ABSTRACT

XU ZHOU. Dilute Sulfuric Acid Pretreatment of Genetically-Engineered Switchgrass for Improved Sugar Production. (Under the direction of Jay J. Cheng.)

Conventional Alamo switchgrass and its transgenic counterparts with reduced/modified lignin were subjected to dilute sulfuric acid pretreatment for improved sugar production. At 150 °C, the effects of acid concentration (0.75, 1, 1.25%) and residence time (5, 10, 20, 30 min) on sugar productions in pretreatment and enzymatic hydrolysis were investigated. And the optimal pretreatment conditions were determined for each switchgrass genotype based on total sugar yield and the amounts of sugar degradation products generated during the pretreatment. The results show that genetic engineering, although did not cause an appreciable lignin reduction, resulted in a substantial increase in the ratio of acid soluble lignin:acid insoluble lignin, which led to considerably increased sugar productions in both pretreatment and enzymatic hydrolysis. At an elevated threshold concentration of combined 5-hydroxyfuranmethal and furfural (2.0 g/L), the overall carbohydrate conversions of conventional switchgrass and its transgenic counterparts, 10/9-28,10/9-33,10/9-40,11/5-41 and 11/5-47 reached 77.3, 82.3,82.6, 84.4 and 82.2%, respectively.
Dilute Sulfuric Acid Pretreatment of Genetically-Engineered Switchgrass for Improved Sugar Production

by
Xu Zhou

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

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APPROVED BY:

____________________________________  __________________________________
Jay Cheng                                    Ratna R. Sharma-Shivappa
Committee Chair

____________________________________
Jason Osborne
DEDICATION

This thesis is dedicated to my parents, who never fail to provide me unconditional financial and moral support.
BIOGRAPHY

In fall of 2009, Xu Zhou attended “3+1” program, which is aimed to help Zhejiang University students have abroad study opportunities. At the last year of his undergraduate stage, Xu Zhou began his program as a graduate student in the Department of Biological and Agriculture Engineering at North Carolina State University. In July 2010, he achieved his bachelor degree from Zhejiang University in China. Under the guidance of Dr. Cheng, Jiele Xu and Ziyu Wang, Xu Zhou not only completed his own studies, but also gained experience working in a variety of projects in the group.
ACKNOWLEDGMENTS

Foremost, I would like to thank my advisor, Dr. Jay J. Cheng. Without your patience and press, my written and spoken English will not make such progress in short time. I also thank you for your trust in my ability to give the speech on ASABE meeting in Louisville, Kentucky. Additionally thank you for your invitation to your house, where I spent happy time like in a big family in the United State. I also enjoyed very much in the beer party after group meeting sometime.

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CHAPTER 1

INTRODUCTION

1.1 Background

The US ethanol industry has been developing very fast during the last decade to meet the growing demand for biofuel to power the transportation sector. It is reported that the total ethanol production in 2010 reached 13 billion gallons, which is seven times greater than that a decade ago and resulted in a record oil import displacement of 445 million barrels (Renewable Fuels Association, 2011). However, the dominant feedstock for ethanol production is corn in the US, which has raised much concern over the sustainability of the ethanol industry because corn is also an important raw material for food and feed production (Sun and Cheng, 2002). Lignocellulosic biomass including dedicated energy crops, agricultural residues, and forest wastes is considered as a potential solution to this dilemma as it is abundant with a variety of feedstocks (Xu et al., 2010a).

Switchgrass (Panicum virgatum L.) is a perennial warm-season C4 grass native to North America. Adapted ecotypes of switchgrass inhabit regions throughout the US due to its tolerance to heat, cold, and draught (Casler et al., 2007). Because of its high productivity, low requirements for agricultural inputs, suitability for marginal land quality, and environmental benefits, switchgrass was selected as a model herbaceous energy crop by the US Department of Energy (McLaughlin, 1993; McLaughlin et al., 1999). Like other lignocellulosic biomass, however, switchgrass is not susceptible to
biochemical conversion due to its complex and recalcitrant structure, especially the presence of lignin barrier. Without structural modification, the conversion of switchgrass to liquid fuel would be much less promising than other pathways like direct burning or pyrolysis. To improve biomass-to-ethanol conversion, a pretreatment step is required for switchgrass. An effective biomass pretreatment causes the breakup of lignocellulosic structure, thus making the carbohydrates more accessible during the subsequent enzymatic saccharification (Xu et al., 2010b). Dilute acid pretreatment is one of the most extensively studied pretreatment methods. It results in an effective solubilization of hemicellulose and a reduction in the crystallinity of cellulose by disrupting covalent bonds, hydrogen bonds, and van der Waals forces that hold together the biomass components (Sun and Cheng, 2002; Li et al., 2010). Genetic engineering is another tool that can be used to improve the saccharification of lignocellulose. Since lignin is the major barrier limiting the conversion of biomass carbohydrates to fermentable sugars in enzymatic hydrolysis, one of the most direct and effective approaches to improve biomass conversion is to down-regulate the enzymes involved in lignin biosynthesis to reduce lignin content or modify its composition/structure (Ralph et al., 2006; Chapple et al., 2007; Chen and Dixon, 2007). A lot of successful work has been done in this direction (Hisano et al., 2009). It is expected, therefore, that the genetically engineered switchgrass with reduced/modified lignin might serve as a very promising feedstock for ethanol production.

In this research, the conventional and genetically engineered switchgrass obtained
from the Department of Crop Science at North Carolina State University were used as feedstock. Dilute sulfuric acid pretreatment of switchgrass was conducted at 150 °C to improve sugar production in enzymatic hydrolysis. The effects of acid concentration and residence time on glucose and xylose yields of switchgrass were investigated and the optimal pretreatment conditions for both conventional and genetically engineered switchgrass were determined based on total sugar recovered from prehydrolysate and hydrolysate as well as the amounts of sugar degradation products generated during the pretreatment.

1.2 Objectives of this Study

This research was aimed to investigate genetically engineered switchgrass for improved sugar generation, including composition analysis and scanning electron microscope (SEM) which provide morphology changes information. Furthermore, this study also provides the information of different performance from various types of switchgrass after pretreatment.

The specific objectives of this project were to: 1) Compare the effects of pretreatment conditions on fermentable sugar production from transgenic switchgrass and conventional switchgrass; 2) Investigate the effect of the dilute sulfuric acid (H₂SO₄) pretreatment and then optimize the pretreatment of both conventional and transgenic switchgrass at high temperature (150°C) for improved sugar yield on the subsequent enzymatic hydrolysis.
REFERENCES


CHAPTER 2
Pretreatment for Lignocellulosic Materials for an Enhanced Bioethanol Production: A Review

2.1 INTRODUCTION

2.1.1 Need for renewable bioenergy

Renewable bioenergy is increasingly attracting attention from policy makers and scientists, as fossil fuels become more costly and harder to find and extract, and cause a serious environmental concerns such as greenhouse gas emissions. Some factors have enabled the increase in renewable bioenergy resources to supplement or, with increased efficiency, perhaps replace part of the conventional fossil fuel based energy production.

The first factor is the future energy supply and security. Growing evidence has shown that the total oil production from exiting oil fields is declining and the oil peak will certainly happen even taking the undiscovered oil field into account (Kjell, 2006). However, because the overall uncertainty and disagreement on the extent of unexplored crude oil, there is a big range of the certain date of oil peak.

Petroleum plays a significant role in the US energy system, approximately accounting for 40% of the total energy consumption (Figure 2.1), and more than 50% of petroleum is imported from overseas, which results in national energy security and trade deficit issues (EIA, 2010). By 2017, the US alone will need 135 billion liters of renewable fuels as a goal set by the 20 in 10 program (reduce gasoline usage by 20% in
Environmental issue is another factor to consider in exploring alternative sources of energy. The scientific community involved in investigating climate change, agrees that burning fossil fuels contributes to global warming (Crutzen, 2008). Global warming is expected to shift the location of viable agriculture, to harm ecosystems and animal habitats, and to change the timing and magnitude of water supply (Motha, 2007). In the United State, almost all gasoline is blended with at least 10% ethanol because ethanol is a renewable energy source and blending ethanol into gasoline contributes to significantly reducing petroleum use and exhaust green house gas (GHS) emission (Wheals et al., 1999).

The third factor to focus on renewable bioenergy technologies is that new employment would emerge in connection with research, development, and production in the field (Demirbas, 2008). Researchers foresee important possibilities for using the technologies in a commercial context (Marina et al., 2011). In additional, the financial
crisis and downturn of the world economy since 2008 is regarded by some policymakers as a time in which public money should be made available to improve technology that allows for faster breakthrough and jumps ahead (Marina et al., 2011).

The US ethanol industry did a good job on job creation. The fuel ethanol industry employed 400,677 Americans in 2010, of which 83 percent of the employees earned wages exceeding $40,000 with health care and other benefits. It indicates that the ethanol industry is paying well and providing good benefits. In addition, the ethanol industry added $53.6 billion to the Gross Domestic Product (GDP), an effective indicator of U.S. economic activity (RFA, 2011).

2.1.2 Current Ethanol Industry in U.S.

The liquid transportation fuel replacement candidates with the most potential include biomethanol, biobutanol, and bioethanol in which the 'bio' prefix demonstrates these fuels are produced from a biomass source (Redding, 2010) combustion, ethanol has been used as a gasoline additive (10% ethanol v/v) in the U.S. to replace the toxic chemical, methyl tertiary butyl ether (MTBE) (Browner, 2000). In October 2010 a waiver was released by EPA to authorize the blend level to improve from 10% to 15%, indicating that up to 15% of ethanol blended with gasoline can be sold for a model 2007 or newer cars and light pickup trucks (Brent et al., 2010). In January 2011, EPA has expanded the waiver to mandate the E15 use for 2001 and newer passenger vehicles.

The united State has experienced unprecedented interest in developing ethanol
industry in the last decade. Between the years 2000 and 2010 the number of ethanol plants in the United States grew from 56 to 204 (Table 2.1). Over the same period, annual ethanol production capacity increased from about 2 billion gallons to 13.5 billion gallons. The growth rates of 260% in the number of plants and 550% in ethanol production capacity, respectively, mostly relied on the availability of corn as a feedstock.

