192.940
13	90.252	136.094
23	119.379	169.248
33	147.173	187.101
43	155.266	197.101
	Bermudagrass	
11	132.965	207.168
21	140.725	205.221
31	148.296	218.040
41	152.130	216.899
12	145.766	195.691
22	148.570	208.174
32	155.566	204.081
42	174.808	217.302
13	143.411	203.745
23	153.949	206.362
33	158.723	219.181
43	176.952	229.315

[1] Data units are mg/g biomass.

[2] The first digit denotes dilute sulfuric acid concentration (w/w). 1 = 0.6%; 2 = 0.9%; 3 = 1.2%; 4 = 1.5%; The second digit denotes residence time (min). 1 = 30; 2 = 60; 3 = 90.

Table 3. Glucose, cellobiose and total reducing sugars yields during the 72-h saccharification of pretreated rye straw with different enzyme loadings [1].

	Glucose							
Treatment [2]	0 h	2 h	5 h	8 h	12 h	24 h	48 h	72 h
11	1.048	23.675	32.945	40.170	48.145	64.160	80.882	90.913
12	1.289	34.602	50.557	58.747	67.968	88.331	108.436	114.992
13	1.554	40.769	58.361	68.741	77.493	98.784	122.454	134.676
21	7.247	44.832	59.565	63.552	73.846	83.612	96.993	103.735
22	8.981	58.539	72.944	77.570	85.935	100.062	115.625	122.070
23	7.191	61.605	74.758	82.846	93.088	113.907	135.037	141.088
31	11.635	50.337	60.017	67.702	77.402	86.381	100.124	108.484
32	5.512	62.016	73.774	79.699	90.506	100.649	119.735	127.476
33	12.146	67.470	82.427	92.827	100.152	119.677	142.331	147.580
control	37.237	64.443	69.920	77.985	81.394	91.393	95.052	95.722
	Cellobiose							
11	1.943	17.169	16.551	14.238	11.826	9.921	8.464	8.797
12	1.527	16.063	13.911	12.252	9.930	9.052	8.681	8.219
13	0.804	15.179	12.338	10.995	8.674	8.434	8.338	8.116
21	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
22	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
23	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
31	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
32	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
33	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
control	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Total Reducing Sugars							
11	7.806	39.706	50.469	58.405	64.835	78.623	97.547	108.740
12	6.004	48.202	62.625	72.920	80.127	97.622	118.877	129.114
13	11.162	58.709	74.185	84.752	93.073	111.401	132.598	149.881
21	11.287	51.925	66.392	71.707	82.472	91.974	104.622	115.263
22	15.109	65.081	80.562	90.374	97.026	113.113	128.316	138.453
23	15.637	69.512	86.528	96.364	107.669	127.362	147.229	157.755
31	17.035	59.006	71.724	81.077	87.555	98.692	110.735	122.730
32	19.117	70.334	85.037	92.358	104.411	116.022	131.350	145.050
33	16.663	72.527	90.817	102.831	111.101	136.476	152.996	162.197
control	84.065	133.373	148.253	152.712	157.445	163.424	175.493	187.564

[1] Data units are mg/g biomass.

[2] The first digit denotes dilute β-glucosidase loading (CBU/g biomass). 1 = 0; 2 = 25; 3 = 50; The second digit denotes cellulase loding (FPU/g biomass). 1 = 5; 2 = 10; 3 = 15.

Table 4. Glucose, cellobiose and total reducing sugars yields during the 72-h saccharification of pretreated bermudagrass with different enzyme loadings [1].

