ABSTRACT

YANG, YING. Ethanol Production Potential of Acid Pretreated Switchgrass Varieties. (Under the direction of Dr. Ratna R. Sharma-Shivappa.)

Three new experimental switchgrass germplasms (St6-1, St6-3E and St6-3F) containing 22.71 to 30.95% glucan, 13.27 to 19.37% xylan and 17.39 to 20.60% lignin (on dry matter basis) were studied. Oven- or freeze-dried switchgrass whole-plant samples were pretreated with dilute sulfuric acid at 10% solid loading in an autoclave at 121 °C/15 psi. The effect of three acid concentrations (0.5, 1.0 and 1.5% w/v) and residence times (30, 45 and 60 min) on switchgrass composition was investigated. Influence of plant part was also studied for freeze-dried samples that had been separated into stems and leaves prior to drying. The greatest hemicellulose removal observed was 83.58% for oven-dried and 85.87% for freeze-dried samples resulting from intense pretreatment involving greater acid concentration (1.5% w/v H₂SO₄) or longer residence time (60 min). Hemicellulose in leaf was easily solubilized during acid pretreatment and removed from the solids by effective washing. However, delignification was not significant during acid pretreatment and was limited to 10% for all samples investigated. For each oven- or freeze-dried germplasm, pretreatment conditions resulting in the least lignin content or greatest hemicellulose solubilization were selected for hydrolysis and fermentation. The pretreatment conditions selected based on lignin and hemicellulose content were: 60 min/0.5% H₂SO₄ and 60 min/1.0% H₂SO₄ for oven-dried St6-1, 30 min/1.0% H₂SO₄ and 60 min/1.5% H₂SO₄ for oven-dried St6-3E, 30 min/1.0% H₂SO₄ and 45 min/1.5% H₂SO₄ for oven-dried St6-3F, 45 min/1.0% H₂SO₄ (whole plant) and 45 min/1.0% H₂SO₄ (leaf) for freeze-dried St6-1, 60 min/1.0% H₂SO₄ (stem) and 60 min/1.5% H₂SO₄ (leaf) for freeze-dried St6-3F.

A cellulase (Novozymes NS 50013 cellulase complex) and cellobiase (Novozymes NS 50010 β-Glucosidase) mixture at an activity ratio of 1:4 FPU/CMU
was added during hydrolysis at cellulase activities of 0, 15 and 30 FPU/g dry biomass. The effect of xylanase supplementation at 0.25% w/w dry biomass was also tested. Enzymatic hydrolysis was enhanced by acid pretreatments especially those resulting in greater hemicellulose solubilization. The greatest glucan to glucose conversion obtained was 104.70-106.65% for freeze-dried St6-3F leaf samples after being pretreated with 1.0% acid for 60 min at 121°C/15 psi and hydrolyzed by cellulase at 15 FPU/g dry biomass supplemented with xylanase or by cellulase alone at 30 FPU/g dry biomass. Addition of cellulase significantly impacted (P < 0.05) hydrolysis efficiency for all switchgrass samples tested while adding xylanase did not appreciably enhance glucose yield.

Fermentation of switchgrass hydrolyzates by *Saccharomyces cerevisiae* (ATCC 24859) resulted in almost complete utilization of glucose for ethanol production, indicating that dilute acid pretreatment had no inhibitory effect on fermentation if pretreated solids were completely washed. The greatest ethanol yield from the most effective acid pretreatment was 0.082 g/g initial biomass obtained with oven-dried St6-3E and freeze-dried St6-3F whole-plant samples.

A 60% theoretical ethanol yield at 0.092 g/g initial biomass was achieved through a 7-day simultaneous saccharification and fermentation (SSF) of oven-dried St6-3E switchgrass pretreated with 1.5% sulfuric acid for 60 min at 121°C/15 psi. Fermentation of the hydrolyzate from pretreated oven-dried switchgrass with immobilized yeast cells did not enhance ethanol yield compared with conventional fermentation. Based on switchgrass yields of 13450 kg/ha and its capability of producing 0.082 g ethanol/g initial biomass (consider cellulose conversion only), the estimated overall ethanol yield is 1398 L/ha switchgrass (149 gallon/acre), showing its potential as an energy crop for bioethanol production. However, a comprehensive economic analysis of lignocellulose-to-ethanol conversion and further investigation of treatment parameters are needed for facilitating the scale-up of this challenging process.
DEDICATION

This thesis is dedicated to my beloved parents for their constant love and support along the way.
Ying Yang was born in October, 1984 in Sichuan Province, China. The given name ‘Ying’, meaning fluorescence in Chinese, was chosen by her father expecting her daughter to shine more or less somewhere in the future. At the age of three, Ying and her family moved to Wuhan, the capital city of Hubei Province, where she spent the next 15 years with her parents till graduation from high school in July 2002. She got used to the new life style immediately after moving and greatly enjoyed the unique Yangtze River Culture offered in both Sichuan and Hubei. During high school days, Ying became very interested in the atmosphere of the metropolitan capital and dreamed of starting her college life in Beijing. Finally it came true as she attended Beijing Institute of Technology (BIT) in the fall of 2002 with a major in Biological Engineering. The next four years, filled with hard work as well as joy and happiness, were the most unforgettable period in her life till now. Besides classes, she tried lots of different things such as joining a week-long field trip for investigation of the ecology in the less developed areas around Beijing and volunteering at the international meetings for environmental protection. She is an optimistic girl who never stops exploring the life by putting forth her best effort. Upon graduation from BIT in July 2006, she travelled half a world apart from China to the United States for her Master’s degree at North Carolina State University. She worked on a bioethanol project under the direction of Dr. Ratna Sharma-Shivappa in the Department of Biological and Agricultural Engineering.

Ying is quite an easy-going girl and brings her smiles everywhere. Although a little bit introversive to strangers, she is very extroversive to close friends. In her spare time, Ying enjoys travelling very much and considers it as life’s greatest pleasure. She has been to more than half of the provinces in China and was fascinated by the charming sceneries of nature. Here in the United States, she loves exploring
different cultures by visiting various cities and currently, she is strongly attracted to
the culture-rich atmosphere in New England. She also likes watching sports games in
her free time; however, even she does not really remember how this habit actually
grew as it dated back to the mid-90s. Besides, she enjoys swimming, reading and the
most important, getting together with dear family and friends. Ying believes that her
NC State experience by far has been positive and fulfilling and she is looking forward
to new challenges that life will bring.
ACKNOWLEDGEMENTS

Many people have assisted me in the past two years to complete this thesis for a Master’s Degree. First of all, I would like to express my overwhelming thanks to my advisor, Dr. Ratna Sharma-Shivappa, for her guidance and support during my Master study. She is extremely approachable and patient to offer help not only in research, but also in my personal development. She gave me the freedom to learn what I am interested in, encouraged me to enthusiastically participate in the Master project, and provided valuable suggestions on how to make smooth transitions in life as an international graduate student. Without these, my academic and personal progress over the past two years would not have been possible.

I gratefully acknowledge other three committee members Drs. George Allen, Jay Cheng and Joseph Burns for their guidance to help me to complete this project. Dr. Allen introduced me to the world of plant biotechnology in his classes, and in my opinion, he is a professor who is always fascinated by his research and ready to help the students. Dr. Cheng offered lots of insightful suggestions on my research and encouraged me to think critically beyond finishing the work in the laboratory. Dr. Burns kindly provided lots of useful information for this thesis and gave important feedbacks after reviewing the section which I was not familiar with.

Additional thanks go to Dr. Mari Chinn, Dr. Lingjuan Wang and Dr. Dan Willits for their valuable assistance and encouragement. Besides, I would like to thank Dr. Jason Osborne who provided a tremendous help on composing the codes for one statistical analysis section in this research. Huge thanks go to all brilliant instructors, especially Dr. Kimberly Weems (Statistics) and Dr. Joanna Miller (Biotechnology Program), who offered interesting courses that greatly inspired me during my Master study.
I would like to express my sincere appreciation to Jian Shi who helped me with both lab work and data analysis. Special thanks go to the current students working in the Bioprocessing Lab of the BAE Department: Jiele Xu, Ziyu Wang, Deepak Keshwani, Billy Duvernay, and Arthur Redding. Besides, I would like to thank all the colleagues, friends and recent graduates of Weaver Labs, especially Gabrielle Skipper, Sara Johnson, Cyrus Yunker, Kelly Collins, Shuhai Li, Haiyan Yao and Zifei Liu. Though Weaver is an old building, it is filled up with plenty of fresh ideas and active interactions between faculty and students. I am fortunate enough to have spent the past two years studying and working here in the BAE Department of North Carolina State University at Raleigh, NC, and I am sure my pleasant study and work experience, the southern hospitality, and the comfortable weather will be some of the nicest memories ever.

In addition, I would like to thank all my loveliest friends back in China, here in the US, or currently pursuing their advanced degrees somewhere else on the planet for their continuous support in different ways.

Last but not the least, extremely huge thanks to my family for their endless love and encouragement without which nothing would have been possible for me to come along the way.
TABLE OF CONTENTS

LIST OF TABLES ............................................................................................................ x
LIST OF FIGURES ......................................................................................................... xiii
CHAPTER 1  Introduction .............................................................................................. 1
  1.1 Background ........................................................................................................... 1
  1.2 Objectives .......................................................................................................... 3
CHAPTER 2  Literature Review ................................................................................. 5
  2.1 Energy Demand and Environmental Issues ...................................................... 5
  2.2 From Biomass to Biofuels ............................................................................... 6
    2.2.1 Biomass as renewable energy resource ..................................................... 6
    2.2.2 Current trends in biofuel production ......................................................... 8
    2.2.3 Why choose switchgrass ....................................................................... 10
  2.3 Composition of Natural Cellulosic Feedstocks .............................................. 13
    2.3.1 Structure of cell wall ............................................................................. 13
    2.3.2 Cellulose ............................................................................................ 14
    2.3.3 Hemicellulose .................................................................................... 14
    2.3.4 Lignin ................................................................................................. 15
  2.4 Overview of Cellulosic Biomass to Ethanol Conversion .................................. 16
  2.5 Pretreatment ..................................................................................................... 18
    2.5.1 Pretreatment introduction ................................................................. 18
    2.5.2 Physical pretreatment ........................................................................ 18
    2.5.3 Chemical pretreatment .................................................................... 21
    2.5.4 Biological pretreatment .................................................................. 29
  2.6 Hydrolysis ......................................................................................................... 31
    2.6.1 Chemical hydrolysis ........................................................................ 31
    2.6.2 Enzymatic hydrolysis ....................................................................... 31
  2.7 Fermentation ..................................................................................................... 35
    2.7.1 Principles of ethanol fermentation ...................................................... 35
    2.7.2 Simultaneous saccharification and fermentation (SSF) ......................... 36
    2.7.3 Yeast cell immobilization during fermentation ..................................... 37
    2.7.4 Fermentation of pentose .................................................................. 38
    2.7.5 Ethanol collection ............................................................................. 40
  2.8 From Lab Research to Industrial Scale-up ....................................................... 41
  2.9 Summary .......................................................................................................... 41
CHAPTER 3  Dilute Acid Pretreatment of Oven-dried New Switchgrass
  Germplasms for Bioethanol Production ................................................................. 43
3.1 Introduction .................................................................................................... 43
3.2 Materials and Methods ..................................................................................... 45
  3.2.1 Switchgrass feedstock .............................................................................. 45
  3.2.2 Composition analysis .............................................................................. 46
  3.2.3 Dilute acid pretreatment .......................................................................... 54
  3.2.4 Enzymatic hydrolysis ............................................................................. 57
  3.2.5 Fermentation ......................................................................................... 59
  3.2.6 General statistical analysis ................................................................... 60
3.3 Results and Discussion .................................................................................... 61
  3.3.1 Composition of switchgrass .................................................................. 61
  3.3.2 Effect of dilute acid pretreatment .......................................................... 63
  3.3.3 Enzymatic hydrolysis ........................................................................... 70
  3.3.4 Fermentation ......................................................................................... 75
3.4 Conclusion ...................................................................................................... 80
References ............................................................................................................. 82

CHAPTER 4  Saccharification and Fermentation of Dilute Acid Pretreated
Freeze-dried Switchgrass Germplasms ................................................................. 86
4.1 Introduction ..................................................................................................... 86
4.2 Materials and methods ................................................................................... 88
  4.2.1 Switchgrass feedstock ........................................................................... 88
  4.2.2 Composition analysis ........................................................................... 89
  4.2.3 Dilute acid pretreatment ....................................................................... 90
  4.2.4 Enzymatic hydrolysis .......................................................................... 92
  4.2.5 Fermentation ....................................................................................... 92
  4.2.6 General statistical analysis .................................................................. 92
4.3 Results and Discussion .................................................................................. 93
  4.3.1 Composition of switchgrass ................................................................ 93
  4.3.2 Effect of dilute acid pretreatment ........................................................... 96
  4.3.3 Enzymatic hydrolysis ........................................................................... 106
  4.3.4 Fermentation ...................................................................................... 113
4.4 Conclusion ................................................................................................... 117
References .......................................................................................................... 119

CHAPTER 5  Simultaneous Saccharification and Fermentation (SSF) and
Immobilized Yeast Cell Fermentation of Dilute Acid Pretreated Switchgrass ....... 124
5.1 Introduction .................................................................................................. 124
5.2 Materials and Methods ................................................................................. 126
  5.2.1 Biomass feedstock ............................................................................... 126
  5.2.2 Dilute acid pretreatment ...................................................................... 127
  5.2.3 Yeast culture inoculum preparation .................................................... 127
  5.2.4 Simultaneously saccharification and fermentation (SSF) .................. 128
## TABLES

**Table 2.1** Chemical composition of untreated and pulped spring harvested switchgrass samples (on percent dry matter basis)\(^A\) .................................................. 11

**Table 3.1** Composition of the three oven-dried switchgrass germplasms .... 61

**Table 3.2** Composition of St6-1 oven-dried switchgrass after acid pretreatment at different conditions (expressed as g/100g unpretreated dry switchgrass) ................................................................. 64

**Table 3.3** Composition of St6-3E oven-dried switchgrass after acid pretreatment at different conditions (expressed as g/100g unpretreated dry switchgrass) ................................................................. 65

**Table 3.4** Composition of St6-3F oven-dried switchgrass after acid pretreatment at different conditions (expressed as g/100g unpretreated dry switchgrass) ................................................................. 66

**Table 3.5** P-values of ANOVA type III test for lignin content in dilute acid pretreated oven-dried switchgrass ................................................................. 67

**Table 3.6** P-values of ANOVA type III test for percent hemicellulose solubilization in dilute acid pretreated oven-dried switchgrass ........ 68

**Table 3.7** Amount of reducing sugars released in the filtrate during dilute acid pretreatment of oven-dried switchgrass feedstocks (expressed as mg/g of dry initial switchgrass) ................................................................. 69

**Table 3.8** P-values of ANOVA type III test for hydrolysis of the least-lignin-content dilute acid pretreated oven-dried switchgrass .. 71

**Table 3.9** P-values of ANOVA type III test for hydrolysis of the greatest-hemicellulose-solubilization dilute acid pretreated oven-dried switchgrass ................................................................. 74

**Table 3.10** Xylan conversion efficiency (%) during enzymatic hydrolysis . 75
TABLE 3. 11 P-VALUES OF ANOVA TYPE III TEST FOR FERMENTATION OF THE LEAST-LIGNIN-CONTENT DILUTE ACID PRETREATED OVEN-DRIED SWITCHGRASS...

TABLE 3. 12 P-VALUES OF ANOVA TYPE III TEST FOR FERMENTATION OF THE GREATEST-HEMICELLULOSE-SOLUBILIZATION DILUTE ACID PRETREATED OVEN-DRIED SWITCHGRASS..........................................................79

TABLE 4. 1 COMPOSITION OF THREE FREEZE-DRIED SWITCHGRASS GERMLASMS AND THEIR PLANT PARTS.................................................................94

TABLE 4. 2 COMPOSITION OF ST6-1 FREEZE-DRIED SWITCHGRASS AFTER ACID PRETREATMENT AT DIFFERENT CONDITIONS (EXPRESSED AS G/100G UNPRETREATED DRY SWITCHGRASS)........................................97

TABLE 4. 3 COMPOSITION OF ST6-3E FREEZE DRIED SWITCHGRASS AFTER ACID PRETREATMENT AT DIFFERENT CONDITIONS (EXPRESSED AS G/100G UNPRETREATED DRY SWITCHGRASS)........................................99

TABLE 4. 4 COMPOSITION OF ST6-3F FREEZE-DRIED SWITCHGRASS AFTER ACID PRETREATMENT AT DIFFERENT CONDITIONS (EXPRESSED AS G/100G UNPRETREATED DRY SWITCHGRASS)........................................101

TABLE 4. 5 P-VALUES OF ANOVA TYPE III TEST FOR LIGNIN CONTENT IN DILUTE ACID PRETREATED FREEZE-DRIED SWITCHGRASS.................................104

TABLE 4. 6 P-VALUES OF ANOVA TYPE III TEST FOR PERCENT HEMICELLULOSE SOLUBILIZATION IN DILUTE ACID PRETREATED FREEZE-DRIED SWITCHGRASS.....104

TABLE 4. 7 P-VALUES OF ANOVA TYPE III TEST FOR HYDROLYSIS OF THE LEAST-LIGNIN-CONTENT DILUTE ACID PRETREATED FREEZE-DRIED SWITCHGRASS .................................................................................................................................108

TABLE 4. 8 P-VALUES OF ANOVA TYPE III TEST FOR HYDROLYSIS OF THE GREATEST-HEMICELLULOSE-SOLUBILIZATION DILUTE ACID PRETREATED FREEZE-DRIED SWITCHGRASS.................................................................111

TABLE 4. 9 XYLAN CONVERSION EFFICIENCY (%) DURING ENZYMATIC HYDROLYSIS .112

TABLE 4. 10 P-VALUES OF ANOVA TYPE III TEST FOR FERMENTATION OF THE
LEAST-LIGNIN-CONTENT DILUTE ACID PRETREATED FREEZE-DRIED SWITCHGRASS

Table 4. 11 P-values of ANOVA Type III test for fermentation of the greatest-hemicellulose-solubilization dilute acid pretreated freeze-dried switchgrass

Table 5. 1 Reducing sugar and ethanol contents in fermentation samples from SSF flasks sampled intermittently (System A, sampled every 24 h) and non-intermittently (System B, sampled at 0 h and 168 h).............. 134

Table 5. 2 Reducing sugar and ethanol contents in intermittently (System A, sampled every 24 h) and non-interruptently (System B, sampled at 0 h and 168 h) sampled immobilized yeast fermentation systems ............. 137
LIST OF FIGURES

FIGURE 2.1 Structure of cellulose microfibrils with hydrogen bonds linking neighboring molecules and chains (modified from: http://www.lsbu.ac.uk/water/hycel.html) ................................................. 14

FIGURE 2.2 Flowchart for the conversion of lignocellulose to bioethanol. Steps marked with * were performed or analyzed in this study. .......... 17

FIGURE 3.1 The Soxhlet extraction units ............................................................. 48

FIGURE 3.2 Siphoning during the extraction process ............................................. 49

FIGURE 3.3 The HPLC equipment for carbohydrate analysis ............................... 52

FIGURE 3.4 Buchner funnel filtration assembly for solid and liquid separation .................................................................................................. 55

FIGURE 3.5 Yeast culture dilution and incubation using the spread-plate technique .......................................................................................... 60

FIGURE 3.6 Hydrolysis of unpretreated and pretreated least-lignin-content oven-dried samples. Columns are grouped by the same cellulase activity levels for each switchgrass germplasm ........................................... 71

FIGURE 3.7 Hydrolysis of unpretreated and pretreated greatest-hemicellulose-solubilization oven-dried samples. Data for unpretreated samples are the same as those in Figure 3.6. Columns are grouped by the same cellulase activity levels for each switchgrass germplasm ................................................................. 73

FIGURE 3.8 Ethanol yields of unpretreated and pretreated oven-dried samples with the least lignin content after pretreatment for each germplasm. Columns are grouped by the same cellulase activity levels during hydrolysis for each switchgrass germplasm ........................................... 76
FIGURE 3.9 ETHANOL YIELDS OF UNPRETREATED AND PRETREATED OVEN-DRIED SAMPLES WITH THE GREATEST HEMICELLULOSE SOLUBILIZATION AFTER PRETREATMENT FOR EACH GERMPLASM. DATA FOR UNPRETREATED SAMPLES ARE THE SAME AS THOSE IN FIGURE 3.8. COLUMNS ARE GROUPED BY THE SAME CELLULASE ACTIVITY LEVELS DURING HYDROLYSIS FOR EACH SWITCHGRASS GERMPLASM.

FIGURE 4.1 HYDROLYSIS OF UNPRETREATED AND PRETREATED LEAST-LIGNIN-CONTENT FREEZE-DRIED SAMPLES. COLUMNS ARE GROUPED BY THE SAME CELLULASE ACTIVITY LEVELS FOR EACH SWITCHGRASS GERMPLASM.

FIGURE 4.2 HYDROLYSIS OF UNPRETREATED AND PRETREATED GREATEST-HEMICELLULOSE-SOLUBILIZATION FREEZE-DRIED SAMPLES. COLUMNS ARE GROUPED BY THE SAME CELLULASE ACTIVITY LEVELS FOR EACH SWITCHGRASS GERMPLASM.

FIGURE 4.3 ETHANOL YIELDS OF UNPRETREATED AND PRETREATED FREEZE-DRIED SAMPLES WITH THE LEAST LIGNIN CONTENT AFTER PRETREATMENT WITHIN EACH GERMPLASM. COLUMNS ARE GROUPED BY THE SAME CELLULASE ACTIVITY LEVELS DURING HYDROLYSIS FOR EACH SWITCHGRASS GERMPLASM.

FIGURE 4.4 ETHANOL YIELDS OF UNPRETREATED AND PRETREATED FREEZE-DRIED SAMPLES WITH THE GREATEST HEMICELLULOSE SOLUBILIZATION AFTER PRETREATMENT WITHIN EACH GERMPLASM. COLUMNS ARE GROUPED BY THE SAME CELLULASE ACTIVITY LEVELS DURING HYDROLYSIS FOR EACH SWITCHGRASS GERMPLASM.

FIGURE 5.1 EQUIPMENT FOR SSF EXPERIMENT

FIGURE 5.2 YEAST CULTURE IMMOBILIZATION IN CA-ALGINATE BEADS

FIGURE 5.3 EQUIPMENT FOR IMMOBILIZED YEAST CELL FERMENTATION

FIGURE 5.4 CHANGE IN GLUCOSE, XYLOSE AND ETHANOL CONTENTS DURING SIMULTANEOUS SACCHARIFICATION AND FERMENTATION (SSF) OF ACID PRETREATED SWITCHGRASS OVER 7 DAYS.
FIGURE 5. CHANGE IN GLUCOSE, XYLOSE AND ETHANOL CONTENTS DURING IMMobilized YEAST CELL FERMENTATION OF ACID PRETREATED AND ENZYMATIC HYDROlyZED SWitcHGRASS OVER 7 DAYS. ........................................................ 135
CHAPTER 1  Introduction

1.1 Background

Due to the increasing demand for energy and serious concerns over greenhouse gas emissions, alternative energy resources have become highly preferred. In the newly released report of International Energy Outlook 2007 (IEO2007), scientists project continuous robust growth of worldwide energy demand over the period from 2004 to 2030. It is forecasted that the total world energy consumption will increase from 471 quadrillion (10^{15}) Kilojoules in 2004 to 740 quadrillion Kilojoules in 2030, resulting in a 57% growth. Among all the categories of energy sources, crude oil is the most essential to industrialized nations such as the United States. Though United States is currently the world’s largest energy consumer, about 60% of the crude oil consumed in the country is imported (Gray et al., 2006).

Additionally, burning of fossil fuels contributes to global warming and air quality degradation, which is disadvantageous for sustainable development. Therefore, environmental concerns, uncertain future availability of fossil fuels and the strong awareness of self-reliance of energy supply, have prompted extensive investigation of renewable energy resources since the late 20th century.

Biomass, which refers to renewable organic materials derived from plant and animal sources through various natural or human activities, has recently surpassed hydropower to become the No. 1 renewable energy resource in the US (Perlack et al., 2005). Biomass can be converted to biofuels thus giving mobility to everyday life. A significant advantage of the biomass-to-biofuel process is the low net emission of carbon dioxide. Since the carbon dioxide generated during utilization of biofuels is recaptured mainly by photosynthesis during the regrowth of harvested biomass, a nearly closed carbon loop can be created (Wyman, 1999; Fatsikostas et al., 2002). According to the report by Perlack et al. (2005), the US has the potential to produce
over 1.3 billion dry tons of biomass feedstock per year, which is sufficient to produce biofuels for replacing one third of the current demand for transportation fuels.

Generally, biofuels are divided into two categories: biodiesel and bioethanol. Normally, raw materials for biodiesel production include vegetable oils (for example, soybean or rapeseed) or animal fats (Ma and Hanna, 1999); whereas biomass-to-ethanol conversion utilizes structural sugar components contained in biomass feedstocks such as grain or sugar crops, energy crops, agricultural residues and municipal wastes. Biodiesel, chemically recognized as a fatty acid alkyl ester, is derived from triglyceride by transesterification with acyl acceptors such as methanol (Fukuda et al., 2001). Currently, the major feedstocks for ethanol production are corn kernels (starch) in the US and sugarcane (sucrose) in Brazil (Parikka, 2004; Gray et al., 2006). Technologies for these two feedstocks are well developed and the corresponding commercial productions have been realized for years. However, these two feedstocks are not in sufficient supply to meet the worldwide demand for biofuels; for example, only 12% of the gasoline demand can be met by dedicating all the US corn for bioethanol production (Hill et al., 2006). Besides, their elementary roles as food source (specifically grain) as well as the regional limitations cause additional problems in sustainable biofuel production. Therefore, other categories of biomass, especially lignocellulose, are being considered for bioethanol production.

Lignocellulose is the main component of the plant cell wall and is made up of cellulose, hemicellulose and lignin. The general process for bioethanol production using lignocellulosic biomass involves pretreatment, hydrolysis and fermentation. Natural lignocellulosic biomass is resistant to hydrolysis because of the crystallinity of cellulose, protection of cellulose by lignin, cellulose sheathing by hemicellulose and the limited accessible surface area of the biomass matrix (Mosier et al., 2005). Therefore, pretreatment, the most challenging step, is essential to break down the close knit structure of lignocellulosic biomass thus providing hydrolysis agents with a more direct access to the feedstock (Wooley et al., 1999; Wyman et al., 2005).
Different pretreatment approaches (including physical, chemical, biological and their combinations) have been extensively investigated over the past decade and tailoring of an appropriate pretreatment for specific biomass feedstocks is of great interest to researchers (Ragauskas et al., 2006).

The biomass feedstock used in this study was switchgrass (*Panicum virgatum* L.), a warm season, perennial grass native to North America which provides high biomass yields with comparatively low input for both establishment and maintenance (van den Oever et al., 2003). Switchgrass has been identified as a lignocellulosic bioenergy crop due to its high yield and good compatibility with current farming practices; also, significant gains in terms of energy return can be achieved in switchgrass-to-bioethanol conversion (McLaughlin, 1992; McLaughlin et al., 1999). Additionally, the high cellulose (approximately 30%) content in switchgrass is advantageous for bioethanol production (McLaughlin et al., 1999).

1.2 Objectives

Results from previous research support the potential of switchgrass for bioethanol production through proper pretreatment, hydrolysis and fermentation (McLaughlin et al., 1999; Chang et al., 2001a; Alizadeh et al., 2005). However, the effects of different germplasms, plant parts and preparation prior to pretreatment have not been investigated. Therefore, this study was undertaken with the following objectives:

1. Determine the chemical composition of new germplasms of switchgrass;
2. Investigate and optimize the processing conditions (acid concentration, time) during acid pretreatment for enhancing enzymatic hydrolysis;
3. Study the effects of enzyme loadings and combinations on enzymatic hydrolysis to determine the effectiveness of pretreatment;
4. Study simultaneous saccharification and fermentation (SSF) and yeast cell immobilization during fermentation to test the improvement in ethanol yield;
(5) Evaluate the ethanol production potential of acid pretreated switchgrass.

The most efficient processing conditions identified in this study can be instrumental in improving the conversion process for similar bioethanol research on other feedstocks. In addition, comprehensive results obtained can be referred to in future scale-up after accounting for concerns over economics and environment issues.
CHAPTER 2  Literature Review

2.1 Energy Demand and Environmental Issues

Energy is one of the most significant building blocks for the world’s economy and contributes to the improvement of life quality (Kaygusuz, 2002). Fossil fuel, a conventional but principal energy resource, has accelerated global industrial and social development for centuries by providing electricity, heat and fueling the transportation system for everyday life. During the 20th century, energy crisis or oil price fluctuation has been viewed as the driving force for developing conservation measures on fossil fuel consumption (Turner, 1999); but generally, energy demand has not shown a downward trend. According to the report released by the Energy Information Administration of US Department of Energy (International Energy Outlook 2007), a 57% increase in global energy demand is expected over the period from 2004 to 2030. North America (US and Canada) currently consumes nearly one fourth of the world’s energy at 127.4 quadrillion \((10^{15})\) Kilojoules out of the world total of 470.8 quadrillion Kilojoules. In the US, however, almost two thirds of petroleum consumed is imported, thus creating a balance of trade deficit as well as concerns over nation security (Wyman et al., 2005). In the ensuing years, global energy demand is projected to grow with much of the increase coming from rapidly developing countries in Asia and Africa. Thus, due to the uncertain future availability of nonrenewable resources, powering the world entirely by fossil fuel is not a reliable long-term policy.

Environmental concern is another issue worthy of in-depth thought. Fossil fuel consumption is responsible for majority of the greenhouse gas emission and carbon dioxide, a dominant component of greenhouse gas, causes ambient temperature to increase. The existence of greenhouse gas is essential to all living beings on earth without which the ambient temperature would be 30 °C lower than it
is now. But human activities today, especially consumption of fossil fuel and the change in land use patterns, are causing concentration of greenhouse gas to increase rapidly thus resulting in a continuous warming of the global climate (Joint Science Academies, 2005). According to the Assessment Report given by Intergovernmental Panel on Climate Change (IPCC, 2007), the linear warming shift over the last 50 years from 1956 to 2005 has reached 0.13 °C per decade which is almost twice as that for the last 100 years as a whole. In addition, other emissions from direct or indirect combustion of fossil fuel, such as nitrogen oxides and sulfur oxides, could also trigger environmental problems. For example, acid rain and photochemical smog are the most common outcomes directly related to the decline of air quality. Sometimes, environmental concerns, rather than the shortage of energy supply, are the driving forces for developing alternative energy resources (Law et al., 2001). Therefore, due to the challenges of minimizing emission of carbon dioxide and other pollutants, as well as overcoming the shortage of power supply, there is an urgent need to develop alternative cost-effective approaches that are capable of powering everyday life without bringing harmful environmental changes while providing a permanent renewable energy supply.

2.2 From Biomass to Biofuels

2.2.1 Biomass as renewable energy resource

Biomass refers to renewable organic materials such as plant or animal matter derived from various natural or human activities. Recently, biomass is more commonly defined as the plant matter grown specifically for producing biofuels thus allowing conversion of solar-derived chemical energy into other categories of convenient energy sources. Other categories of biomass, including residues from agriculture or forestry, organic components of municipal and industrial wastes, and dedicated energy crops, are also considered as the feedstock for global energy supply.
(Gray et al., 2006; NREL, 2006). A conservative estimate of global biomass is approximately 10 oven-dry tons/hectare/year, although some small-scale field trials have reported a much higher yield (Berndes et al., 2001; Perlack et al., 2005). Biomass has been used for energy ever since mankind started burning wood to keep warm and cook food. In 2003, nearly 3.1 quadrillion \(10^{15}\) Kilojoules of total US energy supply was derived from biomass, which represented 3% of the overall 103 quadrillion Kilojoules. Additionally, biomass has recently surpassed hydropower to become the No. 1 renewable energy source in the US (Perlack et al., 2005).

Utilization of fuel from biomass releases carbon dioxide which is primarily balanced by the carbon dioxide captured during the regrowth of plants through photosynthesis (Spatari et al., 2005). This is the basic difference in carbon dioxide emission between biomass and fossil fuels; because the latter yields carbon dioxide that was trapped millions of years ago as “new” greenhouse gases to the atmosphere. Besides, biomass consumption can be beneficial to local and national agriculture and industry since biomass-to-energy production could make use of agricultural or forestry surplus as well as industrial residue thus solving disposal problems. In addition, large scale biofuel production can greatly reduce the nation’s dependence on imported crude oil thus enhancing self-reliance for energy (NREL, 2006). Therefore, biomass is widely considered as one of the most promising energy resources following the era of coal, oil and natural gas and would probably play a vital role in energy supply in the near future (Klass, 1998).

Biomass can be divided into two categories on the basis of products: starch/cellulosic biomass for sugar-to-bioethanol conversion, and animal fat or vegetable oil for biodiesel production. The present industrial scale production of bioethanol largely relies on the fermentation of starch from corn kernels in the US and of sucrose from sugarcane in Brazil (Parikka, 2004; Gray et al., 2006). While, biodiesel production mainly uses soybean oil, and various animal fats or recycled
frying oils are also available and competitive (Ma and Hanna, 1999). Significant reduction in greenhouse gas emissions to the level of 12% by producing and combusting bioethanol or 41% with biodiesel can be achieved compared with burning traditional fossil fuels (Hill et al., 2006). Relative to energy input and output, Hill et al. (2006) positively reported that ethanol yields 25% more energy than the energy invested for the growth and maintenance of corn, while biodiesel from soybeans yielding 93% more. However, this is an arguable topic since several energy inputs which can lead to a shrink in the net energy return are normally omitted in such estimations; these inputs could come from the production and repair of farm machinery as well as the equipment required for fermentation and distillation (Pimentel and Patzek, 2005).

Since the United States has the potential to produce over 1 billion tons of biomass each year, US Department of Agriculture and Department of Energy have targeted replacing 30% of the liquid petroleum transportation fuel (about 300 billion liter) with biofuels by 2025 (Perlack et al., 2005). In addition, the 2007 Federal Budget request calls for a 60% increase in funding for the biofuel initiative (EERE, 2006). These administrative interests in facilitating biofuel production show its tremendous potential to be an important energy source in the nation’s future.

2.2.2 Current trends in biofuel production

Natural cellulose is the most abundant renewable organic compounds on earth (Mandels et al., 1974). Lignocellulosic feedstock refers to agricultural residues, forestry wastes, municipal solid wastes and some specifically grown energy crops that primarily contain cellulose, hemicellulose and lignin. Solar energy is captured through photosynthesis by raw lignocellulosic material and stored as chemical energy. However, only a very small part of this energy is currently being used as crops, forages, papermaking raw materials etc. Most of the lignocellulosic feedstocks are efficiently decomposed by naturally occurring microbes, mainly fungi and
bacteria, playing an important role in completing the global carbon cycle (Ohkuma, 2003).

Biofuel derived from wood resources has a long history through direct burning in the open air in spite of little heat collection. Besides being very hazardous to air quality and a waste of potential energy, open-field burning can cause other problems such as destroying ecological balance and decreasing the fertility of soil (Law et al., 2001). Therefore, this relatively convenient practice is severely restricted and may be eventually prohibited (Kadam et al., 2000). Currently the use of sawdust, wood chips and bark in industrialized countries for power generation or system heating is significant. However, there is limited scope for expansion of these applications of wood without causing extra environmental stresses (Samson et al., 2005). Alternatively, crop residues, the non-edible plant parts that are left in the fields after harvest, such as corn stovers and wheat straws, are another group of lignocellulosic feedstocks which widely differ in properties. They are considered effective in enhancing, maintaining and sustaining soil quality as well as being an alternative source for expanding the production of biofuels (Lal, 2005), though the economic and environmental consequences of biofuel production from crop residues need to be further evaluated. Additionally, there is a need to grow dedicated energy crops on specifically identified lands to supplement the energy output of crop residues, thus further reducing the dependence on fossil fuels (Lal, 2005). This however involves overcoming challenges such as the willingness of farmers to grow energy crops while maintaining the same economic level as conventional crops. In addition, when biofuel production is conducted on a large scale, it is essential to compare the operating cost and product quality with fossil fuels to test if it is really environmental-friendly and energy-saving (McLaughlin et al., 1999).

The most significant challenge for biofuel production is to develop feasible and efficient treatment suitable for each specific biomass feedstock while enhancing
biomass yields by at least two fold (Ragaukas et al., 2006). Currently, some of the technologies for biomass-to-value added products conversion are well established, such as producing ethanol from corn grains, while most others are still being tested and assessed due to numerous technical and economical impediments (Gray et al., 2006). For example, McAloon et al. (2000) estimated that the production cost of corn-derived ethanol was 0.23 US dollars per liter ($0.88 per gallon) for dry mill operations and the capital cost of dry mill ethanol plants with the necessary utilities were between 0.33 to 0.40 US dollars per liter ($1.25-1.50 per gallon), showing a significant decrease in costs compared with those in the 1970s when ethanol fuel industry first started. For lignocellulose based biofuel production, the capital cost per liter was estimated at almost 1.44 US dollars ($5.44 per gallon) for the plant. Though the difference in costs exists, corn grain is not considered a sustainable feedstock for bioethanol production because of its primary role as a food source and its requirement for fertile land. Therefore, the conversion process for lignocellulosic feedstocks needs to be further improved to be rewarding for sustainable economic and social development.

2.2.3 Why choose switchgrass

Switchgrass (Panicum virgatum L.) is a warm-season, perennial (C₄) grass which has a wide range of geographic adaptation because of its well-developed extensive root system and high water use efficiency (Black, 1971). It is natively and widely grown within North America, from Maine to Saskatchewan, in the south from Florida to Arizona and Costa Rica, and also in the West Indies (Phillips and Rix, 2002). Switchgrass provides high yields with relatively low input for both establishment and maintenance and has been used for forage production, soil conservation and as an ornamental crop for quite a long time (van den Oever et al., 2003).

Numerous studies have been conducted on analyzing the chemical
characteristics of switchgrass according to the TAPPI standard methods widely used in the field of pulping and papermaking (Radiotis et al., 1996; Law et al., 2001; van den Oever et al., 2003). Generally, results show that switchgrass is relatively rich in holocellulose (cellulose and hemicellulose) and contains less ash, lignin and extractives, making it a good source of fiber for various uses (van den Oever et al., 2003; Dien et al., 2006). The chemical characteristics of two switchgrass varieties, named ‘Kanlow’ and ‘Cave-in-rock’, are presented in Table 2.1.

Table 2.1 Chemical composition of untreated and pulped spring harvested switchgrass samples (on percent dry matter basis)\textsuperscript{a}

<table>
<thead>
<tr>
<th></th>
<th>Kanlow</th>
<th>Cave-in-rock</th>
<th>pulped Cave-in-rock</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ash</td>
<td>1.9</td>
<td>1.8</td>
<td>2.6 \textsuperscript{b}</td>
</tr>
<tr>
<td>Extractives</td>
<td>10.4</td>
<td>9.5</td>
<td>—</td>
</tr>
<tr>
<td>Lignin</td>
<td>18.9</td>
<td>19.5</td>
<td>22.5</td>
</tr>
<tr>
<td>Cellulose</td>
<td>30.5</td>
<td>28.8</td>
<td>33.6</td>
</tr>
<tr>
<td>Hemi-cellulose</td>
<td>30.4</td>
<td>31.2</td>
<td>31.5</td>
</tr>
<tr>
<td>Pectin</td>
<td>1.4</td>
<td>1.3</td>
<td>1.7</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Source: van den Oever et al., 2003.
\textsuperscript{b} Pulp characterized without prior extraction.

Switchgrass is considered as a competitive dedicated lignocellulosic energy crop due to its specific advantages over other raw materials. It effectively reduces feedstock cost on an equivalent yield basis due to the easy production techniques compared to other biomass crops, such as willow and miscanthus (Girouard et al., 1996). Unlike short rotation plants, the role of switchgrass as a perennial plant helps to reduce the cost for annual establishment (Samson et al., 2005). Moreover, the adaptability of switchgrass to less fertile marginal land effectively avoids its competition with food production on better quality cropland (McLaughlin et al., 1999). Relative to the energy budgets, switchgrass is arguably a competitive herbaceous crop with a higher energy output/input ratio than corn grains which are currently taking up almost the entire US biofuel industry (McLaughlin and Walsh, 1999).
The bioenergy-crop to energy conversion generally involves three pathways: liquid fuel production, combustion alone or with other fossil fuels to produce heat or electricity, and gasification to simpler gas products for further applications. A comprehensive analysis of switchgrass’ chemical composition, energy content and combustion properties indicates that it is a versatile feedstock capable of meeting the requirements of all these endpoint processes (McLaughlin et al., 1996; McLaughlin et al., 1999).

During bioethanol production, carbohydrates from biomass feedstocks are fermented to ethanol after being broken down into small segments of oligo- or monosaccharides (Radiotis et al., 1996). The high holocellulose content in switchgrass is a desirable property for bioethanol feedstock. Yield, or general productivity, is another decisive and predominant factor when evaluating the feasibility of a feedstock for biofuel production (Bush et al., 2001). Switchgrass, as a lignocellulosic energy crop, has substantially higher yield than other feedstocks in North America, especially in the southeastern region. For example, the switchgrass cultivar, Cave-in-rock, has an average yield of 12.6 oven-dry tons/hectare/year (odt/ha/yr) compared to general crop yields of less than 10 odt/ha/yr (Girouard and Samson, 1998). In warmer and more humid areas such as Alabama, the yield could reach as much as 25 odt/ha/yr (Girouard et al., 1996).

Studies have been conducted to investigate the compositional differences among various parts of grass though in-depth investigation on bioethanol yields is limited. Lanning and Electerius (1987) found that silica levels in Kansas prairie stands were 1.03%, 3.85%, 3.41% and 5.04% in stems, leaf sheaths, leaf blades and inflorescences, respectively. They also reported that as a major component of ash, greater silica content was disadvantageous to the biomass-to-ethanol conversion. Though the stem fraction of grass was found to contain the least silica, the cellulose...
content of switchgrass was reported to be greatest in the stem fraction (Radiotis et al., 1996). Therefore, selection of greater stem content is likely to assist in reducing ash, while promoting the desirable characteristics of the feedstock for bioethanol production (Samson and Mehdi, 1998).

2.3 Composition of Natural Cellulosic Feedstocks

2.3.1 Structure of cell wall

The plant cell wall is the frontier zone of a plant cell, which provides the cell with strength and shape. It also controls cell expansion and intercellular transport while protecting the cell from a majority of potential pathogenic organism. Therefore, it plays an important role in both structure and physiology within a plant. The plant cell wall is made up of a series of cell layers. Besides middle lamella and primary cell wall, which are two main layers in a cell wall, certain specialized cells proceed to develop a secondary wall when differentiation occurs (Brett and Waldron, 1996).