Table 2.1 U.S. Ethanol Plant Production Capacity 2000-2010 (RFA,2011)

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Capacity(MGY)</td>
<td>13508</td>
<td>11,887</td>
<td>10,569</td>
<td>7888</td>
<td>5493</td>
<td>4336</td>
<td>3644</td>
<td>3101</td>
<td>2707</td>
<td>2348</td>
<td>1922</td>
</tr>
<tr>
<td>Change in Total Capacity</td>
<td>1,621</td>
<td>1,318</td>
<td>2,681</td>
<td>2,395</td>
<td>1,157</td>
<td>692.7</td>
<td>543</td>
<td>394</td>
<td>360</td>
<td>425</td>
<td>N/A</td>
</tr>
<tr>
<td>% Change in Total Capacity</td>
<td>12.0%</td>
<td>11.1%</td>
<td>25.4%</td>
<td>30.4%</td>
<td>21.1%</td>
<td>16.0%</td>
<td>14.9%</td>
<td>12.7%</td>
<td>13.3%</td>
<td>18.1%</td>
<td>N/A</td>
</tr>
<tr>
<td>Number of Plants</td>
<td>204</td>
<td>189</td>
<td>170</td>
<td>139</td>
<td>110</td>
<td>95</td>
<td>81</td>
<td>72</td>
<td>68</td>
<td>61</td>
<td>56</td>
</tr>
<tr>
<td>Change in Number of Plants</td>
<td>15</td>
<td>19</td>
<td>31</td>
<td>29</td>
<td>15</td>
<td>14</td>
<td>9</td>
<td>4</td>
<td>7</td>
<td>5</td>
<td>N/A</td>
</tr>
<tr>
<td>% in the Number of Plants</td>
<td>7.4%</td>
<td>10.1%</td>
<td>18.2%</td>
<td>20.9%</td>
<td>13.6%</td>
<td>14.7%</td>
<td>11.1%</td>
<td>5.6%</td>
<td>10.3%</td>
<td>8.2%</td>
<td>N/A</td>
</tr>
</tbody>
</table>

The First and second Renewable Fuel Standards (RFS) acts were the main engines to the changes in U.S. energy policy. They were part of the EPAct (Energy Policy Act) of 2005 and EISA (Energy Independence and Security Act) of 2007, respectively. The EISA is stated to reduce U.S. energy dependence and greenhouse gas emissions, and the second RFS was an expanded version of the first RFS. Table 2.2 shows the requirements of second RFS mandates.

The EISA has authorized the U.S. Environmental Protection Agency (EPA) to develop and implement regulations to ensure that transportation fuel sold in the United States contains a minimum volume of renewable fuel. Based on the data from the Energy
Information Administration (EIA), EPA is required to set the cellulosic biofuel standard each year based on the projected available volume for the following year. If the projected available volume of cellulosic biofuel is less than the required volume specified, EPA has the authorization to lower the required volume in the RFS2 to the projected available volume. EPA has set the standard for 2012 which is showed in Table 2.3.

Table 2.2 Second Renewable Fuel Standard 2008-2022 (Energy Independence and Security Act of 2007)

(Source: Energy Independence and Security Act of 2007)

<table>
<thead>
<tr>
<th>Year</th>
<th>Renewable Biofuel (BGY)</th>
<th>Advanced Biofuel (BGY)</th>
<th>Cellulosic Biofuel (BGY)</th>
<th>Biomass-based Diesel (BGY)</th>
<th>Undifferentiated Advanced Biofuel (BGY)</th>
<th>Total RFS (BGY)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2008</td>
<td>9.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9.0</td>
</tr>
<tr>
<td>2009</td>
<td>10.5</td>
<td>.6</td>
<td>.5</td>
<td>0.1</td>
<td></td>
<td>11.1</td>
</tr>
<tr>
<td>2010</td>
<td>12</td>
<td>.95</td>
<td>.1</td>
<td>.65</td>
<td>0.2</td>
<td>12.95</td>
</tr>
<tr>
<td>2011</td>
<td>12.6</td>
<td>1.35</td>
<td>.25</td>
<td>.8</td>
<td>0.3</td>
<td>13.95</td>
</tr>
<tr>
<td>2012</td>
<td>13.2</td>
<td>2</td>
<td>.5</td>
<td>1</td>
<td>0.5</td>
<td>15.2</td>
</tr>
<tr>
<td>2013</td>
<td>13.8</td>
<td>2.75</td>
<td>1</td>
<td>1.75</td>
<td>1.75</td>
<td>16.55</td>
</tr>
<tr>
<td>2014</td>
<td>14.4</td>
<td>3.75</td>
<td>1.75</td>
<td>2</td>
<td></td>
<td>18.15</td>
</tr>
<tr>
<td>2015</td>
<td>15</td>
<td>5.5</td>
<td>3</td>
<td>2.5</td>
<td></td>
<td>20.5</td>
</tr>
<tr>
<td>2016</td>
<td>15</td>
<td>7.25</td>
<td>4.25</td>
<td>3.0</td>
<td></td>
<td>22.25</td>
</tr>
<tr>
<td>2017</td>
<td>15</td>
<td>9</td>
<td>5.5</td>
<td>3.5</td>
<td></td>
<td>24</td>
</tr>
<tr>
<td>2018</td>
<td>15</td>
<td>11</td>
<td>7</td>
<td>4.0</td>
<td></td>
<td>26</td>
</tr>
<tr>
<td>2019</td>
<td>15</td>
<td>13</td>
<td>8.5</td>
<td>4.5</td>
<td></td>
<td>28</td>
</tr>
<tr>
<td>2020</td>
<td>15</td>
<td>15</td>
<td>10.5</td>
<td>4.5</td>
<td></td>
<td>30</td>
</tr>
<tr>
<td>2021</td>
<td>15</td>
<td>18</td>
<td>13.5</td>
<td>4.5</td>
<td></td>
<td>33</td>
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<tr>
<td>2022</td>
<td>15</td>
<td>21</td>
<td>16</td>
<td>5</td>
<td></td>
<td>36</td>
</tr>
</tbody>
</table>

BGY=billion gallon per year
Table 2.3: Proposed Volume and Percentage of Renewable Fuels for 2012 (EPA, 2011).

<table>
<thead>
<tr>
<th>Final Volumes for 2012</th>
<th>Actual Volume</th>
<th>Ethanol Equivalent Volume</th>
<th>Percentage in total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cellulosic biofuel</strong></td>
<td>3.45 - 12.9 million gal.</td>
<td>3.55 - 15.7 million gal.</td>
<td>0.002 to 0.010%</td>
</tr>
<tr>
<td><strong>Biomass-based diesel</strong></td>
<td>1.0 billion gal.</td>
<td>1.5 billion gal.</td>
<td>0.91%</td>
</tr>
<tr>
<td><strong>Advanced biofuel</strong></td>
<td>2.0 billion gal.</td>
<td>2.0 billion gal.</td>
<td>1.21%</td>
</tr>
<tr>
<td><strong>Renewable fuel</strong></td>
<td>15.2 billion gal.</td>
<td>15.2 billion gal.</td>
<td>9.21%</td>
</tr>
</tbody>
</table>

Currently, ethanol is mainly produced from sugar or starch in industry. According to the data from RFA 2011, 36% of corn in the US is used to produce ethanol. However, the demand for corn as the raw material, which is also a food source, makes the industry and government in a dilemma of food vs fuel (Galbe et al., 2006). Lignocellulosic materials as an almost ubiquitous resource on earth, offer a good opportunity for local bioethanol. Lignocellulosic biomass is renewable, cheap and readily available with 180 million tons produced per year in the United States (Kim, 2004), and 10-50 billion tons produced per year globally (Green, 2004).

### 2.1.3 Chemical Composition of Lignocellulosic Biomass

The term "lignocellulosic biomass" is used when referring to grasses, softwood or hardwood and agricultural residuals. Lignocellulosic biomass is primarily composed of cellulose, hemicellulose and lignin that are closely twisted together in a complex structure (Figure 2.2).
2.1.3.1 Cellulose

Cellulose, a glucose polymer, consists of anhydro-glucose molecules linked with β(1-4”) glycosidic bonds (Figure 3) (Goldstein, 1991). It is the most plentiful polymer in the world.

Hydroxy groups form hydrogen bonds bundling cellulose chains together to form hard, stable crystalline regions. Crystalline cellulose is dewatering because the hydrogen bonds are in interchain bonding (Goldstein, 1991). Amorphous cellulose is another form during cellulose formation.

Normally, the average length of a cellulose polymer is approximant 2.5-5 μm, with a high degree of polymerization about n=10,000 and the diameter is 8 Å (Bowyer et al., 2003; Panshin and Zeeuw, 1964). The crystallinity of cellulose fibers is one of the major hurdles for efficient enzymatic hydrolysis. (Vinod, 1984)
2.1.3.2 Hemicellulose

The hemicellulose is mainly composed of five-carbon (xylose, arabinose) and six-carbon sugars (galactose, mannose), and its degree of polymerization is around 100. Arabinoxylan and glucomannan are two types of hemicelluloses in grasses such as switchgrass. Arabinoxylan is the major hemicellulose, and it consists of a xylan backbone made up of xylose units β (1-4”), building a linear chain with occasional branches at C2 and C3. Compared with cellulose, hemicellulose has lower crystallinity because of the presence of occasional branches minimizing hydrogen bonding, although the structure of hemicellulose is similar to cellulose. The minor hemicellulose is glucomannan, which is mainly a straight chain composed of D-mannose and D-glucose in a ratio of 1.6:1. Small amounts of occasional branching also help to keep the low crystallinity.
2.1.3.3 Lignin

Lignin is an aromatic polymer composed of phenylpropane units connected together with ether or carbon-carbon linkages (Goldstein, 1991). There are three types of phenolic acids: p-coumaryl alcohol, coniferyl alcohol and synapyl alcohol, which are called lignin monomers. The assembly of lignin monomers forms a three-dimensional amorphous network polymer, which is difficult to depict because of the highly diverse connection between monomers. In general, grasses such as switchgrass typically contain equal amounts of all three lignin monomers. The carbon-carbon bonds and ether bonds unify the fibers together to compose a physico-chemical barrier against microbial attack and to
provide rigidity to the cell wall (Jeronimidis, 1980; Northcote, 1989). Lignin is a major
barrier to the utilization of the grasses for ethanol production in the biochemical
conversion (Chiang, 2002; Lee, 1997). In order to reduce energy and chemical
consumption in the bioconversion process, it is necessary to remove lignin and/or
produce low lignin feedstock.