	Glucose							
Treatment [2]	0 h	2 h	5 h	8 h	12 h	24 h	48 h	72 h
11	3.204	19.873	37.529	51.473	64.470	99.198	117.352	126.248
12	3.739	34.434	66.128	82.277	92.862	117.556	138.554	146.366
13	6.048	51.766	83.061	104.796	111.980	136.717	150.676	153.190
21	13.361	62.898	90.782	99.395	112.326	125.466	139.418	147.464
22	11.771	83.764	110.340	120.066	128.292	144.830	154.222	160.460
23	11.934	94.790	121.146	129.516	139.900	151.365	163.271	166.662
31	14.140	67.602	95.037	104.607	116.740	133.295	146.903	152.541
32	14.868	88.707	114.233	123.621	131.367	148.414	157.225	162.409
33	14.654	99.621	129.991	137.112	147.740	158.255	167.681	168.912
control	25.532	51.218	67.801	74.487	79.759	92.743	109.633	114.107
	Cellobiose							
11	2.757	28.331	31.095	31.297	27.462	20.241	16.607	14.940
12	2.305	31.097	29.867	28.115	22.918	16.305	12.412	11.618
13	1.869	26.595	24.222	20.532	15.376	13.142	8.818	8.079
21	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
22	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
23	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
31	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
32	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
33	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
control	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Total Reducing Sugars							
11	13.244	59.371	78.014	92.354	100.108	117.999	135.961	143.789
12	12.250	75.291	101.373	112.550	121.658	139.459	152.787	163.323
13	16.445	90.913	117.348	128.072	136.547	155.203	170.058	170.794
21	27.663	83.627	112.157	123.530	133.195	146.278	160.655	164.739
22	21.541	102.699	126.566	139.845	149.086	159.364	170.949	174.848
23	23.033	111.823	137.798	149.811	157.140	169.242	177.184	183.980
31	25.736	85.706	111.754	127.278	135.851	147.682	160.705	168.597
32	27.415	106.490	132.383	145.489	152.221	163.376	172.978	180.317
33	26.109	117.637	144.063	158.939	169.026	174.457	183.024	186.568
control	41.647	99.136	114.753	126.925	138.202	148.584	166.940	178.950

[1] Data units are mg/g biomass.

[2] The first digit denotes dilute β-glucosidase loading (CBU/g biomass). 1 = 0; 2 = 25; 3 = 50; The second digit denotes cellulase loding (FPU/g biomass). 1 = 5; 2 = 10; 3 = 15.

Table 5. Endoglucanase activity, total soluble protein, and percent of E1 in the extracts of transgenic duckweed pCel25-IX15.

Treatment [1]	activity (μmol g^{-1} min^{-1})	total soluble protein (mg g^{-1})	% of maximum E1 extracted
011	ND[2]	1.157	
111	0.239	1.337	67.8
121	0.307	0.306	72.4
112	0.319	4.399	75.2
122	0.426	1.173	75.8
113	0.517	9.585	96.4
123	0.555	5.783	100

[1] The first digit denotes the duckweed. 0 = wild type; 1 = transgenic duckweed pCel25-IX15; The second digit denotes the extraction conditions. 1 = the crude extracts were not heated; 2 = the crude extracts were heated at 65°C for 5 min; The third digit denotes the extraction buffer used. 1 = sodium citrate buffer (50 mM, pH 4.8); 2 = sodium acetate buffer (50 mM, pH 5); 3 = HEPES buffer (50 mM, pH 8).

[2] ND = not detected.

Table 6. Endoglucanase activity of transgenic duckweed pCel25-IX15 at different pH and temperature.

Temperature (°C)	Endoglucanase activity (μmol g^{-1} min^{-1})			
	pH 4	pH 5	pH 6	pH 7
60	0.0377	0.139	0.150	0.128
70	0.0551	0.197	0.203	0.182
80	0.110	0.363	0.353	0.253
90	0.0698	0.398	0.159	0.0541
95	0.0469	0.139	0.0786	0.0335

Table 7. Percent of E1 maximum activity after heat treatment.

Heating Time (min)	% of maximum activity			
	60°C	70°C	80°C	90°C
0	100.0	100.0	100.0	100.0
15	97.4	78.6	64.5	3.3
30	96.1	73.7	57.0	1.9
45	97.4	72.5	49.4	0.0
60	96.5	74.8	38.3	0.0
120	99.3	74.4	13.0	0.0
180	95.7	74.1	2.1	0.0
240	98.2	75.6	0.0	0.0
300	100.4	73.4	0.0	0.0
360	98.8	72.2	0.0	0.0