Each cell layer is composed of two phases — a microfibrillar phase and a matrix phase. The microfibrillar phase consists of microfibrils which are made up of parallel-aligned cellulose molecules and displays a high degree of crystallinity. However, the chemical structure of the matrix phase is extremely complex which contains different varieties of polysaccharides, proteins and phenolic compounds and the combination of these compounds differs from cell to cell (Brett and Waldron, 1996). The so-called lignocellulose mainly comprises three fractions, namely cellulose, hemicellulose and lignin. In contrast to starch crops, the complex structure of lignocellulosic feedstock causes difficulties in the biomass-to-ethanol conversion since an extra step for structure disruption is needed before the degradation of cellulose (Zaldivar et al., 2001).
2.3.2 Cellulose

Found in the cell wall of all plants, cellulose is reported as the structural component of plants. It consists of linear $\beta$-1,4-linked D-glucopyranose residues with a degree of polymerization up to at least 15000 without branches. Hydrogen bonds between cellulose molecules enable the neighboring parallel or anti-parallel linear chains to become condensed to form an extremely long and thin structure called microfibrils as shown in Figure 2.1, hence building up the main microfibrillar phase (Brett and Waldron, 1996; Vries and Visser, 2001). The crystalline structure of cellulose chain gives rise to its considerable tensile strength with minimal flexibility and water insolubility. Therefore, it is fairly resistant to biological attack, which is a major limitation to cell wall hydrolysis (Gray et al., 2006).

![Figure 2.1 Structure of cellulose microfibrils with hydrogen bonds linking neighboring molecules and chains](http://www.lsbu.ac.uk/water/hycel.html)

2.3.3 Hemicellulose

Hemicellulose is the second abundant polysaccharide in plant cell wall, constituting about 20-35% of the plant materials (Saha, 2003). Unlike the comparatively uniform composition of cellulose, hemicellulose varies tremendously among different cell types and species. Also, the degree of branching and the characteristic of the minor sugars within hemicellulose differ from plant to plant.
(Gray et al., 2006). But for a certain plant, there is usually one kind of hemicellulose that is predominant (Brett and Waldron, 1996). The major hemicellulose in cereals and hardwood is xylan, while another hemicellulose that is popular in soft- and hardwood is named galactoglucosamannan (Vries and Visser, 2001). Xylan generally contains a backbone of β-1, 4-linked xylose residues. This structure is occasionally substituted by α-linked 4-O-methylglucoronic acid on C2, α-linked arabinose or acetyl esters on C2 or C3 of some xylose residues (Brett and Waldron, 1996). Hemicellulose non-covalently links onto cellulose with hydrogen-bonds and its C-5 sugar ring, which shows an asymmetric configuration, assists cellulosics to form the liquid crystalloid (Vincent, 1999). However, xylan itself does not have a crystalline structure because it lacks the ability to form hydrogen bonds between neighboring polysaccharide chains. Within some plant cell walls, cellulose and xylan are in nearly the same amount; but since xylan is able to form hydrogen bonds only on one side, just one half of xylan could be found to directly interact with cellulose, which assists in constructing the structural backbone for the plant cell wall. This kind of intersection is sometimes identified as the sheathing of cellulose by hemicellulose, which is also considered an impediment for cellulose digestion (Neville, 1993; Brett and Waldron, 1996).

2.3.4 Lignin

Certain differentiated cell types contain lignin, the most abundant aromatic polymer in nature that is gradually laid down when cell elongation occurs and normally constitutes about 10-25% of the plant material (Zaldivar et al., 2001; Saha, 2003). Three different alcohols are precursors for lignin synthesis — ρ-Hydroxyphenyl alcohol, guaiacyl alcohol and syringyl alcohol. They link with each other by a wide variety of bonds to form a huge network in the final phenolic compound (Brett and Waldron, 1996; Sakakibara, 1980). In addition, the lignin network continues to expand as long as the precursors are available, hence filling up
the spaces that have not been occupied by other components and greatly replacing water. Within the complicated meshwork, lignin has a considerable impact on other existing links. The most important effect is the enhancement of the strength of hydrogen-bonds between polysaccharides, which in turn increases the stability and rigidity of the cellulose-hemicellulose structure. This type of protection successfully reduces the chance of penetration of wall-degrading enzymes, and serves as an effective barrier to pests and diseases to protect the plant body (Brett and Waldron, 1996). However, this protection is not preferred in bioprocessing since it limits enzyme accessibility to the internal polysaccharides.

### 2.4 Overview of Cellulosic Biomass to Ethanol Conversion

The biological conversion of cellulosic biomass to ethanol involves steps from size reduction of biomass to the final distillation and dehydration of ethanol. During the conversion process, there are three constitutive steps, namely pretreatment, hydrolysis and fermentation. The feedstock matrix becomes more accessible to enzymes after pretreatment; and polysaccharides, mainly cellulose, can be broken down to reducing sugars which are the substrate for microbial fermentation. Finally, purification in terms of distillation and dehydration is needed to meet fuel market requirement.

The complex structure of plant cell wall involving various interactions between cellulose, hemicellulose and lignin makes the conversion of lignocellulosic biomass to ethanol problematic. Cellulose is the component of prime interest and can be chemically (by acid) or enzymatically hydrolyzed to glucose which is the major substrate in ethanol fermentation. Hemicellulose has a less compact structure than cellulose and can be significantly degraded or solubilized during pretreatment. If hemicellulose is successfully removed from the complex and degraded to oligo or mono xylose, these smaller molecules can be further converted to other byproducts.
such as xylitol which are of certain commercial value (Converti et al., 2000).

An outline of lignocellulose-to-ethanol conversion including dilute acid pretreatment is illustrated in Figure 2.2.

---

Figure 2.2 Flowchart for the conversion of lignocellulose to bioethanol. Steps marked with * were performed or analyzed in this study.
2.5 Pretreatment

2.5.1 Pretreatment introduction

The efficiency of lignocellulosic biomass to ethanol conversion largely depends on enzymes obtaining access to the plant polysaccharides to yield fermentable sugars; therefore, altering the original structure of the plant cell wall can bring about favorable results (Ragauskas, et al., 2006). This process is identified as pretreatment with the overall objective of breaking the lignin seal as well as hemicellulose sheathing, and disrupting the crystalline structure of cellulose, thus making the overall conversion feasible (Mosier et al., 2005). Pretreatment of lignocellulose is one of the most challenging steps during lignocellulosic biomass-to-ethanol production compared to the relatively simplified corn-to-ethanol conversion, possibly because the substrate for the latter is starch which has a less compact structure and allows the hydrolysis agents with better access to the biomass matrix. In addition, pretreatment is among the most expensive steps and it also affects the cost of other operations including prior size reduction and subsequent enzymatic hydrolysis and fermentation (Wooley et al., 1999; Wyman et al., 2005).

Numerous pretreatment methods have been evaluated extensively. Some of the methods have already undergone scale-up while others are still limited to lab research due to less developed technology or excessive cost. The overall objective of various studies on pretreatment is to find the most suitable method for each of the feedstock, thus providing an approach for economic success upon scaling up (Ragauskas et al., 2006). There are three main categories of pretreatments, namely physical, chemical and biological, some of which are promising though their specific effect on the biomass feedstock differs considerably.

2.5.2 Physical pretreatment

Physical pretreatment includes mechanical comminution, steam explosion,
microwave, radiation etc.

1. Mechanical comminution

   Mechanical comminution can result in significant changes in the physical characteristics of biomass, including smaller size, as well as a lesser degree of both crystallinity and polymerization. It has been widely applied in the corn-to-ethanol process, making corn kernels more open to enzymatic hydrolysis (Kim and Dale, 2002). Lignocellulosic biomass, however, requires much more mechanical energy input than corns, thus making comminution a less economically competitive approach for ethanol production (Graf and Koehler, 2000). Although power input for this process greatly depends on the final size requirement and the physical characteristic of the selected feedstock (Sun and Cheng, 2002), generally speaking, the energy requirement for comminution is significant.

2. Steam explosion (autohydrolysis)

   Steam explosion provides swift thermal expansion which opens up the structure of the target biomass. It is recognized as a suitable pretreatment method for hardwoods and agricultural residues, but less effective for softwoods (Clark and Mackie, 1987). This kind of pretreatment is typically initiated at a temperature of 160-260 °C (corresponding pressure 0.69-4.83 MPa) and the residence time varies from seconds to a few minutes (Sun and Cheng, 2002). The main factors affecting the results of steam explosion are residence time, temperature, particle size as well as moisture content (Duff and Murray, 1996); generally, overall recovery of dry matter decreases as the residence time and temperature increase (Weil et al., 1994). Steam explosion mainly contributes to hemicellulose degradation, and is currently used to commercially hydrolyze hemicellulose for the manufacturing of fibre-board or other byproducts (Mosier et al., 2005). In addition, since steam explosion is a chemical-free process, no environmental cost in terms of chemical recycling is
needed, thus reducing the operating cost. However, during the steam explosion process, some intermediate compounds generated are inhibitory to subsequent processes such as microbial fermentation (Mackie et al., 1985). Therefore, a water wash is usually applied to solve this problem, although some of the soluble sugars may also be washed off during this step (McMillan, 1994).

Recent literature has indicated a growing interest in introducing dilute-acid impregnation before steam explosion to promote the performance of pretreatment. In such a process, steam explosion is modified to be coupled with SO$_2$ catalysis by impregnating the substrate with SO$_2$ prior to the standard process (Tengborg et al., 1998). Dilute acid-added steam explosion can solubilize a significant amount of hemicellulose component in lignocellulosic feedstocks thus improving enzyme accessibility to cellulose during hydrolysis. In general, pretreatment temperature ranges from 140 to 180 $^\circ$C for normal feedstock treatments. Higher temperature with shorter residence time results in enhanced hemicellulose removal and thus a better enzymatic cellulose digestibility as shown in studies on corn stover (Melvin et al., 2003).

3. Microwave

The principle of microwave pretreatment relies on the effect of temperature. Since the temperature required is usually above 160-180$^\circ$C, it is sufficient to soften the main components of the cell wall and decrease the crystallinity of cellulose. Former studies using rice straw have verified the effects that microwave could change the crystalline structure of cellulose, degrade lignin and hemicellulose, thus increasing the enzymatic accessibility (Kitchaiya et al., 2003; Zhu et al., 2005). But elevated temperatures may also cause some useful components in the feedstock to decompose. Therefore, microwave pretreatment has been studied in conjunction with chemical reagents for pretreatment at lower temperature while maintaining effective results (Zhu et al., 2005). Keshwani et al. (2007) investigated the effect of
microwave pretreatment on switchgrass and indicated that higher enzymatic hydrolysis efficiencies could be obtained when pretreatment was performed at lower power levels. The greatest yield of reducing sugars was observed during microwave exposure of switchgrass immersed in 3% (w/v) sodium hydroxide solution for 10 minutes at 250 watts. However, this approach is currently limited to lab studies due to cost and safety concerns.

4. Radiation

Radiation is another physical method with the objective of achieving the expected degree of both polymerization and reactivity of cellulose using α-ray or electronic ray. Pretreatments of wheat straw using an electron beam accelerator in the presence of peracetic acid solutions were carried out by Lu and Kumakura (1995) for investigation of the effect of radiation. It was proposed that as the irradiation dose increased to 500 kGy (KiloGray) or above, pretreatment with a combination of peracetic acid and irradiation can significantly enhance enzymatic hydrolysis. Yang et al. (2008) studied the effect of γ radiation pretreatment on enzymatic hydrolysis of wheat straw and indicated that glucose yield increased with elevated irradiation doses and achieved the maximum (13.4%) at 500 kGy. However, radiation, like microwave pretreatment, is currently limited to lab scale because of its considerable cost and security concerns.

2.5.3 Chemical pretreatment

Chemical pretreatment, mainly employing chemical agents such as acids and alkalis, can enhance hydrolysis and improve glucose recovery from cellulose because of the removal of hemicellulose or lignin (Mosier et al., 2005). However, anti-corrosion equipment and chemical recycling are required if such processes are scaled up.
1. Acid pretreatment

Acid pretreatment has received considerable attention over years (Mosier et al., 2005). Dilute acid pretreatment has been well developed recently to successively avoid problematic issues with toxicity, acid recovery and anti-corrosion equipment maintenance associated with concentrated acid pretreatment (Sivers and Zacchi, 1995; Sun and Cheng, 2002). Sulfuric acid is the most commonly used agent in dilute acid pretreatment because of its comparatively lesser cost and greater efficiency (up to 90%) for both hemicellulose hydrolysis and glucose yield (Hsu, 1996; Nguyen et al., 2000), though other acids such as phosphoric acid (Israilides et al., 1978) and carbonic acid (van Walsum and Shi, 2004) have also been tested in several studies.

The main function of dilute acid pretreatment is to effectively remove the hemicellulose sheathing over cellulose, while at the same time loosening the structure of lignin and decreasing the crystallinity of digestible cellulose. The dissolved hemicellulose in the liquid phase is removed from the solid biomass residues and may be separately hydrolyzed to xylose and other 5C or 6C sugars, or eventually broken down to furfural (Mosier et al., 2005). Furfural can be recovered from distillation and is widely applicable in the field of industry mainly as a solvent in petrochemical refining (Paturau, 1987). It is reported that glucose yield subsequent to complete removal of hemicellulose can reach as high as 100% (Knappert et al., 1981). During acid pretreatment, two reactions occur simultaneously relative to lignin: degradation and accumulation (Pearl, 1967); therefore, the changes in lignin content greatly depend on which reaction is stronger. Although lignin removal is not significant in acid pretreatment, it has been stated that the structure of lignin is interrupted thus making the carbohydrates more accessible to enzymes (Yang and Wyman, 2004).

Acid pretreatment has been studied on numerous biomass feedstocks including hardwoods, grasses and agricultural residues. Most of the feedstocks
displayed satisfying performance with apparent solubilization of hemicellulose being the main effect (Wyman et al., 2005). Liao et al. (2007) reported that acid concentration was the most important factor to alter sugar components (cellulose and hemicellulose) in dairy manure. Two other individual factors, residence time and temperature, also had significant influences on the compositional changes of the lignocellulosic material. Torget et al. (1990) reported that about 92% of the xylan was solubilized and 75% enzymatic digestibility was observed when herbaceous crops such as switchgrass and weeping lovegrass were pretreated with 0.5% (v/v) sulfuric acid for 60 min at 140 °C or 10 min at 160 °C. Upon further increasing the temperature to 180 °C, Esteghlalian et al. (1996) found that about 90% of the xylan could be solubilized in the first minute of pretreatment using 0.9% (w/w) sulfuric acid for lignocellulosic biomass such as corn stover, poplar and switchgrass. Additionally, Lloyd and Wyman (2005) investigated dilute sulfuric acid pretreatment of corn stover and found that the acid pretreatment conditions that maximized xylan removal and those that maximized total sugar (glucan and xylan) yield were different, showing the importance of clearly stating the pretreatment goals when optimizing acid pretreatment conditions. Though the effect of removing glucan from the biomass matrix is not as significant as xylan solubilization, it is also of importance to examine the change in glucan content during acid pretreatment since glucan is usually the main substrate for enzymatic hydrolysis. Dien et al. (2006) studied the glucose yield from dilute sulfuric acid (0-2.5% w/v, 121 and 150 °C, 1 h) pretreatment and found that glucose yields of different switchgrass samples were similar at about 50 g/kg dry matter with the exception of an approximate two-fold greater glucose yield from the switchgrass cut at the anthesis stage. Also, at the anthesis stage, switchgrass produced a lesser glucose yield at 150 °C than 121 °C, which showed an opposite trend compared to switchgrass samples harvested at other stages such as pre-boot or post-frost. Therefore, even for one lignocellulosic
feedstock, besides reaction conditions, other factors such as the plant maturity level may affect the pretreatment result. Additionally, innovative equipment is now available for very dilute sulfuric acid pretreatment with the application of a flow-through reactor configuration. The concentration of acid needed in this type of treatment is as less as 0.07% compared to the conventional concentration range of 0.7-3.0% (Mosier et al., 2005).

One alternative reagent for acid pretreatment is carbonic acid which can bring benefits to the acid effect without the drawback of sulfuric acid. The pH of carbonic acid is determined by the partial pressure of carbon dioxide in contact with water, and it can be adjusted by altering the environmental pressure thus providing an easy approach for neutralization. This kind of pH control successfully avoids the high corrosiveness of sulfuric acid and greatly minimizes the problem of neutralization (van Walsum and Shi, 2004). Compared with sulfuric acid, carbonic acid offers relatively weaker hydrolytic capability and exhibits catalytic effect on xylan hydrolysis at a temperature of approximately 200 °C (van Walsum, 2001).

2. Alkali pretreatment

Alkali pretreatment is conducted under milder conditions at lower temperature and pressure compared with acid pretreatment. However, alkali pretreatment is much more time consuming and the reaction time greatly depends on the operation temperature selected (Mosier et al., 2005; Wyman et al., 2005). The major effect of alkali pretreatment is the saponification of intermolecular ester bonds which crosslink lignin and carbohydrates, thus increasing porosity and internal surface area of the biomass matrix as well as decreasing the degree of crystallinity of cellulose (Sun and Cheng, 2002). Lignin can also be disrupted and removed from the biomass matrix, resulting in improved susceptibility of the remaining polysaccharides to enzyme attack during hydrolysis. One limitation related to alkali pretreatment is the formation of unrecoverable salts within the biomass feedstock.
Sodium hydroxide is commonly chosen for alkali pretreatment and works well on delignification. Sharma et al. (2002) investigated the alkali pretreatment on sunflower stalks and reported that sodium hydroxide at 0.5% (w/v) along with autoclaving for 1.5 h at 1.05 kg/cm² was the most effective processing condition as evaluated by the following-up enzymatic hydrolysis. Sodium hydroxide pretreatment is suitable for less-lignified cellulosic materials, but it has little effect on softwood with a lignin content greater than 26% (Millet et al., 1976; Bjerre et al., 1996). Additionally, research has been conducted on modifying the conventional heating with radio frequency (RF)-based dielectric heating in alkali (NaOH) pretreatment of switchgrass (Hu et al., 2008). It was found that at 20% solid loading, RF-assisted alkali pretreatment (at 0.1 g NaOH/g switchgrass loading and 90 °C) resulted in greater xylose yields and also led to elevated glucose yields in subsequent hydrolysis than the conventional heating pretreatment. The effect of delignification was enhanced by increasing alkali loading from 0 to 0.2 g NaOH/g biomass, and it leveled off between 0.2 and 0.3 g NaOH/g biomass. Additionally, the impact of biomass particle size (from less than 0.25 mm to 2.0 mm) on delignification was also investigated and no significant effect was detected as percent lignin removal fell between 55 and 70 for all switchgrass samples with different particle sizes.

Besides sodium hydroxide, calcium hydroxide (lime) is an effective pretreatment agent which is the least expensive chemical among all hydroxides. Furthermore, calcium can be recovered from the reaction system by introducing carbon dioxide for calcium hydroxide regeneration (Karr and Holtzapple, 2000). Generally, lime pretreatment is able to remove at least 30% of lignin contained in the feedstock (Wyman et al., 2005). Lime pretreatment can be conducted at a wide range of temperatures, but preferred under 100°C since pressure vessels are not required for reactions at lower temperatures. For low-lignin herbaceous feedstocks such as
switchgrass, pretreatment at this temperature level is sufficient to enhance the feedstock’s accessibility to enzymes (Kaar and Holtzapple, 2000). Chang et al. (1997) recommended a loading of 0.1 g Ca(OH)$_2$/g dry biomass at 100 or 120 °C for lime pretreatment of switchgrass. Under the recommended pretreatment conditions, the reducing sugar yield can reach five times that of the untreated switchgrass after a 3-day treatment. Lime pretreatment has also been performed on other lignocellulosic feedstocks such as wheat straw (Chang et al., 1998), poplar wood (Chang et al., 2001b) and corn stover (Kaar and Holtzapple, 2000). But for highly lignified materials such as poplar wood, lime is usually supplemented with either oxygen or air during pretreatment for better performance in delignification. It is proposed that the efficiency of lignin removal in this modified lime pretreatment can reach as much as 80% (Chang and Holtzapple, 2000).

3. Cellulose solvent

Cellulose solvent is another chemical additive used for pretreatment (Mosier et al., 2005). This kind of solvent, such as alkaline H$_2$O$_2$, ozone, and glycerol, can disrupt the structure of cellulose within biomass feedstocks thus improving the enzyme digestibility during hydrolysis (Ladisch et al., 1978; Wood and Saddler, 1988). After pretreatment by cellulose solvent, cellulose is released into the liquid phase with a decreased crystallinity and looser interaction with hemicellulose and lignin, which is advantageous to the subsequent hydrolysis. However, these chemical additives are too expensive to be used at a large scale. Solvent recycle is normally required to make this approach economically feasible (Mosier et al., 2005).

4. Supercritical fluids (hydrothermolysis)

Water is normally used as the reactant for supercritical fluids pretreatment. It is arguably the most environmentally friendly and food safe solvent that enjoys a wide variety of applications in biochemical processes (Ragauskas et al., 2006).
Water is maintained in liquid state under certain pressure at elevated temperatures, and it can penetrate the cell wall of biomass feedstock, hydrate cellulose and remove hemicellulose by disrupting the linkages between these structural components (Wyman et al., 2005).

Water pretreatment avoids the requirement for neutralization or environment conditioning and it allows repeated use since there are no additives contained in the reaction system. Any size reduction of the incoming biomass is unnecessary since the lignocellulosic particles can break down themselves when mixed with supercritical water (Kohlman et al., 1995; Weil et al., 1997). All these advantages make water pretreatment a competitive method for lignocellulose-to-ethanol conversion. Mok and Antal (1992) reported that by mixing biomass materials including switchgrass with the hot compressed liquid water for up to 15 min at temperatures between 200 and 230 °C, about half of the total biomass can be dissolved, within which 4-22% of the cellulose, 35-60% of the lignin and approximately 100% hemicellulose can be dissolved and removed during the process (Mok and Antal, 1992). This study also suggests that in supercritical fluid pretreatment, the physical and chemical characteristics of biomass, rather than the reaction conditions such as reaction temperature and time, have significant influences on the experimental results.

5. Ammonia pretreatment

Pretreatment with ammonia has been shown to be effective in improving the accessibility of enzymes with ammonia being more easily recyclable compared to other alkalis (Wyman et al., 2005). Ammonia fiber explosion (AFEX) pretreatment can disrupt the crystalline structure of cellulose and deacetylate acetyl linkages, thus greatly increasing the efficiency of enzymatic hydrolysis (Mitchell et al., 1990; Gollapalli et al., 2002). Although the removal of hemicellulose or lignin is not as significant for AFEX pretreatment as for acid or alkali pretreatment, respectively, the
structure of lignin is modified or altered during the process and the hemicellulose is also depolymerized by interacting with ammonia (Martinez et al., 1991; Wyman et al., 2005). Therefore, pretreated cellulose can be more easily and quickly hydrolyzed to glucose even when the enzyme loading is not high (Dale et al., 1996; Moniruzzaman et al., 1997; Foster et al., 2001). AFEX is reported to be very effective on herbaceous and agricultural residues, while less efficient when applied on softwoods (Kim et al., 2000; McMillan, 1994). Alizadeh et al. (2005) reported that the cellulose hydrolysis efficiency of AFEX-treated switchgrass reached as much as 93% compared to that of untreated samples which only showed a cellulose conversion of 16%. Kurakake et al. (2001) studied the sugar yields in enzymatic hydrolysis after ammonia water pretreatment of two varieties of switchgrass (Miscanthus sinensis and Solidago altissima L.) with a loading of 2 ml ammonia water/g dry matter at 120 °C for 20 minutes. Compared with the unpretreated samples which had a total sugar yield of less than 100 mg/ g raw biomass for both varieties, ammonia-treated switchgrass could yield 331.2 and 493.6 mg reducing sugars out of 1 g of raw Solidago altissima L. and Miscanthus sinensis, respectively.

In addition, a method known as ammonia recycled percolation (ARP) is commonly applied by passing aqueous ammonia (5-15% w/w) through biomass feedstock at elevated temperatures (160-180°C) and then separating the ammonia for recycle (Kim and Lee, 1996; Kim et al., 2003). Under such conditions, aqueous ammonia swells the biomass, degrades lignin and interrupts the interactions between lignin and carbohydrates (Mosier et al., 2005). Besides, residual ammonia in the pretreated products has no inhibitory effect on downstream processes and it is compatible with microorganisms without extra conditioning. Furthermore, it is reported that ammonia can even have some advantageous influence on fermentation (Dale et al., 1985). The cost of AFEX pretreatment is affected by the cost of ammonia as well as the extent of chemical recycle (Holtzapple et al., 1992).
2.5.4 Biological pretreatment

Biological pretreatment utilizes microbial metabolism or genetic modification to remove lignin or hemicellulose from the plant cell wall. Compared with physical or chemical processes, biological pretreatment involves more complicated reactions and is more time-consuming.

1. Microbial digestion

In biological pretreatment, microorganisms, mostly fungi, are used to digest lignin and hemicellulose in waste materials (Schurz, 1978). White-rot fungi such as *Pleurotus ostreatus* and *Pycnoporus cinnabarinus* are preferred for biological pretreatment because of its high efficiency in degrading or modifying the lignin content in lignocellulosic biomass (Hatakka, 1983). Besides, some modifications have been made to fungal cultivation to improve the digestion of lignin and avoid degradation of cellulose. For example, construction of fungal mutants with weaker cellulase-producing ability has been tested to be applicable (Ander and Eriksson, 1977); restricting the availability of certain nutrients which are necessary for normal metabolism of fungi is also an effective approach which leads to the production of expected lignolytic enzymes useful in lignin digestion (Boominathan and Reddy, 1992).

2. Ensiling

Silage is a traditional technology used to preserve large quantities of cellulosic materials harvested for storage in a year-round system. Through the ensiling process, the rate of carbohydrate degradation is strictly controlled by creating a disadvantageous anaerobic environment in which microbes favor acetic and lactic acid fermentation. Therefore, pH is greatly reduced within the system (Ren, 2006).

Ensiling is not only a storage method for crops or ruminant feedings, it also
contributes to the saccharification of plant cell wall and mixed-acid fermentation. During these processes, the structure of cellulosic biomass is broken down and the degradability of the biomass matrix is greatly improved (Richard et al., 2001). Chen et al. (2007) investigated the potential of using ensiling as a cost-effective pretreatment for bioethanol production from agricultural residues such as cotton stalk and wheat straw. Ensiling was found to have limited side effect on holocellulose (cellulose and hemicellulose) losses (less than 10%) and resulted in significant increases in holocellulose-to-reducing sugars conversion during subsequent hydrolysis with cellulolytic and xylanolytic enzymes.

3. Molecular modification

In addition to the traditional biological pretreatment methods, recently there is increasing interest in improving pretreatment at a microscopic level. Molecular modifications have been applied to alter the intrinsic characteristic of cellulosic feedstocks thus making the biomass matrix more digestible (Ragauskas et al., 2006). Because the efficiency of biomass conversion depends on enzymes obtaining access to the carbohydrate substrate, appropriate modification of the plant cell wall structure can bring advantageous effects for enzyme digestion (Ragauskas et al., 2006). One intriguing research area is the modification of cinnamoyl-CoA reductase (CCR) gene which is responsible for lignin biosynthesis. Upon appropriate expression of the modified CCR gene, the interaction between lignin and holocellulose is weakened and twice the amount of monomeric sugar yield can be obtained during hydrolysis compared with that of the natural feedstock (Boudet et al., 2003).

Another type of modification defined as molecular farming has also been tested for biofuel production. During this process, plants are capable of producing polysaccharide hydrolyase enzymes and depolymerizing cellulose ‘in situ’ (Rishi et al., 2001). The cellulase gene on which a signal sequence from the cell wall protein
is spliced is applied to ensure that the freshly generated cellulase can be localized to the plant cell wall. The coding region of cellulase sequence is attached to a chemically induced promoter, which is responsible for switching on the specific cellulase gene when required. After the host plant is genetically modified by this exogenous gene segment, the way of plant cultivation is kept unchanged. However, once the crop is sprayed with the chemical inducer before harvest, cellulase can be actively generated and transported to the plant cell wall where cellulose depolymerization takes place thereafter (Ragauskas et al., 2006). The degradation of polysaccharides ‘in situ’ can reduce the burden of downstream processing in advance thus facilitating the whole bioconversion procedure.

2.6 Hydrolysis

2.6.1 Chemical hydrolysis

Hydrolysis is used to facilitate the dissolution of chemicals by reaction with water, and it is especially effective on some organic compounds that are relatively resistant to solubilization and degradation. Normally hydrolysis is chemical based, catalyzed either by acid or alkali; however, in order to break down certain polysaccharides, hydrolase enzymes are frequently chosen as alternatives to degrade these polymer sugars. Enzymatic hydrolysis is an environment-friendly method which effectively avoids problems of chemical recovery, disposal and equipment maintenance when employed on a large scale. Though chemical hydrolysis is still effective at depolymerizing certain polysaccharides such as hemicellulose, enzymatic hydrolysis is a more competitive choice for holocellulose degradation during the biomass-to-ethanol conversion.

2.6.2 Enzymatic hydrolysis

Enzymatic hydrolysis is attractive since it is possible to yield fermentable
sugars from holocellulose at near theoretical levels under mild conditions, which is important to economical feasibility (Wyman et al., 2005). However, enzymes used in the process of cellulosic biomass conversion need to be more effective and economical in order to be cost-competitive with traditional ethanol product derived from corn and sugarcane (Gray et al., 2006).

1. Introduction of enzymes

Both bacteria and fungi are able to yield cellulases suitable for digestion of the plant cell wall polysaccharides, although some of these microorganisms vary significantly in characteristics. Cellulomonas fini and Thermomonospora fusca are the most extensively studied bacteria; while Trichoderma and Aspergillus are two fungal genera that are of great interest to researchers (Vries and Visser, 2001; Sun and Cheng, 2002).

Cellulase is a complex mainly containing three categories of enzymes: endoglucanases, which act on internal β-1,4-glucosidic bonds of polysaccharides; exoglucanases, which continually cleave the reducing and non-reducing ends of cellulose chains to generate short-chain cello-oligosaccharides; and β-glucosidases, which finally break cello-oligosaccharides such as cellobiose to release glucose units (Gray et al., 2006). Generally, the major reaction products from cellulose hydrolysis catalyzed by cellulase complex are cellobiose and glucose. Since cellobiose is not a monomeric sugar ready to be fermented, cellulase is usually supplemented with pure cellobiase (β-glucosidase) to maximize fermentable sugar availability. Recent studies show that cellobiase and cellulase can be added to the pretreated wet biomass at an activity ratio of CBU:FPU ranging from 1.75 (Yang and Wyman, 2005; Lu et al., 2002) to 4 (Ingesson et al., 2001; Bura et al., 2002) in order to avoid end-product inhibition brought on by the accumulation of cellobiose.

Hemicellulase, mainly xylanase, randomly acts on the β-1,4-backbone between xylose residues and on other side chains in the plant cell wall complex
(Collins et al., 2005). However, commercial development of hemicellulases for lignocellulosic bioconversion is not as advanced as that of cellulases because the majority of the hemicellulose in feedstocks has already been removed during acid hydrolysis which is the mostly selected pretreatment method (Gray et al., 2006).

New cellulases and hemicellulases are continually isolated from both bacteria and fungi sources (Shallom and Shoham, 2003; Hilden and Johansson, 2004), and researches on regulating the specific microorganism for cellulase production have also been extensively conducted (Aro et al., 2005). Several approaches have been tested to improve the efficiency of cellulase, thus reducing the enzyme loading for polysaccharides degradation. For example, some mutants of certain microorganisms have been constructed with new characteristics of better activity and thermal stability (Teter et al., 2004).

The mechanism of enzyme-substrate system is also a field of growing interest. Cellulase often contains carbohydrate-binding modules (CBMs) which play an important role in enhancing the enzyme’s interaction with the substrate surface. Once cellulase recognizes the specific substrate, an effective accumulation of cellulase occurs on the surface. Besides assisting in the targeting of cellulase to polysaccharide substrate, CBMs have been reported to have some extra effect on disrupting the structure of polysaccharides, which in turn facilitates the hydrolysis (Gray et al., 2006).

2. Enzymatic hydrolysis improvement

Enzymatic hydrolysis is affected by a variety of factors, including properties of substrates, enzyme activities and reaction conditions such as pH, temperature, time, etc. In addition, enzyme recycling is important in controlling the reaction rate and cost (Sun and Cheng, 2002).

The characteristics of the biomass substrate are of great importance to hydrolysis optimization. The susceptibility of cellulosic substrates to cellulases
mainly depends on the degree of crystallinity and polymerization of cellulose, availability of the surface area as well as lignin content (Sun and Cheng, 2002). Lignin can form non-productive binding with cellulase to make the enzyme inactive, so the prior removal of lignin is greatly preferred (Gray et al., 2006). Berlin et al. (2005) proposed a promising approach to minimize the effect of the non-productive interaction between enzymes and lignin by using the weak lignin-binding enzymes. Palonen (2004) reported that the location and structure of lignin, rather than the absolute amount of lignin, greatly affected the hydrolysis efficiency. Further studies are needed to better clarify the interaction mechanism of cellulase with all major compounds contained in the lignocellulosic feedstocks.

Enzyme loading is another factor which is crucial to hydrolysis efficiency. Because the amount of enzyme directly affects the operating cost, simply increasing enzyme loading is not a reasonable approach to facilitate the reaction and improve sugar yields. Gregg and Saddler (1996) suggested that cellulase dosage of 10 FPU/g cellulose is practical for lab-scale research since it is sufficient to provide great glucose yield within an acceptable duration at a reasonable cost. Lloyd and Wyman (2005) experimented on enzyme loading to investigate its effect on hydrolysis of the pretreated corn stover. They chose four enzyme loadings of 3, 7, 15 and 60 FPU respectively of Spezyme CP/g original glucan in the pretreated solids with Novozym 188 added at a ratio of 2:1 CBU to FPU. The experiment result obtained after digestion showed that reduction in sugar yields was not directly correlated to the reduction in enzyme loading. Less than 1% decrease in total reducing sugar (glucose and xylose) yield was observed when the enzyme loading was decreased from 60 to 15 FPU/g original glucan and about 3% reduction was with a decrease from 60 to 7 FPU/g original glucan. In addition, Chen et al. (2007) reported that no significant enhancement in glucan conversion was observed when increasing the enzyme loading from 40 FPU/g glucan to 60 FPU/g glucan. Therefore, if two different
enzyme loadings do not make a statistical difference in final glucose yields, the lesser enzyme dosage is preferred. Relative to xylan conversion, since cellulase and cellobiase also contain certain xylanolytic activity, the addition of xylanase does not always lead to an improvement in xylan-to-xylose conversion during enzymatic hydrolysis (Saha et al., 2005a; Chen et al., 2007).

2.7 Fermentation

2.7.1 Principles of ethanol fermentation

Ethanol fermentation begins with the completion of glycolysis, which is also termed the EMP (Embden-Meyerhoff-Parnas) pathway (Dien et al., 2003). The EMP is the most common reaction for oxidizing glucose to pyruvate which is an important intermediate metabolite for most living organisms. The EMP pathway is composed of three stages, namely activation of glucose, hexose splitting and energy extraction; the overall reaction formula for the EMP is summarized in Equation 2.1 as (Fermentation, online Microtextbook from University of Wisconsin-Madison):

\[
2 \text{ ATP} + \text{glucose} + 4 \text{ ADP} + 2\text{Pi} + 2 \text{ NAD}^+ \rightarrow 2 \text{ ADP} + 2 \text{ pyruvate} + 4 \text{ ATP} + 2 \text{ NADH} + 2 \text{ H}^+ \tag{2.1}
\]

Ethanol is formed as an end product of the EMP pathway. First, acetaldehyde is produced from pyruvate by reducing a molecule of CO₂ out of pyruvate, and then acetaldehyde is reduced to ethanol along the redox reaction between NADH and NAD⁺. Besides ethanol, lactic acid is another end product of microbial fermentation.

Since certain types of inhibitors are formed during acid pretreatment such as acetic acid, furfural, and lignin-derived compounds (Sreenath and Jeffries, 2000), commercial organisms capable of fermenting reducing sugars must be able to
function normally in the large-scale environment and tolerate various toxic compounds contained in the hydrolyzate (Gray et al., 2006).

By studying the natural yeast strains that can convert hexoses and pentoses to ethanol, researchers are trying to optimize the conditions for cell growth and fermentation, including hydrolyzate composition, pH and acetate concentration. Sreenath and Jeffries (2000) conducted a study that focused on the fermentation ability of *Candida shehatae* and *Pichia stipitis* strains on wood hydrolyzate with a 1:1 mixture of glucose and xylose. It was reported that the optimum ethanol production rate was obtained with a pH range between 5.5 and 6.0, and an ethanol yield of 0.41-0.46 g/g glucose and xylose can be achieved within these optimized batches. Additionally, the loading of microbial cells and cell recycle are also of great importance in enhancing ethanol production.

### 2.7.2 Simultaneous saccharification and fermentation (SSF)

Although the addition of cellobiase during hydrolysis reduces cellobiose accumulation which may inhibit cellulase activity as hydrolysis proceeds (Sun and Cheng, 2002), the increased glucose content in hydrolyzate has negative effect on both cellobiase and cellulase activities (Xiao et al., 2004). In order to avoid various end-product inhibitions, the approach of simultaneous saccharification and fermentation (SSF) has been developed and applied for years.

Cellulosic hydrolysis carried out in the presence of the fermentative microorganism is termed as simultaneous saccharification and fermentation (SSF). SSF combines cellulose hydrolysis and hexose fermentation in the same vessel compared to traditional SHF (separate hydrolysis and fermentation). The main objective of SSF is to solve the problem triggered by product inhibition in enzyme-catalyzed hydrolysis. Reducing sugars produced during enzymatic hydrolysis can be consumed immediately by yeast in fermentation, which greatly reduces the disadvantageous effect of the intermediate products.
However, when combining hydrolysis and fermentation together, reaction conditions need to be reset to compromise between different requirements of the two individual operations. The optimal temperature of SSF is proposed to be around 38°C which is between the normal temperature of 45-50°C for hydrolysis and 30°C for fermentation (Philippidis, 1996). Since hydrolysis is considered the rate-limiting step in SSF, microorganisms capable of tolerating high temperatures are preferred in SSF in order to keep a suitable environment for hydrolysis (Philippidis and Smith, 1995). Alizadeh et al. (2005) reported that when the optimally AFEX-treated switchgrass samples were subjected to SSF, glucose produced by cellulosic hydrolysis was almost completely consumed by yeast and converted to ethanol although the amount of xylose leveled off after the first 24 h. In addition, the final ethanol yield of the optimally treated switchgrass could reach 0.2 g ethanol/g dry biomass. Chang et al. (2001a) investigated SSF on lime-treated switchgrass and concluded that the ethanol yield was 72% of theoretical; they also mentioned that if less ethanol yield was observed, less cellulose digestibility rather than inhibitors produced during pretreatment would be most likely the cause. However, SSF is not always superior over SHF. Saha et al. (2005b) reported that SSF performed unsatisfactorily in comparison with SHF when applied to rice hull hydrolyzates, with final ethanol yield of 0.05 g/g hull for SSF compared to 0.13 g/g hull for SHF.

Besides the most significant benefit of decreasing the chances of product inhibition, SSF is preferable because it also helps to reduce enzyme dosage and facilitate the overall process with a greater yield at lesser energy consumption (Alzate et al., 2006). However, further improvements are required to better optimize the temperature differences between stages and to modify the fermenting microorganisms with greater ethanol tolerance (Sun and Cheng, 2002).

2.7.3 Yeast cell immobilization during fermentation

Compared with free cells, immobilized cells display specific advantages
such as convenient product separation, easy cell recycle, high productivity in fixed reaction volume, and low risk of cell contamination (Göksungur and Zorlu, 2001). Four different immobilization methods can be applied, namely carrier-binding, cross-linking, entrapping and a combination of these three individual techniques (Vullo and Wachsman, 2005). Among various categories of immobilizations, cell entrapment in calcium alginate gel is a well established technique which has been extensively studied because of its simplicity and non-toxicity. Cell entrapment is completed by the drop-wise addition of cell suspended in sodium alginate into the calcium chloride solution, where beads are immediately formed in the calcium alginate gel (Göksungur and Zorlu, 2001). Taherzadeh et al. (2001) reported that when pure glucose was used as both carbon and energy sources for Ca-alginate immobilized *Saccharomyces cerevisiae*, ethanol was the major metabolite with yields of 0.39 g/g glucose and mannose and glycerol was another metabolite with the productivity of 0.093 g/g glucose and mannose. Besides traditional batch fermentation, continuous cultivation of glucose-contained hydrolyzates has also been studied using a dilution rate of between 0.2-0.6 h\(^{-1}\). This rate is kept constant over the fermentation process to ensure that original fermentation capacity is maintained and no “wash-out” occurs during fermentation (Taherzadeh et al., 2001; Göksungur and Zorlu, 2001). According to the research conducted by Abbi et al. (1996) on batch fermentation of xylose and rice straw hydrolyzate, immobilized cells exhibited better capability of converting sugar substrate to ethanol, which was probably due to the protection of yeast cells from inhibitors by the gel support.

### 2.7.4 Fermentation of pentose

Currently, fermentation of a mixture of hexoses and pentoses is inefficient. This is one of the major restrictions for the commercial scale-up of bioethanol production from cellulosic feedstocks, since the hydrolyzate of these feedstocks comprises of both hexoses and pentoses in the same broth rather than merely hexoses
as in corn and sugarcane. Natively, the yeast *Saccharomyces cerevisiae* is only able to metabolize hexoses, such as glucose, galactose and mannose as substrates for fermentation. Pentoses, however, are not consumed by *Saccharomyces cerevisiae* to yield ethanol. Therefore, the presence of pentose complicates the fermentation process (Gray et al., 2006).

The metabolism of D-xylose by some natural occurring bacteria and other yeasts (such as *Candida shehatae*, *Pichia stipitis* and *Pachysolen tannophilus*) has been extensively studied (Preez et al., 1984; Delgenes et al., 1996). The main procedure of pentose conversion is indicated in Equation 2.2:

\[
\text{Xylose} \xrightarrow{\text{xylose reductase}} \text{xylitol} \xrightarrow{\text{xyitol dehydrogenase}} \text{xylulose} \\
\xrightarrow{\text{xylulokinase}} \text{xulose-5-phosphate} \xrightarrow{\text{PPP}} \text{fructose-6-phosphate}
\]

Bacteria can directly convert xylose to xylulose with xylose isomerase (Jeffries, 1983). However, yeasts that are capable of consuming pentose, first reduce xylose to xylitol with NADPH-dependent xylose reductase, and then convert xylitol to xylulose with NAD\(^+\)-dependent xylitol dehydrogenase. Thereafter, xylulose is converted to xylulose-5-phosphate by xylulokinase. Through the pentose phosphate pathway (PPP), xylulose-5-phosphate, the central metabolite, can be converted to fructose-6-phosphate. Then via glycolysis, pyruvate is produced as an intermediate in ethanol production (Freer et al., 1997). However, wild-type strains of *Saccharomyces cerevisiae* are unable to ferment D-xylose directly and can only act on xylulose to produce ethanol when exogenous xylose isomerase is introduced to the D-xylose containing system (Preez, 1994).

Various approaches have been extensively tested to genetically modify the gene of *Saccharomyces cerevisiae* to endow it with the ability to ferment both 5C and 6C sugars. For example, bacteria xylose isomerase has been tried to be cloned into *Saccharomyces cerevisiae*, but the consequent gene expression was not
satisfactory (Sarthy et al., 1987). Transformed *Saccharomyces cerevisiae* with the yeast genes of xylose reductase and xylitol dehydrogenase tend to work on D-xylose in a fairly inefficient way. Moreover, certain amount of xylitol is also produced along with the yield of ethanol. This problem is caused by the existence of redox cofactor imbalance — NADPH and NAD$^+$, which are linked with xylose reductase and xylitol dehydrogenase, respectively (Freer et al., 1997). In addition, respiration also impacts the performance of both native xylose-fermenting yeasts and recombinant *Saccharomyces cerevisiae*. It is reported that by limiting the respiration rate, ethanol production can be greatly enhanced (Jeffries and Jin, 2004).