Figure 2.5. p-Coumaric acid, ferulic acid, Guaiacyl and Syringyl lignin monomers (Bowyer et al.
2007)

2.2 Pretreatment of Lignocellulosic Biomass

The mission of bioprocessing lignocellulose to fermentable sugars is still technically
problematic due to the naturally resistant carbohydrate-lignin barrier which limits the
accessibility of enzymes to the cellulose and hemicellulose (Moiser et al., 2005). Due to
many physical-chemical and structural factors, a pretreatment step is required to improve
biomass-to-ethanol conversion. An effective biomass pretreatment causes the breakup of
lignocellulosic structure, thus making the carbohydrates more accessible during the
subsequent enzymatic saccharification (Xu et al., 2010).
Pretreatment is not only considered as a crucial step in bio-conversion process, also it has been described as one of the most costly single step, accounting up to 18% of the total projected cost (Yang et al., 2008).

Due to the different structural and composition properties in different lignocellulosic materials, the development of suitable pretreatment technologies is adopted based on the unique characteristics of different types of biomass. Besides, the choice of certain pretreatment technology has great influence on the subsequent steps. For example, the production of inhibitory compounds such as furfural affects ethanol fermentation. (Galbe and Zacchi, 2007).

2.2.1 Key Attributes to An Effective and Practical Pretreatment

Given the big impact of pretreatment on process cost, there are many key attributes to take into consideration for low-cost, advanced pretreatment technologies (Yang and Wyman, 2008):

A) Low biomass size reduction cost
Pretreatment technologies that require no or limited size reduction are desirable, since it is energy-intensive and expensive to mill or grind (mechanical comminution) raw biomass to small particle sizes.

B) Reasonable cost in reactors for operation

C) The reactor cost relates to its material, size and operational environment such as pressure and temperature. As a result, these factors need to be considered when
exploring appropriate pretreatment conditions.

D) No significant carbohydrate degradation or loss after pretreatment

E) High digestibility of pretreated biomass

Cellulose after the pretreatment should be highly digestible with greater yields and with lower cellulase requirement in the subsequent enzymatic hydrolysis.

F) Minimum formation of toxic compounds

The harsh pretreatment conditions result in carbohydrate and lignin degradation to produce inhibitory compounds which potentially affect the subsequent hydrolysis and fermentation steps (Palmqvist et al., 2000). It is necessary to minimize the concentration of the inhibitory compounds to avoid inhibition in the following hydrolysis and fermentation.

2.2.2 Pretreatment Options for Lignocellulosic Biomass Conversion

Pretreatment technologies for lignocellulosic biomass can be categorized as biological, physical, chemical, and thermal processes.

2.2.2.1 Biological Pretreatment

In general, biological pretreatment employ microorganisms such as white, brown and soft rot-fungi to degrade hemicellulose and lignin. The advantages of biological pretreatment include low energy cost, no chemical consumption, low capital cost and mild operational conditions. However, the slow rate of biological hydrolysis obtained in the biological pretreatment process is the main drawback, compared to other pretreatment
technologies (Sun and Cheng, 2002).

Fungi such as *Cyathus stercorarius*, and *Pleurotus ostreaus* have been examined on different lignocellulosic biomass, but their low efficiency and slow growth rate make them far away from a suitable pretreatment process for commercial purpose (Shi et al., 2009).

Much work has been done with *Phanerochaete chrysosporium*, belonging to white-rot fungi (Lewis et al., 1987; Blanchette, 1991; Hatakka, 1983; 1988; Orth et al., 1991; Kondo et al., 1994; Leštan et al., 1994; Keller et al., 2003). Due to its lignin-degrading enzyme system, an array of peroxidases and oxidases is generated to carry out spontaneous cleavage reactions (Shi et al., 2009). *P. chrysosporium* has nearly no influence on cellulose of the wood, which makes it very promising for lignocellulosic biomass pretreatment. Other white-rot fungi such as *Ceriporia lacerata*, *Ceriporiopsis subvermispora*, *Pycnoporus cinnarbarinus*, also have been examined on different biomass indicating relative high efficiency (Kumar et al., 2009). Another study has examined seventeen *Cyathus stercoreus* isolates to test the suitability of pretreatment on rice straw for improved enzymatic saccharification (Yamagishi et al, 2011).

It was reported that two novel two-step pretreatments for enzymatic hydrolysis of rice hull (RH) were proposed to lower the time of pretreatment. A mild physical (ultrasonic) or chemical step (H₂O₂) was combined with a subsequent biological treatment (*Pleurotus ostreatus*). The combined pretreatments led to significant increases
of the lignin degradation and the net total sugar yield. Meanwhile, it is possible to shorten the pretreatment time from 60 days with one step biological pretreatment to 18 days with combined pretreatment (Yu et al., 2008).

2.2.2.2 Physical pretreatment

Physical pretreatment of lignocellulosic biomass normally involves size reduction by grinding, milling or chipping. In this review, ultrasonic pretreatment and microwave pretreatment are also included.

2.2.2.2.1 Mechanical Comminution

The main objective of mechanical pretreatment is to reduce particle size. It can be realized by a combination of chipping, grinding and/or milling. The size of the materials is usually 10-30 mm after chipping and 0.2-2 mm after milling or grinding (Sun and Cheng, 2002). Mechanical pretreatment is usually the first processing step, and the desired particle size depends on the following steps, i.e. the enzymatic hydrolysis and fermentation. Generally, the power demand highly depends on the particle size and biomass properties. It also showed that Biomass moisture also has significant influence on comminution energy consumption, especially for fine size reduction.
2.2.2.2 Ultrasound Pretreatment

Ultrasound technique is used often on waste water treatment, and it is also studied for the treatment of lignocellulosic biomass. A hydrodynamic shear force in aqueous phase is generated by ultrasound because of the rapid collapse of micro-bubbles formed during cavitations (Kuttruff, 1991). The hydrodynamic shear force contributes to increasing the surface area accessible for enzyme attacking. It was found in a study that ultrasound pretreatment resulted in 10 to 20-fold reduction of corn particles in a dry ethanol mill and improved the fermentable sugar release during enzymatic hydrolysis (Khanal et al., 2007). In another study, ultrasound pretreatment of cassava chip slurries increased the reducing sugar yield by 180% (Nitayavardhana et al., 2008). The same group also found that an ethanol yield of 28% higher was achieved through the pretreatment (Nitayavardhana et al., 2010).

2.2.2.2.3 Microwave Pretreatment

Like ultrasonic pretreatment, microwave irradiation also needs to operate in aqueous environments. The irradiation rapidly changes the electric and magnetic field components at the rate of 2.4-109 times per second, resulting in the generation of heat and extensive intermolecular collisions. The effect enhanced the chemical, biological, and physical processes on lignocelluloses materials (Kashwani et al., 2010). An optimal pretreatment time of 5 min was determined on corn for ethanol production. The sugar yield increased by 8.48% after pretreatment, and the theoretical ethanol yield of 92.27% were obtained.
after 44 h of the Simultaneous Saccharification and Fermentation (SSF) process of corn meal with prior microwave treatment (Svetlana et al., 2011).

2.2.2.3 Physical-Chemical Pretreatment

Typical physico-chemical pretreatments include steam explosion and ammonia fiber explosion (AFEX), which apply physical and chemical treatments to break up the lignocellulosic structure.

2.2.2.3.1 Steam Explosion

Steam explosion is one of the most commonly applied technologies in the pretreatment of lignocellulosic materials due to its low use of chemicals and limited energy requirement (Ballesteros et al., 2000). During the process, the biomass is rapidly heated by high-pressure saturated steam in a reactor. With the temperature between 160-260 ºC, the high pressure causes the hemicellulose degradation and lignin disruption (Brownell and Saddler, 1984; Heitz et al., 1991; Ramos et al., 1992; Avellar and Glasser, 1998). The residence time, temperature, particle size and moisture content are major parameters that affects the effectiveness of steam explosion pretreatment. Some inhibitor products generated in the process may limit the subsequent fermentation step (Mackie et al., 1985). Steam explosion applied on wheat straw led to 80% hemicelluloses recovery and 10% lignin removal, with 70% enzymatic hydrolysis yield (Chen et al., 2008).
2.2.2.3.2. Liquid Hot Water Pretreatment

The purpose of liquid hot water pretreatment of lignocellulosic biomass is to hydrate the more chemically resistant regions of cellulose to eventually enhance enzymatic hydrolysis (Weil et al., 1998). Three types of reactors are applied including concurrent, countercurrent, and flow through (Mosier et al., 2005). Controlled pH liquid hot water pretreatment (LHW) applied to treat distiller’s grains derived from dry mill has been investigated (Youngmi et al., 2008). The optimal pretreatment condition was determined to be 184 °C and 24 min for wheat straw (Pérez et al., 2008). A step-change flow rate liquid hot water (SCFLHW) process was developed to increase sugar recovery from sweet sorghum bagasse (SSB). This study was focused on comparing the effects of different hot water flow rates on sugar release from the biomass (Yu et al., 2008).

In the same group, two step hot water pretreatment has been developed on Eucalyptus grandis. The highest yield of total xylose obtained was 86.4% after 20 min at 180 °C (Yu et al., 2010). The group also used metal salts in the liquid hot water to decompose of hemicellulose and lignin from sweet sorghum bagasse to enhance the sugar recovery (Yu et al., 2011).

2.2.2.3.3 Ammonia Fiber Explosion (AFEX)

In the AFEX process, lignocellulosic biomass is exposed to liquid ammonia at a temperature ranging from 90 to 100 °C and under high pressure for 5-15 min. After a period of time, the pressure is swiftly reduced (Sun and Cheng, 2002). The swift pressure
release causes fiber structure being disrupted, resulting in increasing the accessible surface area of the biomass to enzymes (Reshamwala et al., 1995). The ammonia can be recycled after the pretreatment. The AFEX pretreatment has been proven an effective pretreatment for corn stover, rice straw, and switchgrass, but the ammonia loading and the cost of ammonia recovery processes are the two keys limiting the commercial potential of the AFEX process (Sendich et al., 2008).

Autohydrolysis and AFEX pretreatment processes have been applied to coastal Bermuda grass to compare the performance of pretreatment on the fermentable sugar generation. After 100 °C for 30 min AFEX pretreatment, the sugar yield reached 94.8% at with 30 FPU/g enzyme loading. While for the 170 °C with 60 min autohydrolysis pretreatment, only 55.4% sugar yield reached to with the same enzymatic loading (Jung et al., 2010).

The AFEX pretreatment followed by a Consolidated Bioprocessing (CBP) has been considered as a promising and mid-term ethanol conversion process. However, it is still on the way to be developed (Sabrina et al., 2009). The combination of AFEX and CBP technology has been applied on corn stover to test the potential compared with the current simultaneous saccharification and fermentation (SSF) (Xiong et al., 2011). The technology is also taken as a practical way in mature biomass refining (Mark et al., 2009).

2.2.2.4 Chemical pretreatment

Chemical pretreatments involve using alkali reagents, dilute acids, peroxide, organic solvents, or ozone to alter the structure of lignocellulosic biomass (Sun and Cheng, 2002).
2.2.2.4.1 Dilute acid pretreatment

There are mainly two approaches of dilute acid pretreatment processes: 1. low solids loading (5-10% [w/w]), high temperature (T > 160°C), continuous-flow processes; 2. high solids loading (10-40% [w/w]), lower temperature (T < 160°C), batch processes (Silverstein et al., 2008). Generally, higher reaction temperatures and shorter residence times result in higher soluble xylose recovery yields and enzymatic cellulose digestibility. Higher-temperature dilute acid pretreatment has been shown to increase cellulose digestibility of pretreated residues (Tucker et al., 2008).

Dilute acid pretreatment has many benefits to make it a more competitive choice compared to other pretreatment options. As a result, dilute acid pretreatment has a great potential for commercial application in the production of bioethanol from lignocellulosics (Taherzadeh and Karimi, 2008). First, dilute acid will produce separable streams: (1) Prehydrolyzate, a liquid pre-hydrolysis stream containing a majority of xylose; (2) Hydrolyzate, a liquid post-hydrolysis stream containing a majority of glucose, and (3) a solid stream containing a majority of lignin. Different microorganisms are needed in the fermentation step for the different sugar in prehydrolysate and hydrolysate. The most applied microorganism (*Saccharomyces cerevisiae*) in industry is more specific in six-carbon sugar fermentation. Consequently, the separable streams would benefit for the fermentation of xylose with a more specific microorganism for xylose. In addition, acids, such as sulfuric acid are relatively cheap compared to other chemicals, most specifically alkalis, like sodium hydroxide (Mosier et al., 2005; Hu et al., 2008).
The main objective of the acid pretreatments is to solubilize the hemicellulosic fraction of the biomass and to make the cellulose more accessible to enzymes consequently for high yields of sugars from lignocellulosic materials. Acid pretreatment involves the use of sulfuric, nitric, or hydrochloric acids to remove hemicellulose components and expose cellulose for enzymatic digestion (Silverstein et al., 2008).

The acid pretreatment can be performed either under a high temperature and low acid concentration (dilute acid pretreatment) or under a low temperature and high acid concentration (concentrated acid pretreatment). However, the formation of inhibitory compounds reduced the attraction of utilizing concentrated acid for ethanol production (Taherzadeh and Karimi, 2008). Depending on the process temperature, some sugar degradation compounds such as furfural and 5-hydroxymethylfurfural (HMF) and aromatic lignin degradation compounds are formed during the pretreatment and they may inhibit the subsequent fermentation process (Saha et al., 2005).

Furthermore, equipment corrosion problems and acid recovery are important drawbacks when using concentrated acid pretreatments. The high operational and maintenance costs keep it away from applying the concentrated acid pretreatment from commercial scale (Wyman, 1996).

Considerable research attention has been paid to dilute acid hydrolysis for pretreatment of lignocellulosic materials. The dilute acid pretreatment works fairly well on a wide range of agricultural feedstocks, such as corn stover and rice/wheat straws (Zhu et al., 2008).
High hydrolysis yields have been reported when processing lignocellulosic materials with diluted H$_2$SO$_4$ which is the most studied acid for pretreatment. Hydrochloric acid (Israilides et al., 1978; Goldstein et al., 1983; Goldstein and Easter, 1992), phosphoric acid (Israilides et al., 1978) and nitric acid (Mosier et al., 2005) have also been reported for the pretreatment of lignocellulosic biomass.

The concentration of sulfuric acid at usually below 4% (wt) has been most attractive for the pretreatment as it is low cost and effective (Kumar, 2009). Hydrolysis yield of 74% was shown when wheat straw was pretreated with 0.75% v/v of H$_2$SO$_4$ at 121°C for 1 h (Saha et al., 2005). Olive tree biomass was subjected to 1.4% H$_2$SO$_4$ at 210°C, resulting in 76.5% of sugar yield (Cara et al., 2008). Cashew apple bagasse was pretreated with diluted H$_2$SO$_4$ at 121°C for 15 min, and the ethanol yield reached as high as 0.47 g/g glucose in fermentation tests (Rocha et al., 2009). Eggeman (2005) reported dilute sulfuric acid pretreatment as lower cost per gallon of ethanol produced than sodium hydroxide, lime, or AFEX.

Although dilute acid pretreatment greatly increases the success of enzymatic hydrolysis, disadvantages include the formation of inhibitory compounds, expensive equipment made from stainless steel to resist corrosion, and downstream neutralization of the acid prior to fermentation because a low pH is inhibitory to the growth of yeast and other microorganisms (Mosier et al., 2005; Sun and Cheng, 2002). Therefore, a balance is needed for optimizing the fermentable sugars and the inhibitor compounds for ultimately improved ethanol yield.
Since the dilute acid pretreatment impact slightly on the lignin removal, the methods which help to reduce total lignin content in the biomass would be applied like the genetic engineering. A study modified six genotypes of alfalfa lines to compare the lignin removal with wild type, and the most significant type show that the 50% lignin has been removed. In addition, acid pretreatment and enzymes hydrolysis are conducted for each line, as a result, some transgenic lines yield nearly twice as much sugar as wild-type plants (Chen et al., 2007).

2.2.2.4.2 Alkali Pretreatment

Sodium hydroxide (NaOH) and lime (Ca(OH)$_2$) were commonly used in alkaline pretreatment of lignocellulosic materials.

Rice straw was tested by microwave-assisted alkali to improve saccharification in enzymatic hydrolysis. The maximum reducing sugar yield of 69.3 g/100 g raw dry biomass was obtained when 50 g/l rice straw was pretreated by microwave heating for 15 min at 140 °C in 0.5% NaOH solution and then enzymatically hydrolyzed for 96 h (Jun, 2010).

A novel lime pretreatment has been developed (CaCCO process) that did not require a solid–liquid-separation step in which the final pH was kept by carbonation at 6 (Jeung et al., 2010). In the CaCCO process, most of monomeric sugars were kept in the vessel
including xylan, starch and even sucrose. The ethanol yield obtained finally after simultaneous saccharification and fermentation (SSF) of pretreated rice straw was 19.1 g L\(^{-1}\) ethanol that was 74% of the theoretical yield from glucose and xylose.

### 2.2.2.4.3 Room Temperature Ionic Liquids

Room temperature ionic liquids (RTILs) are emerging for lignocellulosic biomass pretreatment. An RTIL is a salt whose melt point is below 100°C. In comparison to traditional molecular solvents, it exhibits a lot of special interesting properties such as high thermal stability, high electrical conductivity, broad liquidus regions, and negligible vapor pressure. (Brennecke and Maginn, 2001). The combination of anion and cation significantly affects the tunable characteristics of RTILs (e.g., viscosity, melting point, polarity, and hydrogen bond basicity) (Wasserscheid, 2003). In addition, when the RTIL is applied in lignocellulosic biomass pretreatment, the toxicity and corrosivity need to be considered (Maki et al., 2010).

The unique solvating properties of RTILs disrupt the 3D network structure of lignin, cellulose, and hemicelluloses. As a result, several RTILs are prompted to dissolve many biomaterials like cellulose (Fukaya et al., 2006; Fukaya et al., 2008) and lignin (Kilpelainen et al., 2007). The addition of anti-solvents like water helps to separate the dissolved cellulose from the lignin and hemicelluloses (Fort et al., 2006). The tremendous compositional breadth allows RTILs used as solvents for lignocellulosic biomass pretreatment (Brandt et al., 2010; Dadi et al., 2006; Lee et al., 2009; Nguyen et al., 2010;
Pezoa et al., 2010; Singh et al., 2009; Zavrel et al., 2009; Zhao et al., 2009a).

The sugar yield reported and final ethanol produced are really desirable, but the high cost of RTILs may impede the commercial usage for pretreatment at this time. Meanwhile, the issue of RTILs preserving enzyme to make it inactive should be addressed. The recycle and regeneration of RTILs will make the technology more competitive (Mauricio et al., 2011). The summary of RTILs is in Table 2.4.

Table 2.4. RTILs used for the dissolution of lignocellulosic biomass subcomponents and biomass pretreatment (adapted from Mauricio et al., 2011).

<table>
<thead>
<tr>
<th>RTIL Structure</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Ethyl-3-methylimidazolium acetate [EMIM]OAc</td>
<td>Dissolution of cellulose (Kosan et al., 2008; Vitz et al., 2009; Zavrel et al., 2009) Extraction of lignin from maple wood flour (Lee et al., 2009) Dissolution of a variety of carbohydrates such as sugars, starch, and cellulose (Zhao et al., 2008)</td>
</tr>
<tr>
<td>1-Ethyl-3-methylimidazolium chloride [EMIM]Cl</td>
<td>Dissolution of cellulose (Kosan et al., 2008; Vitz et al., 2009; Zavrel et al., 2009)</td>
</tr>
<tr>
<td>1-Allyl-3-methylimidazolium chloride [AMIM]Cl</td>
<td>Dissolution of cellulose (Fukaya et al., 2008; Zavrel et al., 2009; Zhang et al., 2005)</td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td></td>
<td>Dissolution of hard wood and softwoods (Kilpelainen et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>Extraction of lignin from maple wood flour (Lee et al., 2009)</td>
</tr>
<tr>
<td>1-Butyl-3-methylimidazolium bromide [BMIM]Br</td>
<td>Dissolution of cellulose (Swatloski et al., 2002; Vitz et al., 2009; Zavrel et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Dissolution of lignin (Pu et al., 2007)</td>
</tr>
<tr>
<td>1-Butyl-3-methylimidazolium tetrafluoroborate [BMIM]BF4</td>
<td>Dissolution of cellulose (Swatloski et al., 2002; Zavrel et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Extraction of lignin from maple wood flour (Lee et al., 2009)</td>
</tr>
<tr>
<td>1-Butyl-3-methylimidazolium hexafluorophosphate [BMIM]PF6</td>
<td>Dissolution of cellulose (Swatloski et al., 2002; Zavrel et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Extraction of lignin from maple wood flour (Lee et al., 2009)</td>
</tr>
</tbody>
</table>
2.2.2.4 Green Liquor

Green liquor is an alkaline intermediate in Kraft pulping. It contains approximately of 75% NaCO₃ and 25% Na₂S, and its recovery process in the mill has been mature in industry for many years. The green liquor process is a potential alternative for pretreatment of lignocellulosic feedstock for ethanol production. It is reported that the fermentable sugar yield can reach up to 80% in hardwood and 70% in softwood (Jin et al., 2010; Wu et al, 2010). Currently, it is mostly applied on the wood feedstock and an economic analysis of the technology has been conducted with the conclusion that the ethanol price is estimated at $2.51 per gallon, which is quite competitive (Gonzalez et al., 2011).