Besides, the implementation of the process combining simultaneous saccharification with co-fermentation of both hexoses and pentoses is termed SSCF. Since the production of ethanol from mixed sugar hydrolyzates has not reached a commercially acceptable yield as of now (Gray et al., 2006), more research is needed for further improvement. Ho and Tsao (1995) constructed a *Saccharomyces cerevisiae* strain that contained several categories of enzymes needed; the recombinant strain was reported to have the ability to effectively ferment both glucose and xylose, which helped to make cellulosic biomass-to-ethanol technology closer to commercialization.

### 2.7.5 Ethanol collection

After fermentation, the culture broth needs to be separated for ethanol recovery. Conventionally, concentration and rectification columns are used to distill the incoming aqueous solutions, and further dehydration is performed to generate the anhydrous ethanol product. However, these processes are energy intensive, thus not cost-effective. Alternatively, anhydrous ethanol can be separated through modified azeotropic distillation with benzene as entrainer or using the pervaporation system with multiple membrane modules. The energy costs associated with these two advanced processes are reported to be approximately 1/5 of those for traditional
azeotropic distillation and the quality of final ethanol product (> 99.5% w/w) can still be maintained (Alzate et al., 2006).

2.8 From Lab Research to Industrial Scale-up

With regard to energy balance calculated in integrated flowsheets of lignocellulosic biomass-to-ethanol conversion, Alzate et al. (2006) proposed that simultaneous saccharification and co-fermentation (SSCF) process using water recycling gave the best results, with an energy consumption of 41.96 MJ/L ethanol (anhydrous). In addition, energy efficiency of lignocellulose-derived ethanol was also obtained showing a net energy value (NEV) of 17.65-18.93 MJ/L ethanol. Compared to the corresponding NEV of corn and sugarcane being 5.57-6.99 MJ/L (Wang et al., 1999) and 11.39 MJ/L (Prakash et al., 1998) respectively, it is favorable to design a biomass-to-ethanol conversion system on an industrial scale.

However, when lab work is scaled up into an actual plant, new problems may occur due to the introduction of new parameters in reaction conditions and new concerns over project design. Besides, the most important issue is to establish a trade-off between operating cost and product quality; therefore, completing a comprehensive economic analysis of the lignocellulosic biomass-to-ethanol process in advance is highly recommended. The optimal conditions proposed in these studies can not be directly adopted in real industrial processes; further adjustment is needed to make the whole procedure more economically competitive.

2.9 Summary

Biofuels such as ethanol are competitive renewable energy sources. Lignocellulosic biomass such as switchgrass has great potential for ethanol production and serves as an alternative to corn grain and sugarcane. Due to the
complicated interaction between components in the plant cell wall, three constitutive steps are needed for lignocellulosic biomass-to-ethanol conversion, namely pretreatment, hydrolysis and fermentation. Numerous pretreatment methods have been tested to evaluate the results such as hemicellulose or lignin removal, among which dilute sulfuric acid pretreatment is considered an effective approach. Optimizations of conditions have been extensively studied at a lab scale for each of the steps during lignocellulosic biomass-to-ethanol conversion, but further modifications are needed before these experimental operations proceed to industrial application.
CHAPTER 3 Dilute Acid Pretreatment of Oven-dried New Switchgrass Germplasms for Bioethanol Production

3.1 Introduction

The continuous increase in world energy consumption and concerns over diminishing fossil fuels have resulted in an urgent need for alternative energy sources that sustainably power the world. Biomass is a competitive candidate capable of producing significant amounts of renewable energy while maintaining a cost efficiency through process optimization. The polysaccharides contained in biomass, either starch or cellulose, can be broken down to monosaccharide and fermented to produce bioethanol (McAlloon et al., 2000).

Currently in the United States, commercial production of bioethanol primarily relies on corn grain grown predominantly in the Midwest. According to the report by US Department of Agriculture (Interagency Agricultural Projections Committee, 2008), rapid growth in ethanol production has been witnessed over the past several years with production increasing from less than 11 billion liters in 2003 to more than 22 billion liters in 2007. This development trend is projected to continue and exceed 45 billion liters by 2010. Although the contribution of bioethanol to the overall gasoline market is small, such enormous growth is believed to significantly impact the current structure of corn market and promote bioethanol production from alternative biomass feedstocks (Westcott, 2007; Interagency Agricultural Projections Committee, 2008).

Lignocellulose, a major component of the plant cell wall, is an abundant biomass resource which shows great potential as a substrate for bioethanol production. It is comprised of three main components, namely cellulose, hemicellulose and lignin. A variety of lignocellulosic feedstocks such as forestry or agriculture residues, organic components of municipal and industrial wastes, and specific energy crops
have been investigated for bioethanol production. Among these, switchgrass is an
ergy crop which has received considerable attention in recent years (NREL, 2006;
Gray et al., 2006; van den Oever et al., 2003). However, the conversion of
lignocellulose to ethanol is more challenging than the corn-to-ethanol process because
of the complex structure of plant cell walls. Therefore, besides general
saccharification and fermentation, an initial pretreatment step is needed to make the
cellulose matrix more susceptible to cellulolytic enzymes. This is achieved by
breaking the lignin seal and hemicellulose sheathing over cellulose, and disrupting the
crystalline structure of cellulose (Mosier et al., 2005). In recent years, numerous
pretreatment methods involving physical, chemical and biological mechanisms have
been extensively studied. Dilute acid pretreatment has been widely explored because
of its effectiveness in hemicellulose solubilization and ease of availability (Lee et al.,
1999; Sun and Cheng, 2005). In dilute acid pretreatment, the hemicellulose
component of the lignocellulosic matrix can be effectively solubilized into monomeric
sugars, thus opening up the overall lignin-polysaccharides network to facilitate
subsequent cellulose conversion.

Studies have shown the potential of switchgrass for bioethanol production by
dilute acid pretreatment and enzymatic saccharification. Dien et al. (2006)
investigated the optimal sulfuric acid loading which yielded greatest glucose and
hemicellulose sugars (xylose, arabinose and mannose) by treating switchgrass at 10%
solid loading and autoclaving at 121 °C for 60 min. They reported that maximum
sugar yields appeared to level off beginning at an acid concentration of 1.25% (w/v).
Esteghlalian et al. (1997) found that xylan, the major component of hemicellulose in
switchgrass, could be dissolved within the first minute of reaction when using 0.9%
(w/w) sulfuric acid at a temperature of 180 °C.

Although extensive research has been conducted on dilute sulfuric acid
pretreatment of common lignocellulosic feedstocks such as switchgrass, there is a
need to better understand its effect on switchgrass due to differences in germplasms as
well as reaction conditions such as acid concentration and treatment time. The subsequent enzymatic hydrolysis also needs to be investigated in depth to study the effect of enzyme loadings and combinations. In addition, the primary focus of many previous studies concerning dilute acid pretreatment has been the optimization of the monomeric sugar yields during hydrolysis, not necessarily the final ethanol yield from fermentation. However, fermentation is crucial in determining the inhibitory effects of acid pretreatment and is the key to evaluate the overall process of cellulosic ethanol production.

Hence, the objective of this research was to investigate the effect of dilute sulfuric acid pretreatment on solubilization of hemicellulose as well as its influence on lignin degradation in oven-dried samples of two new cultivars and an improved germplasm of switchgrass (generally recognized as three germplasms afterward). The impact of enzyme loading during hydrolysis with cellulase, cellobiase and xylanase was also studied. The hydrolyzates were fermented by yeast (Saccharomyces cerevisiae) to estimate the overall ethanol yield potential of switchgrass. Results of this bench-scale study can provide useful insight for optimizing the switchgrass-to-bioethanol process in future scale-up systems.

3.2 Materials and Methods

3.2.1 Switchgrass feedstock

Three switchgrass germplasms, well adapted to the Southeastern US, were obtained from the Central Crops Research Station, near Clayton, NC for this study. Each switchgrass field was a well established stand representing one of the three germplasms designated as St6-1, St6-3E and St6-3F. Since sampling, St6-3E has been released under the name of ‘BoMaster’ and St6-3F as ‘Performer’ (Burns et al., 2008a; Burns et al., 2008b). Germplasms St6-1 and Bomaster were selected for high dry matter yield; whereas Performer, preferable as cattle feed, was selected for improved
nutritive value and digestibility. Performer is reported to be 5 units more digestible than Bomaster as determined by ‘in vitro dry matter digestion (IVDMD)’. Digestibility and dry matter yield are normally not positively associated (Burns et al., 2008b); however, at the time of sampling for this study, all three germplasms provided comparable dry matter yield which was approximately 13450 kg/ha (12000 lbs/acre).

Switchgrass germplasms were harvested to about a 6-inch stubble on July 30, 2007. A harvest strip was taken randomly from each quarter of each field (four strips for one germplasm), weighed, and a sub-sample taken from each strip was used to determine the moisture content and therefore to calculate dry matter yield per unit land area. The four strips, from the same field for one germplasm, were combined to form one bulk sample. Three bulk samples, each representing one specific germplasm, were placed onto a cloth sheet, wrapped and transported to a field laboratory where they were transferred into cloth bags and dried in a forced-air oven at 70 °C until constant weight was obtained (at least 3 days). These grass samples were then reduced in size by grinding through a Wiley mill fitted with a 2 mm screen. Ground samples were collected in tightly sealed zip-locked plastic bags and delivered to the Biological and Agricultural Engineering Department at North Carolina State University, Raleigh, NC where they were stored at room temperature until analyzed.

3.2.2 Composition analysis

Composition analysis of all three switchgrass germplasms was conducted in triplicate. Six characteristics of the biomass feedstock, namely total solids, ash, extractives, acid insoluble lignin (AIL), acid soluble lignin (ASL), and structural carbohydrates which are mainly made up of glucan, xylan and arabinan, were measured. All measurements except extractives were performed according to the below described Laboratory Analytical Procedures (LAPs) released by the National Renewable Energy Laboratory (NREL). Extractives were measured based on the
method described by Han and Rowell (1996).

1. Total solids

Total solids were measured for both original and pretreated biomass according to the protocol ‘Determination of Total Solids in Biomass’ (Sluiter, 2005a). Approximately 1.0 g switchgrass was placed in the pre-weighed dry aluminum dishes and weighed prior to keeping in a 105 °C convection oven for 15 h. The samples were removed from the convection oven and allowed to cool to room temperature in a desiccator till constant weight was achieved and the total weights of both dish and oven-dried sample were recorded. The percentage of total solids on a 105 °C dry weight basis was calculated as:

\[
\text{Percent Total Solids} = \frac{W_3 - W_1}{W_2} \times 100
\]

Where:
- \(W_1\) = weight of aluminum weighing dishes (g);
- \(W_2\) = weight of the initial switchgrass sample (g);
- \(W_3\) = total weight of dish and sample after 105 °C drying (g).

2. Ash

Ash content for untreated switchgrass was measured based on the procedure provided by NREL (Sluiter, 2005b). Crucibles were placed in a furnace (Barnstead Thermolyne 1400 Furnace) at 575 °C for 24 h and then placed directly in a desiccator to be weighed after cooling to room temperature. Approximately 1.0 g of the switchgrass sample was placed into the pre-weighed crucible, weighed, and ashed in the same furnace at 575 °C for 24 hours. The crucible was transferred to the desiccator and allowed to cool before weighing. The ash content was calculated as:

\[
\text{Percent Ash} = \frac{W_2 - W_1}{W \times \% \text{Total Solid}} \times 100
\]

Where:
- \(W\) = weight of initial switchgrass sample (g);
- \(W_1\) = weight of crucible (g);
3. Extractives

Extractives, which can be removed through a series of extraction procedures, are cell wall chemicals composed of fats, fatty acids, fatty alcohols, and waxes, etc. In this study, extraction was performed for initial switchgrass samples based on the analytical procedures described by Han and Rowell (1996). Extraction thimbles were first placed in a vacuum oven at 40 °C for 24 h, cooled in a desiccator for 1 h and weighed. Approximately 2.0 g of the pre-weighed biomass sample was added to the extraction thimbles which were then placed in the pre-installed Soxhlet extraction units. Two hundred and twenty-five milliliters of 2:1 toluene: ethanol (v/v) mixture was prepared in a 500 ml round bottom flask along with several boiling chips to avoid bumping. Extraction was carried out in the fume hood for 24 h with siphoning from the extractor being no less than four times per hour. The equipment for extraction and the phenomenon of siphoning are shown below in Figures 3.1 and 3.2, respectively.
After extraction, thimbles were removed from the extraction system and placed in a vacuum oven at 40 °C for 24 h, cooled in a desiccator for 1 h and weighed. The sample, considered moisture and extractives free at this point, was then transferred into zip-lock plastic bags for future use. The extractives were calculated as:

\[
\text{Percent Extractives} = \frac{W_1 \times \% \text{Total Solid} - (W_2 - W)}{W_1 \times \% \text{Total Solid}} \times 100
\]  

(3.3)

Where \( W \) = weight of thimble (g);

\( W_1 \) = weight of initial switchgrass sample (g);

\( W_2 \) = total weight of thimble and sample after 24 h extraction and subsequent drying (g);

‘% total solid’ was measured using Equation 3.1.

4. Acid insoluble lignin

Acid insoluble lignin (AIL) and acid soluble lignin (ASL) in initial and pretreated biomass were measured according to the NREL procedure entitled ‘Determination of Structural Carbohydrates and Lignin in Biomass’ (Sluiter, 2006).
Approximately 0.3 g moisture-free (and extractive-free for the unpretreated biomass) switchgrass feedstock was weighed and placed in 100 ml serum bottles in triplicate. Three milliliters of 72% sulfuric acid was added to each serum bottle and the acid-soaked biomass was incubated for 1 h in a water bath at 30 °C and 50 rpm. The mixture was stirred at the beginning and occasionally during the incubation process to ensure complete contact between acid and biomass particles. Upon completion of incubation, serum bottles were removed from the water bath and acid was diluted by adding 84 ml deionized water to reduce the concentration to 4%. All serum bottles were sealed, crimped and autoclaved for 1 h at 121 °C/15 psi in standard liquid cycle. Samples, allowed to cool to room temperature after autoclaving, were then vacuum filtered through pre-weighed filtering crucibles. The filtrate was saved separately in two 15 ml polypropylene tubes at 4 °C and – 80 °C for subsequent acid soluble lignin and carbohydrate analyses, respectively. Solids remaining in crucibles were rinsed with 40 ml deionized water by vacuum filtration and dried in a convection oven at 105 °C for 15 h. The crucibles were transferred to the desiccator, weighed and placed in the furnace at 575 °C for 24 h. The total weight of crucibles together with the residual ash was measured. Acid insoluble lignin was calculated using Equation 3.4:

\[
\text{Percent Acid Insoluble Lignin} = \left( \frac{W_1 - W_2}{W} \right) \times 100
\]  

(3.4)

Where \( W = \) weight of the moisture and extractive free sample obtained after extraction if initial grass sample was analyzed (g), or weight of the moisture free sample (without being extracted) determined by multiplying 40 °C oven-dried sample weight (g) by ‘% total solids’ (as described later in Equation 3.9) if pretreated grass was analyzed (g);

\( W_1 = \) total weight of crucible and the insoluble residue after drying at 105 °C in the oven (g);

\( W_2 = \) total weight of crucible and ash after burning at 575 °C in the furnace.
5. Acid soluble lignin

The filtrate saved during acid insoluble lignin analysis was used immediately for acid soluble lignin measurement in a quartz cuvette with a spectrophotometer (PharmaSpec UV-1700, Shimadzu). The wavelength was set at 205 nm (Ehrman, 1996) and the filtrate was diluted 10-fold to bring the absorbance between 0.1 and 1.0. Four percent sulfuric acid was also diluted 10-fold and used as blank in spectrophotometric measurement. Acid soluble lignin was calculated as follows:

\[
\text{Percent Acid Soluble Lignin} = \frac{\text{UV}_{\text{abs}} \times \text{Filtrate Volume} \times \text{Dilution}}{W} \times \frac{1L}{1000ml} \times 100
\]

(3.5)

Where \( \text{UV}_{\text{abs}} = \frac{A}{b \times a} \),
\( A = \) absorbance at 205 nm,
\( b = \) cell path length (1 cm),
\( a = \) absorptivity which equals 110 L/(g·cm);

Filtrate volume = 87 ml;
Dilution = 10;

\( W = \) weight of the moisture and extractive free sample for initial grass or weight of the moisture free sample for the pretreated grass (g).

6. Carbohydrate analysis

The filtrate from acid insoluble lignin analysis, saved in the freezer at -80 °C, was analyzed for carbohydrate content. The analysis process was based on the procedure titled ‘Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples’ (Sluiter, 2005c).

A major portion of the biomass feedstock is made up of carbohydrates, which are polysaccharides primarily composed of glucose, xylose and arabinose monomeric subunits. During acid insoluble lignin measurement, a large portion of polysaccharides were hydrolyzed to monomeric subunits and released into the
solution in the presence of concentrated sulfuric acid. These soluble sugars contained in the liquid fraction were quantified by High Performance Liquid Chromatography (HPLC, Figure 3.3). HPLC analysis, performed on both initial and pretreated biomass, can provide important information for evaluating the impact of pretreatment on carbohydrate solubilization. The hydrolyzates and fermented samples were also chromatographically analyzed to determine the availability of both reducing sugars and ethanol.

Figure 3.3 The HPLC equipment for carbohydrate analysis

Aliquots of the acid insoluble lignin analysis filtrates from all initial and pretreated biomass were neutralized with calcium carbonate (approximately 0.5–0.7 g) to a pH between 5 and 6. After precipitates settled out, the clear liquid was decanted and passed through a 0.2 μm syringe filter into an autosampler vial for HPLC analysis. Samples from hydrolysis and fermentation were prepared in vials directly without neutralization.

For carbohydrate analysis, high purity calibration standard solutions at three concentrations were prepared by mixing equal amounts of glucose, xylose and arabinose at concentrations of 1, 2 and 4 g/L. An independent calibration verification standard (CVS) was also prepared which was a solution containing the same three
sugars at a concentration of 2.5 g/L. CVS was analyzed in HPLC together with samples at regular intervals throughout the sequence to verify the stability of calibration for each sequence run. All sugar standards and CVS were passed through a 0.2 μm syringe filter into an autosampler vial. Calibration standards for hydrolysis samples were performed by mixing cellobiose at 1, 2 and 4 g/L along with the above mentioned three sugars. Calibration standards for fermentation samples were prepared by supplementing the 1, 2 and 4 g/L three-sugar-solution with 200 proof ethanol (HPLC/spectrophotometric grade, Aldrich Chemical Co., Inc.) at concentrations of 1, 4 and 16 g/L, respectively. Other two monomeric sugars contained in hemicellulose, namely mannose and galactose, could not be separated from xylose according to the chromatography as these three sugars (xylose, mannose and galactose) were eluted at similar time intervals if Biorad Aminex HPX-87H column was used in HPLC. Therefore, if mannose and galactose were included in the calibration standards besides glucose, xylose and arabinose, further correction for xylose was needed according to the contribution of xylose in the elution peak which actually represented a combination of xylose, mannose and galactose. In this study, the proportion of xylose was found to be 0.328 (approximately 1/3).

A set of sugar recovery standards (SRS) was also prepared and used to correct for losses due to decomposition of sugars under acidic conditions during autoclaving. Three monomeric sugars, glucose, xylose and arabinose, were dissolved in 10.0 ml HPLC grade water in serum bottles with the final concentrations being 1 g/L, 2 g/L and 4 g/L. Three hundred and forty-eight microliter of 72% sulfuric acid was added to each SRS before bottles were stoppered and aluminum seals were crimped into place. All sealed liquid samples were autoclaved for 1 h at 121 °C/15 psi under standard liquid setting, neutralized to a pH between 5 and 6, and injected through the 0.2 μm syringe filter into autosampler vials for HPLC analysis. The final concentration of each component sugar recovered after autoclaving in SRS was compared with their original concentrations (1 g/L, 2 g/L or 4 g/L). An average
percentage of sugar reduction \( (%R_{\text{sugar}}) \) was calculated for each individual sugar of interest as shown in Equation 3.6:

\[
\text{Percent Sugar Reduction} \ (\%R_{\text{sugar}}) = \frac{\text{conc. detected by HPLC, g/L}}{\text{known conc. of sugar before autoclaving, g/L}} \times 100
\]

(3.6)

The percentage of sugar reduction \( (%R_{\text{sugar}}) \) obtained from HPLC analysis was essentially a conversion constant and was applicable to all sugar samples obtained from the composition analyses of initial and pretreated biomass.

The amount of monosaccharides (glucose, xylose and arabinose), cellobiose and ethanol contained in the filtered samples were analyzed with an HPLC (Shimadzu, Kyoto, Japan) equipped with a refractive index detector (Shimadzu RID-10A). A Biorad Aminex HPX-87H column was maintained at a working temperature of 65 °C with a corresponding guard column. The mobile phase used was 5 mM H\(_2\)SO\(_4\) at a flow rate of 0.6 ml/min.

3.2.3 Dilute acid pretreatment

Sulfuric acid at 0.5, 1.0 and 1.5% (w/v) was used for chemical pretreatment of the oven-dried switchgrass samples. A solid-to-liquid ratio of 1:10 was applied by mixing 3.0 g of the grass samples with 30 ml of dilute acid solutions in 100 ml serum bottles for all pretreatments. The mixtures were autoclaved at 121 °C/15 psi under standard liquid cycle for residence times of 30, 45 and 60 min.

Upon completion of the autoclave cycle, samples were removed and allowed to cool to room temperature. The pretreated slurry was filtered through a porcelain Buchner funnel using the filtration assembly shown in Figure 3.4.

The residual acid was washed from the surface of the pretreated biomass with 250 ml hot deionized water, which was also used to ensure the complete transfer of biomass from the serum bottle to the Buchner funnel. For each pretreatment condition performed in triplicate, one filtrate sample was collected in a 15 ml polypropylene
The wet biomass sample left on the Buchner funnel was completely transferred to a pre-weighed zip-locked plastic bag which was then firmly sealed; and the total weight of zip-locked bag plus the wet biomass sample was recorded. Two 1.5 g sub-sets of the wet biomass were drawn from each replicate and dried in pre-weighed aluminum weighing dishes at 105 °C convection oven and 40 °C vacuum oven, respectively. Upon completion of drying for 15 h, the total weight of weighing dishes plus 105 °C dry pretreated biomass was recorded and the moisture content of the pretreated biomass was calculated as:

\[
\text{Percent Moisture} = \left(1 - \frac{W_2 - W}{W_1}\right) \times 100
\]

(3.7)

Where \(W\) = weight of the aluminum weighing dish (g);

\(W_1\) = weight of the small portion of wet pretreated biomass to be oven dried (g);

\(W_2\) = total weight of the weighing dish and the pretreated biomass after 105 °C drying for 15 h (g).

Percent moisture determined in Equation 3.7 was used to calculate the solid recovery for each pretreatment process. Normally, solid loss due to incomplete
transfer of biomass (residue on the filter paper in Buchner funnel) was estimated to be approximately 1% based on tests conducted by weighing the dry filter paper before and after each filtration. Therefore, solid recovery was calculated as:

\[
\text{Percent Solid Recovery} = \left(\frac{W_2 - W_1}{W}\right) \times (1 - \%_{\text{moisture}}) \times 100 + 1^* \quad (3.8)
\]

Where

- \(W_1\) = weight of the zipper bag (g);
- \(W_2\) = total weight of the zipper bag and wet pretreated biomass (g);
- \(W\) = weight of the initial biomass used for pretreatment (g, usually = 3.0);
- * represents the solid loss within the Buchner filtering kit.

Since equally weighted wet sub-samples (approximately 1.5 g) were drawn and oven dried at 40 or 105 °C for each pretreated biomass and 105 °C oven dried biomass was considered completely moisture free, percent total solid in 40 °C oven dried pretreated sample was determined as:

\[
\text{Percent Total Solids} = \frac{W_2 - W_1}{W_4 - W_3} \times 100\% \quad (3.9)
\]

Where

- \(W_1\) = weight of aluminum dish for sample drying at 105 °C (g);
- \(W_2\) = total weight of aluminum dish and pretreated biomass after 105 °C oven drying (g);
- \(W_3\) = weight of aluminum dish for sample drying at 40 °C (g);
- \(W_4\) = total weight of aluminum dish and pretreated biomass after 40 °C oven drying (g).

Composition analyses for determination of lignin (both acid insoluble and acid soluble) and carbohydrate were performed on 0.3 g of the 40 °C oven-dried pretreated sample (with ‘total solids’ being approximately 95%) according to the procedures described earlier. The 105 °C oven dried samples were not used due to the possibility that structural components contained in the biomass may be disrupted during heating at elevated temperature. The lignin and carbohydrate (represented by
monomeric sugars) contents in the pretreated biomass were expressed on the basis of initial biomass by multiplying the calculated value with the corresponding ‘percent solid recovery’. Therefore, the changes in composition due to acid pretreatment can be estimated by comparing values obtained before and after each pretreatment.

The most effective pretreatment conditions for each germplasm were identified based on least lignin content and greatest percent hemicellulose solubilization. Six pretreatment conditions (2 within each germplasm) were selected and biomass samples pretreated under these conditions were hydrolyzed and fermented for ethanol production.

### 3.2.4 Enzymatic hydrolysis

Samples for enzymatic hydrolysis were prepared by repeating the selected pretreatments to provide enough pretreated biomass. All three enzymes, cellulase (NS 50013 cellulase complex, density 1.20 g/ml), cellobiase (NS 50010 β-glucosidase, density 1.24 g/ml) and xylanase (NS50030 xylanase, density 1.09 g/ml), kindly provided by Novozymes North America Inc., Franklinton, NC, were used for this study. Enzyme assays were performed indicating that the activities of cellulase and cellobiase were at 75.5 FPU (filter paper units)/ml and 634.2 CBU (cellobiase units)/ml, respectively (Appendix Ia, Ib).

Hydrolysis was performed at 5% solid loading by mixing wet pretreated biomass equivalent to 1 g dry basis (determined by moisture content) with 20 ml 0.05 M citrate buffer (pH 4.8) containing 40 μg/ml tetracycline hydrochloride to avoid microbial contamination. Thereafter, enzymes were added to the hydrolysis mixture at three different levels with cellulase activities being 0 FPU (0 μl), 15 FPU (199 μl) and 30 FPU (397 μl) /g dry biomass, respectively. Cellobiase was supplemented into the solution at an activity ratio of 4 CBU: 1 FPU (cellobiase: cellulase) to avoid end-product inhibition due to cellobiose accumulation (Ingesson et al., 2001; Bura et al., 2002), and the volumes of cellobiase added were correspondingly 0, 93 and 185 μl.
The effect of xylanase on reducing sugar generation was also studied by adding xylanase at a loading of 0.25% w/w dry biomass in combination with cellulase and cellobiase. Additionally, unpretreated switchgrass samples were hydrolyzed as control at similar enzyme loadings. Samples (unpretreated or pretreated) soaked in buffer solution without enzymes (at 0 FPU/g dry biomass) were prepared to study the impact of soaking on polysaccharide solubilization. All samples were incubated in 50 ml polypropylene centrifuge tubes in a water bath at 55 °C and 150 rpm for 72 hours. Upon completion, hydrolyzates were centrifuged at 4 °C and 4000 rpm for 10 minutes in an Eppendorf 5810R centrifuge. One milliliter of the supernatant was drawn and diluted 6 fold for the unpretreated samples or 10 fold for the pretreated samples and filtered through 0.2 μm syringe filters into autosampler vials for fermentable sugar analysis in HPLC. The remaining hydrolyzates were autoclaved at standard liquid cycle (121 °C/15 psi) for 15 minutes to inactivate any contaminating microorganisms and degrade the antibiotic before fermentation. The conversion efficiency of hydrolysis was calculated by comparing the glucose yield (g) after hydrolysis with the initial glucose content (g, or presented as 1.1 times the initial glucan content) in the pre-hydrolysis biomass (unpretreated or pretreated), and it was expressed in terms of the percentage of cellulose enzymatically converted to glucose (%ECC) using Equation 3.10 (Varga et al., 2004):

\[
\%\text{ECC} = \frac{C \times V \times a \times \frac{1L}{1000ml}}{m \times \%\text{Cellulose} \times 1.1} \times 100\%
\]  

(3.10)

Where C = concentration of glucose after enzymatic hydrolysis detected by HPLC (g/L);

V = the volume of hydrolyzate (ml);

a = the dilution rate of hydrolyzate for HPLC sugar analysis;

m = weight of dry biomass (unpretreated or pretreated) before hydrolysis (g);

‘% cellulose’ was determined by the carbohydrate analysis as described in 3.2.2 on the unpretreated or pretreated biomass.
3.2.5 Fermentation

*Saccharomyces cerevisiae* (ATCC 24859), obtained from Dr. Demirci’s Microbiological Engineering Laboratory in the Agricultural and Biological Engineering Department at Pennsylvania State University, was used for fermentation in this study. The yeast medium was prepared by dissolving 20 g glucose, 8.5 g yeast extract, 1.32 g NH₄Cl, 0.11 g MgSO₄, and 0.06 g CaCl₂ in 1 L of deionized water (Chen et al., 2007). For inoculation, approximately 1.0 ml of the 30% glycerol yeast stock kept in the – 80 °C freezer was added to the 100 ml media. The yeast was allowed to grow aerobically in a water bath at 30 °C and 150 rpm for 48–72 hours till the culture partially covered the bottom of the 250 ml centrifuge bottle. Time for activation differed as the growing trend of yeast cells varied for each inoculation. Completely activated yeast cells were harvested by centrifugation at 4000 rpm at 4 °C for 10 minutes, washed twice with 50 ml 0.1% sterilized peptone water to remove residual media and resuspended in 20 ml peptone water before use. One milliliter aliquot of the yeast solution was taken to perform serial dilutions up to 10⁵ and 100 μl of the diluted culture was spread-plated onto yeast extract peptone dextrose (YEPD) medium prepared by adding 10 g yeast extract, 20 g peptone, 20 g glucose and 16 g agar per liter of deionized water (Süssmuth et al., 1979). Plates were placed in the incubator at 30 °C for 72 hours and yeast colonies were counted to ensure that each time the initial inoculation stayed at approximately 6.3 ×10⁷ cfu/ml corresponding to 11 g dry weight/L. Fig. 3.5 shows how the serial dilutions and plate counts were conducted.

Two hundred and fifty microliter of the yeast suspension was added to each tube containing 19 ml of sterilized hydrolyzate for fermenting in the incubator at 30 °C for 48 hours under anaerobic conditions. Upon completion of the fermentation, samples were centrifuged at 4 °C and 4000 rpm for 10 minutes in the Eppendorf
5810R centrifuge. Appropriate amount of liquid sample was drawn from each fermentation batch and filtered through 0.2 μm syringe filters for HPLC analysis of ethanol and residual/unfermented sugar availability. Ethanol yield was calculated by dividing the total amount of ethanol produced (g ethanol) in the fermentation broth by the starting weight of initial switchgrass (g initial biomass). It can also be expressed as percent ethanol yield as compared with the theoretical value using Equation 3.11:

\[
\text{Percent Ethanol Yield} = \frac{\text{Ethanol(g)} / \text{biomass(g)}}{0.511 \times \text{Glucose(g)} / \text{biomass(g)}}
\]  

(3.11)

Where 0.511 represents the theoretical ethanol yield (g) generated per gram of glucose (Thomas et al., 1996);

- Ethanol (g)/biomass (g) = the ethanol yield (g) per 1 g of initial biomass;
- Glucose (g)/biomass (g) = the glucose content (g) in 1 g of initial biomass (can be obtained from the composition analysis of raw biomass).

### 3.2.6 General statistical analysis

Multiple Comparison with the Best (Hsu, 1996) was conducted using SAS® (version 9.1.3, Cary, NC) to identify the most effective pretreatment condition for each germplasm based on least lignin content and greatest hemicellulose solubilization. Triplicate data from the 9 pretreatment conditions (3 acid
concentrations × 3 residence times) for each of the 3 germplasms was used for the analysis. Samples from the 6 pretreatments identified were further hydrolyzed. Analysis of variance (ANOVA) tables were generated to determine the effects of various factors on pretreatment, hydrolysis and fermentation. A 95% confidence level was applied to all analyses performed in this study. Additionally, Tukey simultaneous tests were conducted to test the statistical differences between treatments.

3.3 Results and Discussion

3.3.1 Composition of switchgrass

Oven-dried switchgrass samples of three different germplasms were analyzed for key components (Table 3.1), and were found to contain 26.65 to 29.28% glucan, 17.92 to 19.37% xylan, 2.02 to 2.76% arabinan and 17.74 to 19.23% lignin on dry matter basis. Besides, oven-drying resulted in a moisture content of 1.24 to 1.54% in the biomass.

Table 3.1 Composition of the three oven-dried switchgrass germplasms

<table>
<thead>
<tr>
<th>Composition¹</th>
<th>St6-1 whole grass</th>
<th>St6-3E whole grass</th>
<th>St6-3F whole grass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ash</td>
<td>3.07</td>
<td>2.98</td>
<td>3.88</td>
</tr>
<tr>
<td>Extractives</td>
<td>6.40</td>
<td>5.38</td>
<td>4.87</td>
</tr>
<tr>
<td>Acid insoluble lignin</td>
<td>15.93</td>
<td>15.50</td>
<td>13.70</td>
</tr>
<tr>
<td>Acid soluble lignin</td>
<td>3.30</td>
<td>3.48</td>
<td>4.04</td>
</tr>
<tr>
<td>Glucan</td>
<td>26.65</td>
<td>27.24</td>
<td>29.28</td>
</tr>
<tr>
<td>Xylan</td>
<td>18.06</td>
<td>17.92</td>
<td>19.37</td>
</tr>
<tr>
<td>Arabinan</td>
<td>2.10</td>
<td>2.02</td>
<td>2.76</td>
</tr>
<tr>
<td>Others</td>
<td>24.49</td>
<td>25.48</td>
<td>22.10</td>
</tr>
</tbody>
</table>

Note. ¹Values reported as average percentages on dry matter basis.

Based on the information provided online in the Biomass Feedstock Composition and Property Database (US Department of Energy, EERE), it was noticed that compared with varieties like Alamo and Cave-in-Rock that are widely
available in the market, both ash and extractives were less in the three germplasms studied. Acid insoluble lignin contributed approximately 80% of the total lignin contained in the biomass. In switchgrass cell wall, glucan is the dominant polysaccharide while xylan is the second most abundant sugar. For the three germplasms studied, glucan and xylan contributed approximately 50% of the total dry matter. Arabinan, another polysaccharide, was measured at 2-3% of the total dry matter of switchgrass.

Mass closure (by summating all components on dry matter basis) was 75% on average, with the remaining undefined components possibly being organic compounds such as uronic acid, acetyl groups and several other minerals (Kaar and Holtzapple, 2000; Samson, 1998; Samson, 2005). Additionally, it is difficult to ensure that the extraction process is effective enough to remove all extractives from the initial switchgrass sample since a mixture of very diverse materials such as waxes, fats, gums, starches, resins and essential oils is present in plants (Kuhad and Singh, 1993). The composition of lignocellulosic material can change as cell wall carbohydrates are affected by external factors such as seasonal time of harvest or the contribution of stem or leaf proportions to the bulk sample. Adler et al. (2006) reported that carbohydrate contents showed an increasing trend over winter since sugar contents were greater in spring harvested plants than those harvested in fall. Also, Dien et al. (2006) found that carbohydrate and lignin contents were less in early maturity lignocellulosic samples compared with the more mature ones.

Compositions of St6-1 and St6-3E were more comparable for sugar and lignin contents compared with St6-3F. Since St6-3F is a hybrid germplasm developed for high digestibility and St6-1 and St6-3E are intended for greater dry biomass yield, compositional differences among them were reasonable. According to Table 3.1, oven-dried St6-3F switchgrass shows greater potential for grass-to-ethanol conversion as it is less in acid insoluble lignin and greater in glucan; and both these characteristics are advantageous for releasing a larger amount of glucose during
hydrolysis.

3.3.2 Effect of dilute acid pretreatment

Solids remaining after pretreatment of switchgrass germplasms with dilute sulfuric acid at 0.5, 1.0 and 1.5% (w/v) for 30, 45 and 60 min were analyzed for sugar and lignin contents and the results are summarized in Tables 3.2, 3.3 and 3.4.

Sulfuric acid pretreatment resulted in 51.97–72.56% solid recovery for the three switchgrass germplasms. Solid recovery was significantly affected by germplasm, treatment time and acid concentration (P < 0.05) with acid concentration being the most decisive factor according to the F-value obtained (Appendix Table IIA). Solid recovery decreased as the acid concentration and residence time increased; therefore, more severe the pretreatment condition, less the amount of solid recovered.

1. Effect of pretreatment on lignin degradation

Sulfuric acid pretreatment did not greatly remove lignin from switchgrass samples. Statistically, for each germplasm, acid concentration, residence time and their interaction term (acid*time) did not have significant impacts on lignin content in the pretreated samples (P > 0.05) except the interaction term for St6-1 (P < 0.05, Table 3.5).

For St6-1, St6-3E and St6-3F switchgrass samples, the greatest lignin removal rates were limited to 5.46%, 8.12% and 6.54%, respectively. These reaction conditions were not combinations of greatest acid concentration and longest residence time, thus indicating that lignin removal was not positively associated with the intensity of dilute acid pretreatment. Lignin has been reported to be very sensitive to sulfuric acid and two opposite mechanisms, degradation (depolymerization) and accumulation (repolymerization), occur simultaneously when lignin interacts with sulfuric acid (Pearl, 1967; Sjostrom, 1993). It is possible that lignin accumulation was greater than lignin degradation during acid pretreatment of the switchgrass.
Table 3. 2 Composition of St6-1 oven-dried switchgrass after acid pretreatment at different conditions (expressed as g/100g unpretreated dry switchgrass)

<table>
<thead>
<tr>
<th>Pretreatment conditions</th>
<th>Time (min)</th>
<th>Acid concentration (%)</th>
<th>Total solid</th>
<th>Composition of solid fractions (%)</th>
<th>Hemicellulose solubilization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lignin</td>
<td>Glucose</td>
</tr>
<tr>
<td>Initial sample (unpretreated)</td>
<td>100</td>
<td>19.22 (0.11)</td>
<td>29.31 (4.88)</td>
<td>19.87 (2.93)</td>
<td>2.31 (0.46)</td>
</tr>
<tr>
<td>30</td>
<td>0.5</td>
<td>19.14 (0.38)</td>
<td>25.54 (3.77)</td>
<td>13.68 (3.50)</td>
<td>0.79 (0.16)</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>18.38 (0.55)</td>
<td>22.39 (6.78)</td>
<td>7.59 (3.15)</td>
<td>0.30 (0.15)</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>18.53 (0.48)</td>
<td>25.37 (8.22)</td>
<td>5.90 (1.37)</td>
<td>0.31 (0.12)</td>
</tr>
<tr>
<td>45</td>
<td>0.5</td>
<td>18.80 (0.32)</td>
<td>20.95 (6.21)</td>
<td>9.65 (3.10)</td>
<td>0.38 (0.13)</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>18.50 (0.38)</td>
<td>28.88 (7.93)</td>
<td>6.99 (1.57)</td>
<td>0.38 (0.18)</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>19.05 (0.46)</td>
<td>28.73 (11.00)</td>
<td>5.92 (2.06)</td>
<td>0.28 (0.14)</td>
</tr>
<tr>
<td>60</td>
<td>0.5</td>
<td>18.17 (0.72)</td>
<td>22.74 (9.33)</td>
<td>8.84 (4.54)</td>
<td>0.42 (0.30)</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>18.48 (0.57)</td>
<td>21.57 (3.60)</td>
<td>4.80 (0.86)</td>
<td>0.24 (0.11)</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>19.03 (0.42)</td>
<td>28.77 (6.00)</td>
<td>4.95 (0.71)</td>
<td>0.30 (0.09)</td>
</tr>
</tbody>
</table>

Note. 1Expressed as Mean value (Standard deviation).
2Least lignin availability observed.
3Greatest hemicellulose solubilization observed.
Table 3. Composition of St6-3E oven-dried switchgrass after acid pretreatment at different conditions (expressed as g/100g unpretreated dry switchgrass)

<table>
<thead>
<tr>
<th>Pretreatment conditions</th>
<th>Acid concentration (%)</th>
<th>Total solid¹</th>
<th>Lignin</th>
<th>Glucose</th>
<th>Xylose</th>
<th>Arabinose</th>
<th>Hemicellulose¹ solubilization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial sample (unpretreated)</td>
<td>100</td>
<td>18.97 (0.38)</td>
<td>29.96 (0.38)</td>
<td>19.71 (0.39)</td>
<td>2.22 (0.19)</td>
<td>36.45 (3.34)</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>0.5</td>
<td>70.65 (2.44)</td>
<td>17.94 (0.44)</td>
<td>24.05 (0.72)</td>
<td>13.26 (0.70)</td>
<td>0.69 (0.03)</td>
<td>65.43 (19.20)</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>60.29 (1.96)</td>
<td>17.43 (0.46)²</td>
<td>23.36 (7.76)</td>
<td>7.25 (3.98)</td>
<td>0.33 (0.24)</td>
<td>65.24 (0.43)</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>56.61 (0.59)</td>
<td>17.74 (0.68)</td>
<td>32.18 (4.64)</td>
<td>7.26 (0.09)</td>
<td>0.37 (0.01)</td>
<td>65.43 (0.43)</td>
</tr>
<tr>
<td>45</td>
<td>0.5</td>
<td>64.60 (5.50)</td>
<td>17.56 (0.05)</td>
<td>31.28 (7.42)</td>
<td>13.66 (3.43)</td>
<td>0.66 (0.32)</td>
<td>34.77 (17.08)</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>58.48 (0.99)</td>
<td>17.60 (0.34)</td>
<td>25.62 (4.26)</td>
<td>6.68 (1.23)</td>
<td>0.28 (0.13)</td>
<td>68.26 (6.22)</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>55.48 (1.02)</td>
<td>18.09 (0.19)</td>
<td>27.28 (4.04)</td>
<td>5.35 (1.23)</td>
<td>0.28 (0.13)</td>
<td>74.35 (6.20)</td>
</tr>
<tr>
<td>60</td>
<td>0.5</td>
<td>63.22 (0.56)</td>
<td>18.30 (0.02)</td>
<td>28.60 (8.70)</td>
<td>10.22 (3.17)</td>
<td>0.55 (0.24)</td>
<td>50.94 (15.53)</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>55.47 (0.49)</td>
<td>17.51 (0.94)</td>
<td>28.86 (0.97)</td>
<td>6.09 (0.12)</td>
<td>0.36 (0.01)</td>
<td>70.58 (0.58)</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>53.63 (0.37)</td>
<td>18.07 (0.43)</td>
<td>20.27 (5.59)</td>
<td>3.43 (1.11)</td>
<td>0.17 (0.00)</td>
<td>83.58 (5.07)³</td>
</tr>
</tbody>
</table>

Note. ¹Expressed as Mean value (Standard deviation).
²Least lignin availability observed.
³Greatest hemicellulose solubilization observed.
Table 3.4 Composition of St6-3F oven-dried switchgrass after acid pretreatment at different conditions (expressed as g/100g unpretreated dry switchgrass)

<table>
<thead>
<tr>
<th>Pretreatment conditions</th>
<th>Acid concentration (%)</th>
<th>Time (min)</th>
<th>Total solid&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Lignin</th>
<th>Glucose</th>
<th>Xylose</th>
<th>Arabinose</th>
<th>Hemicellulose&lt;sup&gt;1&lt;/sup&gt; solubilization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Initial sample (unpretreated)</td>
<td>100</td>
<td>17.74 (0.25)</td>
<td>32.21 (0.97)</td>
<td>21.31 (0.89)</td>
<td>3.04 (0.03)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>0.5</td>
<td>71.91 (2.31)</td>
<td>17.55 (0.24)</td>
<td>27.76 (2.82)</td>
<td>15.39 (0.19)</td>
<td>0.94 (0.04)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>58.57 (1.42)</td>
<td>16.58 (0.50)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>28.28 (8.49)</td>
<td>8.72 (2.37)</td>
<td>0.44 (0.11)</td>
<td>62.36 (10.12)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>54.78 (1.46)</td>
<td>16.86 (0.21)</td>
<td>23.15 (10.01)</td>
<td>5.46 (3.22)</td>
<td>0.32 (0.21)</td>
<td>76.21 (14.07)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>45</td>
<td>0.5</td>
<td>65.98 (2.77)</td>
<td>16.85 (0.55)</td>
<td>29.68 (8.26)</td>
<td>12.41 (3.59)</td>
<td>0.62 (0.27)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>55.65 (2.06)</td>
<td>17.41 (0.34)</td>
<td>31.39 (7.17)</td>
<td>7.87 (1.81)</td>
<td>0.46 (0.14)</td>
<td>65.79 (8.03)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>54.41 (0.73)</td>
<td>16.80 (0.00)</td>
<td>22.28 (5.47)</td>
<td>4.34 (1.60)</td>
<td>0.24 (0.10)</td>
<td>81.20 (6.92)&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>0.5</td>
<td>62.10 (1.09)</td>
<td>17.18 (0.18)</td>
<td>27.60 (7.41)</td>
<td>10.76 (3.13)</td>
<td>0.54 (0.24)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>54.62 (1.15)</td>
<td>17.10 (0.74)</td>
<td>27.10 (4.78)</td>
<td>5.79 (0.77)</td>
<td>0.30 (0.10)</td>
<td>75.02 (3.55)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>51.97 (0.34)</td>
<td>16.71 (0.37)</td>
<td>27.71 (6.73)</td>
<td>4.72 (0.93)</td>
<td>0.23 (0.10)</td>
<td>79.69 (4.24)</td>
</tr>
</tbody>
</table>

Note. <sup>1</sup>Expressed as Mean value (Standard deviation).
<sup>2</sup>Least lignin availability observed.
<sup>3</sup>Greatest hemicellulose solubilization observed.
Table 3. 5 P-values of ANOVA type III test for lignin content in dilute acid pretreated oven-dried switchgrass

<table>
<thead>
<tr>
<th>Factors</th>
<th>St6-1</th>
<th>St6-3E</th>
<th>St6-3F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>0.5381</td>
<td>0.0674</td>
<td>0.9560</td>
</tr>
<tr>
<td>Acid concentration</td>
<td>0.3601</td>
<td>0.5038</td>
<td>0.2250</td>
</tr>
<tr>
<td>Time * Acid concentration</td>
<td>0.0380</td>
<td>0.4154</td>
<td>0.1747</td>
</tr>
</tbody>
</table>

Yang and Wyman (2004) described that lignin removal during dilute acid pretreatment in a batch process was less than that with hot water treatment alone at the same treatment temperature. Also, Liao et al. (2007) reported that lignin content increased from the original 0.1211 g per gram of initial biomass to a range between 0.1601 and 0.1796 g lignin/g initial biomass as dilute acid pretreatment conditions of time, temperature and acid concentration changed from the mildest (1 h, 110 °C and 1% acid) to the most severe (3 h, 130 °C and 3% acid). Besides, since the extractives were not removed from the pretreated switchgrass samples before composition analysis, it is possible that those chemicals (mostly the organic compounds) condensed together with lignin during 72% H$_2$SO$_4$ treatment and were measured as acid insoluble lignin in the post-pretreatment analysis (Agblevor et al., 2003; Thammasouk et al., 1997). Another factor possibly affecting lignin measurement is the absorbance read from the spectrophotometer during acid soluble lignin analysis. Presence of any component besides acid soluble lignin in the liquid fraction can impact the absorption of light at the wavelength of 205 nm, leading to an over estimation of the lignin content (Ehrman, 1996). Although little lignin degradation was observed, it can be inferred that the structure of lignin was disrupted, thus increasing the prospect of interactions between cellulose and cellulolytic enzymes in subsequent hydrolysis (Yang and Wyman, 2004).