2.2.3 Summary

Table 2.5 summarizes some of current pretreatment approaches along with their key advantages and disadvantages. While all of the listed pretreatments result in enhanced enzymatic saccharification of the pretreated biomass, an effective process that is economically suitable with minimal impact on downstream processing has yet to be developed.
<table>
<thead>
<tr>
<th>Pretreatment process</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mechanical</strong></td>
<td>1. Reduces cellulose crystallinity</td>
<td>1. Power consumption usually high</td>
</tr>
<tr>
<td><strong>Commination</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Steam explosion</strong></td>
<td>1. Causes hemicellulose degradation and lignin transformation</td>
<td>1. Destruction of a portion of the xylan fraction; incomplete disruption of the lignin-carbohydrate matrix</td>
</tr>
<tr>
<td></td>
<td>2. High Cost performance</td>
<td>2. Generation of compounds inhibitory to microorganisms</td>
</tr>
<tr>
<td><strong>AFEX</strong></td>
<td>1. Increases accessible surface area, removes lignin and hemicellulose to an extent</td>
<td>1. Not efficient for biomass with high lignin content</td>
</tr>
<tr>
<td></td>
<td>2. Low formation of inhibitors</td>
<td></td>
</tr>
<tr>
<td><strong>CO2 Explosion</strong></td>
<td>Increases accessible surface area;</td>
<td>Does not modify lignin or hemicelluloses</td>
</tr>
<tr>
<td><strong>Acid Hydrolysis</strong></td>
<td>1. Hydrolyzes hemicellulose to xylose and other sugars</td>
<td>Generation of inhibitory products</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low sugar concentration in exit stream</td>
</tr>
</tbody>
</table>
2.3 Conclusions and Perspectives

For biochemical conversion of lignocellulosic biomass, pretreatment plays a crucial role and it is significant cost deterrence to the commercialization of the conversion technologies. In the recent years, different pretreatment technologies have been emerging and widely studied to enhance ethanol production from the lignocellulosic materials. An ideal pretreatment approach should follow the keys discussed in this few. However, currently not all pathways allow the conversion from biomass to sugar at high sugar yield while keeping economics viable.

Among the different technologies, chemical and physical-chemical pretreatments are currently the most practical and promising technologies for industrial applications. However, it is too costly to use some chemical such as wet oxidation, solvents and metal complexes in the pretreatment step when the values of the glucose or ethanol production are relatively low. Combination of different pretreatments may be a good way to address the efficiency and cost dilemma, eventually to make the technology profitable. In addition, there is no significantly difference on projected economics among many promising approaches such as steam pretreatment, lime pretreatment, liquid hot water LHW systems, AFEX, dilute acid and alkaline pretreatments.

It is promising to integrate the pretreatment with enzymatic hydrolysis and fermentation steps to decrease pretreatment severity, total enzyme loading, and eventually to make the whole process economically viable. Consolidated bioprocessing (CBP) which combines hydrolysis and fermentation in one step, is earning more and
more attention to use some thermophilic bacteria to produce ethanol. These thermophilic bacteria are proven being able to tolerate higher concentration of ethanol (Larsen et al., 1997).

Another promising approach is genetic improvement of perennial species to reduce intrinsic recalcitrance to pretreatment and fermentative bioprocessing, eventually to improve biofuel and chemical production processes and have a profound positive impact on bioenergy industry. Exploration of natural variation in alfalfa, switchgrass and canarygrass, has illustrated that decreased lignin levels contributed to improving in vitro enzyme hydrolysis (Dien et al., 2006). But to date, very few reports are published regarding pretreatment and fermentation of improved plants for ethanol production in any genetically modified perennial grasses like switchgrass as a model biofuel crop. The integration of genetic engineering on feedstock and current dominant pretreatment technologies are worthy further exploring.

The effect of the pretreatments is mainly dependent on the biomass composition, structure and operating conditions. All these pretreatments have their advantages and disadvantages and further research is needed for optimization.
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CHAPTER 3

Dilute Sulfuric Acid Pretreatment of Transgenic Switchgrass for Sugar Production

3.1 Materials and Methods

3.1.1 Biomass Preparation

Five genetically engineered Alamo switchgrass which include 10/9-28, 10/9-33, 10/9-40, 11/5-41 and 11/5-47, and the conventional Alamo switchgrass were investigated in this study. Switchgrass biomass was obtained from greenhouse of Department of Crop Science at North Carolina State University in Oct 2010. The harvested switchgrass plants were dried in an oven at 50 °C for 72 h and the dried plants were subjected to size reduction using a Wiley Laboratory Mill (Thomas, Model No. 4) fitted with a 2 mm sieve. The ground biomass was collected in sealed plastic bags and stored at room temperature before composition analysis and acid pretreatment.

3.1.2 Pretreatment

Based on our previous work on dilute sulfuric acid pretreatment of coastal Bermuda grass (Redding et al., 2011), the pretreatment temperature was set at 150 °C in this study, and the acid concentrations of 0.75, 1, and 1.25 % (w/v) and residence times of 5, 10, 20, and 30 min were investigated. Pretreatments were carried out in vessels made from 306 stainless steel pipe nipples (4 inches long by 0.75 inches inner diameter) and pipe caps.
purchased from McMaster-Carr (Santa Fe Springs, CA, USA). Dilute sulfuric acid of desired concentration and switchgrass biomass was mixed in the vessel at a ratio of 10:1 (w/w) (30 ml acid and 3 g biomass). After loaded with acid and biomass, the vessels were sealed with pipe caps using Teflon tapes. All the pretreatments were conducted in a Fisher Scientific High-Temp Bath (Model 160A, Fisher Scientific, Dubuque, IA, USA) filled with silicon oil as a heat transfer fluid. Since the difference between the temperature of the outer surface of the vessel and that of the slurry in the vessel was neglectable according to our preliminary experiment, the temperature of the slurry in the vessel was monitored by a plug k-type thermocouple (Model TC-K-NPT-G-72-SMP, Omega, Stamford, CT, USA) attached to the outer surface of the vessel. An Extech Easyview Thermometer (Model 11A, Extech, Waltham, MA, USA) was connected to the temperature probe to display temperature readings. After the vessels were put into the oil bath, it took 10 ± 1 min for the temperature of the slurry to reach 150 °C. Once the temperature reading reached 150 °C, the residence time began. After the desired residence time elapsed, the vessels were quickly removed from oil and put into tap water for cooling. After pretreatment, the pretreated biomass was recovered by filtration. Sixty milliliters of deionized (DI) water was used to wash the inner surface of the vessel to ensure the collection of all the biomass. The total volume of prehydrolysate was measured with a 100 ml measuring cylinder and 10 ml of the prehydrolysate was transferred to a 15 ml centrifuge tube and stored at -20 °C for the analyses of monomeric sugars and sugar degradation products at a later time. After the prehydrolysate was collected, 140 ml of additional DI water was used to rinse the biomass.
to remove residual acid. The pretreated biomass was collected in sample bags and stored at 4 °C for enzyme hydrolysis at a later time.

3.1.4 Enzymatic Hydrolysis

Enzymatic hydrolysis was conducted in 50 ml centrifuge tubes in an automated shaking water bath (Model C76, New Brunswick Scientific, Edison, NJ, USA) set at 50 °C and 150 rpm agitation for 72 hours. Biomass (0.5 g, dry basis) was immersed into 0.05 M sodium citrate buffer (pH 4.8) in the centrifuge tube. Enzyme Cellic CTec2 provided by Novozymes North America (Franklinton, NC, USA) was used for hydrolysis. An excessive enzyme loading of 40% (g enzymes /g dry biomass) was applied to avoid the effect of enzyme limitation on sugar production. Sodium azide of 0.3% (w/v) was applied to inhibit microbial growth during the hydrolysis. The total volume of the slurry in each tube was made 15 ml by adjusting the buffer addition.

3.1.5 Analytical Methods

Moisture, lignin, and ash contents of the raw and pretreated biomass were determined using the National Renewable Energy Laboratory (NREL) procedures (Sluiter et al., 2005a; Sluiter et al., 2005b; Sluiter et al., 2008). The profile of monomeric sugars in composition analysis sample was determined using an ion-exchange chromatography (IC) (Dionex ICS-5000, Dionex Corporation, Sunnyvale, CA, USA). The IC was equipped with a pulsed electrochemical detector. The samples were prepared from the pH 4.8 hydrolyzate, and filtered through a syringe filter. The column used was a CarboPac PA1 (4 x 250 mm)
column operated at 18 °C with 0.018 M potassium hydroxide as the mobile phase at a flow rate of 0.9 ml/min. Sample injection volume was 5 μl with 25 min run time. Monomeric sugars and sugar degradation products including 5-hydroxyfuranmethal (HMF) and furfural in prehydrolysate and monomeric sugars in hydrolysate were determined using a high performance liquid chromatography system (HPLC) (Dionex UltiMate 3000, Dionex Corporation, Sunnyvale, CA, USA). The HPLC system was equipped with a Bio-Rad Aminex HPX-87H column (300 mm x 7.8 mm), a Bio-Rad Micro-Guard column, a thermostatted autosampler, an isocratic analytical pump, and a refractive index detector. The analytical column was operated at 65 °C with 0.005 M H₂SO₄ as the mobile phase at a flow rate of 0.6 ml/min. Sample injection volume was 10μl with 50 min runtime. The yields of total sugars, monomeric sugars, and sugar degradation products were calculated based on per gram of raw biomass.