2. Effect of pretreatment on hemicellulose solubilization

Unlike lignin, the amount of hemicellulose in the pretreated samples
decreased as the intensity of pretreatments increased during dilute acid pretreatment. ANOVA demonstrated that acid concentration, with a P-value less than 0.05 for all three germplasms, had the greatest influence on solubilization of hemicellulose. However, residence time and the interaction term (acid*time) were not significant for any of the germplasms (P > 0.05, Table 3.6).

<table>
<thead>
<tr>
<th>Factors</th>
<th>St6-1</th>
<th>St6-3E</th>
<th>St6-3F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>0.1056</td>
<td>0.1884</td>
<td>0.0690</td>
</tr>
<tr>
<td>Acid concentration</td>
<td>0.0021</td>
<td>0.0009</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Time * Acid concentration</td>
<td>0.5418</td>
<td>0.8718</td>
<td>0.6655</td>
</tr>
</tbody>
</table>

The degree of hemicellulose solubilization increased significantly from 32.96-36.45% when treated with the mildest condition (30 min and 0.5% H2SO4) to 76.32-83.58% when subjected to the most severe pretreatment (60 min and 1.5% H2SO4). The treatment conditions under which greatest hemicellulose solubilization was obtained involved either greatest acid concentration or longest residence time or both, indicating that hemicellulose solubilization was positively associated with the intensity of dilute acid pretreatment. Esteghlalian et al. (1997) pretreated switchgrass at 180 °C with 0.9% sulfuric acid and obtained approximately 90% solubilization of xylan within the first minute of the reaction. Moreover, other studies on dilute acid pretreatment have shown that hemicellulose in the lignocellulosic feedstock can be completely solubilized during acid pretreatment if the reaction conditions became very intense. It has been demonstrated that hemicellulose was the only component in plant fiber that could be thoroughly removed by dilute acid pretreatment (Nguyen, 1998; Roberto et al., 2003). A greater level of hemicellulose solubilization results in enhanced digestibility of cellulose in residual solids which is essential for the increase in enzymatic conversion efficiency (Mosier et al., 2005).
The filtrate from washing of pretreated switchgrass samples prior to hydrolysis was analyzed for sugar availability. The amount of reducing sugars detected by chromatography is summarized in Table 3.7.

Table 3.7 Amount of reducing sugars released in the filtrate during dilute acid pretreatment of oven-dried switchgrass feedstocks (expressed as mg/g of dry initial switchgrass)

<table>
<thead>
<tr>
<th>Monosugars released</th>
<th>St6-1</th>
<th>St6-3E</th>
<th>St6-3F</th>
</tr>
</thead>
<tbody>
<tr>
<td>xylose</td>
<td>22.79–127.88</td>
<td>28.41–147.57</td>
<td>24.94–133.66</td>
</tr>
</tbody>
</table>

Comparing the sugar content of the filtrate (Table 3.7) with that of the initial switchgrass (Table 3.1), it can be inferred that the predominant effect of dilute acid pretreatment was solubilization/degradation of hemicellulose (xylose and arabinose) irrespective of operating conditions. Besides the three standard monomeric sugar peaks, many other components were eluted as evident from the multiple unidentified peaks in HPLC chromatogram for filtrate analysis. These peaks may represent even smaller components such as furfural or other byproducts derived from the further degradation of monomeric sugars or lignin released during acid pretreatment (Söderström et al., 2003; Sun and Cheng, 2005; Mosier et al., 2005). A control pretreatment wherein St6-3F switchgrass with deionized water alone was autoclaved for 30 min showed that with a solid recovery over 80%, only 6.56 mg glucose and 5.72 mg xylose per gram of initial St6-3F switchgrass which contained 292.8 mg glucan and 193.7 mg xylan was released in the filtrate after pretreatment.

3. Effect of pretreatment on other cell wall compounds

Majority of the cellulose was retained in the solid residue as the main substrate for subsequent enzymatic hydrolysis. The percentage of cellulose released to the liquid fraction during acid pretreatment was limited to 28.52%, 32.34% and 30.83%
for St6-1, St6-3E and St6-3F switchgrass samples, respectively. Statistical analysis elucidated that acid concentration, residence time and their interaction term did not have significant effects (P > 0.05) on the cellulose content in pretreated switchgrass for all three germplasms.

Apart from lignin, hemicellulose and cellulose, plant cells contain proteins and extractives like waxes. It has been reported that extractives can be removed simultaneously with hemicellulose during acid pretreatment (Nguyen et al., 2000). Also, the protein content decreases as acid concentration, reaction temperature and time increase during dilute sulfuric acid pretreatment (Liao et al., 2007). In this study, extractives and proteins remaining in the pretreated solids were not quantified. However, as evident from the post-pretreatment composition analysis (Table 3.2, 3.3 and 3.4), the sum of lignin and all three sugars approximately equaled the total solids (after pretreatment) as treatment conditions become more intense, thus indicating that proteins and extractives may have been removed from the solid biomass.

3.3.3 Enzymatic hydrolysis

Hydrolysis was conducted on two sets of pretreated biomass based on the extent of delignification or hemicellulose solubilization. Samples with least lignin and greatest hemicellulose solubilization for each germplasm were hydrolyzed with cellulase, cellobiase and xylanase at various activity levels.

1. Hydrolysis of the pretreated least-lignin-content biomass

The three pretreatments which provided least lignin content — 60 min and 0.5% acid for St6-1, and 30 min and 1.0% acid for both St6-3E and St6-3F were selected on the basis of a multiple comparison with the best (SAS® 9.1.3, Cary, NC) for enzymatic hydrolysis and fermentation (Appendix Tables IIb, IIc and IIId). The cellulose conversion efficiency for the hydrolyzed samples is summarized in Figure 3.6 which shows the effect of pretreatment, cellulase/cellobiase addition and loading as well as the addition of xylanase on reducing sugar yield.
Figure 3.6 Hydrolysis of unpretreated and pretreated least-lignin-content oven-dried samples. Columns are grouped by the same cellulase activity levels for each switchgrass germplasm.

The enzymatic conversion of pretreated cellulose was between 39.60 and 68.02% compared with 30.55 to 47.56% for unpretreated biomass. The effect of four experimental factors including (1) untreated vs. pretreatment, (2) cellulase loading (0, 15 and 30 FPU/g dry biomass), (3) presence or absence of xylanase and (4) the interaction between cellulase loading and xylanase (FPU*xylanase) studied for hydrolysis are summarized in Table 3.8.

Table 3.8 P-values of ANOVA type III test for hydrolysis of the least-lignin-content dilute acid pretreated oven-dried switchgrass

<table>
<thead>
<tr>
<th>Germplasm</th>
<th>P values in ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>St6-1</td>
</tr>
<tr>
<td>Pretreatment</td>
<td>0.0590</td>
</tr>
<tr>
<td>Xylanase addition</td>
<td>0.2054</td>
</tr>
<tr>
<td>Cellulase addition (FPU)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Xylanase addition * FPU</td>
<td>0.0183</td>
</tr>
</tbody>
</table>

Adding cellulase significantly increased the hydrolysis efficiency compared
with the non-cellulase control for all three germplasms, however, no statistical differences were observed between the loadings of 15 FPU and 30 FPU (P > 0.05) except for St6-3E samples. Pretreatment did not have a significant impact on hydrolysis efficiency (P > 0.05), especially for St6-3E germplasm. Öhgren et al. (2007) showed that supplementation of the cellulase mixture with xylanase at a concentration of 0.06 g protein/g cellulose increased the hemicellulose hydrolysis with the xylose yield increasing from 65% to 90% of theoretical, thus making cellulase more accessible to cellulose. However, in this study, adding xylanase did not significantly improve cellulose conversion efficiency (P > 0.05), especially at the cellulase loading of 15 FPU. At the cellulase loading of 30 FPU, xylanase was found to significantly promote (P < 0.05) hydrolysis efficiency for the St6-1 germplasm.

Almost no cellobiose was detected in the hydrolyzate, indicating that the cellobiose produced during hydrolysis was effectively hydrolyzed by cellobiase, thus preventing end-product inhibition. In the hydrolyzate from control samples without enzymes, pretreated switchgrass did not generate any monomeric sugars (glucose, xylose and arabinose). However, 30.55–33.24% cellulose conversion was detected in the control in which unpretreated switchgrass was soaked in citrate buffer without adding any cellulolytic or xylolytic enzymes. Perhaps this was because all free monomeric sugars were completely transferred from the pretreatment liquid into the filtrate by deionized water washing after dilute acid pretreatment, thus leaving no free monomeric sugars available on the surface of the pretreated grass samples.

2. Hydrolysis of the pretreated greatest-hemicellulose-solubilization biomass

For each germplasm, the pretreatment condition which led to the greatest percent hemicellulose (both xylose and arabinose) solubilization based on a multiple comparison analysis (SAS® 9.1.3, Cary, NC) was selected for subsequent hydrolysis. The treatment conditions were 60 min and 1.0% acid for St6-1; 60 min and 1.5% acid for St6-3E; and 45 min and 1.5% acid for St6-3F (Appendix Tables IIe, IIf and IIg).
The cellulose conversion efficiency for samples with greatest hemicellulose solubilization during acid pretreatment is summarized in Figure 3.7.

Figure 3.7 Hydrolysis of unpretreated and pretreated greatest-hemicellulose-solubilization oven-dried samples. Data for unpretreated samples are the same as those in Figure 3.6. Columns are grouped by the same cellulase activity levels for each switchgrass germplasm.

Compared to unpretreated samples that showed a maximum cellulose conversion of 47.56%, pretreated samples had a cellulose conversion greater than 64%, with the greatest being 91.75% for St6-3E pretreated samples hydrolyzed with cellulase at 30 FPU/g dry biomass plus xylanase. Pretreatment enhanced cellulose conversion for all three germplasms and the increase was statistically significant (P < 0.05) for St6-3E. Compared with delignification, it is inferred that removing hemicellulose sheathing from the lignocellulosic matrix is a more effective approach for improving the cellulose conversion efficiency during enzymatic hydrolysis. The addition of cellulase significantly improved the hydrolysis efficiency (P < 0.05) for all three germplasms; while the differences between 15 and 30 FPU were not significant (P > 0.05). Xylanase addition was not a significant factor in improving cellulose conversion efficiency for all three germplasms (Table 3.9). Specifically, by
comparing the hydrolysis efficiency between samples hydrolyzed with and without xylanase at two cellulase activity levels (15 and 30 FPU), no improvement (P > 0.05) in cellulose conversion was observed statistically.

Table 3. 9 P-values of ANOVA type III test for hydrolysis of the greatest-hemicellulose-solubilization dilute acid pretreated oven-dried switchgrass

<table>
<thead>
<tr>
<th>Factors</th>
<th>St6-1</th>
<th>St6-3E</th>
<th>St6-3F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment</td>
<td>0.0695</td>
<td>0.0119</td>
<td>0.0597</td>
</tr>
<tr>
<td>Xylanase addition</td>
<td>0.4170</td>
<td>0.9254</td>
<td>0.7448</td>
</tr>
<tr>
<td>Cellulase addition (FPU)</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Xylanase addition * FPU</td>
<td>0.3552</td>
<td>0.0902</td>
<td>0.0693</td>
</tr>
</tbody>
</table>

No monomeric sugars were detected in the liquid fraction of the control in which pretreated samples were soaked in the hydrolysis buffer without adding any enzymes. This might be due to the complete removal of free monomeric sugars from the biomass surface by washing with deionized water after acid pretreatment.

3. Xylan conversion during enzymatic hydrolysis

The conversion efficiency of xylan to xylose during hydrolysis was studied and the corresponding conversion levels are presented in Table 3.10.

During hydrolysis of unpretreated biomass, xylose was detected in the hydrolyzate with xylan conversion efficiency ranging from 31.31 to 41.22%. However, it was interesting to notice that the xylan conversion efficiency of pretreated samples was not significantly different (P > 0.05) from that of the unpretreated samples, except for the pretreated least-lignin-content St6-1 and St6-3F germplasms. The addition of xylanase did not significantly enhance xylan conversion (P > 0.05), whereas the addition of cellulase statistically affected xylan hydrolysis (P < 0.05). This could be because cellulase enzyme complex may contain sufficient
Table 3. 10 Xylan conversion efficiency (%) during enzymatic hydrolysis

<table>
<thead>
<tr>
<th>Hydrolysis conditions</th>
<th>Germlasms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>St6-1</td>
</tr>
<tr>
<td>0 FPU, unpretreated</td>
<td>38.68¹</td>
</tr>
<tr>
<td>15 FPU, unpretreated</td>
<td>32.54</td>
</tr>
<tr>
<td>30 FPU, unpretreated</td>
<td>31.31</td>
</tr>
<tr>
<td>0 FPU, pretreated</td>
<td>0.00</td>
</tr>
<tr>
<td>15 FPU, pretreated</td>
<td>26.29</td>
</tr>
<tr>
<td>30 FPU, pretreated</td>
<td>21.91</td>
</tr>
<tr>
<td>0 FPU+xylanase, pretreated</td>
<td>0.00</td>
</tr>
<tr>
<td>15 FPU+xylanase, pretreated</td>
<td>26.29</td>
</tr>
<tr>
<td>30 FPU+xylanase, pretreated</td>
<td>40.90</td>
</tr>
</tbody>
</table>

Least lignin content switchgrass

Greatest hemicellulose solubilized switchgrass

<table>
<thead>
<tr>
<th>Hydrolysis conditions</th>
<th>Least lignin content switchgrass</th>
<th>Greatest hemicellulose solubilized switchgrass</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>St6-1</td>
<td>St6-3E</td>
</tr>
<tr>
<td>0 FPU, pretreated</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>15 FPU, pretreated</td>
<td>26.29</td>
<td>44.43</td>
</tr>
<tr>
<td>30 FPU, pretreated</td>
<td>21.91</td>
<td>71.76</td>
</tr>
<tr>
<td>0 FPU+xylanase, pretreated</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>15 FPU+xylanase, pretreated</td>
<td>26.29</td>
<td>44.43</td>
</tr>
<tr>
<td>30 FPU+xylanase, pretreated</td>
<td>40.90</td>
<td>76.89</td>
</tr>
</tbody>
</table>

Note. ¹ All values are expressed as percent xylan conversion to xylose.

xylanase activities for the amount of substrate (xylan) available and any subsequent xylanase addition is not required. It has been reported in previous studies that high xylan conversion efficiency could be achieved by cellulase digestion alone without xylanase during hydrolysis (Alizadeh et al., 2005; Chen et al., 2007). Soaking the unpretreated switchgrass in citrate buffer resulted in 38.68-41.22% xylan conversion potentially due to the release of free xylose molecules from the surface of the initial biomass.

3.3.4 Fermentation

All hydrolyzate samples were anaerobically fermented and analyzed chromatographically to evaluate the potential of bioethanol yield from switchgrass. Ethanol yield was calculated and is presented as ‘g ethanol/g initial biomass’ in Figures 3.8 and 3.9.

1. Fermentation of hydrolyzates from the pretreated least-lignin-content biomass

Ethanol yields ranging from 0.0493 to 0.0807 g/g initial biomass were
obtained from hydrolyzates of least-lignin-content pretreated switchgrass samples. No ethanol was detected in hydrolyzates of pretreated samples hydrolyzed in the absence of enzymes (Figure 3.8).

![Figure 3.8 Ethanol yields of unpretreated and pretreated oven-dried samples with the least lignin content after pretreatment for each germplasm. Columns are grouped by the same cellulase activity levels during hydrolysis for each switchgrass germplasm.](image)

Pretreatment had a significant effect on ethanol yield for St6-3F (P < 0.05). For St6-3E samples, improvement in ethanol yield was also noticed in pretreated samples when cellulolytic enzymes were present for hydrolysis, however the effect of pretreatment was not significant. Acid-treated St6-3F switchgrass produced approximately 2-5 times more ethanol than the unpretreated samples, indicating that increasing the digestibility of the biomass through dilute acid pretreatment was effective at enhancing ethanol yield for this specific germplasm.

The addition of cellulase statistically enhanced the final ethanol yield (P < 0.05, Table 3.11). Although the effects of increasing cellulase loading from 15 to 30 FPU/g dry biomass and the addition of xylanase did not significantly improve the production of ethanol (P > 0.05); within each germplasm, the greatest ethanol yield was obtained with pretreated biomass that was hydrolyzed at a cellulase loading of 30
FPU/g dry biomass with the supplementation of xylanase.

Table 3. 11 P-values of ANOVA type III test for fermentation of the least-lignin-content dilute acid pretreated oven-dried switchgrass

<table>
<thead>
<tr>
<th>Factors</th>
<th>St6-1</th>
<th>St6-3E</th>
<th>St6-3F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment</td>
<td>0.0147$^1$</td>
<td>0.1470</td>
<td>0.0029</td>
</tr>
<tr>
<td>Xylanase addition</td>
<td>0.5446</td>
<td>0.6673</td>
<td>0.7733</td>
</tr>
<tr>
<td>Cellulase addition (FPU)</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

Note. $^1$The significant difference in ethanol yield between non-pretreated and pretreated samples when no enzymes were added greatly contributes to reducing this p-value (P < 0.05). In the presence of enzymes at either 15 or 30 FPU, pretreatment is not a significant factor for St6-1 switchgrass sample.

HPLC analysis indicated that for all the acid-treated samples, glucose released during enzymatic hydrolysis was almost completely consumed by yeast after 48 h of fermentation, but there was no obvious change in xylose content during fermentation. Therefore, the yeast being used in this study (Saccharomyces cerevisiae, ATCC 24859) was considered a robust culture which was able to efficiently consume the glucose substrate to produce ethanol although it was not capable of simultaneously converting xylose to ethanol. Additionally, in this study, all pretreated switchgrass samples displayed high fermentation efficiency possibly due to the effective washing of biomass after acid pretreatment. The complete water wash seemed to have removed the potential fermentation inhibitors such as furfurals which were generated during acid pretreatment.

2. Fermentation of hydrolyzates from the pretreated greatest-hemicellulose-solubilization biomass

Ethanol yields ranging from 0.0350 to 0.0819 g/g initial biomass were obtained from hydrolyzates of high hemicellulose solubilized pretreated switchgrass samples. Almost no ethanol was detected in hydrolyzates of pretreated samples in the
absence of enzymes except that of St6-3F pretreated samples, 0.45 g/L ethanol was detected (Figure 3.9). Since no glucose substrate was found in this hydrolyzate before yeast fermentation, the elution peak which was present in the chromatogram at similar elution time as ethanol could possibly be due to other unidentified components available in the fermentation broth, and it requires further investigation.

Acid pretreatment, which effectively removes hemicellulose from plant cell wall, enhanced the biomass-to-bioethanol conversion for St6-3E and St6-3F switchgrass. The improvement was statistically significant for St6-3F (P < 0.05) as the ethanol yield from the hydrolyzate obtained with cellulase at 30 FPU/g dry biomass reached 0.079 g/g initial biomass compared with only 0.010 g of ethanol obtained from unpretreated biomass hydrolyzed at similar cellulase loadings. The addition of cellulase significantly influenced the ethanol yield for all three germplasms (P < 0.05). Furthermore, increasing cellulase activity from 15 to 30 FPU/g dry biomass during hydrolysis significantly promoted ethanol production for
the St6-3F germplasm (P < 0.05). Adding xylanase during hydrolysis did not impact the final ethanol yield statistically (P > 0.05, Table 3.12). Apart from the presence of adequate amount of xylanase activity in the cellulase complex already, this effect may be attributed to the inability of conventional yeast to ferment xylose due to which generation of any additional xylose during hydrolysis does not necessarily improve ethanol yield.

Table 3. 12 P-values of ANOVA type III test for fermentation of the greatest-hemicellulose-solubilization dilute acid pretreated oven-dried switchgrass

<table>
<thead>
<tr>
<th>Factors</th>
<th>P values in ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>St6-1</td>
</tr>
<tr>
<td>Pretreatment</td>
<td>0.0066</td>
</tr>
<tr>
<td>Xylanase addition</td>
<td>0.9703</td>
</tr>
<tr>
<td>Cellulase addition (FPU)</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

Note. ¹The significant difference in ethanol yield between non-pretreated and pretreated samples when no enzymes were added greatly contributes to reducing this p-value (P < 0.05). In the presence of enzymes at either 15 or 30 FPU, pretreatment is not a significant factor for St6-1 switchgrass sample.

It was interesting to notice that 0.052–0.059 g ethanol could be produced from 1 g of raw St6-1 switchgrass through direct hydrolysis and fermentation without dilute acid pretreatment, and this yield was similar to that obtained from the pretreated St6-1 switchgrass sample. Comparing the ethanol yield from acid pretreated samples with the least lignin content and greatest hemicellulose solubilization, it was found that these two optimized pretreatments resulted in similar ethanol yield (0.070–0.080 g ethanol/g initial biomass) for St6-3F switchgrass when hydrolyzed by cellulase at 30 FPU/g dry biomass. However, the most severe pretreatment condition (60 min and 1.5% H2SO4), under which the greatest hemicellulose solubilization was achieved for St6-3E switchgrass (and also the greatest for all germplasms), resulted in a much greater ethanol yield (0.075-0.082 g ethanol/g initial biomass) compared with the
least-lignin-content counterpart (0.052-0.062 g ethanol/g initial biomass) within this germplasm.

3. Ethanol production evaluation

Among all three new switchgrass germplasms, St6-3E and St6-3F displayed good potential for bioethanol production if appropriate dilute acid pretreatment is applied to the oven-dried samples before hydrolysis and fermentation.

The greatest ethanol yield in this study was 0.082 g ethanol/g initial biomass (considering glucose fermentation only). It was less than the optimized yield (0.2 g ethanol/g dry biomass) reported by Alizadeh et al. (2005) in which the switchgrass feedstock was first pretreated by ammonia fiber explosion (AFEX) and then simultaneously saccharified and fermented (SSF) with a non pentose fermenting yeast strain. Regarding each germplasm, the optimal ethanol yield from acid-pretreated switchgrass for St6-1, St6-3E and St6-3F were 40.7%, 53.5% and 49.0% of theoretical, respectively. These were less than 72% of the theoretical ethanol yield obtained from SSF on lime-pretreated switchgrass (Chang et al., 2001). Apart from the difference that this study evaluated separate hydrolysis and fermentation, the less ethanol yield could be due to less lignin removal and some loss of cellulose during dilute acid pretreatment.

3.4 Conclusion

This study investigated the potential of three new switchgrass germplasms for bioethanol production through dilute sulfuric acid pretreatment and subsequent enzymatic hydrolysis. The three germplasms contained 26.65-29.28% glucan, 17.92-19.37% xylan and 17.74-19.23% lignin.

The most effective dilute acid pretreatment conditions selected for subsequent hydrolysis were based on least lignin content or greatest hemicellulose solubilization as a result of various acid pretreatments (3 acid concentrations × 3
residence times) for each germplasm. The pretreatments yielding greatest
ehemicellulose solubilization generally involved severe conditions such as longer
residence time (60 min) and greater acid concentration (1.5% H₂SO₄), whereas those
providing least lignin contents were less intense with the effect of acid pretreatment
on delignification not being apparent. Hydrolysis and fermentation of the pretreated
biomass indicated that generally, the selected pretreatments were effective at
enhancing the subsequent enzymatic hydrolysis and final yield of ethanol. Glucose
produced during enzymatic hydrolysis was almost completely consumed during
fermentation of the hydrolyzate from acid-treated biomass and the greatest ethanol
yield of 0.08 g ethanol/g initial St6-3E or St6-3F biomass averaged approximately 50%
of theoretical value. These results demonstrate that the new switchgrass cultivars
being studied can be potential energy crops for producing bioethanol through dilute
sulfuric acid pretreatment. However, scale up studies and economic analysis is
required to draw further conclusions.
References


CHAPTER 4 Saccharification and Fermentation of Dilute Acid Pretreated Freeze-dried Switchgrass Germplasms

4.1 Introduction

Due to the rapid growth in global energy demand and serious concerns over greenhouse gas emissions, there is an urgent need to develop renewable energy sources and improve the efficiency of energy generation processes. Since liquids are still considered the most important fuels for transportation, the development of alternative environment-friendly fuels capable of widely competing with petroleum-based liquids is being considered as a breakthrough that can facilitate the world’s going green. Among the various types of renewable energy sources such as biomass, solar, wind and hydro power, biomass-derived biofuels have emerged as competitive candidates which met over 3 percent of the United States energy needs in 2005, thus surpassing hydropower to become the No. 1 renewable energy resource in the US (Perlack et al., 2005).

Biomass refers to all plant and animal-derived materials which show potential as sources for generating renewable energy to fuel the world. Besides its role as a food source, plant biomass can be converted to biofuels to reduce the burden on fossil fuels and meet the continuously increasing world energy demand. Biomass-based fuels broadly refer to biodiesel and bioethanol. According to the reports released by USDA and National Biodiesel Board in 2008, biodiesel production in the US, which is primarily dependent on soybean oil, reached approximately 900 million liters in 2006 and is expected to maintain an upward trend to achieve more than 2 billion liters by 2013. Although currently the commercial production of bioethanol relies largely on conversion of starch from corn kernels in the US and sucrose from sugarcane in Brazil, these two feedstocks tap into food supply chains and are limited by fertile land occupation as well as rigid requirements for climate
An alternative to these feedstocks can be the lignocellulosic biomass which is capable of making bioethanol production more cost-effective due to its significant availability with relatively low input (Von Sivers and Zacchi, 1996). Lignocellulosic feedstocks can be agricultural or forestry residues as well as bioenergy crops such as switchgrass.

Switchgrass is a warm season, perennial grass native to North America and well adapted to the southeast region in the US. It is relatively rich in cellulose (approximately 30% on dry basis) and can provide high biomass yield at a low cost, thus making it suitable for bioethanol production (McLaughlin et al., 1999; van den Oever et al., 2003).

Conversion of switchgrass to bioethanol is made up of three steps, namely pretreatment to interrupt the tight interaction between the main components in the plant cell wall including lignin, hemicellulose and cellulose, hydrolysis to break polysaccharides into reducing sugars and fermentation to convert reducing sugars to ethanol. Dilute sulfuric acid pretreatment at elevated temperature is an extensively investigated method which is used to effectively hydrolyze the hemicellulose component in lignocellulosic biomass to soluble sugars, thus increasing the efficiency of subsequent enzymatic digestibility of cellulose (Esteghlalian et al., 1997). Varga et al. (2004) reported that the reduction of hemicellulose in the biomass feedstock such as corn stover could reach 80% during high temperature sulfuric acid pretreatment, while the solubilization and/or degradation of lignin was not as significant as with other chemical pretreatments such as alkaline (Sun and Cheng, 2002; Wyman et al., 2005). Relative to switchgrass, Torget et al. (1990) proposed that approximately 92% of the xylan (the main component of hemicellulose) in switchgrass could be solubilized when pretreated with 0.5% (v/v) sulfuric acid at 140 °C for 60 min or 160 °C for 10 min.

Though acid pretreatment of lignocellulosic biomass has been widely studied, limited work has been done on studying the effect of dilute sulfuric acid pretreatment
on freeze-dried switchgrass germplasms and plant parts. Additionally, the effects of enzyme loadings and combinations on hydrolysis and fermentation as well as inhibitory effects of acid pretreatment need to be studied in depth. Therefore, the objectives of the presented work were to (1) study the effects of dilute sulfuric acid pretreatment on lignin and hemicellulose removal in two new cultivars and an improved germplasm of switchgrass (generally recognized as three germplasms afterward), either as whole plants or specific plant parts (stem and leaf), (2) investigate the effects of cellulolytic enzyme loadings and combinations during enzymatic hydrolysis of least-lignin-content or greatest-hemicellulose-solubilization pretreated samples, and (3) compare the final ethanol yields from all hydrolyzates for an evaluation of the complete switchgrass-to-ethanol process.

4.2 Materials and methods

4.2.1 Switchgrass feedstock

Three germplasms of switchgrass, well adapted to the Southeastern US, were harvested on August 12, 2005 from the Central Crops Research Station (Clayton, NC) for this study. Besides, whole-plant switchgrass samples harvested on July 30, 2007 which showed no statistical differences in composition from those cut in 2005 were also used in some of the experiments. Each switchgrass field was a well established stand representing one of the three germplasms designated as St6-1, St6-3E and St6-3F. Since sampling, St6-3E has been released under the name of ‘BoMaster’ and St6-3F as ‘Performer’ (Burns et al., 2008a; Burns et al., 2008b). Germplasms St6-1 and Bomaster were selected for high dry matter yield; whereas Performer, preferable as cattle feed, was developed for improved nutritive value and digestibility. Performer is reported to be 5 units more digestible than Bomaster as determined by ‘in vitro dry matter digestion (IVDMD)’. Digestibility and dry matter yield are normally not positively associated (Burns et al., 2008b); however, at the time of sampling for this
study, all three entries provided comparable dry matter yield which was approximately 13450 kg/ha (12000 lbs/acre).

The switchgrass harvest strip was taken randomly from each quarter of each field (four strips for one germplasm), weighed, and a sub-sample taken from each strip was used to determine the moisture content and therefore to calculate dry matter yield per unit land area. The four strips, from the same field for one germplasm, were combined to form one bulk sample. Three bulk samples, each representing one specific germplasm, were placed onto a cloth sheet, wrapped and transported to a field laboratory for further processing. Each bulk sample was placed onto a table, thoroughly mixed, and divided into two sub-samples. One sub-sample was left intact and retained as a whole-plant bulk, while the other one was further separated into stem and leaf, thus making two plant-part samples available. The whole, stem and leaf bulk samples were placed into tightly sealed zip-locked plastic bags and submerged into liquid nitrogen for immediate freezing and the cessation of respiration. Samples were removed after about 30 minutes, transferred into a freezer at – 20 °C and held for about 6 days until they were freeze dried. Upon completion of freeze drying, grass samples were reduced in size by grinding through a Wiley mill fitted with a 2 mm screen. Ground samples were collected in tightly sealed zip-locked plastic bags and delivered to the Biological and Agricultural Engineering Department at North Carolina State University, Raleigh, NC where they were stored in a freezer at – 80 °C until analyzed.

4.2.2 Composition analysis

Composition analysis of the whole plant and two plant-part samples for each of the three switchgrass germplasms (totally 9 biomass samples) was conducted in triplicate. Six characteristics of the biomass feedstock were measured as described in 3.2.2 in Chapter 3, namely total solids, ash, extractives, acid insoluble lignin (AIL), acid soluble lignin (ASL), and structural carbohydrates which are mainly made up of
glucan, xylan and arabinan.

4.2.3 Dilute acid pretreatment

Sulfuric acid at 0.5, 1.0 and 1.5% (w/v) was used for chemical pretreatment of the switchgrass whole-plant, stem and leaf samples. A solid-to-liquid ratio of 1:10 was applied by mixing 3.0 g of the grass samples with 30 ml of dilute acid solutions in 100 ml serum bottles for all pretreatments. The mixtures were autoclaved at 121 °C/15 psi under standard liquid cycle for residence times of 30, 45 and 60 min.

Upon completion of the autoclave cycle, samples were removed and allowed to cool to room temperature. The pretreated slurry was filtered through a porcelain Buchner funnel using a filtration assembly. The residual acid was washed from the surface of the pretreated biomass with 250 ml hot deionized water, which was also used to ensure the complete transfer of biomass from the serum bottle to the Buchner funnel. The wet biomass sample left on the Buchner funnel was completely transferred to a pre-weighed zip-locked plastic bag which was then firmly sealed; and the total weight of zip-locked bag plus the wet biomass sample was noted. Two 1.5 g sub-sets of the wet biomass were drawn from each replicate and dried in pre-weighed aluminum weighing dishes in a 105 °C convection oven and 40 °C vacuum oven, respectively. Upon completion of drying after 15 h, the total weight of weighing dishes plus 105 °C dry pretreated biomass was recorded and the moisture content of the pretreated biomass was calculated as:

\[
\text{Percent Moisture} = \left(1 - \frac{W_2 - W}{W_1}\right) \times 100
\]  

(4.1)

Where \(W\) = weight of the aluminum weighing dish (g);

\(W_1\) = weight of the small portion of wet pretreated biomass to be oven dried (g);

\(W_2\) = total weight of the weighing dish and the pretreated biomass after 105 °C drying for 15 h (g).
Percent moisture determined in Equation 4.1 was used to calculate the solid recovery for each pretreated sample. Normally, solid loss due to incomplete transfer of biomass (residue on the filter paper in Buchner funnel) was estimated to be approximately 1% based on tests conducted by weighing the dry filter paper before and after each filtration. Therefore, solid recovery was calculated as:

\[
\text{Percent Solid Recovery} = \left( \frac{W_2 - W_1}{W} \right) \times (1 - \% \text{moisture}) \times 100 + 1^*
\]  

(4.2)

Where \( W_1 \) = weight of the zipper bag (g);
\( W_2 \) = total weight of the zipper bag and wet pretreated biomass (g);
\( W \) = weight of the initial biomass used for pretreatment (g, usually = 3.0);
* represents the solid loss within the Buchner filtering kit.

Since equally weighted wet sub-samples (approximately 1.5 g) were drawn and oven dried at 40 or 105 °C for each pretreated biomass and 105 °C oven dried biomass was considered completely moisture free, percent total solid in the 40 °C oven dried pretreated sample was determined as:

\[
\text{Percent Total Solids} = \frac{W_2 - W_1}{W_4 - W_3} \times 100\% 
\]  

(4.3)

Where \( W_1 \) = weight of aluminum dish for sample drying at 105 °C (g);
\( W_2 \) = total weight of aluminum dish and pretreated biomass after 105 °C oven drying (g);
\( W_3 \) = weight of aluminum dish for sample drying at 40 °C (g);
\( W_4 \) = total weight of aluminum dish and pretreated biomass after 40 °C oven drying (g).

Composition analyses for determination of lignin (both acid insoluble and acid soluble) and carbohydrate were performed on 0.3 g of the 40 °C oven dried pretreated sample based on the procedures described in 3.2.2 of Chapter 3. The 105 °C oven dried samples were not used due to the possibility of disruption of
structural components during heating at elevated temperature. The lignin and carbohydrate (represented by monomeric sugars) contained in the pretreated biomass were expressed on the basis of initial biomass by multiplying the calculated value with the corresponding 'percent solid recovery'. Therefore, the changes in composition due to acid pretreatment could be estimated by comparing values obtained before and after each pretreatment.

Multiple Comparison with the Best (Hsu, 1996) was conducted using SAS® (version 9.1.3, Cary, NC) to identify the most effective pretreatment condition for each germplasm based on least lignin content and greatest hemicellulose solubilization. Triplicate data from the 9 pretreatment conditions (3 acid concentrations × 3 residence times) for each whole-plant or plant part samples of the 3 germplasms was used for the analysis. Six pretreatment conditions based on lignin or hemicellulose (2 within each germplasm) were selected and the specific biomass samples pretreated under these conditions were hydrolyzed and fermented for ethanol production.

4.2.4 Enzymatic hydrolysis

Samples for enzymatic hydrolysis were prepared by repeating the most effective pretreatments selected in this study on the specific switchgrass samples to provide enough pretreated biomass. Procedures for hydrolysis are the same as described in 3.2.4 in Chapter 3.

4.2.5 Fermentation

All the hydrolyzates were anaerobically fermented by *Saccharomyces cerevisiae* (ATCC 24859) as described in 3.2.5 in Chapter 3.

4.2.6 General statistical analysis

Besides ‘Multiple Comparison with the Best (Hsu, 1996)’, analysis of
variance (ANOVA) tables were generated using SAS® (version 9.1.3, Cary, NC) to determine the effects of various factors on pretreatment, hydrolysis and fermentation. A 95% confidence level was applied to all analyses performed in this study. Additionally, Tukey simultaneous tests were performed to test the statistical differences between treatments.

4.3 Results and Discussion

4.3.1 Composition of switchgrass

Three germplasms of freeze-dried switchgrass each with one whole-plant and two plant parts samples (stem and leaf) were analyzed for major compositions (Table 4.1). Besides a moisture content of 1.37 to 2.26%, these switchgrass samples were found to contain 22.71 to 30.95% glucan, 13.27 to 18.58% xylan, 1.54 to 2.46% arabinan and 17.39 to 20.60% lignin on dry matter basis.

Compositions of the stem samples were found to be similar to the whole-plant due to a greater contribution of stem than leaf in the whole-plant sample. It has been reported that the leaf/stem ratio can change with the maturity level of perennial grass and the ratio normally decreases in mature grasses like the samples used in this study (Stone, 1994). Ash and extractives were greater in leaf than in stem; and the germplasm St6-3F had a greater fraction of these components than St6-1 and St6-3E. However, ash and extractives content of the three germplasms was less than commercial switchgrass cultivars like Alamo and Cave-in-Rock as reported in the Biomass Feedstock Composition and Property Database (US Department of Energy, EERE); specifically, extractives determined in this study were approximately 50% of the value given in the database. This difference may be due to difficulties in achieving
<table>
<thead>
<tr>
<th>Composition</th>
<th>St6-1</th>
<th>St6-3E</th>
<th>St6-3F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ash</td>
<td>2.63</td>
<td>2.39</td>
<td>3.97</td>
</tr>
<tr>
<td>Extractives</td>
<td>4.05</td>
<td>5.25</td>
<td>4.33</td>
</tr>
<tr>
<td>Acid insoluble lignin (AIL)</td>
<td>17.14</td>
<td>18.45</td>
<td>16.99</td>
</tr>
<tr>
<td>Acid soluble lignin (ASL)</td>
<td>2.32</td>
<td>1.95</td>
<td>3.61</td>
</tr>
<tr>
<td>Glucan</td>
<td>30.95</td>
<td>30.85</td>
<td>23.21</td>
</tr>
<tr>
<td>Xylan</td>
<td>18.58</td>
<td>18.06</td>
<td>14.82</td>
</tr>
<tr>
<td>Arabinan</td>
<td>2.15</td>
<td>1.95</td>
<td>2.34</td>
</tr>
<tr>
<td>Others</td>
<td>22.18</td>
<td>21.10</td>
<td>30.73</td>
</tr>
</tbody>
</table>

Note. 1Values reported as average percentages on dry matter basis.
complete extraction as extractives in lignocellulosic feedstock are made up of very diverse materials such as waxes, fats, gums, starches, resins and essential oils (Kuhad and Singh, 1993). In addition, the extraction process relies on external factors such as the temperature of the condensers and the siphon rate during extraction (Sluiter et al., 2005). Acid insoluble lignin (AIL), which provides strength and protection for the plant (Brett and Waldron, 1996), formed a predominant portion of the total lignin. Although total lignin content was approximately 18–20% on dry matter basis in all switchgrass samples, stem contained greater AIL and less acid soluble lignin (ASL) than stem. It has been reported that lignin accumulation takes place as stem elongates, and elevated lignin content indicates increased maturity and reduced whole plant digestibility (Walker et al., 1990). Comparing germplasms, total lignin in St6-1 was greater than that in St6-3E and St6-3F.

Glucan and xylan, with a ratio of about 3:2, were two of the most abundant polysaccharides synthesized from glucose and xylose in switchgrass samples and contributed to approximately 40–50% of the dry matter. St6-1 had greatest sugar content while St6-3F contained the least. Within each germplasm, sugar content in stem was similar to that in the whole plant; whereas leaf contained less glucan and xylan but was slightly greater in arabinan, another polysaccharide less than 3% on dry matter basis. It has been reported that although leaf contains smaller quantities of cell wall polysaccharides than stem, elevated extent of plant digestibility can be achieved with a higher leaf-to-stem ratio (Bourquin and Fahey, 1994). Several other components in switchgrass feedstocks were not quantified such as uronic acid, acetyl groups and different minerals (Kaar and Holtzapple, 2000; Samson, 1998; Samson, 2005). In addition, since the composition of switchgrass also depends on factors like harvest time and maturity level (Adler et al., 2006; Dien et al., 2006), certain compositional variances were considered reasonable.
4.3.2 Effect of dilute acid pretreatment

Solids remaining in the nine switchgrass samples (3 germplasms × 3 sample types (1 whole-plant + 2 plant part samples)) after pretreatment with dilute sulfuric acid at 0.5–1.5% (w/v) for 30–60 min were analyzed for sugar and lignin contents and the results are summarized in Tables 4.2, 4.3 and 4.4.

Sulfuric acid pretreatment resulted in 52.13–77.80% solid recovery for all switchgrass samples tested. Generally, solid recovery decreased as acid concentration and treatment time increased. Relative to plant parts, stem samples gave the greatest solid recovery while leaves were the most digestible during acid pretreatment. According to statistical analysis, solid recovery was significantly affected by all four factors: germplasm, plant part, treatment time and acid concentration (P < 0.05) with the effect of acid concentration being the greatest based on the F-value (Appendix Table IIh).

1. Effect of pretreatment on lignin degradation

Lignin has been reported to be very sensitive to sulfuric acid even under mild conditions and both lignin degradation and accumulation can take place simultaneously when lignin and acid interact (Pearl, 1967; Sjostrom, 1993). The acid pretreatment conditions (intense or weak) under which the trend of delignification predominates have been discussed in other studies (Sarkanen and Ludwig, 1971; Liao et al., 2007). Experiments on dilute acid pretreatment of dairy manure showed that lignin accumulation was greater than lignin degradation under all conditions selected (110–130 °C, 1–3 h and 1–3% acid). Lignin content increased from the initial 121.1 mg per gram of raw dairy manure to 160.1 – 179.6 mg/g initial biomass when treatment conditions of acid concentration, temperature and time changed from 1% acid, 110 °C and 1 h to 3% acid, 130 °C and 3 h (Liao et al., 2007). In this study, acid pretreatment of switchgrass resulted in both lignin degradation and accumulation with accumulation being more predominant among the various samples.
Table 4.2 Composition of St6-1 freeze-dried switchgrass after acid pretreatment at different conditions (expressed as g/100g unpretreated dry switchgrass)

<table>
<thead>
<tr>
<th>Plant (part)</th>
<th>Pretreatment conditions</th>
<th>Acid concentration (%)</th>
<th>Time (min)</th>
<th>Total solid</th>
<th>Lignin</th>
<th>Glucose</th>
<th>Xylose</th>
<th>Arabinose</th>
<th>Hemicellulose solubilization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole</td>
<td>Initial sample (unpretreated)</td>
<td>100</td>
<td>30</td>
<td>0.5</td>
<td>73.35 (0.46)</td>
<td>19.48 (0.28)</td>
<td>34.05 (1.03)</td>
<td>20.44 (0.55)</td>
<td>2.37 (0.18)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.0</td>
<td>65.93 (1.13)</td>
<td>18.80 (0.16)</td>
<td>36.18 (3.39)</td>
<td>9.59 (0.77)</td>
<td>0.49 (0.12)</td>
<td>55.81 (3.82)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.5</td>
<td>64.06 (1.67)</td>
<td>19.44 (0.53)</td>
<td>29.84 (0.91)</td>
<td>8.22 (2.75)</td>
<td>0.11 (0.15)</td>
<td>63.50 (11.66)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>45</td>
<td>0.5</td>
<td>69.92 (0.74)</td>
<td>18.90 (0.19)</td>
<td>33.05 (1.91)</td>
<td>12.29 (1.16)</td>
<td>0.63 (0.13)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.0</td>
<td>63.97 (0.56)</td>
<td>18.34 (0.39)</td>
<td>27.97 (3.04)</td>
<td>6.51 (0.82)</td>
<td>0.40 (0.01)</td>
<td>69.70 (3.63)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.5</td>
<td>60.07 (0.10)</td>
<td>18.59 (0.12)</td>
<td>29.33 (4.26)</td>
<td>5.48 (1.11)</td>
<td>0.33 (0.11)</td>
<td>74.53 (5.30)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>60</td>
<td>0.5</td>
<td>67.75 (2.65)</td>
<td>18.82 (0.00)</td>
<td>38.81 (3.66)</td>
<td>14.65 (1.23)</td>
<td>0.58 (0.19)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.0</td>
<td>61.08 (0.22)</td>
<td>18.65 (0.03)</td>
<td>31.62 (0.35)</td>
<td>5.83 (0.19)</td>
<td>0.27 (0.06)</td>
<td>73.22 (1.11)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.5</td>
<td>57.93 (0.96)</td>
<td>20.93 (0.19)</td>
<td>32.93 (3.68)</td>
<td>4.86 (0.74)</td>
<td>0.22 (0.16)</td>
<td>77.69 (2.59)</td>
</tr>
<tr>
<td>Stem</td>
<td>Initial sample (unpretreated)</td>
<td>100</td>
<td>30</td>
<td>0.5</td>
<td>74.72 (1.11)</td>
<td>20.41 (0.30)</td>
<td>33.93 (2.39)</td>
<td>19.87 (1.42)</td>
<td>2.14 (0.31)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.0</td>
<td>68.89 (1.92)</td>
<td>20.76 (0.15)</td>
<td>30.86 (3.76)</td>
<td>11.07 (0.93)</td>
<td>0.50 (0.01)</td>
<td>58.80 (12.67)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.5</td>
<td>66.15 (1.03)</td>
<td>19.59 (0.06)</td>
<td>32.44 (9.73)</td>
<td>8.55 (2.65)</td>
<td>0.51 (0.13)</td>
<td>60.32 (5.18)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>45</td>
<td>0.5</td>
<td>73.50 (0.33)</td>
<td>19.89 (0.18)</td>
<td>31.48 (9.88)</td>
<td>11.71 (3.15)</td>
<td>0.49 (0.01)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.0</td>
<td>67.33 (0.73)</td>
<td>19.62 (0.13)</td>
<td>29.23 (1.99)</td>
<td>6.69 (0.19)</td>
<td>0.42 (0.01)</td>
<td>67.66 (0.81)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.5</td>
<td>64.59 (0.61)</td>
<td>19.55 (0.27)</td>
<td>29.82 (2.51)</td>
<td>5.44 (0.47)</td>
<td>0.31 (0.15)</td>
<td>73.84 (2.80)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>60</td>
<td>0.5</td>
<td>71.37 (1.19)</td>
<td>18.47 (1.27)</td>
<td>39.28 (4.63)</td>
<td>12.31 (1.34)</td>
<td>0.46 (0.01)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.0</td>
<td>63.49 (0.93)</td>
<td>19.72 (0.40)</td>
<td>29.44 (0.95)</td>
<td>5.58 (0.32)</td>
<td>0.29 (0.06)</td>
<td>73.36 (1.72)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.5</td>
<td>61.24 (1.32)</td>
<td>21.30 (0.04)</td>
<td>36.50 (1.61)</td>
<td>5.26 (0.05)</td>
<td>0.22 (0.18)</td>
<td>75.09 (1.00)</td>
</tr>
</tbody>
</table>
Table 4.2 — Continued

<table>
<thead>
<tr>
<th>Plant (part)</th>
<th>Pretreatment conditions</th>
<th>Acid concentration (%)</th>
<th>Time (min)</th>
<th>Total solid(^1)</th>
<th>Composition of solid fractions (%)</th>
<th>Hemicellulose(^1) solubilization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lignin (0.95)</td>
<td>Glucose (6.23)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Xylose (2.13)</td>
<td>Arabinose (0.27)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf</td>
<td>Initial sample (unpretreated)</td>
<td>100</td>
<td>20.60 (0.39)</td>
<td>25.53 (0.95)</td>
<td>16.30 (0.54)</td>
<td>2.57 (0.18)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.5</td>
<td>70.70 (1.09)</td>
<td>22.54 (0.18)</td>
<td>29.68 (6.23)</td>
<td>10.92 (2.13)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>63.03 (0.47)</td>
<td>21.51 (0.13)</td>
<td>18.82 (1.38)</td>
<td>4.43 (0.58)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>60.09 (2.56)</td>
<td>21.54 (0.11)</td>
<td>26.53 (3.38)</td>
<td>5.10 (1.82)</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>0.5</td>
<td>74.57 (0.58)</td>
<td>21.80 (0.20)</td>
<td>24.74 (0.86)</td>
<td>12.46 (0.43)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>62.69 (0.75)</td>
<td>21.31 (0.20)</td>
<td>19.03 (6.14)</td>
<td>3.91 (1.28)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>59.34 (0.37)</td>
<td>21.13 (0.24)</td>
<td>24.59 (0.45)</td>
<td>4.15 (0.07)</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.5</td>
<td>71.43 (1.15)</td>
<td>21.39 (0.07)</td>
<td>34.84 (4.33)</td>
<td>14.21 (2.19)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>58.92 (0.37)</td>
<td>21.12 (0.15)</td>
<td>22.90 (2.83)</td>
<td>4.05 (0.64)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>57.14 (0.80)</td>
<td>21.86 (0.97)</td>
<td>33.32 (1.60)</td>
<td>4.75 (0.20)</td>
</tr>
</tbody>
</table>

Note. \(^1\)Expressed as Mean value (Standard deviation).
\(^2\)Least lignin availability observed for this germplasm after pretreatment.
\(^3\)Greatest hemicellulose solubilization observed for this germplasm after pretreatment.
Table 4. Composition of St6-3E freeze dried switchgrass after acid pretreatment at different conditions (expressed as g/100g unpretreated dry switchgrass)

<table>
<thead>
<tr>
<th>Plant (part)</th>
<th>Pretreatment conditions</th>
<th>Time (min)</th>
<th>Acid concentration (%)</th>
<th>Total solid</th>
<th>Lignin</th>
<th>Glucose</th>
<th>Xylose</th>
<th>Arabinose</th>
<th>Hemicellulose solubilization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole</td>
<td>Initial sample (unpretreated)</td>
<td>100</td>
<td>17.93 (0.24)</td>
<td>31.55 (3.10)</td>
<td>19.92 (2.13)</td>
<td>2.35 (0.16)</td>
<td>57.09 (15.56)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.5</td>
<td>71.24 (1.22)</td>
<td>19.68 (0.58)</td>
<td>22.46 (9.24)</td>
<td>9.16 (3.21)</td>
<td>0.39 (0.26)</td>
<td>57.02 (8.04)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>65.40 (1.11)</td>
<td>19.05 (0.30)</td>
<td>31.25 (5.90)</td>
<td>8.93 (1.58)</td>
<td>0.64 (0.21)</td>
<td>57.02 (8.04)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>62.57 (0.25)</td>
<td>19.00 (0.64)</td>
<td>26.37 (6.73)</td>
<td>6.35 (1.26)</td>
<td>0.34 (0.11)</td>
<td>69.96 (6.13)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>0.5</td>
<td>71.06 (1.27)</td>
<td>18.83 (0.36)</td>
<td>29.95 (2.74)</td>
<td>10.06 (1.84)</td>
<td>0.55 (0.13)</td>
<td>52.37 (8.42)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>61.50 (1.01)</td>
<td>18.70 (0.11)</td>
<td>30.16 (1.78)</td>
<td>7.11 (0.32)</td>
<td>0.48 (0.11)</td>
<td>65.91 (1.97)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>57.58 (1.34)</td>
<td>18.08 (0.06)</td>
<td>25.06 (2.09)</td>
<td>4.37 (0.13)</td>
<td>0.20 (0.01)</td>
<td>79.45 (0.64)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.5</td>
<td>71.68 (4.42)</td>
<td>18.17 (0.55)</td>
<td>30.25 (0.82)</td>
<td>19.22 (0.54)</td>
<td>1.92 (0.00)</td>
<td>56.57 (4.40)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>69.09 (0.60)</td>
<td>18.64 (0.11)</td>
<td>32.56 (2.47)</td>
<td>5.75 (0.32)</td>
<td>0.30 (0.00)</td>
<td>72.86 (1.42)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>59.75 (0.98)</td>
<td>19.44 (0.51)</td>
<td>30.86 (7.08)</td>
<td>4.26 (1.00)</td>
<td>0.11 (0.12)</td>
<td>80.39 (3.91)</td>
<td></td>
</tr>
<tr>
<td>Stem</td>
<td>Initial sample (unpretreated)</td>
<td>100</td>
<td>17.93 (0.24)</td>
<td>31.55 (3.10)</td>
<td>19.92 (2.13)</td>
<td>2.35 (0.16)</td>
<td>57.09 (15.56)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.5</td>
<td>73.15 (0.49)</td>
<td>19.25 (0.35)</td>
<td>20.59 (2.17)</td>
<td>8.85 (0.79)</td>
<td>0.33 (0.14)</td>
<td>54.21 (9.63)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>66.95 (0.56)</td>
<td>18.32 (0.41)</td>
<td>31.71 (5.94)</td>
<td>9.11 (1.93)</td>
<td>0.57 (0.12)</td>
<td>67.46 (4.83)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>63.44 (1.07)</td>
<td>18.18 (0.25)</td>
<td>25.03 (3.81)</td>
<td>6.54 (0.91)</td>
<td>0.34 (0.11)</td>
<td>74.33 (1.24)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>0.5</td>
<td>69.89 (0.46)</td>
<td>18.35 (0.04)</td>
<td>30.64 (4.02)</td>
<td>12.33 (1.49)</td>
<td>0.63 (0.13)</td>
<td>38.69 (7.58)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>63.31 (1.08)</td>
<td>18.09 (0.29)</td>
<td>30.69 (1.11)</td>
<td>7.49 (0.43)</td>
<td>0.42 (0.00)</td>
<td>62.60 (2.06)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>59.95 (0.43)</td>
<td>18.68 (0.56)</td>
<td>28.37 (1.74)</td>
<td>5.04 (0.27)</td>
<td>0.38 (0.01)</td>
<td>74.33 (1.24)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.5</td>
<td>70.11 (5.05)</td>
<td>18.14 (0.58)</td>
<td>40.12 (4.40)</td>
<td>14.67 (4.18)</td>
<td>0.71 (0.28)</td>
<td>27.25 (21.03)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>59.85 (0.51)</td>
<td>16.90 (0.89)</td>
<td>23.93 (0.74)</td>
<td>5.29 (0.21)</td>
<td>0.27 (0.06)</td>
<td>73.71 (1.11)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>59.44 (1.45)</td>
<td>18.50 (0.68)</td>
<td>29.31 (5.37)</td>
<td>4.29 (0.68)</td>
<td>0.26 (0.01)</td>
<td>78.50 (3.30)</td>
<td></td>
</tr>
</tbody>
</table>
Table 4.3 — Continued

<table>
<thead>
<tr>
<th>Plant (part)</th>
<th>Pretreatment conditions</th>
<th>Acid concentration (%)</th>
<th>Time (min)</th>
<th>Total solid</th>
<th>Lignin</th>
<th>Glucose</th>
<th>Xylose</th>
<th>Arabinose</th>
<th>Hemicellulose solubilization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf</td>
<td>Initial sample (unpretreated)</td>
<td>100</td>
<td></td>
<td></td>
<td>17.76 (1.13)</td>
<td>27.17 (2.22)</td>
<td>16.51 (1.59)</td>
<td>2.71 (0.27)</td>
<td>19.22 (2.46)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.5</td>
<td>78.23 (1.60)</td>
<td>21.50 (0.31)</td>
<td>26.93 (1.46)</td>
<td>14.25 (0.30)</td>
<td>1.27 (0.17)</td>
<td>19.22 (2.46)</td>
<td>69.73 (12.59)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>60.42 (0.62)</td>
<td>19.38 (0.20)</td>
<td>21.00 (7.94)</td>
<td>5.42 (2.23)</td>
<td>0.40 (0.20)</td>
<td>69.73 (12.59)</td>
<td>76.12 (7.05)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>57.76 (0.14)</td>
<td>20.01 (0.39)</td>
<td>20.90 (5.11)</td>
<td>4.34 (1.30)</td>
<td>0.25 (0.11)</td>
<td>76.12 (7.05)</td>
<td>76.62 (2.16)</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>0.5</td>
<td>72.71 (0.49)</td>
<td>20.27 (0.11)</td>
<td>24.96 (1.74)</td>
<td>11.30 (1.15)</td>
<td>0.89 (0.28)</td>
<td>36.60 (7.33)</td>
<td>70.25 (1.19)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>60.87 (3.71)</td>
<td>19.20 (0.05)</td>
<td>25.07 (1.23)</td>
<td>5.38 (0.15)</td>
<td>0.34 (0.11)</td>
<td>70.25 (1.19)</td>
<td>48.28 (10.96)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>54.89 (0.53)</td>
<td>19.92 (0.10)</td>
<td>26.42 (0.79)</td>
<td>4.20 (0.31)</td>
<td>0.30 (0.10)</td>
<td>76.62 (2.16)</td>
<td>74.92 (1.99)</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.5</td>
<td>61.58 (4.59)</td>
<td>19.80 (0.30)</td>
<td>32.93 (2.62)</td>
<td>9.24 (2.12)</td>
<td>0.70 (0.18)</td>
<td>48.28 (10.96)</td>
<td>83.99 (2.95)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>55.34 (0.41)</td>
<td>19.44 (0.25)</td>
<td>26.89 (2.25)</td>
<td>4.50 (0.33)</td>
<td>0.32 (0.05)</td>
<td>74.92 (1.99)</td>
<td>83.99 (2.95)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>53.05 (0.16)</td>
<td>19.72 (0.38)</td>
<td>23.72 (3.65)</td>
<td>2.91 (0.52)</td>
<td>0.17 (0.04)</td>
<td>83.99 (2.95)</td>
<td>83.99 (2.95)</td>
</tr>
</tbody>
</table>

Note. ¹Expressed as Mean value (Standard deviation).
²Least lignin availability observed for this germplasm after pretreatment.
³Greatest hemicellulose solubilization observed for this germplasm after pretreatment.
Table 4.4 Composition of St6-3F freeze-dried switchgrass after acid pretreatment at different conditions (expressed as g/100g unpretreated dry switchgrass)

<table>
<thead>
<tr>
<th>Plant (part)</th>
<th>Pretreatment conditions</th>
<th>Acid concentration (%)</th>
<th>Total solid(^{1}) (%)</th>
<th>Composition of solid fractions(^{1}) (%)</th>
<th>Hemicellulose(^{1}) solubilization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole</td>
<td>Initial sample (unpretreated)</td>
<td>100</td>
<td>17.39 (0.08)</td>
<td>26.77 (1.79) 16.15 (1.05) 2.20 (0.17)</td>
<td>36.81 (7.49)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.5</td>
<td>71.91 (0.40)</td>
<td>18.66 (0.50) 22.18 (2.15) 11.02 (1.26)</td>
<td>0.57 (0.12) 36.81 (7.49)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>60.24 (1.20)</td>
<td>16.17 (0.24) 24.98 (3.61) 6.98 (1.04)</td>
<td>0.45 (0.09) 59.51 (6.14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>57.93 (0.11)</td>
<td>16.72 (0.38) 25.75 (3.06) 5.62 (0.46)</td>
<td>0.38 (0.02) 67.31 (2.58)</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>0.5</td>
<td>63.94 (1.35)</td>
<td>16.06 (0.17) 20.30 (2.14) 7.51 (0.90)</td>
<td>0.35 (0.12) 57.14 (5.55)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>58.02 (0.69)</td>
<td>16.08 (0.07) 21.63 (2.82) 5.10 (0.73)</td>
<td>0.26 (0.12) 70.82 (4.60)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>55.77 (1.09)</td>
<td>15.95 (0.23) 23.58 (2.17) 4.33 (0.36)</td>
<td>0.19 (0.00) 75.41 (2.00)</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.5</td>
<td>59.80 (2.08)</td>
<td>18.63 (0.38) 18.87 (1.62) 4.10 (0.48)</td>
<td>0.20 (0.01) 76.56 (2.67)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>59.50 (0.47)</td>
<td>16.86 (0.14) 21.46 (1.02) 3.84 (0.32)</td>
<td>0.19 (0.00) 78.01 (1.72)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>54.23 (0.45)</td>
<td>17.22 (0.26) 31.07 (1.08) 4.20 (0.15)</td>
<td>0.25 (0.01) 75.75 (0.87)</td>
</tr>
<tr>
<td>Stem</td>
<td>Initial sample (unpretreated)</td>
<td>100</td>
<td>17.81 (0.22)</td>
<td>26.73 (1.57) 16.67 (1.06) 1.69 (0.34)</td>
<td>40.87 (22.53)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.5</td>
<td>76.76 (0.28)</td>
<td>19.50 (0.08) 19.76 (6.92) 10.32 (3.88)</td>
<td>0.54 (0.27) 40.87 (22.53)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>64.89 (0.34)</td>
<td>17.48 (0.25) 23.36 (5.15) 6.90 (1.56)</td>
<td>0.35 (0.12) 60.49 (9.09)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>62.75 (0.48)</td>
<td>17.48 (0.14) 30.52 (7.29) 7.47 (2.09)</td>
<td>0.46 (0.11) 56.81 (11.95)</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>0.5</td>
<td>70.77 (0.43)</td>
<td>18.56 (0.26) 22.73 (1.58) 9.74 (0.60)</td>
<td>0.46 (0.02) 44.47 (3.18)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>62.20 (0.85)</td>
<td>17.79 (0.24) 31.42 (3.05) 7.42 (0.58)</td>
<td>0.40 (0.00) 57.41 (3.16)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>60.18 (0.89)</td>
<td>18.34 (0.34) 29.12 (1.66) 5.56 (0.35)</td>
<td>0.39 (0.01) 67.57 (1.93)</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.5</td>
<td>64.28 (0.58)</td>
<td>18.88 (0.25) 19.79 (3.77) 5.27 (1.21)</td>
<td>0.29 (0.13) 69.75 (7.30)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>59.78 (0.90)</td>
<td>17.81 (0.08) 19.20 (1.81) 3.73 (0.06)</td>
<td>0.20 (0.00) 78.57 (0.35)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>57.49 (1.12)</td>
<td>17.64 (0.29) 26.49 (4.97) 3.78 (0.67)</td>
<td>0.15 (0.13) 78.60 (4.07)</td>
</tr>
<tr>
<td>Plant (part)</td>
<td>Pretreatment conditions</td>
<td>Acid concentration (%)</td>
<td>Total solid</td>
<td>Composition of solid fractions (%)</td>
<td>Hemicellulose solubilization (%)</td>
</tr>
<tr>
<td>-------------</td>
<td>-------------------------</td>
<td>------------------------</td>
<td>-------------</td>
<td>-----------------------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td></td>
<td>Time (min)</td>
<td></td>
<td></td>
<td>Lignin</td>
<td>Glucose</td>
</tr>
<tr>
<td>Leaf</td>
<td>Initial sample (unpretreated)</td>
<td>100</td>
<td>17.90 (1.22)</td>
<td>24.98 (2.40)</td>
<td>14.60 (1.36)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.5</td>
<td>77.80 (0.34)</td>
<td>21.73 (0.29)</td>
<td>18.66 (2.03)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>59.20 (0.59)</td>
<td>18.19 (0.10)</td>
<td>16.74 (2.24)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>56.76 (0.52)</td>
<td>18.38 (0.46)</td>
<td>22.84 (6.58)</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>0.5</td>
<td>59.30 (0.44)</td>
<td>16.08 (0.45)</td>
<td>16.97 (2.16)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>57.46 (0.36)</td>
<td>17.85 (1.22)</td>
<td>25.24 (1.70)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>54.24 (0.15)</td>
<td>19.09 (0.28)</td>
<td>23.54 (3.01)</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.5</td>
<td>66.96 (6.03)</td>
<td>20.09 (0.34)</td>
<td>17.70 (2.36)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>55.11 (0.20)</td>
<td>18.46 (0.15)</td>
<td>15.09 (0.26)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>52.13 (0.59)</td>
<td>18.66 (0.37)</td>
<td>22.17 (2.37)</td>
</tr>
</tbody>
</table>

Note. 1Expressed as Mean value (Standard deviation).
2Least lignin availability observed for this germplasm after pretreatment.
3Greatest hemicellulose solubilization observed for this germplasm after pretreatment.
The greatest lignin removal was limited to 5.85%, 6.99% and 8.28% for St6-1 whole plant, St6-3E stem and St6-3F whole plant, respectively. Under many circumstances, the lignin content in pretreated biomass especially leaf samples increased slightly. Lignin accumulation (9.42–21.40%) was usually observed when pretreating leaf samples with the least dilute acid (0.5% H₂SO₄) for the shortest residence time (30 min). In studies conducted by Agblevor et al. (2003), it was reported that other organic and inorganic materials present in the biomass can condense with lignin during a 72% H₂SO₄ treatment. These acid insoluble materials, normally classified as lignin during analysis, may cause the observed lignin content to be greater than the actual value. Since leaf samples of switchgrass contained greatest extractives which may not have been completely removed during mild acid pretreatment, it is possible that the extractives condensed with lignin as the acid insoluble materials and resulted in increasing AIL values.

Another factor possibly impacting the measurement of lignin was spectrophotometric absorbance measurement during acid soluble lignin analysis. Any component, besides acid soluble lignin, contained in the liquid fraction used for ASL determination which can absorb light at a wavelength of 205 nm might contribute to an over estimation of the lignin value (Ehrman, 1996).

The pretreatment conditions under which greater lignin degradation was obtained were not the most intense, and were different for the three germplasms. Statistically, type of plant sample (whole, stem or leaf), acid concentration, residence time and the interaction between two numerical variables (acid*time) all had significant influence (P < 0.05) on lignin content in pretreated samples within each switchgrass germplasm (Table 4.5).

Although little lignin degradation was observed during acid pretreatment, it can be inferred that the structure of lignin was disrupted, thus promoting the interactions between cellulose and cellulolytic enzymes in subsequent saccharification
Table 4.5 P-values of ANOVA type III test for lignin content in dilute acid pretreated freeze-dried switchgrass

<table>
<thead>
<tr>
<th>Factors</th>
<th>St6-1</th>
<th>St6-3E</th>
<th>St6-3F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of plant sample</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Time</td>
<td>0.0005</td>
<td>0.0004</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Acid concentration</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Time * Acid concentration</td>
<td>&lt;.0001</td>
<td>0.0104</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

2. Effect of pretreatment on hemicellulose solubilization

Unlike lignin, hemicellulose (both xylan and arabinan) solubilization was promoted when acid concentration increased and residence time extended. Acid concentration and the interaction term (acid*time) had significant effect on hemicellulose solubilization for all three germplasms (P < 0.05). For St6-3F, the type of plant sample and the treatment time were also critical for predicting the degree of hemicellulose solubilization (P < 0.05, Tables 4.6).

Table 4.6 P-values of ANOVA type III test for percent hemicellulose solubilization in dilute acid pretreated freeze-dried switchgrass

<table>
<thead>
<tr>
<th>Factors</th>
<th>St6-1</th>
<th>St6-3E</th>
<th>St6-3F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of plant sample</td>
<td>0.8920</td>
<td>0.2690</td>
<td>0.0004</td>
</tr>
<tr>
<td>Time</td>
<td>0.0958</td>
<td>0.2712</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Acid concentration</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Time * Acid concentration</td>
<td>0.0048</td>
<td>0.0417</td>
<td>0.0012</td>
</tr>
</tbody>
</table>

Percent hemicellulose solubilization in pretreated samples ranged from 19.32 to 78.21 for St6-1; from 19.22 to 83.99 for St6-3E; and from 36.81 to 85.87 for St6-3F. Solubilization observed for leaf samples were greater than stem. It was observed that approximately 80% of the hemicellulose could be removed from the
lignocellulosic matrix by increasing the intensity of pretreatment to the greatest level (1.5% acid and 60 min), indicating that removal of hemicellulose was much more significant than lignin degradation during acid pretreatment. Studies have demonstrated that theoretically, hemicellulose in the lignocellulosic feedstock can be completely solubilized during high temperature (above 160 °C) dilute acid pretreatment, indicating that hemicellulose is the only component in plant fiber that can be completely solubilized by this specific chemical pretreatment (Nguyen, 1998; Roberto et al., 2003). Studies investigating the effect of temperature during acid pretreatment of switchgrass have shown that when a higher temperature (> 121 °C) is applied, hemicellulose can be more effectively and efficiently removed through greater solubilization (> 90%) within a shorter period (< 10 min) (Torget et al., 1990; Esteghlalian et al., 1997).

The hemicellulose solubilized during acid pretreatment was removed by filtration of the acid solution from the residual pretreated solids. Although the filtrate was not chromatographically analyzed, similar analysis on oven-dried switchgrass samples (Chapter 3) showed that the filtrate was rich in xylose. Since the sheathing of hemicellulose over cellulose forms a physical barrier to enzyme attack, effective hemicellulose solubilization can lead to enhanced digestibility of cellulose, thus increasing enzymatic conversion efficiency during hydrolysis (Hsu, 1996; Mosier et al., 2005).

3. Effect of pretreatment on other cell wall compounds

At least 70% of the cellulose (the main substrate for saccharification to release fermentable sugars) in the residual solids after acid pretreatment was recovered. One exception was 60.4% cellulose (based on glucose analysis) recovery in St6-3F leaf samples. It is assumed that the pretreated biomass contained cellulose with a lower degree of crystallinity and a weaker interaction with lignin and hemicellulose compared with the initial biomass. Generally, glucose content in
pretreated samples decreased as the treatment conditions became more intense relative to acid concentration and treatment time. The type of plant sample and the treatment time significantly ($P < 0.05$) affected glucose content after pretreatment for all three germplasms. Although acid concentration had a significant effect on glucose content in St6-1 and St6-3F, the ‘acid concentration*time’ interaction term was not significant for the pretreated St6-1 switchgrass samples ($P > 0.05$).

Apart from lignin, hemicellulose and cellulose, plant cells contain proteins, ash and extractives such as waxes. In this study, extractives, proteins and ash remaining in the pretreated solids were not quantified but it has been reported that protein content decreases as acid, reaction temperature and time increase during dilute sulfuric acid pretreatment (Liao et al., 2007). Also, certain extractives are removed simultaneously with hemicellulose during acid pretreatment (Nguyen et al., 2000). Chung et al. (2005) have reported that acid pretreatment does not change the percent ash content of pretreated switchgrass as compared with the untreated biomass. Post-pretreatment composition analysis (Table 4.2, 4.3 and 4.4) shows that, the sum of lignin and all three sugars adds up to the total solids (after pretreatment) more closely as treatment conditions become more intense.

4.3.3 Enzymatic hydrolysis

Hydrolysis was conducted on two sets of pretreated samples based on the extent of delignification or hemicellulose solubilization. Samples from each germplasm, irrespective of whether they were whole, stem or leaf, with least lignin and greatest percent hemicellulose solubilization were hydrolyzed by cellulase, cellobiase and xylanase at various activity levels. The ultimate goal of enzymatic hydrolysis in this study was to release the greatest percentage of glucose from the cellulose matrix since glucose is the only monomeric sugar suitable for traditional wild type yeast fermentation.
1. Hydrolysis of the pretreated least-lignin-content biomass

The three pretreatments which provided least lignin content were selected on the basis of a multiple comparison with the best (SAS® 9.1.3, Cary, NC) (Appendix Tables IIi, IIj and IIk). The conditions selected were 45 min and 1.0% acid for St6-1 whole plant; 60 min and 1.0% acid for St6-3E stem; and 45 min and 1.5% acid for St6-3F whole plant. The enzymatic cellulose conversion (ECC%) representing the degree of cellulose to glucose conversion during hydrolysis is summarized in Figure 4.1 which shows the effect of pretreatment, cellulase/cellobiase loadings as well as xylanase addition on reducing sugar yield.

The enzymatic conversion of cellulose in pretreated samples with the least amount of lignin was between 34.62 and 61.18% compared with that ranging from 11.04 to 39.01% for unpretreated biomass. The greatest cellulose conversion observed was only slightly above 60%, and the small value may be because the overall low levels of delignification (< 10%) did not allow sufficient access to the biomass by
enzymes.

No cellobiose was detected in the hydrolyzates, indicating that the cellobiose produced during cellulose hydrolysis was effectively hydrolyzed by cellobiase, therefore preventing end-product inhibition of cellulase. Among the various factors involved in hydrolysis which included (1) untreated vs. pretreatment, (2) cellulase loading (0, 15 and 30 FPU/g dry biomass), (3) addition of xylanase, and (4) the interaction between cellulase loading and presence of xylanase (FPU*xylanase), addition of cellulase significantly affected the cellulose conversion efficiency for each germplasm with P-value less than 0.0001. Pretreatment was a significant factor for St6-3F samples (P < 0.05), and the interaction between cellulase activity levels (FPU) and the addition of xylanase for cellulose conversion efficiency was significant for St6-1 samples (P < 0.05). Although adding cellulase can greatly improve the hydrolysis of cellulosic substrate, significant differences in cellulose conversion efficiency between 15 and 30 FPU/g dry biomass were not observed (P > 0.05) except for the St6-1 whole plant. Addition of xylanase alone did not improve cellulose hydrolysis efficiency statistically (P > 0.05, Table 4.7); however, adding xylanase significantly improved cellulose conversion efficiency from 34.62% to 46.98% when cellulase was added at 15 FPU (P < 0.05) for St6-1 samples. Although the majority

<table>
<thead>
<tr>
<th>Factors</th>
<th>Germplasm</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>St6-1</td>
<td>St6-3E</td>
<td>St6-3F</td>
</tr>
<tr>
<td>Pretreatment</td>
<td>0.0674</td>
<td>0.4443</td>
<td>0.0057</td>
<td></td>
</tr>
<tr>
<td>Xylanase addition</td>
<td>0.2610</td>
<td>0.8383</td>
<td>0.6017</td>
<td></td>
</tr>
<tr>
<td>Cellulase addition (FPU)</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td></td>
</tr>
<tr>
<td>Xylanase addition * FPU</td>
<td>0.0202</td>
<td>0.0733</td>
<td>0.1724</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.7 P-values of ANOVA type III test for hydrolysis of the least-lignin-content dilute acid pretreated freeze-dried switchgrass.
of the hemicellulose had been removed from the biomass matrix during dilute acid pretreatment, the inhibitory effect of residual hemicellulose on cellulose can act as a physical barrier to cellulose conversion (Öhgren et al., 2007). Therefore, xylanase supplementation can be useful in increasing hydrolysis efficiency by reducing inhibitory effect, especially in samples pretreated by non-acidic methods that contain greater hemicelluloses fractions. The cellulase used in this study was a mixture of enzymes containing some xylanase activity which was probably sufficient for hydrolyzing the hemicelluloses to the maximum extent possible. Hence, addition of supplemental xylanase did not improve cellulose hydrolysis.

In the hydrolyzate from control samples without enzymes, pretreated switchgrass did not generate any monomeric sugars (glucose, xylose and arabinose). However, 11.04–14.28% cellulose conversion was detected in the unpretreated switchgrass control samples soaked in citrate buffer without adding any cellulolytic or xylolytic enzymes. This was potentially due to the transfer of free monomeric sugars into the pretreatment liquid and filtrate generated by deionized water washing of residual solids after dilute acid pretreatment, thus leaving no free monomeric sugars to be released from the surface of the pretreated samples during hydrolysis.

2. Hydrolysis of the pretreated greatest-hemicellulose-solubilization biomass

For each germplasm, the pretreatment condition which led to the greatest hemicellulose (sum of xylose and arabinose) solubilization based on a multiple comparison analysis (SAS® 9.1.3, Cary, NC) was selected for subsequent hydrolysis. The treatment conditions were 45 min and 1.0% acid for St6-1 leaf, 60 min and 1.5% acid for St6-3E leaf and 60 min and 1.0% acid for St6-3F leaf (Appendix Tables III, IIIm and IIIn). The efficiency of cellulose conversion for samples with greatest hemicellulose solubilization during acid pretreatment is summarized in Figure 4.2.
Figure 4.2 Hydrolysis of unpretreated and pretreated greatest-hemicellulose-solubilization freeze-dried samples. Columns are grouped by the same cellulase activity levels for each switchgrass germplasm.

Compared with the unpretreated samples that showed a maximum cellulose conversion of 22.82% through hydrolysis of the St6-3F leaf sample with cellulase at 30 FPU/g dry biomass supplemented with xylanase, a much greater cellulose hydrolysis efficiency was observed for all pretreated freeze-dried samples with the greatest value being 106.65%. Yields over 100% can be attributed to inexact measurements during the various experimental analyses and have also been reported in other studies (Öhgren et al., 2007; Chen et al., 2007). The greater cellulose conversion efficiency demonstrated that cellulolytic enzymes were not inhibited by the accumulation of glucose. Statistically, pretreatment significantly (P < 0.05) improved the hydrolysis efficiency of all three germplasms (Table 4.8). Besides acid pretreatment, addition of cellulase (FPU) had a significant influence on cellulose conversion efficiency in all tests conducted (P < 0.05). However, the addition of xylanase and the interaction between cellulase activity and xylanase (FPU*xylanase) did not alter the cellulose hydrolysis (P > 0.05). Although adding cellulase enhanced the hydrolysis, no significant differences (P > 0.05) in the efficiency of cellulose
conversions were observed between 15 and 30 FPU (P > 0.05).

Table 4. 8 P-values of ANOVA type III test for hydrolysis of the greatest-hemicellulose-solubilization dilute acid pretreated freeze-dried switchgrass

<table>
<thead>
<tr>
<th>Factors</th>
<th>St6-1</th>
<th>St6-3E</th>
<th>St6-3F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment</td>
<td>&lt;.0001</td>
<td>0.0001</td>
<td>0.0003</td>
</tr>
<tr>
<td>Xylanase addition</td>
<td>0.5231</td>
<td>0.5304</td>
<td>0.7477</td>
</tr>
<tr>
<td>Cellulase addition (FPU)</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Xylanase addition* FPU</td>
<td>0.1475</td>
<td>0.1761</td>
<td>0.0710</td>
</tr>
</tbody>
</table>

Compared to the hydrolyzates derived from least-lignin-content pretreated biomass, cellulose hydrolysis efficiencies obtained for greatest-hemicellulose solubilized samples were greater (Figure 4.2), indicating that hemicellulose removal rather than delignification was more effective in enhancing cellulose hydrolysis in acid-pretreated switchgrass. Plant part also impacted hydrolysis efficiency. Though slightly less in cellulose than stem, leaf samples showed greater hemicellulose solubilization during acid pretreatment and gave better cellulose hydrolysis efficiency.

No monomeric sugars were detected in the hydrolyzates of pretreated controls soaked in the hydrolysis buffer without enzymes. This might be due to the removal of free monomeric sugars from the biomass surface in the pretreatment liquid and by washing with deionized water after acid pretreatment. One exception was that 0.22% of cellulose-to-glucose conversion was detected in the hydrolyzate of one of the triplicates from soaking pretreated St6-1 leaves in hydrolysis buffer with xylanase but without cellulolytic enzymes.

3. Xylan conversion during enzymatic hydrolysis

The conversion efficiency of xylan to xylose was also analyzed along with cellulose conversion and the results are presented in Table 4.9.
# Table 4: 9 Xylan conversion efficiency (%) during enzymatic hydrolysis

<table>
<thead>
<tr>
<th>Hydrolysis conditions</th>
<th>Germplasms and plant part</th>
<th>St6-1 whole</th>
<th>St6-3E stem</th>
<th>St6-3F whole</th>
<th>St6-1 leaf</th>
<th>St6-3E leaf</th>
<th>St6-3F leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 FPU, unpretreated</td>
<td></td>
<td>25.07&lt;sup&gt;1&lt;/sup&gt;</td>
<td>25.39</td>
<td>21.91</td>
<td>6.74</td>
<td>11.08</td>
<td>16.29</td>
</tr>
<tr>
<td>15 FPU, unpretreated</td>
<td></td>
<td>26.26</td>
<td>34.28</td>
<td>27.95</td>
<td>8.23</td>
<td>12.56</td>
<td>17.55</td>
</tr>
<tr>
<td>30 FPU, unpretreated</td>
<td></td>
<td>27.46</td>
<td>34.28</td>
<td>29.46</td>
<td>10.48</td>
<td>11.82</td>
<td>16.29</td>
</tr>
<tr>
<td>Least lignin content switchgrass</td>
<td>Greatest hemicellulose solubilized switchgrass</td>
<td>0 FPU, pretreated</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>15 FPU, pretreated</td>
<td></td>
<td>23.97</td>
<td>39.06</td>
<td>47.23</td>
<td>52.14</td>
<td>51.95</td>
<td>74.75</td>
</tr>
<tr>
<td>30 FPU, pretreated</td>
<td></td>
<td>41.94</td>
<td>41.36</td>
<td>44.60</td>
<td>58.65</td>
<td>55.66</td>
<td>89.71</td>
</tr>
<tr>
<td>0 FPU+xylanase, pretreated</td>
<td></td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>15 FPU+xylanase, pretreated</td>
<td></td>
<td>35.95</td>
<td>41.36</td>
<td>44.60</td>
<td>55.40</td>
<td>48.24</td>
<td>89.71</td>
</tr>
<tr>
<td>30 FPU+xylanase, pretreated</td>
<td></td>
<td>41.94</td>
<td>41.36</td>
<td>47.23</td>
<td>58.65</td>
<td>55.66</td>
<td>79.74</td>
</tr>
</tbody>
</table>

Note. <sup>1</sup> All values are expressed as percent xylan conversion to xylose.

During hydrolysis of unpretreated switchgrass, xylose was detected in the hydrolyzate with xylan conversion efficiency being less than 35%. Xylan contained in unpretreated samples was less easily hydrolyzed in the presence of cellulolytic enzymes alone. Relative to plant parts, it was found that xylan hydrolysis was less in leaf samples.

For least-lignin-content pretreated samples, xylan conversion was not significantly different from the unpretreated samples (P > 0.05), however, for greatest-hemicellulose-solubilized pretreated samples, pretreatment had a significant effect on xylose yield (P < 0.05). Also, it was found that the addition of cellulase rather than the addition of xylanase greatly affected (P < 0.05) the hydrolysis of xylan to yield xylose. Since a large portion of hemicellulose had been removed from the switchgrass feedstock during acid pretreatment, xylanase activities contained in the cellulolytic enzymes may be sufficient to convert xylan to xylose. It has been reported in previous studies that high xylan conversion could be achieved during hydrolysis.
with cellulolytic enzymes alone (Alizadeh et al., 2005; Chen et al., 2007). To optimize xylose yield from acid pretreatment of the lignocellulosic feedstock, a mass balance calculation which accounts for compositions of both solid and liquid fractions collected after pretreatment is recommended (Chung al., 2005). Damasco et al. (2004) reported that with alkali or thermal pretreatments in which xylan removal was not as significant as with acid pretreatment, xylan hydrolysis by xylanase was enhanced after pretreatment. Soaking the unpretreated biomass in citrate buffer resulted in 6.74-25.39% xylan conversion potentially due to the release of free xylose molecules from the surface of the raw biomass.

4.3.4 Fermentation

All hydrolyzate samples were anaerobically fermented and analyzed chromatographically to evaluate the potential bioethanol yield from switchgrass. Ethanol yield was calculated and presented as ‘g ethanol/g initial biomass’.