3.1.6 Statistical analysis

All treatments in this study were conducted in triplicate. But due to unequal heating during the pretreatment, enzymatic hydrolysis error, or analysis error, there some outliers were produced. Also some samples were negligently smashed. As a result, a few triplicates had only two sets closed and another one with larger difference. In this case, the data with clear difference was removed and it would be recognized as a missing data in SAS system. The GLM (General Linear Model) procedure with Tukey adjustment in SAS 9.1 software (SAS Institute Inc., Cary, NC) was used for significant difference of total
lignin content and lignin composition between conventional switchgrass and transgenic switchgrass where \( p<0.05\) (\( \alpha=0.01 \)). An analysis of various (ANOVA) was performed to identify significant effect from each of factors as well as interactions of factors on total sugar yield.

### 3.2 Results and Discussion

#### 3.2.1 Characterization of Switchgrass

Both conventional and transgenic Alamo switchgrass contain high carbohydrates (Table 3.1). Glucan and xylan are the dominant sugar polymers in switchgrass, which consists 92.6-94.7% of total carbohydrates, and no significant (\( P>0.05 \)) difference was found between their contents in conventional and transgenic switchgrass. Arabinan and galactan are the two minor sugar polymers derived from hemicellulose and their contents were significantly (\( P<0.05 \)) higher in transgenic switchgrass, especially in switchgrass 10/9-33. However, due to their neglectable contents compared with glucan and xylan, there is no significant (\( P>0.05 \)) difference in total carbohydrates between the conventional and the transgenic switchgrass. Since lignin reduction/modification is the major purpose of genetic engineering of switchgrass, the changes of lignin content and its composition deserves more attention. After genetic modification, the content of acid soluble lignin (ASL) in switchgrass increased significantly (\( P<0.05 \)), while that of the acid insoluble lignin (AIL) decreased significantly (\( P<0.05 \)). Although there are significant changes in ASL and AIL contents of the transgenic switchgrass in comparison with the conventional switchgrass, the
total lignin contents in the conventional and the transgenic switchgrass are very close. According to the Tukey process for multiple comparison (Table 3.2), only 10/9-40 had a total lignin content which was significantly different from the control and other transgenic switchgrass genotypes. However, the ASL:AIL ratio substantially increased after genetic modification. Based on the statistic analysis, the ASL:AIL ratio for each genotype of transgenic switchgrass is significantly different from the conventional switchgrass. Besides, among the transgenic switchgrass, the ASL:AIL ratios all significantly differ from each other except between 10/9-28 and 10/9-40, 10/9-33 and 11/5-47 (Table 3.3). According to the literature (Bose et al., 2009; Davison et al., 2006; Li et al., 2003; ), syringyl (S) unit and guaiacyl (G) unit are the two important monolignol incorporated into the lignin polymer and the S:G ratio is positively associated with the lignin reactivity and, consequently, the biomass susceptibility to chemical conversion. On the other hand, research also shows that, compared with guaiacyl (G) lignin, syringyl (S) lignin is much more readily dissolved in 72% sulfuric acid and measured as ASL (Yasuda et al., 2001). Therefore, a high ASL:AIL ratio is a good indicator for the improved lignin reactivity resulted from genetic modification. It seems that, instead of resulting in considerable lignin reduction, the genetic engineering technology applied on Alamo switchgrass led to an improvement in lignin composition/structure, which would potentially favor biomass conversion.
### Table 3.1. Composition of conventional and transgenic switchgrass.

<table>
<thead>
<tr>
<th>Component content (% weight of dry biomass)</th>
<th>Control</th>
<th>Al 10/9-28</th>
<th>Al 10/9-33</th>
<th>Al 10/9-40</th>
<th>Al 11/5-41</th>
<th>Al 11/5-47</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucan</td>
<td>40.1 (0.32)*</td>
<td>40.19 (0.76)</td>
<td>40.76 (0.64)</td>
<td>41.4 (0.24)</td>
<td>40.34 (0.47)</td>
<td>40.2 (0.57)</td>
</tr>
<tr>
<td>Xylan</td>
<td>22.1 (1.19)</td>
<td>22.64 (0.26)</td>
<td>22.53 (0.18)</td>
<td>22.2 (0.21)</td>
<td>21.72 (0.64)</td>
<td>21.4 (0.27)</td>
</tr>
<tr>
<td>Arabinan</td>
<td>2.49 (0.04)</td>
<td>3.81 (0.08)</td>
<td>3.29 (0.03)</td>
<td>3.18 (0.06)</td>
<td>2.81 (0.01)</td>
<td>3.53 (0.04)</td>
</tr>
<tr>
<td>Galactan</td>
<td>0.97 (0.05)</td>
<td>1.39 (0.03)</td>
<td>1.47 (0.04)</td>
<td>1.14 (0.02)</td>
<td>1.24 (0.05)</td>
<td>1.35 (0.03)</td>
</tr>
<tr>
<td>Total carbohydrate</td>
<td>65.7 (1.74)</td>
<td>65.6 (1.74)</td>
<td>68.0 (0.428)</td>
<td>67.9 (1.11)</td>
<td>66.1 (0.18)</td>
<td>66.5 (0.87)</td>
</tr>
<tr>
<td>Acid insoluble lignin</td>
<td>2.71 (0.01)</td>
<td>3.02 (0.01)</td>
<td>3.57 (0.006)</td>
<td>2.95 (0.01)</td>
<td>3.97 (0.05)</td>
<td>3.57 (0.02)</td>
</tr>
<tr>
<td>Acid soluble lignin</td>
<td>18.70 (0.10)</td>
<td>18.27 (0.03)</td>
<td>17.53 (0.14)</td>
<td>17.3 (0.04)</td>
<td>17.15 (0.20)</td>
<td>17.6 (0.01)</td>
</tr>
<tr>
<td>Total lignin</td>
<td>21.4 (0.16)</td>
<td>21.29 (0.18)</td>
<td>21.1 (0.20)</td>
<td>20.2 (0.26)</td>
<td>21.12 (0.24)</td>
<td>21.2 (0.04)</td>
</tr>
<tr>
<td>ASL:AIL</td>
<td>0.14 (0.001)</td>
<td>0.1653 (0.004)</td>
<td>0.2037 (0.002)</td>
<td>0.17 (0.002)</td>
<td>0.2315 (0.006)</td>
<td>0.20 (0.002)</td>
</tr>
</tbody>
</table>

* The number in parentheses is standard deviation of triplicate samples
3.2. Multiple comparison for total lignin content between each genotype*

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Difference Between Means</th>
<th>Simultaneous 95% Confidence Limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control - 10/9-28</td>
<td>0.1712</td>
<td>-0.3235</td>
</tr>
<tr>
<td>Control - 11/5-47</td>
<td>0.2909</td>
<td>-0.1515</td>
</tr>
<tr>
<td>Control - 11/5-41</td>
<td>0.3424</td>
<td>-0.1000</td>
</tr>
<tr>
<td>Control - 10/9-33</td>
<td>0.3564</td>
<td>-0.0861</td>
</tr>
<tr>
<td>Control - 10/9-40</td>
<td>1.2441</td>
<td>0.8017</td>
</tr>
</tbody>
</table>

* The GLM Procedure: Tukey's Studentized Range (HSD) Test for lignin_content.  
**Comparisons significant at the 0.05 level.

Table 3.3. Multiple comparison for ASL: ASIL ratio between each genotype*

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Difference Between Means</th>
<th>Simultaneous 95% Confidence Limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control - 11/5-41</td>
<td>-0.086799</td>
<td>-0.094744</td>
</tr>
<tr>
<td>Control - 10/9-33</td>
<td>-0.059509</td>
<td>-0.067454</td>
</tr>
<tr>
<td>Control - 11/5-47</td>
<td>-0.058225</td>
<td>-0.066170</td>
</tr>
<tr>
<td>Control - 10/9-40</td>
<td>-0.026775</td>
<td>-0.034720</td>
</tr>
<tr>
<td>Control - 10/9-28</td>
<td>-0.020781</td>
<td>-0.029664</td>
</tr>
</tbody>
</table>

* The GLM Procedure: Tukey's Studentized Range (HSD) Test for ASL: ASIL.  
**Comparisons significant at the 0.05 level.
3.2.2 Acid Pretreatment

Acid pretreatment of switchgrass generated two output streams, the prehydrolysate liquor and the pretreated biomass. The prehydrolysate liquor contains a large amount of cellulose/hemicellulose derived sugars and their degradation products including HMF and furfural. Since hemicellulose is the major target of acid prehydrolysis, xylose is normally the most abundant monomeric sugar in prehydrolysate. Figure 3.1 shows the xylose yields of switchgrass after pretreatment at different conditions. Generally, 0.75% H$_2$SO$_4$ was not strong enough for pretreatments at residence times less than 20 min, while applying 1.25% H$_2$SO$_4$ resulted in less xylose yields in many cases due to the increased xylose degradation under overly intense pretreatment conditions. For both conventional and transgenic switchgrass, 1% H$_2$SO$_4$ was sufficient to maximize xylose yield. Similarly, applying the most extended residence time did not necessarily result in the maximum xylose yield due to the increased xylose degradation. Compared with those of conventional switchgrass, pretreatments of transgenic switchgrass resulted in higher xylose yields. The maximum xylose yields of transgenic switchgrass 10/9-28, 10/9-33, 10/9-40, 11/5-41 and 11/5-47 were 153.9, 183.4, 172.7, 167.2, and 173.5 mg/g raw biomass, respectively, which were 19.5%, 21.5%, 14.4%, 10.8%, and 14.9% higher than that of conventional switchgrass, respectively.

Although the semicrystalline structure makes cellulose less susceptible to acid attack, solubilization of cellulose is inevitable at harsh pretreatment conditions. Table 3.4 shows the glucose detected in the prehydrolysate. Generally, glucose yield increased with the elevation of H$_2$SO$_4$ concentration and the extension of residence time. This follows the
logic that the more intense pretreatment should release more glucose from cellulose. In addition, the improved hemicellulose degradation at harsher pretreatment conditions would expose more cellulose to acid prehydrolysis. Based on statistic analysis, the switchgrass genotype did not have a significant (P>0.05) impact on glucose yield.