1. Fermentation of hydrolyzates from the pretreated least-lignin-content biomass

Ethanol yields ranging from 0.0562 to 0.0826 g/g initial biomass were obtained from hydrolyzates of least lignin content pretreated switchgrass samples. The greatest ethanol yield was observed for St6-3F whole plant which was pretreated at 121 °C/15 psi for 45 min with 1.5% acid and hydrolyzed by 30 FPU cellulase/g dry biomass supplemented with 0.25% (w/w) xylanase. No ethanol was detected in hydrolyzates of pretreated samples generated in the absence of enzymes (control) as no glucose was available (Figure 4.1, 4.3). It was observed that the yeast culture used in this study (Saccharomyces cerevisiae, ATCC 24859) effectively consumed almost all the glucose in the hydrolyzate. The fermentation efficiency also indicated that almost no inhibitory effect on yeast fermentation occurred in this study possibly due to the effective washing of solids after dilute acid pretreatment. Washing is believed to have removed a diverse variety of potential pretreatment-derived inhibitors such as
furfural and 5-hydroxymethylfurfural (5-HMF) (Larsson et al., 1999; Luo et al., 2002).

Figure 4. 3 Ethanol yields of unpretreated and pretreated freeze-dried samples with the least lignin content after pretreatment within each germplasm. Columns are grouped by the same cellulase activity levels during hydrolysis for each switchgrass germplasm.

The addition of cellulase significantly affected the ethanol yield for all three germplasms (P < 0.05). However, although the unpretreated St6-3F samples showed greater cellulose conversion efficiency at a cellulase loading of 30 FPU/g dry biomass compared to 15 FPU/ g dry biomass (Figure 4.1) and correspondingly, greater amount of glucose was generated in hydrolyzates when more cellulase was applied, fermentation results suggested that ethanol production was inefficient at the greater cellulase level. This might be due to a steric hindrance effect between the unpretreated sample and the cellulolytic enzymes (Damasco et al., 2004). It was interesting to note that unpretreated St6-3E stem samples were capable of producing 0.034 g ethanol/g initial biomass upon soaking in the hydrolysis buffer without any enzymes. Presence of cellulolytic enzymes increased ethanol yield to 0.063–0.068 g ethanol/g initial
biomass, which was significantly greater (P < 0.05) than that from the pretreated St6-3E stem samples. The addition of xylanase as a supplement to cellulolytic enzymes did not greatly affect the production of ethanol (P > 0.05, Table 4.10).

<table>
<thead>
<tr>
<th>Factors</th>
<th>St6-1</th>
<th>St6-3E</th>
<th>St6-3F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment</td>
<td>0.4137</td>
<td>0.0003</td>
<td>0.0614</td>
</tr>
<tr>
<td>Xylanase addition</td>
<td>0.5585</td>
<td>0.9263</td>
<td>0.7994</td>
</tr>
<tr>
<td>Cellulase addition (FPU)</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

Note. The significant difference in ethanol yield (P < 0.05) indicates that a greater amount of ethanol can be generated from the unpretreated samples than those pretreated ones for this specific switchgrass sample (St6-3E stem).

2. Fermentation of hydrolyzates from the pretreated samples with greatest hemicellulose solubilization

Ethanol yields from the hydrolyzates of greatest hemicellulose solubilized pretreated freeze-dried biomass (all of them were leaf samples) fell between 0.0576 and 0.0720 g ethanol/g initial biomass, and were similar among three germplasms. However, for the unpretreated sample, the greatest ethanol yield was limited to 0.0282 g/g initial biomass (Figure 4.4). No ethanol was detected in hydrolyzates of pretreated samples hydrolyzed in the absence of enzymes because no glucose, the substrate for fermentation, was released during hydrolysis.

Pretreatment and addition of cellulase significantly affected the ethanol yield (P < 0.05) for all three germplasms. However, statistical differences in ethanol yield between 15 and 30 FPU cellulase level in hydrolysis were not observed (P > 0.05). The addition of xylanase along with the cellulolytic enzymes gave no statistical differences in the fermentation products generated (P > 0.05, Table 4.11).
Figure 4.4 Ethanol yields of unpretreated and pretreated freeze-dried samples with the greatest hemicellulose solubilization after pretreatment within each germplasm. Columns are grouped by the same cellulase activity levels during hydrolysis for each switchgrass germplasm.

Table 4.11 P-values of ANOVA type III test for fermentation of the greatest-hemicellulose-solubilization dilute acid pretreated freeze-dried switchgrass

<table>
<thead>
<tr>
<th>P values in ANOVA</th>
<th>Germplasm</th>
<th>St6-1</th>
<th>St6-3E</th>
<th>St6-3F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factors</td>
<td></td>
<td>&lt;.0001</td>
<td>0.0002</td>
<td>0.0022</td>
</tr>
<tr>
<td>Pretreatment</td>
<td></td>
<td>0.9666</td>
<td>0.8146</td>
<td>0.5128</td>
</tr>
<tr>
<td>Xylanase addition</td>
<td></td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Cellulase addition (FPU)</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td></td>
</tr>
</tbody>
</table>

In this study, all the glucose present in the hydrolyzate was almost depleted by yeast during fermentation. The high fermentation efficiency was due to the effective wash of the acid pretreated solids before hydrolysis and fermentation.

3. Ethanol production evaluation

All three switchgrass germplasms show potential for bioethanol production with the final ethanol yield being around 0.07 g ethanol/g initial biomass. Appropriate...
dilute acid pretreatment is needed for improving the ethanol yield especially when greater hemicellulose solubilization is targeted during pretreatment.

The greatest ethanol yield in this study was 0.083 g ethanol/g initial biomass obtained from the St6-3F whole plant. This is approximately the same as the optimum ethanol yield (0.082 g from St6-3E whole plant, Chapter 3) from oven-dried switchgrass with the same three germplasms being tested.

In this study, the optimum ethanol yield from acid-pretreated switchgrass for oven- and freeze-dried St6-1, St6-3E and St6-3F were found to be 53.8%, 49.3% and 64.7% of theoretical, respectively. Though the optimum ethanol yield from freeze-dried switchgrass was approximately the same as that of the oven-dried, the ethanol production efficiencies relative to theoretical yield were generally greater for freeze-dried samples than for oven dried ones. This might be due to the ability of freeze drying technology to better maintain the biomass cell structure and less degradation of carbohydrates/sugars as with heating. Ethanol production potential of switchgrass has also been discussed in other studies. Chang et al. (2001) reported an ethanol yield of 72% theoretical when switchgrass feedstock was first pretreated by lime and then simultaneously saccharified and fermented (SSF). Additionally, Alizadeh et al. (2005) proposed that 0.2 g ethanol/g dry switchgrass could be obtained by SSF following pretreatment by ammonia fiber explosion (AFEX). The differences in final ethanol yields may be attributed to variations in lignin removal and cellulose loss during dilute acid pretreatment as well as the potential differences between SSF and separate hydrolysis and fermentation (SHF).

4.4 Conclusion

This study investigated the potential of three new switchgrass germplasms for ethanol production by determining the most effective reaction conditions in dilute acid pretreatment and enzymatic hydrolysis. The whole plant of these switchgrass
germplasms was found to contain 24.34–30.95% glucan, 14.68–18.58% xylan and 17.39–17.93% lignin. Compared with stem, leaf samples had slightly less carbohydrate content.

The optimal pretreatment conditions for this study were identified within each germplasm based on the criteria of obtaining least lignin content or greatest hemicelluloses solubilization in biomass upon completion of pretreatment. Acid pretreatment did not result in significant delignification due to the presence of acid-lignin interaction which may lead to simultaneous lignin accumulation and degradation. However, more than 80% of the hemicellulose could be solubilized when relatively intense pretreatment was applied, especially for leaf samples. It has been suggested in other studies that perennial grasses which contain a large stem fraction are more desirable for ethanol production because stems are relatively greater in cellulose and less in ash (Radiotis et al., 1996; Samson and Mehdi, 1998). Based on the present study, which targeted greater hemicellulose removal during acid pretreatment, leaf was preferred since it showed greater digestibility than stem. Generally, the cellulose conversion efficiency was improved by acid pretreatment. Glucose produced during enzymatic hydrolysis was almost completely consumed during fermentation of the acid-treated biomass, indicating that no apparent inhibitors were present during Saccharomyces cerevisiae fermentation if the pretreated biomass was completely washed before hydrolysis and fermentation. The greatest ethanol yield obtained was 0.083 g ethanol derived from 1g of initial St6-3F switchgrass whole plant, which is approximately 65% of theoretical. These results demonstrate that the new switchgrass cultivars being studied can be potential energy crops for producing bioethanol through dilute sulfuric acid pretreatment.
References


Damasco M. C. T., de Castro, A. M., Castro, R. M., Andrade, C., and Pereira, N.,


reactor. Industrial Crops and Products 17, 171-176.


CHAPTER 5  Simultaneous Saccharification and Fermentation (SSF) and Immobilized Yeast Cell Fermentation of Dilute Acid Pretreated Switchgrass

5.1 Introduction

Lignocellulose, the key component of the plant cell wall, is a promising substrate for bioethanol production because of its wide availability and high holocellulose (both cellulose and hemicellulose) content. General lignocellulose-to-ethanol conversion involves three steps, namely pretreatment to disrupt the tight interaction between lignin, hemicellulose and cellulose in the plant cell wall, hydrolysis to break down polysaccharides into reducing sugars and fermentation to convert reducing sugars to ethanol. A variety of lignocellulosic agricultural and forestry feedstocks including crops, residues and energy crops are currently being investigated for their ethanol production potential.

Switchgrass (*Panicum virgatum* L.) is a warm-season, perennial (*C*₄) lignocellulosic grass which has a wide range of geographic adaptation (Phillips and Rix, 2002) and can provide high yields with relatively little input for both establishment and maintenance (van den Oever et al., 2003). Therefore, besides being traditionally used for forage production, switchgrass is being considered as a dedicated lignocellulosic energy crop for bioethanol production (Walsh, 1998).

Numerous studies have been conducted on optimizing the reaction conditions for pretreatment and hydrolysis of lignocellulosic feedstocks, including switchgrass, for greater reducing sugar yields. Dilute acid pretreatment has been shown to effectively remove hemicellulose from the lignocellulose matrix through carbohydrate solubilization, thus improving digestibility of the residual cellulose in pretreated solids (Mosier et al., 2005; Wyman et al., 2005). Increasing the cellulase activity to a certain extent during subsequent hydrolysis can further improve the process by
increasing glucose yield. However, greater cellulase dosage increases cost of hydrolysis which is not advantageous for the processing economy (Sun and Cheng, 2002) and the balance between process efficiency and cost needs to be maintained.

Though pretreatment and hydrolysis have been extensively studied relative to process optimization, fermentation of the lignocellulose hydrolyzate ultimately determines the ethanol yield from the feedstock and reflects any fermentation inhibitions caused by the preceding pretreatment. Normally, fermentation of the hydrolyzate is performed at 30 °C for 48 h with active yeast inoculation (Chapter 3 and 4). Alternative ethanol production methods, namely simultaneous saccharification and fermentation (SSF) and immobilized yeast cell fermentation of hydrolyzates from dilute acid pretreated feedstocks, may be employed to enhance ethanol yields.

SSF combines enzymatic hydrolysis and yeast fermentation into a single step and avoids potential inhibition of cellulolytic enzymes by accumulation of monomeric reducing sugars released during enzymatic hydrolysis as they are simultaneously consumed by yeast to produce ethanol (Saha et al., 2005; Xiao et al., 2004). Wingren et al. (2003) reported that for softwood, overall ethanol yield from SSF was greater than that from separate hydrolysis and fermentation (SHF) with the production costs for SSF and SHF base cases being 0.57 and 0.63 USD/L, respectively. SSF has been applied to switchgrass samples pretreated by various chemicals. For example, the ethanol yield after optimized ammonia fiber explosion (AFEX) pretreatment was about 0.2 g/g dry switchgrass (Alizadeh et al., 2005). Additionally, ethanol yield from lime-pretreated switchgrass has been reported to be 72% of theoretical (Chang et al., 2001). However, certain problems such as (1) difficulties in compromising the operation conditions for this integrated process (Saha et al., 2005) and (2) the inhibition of enzymatic hydrolysis due to ethanol accumulation during SSF have been reported (Wu and Lee, 1997). SSF of dilute acid pretreated switchgrass can facilitate comparison of the ethanol yielding potential with traditional SHF and assess mechanisms for overcoming the above challenges.
Unlike SSF, immobilized yeast cell fermentation is a modified process for fermentation alone. Immobilized cell systems provide greater cell concentrations which lead to increased fermentation efficiency, facilitate separation of the biocatalysts from the liquid phase, and improve protection of the cells against inhibitors (Pilkington et al., 1998). Yeast cell immobilization has been widely used in beer brewing; however, limited researches have been performed on applying this technology to lignocellulose hydrolyzate fermentation.

Therefore, the objectives of this study were to (1) monitor a 7-day-fermentation process to study changes in glucose, xylose and ethanol concentrations for both SSF and immobilized yeast cell fermentation of dilute acid pretreated switchgrass, and (2) compare the ethanol yields obtained in this study with those of separate saccharification and fermentation for evaluating the potential of these alternative fermentation methods in improving the ethanol production process.

5.2 Materials and Methods

5.2.1 Biomass feedstock

The feedstock used for this study was a new switchgrass germplasm obtained from the Central Crops Research Station, near Clayton, NC on July 30, 2007. This new cultivar, designated as St6-3E, was selected for high dry matter yield and has been recently released under the name of ‘BoMaster’ (Burns, 2008). Choice of germplasm and feedstock preparation was based on results of previous studies on oven- or freeze-dried switchgrass-to-bioethanol conversion through acid pretreatment and separate hydrolysis and fermentation, showing that oven-dried St6-3E and freeze-dried St6-3F whole plants gave the highest ethanol yields of 0.0819 and 0.0826 g ethanol/g initial biomass, respectively (Chapters 3 and 4). Since compared with freeze-drying, oven-drying is a more cost-effective method in biomass preparation especially for scaled-up processing, oven-dried St6-3E switchgrass was selected as the
feedstock for this study. After harvest, switchgrass samples were dried in a forced-air oven at 70 °C for appropriately 3 days and reduced in size by grinding through a Wiley mill fitted with a 2 mm screen. Ground samples were collected in tightly sealed zip-locked plastic bags and delivered to the Biological and Agricultural Engineering Department at North Carolina State University, Raleigh, NC where they were stored at room temperature until analyzed. As per composition analysis (Chapter 3), this specific switchgrass cultivar contained 27.24% glucan, 17.92% xylan and 18.98% lignin on dry matter basis.

5.2.2 Dilute acid pretreatment

Switchgrass samples were mixed with 1.5% (w/v) sulfuric acid at a solid loading of 10% and pretreated by autoclaving at standard liquid cycle (121 °C/15 psi) for 60 min. The conditions for pretreatment were determined by analyzing the results of studies on the optimization of dilute acid pretreatment of oven- and freeze-dried switchgrass (Chapter 3 and 4). After pretreatment, the slurry was filtered through a Buchner funnel filtering kit and washed with hot deionized water (approximately 80 ml per gram of dry biomass pretreated) till the filtrate became colorless. The biomass solids remaining in the funnel were collected in zip-lock bags and kept at 4 °C before use. A small portion of the wet solids from each bag were weighed and dried in the 105 °C convection oven to determine the moisture content in acid-treated biomass.

5.2.3 Yeast culture inoculum preparation

Saccharomyces cerevisiae (ATCC 24859) obtained from Dr. Demirci’s Microbiological Engineering Laboratory in the Agricultural and Biological Engineering Department at Pennsylvania State University was used for fermentation in this study. The yeast medium was prepared by dissolving 20 g glucose, 8.5 g yeast extract, 1.32 g NH₄Cl, 0.11 g MgSO₄, and 0.06 g CaCl₂ in 1 L deionized water (Chen et al., 2007). Approximately 1.0 ml of the 30% glycerol yeast stock saved in the –
80 °C freezer was added to 100 ml media and allowed to grow aerobically in a water bath at 30 °C/150 rpm for 48-72 h. Time for activation differed as the growing trend of yeast cells was not consistent for each inoculation. The activated freeze dried cells were harvested by centrifugation at 4 °C/4000 rpm for 10 minutes in an Eppendorf 5810R centrifuge, washed twice with 50 ml 0.1% sterile peptone water to remove residual media and resuspended in 20 ml peptone water. One milliliter of the yeast solution was taken to perform serial dilution up to 10⁵ and 100 μl of the diluted culture was spread-plated onto yeast extract peptone dextrose (YEPD) agar prepared by adding 10 g yeast extract, 20 g peptone, 20 g glucose and 16 g agar per liter of deionized water (Süssmuth et al., 1979). Plates incubated at 30 °C for 72 h and yeast colonies were counted to give an average count of 6.3×10⁷ cfu/ml (approximately 11 g/L).

5.2.4 Simultaneously saccharification and fermentation (SSF)

Simultaneous saccharification and fermentation was performed in 250 ml Erlenmeyer flasks in a water bath at 38 °C and 100 rpm for 7 days. Each flask was equipped with a sterile water trap filled with glycerol to allow CO₂ to escape and avoid air contamination as shown in Figure 5.1.

![Figure 5.1 Equipment for SSF experiment](image)
The volume of the SSF mixture composed of the wet acid-treated grass equivalent to 3 g dry biomass (determined by the moisture content), 6 ml yeast extract-peptone (YP) medium (prepared by dissolving 10 g yeast extract and 20 g peptone per 100 ml deionized water), 750 μl concentrated yeast inoculum (11 g dry weight/L, approximately $6.3 \times 10^7$ cfu/ml) and 50 ml 0.05 M citrate buffer (pH 4.8) was approximately 60 ml (Chang et al., 2001; Dowe and McMillan, 2001). Solid loading during SSF was kept at 5% as per standard hydrolysis protocol (Sun and Cheng, 2005). Enzymes added for hydrolysis were determined from a study on identification of ethanol production potential of oven-dried switchgrass germplasms (Chapter 3). The enzymes added were 1.192 ml cellulase (equivalent to 30 FPU/g dry biomass; NS 50013 cellulase complex, density 1.20 g/ml), 556 μl cellobiase (1:4 FPU:CBU activity ratio, NS 50010 β-glucosidase, density 1.24 g/ml) and 6.9 μl xylanase (0.25% w/w dry biomass loading, NS50030 xylanase, density 1.09 g/ml). All enzymes were kindly provided by Novozymes North America, Inc., Franklinton, NC.

The SSF experiments were conducted in triplicate with a total of 6 flasks. Three flasks were aseptically sampled every 24 h from 0 h to 168 h (7 days) while the other three flasks were sampled twice, at 0 h and 168 h, for evaluation of the overall SSF performance. During sampling, one milliliter of liquid was drawn from the flasks by opening the stoppers, clarified by passing through the 2 mm syringe filter and analyzed by HPLC for concentrations of glucose, xylose and ethanol.

Percent theoretical ethanol yield was calculated using Equation 5.1:

$$\text{Percent Ethanol Yield} = \frac{\text{Ethanol}(g)/\text{biomass}(g)}{0.511 \times \text{Glucose}(g)/\text{biomass}(g)}$$  (5.1)

Where Ethanol (g)/biomass (g) = the ethanol yield (g) per 1 g of initial biomass;

Glucose (g)/biomass (g) = the glucose content (g) in 1 g of initial biomass (can be obtained from the composition analysis of initial biomass).
5.2.5 Yeast cell immobilization for ethanol fermentation

Wet biomass equivalent to 1 g on dry matter basis was mixed at a solid loading of 5% with 20 ml 0.05 M citrate buffer (pH 4.8) containing 40 μg/ml tetracycline hydrochloride to avoid microbial contamination. Enzymes used for hydrolysis were the same as those used during SSF. Cellulase (NS 50013 cellulase complex, density 1.20 g/ml) was added to the system at 30 FPU/g dry biomass and supplemented with cellobiase (NS 50010 β-glucosidase, density 1.24 g/ml) at an activity ratio of 4 CBU:1 FPU. In addition, xylanase (NS 50030 xylanase, density 1.09 g/ml) was introduced at a loading of 0.25% w/w dry biomass. Totally 12 tubes containing the same hydrolysis system were prepared simultaneously to provide enough hydrolyzate for fermentation.

Hydrolysis samples were incubated in 50 ml polypropylene centrifuge tubes in a water bath at 55 °C/150 rpm for 72 h. Upon completion of hydrolysis, samples were centrifuged at 4 °C/4000 rpm for 10 minutes in the Eppendorf 5810R centrifuge. Supernatant collected (approximately 19 ml supernatant/tube) from two tubes each was combined and transferred to six 250 ml sterile Erlenmeyer flasks and autoclaved at 121 °C/15 psi for 15 min to inactivate the antibiotics and sterilize the hydrolyzate.

Twenty-five milliliter of concentrated yeast culture at 6.3×10^7 cfu/ml (approximately 11 g dry weight/L) was mixed with an equal volume of 4% (w/v) Na-alginate (Alginic acid sodium salt, medium viscosity, ICN Biomedicals, USA) solution. The 50 ml aliquot of alginate-cell suspension containing 2% Na-alginate was added drop-wise to 500 ml 2% CaCl₂ solution through sterile 10 ml syringes. Drops solidified and formed beads immediately upon contact with CaCl₂. One milliliter of the yeast-Na alginate suspension generated 25 beads. Yeast beads were allowed to harden for 30 minutes in CaCl₂, washed with sterile saline solution (0.90% NaCl) to remove residual yeast cells and calcium ions, transferred to 0.2% yeast extract solution (Figure 5.2) and stored in the refrigerator at 4 °C until use (Göksungur and
Fermentation was initiated by introducing 25 Ca-alginate beads into each of the hydrolyzate flasks equipped with a water trap filled with glycerol to allow CO₂ to escape and keep air from entering the flask. All flasks were incubated for a 7-day-fermentation at 30 °C (Figure 5.3).

One milliliter of the fermentation broth was drawn from three flasks every 24 h from 0 (the starting point) to 168 h (7 days) by removing the stoppers, clarified by passing through the 2 mm syringe filter, and saved in autosampler vials for
subsequent reducing sugar and ethanol analyses. Aliquotes from the other three flasks were taken at 0 h and 168 h for an overall evaluation of the immobilized yeast cell fermentation. Percent theoretical ethanol yield was calculated using Equation 5.1.

5.2.6 HPLC analysis
Fermentation aliquotes collected in autosampler vials were analyzed by HPLC (Shimadzu, Kyoto, Japan) equipped with a refractive index detector (Shimadzu RID-10A). A Biorad Aminex HPX-87H column was used with an appropriate guard column. The working temperature for the column was 65 °C and the flow rate of eluant (0.005 mol/L H₂SO₄) was set at 0.06 ml/min. Concentrations of glucose, xylose and ethanol were recorded for each run.

5.2.7 General statistical analysis
Analysis of variance (ANOVA) tables were generated using SAS® (version 9.1.3, Cary, NC) to determine the effect of time on ethanol content. A 95% confidence level (P < 0.05) was applied to all analyses performed in this study.

5.3 Results and Discussion
5.3.1 Ethanol yield in SSF
The dilute acid pretreatment resulted in a solid recovery of 53.63%. The concentrations of ethanol and reducing sugars (glucose and xylose) obtained from HPLC analysis were converted on initial dry matter basis and all results are summarized in Figure 5.4. The final pH of all SSF samples was between 4.6 and 4.7, which was within the recommended range of 5.0 ± 0.7 thus avoiding organic acid accumulation (Dowe and McMillan, 2001).
At the initiation of SSF, 0.019 g glucose and 0.002 g xylose per gram of initial biomass was detected in the fermentation mixture. Since previous studies showed that no glucose and xylose were released by soaking acid-pretreated switchgrass in the hydrolysis buffer without adding enzymes (Chapter 3), the presence of monomeric sugars at time 0 might be due to the immediate release of small amounts of reducing sugars by cellulolytic and xylolytic enzymes. Glucose generated during enzymatic hydrolysis was completely consumed by yeast after 24 h, indicating that dilute acid pretreatment did not have an inhibitory effect on yeast \textit{(Saccharomyces cerevisiae, ATCC 24859)} performance after complete wash of the pretreated solids. Additionally, glucose accumulation resulting in end-product inhibition of cellulase and cellobiase hydrolysis (Xiao et al., 2004) was effectively prohibited by performing SSF. Xylose content in the fermentation broth decreased from 0.014 g to 0.009 g at a rate of 0.001 g/g initial biomass per day between Day 1 and Day 6 after a rapid increase during the first 24 h. Ethanol intensively accumulated to reach 0.078 g/g initial biomass within the first day and showed a slight upward trend resulting in 0.083 g/g initial biomass on Day 2. Thereafter, no significant change
(P > 0.05) in ethanol content was observed and the final yield (0.086 g/g initial biomass) was slightly higher than that obtained from separate hydrolysis and fermentation (0.082 g/g initial biomass) (SHF, Chapter 3). Trends of sugar and ethanol contents over the whole process of SSF indicated that the yeast used in this study was effective in converting glucose to ethanol, while its ability of fermenting xylose was limited.

The effect of sampling frequency and technique was evaluated by comparing current ethanol yields with those obtained from SSF flasks which were only sampled at the beginning and the end of 7-day-fermentation; glucose, xylose and ethanol contents in these two fermentation systems are included in Table 5.1.

<table>
<thead>
<tr>
<th>Table 5.1 Reducing sugar and ethanol contents in fermentation samples from SSF flasks sampled intermittently (system A, sampled every 24 h) and non-intermittently (system B, sampled at 0 h and 168 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Compositions of fermentation system A</strong></td>
</tr>
<tr>
<td>Glucose</td>
</tr>
<tr>
<td>Day 0</td>
</tr>
<tr>
<td>Day 7</td>
</tr>
</tbody>
</table>

Note. 1Expressed as Mean value (Standard deviation).

At both Day 0 and Day 7, reducing sugars contained in system A and B were approximately the same. However, upon completion of the 7-day-fermentation, final ethanol yield in system B reached 0.092 g/g initial biomass which was 7% higher than that obtained from system A. Though the increase was found to be not significant at the 95% confidence level (P > 0.05), it indicated the possibility that frequent sampling during the fermentation process affected final ethanol yield negatively. Since in this study, sampling was performed by opening the stopper in the laminar flow hood using aseptic techniques, it is possible that air was introduced into the flasks and therefore...
interfered with the anaerobic fermentation. Compared to SHF, which provided 53% of theoretical ethanol yield, operation processes were simplified for SSF due to the combination of hydrolysis and fermentation, and the production of ethanol was enhanced, resulting in 60% of theoretical yield for the acid pretreated switchgrass.

Though the operating temperature during SSF is a compromise between enzymatic hydrolysis and fermentation, switchgrass has shown high adaptability to SSF and improvements in the corresponding ethanol yield have been reported for samples pretreated by various methods such as acid or alkali and AFEX (ammonia fiber explosion) (Chang, et al., 2001; Alizadeh et al., 2005).

5.3.2 Ethanol yield in immobilized yeast cell fermentation

The final pH of all immobilized yeast cell fermentation samples was between 4.3 and 4.5. The concentrations of ethanol and reducing sugars (glucose and xylose) obtained from immobilized yeast fermentation of switchgrass hydrolyzates calculated on initial dry matter basis are presented in Figure 5.5.

Figure 5.5 Change in glucose, xylose and ethanol contents during immobilized yeast cell fermentation of acid pretreated and enzymatic hydrolyzed switchgrass over 7 days.
Glucose concentration in the medium dropped sharply within the first 24 h of fermentation and stayed at 0 g/g initial biomass thereafter, while xylose continuously decreased from 0.02 to 0.01 g/g initial biomass during the 7-day-fermentation possibly due to the generation of furfural or other smaller components from xylose. Ethanol production reached a maximum of 0.081 g/g initial biomass after 24 h of fermentation and then decreased to 0.064 g upon the completion of fermentation with the effect of time being significant over 7 days (P < 0.05). However, by just investigating the ethanol yield over the first 3 days, no significant decrease in ethanol yield was observed (P > 0.05). One possible explanation for the decrease in ethanol could be that ethanol, as the fermentation product contained in the broth, was drawn out every 24 h during sampling and was not produced after the depletion of glucose. This was different from SSF in which ethanol was continuously generated as long as glucose was released from the polysaccharides during hydrolysis. Another explanation could be that yeasts began to consume ethanol as the carbon source after the depletion of glucose within the first 1 or 2 days. However, this hypothesis needs further investigation. The yeast used in this study was unable to effectively produce ethanol using xylose and the greatest theoretical ethanol yield was 53% at 24 h.

Gel beads situated at the bottom of the fermentation flasks grew slowly in size within the first 3 days of incubation and after Day 4, majority of the immobilized cells became suspended in the fermentation broth possibly because of the reduction in mechanical strength of Ca-alginate following the growth of entrapped yeasts (Eikmeier and Rehm, 1987). Continuous cultivation with proper dilution rate can be a solution for the release of immobilized yeast cells without any loss in yeast activities (Nigam, 2000; Taherzadeh et al., 2001).

Similar to SSF, effect of sampling was also evaluated during immobilized yeast fermentation by comparing the ethanol yields at both Day 0 and 7 through intermittently and non-intermittently sampled systems and the results are summarized in Table 5.2.
Table 5.2 Reducing sugar and ethanol contents in intermittently (system A, sampled every 24 h) and non-intermittently (system B, sampled at 0 h and 168 h) sampled immobilized yeast fermentation systems

<table>
<thead>
<tr>
<th></th>
<th>Glucose</th>
<th>Xylose</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Compositions of fermentation system A</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>0.153 (0.007)(^1)</td>
<td>0.020 (0.000)</td>
<td>0.000 (0.000)</td>
</tr>
<tr>
<td>Day 7</td>
<td>0.000 (0.000)</td>
<td>0.010 (0.000)</td>
<td>0.064 (0.003)</td>
</tr>
</tbody>
</table>

| **Compositions of fermentation system B** |          |          |          |
| Day 0                | 0.156 (0.003) | 0.020 (0.000) | 0.000 (0.000) |
| Day 7                | 0.000 (0.000) | 0.013 (0.000) | 0.079 (0.002) |

Note. \(^1\)Expressed as Mean value (Standard deviation).

Upon completion of the 7-day-fermentation, final ethanol yield in system B reached 0.079 g compared to 0.064 g/g initial biomass obtained from system A, and the difference was significant at a 95% confidence level (P < 0.05). Therefore, frequent sampling may have negatively affected final ethanol yield and compared to SSF, the fermentation system for immobilized yeast cells were more susceptible to sampling. Besides the possibility that ethanol was drawn out during intermittent sampling as described earlier, air might have been introduced to the fermentation system when aseptic sampling was applied by opening the stopper, which could disrupt the anaerobic fermentation process. It is suggested that the system be sparged with nitrogen after sampling or a syringe needle inserted in the stopper be used for sampling in future studies to avoid introduction of aerobic conditions during fermentation.

Although the gel matrix can protect the yeast cells from interacting with inhibitors such as furfurals (Abbi et al., 1996), compared with standard fermentation of the switchgrass hydrolyzate, the modified 7-day-fermentation using Ca-alginate immobilized cells did not exhibit improvement in ethanol yield possibly due to diffusion limitation within the entrapped yeast cells. In addition, the yeast used in this study efficiently consumed glucose to produce ethanol early on, so it is suggested that
the fermentation time be shortened to 48 or 72 h.

5.4 Conclusion

This study investigated two modified fermentation methods for switchgrass-to-bioethanol conversion: (1) simultaneous saccharification and fermentation (SSF) of the acid pretreated biomass and, (2) fermentation of the hydrolyzate using yeast cell immobilization techniques. Compared to the ethanol yield obtained from separate hydrolysis and fermentation (SHF), SSF resulted in a higher yield at 0.092 g ethanol/g initial biomass, while improvement in ethanol yield was not observed for 7-day immobilized yeast cell fermentation.

A 60% theoretical ethanol yield based on total glucose content of acid pretreated switchgrass was achieved during SSF.

Immobilized yeast cell fermentation showed that complete consumption of glucose took place in the first 24 h of incubation with ethanol reaching the maximum yield at 53% of theoretical. Though immobilized cell fermentation is preferred with respect to the advantages of high cell concentration and easy separation of biocatalysts (Carvalho et al., 2002), maintenance of the mechanical structure of the gel matrix usually requires additional attention. Therefore, compared to batch culture, continuous cultivation of the hydrolyzate with regular input of the fresh immobilized cells during fermentation is recommended for ethanol production.

Additionally, frequent sampling during the process of fermentation may have inhibitory effect on final ethanol yield. Once the fermentation system has been built, it is suggested that sampling frequency be reduced while maintaining anaerobic fermentation conditions.
References


CHAPTER 6  Conclusions and Scope for Future Work

In this study, three new germplasms of switchgrass were used as the lignocellulosic feedstock for bioethanol production through dilute sulfuric acid pretreatment, enzymatic hydrolysis and yeast fermentation. The main conclusions of the research are listed below:

(1) The switchgrass feedstocks contained approximately 22.71 to 30.95% glucan, 13.27 to 19.37% xylan and 17.39 to 20.60% lignin. Two germplasms (St6-1 and St6-3E) developed for high biomass yield displayed similar overall composition compared to ST6-3F which has been developed for high digestibility. Relative to plant parts, composition of stem was similar to that of whole plant, but the leaf samples varied.

(2) Acid pretreatments yielding greatest hemicellulose solubilization (77.24 to 85.87%) normally involved severe conditions such as high acid concentration (1.5% w/v H$_2$SO$_4$) and a long residence time (60 min), indicating that hemicellulose removal is the main effect of dilute acid pretreatment and is positively related to the intensity of pretreatment conditions.

(3) Lignin removal was not significant during acid pretreatment and was limited to 10% for both oven- and freeze-dried switchgrass samples.

(4) Pretreatment enhanced the conversion of glucan to glucose during enzymatic hydrolysis. The greatest conversion achieved was approximately 100% for the freeze-dried St6-3F leaf samples after being pretreated with 1.0% (w/v) sulfuric acid for 60 min at 121 °C and hydrolyzed by cellulase at 15 FPU/g dry biomass supplemented with xylanase at 0.25% w/w dry biomass or by cellulase alone at 30 FPU/g dry biomass. Adding cellulase significantly enhanced cellulose conversion efficiency while adding xylanase did not greatly impact the glucose yield.
Almost all the glucose contained in the hydrolyzates was consumed by yeast (*Saccharomyces cerevisiae*, ATCC 24859) during anaerobic fermentation to produce ethanol, indicating that there was no inhibitory effect of dilute acid pretreatment on fermentation since the pretreated solids were completely washed in this study. The greatest ethanol yield from the most efficient acid treatment of switchgrass were 0.0819 and 0.0826 g/g initial biomass for the oven-dried St6-3E and freeze-dried St6-3F whole plant samples, respectively.

A 60% theoretical ethanol yield at 0.092 g/g initial biomass could be achieved through 7-day simultaneous saccharification and fermentation (SSF) of the oven-dried St6-3E whole switchgrass pretreated with 1.5% (w/v) sulfuric acid for 60 min at 121 °C. Fermentation of the hydrolyzate from pretreated oven-dried St6-3E whole switchgrass with immobilized yeast cells did not show improvement in ethanol yield compared to conventional fermentation.

Based on the switchgrass yield of 13450 kg/ha (12000 lbs/acre) and its capability of producing ethanol at 0.082 g/g initial biomass (considering cellulose conversion only), the estimated overall ethanol yield is 1398 L/ha switchgrass (149 gallon/acre).

The new germplasms of switchgrass have potential as energy crops for bioethanol production through dilute acid pretreatment, enzymatic hydrolysis and yeast fermentation. However, further studies are needed to optimize this lab-scale study for future scale-up.

Since a significant amount of hemicellulose is solubilized and removed from the biomass matrix during dilute acid pretreatment, a technically feasible approach needs to be developed to recover and use the dissolved sugars especially when pretreatment is performed on a large scale. Another product generated during pretreatment due to degradation of xylose is furfural which needs to be collected in the liquid fraction after pretreatment for use as a selective solvent in petrochemical refining and also as an intermediate in the production of nylon 6.6 and resins (Paturau,
Additionally, the mechanism of interaction between lignin and acid during pretreatment is another topic of interest; pretreatment conditions which result in a high level of delignification along with significant hemicellulose removal are preferable for both lab research and industrial scale-up.

Effective conversion of cellulose to glucose has been found in the enzymatic hydrolysis of acid-pretreated switchgrass; however, the dosage of cellulolytic enzymes is a factor worthy of further investigation since enzymes significantly contribute to the operating cost of ethanol production from lignocellulosic materials. In addition, end-product inhibition of cellulase due to glucose accumulation, though not obvious in this study, may negatively impact hydrolysis of cellulose to glucose if operated on a large scale. Therefore, effective removal of the monomeric sugars (mainly glucose) during hydrolysis can be achieved through simultaneous saccharification and fermentation (SSF) which requires further study.

Currently, another major challenge faced by the cellulosic ethanol industry is the inefficient fermentation of pentoses since native *Saccharomyces cerevisiae* can only convert 6C sugars to ethanol during anaerobic fermentation. Since the hydrolyzate of lignocellulosic feedstocks comprises of both 6C and 5C sugars unlike corn grain and sugarcane, which contain only 6C sugars, commercial scale-up of bioethanol production from lignocellulosic feedstocks is greatly restricted. Extensive studies have been conducted on genetically modifying *Saccharomyces cerevisiae* to endow it with the ability to ferment both hexoses and pentoses, but limited work has been done on applying the newly developed cultures to the sugar hydrolyzate derived from lignocelluloses. It is predicted that the ethanol yield from lignocellulosic materials can be greatly enhanced if the co-fermentation of both glucose and xylose is realized.

Additionally, a comprehensive economic analysis of the lignocellulosic biomass-to-ethanol conversion needs to be conducted. A trade-off between operating cost and product quality will be finally established to make this conversion
economically feasible.
REFERENCES


Biotechnology 57/58, 147-156.


Yang, B. and Wyman, C. E., 2005. BSA treatment to enhance enzymatic hydrolysis of cellulose in lignin containing substrates. Biotechnology and Bioengineering


APPENDICES
Appendix I: Enzyme Activity Assays

Ia. Measurement of Cellulase Activities

The procedure for measurement of cellulase activity was based on the guidelines established by International Union of Pure and Applied Chemistry (IUPAC) and Laboratory Analytical Procedure (LAP-006) provided by NREL. The cellulase activity was expressed in terms of “filter-paper units” (FPU) per milliliter of original (undiluted) enzyme solution. One unit of FPU is defined as the amount of enzyme that produces 1 μmol of glucose (reducing sugars as glucose) per minute under the assay conditions.

Materials and Methods:

DNS reagent was prepared by adding 10.0 g 3,5-Dinitrosalicylic acid and 20.75 ml 50% w/w (19.1 mol/L) sodium hydroxide to 400 ml DI water. After slightly heating the mixture in order to facilitate dissolving, 300 g Rochelle salts (Na-K tartarate) were added to the mixture before finally making the solution volume up to 1 L by adding appropriate amount of DI water. In this experiment, the DNS reagent was kindly provided by Deepak Keshwani; and the linear standard curve, giving the relation between absorbance and sugar concentration, is presented in Figure 1. The equation (Eqtn. 1) obtained from the standard curve was used to calculate glucose concentration (mg/0.5 ml) of the unknown samples based on absorbance readings.

One mol per liter citrate buffer was made by dissolving 210 g citric acid monohydrate in an appropriate amount of DI water to make the final volume up to 1 L. Then the pH was adjusted to 4.5 by adding sodium hydroxide pellets. This stock solution was diluted 20-fold with DI water to achieve a final concentration of 0.05 M before use.
Equation 1:
\[ y = 0.6406x + 0.0616 \]

Where \( x \) = absorbance value obtained from the spectrophotometer at 540 nm;
\( y \) = glucose concentration expressed as ‘mg glucose/0.5 ml’.

The enzyme stock was made by adding 0.5 ml cellulase (Novozymes NS50013, density 1.20 g/ml) to 9.5 ml 0.05 mol/L citrate buffer (pH 4.8) for a 20-fold dilution (can also be expressed as 1:20 enzyme). The substrate used in this analysis was a 50 mg Whatman No.1 filter paper strip (1.0*6.0 cm). Ten test tubes were divided into three groups: the first four (No. 1-4) were “reaction tubes” in which one piece of filter paper strip was placed for the enzymatic reaction, the next four (No. 5-8) were used as the corresponding enzyme controls where only enzymes were added but not the filter paper substrate, the last two (No. 9-10) were blanks in which only citrate buffer was added.

One milliliter 0.05 mol/L citrate buffer (pH 4.8) was added to each of the ten tubes with filter paper being submerged in the first four tubes as mentioned above. The enzyme stock was diluted into different levels and 0.5 ml out of the 2.0 ml diluted enzyme samples were added to the appropriate test tubes as shown below in Table 1.
Table 1. Preparation of appropriate dilutions for the 1:20 enzyme stock

<table>
<thead>
<tr>
<th>Dilution#</th>
<th>Volume of the 1:20 enzyme (ml)</th>
<th>Volume of citrate buffer (ml)</th>
<th>Total volume (ml)</th>
<th>Test tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.00</td>
<td>2.00</td>
<td>2.00</td>
<td>Two blanks</td>
</tr>
<tr>
<td>1</td>
<td>0.30</td>
<td>1.70</td>
<td>2.00</td>
<td>Test tubes No. 1 &amp; 5</td>
</tr>
<tr>
<td>2</td>
<td>0.40</td>
<td>1.60</td>
<td>2.00</td>
<td>Test tubes No. 2 &amp; 6</td>
</tr>
<tr>
<td>3</td>
<td>0.45</td>
<td>1.55</td>
<td>2.00</td>
<td>Test tubes No. 3 &amp; 7</td>
</tr>
<tr>
<td>4</td>
<td>0.50</td>
<td>1.50</td>
<td>2.00</td>
<td>Test tubes No. 4 &amp; 8</td>
</tr>
</tbody>
</table>

All 10 test tubes were transferred to a water bath to be incubated at 50 °C for exactly 60 min. At the end of the incubation period, they were removed from the 50 °C water bath and the enzyme reactions were stopped immediately by adding 3.0 ml DNS reagent.

All samples, controls and blanks were boiled for exactly 5.0 minutes in a vigorously boiling water bath which contained sufficient water to cover the reaction mixture. Color development was visible during boiling. Following boiling, tubes were transferred to an ice bath.

All tubes (blanks, assays and controls) were diluted in DI water by adding 0.2 ml of color-developed reaction mixture to 2.5 ml DI water in a spectrophotometer cuvette. The absorbance of each diluted sample was measured against the reagent blank at 540 nm by the spectrophotometer (Shimadzu UV-1700) for activity calculation.

Results and Discussions:

The absorbance obtained from the spectrophotometer readings for each tube is listed below.

Table 2. Absorbance of samples in the test tubes

<table>
<thead>
<tr>
<th>No. of tubes</th>
<th>Abs. at 540 nm</th>
<th>No. of tubes</th>
<th>Abs. at 540 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.196</td>
<td>5</td>
<td>0.003</td>
</tr>
<tr>
<td>2</td>
<td>0.261</td>
<td>6</td>
<td>0.005</td>
</tr>
<tr>
<td>3</td>
<td>0.287</td>
<td>7</td>
<td>0.005</td>
</tr>
<tr>
<td>4</td>
<td>0.305</td>
<td>8</td>
<td>0.002</td>
</tr>
</tbody>
</table>
Based on Table 2, the exact absorbance of each assay can be calculated by subtracting the absorbance of enzyme controls from that of the corresponding assay mixtures (for example, tube #5 is the enzyme control for tube #1). Therefore, the actual absorbance of assay No. 1-4 was 0.193, 0.256, 0.282, and 0.303, respectively. According to the equation derived from similar assays on glucose standards using the same stock of DNS reagent, the concentration of glucose can be calculated and expressed as mg glucose per 0.5 ml. The concentration of glucose contained in each assay mixture calculated by the equation derived from standard glucose assay (Eqtn. 1) is listed in Table 3.

Table 3. Glucose concentrations in the appropriately diluted assay mixtures

<table>
<thead>
<tr>
<th>Dilution #</th>
<th>Absorbance</th>
<th>Extra dilution rate before spectrophotometer measurement</th>
<th>Cglucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.193</td>
<td>13.5</td>
<td>2.50068</td>
</tr>
<tr>
<td>2</td>
<td>0.256</td>
<td>13.5</td>
<td>3.04551</td>
</tr>
<tr>
<td>3</td>
<td>0.282</td>
<td>13.5</td>
<td>3.27036</td>
</tr>
<tr>
<td>4</td>
<td>0.303</td>
<td>13.5</td>
<td>3.45197</td>
</tr>
</tbody>
</table>

“Concentration” can be used to represent the proportion of the original enzyme solution present in the diluted assay mixture. Since all enzyme dilutions were made by mixing the 1:20 working stock of enzyme with the appropriate amount of citrate buffer (pH 4.8), the concentration of Dilution #1 was 0.00750 which represented a 3:20 dilution of the 1:20 working stock of enzyme. Based on the same principle, the concentration of Dilution #2, 3 and 4 was 0.01000, 0.01125, and 0.01250, respectively. A graph can be created by plotting glucose (mg/0.5ml) on the X-axis and enzyme concentration on the Y-axis. Hence, the concentration of enzyme which would have released exactly 2.0 mg of glucose can be determined by plotting glucose liberated against enzyme concentration.
Figure 2 Determination of the concentration of enzyme by the amount of glucose released.

Although on a general basis, reducing sugar yield is not a linear function of the quantity of enzyme in the assay mixture; in a very narrow range, it can be considered linear as the $R^2$ value is approximately 1. When the concentration of glucose is 2.0 mg/0.5 ml, the enzyme dilution can be obtained using the derived equation by extrapolating the straight line, thus giving a dilution of 0.0049 (0.0052×2–0.0055=0.0049). Finally, FPU of the cellulase product (Novozymes NS50013) can be calculated using the equation as:

$$FilterPaperActivity = \frac{0.37}{[\text{enzyme}]\text{Releasing } 2.0\text{mg glucose}} \text{ units / ml}$$

$$FPU = \frac{0.37}{0.0049} = 75.5\text{units / ml}$$

References:
Ib. Measurement of Cellobiase Activities

Cellobiase assay was conducted according to the procedures developed by Ghose (1987). One unit of cellobiase activity (CBU) is defined as the amount of enzyme that is capable of generating 2 μmol of glucose per minute from cellobiose.

Materials and Methods:

Substrate solution was made by dissolving 0.2567 g D(+)-cellobiose (98%, MW=342.29, Acros Organics Lot A0252025) in 50 ml 0.05 mol/L citrate buffer (pH 4.8) to achieve a sugar concentration of 15 mmol/L (5.13 g/L).

The enzyme stock was prepared by adding 0.2 ml cellobiase product (Novozymes NS50010, β-glucosidase, density: 1.24 g/L) to 19.8 ml 0.05 mol/L citrate buffer (pH 4.8) to obtain a 100-fold dilution (can also be expressed as 1:100 enzyme).

The 1:100 enzyme was further diluted in the citrate buffer to five different levels as shown below:

| Table 1. Preparation of appropriate dilutions for 1:100 enzyme stock |
|-----------------------------|-----------------------------|-----------------------------|
| Dilution# | Volume of the 1:100 enzyme (ml) | Volume of citrate buffer (ml) | Total volume (ml) |
| 1 | 0.10 | 2.40 | 2.50 |
| 2 | 0.10 | 2.65 | 2.75 |
| 3 | 0.10 | 2.90 | 3.00 |
| 4 | 0.10 | 3.15 | 3.25 |
| 5 | 0.10 | 3.40 | 3.50 |

One milliliter each of the specifically diluted enzyme solution was added to 5 test tubes, respectively. These tubes were preheated in a 50 °C water bath. Then 1.0 ml substrate solution was added to each of the tubes and the mixture was incubated at 50 °C in the water bath for exactly 30 min. Sugar hydrolysis was terminated by immersing the tubes in boiling water for exactly 5.0 min and then tubes were transferred to an ice bath.

Each of the five reaction mixtures were filtered through 0.2 μm syringe filters
and kept in autosampler vials for subsequent HPLC sugar analysis.

**Results and discussions:**

The sugar concentrations were analyzed by HPLC and are summarized in Table 2.

### Table 2 Results of cellobiose hydrolysis for cellobiase assay

<table>
<thead>
<tr>
<th>Dilution #</th>
<th>Enzyme concentration $^1$</th>
<th>Glucose released (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.000400</td>
<td>2.60</td>
</tr>
<tr>
<td>2</td>
<td>0.000364</td>
<td>2.46</td>
</tr>
<tr>
<td>3</td>
<td>0.000333</td>
<td>2.40</td>
</tr>
<tr>
<td>4</td>
<td>0.000308</td>
<td>2.14</td>
</tr>
<tr>
<td>5</td>
<td>0.000286</td>
<td>2.02</td>
</tr>
</tbody>
</table>

Note. $^1$ Concentration = $\frac{1}{\text{Dilution}}$

Results obtained in Table 2 were plotted in Figure 1 to determine the amount of cellobiase required to generate 1.0 mg glucose.

![Figure 1 Determination of the concentration of enzyme by the amount of glucose released](image)

$y = 6849.2x$

$R^2 = 0.8273$

Estimation of the enzyme concentration which would have released exactly
1.0 mg of glucose was made by using the equation obtained in Figure 1.

\[ y = 6849.2x \]

When \( y = 1.0 \), \( x = 1.46 \times 10^{-4} \)

Cellobiase activity CBU

\[
= \frac{0.0926}{\text{enzyme concentration to release 1.0 mg glucose}}
\]

\[
= \frac{0.0926}{1.46 \times 10^{-4}} = 634.2 \text{ units/ml}
\]

References:

## Appendix II: SAS Tables and Codes of ANOVA and Multiple Comparisons

### Table IIa ANOVA for Solid Recovery of Dilute Acid Pretreated Oven-dried Switchgrass (Independent variables: germplasm, minutes and acid concentration; dependent variable: percent solid recovery)

Dependent Variable: SR

<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>6</td>
<td>2609.839007</td>
<td>434.973168</td>
<td>163.00</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Error</td>
<td>72</td>
<td>192.132315</td>
<td>2.668504</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected Total</td>
<td>78</td>
<td>2801.971322</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

R-Square: 0.931430, Coeff Var: 2.728695, Root MSE: 1.633556, SR Mean: 59.86582

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type I SS</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>germplasm</td>
<td>2</td>
<td>89.346163</td>
<td>44.673082</td>
<td>16.74</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>minutes</td>
<td>2</td>
<td>394.784047</td>
<td>197.392024</td>
<td>73.97</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>acidconc</td>
<td>2</td>
<td>2125.708796</td>
<td>1062.854398</td>
<td>398.30</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

<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>germplasm</td>
<td>2</td>
<td>71.267675</td>
<td>35.633837</td>
<td>13.35</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>minutes</td>
<td>2</td>
<td>392.579464</td>
<td>196.289732</td>
<td>73.56</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>acidconc</td>
<td>2</td>
<td>2125.708796</td>
<td>1062.854398</td>
<td>398.30</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>
### Table IIb Multiple Comparison with the Best for Least Lignin Content Selection from Dilute Acid Pretreated Oven-dried St6-1 Switchgrass

<table>
<thead>
<tr>
<th>Obs</th>
<th>Effect</th>
<th>trt</th>
<th>Estimate</th>
<th>StdErr</th>
<th>cllo</th>
<th>clhi</th>
<th>rval</th>
<th>sval</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>trt</td>
<td>301</td>
<td>-19.1433</td>
<td>0.2819</td>
<td>-2.00208</td>
<td>0.05541</td>
<td>0.06480</td>
<td>.</td>
</tr>
<tr>
<td>2</td>
<td>trt</td>
<td>453</td>
<td>-19.0467</td>
<td>0.2819</td>
<td>-1.90541</td>
<td>0.15208</td>
<td>0.09990</td>
<td>.</td>
</tr>
<tr>
<td>3</td>
<td>trt</td>
<td>603</td>
<td>-19.0300</td>
<td>0.2819</td>
<td>-1.88874</td>
<td>0.16874</td>
<td>0.10733</td>
<td>.</td>
</tr>
<tr>
<td>4</td>
<td>trt</td>
<td>451</td>
<td>-18.8033</td>
<td>0.2819</td>
<td>-1.66208</td>
<td>0.39541</td>
<td>0.25803</td>
<td>.</td>
</tr>
<tr>
<td>5</td>
<td>trt</td>
<td>452</td>
<td>-18.5033</td>
<td>0.2819</td>
<td>-1.36208</td>
<td>0.69541</td>
<td>0.58287</td>
<td>.</td>
</tr>
<tr>
<td>6</td>
<td>trt</td>
<td>602</td>
<td>-18.4767</td>
<td>0.2819</td>
<td>-1.33541</td>
<td>0.72208</td>
<td>0.61364</td>
<td>.</td>
</tr>
<tr>
<td>7</td>
<td>trt</td>
<td>302</td>
<td>-18.3800</td>
<td>0.2819</td>
<td>-1.23874</td>
<td>0.81874</td>
<td>0.71910</td>
<td>.</td>
</tr>
<tr>
<td>8</td>
<td>trt</td>
<td>303</td>
<td>-18.3567</td>
<td>0.2819</td>
<td>-1.21541</td>
<td>0.84208</td>
<td>0.74249</td>
<td>.</td>
</tr>
<tr>
<td>9</td>
<td>trt</td>
<td>601</td>
<td>-18.1700</td>
<td>0.2819</td>
<td>-0.84208</td>
<td>1.21541</td>
<td>0.74249</td>
<td>.</td>
</tr>
</tbody>
</table>

Note. See the next page for output explanation.

### Table IIc Multiple Comparison with the Best for Least Lignin Content Selection from Dilute Acid Pretreated Oven-dried St6-3E Switchgrass

<table>
<thead>
<tr>
<th>Obs</th>
<th>Effect</th>
<th>trt</th>
<th>Estimate</th>
<th>StdErr</th>
<th>cllo</th>
<th>clhi</th>
<th>rval</th>
<th>sval</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>trt</td>
<td>601</td>
<td>-18.2967</td>
<td>0.2870</td>
<td>-1.91857</td>
<td>0.19190</td>
<td>0.11589</td>
<td>.</td>
</tr>
<tr>
<td>2</td>
<td>trt</td>
<td>453</td>
<td>-18.0867</td>
<td>0.2870</td>
<td>-1.70857</td>
<td>0.40190</td>
<td>0.25526</td>
<td>.</td>
</tr>
<tr>
<td>3</td>
<td>trt</td>
<td>603</td>
<td>-18.0700</td>
<td>0.2870</td>
<td>-1.69190</td>
<td>0.41857</td>
<td>0.26983</td>
<td>.</td>
</tr>
<tr>
<td>4</td>
<td>trt</td>
<td>301</td>
<td>-17.9367</td>
<td>0.2870</td>
<td>-1.55857</td>
<td>0.55190</td>
<td>0.40285</td>
<td>.</td>
</tr>
<tr>
<td>5</td>
<td>trt</td>
<td>303</td>
<td>-17.7367</td>
<td>0.2870</td>
<td>-1.35857</td>
<td>0.75190</td>
<td>0.63050</td>
<td>.</td>
</tr>
<tr>
<td>6</td>
<td>trt</td>
<td>452</td>
<td>-17.5933</td>
<td>0.2870</td>
<td>-1.21524</td>
<td>0.89524</td>
<td>0.77706</td>
<td>.</td>
</tr>
<tr>
<td>7</td>
<td>trt</td>
<td>451</td>
<td>-17.5650</td>
<td>0.3515</td>
<td>-1.31146</td>
<td>1.01606</td>
<td>0.76013</td>
<td>.</td>
</tr>
<tr>
<td>8</td>
<td>trt</td>
<td>602</td>
<td>-17.5100</td>
<td>0.2870</td>
<td>-1.13190</td>
<td>0.97857</td>
<td>0.84475</td>
<td>.</td>
</tr>
<tr>
<td>9</td>
<td>trt</td>
<td>302</td>
<td>-17.4333</td>
<td>0.2870</td>
<td>-1.01606</td>
<td>1.13190</td>
<td>0.84475</td>
<td>.</td>
</tr>
</tbody>
</table>

Note. See the next page for output explanation.
### Table IIId Multiple Comparison with the Best for Least Lignin Content Selection from Dilute Acid Pretreated Oven-dried St6-3F Switchgrass

<table>
<thead>
<tr>
<th>Obs</th>
<th>Effect</th>
<th>trt</th>
<th>Estimate</th>
<th>StdErr</th>
<th>cllo</th>
<th>clhi</th>
<th>rval</th>
<th>sval</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>trt</td>
<td>301</td>
<td>-17.5500</td>
<td>0.2281</td>
<td>-1.81215</td>
<td>0.00000</td>
<td>0.02233</td>
<td>.</td>
</tr>
<tr>
<td>2</td>
<td>trt</td>
<td>452</td>
<td>-17.4167</td>
<td>0.2281</td>
<td>-1.67882</td>
<td>0.00000</td>
<td>0.04966</td>
<td>.</td>
</tr>
<tr>
<td>3</td>
<td>trt</td>
<td>601</td>
<td>-17.1833</td>
<td>0.2281</td>
<td>-1.44548</td>
<td>0.23215</td>
<td>0.17225</td>
<td>.</td>
</tr>
<tr>
<td>4</td>
<td>trt</td>
<td>602</td>
<td>-17.0933</td>
<td>0.2281</td>
<td>-1.35548</td>
<td>0.32215</td>
<td>0.25816</td>
<td>.</td>
</tr>
<tr>
<td>5</td>
<td>trt</td>
<td>303</td>
<td>-16.8567</td>
<td>0.2281</td>
<td>-1.11882</td>
<td>0.55882</td>
<td>0.57494</td>
<td>.</td>
</tr>
<tr>
<td>6</td>
<td>trt</td>
<td>451</td>
<td>-16.8550</td>
<td>0.2794</td>
<td>-1.21616</td>
<td>0.63400</td>
<td>0.56157</td>
<td>.</td>
</tr>
<tr>
<td>7</td>
<td>trt</td>
<td>453</td>
<td>-16.8000</td>
<td>0.2281</td>
<td>-1.06215</td>
<td>0.61548</td>
<td>0.65528</td>
<td>.</td>
</tr>
<tr>
<td>8</td>
<td>trt</td>
<td>603</td>
<td>-16.7133</td>
<td>0.2281</td>
<td>-0.97548</td>
<td>0.70215</td>
<td>0.76618</td>
<td>.</td>
</tr>
<tr>
<td>9</td>
<td>trt</td>
<td>302</td>
<td>-16.5767</td>
<td>0.2281</td>
<td>-0.70215</td>
<td>0.97548</td>
<td>0.76618</td>
<td>.</td>
</tr>
</tbody>
</table>

Note. In ‘trt’ statement, 30, 45 and 60 indicate treatment time, while the following 1, 2 and 3 correspond to acid concentration of 0.5%, 1.0% and 1.5%, respectively. The pretreatment which results in least lignin content within each germplasm is listed at the bottom (since the original SAS code is designed to select the greatest value based on the complete input, a negative sign (‘-‘) is added in front of each value to make the exactly least (appeared to be the greatest) value be present at the bottom.)
Table IIe Multiple Comparison with the Best for Greatest Hemicellulose
Solubilization Selection from Dilute Acid Pretreated Oven-dried St6-1
Switchgrass

<table>
<thead>
<tr>
<th>Obs</th>
<th>Effect</th>
<th>trt</th>
<th>Estimate</th>
<th>StdErr</th>
<th>cllo</th>
<th>clhi</th>
<th>rval</th>
<th>sval</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>trt</td>
<td>301</td>
<td>34.7400</td>
<td>6.7280</td>
<td>-67.6644</td>
<td>0.0000</td>
<td>0.00148</td>
<td>.</td>
</tr>
<tr>
<td>2</td>
<td>trt</td>
<td>451</td>
<td>54.7700</td>
<td>6.7280</td>
<td>-47.6344</td>
<td>2.6944</td>
<td>0.08260</td>
<td>.</td>
</tr>
<tr>
<td>3</td>
<td>trt</td>
<td>601</td>
<td>58.2550</td>
<td>8.2401</td>
<td>-47.1196</td>
<td>8.4146</td>
<td>0.18283</td>
<td>.</td>
</tr>
<tr>
<td>4</td>
<td>trt</td>
<td>302</td>
<td>64.4000</td>
<td>8.2401</td>
<td>-40.9746</td>
<td>14.5596</td>
<td>0.38321</td>
<td>.</td>
</tr>
<tr>
<td>5</td>
<td>trt</td>
<td>452</td>
<td>66.7567</td>
<td>6.7280</td>
<td>-35.6477</td>
<td>14.6810</td>
<td>0.47810</td>
<td>.</td>
</tr>
<tr>
<td>6</td>
<td>trt</td>
<td>303</td>
<td>71.9933</td>
<td>6.7280</td>
<td>-30.4110</td>
<td>19.9177</td>
<td>0.72889</td>
<td>.</td>
</tr>
<tr>
<td>7</td>
<td>trt</td>
<td>453</td>
<td>72.0500</td>
<td>8.2401</td>
<td>-33.3246</td>
<td>22.2096</td>
<td>0.69816</td>
<td>.</td>
</tr>
<tr>
<td>8</td>
<td>trt</td>
<td>603</td>
<td>76.3200</td>
<td>6.7280</td>
<td>-26.0844</td>
<td>24.2444</td>
<td>0.87998</td>
<td>.</td>
</tr>
<tr>
<td>9</td>
<td>trt</td>
<td>602</td>
<td>77.2400</td>
<td>6.7280</td>
<td>-24.2444</td>
<td>26.0844</td>
<td>0.87998</td>
<td>.</td>
</tr>
</tbody>
</table>

Note. See the next page for output explanation.

Table IIf Multiple Comparison with the Best for Greatest Hemicellulose
Solubilization Selection from Dilute Acid Pretreated Oven-dried St6-3E
Switchgrass

<table>
<thead>
<tr>
<th>Obs</th>
<th>Effect</th>
<th>trt</th>
<th>Estimate</th>
<th>StdErr</th>
<th>cllo</th>
<th>clhi</th>
<th>rval</th>
<th>sval</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>trt</td>
<td>451</td>
<td>34.7650</td>
<td>8.5947</td>
<td>-81.6873</td>
<td>0.0000</td>
<td>0.00570</td>
<td>.</td>
</tr>
<tr>
<td>2</td>
<td>trt</td>
<td>301</td>
<td>36.4500</td>
<td>8.5947</td>
<td>-80.0023</td>
<td>0.0000</td>
<td>0.00715</td>
<td>.</td>
</tr>
<tr>
<td>3</td>
<td>trt</td>
<td>601</td>
<td>50.9367</td>
<td>7.0176</td>
<td>-62.6515</td>
<td>0.0000</td>
<td>0.03784</td>
<td>.</td>
</tr>
<tr>
<td>4</td>
<td>trt</td>
<td>303</td>
<td>65.2350</td>
<td>8.5947</td>
<td>-51.2173</td>
<td>14.5273</td>
<td>0.28951</td>
<td>.</td>
</tr>
<tr>
<td>5</td>
<td>trt</td>
<td>302</td>
<td>65.4267</td>
<td>7.0176</td>
<td>-48.1615</td>
<td>12.6197</td>
<td>0.27675</td>
<td>.</td>
</tr>
<tr>
<td>6</td>
<td>trt</td>
<td>452</td>
<td>68.2600</td>
<td>8.5947</td>
<td>-48.1923</td>
<td>17.5523</td>
<td>0.38581</td>
<td>.</td>
</tr>
<tr>
<td>7</td>
<td>trt</td>
<td>602</td>
<td>70.5800</td>
<td>8.5947</td>
<td>-45.8723</td>
<td>19.8723</td>
<td>0.46835</td>
<td>.</td>
</tr>
<tr>
<td>8</td>
<td>trt</td>
<td>453</td>
<td>74.3450</td>
<td>8.5947</td>
<td>-42.1073</td>
<td>23.6373</td>
<td>0.60838</td>
<td>.</td>
</tr>
<tr>
<td>9</td>
<td>trt</td>
<td>603</td>
<td>83.5800</td>
<td>8.5947</td>
<td>-23.6373</td>
<td>42.1073</td>
<td>0.60838</td>
<td>.</td>
</tr>
</tbody>
</table>

Note. See the next page for output explanation.
**Table IIg Multiple Comparison with the Best for Greatest Hemicellulose Solubilization Selection from Dilute Acid Pretreated Oven-dried St6-3F Switchgrass**

<table>
<thead>
<tr>
<th>Obs</th>
<th>Effect</th>
<th>trt</th>
<th>Estimate</th>
<th>StdErr</th>
<th>cilo</th>
<th>clhi</th>
<th>rval</th>
<th>sval</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>trt</td>
<td>301</td>
<td>32.9600</td>
<td>6.9692</td>
<td>-72.0353</td>
<td>0.0000</td>
<td>0.00025</td>
<td>.</td>
</tr>
<tr>
<td>2</td>
<td>trt</td>
<td>451</td>
<td>46.4600</td>
<td>6.9692</td>
<td>-58.5353</td>
<td>0.0000</td>
<td>0.00435</td>
<td>.</td>
</tr>
<tr>
<td>3</td>
<td>trt</td>
<td>601</td>
<td>53.5867</td>
<td>5.6903</td>
<td>-48.8965</td>
<td>0.0000</td>
<td>0.01125</td>
<td>.</td>
</tr>
<tr>
<td>4</td>
<td>trt</td>
<td>302</td>
<td>62.3600</td>
<td>5.6903</td>
<td>-40.1232</td>
<td>2.4432</td>
<td>0.08555</td>
<td>.</td>
</tr>
<tr>
<td>5</td>
<td>trt</td>
<td>452</td>
<td>65.7900</td>
<td>6.9692</td>
<td>-39.2053</td>
<td>7.7637</td>
<td>0.20273</td>
<td>.</td>
</tr>
<tr>
<td>6</td>
<td>trt</td>
<td>602</td>
<td>75.0200</td>
<td>5.6903</td>
<td>-27.4632</td>
<td>15.1032</td>
<td>0.63459</td>
<td>.</td>
</tr>
<tr>
<td>7</td>
<td>trt</td>
<td>303</td>
<td>76.2733</td>
<td>5.6903</td>
<td>-26.2099</td>
<td>16.3565</td>
<td>0.70351</td>
<td>.</td>
</tr>
<tr>
<td>8</td>
<td>trt</td>
<td>603</td>
<td>79.6933</td>
<td>5.6903</td>
<td>-22.7899</td>
<td>19.7765</td>
<td>0.85590</td>
<td>.</td>
</tr>
<tr>
<td>9</td>
<td>trt</td>
<td>453</td>
<td>81.2000</td>
<td>5.6903</td>
<td>-19.7765</td>
<td>22.7899</td>
<td>.</td>
<td>0.85590</td>
</tr>
</tbody>
</table>

Note. In ‘trt’ statement, 30, 45 and 60 indicate treatment time, while the following 1, 2 and 3 correspond to acid concentration of 0.5%, 1.0% and 1.5%, respectively. The pretreatment which results in greatest hemicellulose solubilization rate within each germplasm is listed at the bottom.
Table IIh ANOVA for Solid Recovery of Dilute Acid Pretreated Freeze-dried Switchgrass (Independent variables: germplasm, plant part, minutes and acid concentration; dependent variable: percent solid recovery)

Dependent Variable: SR

<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>8</td>
<td>8456.87094</td>
<td>1057.10887</td>
<td>147.50</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Error</td>
<td>230</td>
<td>1648.32662</td>
<td>7.16664</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected Total</td>
<td>238</td>
<td>10105.19756</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

R-Square Coeff Var Root MSE SR Mean
0.836883 4.209523 2.677058 63.59527

Source: DF Type I SS Mean Square F Value Pr > F
<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type I SS</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>germplasm</td>
<td>2</td>
<td>794.129397</td>
<td>397.064698</td>
<td>55.40</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>plantpart</td>
<td>2</td>
<td>608.885940</td>
<td>304.442970</td>
<td>42.48</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>minutes</td>
<td>2</td>
<td>1252.891613</td>
<td>626.445807</td>
<td>87.41</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>acidconc</td>
<td>2</td>
<td>5800.963989</td>
<td>2900.481995</td>
<td>404.72</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

Source: DF Type III SS Mean Square F Value Pr > F
<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>germplasm</td>
<td>2</td>
<td>765.839657</td>
<td>382.919828</td>
<td>53.43</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>plantpart</td>
<td>2</td>
<td>643.252129</td>
<td>321.626064</td>
<td>44.88</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>minutes</td>
<td>2</td>
<td>1269.959227</td>
<td>634.979613</td>
<td>88.60</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>acidconc</td>
<td>2</td>
<td>5800.963989</td>
<td>2900.481995</td>
<td>404.72</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>
### Table III: Multiple Comparison with the Best for Least Lignin Content Selection from Dilute Acid Pretreated Freeze-dried St6-1 Switchgrass

<table>
<thead>
<tr>
<th>Obs</th>
<th>Effect</th>
<th>trt</th>
<th>Estimate</th>
<th>StdErr</th>
<th>cllo</th>
<th>clhi</th>
<th>rval</th>
<th>sval</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>trt</td>
<td>L301</td>
<td>-22.5433</td>
<td>0.2132</td>
<td>-5.05575</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>2</td>
<td>trt</td>
<td>L603</td>
<td>-21.8600</td>
<td>0.2611</td>
<td>-4.47303</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>3</td>
<td>trt</td>
<td>L451</td>
<td>-21.8000</td>
<td>0.2611</td>
<td>-4.41303</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>4</td>
<td>trt</td>
<td>L303</td>
<td>-21.5433</td>
<td>0.2132</td>
<td>-4.05575</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>5</td>
<td>trt</td>
<td>L302</td>
<td>-21.5100</td>
<td>0.2132</td>
<td>-4.02242</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>6</td>
<td>trt</td>
<td>L601</td>
<td>-21.3867</td>
<td>0.2132</td>
<td>-3.89908</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>7</td>
<td>trt</td>
<td>L452</td>
<td>-21.3067</td>
<td>0.2132</td>
<td>-3.81908</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>8</td>
<td>trt</td>
<td>S603</td>
<td>-21.3000</td>
<td>0.2132</td>
<td>-3.81242</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>9</td>
<td>trt</td>
<td>L602</td>
<td>-21.1267</td>
<td>0.2132</td>
<td>-3.63908</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>10</td>
<td>trt</td>
<td>L453</td>
<td>-21.1267</td>
<td>0.2132</td>
<td>-3.63908</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>11</td>
<td>trt</td>
<td>W603</td>
<td>-20.9300</td>
<td>0.2132</td>
<td>-3.44242</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>12</td>
<td>trt</td>
<td>S301</td>
<td>-20.7567</td>
<td>0.2132</td>
<td>-3.26908</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>13</td>
<td>trt</td>
<td>S451</td>
<td>-19.8967</td>
<td>0.2132</td>
<td>-2.40908</td>
<td>0.00000</td>
<td>0.00005</td>
<td>.</td>
</tr>
<tr>
<td>14</td>
<td>trt</td>
<td>S303</td>
<td>-19.8900</td>
<td>0.2132</td>
<td>-2.40242</td>
<td>0.00000</td>
<td>0.00006</td>
<td>.</td>
</tr>
<tr>
<td>15</td>
<td>trt</td>
<td>W301</td>
<td>-19.8167</td>
<td>0.2132</td>
<td>-2.32908</td>
<td>0.00000</td>
<td>0.00013</td>
<td>.</td>
</tr>
<tr>
<td>16</td>
<td>trt</td>
<td>S602</td>
<td>-19.7167</td>
<td>0.2132</td>
<td>-2.22908</td>
<td>0.00000</td>
<td>0.00038</td>
<td>.</td>
</tr>
<tr>
<td>17</td>
<td>trt</td>
<td>S452</td>
<td>-19.6200</td>
<td>0.2132</td>
<td>-2.13242</td>
<td>0.00000</td>
<td>0.00105</td>
<td>.</td>
</tr>
<tr>
<td>18</td>
<td>trt</td>
<td>S302</td>
<td>-19.5867</td>
<td>0.2132</td>
<td>-2.09908</td>
<td>0.00000</td>
<td>0.00147</td>
<td>.</td>
</tr>
<tr>
<td>19</td>
<td>trt</td>
<td>S453</td>
<td>-19.5500</td>
<td>0.2611</td>
<td>-2.16303</td>
<td>0.00000</td>
<td>0.00612</td>
<td>.</td>
</tr>
<tr>
<td>20</td>
<td>trt</td>
<td>W303</td>
<td>-19.4400</td>
<td>0.2611</td>
<td>-2.05303</td>
<td>0.00000</td>
<td>0.01453</td>
<td>.</td>
</tr>
<tr>
<td>21</td>
<td>trt</td>
<td>W451</td>
<td>-18.9000</td>
<td>0.2611</td>
<td>-1.51303</td>
<td>0.36475</td>
<td>0.33068</td>
<td>.</td>
</tr>
<tr>
<td>22</td>
<td>trt</td>
<td>W601</td>
<td>-18.8150</td>
<td>0.2611</td>
<td>-1.42803</td>
<td>0.44975</td>
<td>0.44342</td>
<td>.</td>
</tr>
<tr>
<td>23</td>
<td>trt</td>
<td>W302</td>
<td>-18.8033</td>
<td>0.2132</td>
<td>-1.31575</td>
<td>0.38908</td>
<td>0.45049</td>
<td>.</td>
</tr>
<tr>
<td>24</td>
<td>trt</td>
<td>W602</td>
<td>-18.6433</td>
<td>0.2132</td>
<td>-1.15575</td>
<td>0.54908</td>
<td>0.70890</td>
<td>.</td>
</tr>
<tr>
<td>25</td>
<td>trt</td>
<td>W453</td>
<td>-18.5833</td>
<td>0.2132</td>
<td>-1.09575</td>
<td>0.60908</td>
<td>0.79052</td>
<td>.</td>
</tr>
<tr>
<td>26</td>
<td>trt</td>
<td>S601</td>
<td>-18.4667</td>
<td>0.2132</td>
<td>-0.97908</td>
<td>0.72575</td>
<td>0.90575</td>
<td>.</td>
</tr>
<tr>
<td>27</td>
<td>trt</td>
<td>W452</td>
<td>-18.3400</td>
<td>0.2132</td>
<td>-0.72575</td>
<td>0.97908</td>
<td>0.90575</td>
<td>.</td>
</tr>
</tbody>
</table>

Note. In ‘trt’ statement, W, S and L represent whole plant, stem and leaf samples, 30, 45 and 60 indicate treatment time, while the following 1, 2 and 3 correspond to acid concentration of 0.5%, 1.0% and 1.5%, respectively. The pretreatment which results in least lignin content within each germplasm is listed at the bottom (since the original SAS code is designed to select the greatest value based on the complete input, a negative sign (‘-’) is added in front of each value to make the exactly least (appeared to be the greatest) value be present at the bottom.)
Table IIj Multiple Comparison with the Best for Least Lignin Content Selection from Dilute Acid Pretreated Freeze-dried St6-3E Switchgrass

<table>
<thead>
<tr>
<th>Obs</th>
<th>Effect</th>
<th>trt</th>
<th>Estimate</th>
<th>StdErr</th>
<th>cllo</th>
<th>clhi</th>
<th>rval</th>
<th>sval</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>trt</td>
<td>L301</td>
<td>-21.5033</td>
<td>0.2293</td>
<td>-5.51358</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>2</td>
<td>trt</td>
<td>L451</td>
<td>-20.2700</td>
<td>0.2293</td>
<td>-4.28025</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>3</td>
<td>trt</td>
<td>L303</td>
<td>-20.0100</td>
<td>0.2293</td>
<td>-4.02025</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>4</td>
<td>trt</td>
<td>L453</td>
<td>-19.9200</td>
<td>0.2293</td>
<td>-3.93025</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>5</td>
<td>trt</td>
<td>L601</td>
<td>-19.8050</td>
<td>0.2808</td>
<td>-3.92308</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>6</td>
<td>trt</td>
<td>L603</td>
<td>-19.7200</td>
<td>0.2293</td>
<td>-3.73025</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>7</td>
<td>trt</td>
<td>W301</td>
<td>-19.6833</td>
<td>0.2293</td>
<td>-3.69358</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>8</td>
<td>trt</td>
<td>L602</td>
<td>-19.4433</td>
<td>0.2293</td>
<td>-3.45358</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>9</td>
<td>trt</td>
<td>W603</td>
<td>-19.4400</td>
<td>0.2808</td>
<td>-3.55808</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>10</td>
<td>trt</td>
<td>L302</td>
<td>-19.3867</td>
<td>0.2293</td>
<td>-3.39692</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>11</td>
<td>trt</td>
<td>S301</td>
<td>-19.2467</td>
<td>0.2293</td>
<td>-3.25692</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>12</td>
<td>trt</td>
<td>L452</td>
<td>-19.1967</td>
<td>0.2293</td>
<td>-3.20692</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>13</td>
<td>trt</td>
<td>W601</td>
<td>-19.0600</td>
<td>0.2293</td>
<td>-3.07025</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>14</td>
<td>trt</td>
<td>W302</td>
<td>-19.0500</td>
<td>0.2293</td>
<td>-3.06025</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>15</td>
<td>trt</td>
<td>W303</td>
<td>-18.9967</td>
<td>0.2293</td>
<td>-3.00692</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>16</td>
<td>trt</td>
<td>W451</td>
<td>-18.8300</td>
<td>0.2293</td>
<td>-2.84025</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>17</td>
<td>trt</td>
<td>W452</td>
<td>-18.6933</td>
<td>0.2293</td>
<td>-2.70358</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>18</td>
<td>trt</td>
<td>S453</td>
<td>-18.6767</td>
<td>0.2293</td>
<td>-2.68692</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>19</td>
<td>trt</td>
<td>W602</td>
<td>-18.6467</td>
<td>0.2293</td>
<td>-2.65692</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>20</td>
<td>trt</td>
<td>S451</td>
<td>-18.3500</td>
<td>0.2293</td>
<td>-2.36025</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>21</td>
<td>trt</td>
<td>S302</td>
<td>-18.3150</td>
<td>0.2808</td>
<td>-2.43308</td>
<td>0.00000</td>
<td>0.00247</td>
<td>.</td>
</tr>
<tr>
<td>22</td>
<td>trt</td>
<td>S603</td>
<td>-18.2867</td>
<td>0.2293</td>
<td>-2.29692</td>
<td>0.00000</td>
<td>0.00092</td>
<td>.</td>
</tr>
<tr>
<td>23</td>
<td>trt</td>
<td>S303</td>
<td>-18.1833</td>
<td>0.2293</td>
<td>-2.19358</td>
<td>0.00000</td>
<td>0.00246</td>
<td>.</td>
</tr>
<tr>
<td>24</td>
<td>trt</td>
<td>S601</td>
<td>-18.1400</td>
<td>0.2293</td>
<td>-2.15025</td>
<td>0.00000</td>
<td>0.00366</td>
<td>.</td>
</tr>
<tr>
<td>25</td>
<td>trt</td>
<td>S452</td>
<td>-18.0933</td>
<td>0.2293</td>
<td>-2.10358</td>
<td>0.00000</td>
<td>0.00555</td>
<td>.</td>
</tr>
<tr>
<td>26</td>
<td>trt</td>
<td>W453</td>
<td>-18.0750</td>
<td>0.2808</td>
<td>-2.19308</td>
<td>0.00000</td>
<td>0.01525</td>
<td>.</td>
</tr>
<tr>
<td>27</td>
<td>trt</td>
<td>S602</td>
<td>-16.9033</td>
<td>0.2293</td>
<td>0.00000 2.10358</td>
<td>0.00000</td>
<td>0.01525</td>
<td></td>
</tr>
</tbody>
</table>

Note. In ‘trt’ statement, W, S and L represent whole plant, stem and leaf samples, 30, 45 and 60 indicate treatment time, while the following 1, 2 and 3 correspond to acid concentration of 0.5%, 1.0% and 1.5%, respectively. The pretreatment which results in least lignin content within each germplasm is listed at the bottom (since the original SAS code is designed to select the greatest value based on the complete input, a negative sign (‘-’) is added in front of each value to make the exactly least (appeared to be the greatest) value be present at the bottom.)
Table IIk Multiple Comparison with the Best for Least Lignin Content Selection from Dilute Acid Pretreated Freeze-dried St6-3F Switchgrass

<table>
<thead>
<tr>
<th>Obs</th>
<th>Effect</th>
<th>trt</th>
<th>Estimate</th>
<th>StdErr</th>
<th>cllo</th>
<th>clhi</th>
<th>rval</th>
<th>sval</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>trt</td>
<td>L301</td>
<td>-21.7267</td>
<td>0.2122</td>
<td>-6.62261</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>2</td>
<td>trt</td>
<td>L601</td>
<td>-20.0900</td>
<td>0.2122</td>
<td>-4.98594</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>3</td>
<td>trt</td>
<td>S301</td>
<td>-19.4933</td>
<td>0.2122</td>
<td>-4.38928</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>4</td>
<td>trt</td>
<td>L453</td>
<td>-19.0900</td>
<td>0.2598</td>
<td>-4.08540</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>5</td>
<td>trt</td>
<td>S601</td>
<td>-18.8800</td>
<td>0.2122</td>
<td>-3.77594</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>6</td>
<td>trt</td>
<td>L603</td>
<td>-18.6600</td>
<td>0.2122</td>
<td>-3.55594</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>7</td>
<td>trt</td>
<td>W301</td>
<td>-18.6567</td>
<td>0.2122</td>
<td>-3.55261</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>8</td>
<td>trt</td>
<td>W601</td>
<td>-18.6267</td>
<td>0.2122</td>
<td>-3.52261</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>9</td>
<td>trt</td>
<td>S451</td>
<td>-18.5550</td>
<td>0.2598</td>
<td>-3.55040</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>10</td>
<td>trt</td>
<td>L602</td>
<td>-18.4567</td>
<td>0.2122</td>
<td>-3.5261</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>11</td>
<td>trt</td>
<td>L303</td>
<td>-18.3767</td>
<td>0.2122</td>
<td>-3.27261</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>12</td>
<td>trt</td>
<td>S453</td>
<td>-18.3433</td>
<td>0.2122</td>
<td>-3.23928</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>13</td>
<td>trt</td>
<td>L302</td>
<td>-18.1933</td>
<td>0.2122</td>
<td>-3.08928</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>14</td>
<td>trt</td>
<td>L452</td>
<td>-17.8533</td>
<td>0.2122</td>
<td>-2.74928</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>15</td>
<td>trt</td>
<td>S602</td>
<td>-17.8067</td>
<td>0.2122</td>
<td>-2.70261</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>16</td>
<td>trt</td>
<td>S452</td>
<td>-17.7867</td>
<td>0.2122</td>
<td>-2.68261</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>17</td>
<td>trt</td>
<td>S603</td>
<td>-17.6433</td>
<td>0.2122</td>
<td>-2.53928</td>
<td>0.00000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>18</td>
<td>trt</td>
<td>S303</td>
<td>-17.4833</td>
<td>0.2122</td>
<td>-2.37928</td>
<td>0.00000</td>
<td>0.00005</td>
<td>.</td>
</tr>
<tr>
<td>19</td>
<td>trt</td>
<td>S302</td>
<td>-17.4767</td>
<td>0.2122</td>
<td>-2.37261</td>
<td>0.00000</td>
<td>0.00006</td>
<td>.</td>
</tr>
<tr>
<td>20</td>
<td>trt</td>
<td>W603</td>
<td>-16.2233</td>
<td>0.2122</td>
<td>-2.11928</td>
<td>0.00000</td>
<td>0.00092</td>
<td>.</td>
</tr>
<tr>
<td>21</td>
<td>trt</td>
<td>W602</td>
<td>-16.8567</td>
<td>0.2122</td>
<td>-1.75261</td>
<td>0.00000</td>
<td>0.02940</td>
<td>.</td>
</tr>
<tr>
<td>22</td>
<td>trt</td>
<td>W303</td>
<td>-16.7233</td>
<td>0.2122</td>
<td>-1.61928</td>
<td>0.06594</td>
<td>0.08083</td>
<td>.</td>
</tr>
<tr>
<td>23</td>
<td>trt</td>
<td>W302</td>
<td>-16.1700</td>
<td>0.2122</td>
<td>-1.06594</td>
<td>0.61928</td>
<td>0.80234</td>
<td>.</td>
</tr>
<tr>
<td>24</td>
<td>trt</td>
<td>L451</td>
<td>-16.0767</td>
<td>0.2122</td>
<td>-0.97261</td>
<td>0.71261</td>
<td>0.89470</td>
<td>.</td>
</tr>
<tr>
<td>25</td>
<td>trt</td>
<td>W452</td>
<td>-16.0767</td>
<td>0.2122</td>
<td>-0.97261</td>
<td>0.71261</td>
<td>0.89470</td>
<td>.</td>
</tr>
<tr>
<td>26</td>
<td>trt</td>
<td>W451</td>
<td>-16.0600</td>
<td>0.2122</td>
<td>-0.95594</td>
<td>0.72928</td>
<td>0.90724</td>
<td>.</td>
</tr>
<tr>
<td>27</td>
<td>trt</td>
<td>W453</td>
<td>-15.9467</td>
<td>0.2122</td>
<td>-0.72928</td>
<td>0.95594</td>
<td>0.90724</td>
<td>.</td>
</tr>
</tbody>
</table>

Note. In ‘trt’ statement, W, S and L represent whole plant, stem and leaf samples, 30, 45 and 60 indicate treatment time, while the following 1, 2 and 3 correspond to acid concentration of 0.5%, 1.0% and 1.5%, respectively. The pretreatment which results in least lignin content within each germplasm is listed at the bottom (since the original SAS code is designed to select the greatest value based on the complete input, a negative sign (‘-‘) is added in front of each value to make the exactly least (appeared to be the greatest) value be present at the bottom.)
### Table III Multiple Comparison with the Best for Greatest Hemicellulose Solubilization Selection from Dilute Acid Pretreated Freeze-dried St6-I Switchgrass