During high temperature acid pretreatments, monomeric sugars released from cellulose and hemicellulose could be degraded into HMF and furfural which in turns might be further degraded into formic acid and levulinic acid, respectively. According to our previous study (Redding et al., 2010), the formic acid and levulinic acid generated during acid pretreatment of coastal Bermuda grass at temperatures lower than 180 °C were much lower than the reported inhibitory level (> 200 mM) (Palmqvist and Hahn-Hagerdah, 2000). Therefore, only HMF and furfural in the prehydrolysate were measured and reported in this study. Figure 3.2 shows the amounts of HMF and furfural generated during the pretreatment of switchgrass at different conditions. For ease of comparison, the individual amounts of HMF and furfural were combined and presented as a single bar in Figure 2. HMF and furfural are the degradation products of hexoses and pentose during acid pretreatment at high temperatures, respectively. Since xylose is the major sugar in the prehydrolysate, more furfural than HMF was generated in most cases. With the increase of H$_2$SO$_4$ concentration or the extension of residence time, more sugar degradation products were detected. The effect of genetic engineering on the generation of sugar degradation products (combined furfural level) during pretreatment was not significant (P>0.05). Since a high level of sugar degradation products would be inhibitory to the subsequent
fermentation, the amounts of HMF and furfural in the prehydrolysate would be considered later in the determination of the best pretreatment conditions. According to the analysis, the genotypes did not result in significant difference in inhibitory compounds (Table 3.5).

The data were sorted by different genotypes for the GLM (General Linear Model) procedure to determine the contribute of residual time and concentration to the production of sugar degradation products, xylose yield. Table 3.6 shows that time, concentration, and time *concentration provided significant (P<0.05) contribution to the results for each genotype.
Figure 3.1 Xylose in the prehydrolysate after pretreatment of conventional switchgrass, and transgenic switchgrass at different combinations of acid concentration and residence time at 150 °C
Figure 3.2. Combined HMF and furfural in the prehydrolysate after pretreatment of conventional and transgenic switchgrass at different combinations of acid concentration and residence time at 150 °C.
Figure 3.2 Continued

1.25% combined furfural level

Furfural level (g/g raw biomass)

HMF

Furfural

Con 30m
Con 20m
Con 10m
Con 5m
9-28 30m
9-33 30m
9-40 30m
5-41 30m
5-47 30m
9-28 20m
9-33 20m
9-40 20m
5-41 20m
5-47 20m
9-28 10m
9-33 10m
9-40 10m
5-41 10m
5-47 10m
9-28 5m
9-33 5m
9-40 5m
5-41 5m
5-47 5m
Table 3.4. Glucose in the prehydrolysate after pretreatment of conventional and transgenic switchgrass at different combinations of acid concentration and residence time at 150 °C.

<table>
<thead>
<tr>
<th>H₂SO₄ concentration (%)</th>
<th>Residence time (min)</th>
<th>Glucose in prehydrolysate (mg/g raw biomass)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>0.75</td>
<td>5</td>
<td>42.9 (2.17)*</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>71.2 (3.86)</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>72.8 (5.45)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>86.9 (2.34)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>61.1 (0.23)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>88.6 (1.69)</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>94.1 (1.82)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>92.2 (5.21)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>64.9 (4.45)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>72.7 (1.24)</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>86.8 (0.18)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>92.1 (1.51)</td>
</tr>
</tbody>
</table>

* The number in parentheses is standard deviation of triplicate samples
Table 3.5. Test for genotype effect on combined furfural level*

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>5</td>
<td>0.00054186</td>
<td>0.00010837</td>
<td>1.62</td>
<td>0.1564</td>
</tr>
<tr>
<td>Error</td>
<td>210</td>
<td>0.01406065</td>
<td>0.00006696</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected Total</td>
<td>215</td>
<td>0.01460251</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Dependent Variable: Combined Furfural level
Table 3.6. The effect of pretreatment conditions on xylose in prehydrolysate, combined furfural level, glucose in hydrolysate and total sugar sorted by genotype.

<table>
<thead>
<tr>
<th>Switchgrass genotype</th>
<th>Pretreatment conditions</th>
<th>Pr&gt;F</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Xylose</td>
<td>Combined furfural level</td>
<td>Glucose</td>
<td>Total Sugar</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Time</td>
<td>&lt;.0001</td>
<td>0.0003</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Concentration</td>
<td>&lt;.0001</td>
<td>0.0005</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Time*Concentration</td>
<td>&lt;.0001</td>
<td>0.2856</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>Concentration</td>
<td>Time*Concentration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----</td>
<td>--------</td>
<td>---------------</td>
<td>--------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10/9-33</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>0.0014</td>
<td>&lt;.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11/5-41</td>
<td>&lt;.0001</td>
<td>0.0187</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;.0001</td>
<td>0.0680</td>
<td>0.0005</td>
<td>&lt;.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.0012</td>
<td>0.0017</td>
<td>0.0002</td>
<td>0.0004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11/5-47</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;.0001</td>
<td>0.0003</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.2.3 Enzymatic Hydrolysis

After acid pretreatment, the pretreated biomass with high cellulose content was subjected to enzymatic hydrolysis for glucose production. Figure 3.3 shows the glucose yield of switchgrass pretreated at different conditions. Similar to the effect of H$_2$SO$_4$ concentration on xylose yield in the pretreatment, 0.75% H$_2$SO$_4$ was not strong enough to sufficiently increase the enzymatic digestibility of switchgrass, while applying 1.25% H$_2$SO$_4$ resulted in reduced glucose yields due to the increased cellulose solubilization during acid prehydrolysis. For both conventional and transgenic switchgrass, 1% was the optimal H$_2$SO$_4$ concentration for effective biomass pretreatment. Glucose yield increased with the extension of pretreatment residence time until 20 min, beyond which the glucose yield decreased in most cases due to the more intensive cellulose solubilization. This can also be reflected by the increased amount of glucose in the prehydrolysate at extended residence times (or elevated H$_2$SO$_4$ concentrations). Compared with those of conventional switchgrass, pretreatments of transgenic switchgrass also resulted in higher glucose yields. The maximum glucose yields of switchgrass 10/9-28, 10/9-33, 10/9-40, 11/5-41, and 11/5-47 were 279.1, 291.7, 326.5, 290.8, and 285.0 mg/g raw biomass, respectively, which were 1.5%, 6.0%, 18.7%, 5.0%, and 3.2% higher than that of conventional switchgrass, respectively. The maximum glucose yields of conventional switchgrass and switchgrass 10/9-28, 10/9-33, 10/9-40, 11/5-41, and 11/5-47 were achieved at 1% H$_2$SO$_4$ and 30 min, 1.25% H$_2$SO$_4$ and 10 min, 1.25% H$_2$SO$_4$ and 20 min, 1% H$_2$SO$_4$ and 20 min, 1% H$_2$SO$_4$ and 30 min, and 1% H$_2$SO$_4$ and 20 min, respectively.
Although acid pretreatment caused the solubilization of the majority of hemicellulose in switchgrass, there was still a small portion of hemicellulose remained as solids and was converted to xylose in enzymatic hydrolysis. Table 3.7 shows that the xylose yield during enzymatic hydrolysis increased with the reduction of residence time in the pretreatment. This is probably because less hemicellulose was solubilized at shorter residence times in the pretreatment, which resulted in the presence of more hemicellulose residues in the pretreated biomass available for enzymatic hydrolysis. However, the reduced H₂SO₄ concentrations in pretreatment were not necessarily associated with higher xylose yields in enzymatic hydrolysis. This could be a result of reduced biomass digestibility with low H₂SO₄ in the pretreatment, which compromised the enzymatic hydrolysis of hemicellulose to produce xylose. The effect of switchgrass genotype on xylose yield in enzymatic hydrolysis was also not significant (P<0.05).

The effect of pretreatment condition on glucose released in enzymatic hydrolysis for each genotype is shown in Table 3.8. Obviously, time, concentration, and time* concentration significantly (P<0.05) impacts glucose yield in the hydrolysate.
Figure 3.3. Figure 3.3 Glucose in the hydrolysate after enzymatic hydrolysis of conventional switchgrass, and transgenic switchgrass at different combinations of acid concentration and residence time at 150 °C.
Table 3.7. Xylose in the hydrolysate after enzymatic hydrolysis of conventional and transgenic switchgrass pretreated at different combinations of acid concentration and residence time at 150 °C.

<table>
<thead>
<tr>
<th>H₂SO₄ concentration (%)</th>
<th>Residence time (min)</th>
<th>Xylose in hydrolysate (mg/g raw biomass)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Al10/9-28</td>
</tr>
<tr>
<td>0.75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>31.0 (2.26)*</td>
<td>17.6(1.06)</td>
</tr>
<tr>
<td>10</td>
<td>16.8 (1.05)</td>
<td>22.8(1.14)</td>
</tr>
<tr>
<td>20</td>
<td>2.62 (1.08)</td>
<td>2.5(0.77)</td>
</tr>
<tr>
<td>30</td>
<td>0.00 (0.00)</td>
<td>0(0.00)</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>35.2 (1.44)</td>
<td>25.8(1.43)</td>
</tr>
<tr>
<td>10</td>
<td>16.7 (0.85)</td>
<td>23.1(1.28)</td>
</tr>
<tr>
<td>20</td>
<td>23.7 (2.26)</td>
<td>18.6(0.44)</td>
</tr>
<tr>
<td>30</td>
<td>0.96 (0.44)</td>
<td>2.6(0.96)</td>
</tr>
<tr>
<td>5</td>
<td>20.5 (1.43)</td>
<td>32.4(0.26)</td>
</tr>
<tr>
<td>1.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>18.6 (1.13)</td>
<td>28.9(0.36)</td>
</tr>
<tr>
<td>20</td>
<td>11.2 (0.53)</td>
<td>18.2(0.32)</td>
</tr>
<tr>
<td>30</td>
<td>12.6 (1.35)</td>
<td>10.9(0.38)</td>
</tr>
</tbody>
</table>

* The number in parentheses is standard deviation of triplicate sample
3.2.4 Optimal Pretreatment Conditions