<table>
<thead>
<tr>
<th>Obs</th>
<th>Effect</th>
<th>trt</th>
<th>Estimate</th>
<th>StdErr</th>
<th>cllo</th>
<th>clhi</th>
<th>rval</th>
<th>sval</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>trt</td>
<td>L601</td>
<td>19.3167</td>
<td>4.1853</td>
<td>-75.5438</td>
<td>0.0000</td>
<td>0.0000</td>
<td>.</td>
</tr>
<tr>
<td>2</td>
<td>trt</td>
<td>L451</td>
<td>30.0233</td>
<td>4.1853</td>
<td>-64.8371</td>
<td>0.0000</td>
<td>0.0000</td>
<td>.</td>
</tr>
<tr>
<td>3</td>
<td>trt</td>
<td>W601</td>
<td>33.2700</td>
<td>5.1260</td>
<td>-63.5558</td>
<td>0.0000</td>
<td>0.0000</td>
<td>.</td>
</tr>
<tr>
<td>4</td>
<td>trt</td>
<td>L301</td>
<td>37.9733</td>
<td>4.1853</td>
<td>-56.8871</td>
<td>0.0000</td>
<td>0.0000</td>
<td>.</td>
</tr>
<tr>
<td>5</td>
<td>trt</td>
<td>W301</td>
<td>39.6767</td>
<td>4.1853</td>
<td>-55.1838</td>
<td>0.0000</td>
<td>0.0000</td>
<td>.</td>
</tr>
<tr>
<td>6</td>
<td>trt</td>
<td>S601</td>
<td>41.9533</td>
<td>4.1853</td>
<td>-52.9071</td>
<td>0.0000</td>
<td>0.0000</td>
<td>.</td>
</tr>
<tr>
<td>7</td>
<td>trt</td>
<td>W451</td>
<td>43.3633</td>
<td>4.1853</td>
<td>-51.4971</td>
<td>0.0000</td>
<td>0.0000</td>
<td>.</td>
</tr>
<tr>
<td>8</td>
<td>trt</td>
<td>S451</td>
<td>44.5633</td>
<td>4.1853</td>
<td>-50.2971</td>
<td>0.0000</td>
<td>0.0000</td>
<td>.</td>
</tr>
<tr>
<td>9</td>
<td>trt</td>
<td>S301</td>
<td>47.4300</td>
<td>4.1853</td>
<td>-47.4304</td>
<td>0.0000</td>
<td>0.0000</td>
<td>.</td>
</tr>
<tr>
<td>10</td>
<td>trt</td>
<td>W302</td>
<td>55.8100</td>
<td>4.1853</td>
<td>-39.0504</td>
<td>0.0000</td>
<td>0.0039</td>
<td>.</td>
</tr>
<tr>
<td>11</td>
<td>trt</td>
<td>S302</td>
<td>58.0033</td>
<td>4.1853</td>
<td>-36.0571</td>
<td>0.0000</td>
<td>0.0159</td>
<td>.</td>
</tr>
<tr>
<td>12</td>
<td>trt</td>
<td>S303</td>
<td>60.3200</td>
<td>4.1853</td>
<td>-34.5404</td>
<td>0.0000</td>
<td>0.0305</td>
<td>.</td>
</tr>
<tr>
<td>13</td>
<td>trt</td>
<td>W303</td>
<td>63.5000</td>
<td>4.1853</td>
<td>-31.3604</td>
<td>1.9404</td>
<td>0.1008</td>
<td>.</td>
</tr>
<tr>
<td>14</td>
<td>trt</td>
<td>S452</td>
<td>67.6567</td>
<td>4.1853</td>
<td>-27.2038</td>
<td>6.0971</td>
<td>0.3271</td>
<td>.</td>
</tr>
<tr>
<td>15</td>
<td>trt</td>
<td>W452</td>
<td>69.6967</td>
<td>4.1853</td>
<td>-25.1638</td>
<td>8.1371</td>
<td>0.4909</td>
<td>.</td>
</tr>
<tr>
<td>16</td>
<td>trt</td>
<td>L303</td>
<td>71.6967</td>
<td>4.1853</td>
<td>-23.1638</td>
<td>10.1379</td>
<td>0.6566</td>
<td>.</td>
</tr>
<tr>
<td>17</td>
<td>trt</td>
<td>W602</td>
<td>73.2233</td>
<td>4.1853</td>
<td>-21.6371</td>
<td>11.6638</td>
<td>0.7684</td>
<td>.</td>
</tr>
<tr>
<td>18</td>
<td>trt</td>
<td>L603</td>
<td>73.3000</td>
<td>5.1260</td>
<td>-23.5258</td>
<td>13.1379</td>
<td>0.7356</td>
<td>.</td>
</tr>
<tr>
<td>19</td>
<td>trt</td>
<td>S602</td>
<td>73.3633</td>
<td>4.1853</td>
<td>-21.4971</td>
<td>11.8038</td>
<td>0.7776</td>
<td>.</td>
</tr>
<tr>
<td>20</td>
<td>trt</td>
<td>S453</td>
<td>73.8450</td>
<td>5.1260</td>
<td>-22.9808</td>
<td>13.6829</td>
<td>0.3271</td>
<td>.</td>
</tr>
<tr>
<td>21</td>
<td>trt</td>
<td>W453</td>
<td>74.5367</td>
<td>4.1853</td>
<td>-20.3238</td>
<td>12.9771</td>
<td>0.8462</td>
<td>.</td>
</tr>
<tr>
<td>22</td>
<td>trt</td>
<td>L302</td>
<td>75.0567</td>
<td>4.1853</td>
<td>-19.8038</td>
<td>13.4971</td>
<td>0.8716</td>
<td>.</td>
</tr>
<tr>
<td>23</td>
<td>trt</td>
<td>S603</td>
<td>75.0967</td>
<td>4.1853</td>
<td>-19.7638</td>
<td>13.5371</td>
<td>0.8734</td>
<td>.</td>
</tr>
<tr>
<td>24</td>
<td>trt</td>
<td>L453</td>
<td>76.9700</td>
<td>4.1853</td>
<td>-17.8904</td>
<td>15.4104</td>
<td>0.9400</td>
<td>.</td>
</tr>
<tr>
<td>25</td>
<td>trt</td>
<td>L602</td>
<td>77.4433</td>
<td>4.1853</td>
<td>-17.4171</td>
<td>15.8838</td>
<td>0.9514</td>
<td>.</td>
</tr>
<tr>
<td>26</td>
<td>trt</td>
<td>W603</td>
<td>77.6933</td>
<td>4.1853</td>
<td>-17.1671</td>
<td>16.1338</td>
<td>0.9567</td>
<td>.</td>
</tr>
<tr>
<td>27</td>
<td>trt</td>
<td>L452</td>
<td>78.2100</td>
<td>4.1853</td>
<td>-16.1338</td>
<td>17.1671</td>
<td>0.9567</td>
<td>.</td>
</tr>
</tbody>
</table>

Note. In ‘trt’ statement, W, S and L represent whole plant, stem and leaf samples, 30, 45 and 60 indicate treatment time, while the following 1, 2 and 3 correspond to acid concentration of 0.5%, 1.0% and 1.5%, respectively. The pretreatment which results in greatest hemicellulose solubilization within each germplasm is listed at the bottom.
Table IIIm Multiple Comparison with the Best for Greatest Hemicellulose Solubilization Selection from Dilute Acid Pretreated Freeze-dried St6-3E Switchgrass

<table>
<thead>
<tr>
<th>Obs</th>
<th>Effect</th>
<th>trt</th>
<th>Estimate</th>
<th>StdErr</th>
<th>cllo</th>
<th>clhi</th>
<th>rval</th>
<th>sval</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>trt</td>
<td>L301</td>
<td>19.2250</td>
<td>5.8789</td>
<td>-86.1151</td>
<td>0.0000</td>
<td>0.0000</td>
<td>.</td>
</tr>
<tr>
<td>2</td>
<td>trt</td>
<td>S601</td>
<td>27.2467</td>
<td>4.8001</td>
<td>-75.8394</td>
<td>0.0000</td>
<td>0.0000</td>
<td>.</td>
</tr>
<tr>
<td>3</td>
<td>trt</td>
<td>L451</td>
<td>36.5933</td>
<td>4.8001</td>
<td>-66.4927</td>
<td>0.0000</td>
<td>0.0000</td>
<td>.</td>
</tr>
<tr>
<td>4</td>
<td>trt</td>
<td>S451</td>
<td>38.6900</td>
<td>4.8001</td>
<td>-64.3961</td>
<td>0.0000</td>
<td>0.0000</td>
<td>.</td>
</tr>
<tr>
<td>5</td>
<td>trt</td>
<td>W601</td>
<td>39.9467</td>
<td>4.8001</td>
<td>-63.1394</td>
<td>0.0000</td>
<td>0.0000</td>
<td>.</td>
</tr>
<tr>
<td>6</td>
<td>trt</td>
<td>L601</td>
<td>48.2833</td>
<td>4.8001</td>
<td>-54.8027</td>
<td>0.0000</td>
<td>0.0000</td>
<td>.</td>
</tr>
<tr>
<td>7</td>
<td>trt</td>
<td>W451</td>
<td>52.3733</td>
<td>4.8001</td>
<td>-50.7127</td>
<td>0.0000</td>
<td>0.0000</td>
<td>.</td>
</tr>
<tr>
<td>8</td>
<td>trt</td>
<td>S302</td>
<td>54.2100</td>
<td>4.8001</td>
<td>-48.8761</td>
<td>0.0000</td>
<td>0.0000</td>
<td>.</td>
</tr>
<tr>
<td>9</td>
<td>trt</td>
<td>S301</td>
<td>56.5700</td>
<td>4.8001</td>
<td>-46.5161</td>
<td>0.0000</td>
<td>0.0000</td>
<td>.</td>
</tr>
<tr>
<td>10</td>
<td>trt</td>
<td>W302</td>
<td>57.0233</td>
<td>4.8001</td>
<td>-46.0627</td>
<td>0.0000</td>
<td>0.0000</td>
<td>.</td>
</tr>
<tr>
<td>11</td>
<td>trt</td>
<td>W301</td>
<td>57.0933</td>
<td>4.8001</td>
<td>-45.9927</td>
<td>0.0000</td>
<td>0.0000</td>
<td>.</td>
</tr>
<tr>
<td>12</td>
<td>trt</td>
<td>S452</td>
<td>62.5967</td>
<td>4.8001</td>
<td>-40.4894</td>
<td>0.0000</td>
<td>0.0000</td>
<td>.</td>
</tr>
<tr>
<td>13</td>
<td>trt</td>
<td>W452</td>
<td>65.9100</td>
<td>4.8001</td>
<td>-37.1761</td>
<td>0.0161</td>
<td>0.0696</td>
<td>.</td>
</tr>
<tr>
<td>14</td>
<td>trt</td>
<td>S303</td>
<td>67.4633</td>
<td>4.8001</td>
<td>-35.6227</td>
<td>2.5694</td>
<td>0.1144</td>
<td>.</td>
</tr>
<tr>
<td>15</td>
<td>trt</td>
<td>L302</td>
<td>69.7267</td>
<td>4.8001</td>
<td>-33.3594</td>
<td>4.8327</td>
<td>0.2032</td>
<td>.</td>
</tr>
<tr>
<td>16</td>
<td>trt</td>
<td>W303</td>
<td>69.9633</td>
<td>4.8001</td>
<td>-33.1227</td>
<td>5.0694</td>
<td>0.2151</td>
<td>.</td>
</tr>
<tr>
<td>17</td>
<td>trt</td>
<td>L452</td>
<td>70.2500</td>
<td>4.8001</td>
<td>-32.8361</td>
<td>5.3561</td>
<td>0.2301</td>
<td>.</td>
</tr>
<tr>
<td>18</td>
<td>trt</td>
<td>W602</td>
<td>72.8567</td>
<td>4.8001</td>
<td>-30.2294</td>
<td>7.9627</td>
<td>0.3924</td>
<td>.</td>
</tr>
<tr>
<td>19</td>
<td>trt</td>
<td>S602</td>
<td>73.7167</td>
<td>4.8001</td>
<td>-29.3694</td>
<td>8.8227</td>
<td>0.4537</td>
<td>.</td>
</tr>
<tr>
<td>21</td>
<td>trt</td>
<td>L602</td>
<td>74.9233</td>
<td>4.8001</td>
<td>-28.1627</td>
<td>10.0294</td>
<td>0.5421</td>
<td>.</td>
</tr>
<tr>
<td>22</td>
<td>trt</td>
<td>L303</td>
<td>76.1167</td>
<td>4.8001</td>
<td>-26.9694</td>
<td>11.2227</td>
<td>0.6285</td>
<td>.</td>
</tr>
<tr>
<td>23</td>
<td>trt</td>
<td>L453</td>
<td>76.6200</td>
<td>4.8001</td>
<td>-26.4661</td>
<td>11.7261</td>
<td>0.6635</td>
<td>.</td>
</tr>
<tr>
<td>24</td>
<td>trt</td>
<td>S603</td>
<td>78.4967</td>
<td>4.8001</td>
<td>-24.5894</td>
<td>13.6027</td>
<td>0.7812</td>
<td>.</td>
</tr>
<tr>
<td>25</td>
<td>trt</td>
<td>W453</td>
<td>79.4450</td>
<td>5.8789</td>
<td>-25.8951</td>
<td>16.1538</td>
<td>0.7888</td>
<td>.</td>
</tr>
<tr>
<td>26</td>
<td>trt</td>
<td>W603</td>
<td>80.3950</td>
<td>5.8789</td>
<td>-24.9451</td>
<td>17.1038</td>
<td>0.8299</td>
<td>.</td>
</tr>
<tr>
<td>27</td>
<td>trt</td>
<td>L603</td>
<td>83.9900</td>
<td>4.8001</td>
<td>-17.1038</td>
<td>24.5894</td>
<td>0.8299</td>
<td>.</td>
</tr>
</tbody>
</table>

Note. In ‘trt’ statement, W, S and L represent whole plant, stem and leaf samples, 30, 45 and 60 indicate treatment time, while the following 1, 2 and 3 correspond to acid concentration of 0.5%, 1.0% and 1.5%, respectively. The pretreatment which results in greatest hemicellulose solubilization within each germplasm is listed at the bottom.
### Table II: Multiple Comparison with the Best for Greatest Hemicellulose Solubilization Selection from Dilute Acid Pretreated Freeze-dried St6-3F Switchgrass

<table>
<thead>
<tr>
<th>Obs</th>
<th>Effect</th>
<th>trt</th>
<th>Estimate</th>
<th>StdErr</th>
<th>cllo</th>
<th>clhi</th>
<th>rval</th>
<th>sval</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>trt</td>
<td>W301</td>
<td>36.8100</td>
<td>4.1080</td>
<td>-65.4061</td>
<td>0.0000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>2</td>
<td>trt</td>
<td>L301</td>
<td>38.5133</td>
<td>4.1080</td>
<td>-63.7028</td>
<td>0.0000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>3</td>
<td>trt</td>
<td>S301</td>
<td>40.8767</td>
<td>4.1080</td>
<td>-61.3395</td>
<td>0.0000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>4</td>
<td>trt</td>
<td>S451</td>
<td>44.4700</td>
<td>5.0313</td>
<td>-59.6752</td>
<td>0.0000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>5</td>
<td>trt</td>
<td>S303</td>
<td>56.8100</td>
<td>4.1080</td>
<td>-45.4061</td>
<td>0.0000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>6</td>
<td>trt</td>
<td>W451</td>
<td>57.1400</td>
<td>4.1080</td>
<td>-45.0761</td>
<td>0.0000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>7</td>
<td>trt</td>
<td>S452</td>
<td>57.4100</td>
<td>4.1080</td>
<td>-44.8061</td>
<td>0.0000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>8</td>
<td>trt</td>
<td>W302</td>
<td>59.5100</td>
<td>4.1080</td>
<td>-42.7061</td>
<td>0.0000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>9</td>
<td>trt</td>
<td>S302</td>
<td>60.4900</td>
<td>4.1080</td>
<td>-41.7261</td>
<td>0.0000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>10</td>
<td>trt</td>
<td>L451</td>
<td>61.2533</td>
<td>4.1080</td>
<td>-40.9628</td>
<td>0.0000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>11</td>
<td>trt</td>
<td>W303</td>
<td>67.3133</td>
<td>4.1080</td>
<td>-34.9028</td>
<td>0.0000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>12</td>
<td>trt</td>
<td>L601</td>
<td>67.3167</td>
<td>4.1080</td>
<td>-34.8995</td>
<td>0.0000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>13</td>
<td>trt</td>
<td>S453</td>
<td>67.5700</td>
<td>4.1080</td>
<td>-34.6461</td>
<td>0.0000</td>
<td>0.00000</td>
<td>.</td>
</tr>
<tr>
<td>14</td>
<td>trt</td>
<td>L452</td>
<td>68.8200</td>
<td>4.1080</td>
<td>-33.3961</td>
<td>0.0000</td>
<td>0.03763</td>
<td>.</td>
</tr>
<tr>
<td>15</td>
<td>trt</td>
<td>S601</td>
<td>69.7467</td>
<td>4.1080</td>
<td>-32.4695</td>
<td>0.2161</td>
<td>0.05439</td>
<td>.</td>
</tr>
<tr>
<td>16</td>
<td>trt</td>
<td>W452</td>
<td>70.8167</td>
<td>4.1080</td>
<td>-31.3995</td>
<td>1.2861</td>
<td>0.08109</td>
<td>.</td>
</tr>
<tr>
<td>17</td>
<td>trt</td>
<td>L303</td>
<td>72.0633</td>
<td>4.1080</td>
<td>-30.1528</td>
<td>2.5328</td>
<td>0.12450</td>
<td>.</td>
</tr>
<tr>
<td>18</td>
<td>trt</td>
<td>W453</td>
<td>75.4100</td>
<td>4.1080</td>
<td>-26.8061</td>
<td>5.8795</td>
<td>0.31927</td>
<td>.</td>
</tr>
<tr>
<td>19</td>
<td>trt</td>
<td>W603</td>
<td>75.7500</td>
<td>5.0313</td>
<td>-28.3952</td>
<td>7.5911</td>
<td>0.36718</td>
<td>.</td>
</tr>
<tr>
<td>20</td>
<td>trt</td>
<td>W601</td>
<td>76.5633</td>
<td>4.1080</td>
<td>-25.6528</td>
<td>7.0328</td>
<td>0.41039</td>
<td>.</td>
</tr>
<tr>
<td>21</td>
<td>trt</td>
<td>W602</td>
<td>78.0133</td>
<td>4.1080</td>
<td>-24.2028</td>
<td>8.4828</td>
<td>0.53353</td>
<td>.</td>
</tr>
<tr>
<td>22</td>
<td>trt</td>
<td>L453</td>
<td>78.3550</td>
<td>5.0313</td>
<td>-25.7902</td>
<td>10.1961</td>
<td>0.55272</td>
<td>.</td>
</tr>
<tr>
<td>23</td>
<td>trt</td>
<td>S602</td>
<td>78.5700</td>
<td>4.1080</td>
<td>-23.6461</td>
<td>9.0395</td>
<td>0.58112</td>
<td>.</td>
</tr>
<tr>
<td>24</td>
<td>trt</td>
<td>S603</td>
<td>78.6000</td>
<td>4.1080</td>
<td>-23.6161</td>
<td>9.0695</td>
<td>0.58367</td>
<td>.</td>
</tr>
<tr>
<td>25</td>
<td>trt</td>
<td>L302</td>
<td>78.6167</td>
<td>4.1080</td>
<td>-23.5995</td>
<td>9.0861</td>
<td>0.58508</td>
<td>.</td>
</tr>
<tr>
<td>26</td>
<td>trt</td>
<td>L603</td>
<td>85.4333</td>
<td>4.1080</td>
<td>-16.7828</td>
<td>15.9028</td>
<td>0.95814</td>
<td>.</td>
</tr>
<tr>
<td>27</td>
<td>trt</td>
<td>L602</td>
<td>85.8733</td>
<td>4.1080</td>
<td>-15.9028</td>
<td>16.7828</td>
<td>0.95814</td>
<td>.</td>
</tr>
</tbody>
</table>

Note. In ‘trt’ statement, W, S and L represent whole plant, stem and leaf samples, 30, 45 and 60 indicate treatment time, while the following 1, 2 and 3 correspond to acid concentration of 0.5%, 1.0% and 1.5%, respectively. The pretreatment which results in greatest hemicellulose solubilization within each germplasm is listed at the bottom.
Table IIo SAS Codes of Multiple Comparison with the Best for Selecting the Pretreatment Condition Yielding Greatest Hemicellulose Solubilization (Example: freeze-dried St6-1 switchgrass sample)

```sas
options ls=75 ps=1000;

data switch;
  input plantpart$  minutes  ac  hcdegradation;
  ac=round(ac/0.5,1);
  cards;
  W  60  1.5  74.96
  W  60  1.5  80.10
  W  60  1.5  78.02
  S  60  1.5  74.51
  .  .  .
  S  45  1.5  75.82
  L  45  1.5  77.02
  L  45  1.5  76.55
  L  45  1.5  77.34
; run;

data switch;
  set switch;
  length trt $ 20;
  trt=trim(left(plantpart))||trim(left(minutes))||left(ac);
run;

%macro n2cclass(dsin,dsout,class,options = );

  %let clean = 1;
  %let iopt = 1;
  %do %while(%length(%scan(&options,&iopt)));
    %if (%upcase(%scan(&options,&iopt)) = NOCLEAN) %then
      %let clean = 0;
    %else
      %put Warning: Unrecognized option %scan(&options,&iopt).;
    %let iopt = %eval(&iopt + 1);
  %end;

ods listing close;
```

182
ods output Variables=_Var;
proc contents data=&dsin;
run;
ods listing;

%let numc =;
%let nnumc = 0;
data _null_; set _Var;
  _nc = 1; _cvar = trim(left(upcase(scan("&class",_nc))));
do while (_cvar ^= ' ');
  if ( (trim(left(upcase(Variable)))=_cvar ) & (trim(left(Type ))='Num')) then do;
    call symput('numc', trim(left( symget('numc')||' ' ||trim(left(Variable)))))
    call symput('nnumc',trim(left(1+symget('nnumc'))));
  end;
  _nc = _nc + 1; _cvar = trim(left(upcase(scan("&class",_nc))));
end;
run;

data &dsout; set &dsin; run;
%do inumc = 1 %to &nnumc;
  %let numcvar = %scan(&numc,&inumc);
data &dsout; set &dsout;
  _tempc = trim(left(put(&numcvar,best16.))); data &dsout; set &dsout;
  drop &numcvar;
data &dsout; set &dsout;
  rename _tempc=&numcvar;
run;
%end;

%let allv =;
%let nallv = 0;
proc sort data=_Var out=_Var; by Num;
data _null_; set _Var;
  call symput('allv', trim(left( symget('allv')||' ' ||trim(left(Variable)))))
  call symput('nallv',trim(left(1+symget('nallv'))));
run;
data _temp; set &dsout;
data &dsout; if (0); run;
%do ivar = 1 %to &nallv;
   %let var = %scan(&allv,&ivar);
data &dsout; merge &dsout _temp(keep=&var);
   run;
%end;

%if (&clean) %then %do;
   proc datasets library=work nolist;
      delete _Var _temp;
   run;
%end;
%mend;

/*-------------------------------------------------------------------
/*  Constrained MC with the best                                      */
 /*-------------------------------------------------------------------*/
%macro mcb(data            ,
   resp            ,
   mean            ,
   model   = &mean ,
   class   = &mean ,
   alpha   = 0.05  ,
   out     = mcbout,
   options =       );
   /*  Retrieve options.                                              */
   %let print = 1;
   %let clean = 1;
   %let iopt = 1;
   %do %while(%length(%scan(&options,&iopt)));
      %if (%upcase(%scan(&options,&iopt)) = NOPRINT) %then
         %let print = 0;
   %end;
%else %if (%upcase(%scan(&options,&iopt)) = NOCLEAN) %then
   %let clean = 0;
%else
   %put Warning: Unrecognized option %scan(&options,&iopt).;
%let iopt = %eval(&iopt + 1);
%end;

/*
 / Count number of variables in grouping effect.
 /-------------------------------------------------------------------------------
-*/
%let ivar = 1;
%do %while(%length(%scan(&mean,&ivar,*)));
   %let var&ivar = %upcase(%scan(&mean,&ivar,*));
   %let ivar = %eval(&ivar + 1);
%end;
%let nvar = %eval(&ivar - 1);

/*
 / Compute ANOVA and LSMEANS
 /-------------------------------------------------------------------------------
-*/
ods listing close;
proc mixed data=&data;
   class &class;
   model &resp = &model;
   lsmeans &mean;
/*
   make 'LSMeans' out=&out;
*/
ods output LSMeans=&out;
run;
%n2cclass(&out,&out,&class);
ods listing;
data &out; set &out; orig_n = _n_; proc sort data=&out out=&out; by &mean; run;

/*
 / Retrieve the levels of the classification variable.
 /-------------------------------------------------------------------------------
-*/
data &out; set &out;
  drop tvalue probt;
  length level $ 20;

level = ';
%do ivar = 1 %to &nvar;
  level = trim(left(level)) || ' ' || trim(left(&&var&ivar));
%end;
call symput('nlev',trim(left(_n_)));
call symput('lev'||trim(left(_n_)),level);
run;

/*
/ Now, perform Dunnett's comparison-with-control test with each
/ level as the control.
/--------------------------------------------------------------------*/
ods listing close;
proc mixed data=&data;
  class &class;
  model &resp = &model / dfm=sat;
  %do ilev = 1 %to &nlev;
    %let control =;
    %do ivar = 1 %to &nvar;
      %let control = &control "%scan(&&lev&ilev,&ivar)";
    %end;
    lsmeans &mean / diff=controlu(&control) cl alpha=&alpha
      adjust=dunnett;
  %end;

/*
make 'Diffs' out=_mcb;
*/
ods output Diffs=_mcb;
run;
%n2cclass(_mcb,_mcb,&class);
ods listing;
data _mcb; set _mcb;
  length level1 $ 20 level2 $ 20;

level1 = ';
level2 = ';
%do ivar = 1 %to &nvar;
%let v1 = &ivar;&ivar;
%let v2 = _&ivar;&ivar;
%if (%length(&v2) > 8) %then
  %let var2 = %substr(&v2,1,8);
level1 = trim(left(level1)) || ' ' || trim(left(&v1));
level2 = trim(left(level2)) || ' ' || trim(left(&v2));
%end;
run;
/*
   Sort results by first and second level, respectively.
   ---------------------------------------------------------
*/
proc sort data=_mcb out=_tmcb1 by level1 level2;
proc transpose data=_tmcb1 out=_tmcb1 prefix=lo;
   by level1; var AdjLower;
data _tmcb1; set _tmcb1; ilev = _n_;
proc sort data=_mcb out=_tmcb2 by level2 level1;
proc transpose data=_tmcb2 out=_tmcb2 prefix=lo;
   by level2; var AdjLower;
data _tmcb2; set _tmcb2; ilev = _n_;
run;
/*
   From Hsu (1996), p. 94:
   Di+ = +( min_{j!=i} m_i - m_j + d^i*s*sqrt(1/n_i + 1/n_j))^{+}
   = +(-max_{j!=i} m_j - m_i - d^i*s*sqrt(1/n_i + 1/n_j))^{+}
   G = {i : min_{j!=i} m_i - m_j + d^i*s*sqrt(1/n_i + 1/n_j) > 0}
   D_{i-} = 0
   = min_{j!=i} m_i - m_j + d^j*s*sqrt(1/n_i + 1/n_j) otherwise
   ---------------------------------------------------------
*/
data clhi; set _tmcb2; keep level2 clhi ilev;
   rename level2=level;
   clhi = -max(of lo1-lo%eval(&nlev-1));
   if (clhi < 0) then clhi = 0;
data _g; set clhi; if (clhi > 0);
run;

%let ng = 0;
%let g  = 0;
data _null_; set _g;
call symput('ng',_n_);
call symput('g',ilev);
run;

data cllo; set _tmcb1; keep level1 cllo ilev;
   rename level1=level;
   if ((&ng = 1) & (&g = ilev)) then cllo = 0;
   else cllo = min(of lo1-lo%eval(&nlev-1));
run;

data cl; merge cllo clhi;
   by level;
data &out; merge &out cl;
   drop df ilev;
run;

/*
 // Compute RVAL and SVAL. RVAL is just the p-value for Dunnett's
 // test for all means except the best, and SVAL is the maximum RVAL.
 /--------------------------------------------------------------------
-*/
data _slev; set &out; _i_ = _n_;  
proc sort data=_slev out=_slev; by descending estimate;  
%let ibest = 0;  
data _null_; set _slev;  
   if (_n_ = 1) then call symput('ibest',_i_);  
proc sort data=_mcb out=_pval; by level2 adjp;  
proc transpose data=_pval out=_pval prefix=p; by level2; var adjp;  
data _pval; set _pval; keep level2 rval;  
   rename level2=level;  
   if (_n_ = &ibest) then rval = .;  
   else rval = p1;  
proc sort data=_pval out=_spval; by descending rval;  
data _null_; set _spval; if (_n_ = 1) then call symput('sval',rval);  
data _pval; set _pval;  
   if (_n_ = &ibest) then sval = &sval;  
data &out; merge &out _pval; by level; drop level;  
proc sort data=&out out=&out; by orig_n;  
data &out; set &out; drop orig_n;
run;

/*
%if (&print) %then %do;
   proc print uniform data=&out noobs;
   run;
%end;

%if (&clean) %then %do;
   proc datasets library=work nolist;
   delete cllo clhi cl_slev_spval_pval_mcb_tmcb1_tmcb2_g;
   run;
%end;

%mend;

%*-------------------------------------------------------------------
/* Constrained MC with the worst                                     */
%*-------------------------------------------------------------------

%macro mcw(data,
   resp ,
   mean ,
   model  = &mean ,
   class  = &mean ,
   alpha  = 0.05 ,
   out    = mcbout ,
   options =      );

/*
/ Retrieve options.
/--------------------------------------------------------------------
-*/
   %let print = 1;
   %let clean = 1;
   %let iopt = 1;
   %do %while(%length(%scan(&options,&iopt)));
      %if (%upcase(%scan(&options,&iopt)) = NOPRINT) %then
         %let print = 0;
      %else if (%upcase(%scan(&options,&iopt)) = NOCLEAN) %then
         %let clean = 0;
      %end;
   %end;
%let clean = 0;
%else
   %put Warning: Unrecognized option %scan(&options,&iopt).;
%let iopt = %eval(&iopt + 1);
%end;

/*
// Copy the dataset but reverse the sign of the response, so that
// the best is the maximum response.
//---------------------------------------------------------------------
-*/
data _tmpds; set &data; &resp = -&resp; run;
%
mcb(_tmpds,
   &resp ,
   &mean ,
   model = &model ,
   class = &class ,
   alpha = &alpha ,
   out = &out ,
   options = &options);

/*
// Reverse the sign of the results, so that the best is again the
// minimum response.
//---------------------------------------------------------------------
-*/
data &out; set &out;
   rename cllo=cllo;
   rename clhi=clhi;
   estimate = -estimate;
   tvalue = -tvalue;
   _temp = -cllo; cllo = -clhi; clhi = _temp; drop _temp;
run;

/*
// Print and clean up.
//---------------------------------------------------------------------
-*/
%if (&print) %then %do;
   proc print uniform data=&out noobs;
   run;


%if (&clean) %then %do;
   proc datasets library=work nolist;
       delete _tmpds;
   run;
%end;
%mend;

%macro umcb(data,
         resp ,
         mean ,
         model = &mean ,
         class = &mean ,
         alpha = 0.05 ,
         out = mcbout ,
         method = EH ,
         options = );

   /* Retrieve options. */
   %let print = 1;
   %let clean = 1;
   %let iopt = 1;
   %do %while(%length(%scan(&options,&iopt)));
       %if (%upcase(%scan(&options,&iopt)) = NOPRINT) %then
           %let print = 0;
       %else %if (%upcase(%scan(&options,&iopt)) = NOCLEAN) %then
           %let clean = 0;
       %else
           %put Warning: Unrecognized option %scan(&options,&iopt).;
       %let iopt = %eval(&iopt + 1);
   %end;
%end;

/*
/ Count number of variables in grouping effect.
/--------------------------------------------------------------------
-*/
%let ivar = 1;
%do %while(%length(%scan(&mean,&ivar,*)));
  %let var&ivar = %upcase(%scan(&mean,&ivar,*));
  %let ivar = %eval(&ivar + 1);
%end;
%let nvar = %eval(&ivar - 1);

/*
/ Compute ANOVA and LSMEANS
/--------------------------------------------------------------------
-*/
ods listing close;
proc mixed data=&data;
  class &class;
  model &resp = &model;
  lsmeans &mean;
/*
  make 'LSMeans' out=&out;
*/
  ods output LSMeans=&out;
run;
%n2cclass(&out,&out,&class);
ods listing;
data &out; set &out; orig_n = _n_; proc sort data=&out out=&out; by &mean; run;

/*
/ Retrieve the levels of the classification variable.
/--------------------------------------------------------------------
-*/
data &out; set &out;
  drop tvalue probt;
  length level $ 20;

  level = '';
%do ivar = 1 %to &nvar;
  level = trim(left(level)) || ' ' || trim(left(&&var&ivar));
%end;
call symput('nlev',trim(left(_n_)));
call symput('lev'||trim(left(_n_)),level);
run;

%if (%upcase(&method) = TK) %then %do;
  ods listing close;
  proc mixed data=&data;
    class &class;
    model &resp = &model;
    lsmeans &mean / diff=all cl alpha=&alpha adjust=tukey;
    /*
      make 'Diffs' out=_mcb;
    */
    ods output Diffs=_mcb;
  run;
  %n2cclass(_mcb,_mcb,&class);
  ods listing;
  proc sort data=_mcb out=_mcb;
    by &mean _&mean;
  run;
  /*
   / Add reverse differences.
   /--------------------------------------------------------------------
   */
  data _mcb; set _mcb; keep level1 level2 AdjLower AdjUpper adjp;
    length level1 $ 20 level2 $ 20;
    level1 = ' ';
    level2 = ' ';
    %do ivar = 1 %to &nvar;
      %let v1 = &&var&ivar;
      %let v2 = _&&var&ivar;
      %if (%length(&v2) > 8) %then
        %let var2 = %substr(&v2,1,8);
      level1 = trim(left(level1)) || ' ' || trim(left(&v1));
      level2 = trim(left(level2)) || ' ' || trim(left(&v2));
    %end;
  output;
  output;

/*
// Confidence limits are the minimum lower and upper CL's for each
// level.
/---------------------------------------------------------------------
*/
proc sort data=_mcb out=_mcb; by level1 level2;
proc transpose data=_mcb out=cllo prefix=lo;
  by level1; var AdjLower;
proc transpose data=_mcb out=clhi prefix=hi;
  by level1; var AdjUpper;
data cllo; set cllo;
  rename level1=level;
  cllo = min(of lo1-lo%eval(&nlev-1));
data clhi; set clhi;
  rename level1=level;
  clhi = min(of hi1-hi%eval(&nlev-1));
data cl; merge cllo(keep=level cllo) clhi(keep=level clhi);
run;
data &out; merge &out cl; drop level;
run;
%if (&clean) %then %do;
  proc datasets library=work nolist;
    delete _mcb cllo clhi cl;
  run;
%end;
%end;
%else %do;
/*
// Now, perform Dunnett's comparison-with-control test with each
// level as the control.
/---------------------------------------------------------------------
*/
ods listing close;
proc mixed data=&data;
class &class;
model &resp = &model / dfm=sat;
%do ilev = 1 %to &nlev;
  %let control =;
  %do ivar = 1 %to &nvar;
    %let control = &control "%scan(&&lev&ilev,&ivar)"
  %end;
  lsmeans &mean / diff=control(&control) cl alpha=&alpha
          adjust=dunnett;
%end;
/*
  make 'Diffs' out=_mcb;
*/
ods output Diffs=_mcb;
run;
%n2cclass(_mcb,_mcb,&class);
ods listing;
data _mcb; set _mcb;
  length level1 $ 20 level2 $ 20;
  level1 = ''; 
  level2 = ''; 
  %do ivar = 1 %to &nvar;
    %let v1 = &&var&ivar;
    %let v2 = _&&var&ivar;
    %if (%length(&v2) > 8) %then
      %let var2 = %substr(&v2,1,8);
      level1 = trim(left(level1)) || ' ' || trim(left(&v1));
      level2 = trim(left(level2)) || ' ' || trim(left(&v2));
    %end;
proc sort data=_mcb out=_mcb; by level2 level1;
data cl; keep cllo cghi;
  array m(&nlev,&nlev); /* m[i1]-m[i2] - |d|^2s[i1,i2] */
  array p(&nlev,&nlev); /* m[i1]-m[i2] + |d|^2s[i1,i2] */
  array s(&nlev);
  array l(&nlev);
  array u(&nlev);
  do i = 1 to &nlev; do j = 1 to &nlev;
    m[i,j] = .; p[i,j] = .;
  end; end;
  do obs = 1 to %eval(&nlev*(&nlev-1));
set _mcb point=obs;

j = mod((obs-1),%eval(&nlev-1)) + 1;
i2 = int((obs-1)/%eval(&nlev-1)) + 1;
if (j < i2) then i1 = j;
else             i1 = j + 1;

m[i1,i2] = AdjLower;
p[i1,i2] = AdjUpper;
end;

/
/ From Hsu (1996), p. 120:
/  S = \{i : \min_{j \neq i} m_i - m_j + |d|^i s[i,j] > 0\}
/  = \{i : \min_{j \neq i} -(m_j - m_i - |d|^i s[i,j]) > 0\}
/  = \{i : \min_{j \neq i} -m[j,i] > 0\}
/--------------------------------------------------------------------
-*
ns = 0;
do i = 1 to &nlev;
   minmmji = 1e12;
do j = 1 to &nlev; if (j ^= i) then do;
   if (-m[j,i] < minmmji) then minmmji = -m[j,i];
   end;
s[i] = (minmmji > 0);
ns = ns + s[i];
end;

/*
/ From Hsu (1996), p. 115:
/  Lij = (i \neq j) * (m_i - m_j + |d|^j s[i,j])
/    = (i ^= j) * p[i,j]
/  Li  = \min_{j \in S} Lij
/
/  Uij = (i \neq j) * -(m_i - m_j + |d|^j s[i,j])^-
/    = (i ^= j) * \min(0,p[i,j])
/  Ui  = \max_{j \in S} Uij
put "Edwards-Hsu intervals";
do i = 1 to &nlev;
   li = 1e12;
do j = 1 to &nlev; if (s[j]) then do;

if (i = j) then lij = 0;
else
    lij = m[i,j];
if (lij < li) then li = lij;
end; end;

ui = -1e12;
do j = 1 to &nlev; if (s[j]) then do;
    if (i = j) then uij = 0;
    else
        uij = min(0,p[i,j]);
    if (uij > ui) then ui = uij;
end; end;

put li 7.3 " < mu" i 1. " - max_j muj < " ui 7.3;
end;

--------------------------------------------------------------------

-*/

/ From Hsu (1996), p. 120:
/ If S = {i} then
/    Li* = (min_{j!=i} m_i - m_j - |d|^i*s[i,j] )^+
/ = (min_{j!=i} -(m_j - m_i + |d|^i*s[i,j]))^+
/ = (min_{j!=i} -p[j,i])^+
/ Otherwise
/    Li* = min_{j in S,j!=i} m_i - m_j - |d|^j*s[i,j]
/ = min_{j in S,j!=i} m[i,j]

--------------------------------------------------------------------
-*/
do i = 1 to &nlev;
if ((ns = 1) & s[i]) then do;
    minmpji = 1e12;
do j = 1 to &nlev; if (j ^= i) then do;
    if (-p[j,i] < minmpji) then minmpji = -p[j,i];
    end; end;
    l[i] = max(0,minmpji);
end;
else do;
    minpmij = 1e12;
do j = 1 to &nlev; if (s[j] & (j ^= i)) then do;
    if (m[i,j] < minpmij) then minpmij = m[i,j];
    end; end;
    l[i] = minpmij;
From Hsu (1996), p. 120:
If i in S then

\[ U_i^* = \min_{j \neq i} (m_i - m_j + |d|^i s_{i,j}) \]
\[ = \min_{j \neq i} -(m_j - m_i - |d|^i s_{i,j}) \]
\[ = \min_{j \neq i} -m_{j,i} \]

Otherwise

\[ U_i^* = -(\max_{j \in S} (m_i - m_j + |d|^j s_{i,j}))^- \]
\[ = -(\max_{j \in S} p_{i,j})^- \]

----------------------------------

do i = 1 to &nlev;
if (s[i]) then do;

minmmji = 1e12;
do j = 1 to &nlev; if (j ^= i) then do;

if (-m[j,i] < minmmji) then minmmji = -m[j,i];
end; end;
u[i] = minmmji;
end;
else do;
minppij = -1e12;
do j = 1 to &nlev; if (s[j]) then do;

if (p[i,j] > minppij) then minppij = p[i,j];
end; end;
u[i] = minppij;
end;
end;

do i = 1 to &nlev;
cllo = l{i}; clhi = u{i};
output;
end;

stop;
data &out; merge &out cl; drop level;
run;

%if (&clean) %then %do;
    proc datasets library=work nolist;
delete _mcb cl;
run;
%end;

%end;

proc sort data=&out out=&out; by orig_n;
data &out; set &out; drop orig_n;
run;

/*
/ Print and clean up.
/--------------------------------------------------------------------
-*/
%if (&print) %then %do;
   proc print uniform data=&out noobs;
   run;
%end;
%mend;

/*-------------------------------------------------------------------
/* Unconstrained MC with the worst
*/
-------------------------------------------------------------------*/
%macro umcw(data,
             resp ,
             mean,
             model  = &mean ,
             class  = &mean ,
             alpha  = 0.05 ,
             out    = mcbout,
             method = EH ,
             options = );

/*
/ Retrieve options.
/--------------------------------------------------------------------
-*/
%let print = 1;
%let clean = 1;
%let iopt = 1;
%do %while(%length(%scan(&options,&iopt)));
  %if (%upcase(%scan(&options,&iopt)) = NOPRINT) %then
    %let print = 0;
  %else %if (%upcase(%scan(&options,&iopt)) = NOCLEAN) %then
    %let clean = 0;
  %else
    %put Warning: Unrecognized option %scan(&options,&iopt).;
  %let iopt = %eval(&iopt + 1);
%end;

/*
 / Copy the dataset but reverse the sign of the response, so that
 / the best is the maximum response.
 /--------------------------------------------------------------------
 -*/
data _tmpds; set &data; &resp = -&resp; run;

%umcb(_tmpds,
   &resp ,
   &mean ,
   model = &model ,
   class = &class ,
   alpha = &alpha ,
   out = &out ,
   method = &method ,
   options = &options);

/*
 / Reverse the sign of the results, so that the best is again the
 / minimum response.
 /--------------------------------------------------------------------
 -*/
data &out; set &out;
   rename cllo=cllo;
   rename clhi=clhi;
   estimate = -estimate;
   tvalue = -tvalue;
   _temp = -cllo; cllo = -clhi; clhi = _temp; drop _temp;
run;
/*
 * Print and clean up.
 */

%if (&print) %then %do;
   proc print uniform data=&out noobs;
   run;
%end;

%if (&clean) %then %do;
   proc datasets library=work nolist;
      delete _tmpds;
   run;
%end;

%mend;

* %inc '<location of SAS/STAT samples>mcb.sas';
* %mcb(filter,ncolony,brand);
   %mcb(switch,hcdegradation,trt,options=NOPRINT);
   proc sort data=mcbout; by estimate; run;
   proc print data=mcbout; run;
Table IIp SAS Codes of ANOVA for Hydrolysis of the Selected Pretreated Switchgrass Sample
(Example: oven-dried St6-1 switchgrass with least lignin content after pretreatment)

```sas
data St61hydrolysislowlignin;
input pretreatment$ xylanase$ FPU ECC;
/* factors and levels*/
/* 'pretreatment' 2 levels: ptd, unptd */
/* 'xylanase' 2 levels: xyl, noxyl */
/* 'FPU' 3 levels: 0, 15, 30 */
/* 'ECC' enzymatical conversion of cellulose in percentage */
datalines;
unptd noxyl 0 31.53
unptd noxyl 0 32.34
unptd noxyl 15 42.99
.
.
ptd xyl 15 46.29
ptd xyl 30 76.97
ptd xyl 30 50.75
ptd xyl 30 42.94
;
run;
proc GLM;
class pretreatment xylanase FPU;
model ECC = pretreatment xylanase FPU xylanase*FPU;
lsmeans pretreatment xylanase FPU xylanase*FPU/pdiff;
run;
```