The optimal pretreatment conditions for conventional and transgenic switchgrass were determined based on total sugar yield and the generation of sugar degradation products during pretreatment. The total sugar refers to the sum of glucose and xylose that can be recovered from both prehydrolysate and hydrolysate. In the work of Redding et al. (2010), the total amount of sugar degradation products generated during pretreatment was used as an important criterion in determining the best conditions for acid pretreatment of coastal Bermuda grass, and a threshold concentration of 1.0 g/L (10 mg/g raw biomass) was selected for HMF and furfural combined according to the literature (Almeida et al., 2009; Taherzadeh et al., 1997; Delgenes et al., 1996; Navarro, 1994). Using 1.0 g/L as the threshold concentration in this study, the optimal pretreatment conditions were selected and the corresponding total sugar yield and carbohydrate conversion rate were reported in Table 3.11. At the optimal pretreatment conditions, the total sugar yield of transgenic switchgrass 10/9-40 was the highest, which was 9.3 % higher than that of conventional switchgrass. Since strain development technologies including selection, long-term adaption, and genetic engineering are promising tools to increase the tolerance of yeast to HMF and furfural and a remarkable progress has already been made in this area (Palmqvist and Hahn-Hagerdah, 2000), the optimal pretreatment conditions were also determined based on an elevated threshold concentration of 2.0 g/L (20 mg/g raw biomass). At the elevated threshold, the total sugar yields of the conventional switchgrass and the transgenic switchgrass 10/9-28, 10/9-33,
10/9-40, 11/5-41, and 11/5-47 were respectively increased by 4.9%, 0%, 1.2%, 6.9%, 0%, and 8.6%, and all transgenic switchgrass showed considerable advantage over the conventional switchgrass in sugar production. The details were showed in Table 3.11.

In order to examine the influence of different genotypes on the amount of total sugars, a three-way (genotype, time, and concentration) GLM (General Linear Model) procedure is applied for statistic analysis. The results shows that genotype is also a significant (P<0.05) contribute for total sugar yield as time and concentration (Table 3.8). However, the previous three-way model only showed the effect of genotype on total sugars after the pretreatment, it is necessary to explore the performance of different genotypes with untreated biomass. The raw biomass of different genotypes without pretreatment was directly processed with enzymatic hydrolysis under the same conditions. Based on the total sugars released after enzymatic hydrolysis, the ANOVA table which tested for the effect of genotype showed the total sugar release was also significantly (P<0.05) different from one genotype to another (Table 3.9). The sugars released from 10/9-33 and 10/9-40 without pretreatment were even lower than the conventional switchgrass (Table 3.10). However, after pretreatment the transgenic switchgrass 10/9-40 showed the greatest potential of releasing sugars. As a result, the transgenic switchgrass was more susceptible for the acid pretreatment than the conventional switchgrass.
Table 3.8 Three-way test of effect of genotype, time, and concentration on total sugar yield*.

Dependent Variable: TS

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>type</td>
<td>5</td>
<td>0.02546573</td>
<td>0.00509315</td>
<td>12.07</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>time</td>
<td>3</td>
<td>0.48673082</td>
<td>0.16224361</td>
<td>384.63</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>type*time</td>
<td>15</td>
<td>0.09495083</td>
<td>0.00633006</td>
<td>15.01</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>conc</td>
<td>2</td>
<td>0.75535163</td>
<td>0.37767581</td>
<td>895.35</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>type*conc</td>
<td>10</td>
<td>0.03758402</td>
<td>0.00375840</td>
<td>8.91</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>time*conc</td>
<td>6</td>
<td>0.18548399</td>
<td>0.03091400</td>
<td>73.29</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>type<em>time</em>conc</td>
<td>30</td>
<td>0.10464492</td>
<td>0.00348816</td>
<td>8.27</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

* Dependent Variable: Total sugars (TS).

Table 3.9 ANOVA table for effect of genotype on total sugar released without pretreatment

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>5</td>
<td>6432.218251</td>
<td>1286.443650</td>
<td>25.80</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Error</td>
<td>12</td>
<td>598.370818</td>
<td>49.864235</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected Total</td>
<td>17</td>
<td>7030.589070</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Dependent Variable: Total sugar
Table 3.10  Multiple comparison for total sugar release without pretreatment among different switchgrass genotypes.

<table>
<thead>
<tr>
<th>type Comparison</th>
<th>Difference Between Means</th>
<th>Simultaneous 95% Confidence Limits</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control - 11/5-41</td>
<td>-35.189</td>
<td>-54.556</td>
<td>-15.823</td>
</tr>
<tr>
<td>Control - 11/5-47</td>
<td>-34.635</td>
<td>-54.001</td>
<td>-15.269</td>
</tr>
<tr>
<td>Control - 10/9-28</td>
<td>-23.829</td>
<td>-43.195</td>
<td>-4.462</td>
</tr>
<tr>
<td>Control - 10/9-33</td>
<td>5.542</td>
<td>-13.825</td>
<td>24.908</td>
</tr>
<tr>
<td>Control - 10/9-40</td>
<td>10.529</td>
<td>-8.837</td>
<td>29.895</td>
</tr>
</tbody>
</table>

***Comparison of significance at the 0.05 level.
Table 3.11. Optimal pretreatment conditions at 150 °C determined based on total sugar yield and inhibitory level of combined HMF and furfural.

<table>
<thead>
<tr>
<th>Switchgrass genotype</th>
<th>Inhibitory level: 1.0 g/L</th>
<th></th>
<th>Inhibitory level: 2.0 g/L</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Optimal pretreatment</td>
<td>Total sugar yield</td>
<td>Carbohydrate conversion</td>
<td>Optimal pretreatment</td>
</tr>
<tr>
<td></td>
<td>conditions</td>
<td>(mg/g raw biomass)</td>
<td>(%)</td>
<td>conditions</td>
</tr>
<tr>
<td>Control</td>
<td>1% H₂SO₄ 10 min</td>
<td>495.4</td>
<td>71.0</td>
<td>1% H₂SO₄ 20 min</td>
</tr>
<tr>
<td>Al10/9-28</td>
<td>1% H₂SO₄ 10 min</td>
<td>521.8</td>
<td>77.3</td>
<td>1% H₂SO₄ 10 min</td>
</tr>
<tr>
<td>Al10/9-33</td>
<td>1% H₂SO₄ 30 min</td>
<td>551.4</td>
<td>81.2</td>
<td>1% H₂SO₄ 20 min</td>
</tr>
<tr>
<td>Al10/9-40</td>
<td>1% H₂SO₄ 5 min</td>
<td>552.8</td>
<td>77.6</td>
<td>1% H₂SO₄ 20 min</td>
</tr>
<tr>
<td>Al11/5-41</td>
<td>1% H₂SO₄ 10 min</td>
<td>527.9</td>
<td>79.5</td>
<td>1% H₂SO₄ 20 min</td>
</tr>
<tr>
<td>Al11/5-47</td>
<td>1% H₂SO₄ 20 min</td>
<td>507.8</td>
<td>73.6</td>
<td>1.25% H₂SO₄ 10 min</td>
</tr>
</tbody>
</table>


CHAPTER 4

CONCLUSION AND FUTURE RECOMMENDATIONS

4.1 Summary and Conclusion

Compared with conventional switchgrass, genetically engineered switchgrass with altered lignin composition/structure is a more promising feedstock for sugar production. The transgenic switchgrass is proved more vulnerable than conventional switchgrass in the acid pretreatment, especially when the condition is not so severe such as at low acid concentration and short residual time. The substantial elevation of ASL: AIL ratio in transgenic switchgrass may cause appreciable increases in sugar yield in both acid pretreatment and enzymatic hydrolysis. Considerable amounts of HMF and furfural were generated during acid pretreatment at 150 °C, which would potentially inhibit the subsequent fermentation. Nonetheless, if the yeast can tolerate a combined HMF and furfural concentration of 2.0 g/L, more than 82% of the carbohydrates in transgenic switchgrass can be converted to sugars during the biomass conversion.

4.2 Future work

1. S/G ratio testing is necessary to further confirm the effect of genetic engineering on switchgrass for lignin contracture modification.

2. Process simulation on the transgenic switchgrass to final ethanol production to analyze economic viability for large scale operation in current technology scenario.
4.3 Acknowledgements

The authors are grateful to the financial support of this research by the US Department of Energy and the Consortium for Plant Biotechnology Research, Inc. and the generous donation of enzymes from Novozymes North America, Inc. We would also like to thank Dr. Rongda Qu from the Department of Crop Science at North Carolina State University (NCSU) for providing the transgenic switchgrass and Dr. Dhana Savithri from the NCSU Integrated Biomass Research Initiative Program for her assistance in chemical analyses.
Appendix
Appendix A: SAS CODE FOR ANOVA

```sas
data pretreatment;

PROC IMPORT DATAFILE = 'Desktop\Data_reps2.xls' OUT = e1 DBMS=excel REPLACE;
ods html;
ods graphics on;
proc print data=e1;
run;

data f1 (drop =
lignin_contant1 lignin_contant2 lignin_contant3
ASL_AISL1 ASL_AISL2 ASL_AISL3
xylose1 xylose2 xylose3
glucose1 glucose2 glucose3
TS1 TS2 TS3
HMF1 HMF2 HMF3
Furfural1 Furfural2 Furfural3
CF1 CF2 CF3);
set e1 ; if type ne ""
Rep=1; ligninc=lignin_contant1; linginR=ASL_AISL1;Xylose = xylose1;
   Glucose = glucose1 ; TS= TS1;   HMF = HMF1; Furfural=Furfural1;
   CF=CF1;output;
Rep=2; ligninc=lignin_contant2; linginR=ASL_AISL2;Xylose = xylose2;
   Glucose = glucose2 ; TS= TS2;   HMF = HMF2; Furfural=Furfural2;
   CF=CF2;output;
Rep=3; ligninc=lignin_contant3; linginR=ASL_AISL3;Xylose = xylose3;
   Glucose = glucose3 ; TS= TS3;   HMF = HMF3; Furfural=Furfural3;
   CF=CF3;output;
PROC PRINT data = f1;
run;

proc glm data=f1;
class type ligninc linginR;
model Xylose=type;
run;
ods html;
ods graphics on;
proc glm data=f1;
class type ;
model CF=type;
run;
```

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